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SPAWNING OF THE ENDANGERED KILLIFISH, 
APHANIUS DISAR DISPAR (RÜPPEL, 1829), UNDER LABORATORY CONDITIONS

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ABSTRACT

Spawning of the killifish, Aphanius dispar dispers, have been studied in freshwater under controlled laboratory conditions. Two breeding systems, with cages and without cages, were used to study the fish spawning. Five replicates for each system were used. One male and two females were stocked in each tank for ten weeks. The total production of eggs in the tanks with cage system was 144 eggs and the average egg production per tank per week was 2.88 eggs. 59 fries were hatched and the hatchability rate was 41%. No eggs were found in the tanks without cages probably because of predation by their parents. There was a highly significant (P<0.0001) difference between the two systems in egg production. It was also observed that the eggs were infected with fungus in the breeding and incubation tanks which reduced the eggs production and hatching rate, and caused fry mortality. The present study revealed that the tanks with cage system was the best method to prevent predation of fish on eggs and fry, and to increase fry production. The Ultra Violet (UV) system is recommended to disinfect water of breeding tanks and incubation jars to eliminate fungal infection of eggs. This will help in mass production of A. dispers dispers eggs and fry forrestocking of freshwater bodies for biological conservation of the fish to be used for biological control of mosquito larvae and ornamental purpose.

Keywords: Spawning, Killifish, Aphanius dispers, Biological control, Fungal infection, Saudi Arabia

INTRODUCTION

Aphanius dispers dispers (Teleostei, Cyprinodontidae) is one of the dwarf native fishes to Saudi Arabia occupying both fresh and marine waters. A. dispers dispers is a euryhaline fish
found in a wide range of salinities, "from fresh water to 1500‰ (175 ppt) in the dead sea springs" (Plaut, 2000). Lotan (1969, 1971); Lotan and Skadhauge (1972); Skadhage and Lotan (1974) and Fouda (2005) Stated that *A. dispar dispar* can sustain their osmotic pressure and ionic concentration when adapted to salinities 16-105 ppt. According to EPAA, (2003): "*A. dispar dispar* is widely distributed along the east cost of the Mediterranean, coastal region of Bahrain, Djibouti, Egypt, Islamic republic of Iran, Iraq, Northern Somalia, Sudan and the Arabian Peninsula (Oman, Saudi Arabia, Yemen) ,up to Pakistan and the west coast of India where , historically , it is well known for its larvivorous efficiency". *A. dispar dispar* is also found in oasis pools with hypersaline to freshwater. It can breed throughout the year with a peak in the period between April and June and well adapted to environmental conditions. It can be used for biological control of mosquito larvae in areas to which it has well adapted (Haas, 1982; Alkahem and Behnke, 1983 and Krupp, 1983 , 1988). *A. dispar dispar* spawn on roots of Hyacinth and other rooting plants in the subtropical area at temperatures ranging from 16 to 26 ºC (Froese and Pauly, 2009). Al-Akel et. al. (1987) gave a good account on the selective feeding behavior of *A. dispers* in freshwaters of Saudi Arabia; they reported that the fish has high selective feeding capability for food. Frekel and Goren (1997) studied the environmental factors that affect spawning of *A. dispar* especially temperature and the photoperiod.

The population of *A. dispar dispar* is declining as a result of several factors and now the fish is endangered. There is a great need to breed this species for restocking the natural freshwater bodies to save it from extinction as well as to ensure the availability of the fish for the control of mosquito larvae. The declining factors of *A. dispar dispar* in the freshwater environment of Saudi Arabia are summarized by AL-Kahem-Albalawi, et. al. (2008) as a combination of factors affecting the different stages of their life cycle. They include the reduced availability of food, habitat degradation, chemical pollutions, introduction of exotic species, and fish exploitation which lead to declining of such native fish's abundance. Al-Kahem (personal observation), stated that "the population of this species in Al Hasa irrigation canals are declining and in Al Khurj the fish are completely lost due to drying of the water bodies. Several authors stated that *A. dispar dispar* is endangered fish species and there is a need to save their population from extinction. Protecting the fish in general will help in protecting the aquatic biodiversity and biological conservation of fishes of the country ( AL-Kahem-Albalawi, et. al., 2008).

A successful attempts to breed *A. dispar dispar* under controlled conditions for restocking the natural freshwaters will prevent further declining of the fish in freshwater environment, and make them available for biological control and as aquarium fish for the economic and recreational importance.

In this study, two breeding systems ( a cage system and a system without cage) were used to evaluate their reproductive performance.

**MATERIALS AND METHODS**

The killifish , *Aphanius dispar dispar*, (total length 3.2 - 5.3 mm and body weight 1.0 - 2.2 gm) were obtained from irrigation canals of Al-Hasa province and brought to the zoology department laboratory, faculty of science, King Saud university in Riyadh to study the
spawning. The fish were brought in May, 2009 and acclimatized to laboratory conditions for one month. The experiment started on 1/7 2009 lasted for ten weeks on 10/9/ 2009. One mature male and two females were stocked in each tank. Two systems were selected for studying the spawning of the fish. The first system was a suspended mesh cage and the other system without cage. Each system has a set of five cylindrical tanks (25L capacity). The fish were fed with aquarium flakes - aquafire (38% protein). The photoperiod extended to 12 hours a day. Temperature is adjusted to 23-24°C by aquarium heaters. Tanks were aerated by continuous supply of fresh air. The tank water changed weekly and at the same time eggs were collected. Eggs were examined under the microscope for infection and their general conditions. The infected eggs were identified and photographed under light – microscope (fig, 2). A record was maintained for the number of eggs per week per tank. The eggs were incubated in jars (1000 ml) until hatching. Incubators were aerated up to oxygen saturation (7.5-8.0 mg/l) and temperature was adjusted to 23-24ºC. The hatched fry were counted. Hatchability was determined. Then fry were transferred to other jars for feeding with powder feed prepared from aquarium flakes of the brooder fish (38 % protein).

Statistical analysis of T-Test, Two pairs was performed to evaluate the two systems of cage and without cage for breeding performance of A. dispar dispar.

**RESULTS**

After the adjustment of temperature to 23-24 ºC and the photoperiod to 12: 12 hrs (day and night) the fish started to spawn in the breeding tanks. Breeding of Aphanius dispar dispar occurs in the caged system only. No eggs were found in tanks without cages, although the same conditions were maintained for the two systems during the period of the experiment. Data for the eggs produced during the period of study are summarized in Table (1) and figure (1). The total number of eggs produced were 144 and the average number per tank per week were 2.88 eggs. The hatched fry number was 59 and the egg hatching rate was calculated as 41% of the incubated eggs. When fry are stocked together. It was observed that larger fry feed on the small fry and show cannibalistic behavior.

![Figure 1. Average number of Aphanius dispar eggs /tank/week.](image)
The T-test for performance of the two systems (with cages and without cages) shows a highly significant (p<0.005) difference between the cage and without cage systems (table, 2).

The new eggs were transparent, gradually become more opaque towards development of the eggs. At the later stages of development and before egg hatching fry are observed moving inside the eggs. Egg hatched in the hatching jars and the yolk sack is absorbed after 2 days from hatching. The fry now supplied the powder food. Eggs and fry of *A. dispar dispar* are heavier than water: they always settled down on the bottom of the tank.

The some eggs examined under the microscope were found infected with fungus which kills the embryos and (or) stop the development of the eggs (figure 2).

**Table 1. Aphanius dispar dispar eggs collected from the tank cag system during the experimental period (10 weeks)**

<table>
<thead>
<tr>
<th>Weeks of sampling</th>
<th>Tank-1</th>
<th>Tank-2</th>
<th>TankK-3</th>
<th>TankK-4</th>
<th>TankK-5</th>
<th>total</th>
<th>average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Number/week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>7</td>
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<td>2</td>
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<td>3</td>
<td>10</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>18</td>
<td>3.6</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>16</td>
<td>3.2</td>
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<td>5</td>
<td>2</td>
<td>5</td>
<td>15</td>
<td>3.0</td>
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<td>2</td>
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<td>12</td>
<td>2.4</td>
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<td>6</td>
<td>0</td>
<td>2</td>
<td>16</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>12</td>
<td>2.4</td>
</tr>
<tr>
<td>Total</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.88</td>
</tr>
</tbody>
</table>

*Figure 2. An egg of *Aphanius d. dispar* infected with fungus in the breeding tanks.*
Table -2. t-Test: Paired Two Sample for Means of the average eggs of Dispar dispar collected from the two study systems (with cage/ without cage), ( t<0.001)

<table>
<thead>
<tr>
<th></th>
<th>With cages</th>
<th>without cages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
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</tr>
<tr>
<td>Variance</td>
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<td>0</td>
</tr>
<tr>
<td>Observations</td>
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<td>10</td>
</tr>
<tr>
<td>Hypothesized Mean</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>df</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>t Stat</td>
<td>21.4928</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
<td>2.4039E-09</td>
<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.8331</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>4.8078E-09</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.2621</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present study shows that breeding of *Aphanius dispar dispar* occurs only in the tanks with cage system and not in the tanks without cage system, probably, because the fish in the tanks without cage system cannibalize on their eggs and fries, if breeding or hatching had occurred in these tanks. In the tanks with cage system, the fish is prevented from reaching to their eggs or fry residing at the bottom of the tank. Both eggs and fries settled down in the tank and don’t rise up until they acquire a bigger size, therefore cannibalism by their parents is avoided. Similar results of cannibalistic behavior of *A. dispar dispar* were reported by Frenkel and Goren (1999) and advised to use cages to prevent cannibalism on fish fry.

The average egg production per tank per week was 2.88 where as hatchability was recorded 41% in the study made with cage system. Performance can be improved in order to produce a large quantity of eggs and a high hatching rate of the eggs. Both figures are low and this may be attributed to the fungal infection of the eggs which causes a high mortality rate among the embryos. The system can be improved through eradication of the fungi in the breeding tanks and the incubation system of the eggs. Fungal infection can be prevented by using ultra violet light to eradicate the fungus and bacteria which may come as a result of fungal infection. The UV system is being used for a long time to disinfect tilapia eggs which enhance productivity of tilapia fry (Rana and Suliman, 1993). The quantity of *A. dispar dispar* can be increased also by stocking more females in the tank in order to produce more eggs. Formalin can also be used to disinfect the eggs in the hatching jars, but it should be used with care in breeding tanks because it may affect the brooder fishes.

Frenkel and Goren (1997) stated that some environmental factors affect reproduction of *A. dispr dispar* and they found that maturation of oocyte increased by increasing the temperature from 18 to 27 °C. The photoperiod also affects the fish spawning. It is reported that the photoperiod (12:12 hrs) is the most suitable for spawning the fish. So in present study the temperature was adjusted to 23-24 °C which is the most suitable temperature for spawning.
and hatching and the photoperiod was adjusted to 12:12 hrs day and night which is also suitable for enhanced spawning of the fish. Salinity seems has no effect on spawning of *A. dispar*. Oltra and Todoli (2000) studied the breeding of *A. dispar* at different salinities ranging from 5 to 40 ppt and found no significant difference between spawning at different salinities.

We collect the eggs for incubation on weekly basis and the period needed for hatching the eggs is 10 days. This relatively long period may lead to fungal infection of the eggs in breeding tanks and the hatching jars. If the eggs collection is made more frequently some infection may be avoided.

We conclude that *A. dispar* can be bred in freshwater cage system. Breeding tanks and hatching jars should be disinfected with U V lamps to eradicate the fungal and bacteria infection for mass production of *A. dispar* for restocking of freshwater bodies in Saudi Arabia for biological conservation of the fish and to use them in biological control of mosquito larvae and aquarium uses, which in turn give the fish the economic and recreational importance. *A. dispar* fry should be segregated by size to avoid cannibalism and more fish to be stocked for breeding.

**REFERENCES**


Structure of a Glucan and a Heteropolymers from the Cell Wall of the Yeast Phaffia Rhodozyma

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ABSTRACT

Polysaccharides from Phaffia rhodozyma SDS-treated cell walls were successively obtained via cold water, hot water, and dimethylsulfoxide extractions. For this, processed yeast cells were grown under a physiological condition of active production of astaxanthin, its characteristic carotenoid metabolite. Afterwards, the extracted fractions were fractionated via freeze-thawing and precipitation with either Fehling reagent or Cetavlon. An α-D-glucan and a heteropolysaccharide were isolated and structurally investigated using monosaccharide composition determination, methylation analysis, and COSY, TOCSY and HMQC techniques of 13C- and 1H-NMR. The DMSO-extracted and Fehling-purified α-D-glucan was determined to be a linear chain of α-D-(1→3)-glucopyranosyl units. The hot water-extracted and Cetavlon-precipitated heteropolysaccharide showed a complex structure containing α-D-(1→4)-mannopyranosyl units-based main chain with side chains containing β-D-(1→4)-xylopyranosyl units along with D-mannose, D-galactose, and D-glucose units. In addition, these monosaccharides are found to be non-reducing ends of the heteropolysaccharide as well. The cell wall extraction schedule also yielded several minor glucan fractions displaying a positive iodoiphilic reaction which was abolished after pretreatment with crystalline Bacillus subtilis α-amylase. The knowledge of these cell wall features is of importance for the application of whole P. rhodozyma cells as single cell protein, improved digestibility, and astaxanthin release.

Keywords: Phaffia rhodozyma; yeast cell wall; polysaccharide.

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1. INTRODUCTION

The red yeast *Phaffia rhodozyma* was isolated by Herman Phaff in the 1960s [1]. Initially taken as a single genus and species, this yeast taxonomy presents some controversy due to the occurrence of *Phaffia rhodozyma* anamorph(s) and *Xanthophyllomyces dendrorhous* teleomorphs [2]. The yeast has attracted biotechnological interest due to its ability to synthesize as major carotenoid the astaxanthin (3,3’-dihydroxy-β,β-carotene-4,4’-dione) [3], particularly in its unesterified 3R, 3’R stereo form, which is one of the natural forms found in many organisms [4]. Astaxanthin is the carotenoid pigment naturally found in many invertebrate and fish, such as salmonids being responsible for the meat characteristic color. These animals cannot synthesize the carotenoids “de novo”, and depend entirely on dietary supplement to achieve the natural pigmentation [5]. Astaxanthin is the preferred pigment for use as colorant in aquaculture, especially as feed supplement in farmed trout, salmon and prawns since it is incorporated more efficiently than any other carotenoids. However, the yeast rigid cell wall severely reduces the intracellular pigment bioavailability. For instance, incorporation of undisrupted yeast in the diets of rainbow trout resulted in low or no pigmentation [3].

The yeast cell wall is responsible for determining the shape and morphology of the cells while affording mechanical protection from the environmental changes [6]. In general, the cell wall is composed of β(1→3)- and β(1→6)-D-glucans linked together with highly branched mannoproteins in a complex way [7]. The study of the polysaccharides from cell wall may aid the development of a specific rupture method for the industrial production of free carotenoid. In the present work we have isolated and characterized two polysaccharides from the yeast cell wall and they are described below.

2. EXPERIMENTAL

2.1. Microorganism and Culture Conditions

The anamorphic yeast *Phaffia rhodozyma* (ATCC 24202) was grown in a medium consisting of 20g sucrose/L, 1g yeast extract/L, and 5g peptone/L in 250 mL Erlenmeyer flasks that were agitated in a rotatory shaker at 150 rpm for 48 hours at 24°C for inoculum preparation. This culture was employed to inoculate the batch process of cell production. The batch cultures were grown in a 5 L B. Braum Biotech B bioreactor employing a media with the same composition as in the previous phase. The pH was controlled at pH 6.0 ± 0.2 by the automatic addition of M NaOH and M H₂SO₄. The temperature was controlled at 24 ± 0.5°C as well as the agitation rate. Aeration was manually controlled to maintain a dissolved oxygen concentration above 40%. The culture was carried out over a period of 98 hours in the absence of bright light in order to avoid carotenoid photo bleaching. Subsequently, the yeasts were harvested by centrifugation, washed with distilled water and lyophilized.

2.2. Carotenoid Analyses

The carotenoid content of the produced yeast was measured by HPLC. Samples were frozen in liquid nitrogen, pulverized and extracted with acetone in order to remove the
carotenoid. After centrifugation, the supernatant was filtrated in 0.2 μm Teflon (PTFE) membrane. The analyses were performed in a Shimadzu LC-10A liquid chromatograph equipped with C18 Vydac 201TP54 4.6 mm x 250 mm column at 30°C (Shimadzu CTO-10A). Liquid phase was methanol : acetonitrile (9:1, v/v). Carotenoid detection was performed by an UV-VIS Shimadzu SPD-10A detector set at 480 nm. Identification of the main carotenoid peak was accomplished using the elution time obtained from a standard solution of astaxanthin and its characteristic $\lambda_{\text{max}}$ at 480 nm.

2.3. Extraction and Fractionation of Cell Wall Polysaccharides

Dry cells (10 g) were treated with a 2% aqueous SDS (sodium dodecyl sulphate) at room temperature for 12 h (X4) for proteins, nucleic acids, and lipids removal in the supernatant. The resulting precipitate (5 g) was extracted with water at room temperature for 24 h, followed by centrifugation to give a supernatant, which was precipitated with a mixture of ethanol:water (3:1, v/v), as illustrated in Figure 1. The precipitate was dissolved in hot water, dialyzed and freeze-dried (CW1). This process was repeated 2 times more to yield fractions CWII and CWIII. The remaining residue from the initial cold water extraction was successively extracted 3 times with hot water at 80°C for 24 h to give fractions HWI, HWII and HWIII. Finally, fractions DMI, DMII and DMIII were obtained by extracting the remaining residue with DMSO at room temperature for 2 h also in 3 sequential cycles. All these fractions were tested for sugar content according to Dubois [8] and afterwards were frozen and thawed to give precipitate (FTP) and supernatant (FTS) subfractions. Samples which showed a higher yield (HW FTS and DM FTP) were treated with Fehling’s solution (~40 mL) [9]. The resulting complexes (FP) and supernatants (FS) were each neutralized with AcOH, dialyzed against distilled water. This treatment gave rise to the main fraction, originated from mixed DM I, II and III which did not precipitate with Fehling solution (DM FS). The fraction obtained successively from HW I and FS was dissolved in water (~50 mL) and mixed with a 3% solution of Cetavlon in water at pH 7.0 (50 mL). The supernatant of the Cetavlon precipitate was treated with an aq. solution of Na$_2$B$_4$O$_7$ at pH 8.5, which was then adjusted to pH 10.0 and then to 12.0, with aq. NaOH. The resulting precipitates were isolated by centrifugation and decomplexed with 4M aq. NaCl followed by precipitation in ethanol. For the cases where precipitation was observed at pH 8.5, decomplexation was achieved with a 2M aq. AcOH followed by precipitation in ethanol. Here, we have studied the portions that precipitated at pH 10.0 (HW I C10.0).

2.4. Analysis of Monosaccharides Present in Polysaccharides

The polysaccharides were hydrolyzed with 1 M TFA at 100°C for 8 h, followed by evaporation to dryness and successive reduction with NaBH$_4$ and acetylation with Ac$_2$O:pyridine (1:1, v/v) [10, 11]. The resulting alditol acetates were extracted with CHCl$_3$ and analyzed by GC-MS, using a DB-225 or DB-23 capillary column (30 m X 0.25 mm i.d.) using an initial temperature of 50°C, a heating rate of 40°C/min, a final temperature of 220°C, and a FID detector.
The uronic acid content was determined by an improved m-hydroxy-biphenyl colorimetric method [12].

2.5. Methylation Analysis of Polysaccharides

Five milligrams of each purified fraction were per-O-methylated using powdered NaOH in DMSO-iodomethane [13]. The per-O-methylated derivatives were treated with 0.5mL of 50% (v/v) or 72% (w/v) sulfuric acid (1h, 0°C) and the resulting solution diluted with H2O (4mL) and completely hydrolyzed at 100°C for 18h. The solution was neutralized with BaCO3 and filtered. The filtrate was evaporated to dryness and subsequently treated with NaBD4 in order to convert the partially O-methylated aldoses to alditol derivatives, which were analyzed by GC-MS as described in Section 2.4.

2.6. Partial Acid Hydrolysis of Heteropolymer

Twenty milligrams of fraction HW I C10.0 was partially hydrolyzed with 0.1M aq. trifluoroacetic acid, at 100°C for 2h. The acid was removed by freeze-drying. The resulting material was redissolved in distilled water, and then retained on dialysis with a Mw 8kDa cut-off membrane (13 mg).
2.7. HPSEC Analysis

The elution profile of water-soluble fraction was determined by high performance size-exclusion chromatography (HPSEC), using a Waters 510 HPLC pump at 0.6mL/min with four gel permeation columns in series with exclusion sizes of 1X10^6, 4X10^5, 8X10^4, and 5X10^3 Da, using a refraction index detector. The eluent employed was 0.1M/L aq. NaNO₂ containing 200 ppm aq. NaN₃. Prefiltered samples (0.20 μm, Millipore) were injected at the concentration of 1mg/mL.

2.8. Nuclear Magnetic Resonance Spectroscopy

NMR spectra were obtained with a DRX Avance 400MHz Bruker spectrometer. ¹³C (100.6 MHz) and ¹H NMR (400.13) spectra were acquired at 70 or 50°C, with the samples being dissolved in D₂O or in DMSO-d₆ depending on their solubility. Chemical shifts are expressed in δ ppm relative to acetone at δ 30.20 and 2.224 for ¹³C and ¹H signals, respectively.

2.9. Enzymatic Degradation of Iodophilic α-Glucan Minor Fractions

Minor polysaccharide fractions (5 mg/mL in water) were assayed before and after pretreatment with crystalline α-amylase from Bacillus subtilis (Sigma; 2 mg/mL) at 35°C overnight with 1:50 diluted I₂ / KI (1:10, g%) regarding the bluish color given by amyllose-like polysaccharides. The duplicated non stained enzymatic reactions were stopped by the addition of 3 volumes of ethanol and the evaporated supernatants chromatographed in silica gel chromatoplates with isopropanol:ethyl acetate:water (7:1:2, v/v) (1.5 x ascending developments) as mobile phase for the detection of glucose and maltosaccharides after revealing with hot orcinol : sulfuric acid : methanol (1 : 9 : 90).

3. RESULTS AND DISCUSSION

The carotenoid content of the cultivated cells from Phaffia rhodozyma was 163.33 μg astaxanthin/g of dried yeast cell, demonstrating a satisfactory pigment production in the grow medium used [14]. Although carotenoid production is not the aim of this work, this result confirmed the healthy growth of the microorganism, ensuring that the cell wall also corresponded to a full and functional cell envelope. The lyophilized cells had their lipids, proteins and nucleic acids removed with 2% aq. SDS (unpublished results). The remaining material containing cell walls was then subjected to cold water, hot water and DMSO extractions, followed by purification of the component polysaccharides (Figure 1).

3.1. Isolation and Characterization of Glucan Component

The fractions extracted with DMSO showed almost identical contents of xylose, mannose, galactose and glucose (acid monosaccharides were not found), with decreasing and
respective yields of 6.5, 2.8 and 0.7% for fractions I, II and III as referred to the starting material. Glucose was the predominant component (~82 mol%). Therefore, all DMSO fractions were combined and subjected to freeze-thawing cycles. The water insoluble fraction (FTP) was obtained in a higher yield (8.9%), with decreased contents of xylose and galactose in relation to crude fraction (DM).

In order to obtain a purified glucan, FTP was submitted to Fehling fractionation, which yielded 2 portions: soluble (DM FS; 4.5% yield) and precipitated material (DM FP; 3.3% yield). Both fractions showed high contents of glucose, 84.9 and 75.6 mol%, and DM FS consisted of a structure similar to pseudonigeran α-D-glucan, with a typical $^{13}$C NMR spectrum (Figure 2) [15, 16]. It contained six major signals of the glucan at $\delta$ 99.6 (C-1), 82.8 (C-3), 71.9 (C-5), 70.8 (C-2), 69.5 (C-4) and 60.3 (C-6). Methylation analysis showed 2,4,6-tri-O-methyl-glucose as main O-partially methylated derivative (80 mol%).

Glucans have been found in various species of yeasts. It is the first time that this particular structure is described in Phaffia rhodozyma, although other authors have already suggested the presence of an α-glucan in its cell wall [2]. The same glucan was firstly described in Aspergillus niger then isolated with hot water, and also in other fungus species (Polyporus betulinus, Tremella mesenterica, Aspergillus nidulans, Schizophyllum communis and in Cryptococcus and Schizosaccharomyces genus) [17]. In Schizosaccharomyces pombe, the α-(1→3)-glucan was observed in the surface of the cell wall and in regions adjacent to the cell membrane [18].

![Figure 2. 13C NMR spectrum of α(1→3)-D-glucan (DM FS fraction) in Me2SO-d6 at 70ºC: chemical shifts in δ ppm.](image)

3.2. Isolation and Characterization of Heteropolymer Component

The mother liquors of the freeze-thawing process following hot water extraction (HW FTS I, II and III) were further fractionated. They were treated with Fehling solution to give traces of precipitates and the main fraction was found in the supernatant (HW FS I, II, III).
Their monosaccharide compositions were significantly different, where mannose was the principal monosaccharide in the fraction I (57 mol%), and glucose in the fractions II and III (44 and 69 mol% respectively).

Fraction HW FS I was further fractionated with Cetavlon; no precipitation occurring at pH 7.0, agreeing with no acidic monosaccharide being detected. Precipitation occurred mainly at pH 10.0 in the presence of borate.

HPSEC-MALLS analysis of HW I C10.0 gave a homogeneous profile, with MW $8.73 \times 10^3$ g/mol and $dn/dc=0.143$. It contained xylose, mannose, galactose, and glucose in a 22:67:7:4 molar ratio, suggesting a xylomannan main structure.

Methylation analysis of HW I C10.0 (Table 1) showed a highly branched structure based on derived partially $O$-methylated alditol acetates (GC-MS) with a high proportion of nonreducing end-units of Xyl (15.1%), Man (10.9%) and Gal (10.9%), 4-O-substituted (12.0%) Xyl and also 4-O-substituted (17.4%) Man.

The $^{13}$C NMR spectrum of the heteropolymer (Figure 3) corresponded to a highly complex molecule with many C-1 signals, which indicated predominant branched structures with nonreducing end-units of $\alpha$-D-Glc (δ 99.6), $\alpha$-D-Man (δ 101.7), $\beta$-D-Xyl (δ 102.8), and $\beta$-D-Galp (δ 103.4) [15, 19].

In order to elucidate the core of the heteropolymer, a partial acid hydrolysis was carried out and removed the majority of the Xylp units. The product, PH HW I C10, retained on dialysis, contained Xyl (10%), Man (80%), Gal (6%) and Glc (4%). Methylation analysis (Table 1) indicated that the Xylp units were mainly present as nonreducing end units, along with Galp. The increasing content of 2,3,4,6-Me4Man after hydrolysis indicated that the non-reducing end units of Xylp were linked to Manp, which were exposed now as nonreducing end units. Also it was found increased contents of Man 2-O-, Man 4- and Gal 6-O-substituted. These units probably are from the internal core of the original polymer, the increased values resulting from the reduced size of the molecule after partial hydrolysis. $^{13}$C NMR examination (Figure 4) agreed with the removal of Xylp units, as their intensity decreased in relation to $\alpha$-Manp units, when compared with the original polymer (Figure 3). The anomeric carbon $\alpha$-linkage confirmation was obtained by the analysis of the coupled HMQC assay, where it was found a 178 Hz coupling relative to C1 and H1 of the Manp units.

The C-1 region can be interpreted in terms of a principal structure with a (1→4)-linked $\alpha$-D-Manp main chain (C-1: δ 101.7; C-2: δ 69.4; C-3: δ 73.5; C-4: δ 78.5; C-5: δ 75.9; C-6: δ 61.0) partially substituted at O-2 and/or O-6 with Manp (δ 101.7), $\beta$-D-Galp (δ 103.4), $\alpha$-D-Glc (δ 99.3) and internal units of (1→4)-linked Xylp (δ 102.7). These units were assigned in the $^{13}$C NMR spectrum: δ 72.8 (C-2), δ 75.1 (C-3), δ 77.0 (C-4 linked) and δ 65.2 (C-5) [15, 20, 21].

The supposed linkage of main chain are in agreement with the increased value of 2,3,6-Me4Man derivative after hydrolysis (17.4% to 31.2%). Polysaccharides structures containing $\alpha$-(1→2) or $\alpha$-(1→6) Manp units main chains have been described in several Basidiomycete yeast such as Torulopsis apicola, T lactis-condensi and Trichosporon aculeatum [22].

Several minor fractions from the yeast cell wall such as HW FTP, DM FS, DM FP, and HW FS which did not precipitate with Cetavlon, displayed a bluish reaction when assayed with iodine/potassium iodide. This color pattern was completely abolished after pre-incubation with crystalline $\alpha$-amylase from Bacillus subtilis. In order to confirm the $\alpha$-1,4-glucan nature of these fractions the corresponding ethanolic supernantants were
chromatographed aside the pertinent standards. In order of decreasing amounts the fragments of the amylolytic enzyme action were maltotriose, maltopentaose, maltose, maltotetraose, and glucose. Hence, the yeast cell wall despite de extensive washing of cytoplasmatic components with SDS still retains an α-amylose-like component. The alternative explanation is the presence of an iodophylic glucan as a true minor component of the native cell wall. Grün et al [23] have shown that alpha-glucan in fission yeast *Schizosaccharomyces pombe* also contains stretches of α-1,4-glucan.

Table 1. Partially *O*-methylated alditol acetates formed on methylation and GC-MS procedure of heteropolysaccharide (HW I C 10.0 fraction) and its partial hydrolysis product (HP HW I C 10.0 fraction)

<table>
<thead>
<tr>
<th>O-Me-alditol acetate</th>
<th>Fractions (molar %&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Linkage type&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>original</td>
<td>partial hydrolysis</td>
</tr>
<tr>
<td>2,3,4-Me₃-Xyl</td>
<td>15.1</td>
<td>6.6</td>
</tr>
<tr>
<td>2,3,4,6-Me₃-Man</td>
<td>10.9</td>
<td>16.9</td>
</tr>
<tr>
<td>2,3,4,6-Me₄-Glc</td>
<td>5.3</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4,6-Me₄-Gal</td>
<td>10.9</td>
<td>6.4</td>
</tr>
<tr>
<td>2,3-Me₂-Xyl</td>
<td>12.0</td>
<td>5.5</td>
</tr>
<tr>
<td>3,4,6-Me₃-Man</td>
<td>7.3</td>
<td>8.9</td>
</tr>
<tr>
<td>2,4,6-Me₃-Man</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>2,3,6-Me₃-Man</td>
<td>17.4</td>
<td>31.2</td>
</tr>
<tr>
<td>2,3,4-Me₃-Gal</td>
<td>2.4</td>
<td>5.6</td>
</tr>
<tr>
<td>2,3,6-Me₃-Glc</td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td>2,6-Me₂-Man</td>
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<tr>
<td>3,4-Me₂-Man</td>
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<td>3.7</td>
</tr>
<tr>
<td>4-Me₁-Glc</td>
<td>2.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> *O*-Me-alditol acetates obtained by methylation analysis, followed by hydrolysis, reduction with NaBD₄ and acetylation, analyzed by GC-MS (column DB-225).

<sup>b</sup>% of peak area relative to total peak area.

<sup>c</sup> Based on derivated *O*-methylalditol acetates.
Figure 3. 13C NMR spectrum of the heteropolymer (HW I C 10.0 fraction) in D2O at 50ºC: chemical shifts in $\delta$ ppm.

Figure 4. 13C NMR spectrum of the heteropolymer after partial acid degradation (PH HW I C 10.0 fraction) in D2O at 50ºC: chemical shifts in $\delta$ ppm.

4. CONCLUSION

Since astaxanthin accumulation in *Phaffia rhodozyma* is solely an intracellular event and since there is no convincing methodology for cheap rupture of the cell wall when dealing with formulations designated to human kind and animal consumption, knowledge gain on the yeast cell wall is biotechnologically important in order to ensure a prompt release of the antioxidant
astaxanthin and other intracellular and less soluble components. The present molecular characterization of homo- and heteropolysaccharides fulfill such a purpose.

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REFERENCES


ABSTRACT

Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum*/*L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

**Keywords:** *Leishmania chagasi*, canine visceral leishmaniasis, macrophage, spleen, parasite dissemination.
INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a chronic and frequently lethal disease caused by *L. (L) donovani* and *L. (L.) infantum* in the Old World and *L. chagasi* in the Americas (Desjeux, 2001a, b, 2004; Palatnik-de-Sousa *et al.*, 2001; WHO, 1999). Recent evidence shows that the *L. (L) chagasi* strains could not be distinguished from *L. (L.) infantum*, indicating a recent geographical separation, and that *L. (L) infantum* and *L. (L) chagasi* are two names for the same species (Mauricio *et al.*, 2000). VL is mostly a rural disease, with a domestic or peridomestic occurrence. In Brazil, however, urbanization of visceral disease has been found in towns or in the outskirts of large cities such as Belo Horizonte, Montes Claros, Rio de Janeiro, Salvador, and Fortaleza (Palatnik-de-Sousa *et al.*, 2001).

The disease is lethal if not treated early after the onset of the symptoms. Dogs present several signs, and progressive suppression of the cellular immune response. These parasites live inside monocytes and macrophages of lymphoid organs such as the spleen, lymph nodes, bone marrow, and liver. Their biological cycle alternates between the amastigote form in vertebrate host and the promastigote form in the gut of the insect sand fly vector (Baneth and Aroch, 2008). The present review aim to discuss the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL. These mechanisms probably account to development of T-cell unresponsiveness, and disease progression in susceptible dogs.

EPIDEMIOLOGY

The American and European forms of zoonotic VL show several similarities, as both are canid zoonoses that affect mainly children and young human adults (Palatnik-de-Sousa *et al.*, 2001); however, higher seroprevalences have been reported in South America (Ashford *et al.*, 1998; Zerpa *et al.*, 2000). It is well accepted that canids are the most common reservoirs of viscerotropic species causing zoonotic VL in the Mediterranean region, Asia, North Africa, and South America (Moreno and Alvar, 2002), playing a central role in the transmission cycle to humans by phlebotominae sand flies. CVL is a major veterinary and public health problem, and it has also been suggested as a good model for investigating the pathogenesis of human VL (Quinnell *et al.*, 2001b).

CVL has a high prevalence of infection, involving as much as 63%–80% of the dog population, and it is accompanied by a lower rate of apparent clinical disease (Berrahal *et al.*, 1996; Leontides *et al.*, 2002; Solano-Gallego *et al.*, 2004). Previous epidemiologic studies have indicated that about half of the dogs possessing anti-leishmanial antibodies exhibit no clinical signs of disease (Abranches *et al.*, 1991; Acedo-Sanchez *et al.*, 1998; Fisa *et al.*, 1999; Mauricio *et al.*, 1995). Interestingly, in a recent study in Northeast Brazil, a higher prevalence of positive dogs presenting no clinical signs of the disease was detected in the metropolitan when compared to a rural area (Alvar *et al.*, 2004; Berrahal *et al.*, 1996; Queiroz *et al.*, 2008; Solano-Gallego *et al.*, 2001). Cumulative evidence shows that: (1) infection in the canine population in endemic areas is widespread, and the rate of infected dogs is much higher than the fraction that shows clinical illness; (2) infection spreads quickly and
extensively among the dog population when environmental conditions for transmission are optimal (Baneth et al., 2008); and (3) removal or elimination of infected dogs from endemic areas would be followed by immediate substitution of susceptible dogs (Dye, 1996).

Dye (1996) and Burattini and others (1995) have shown that the dog population in endemic areas is composed of four mutually exclusive groups: those susceptible, those resistant, those susceptible that become latent after sand fly bite (asymptomatic), and those infectious to sand flies that emerge from latent dogs at a constant rate (Dye, 1996). Dogs born resistant do not become infectious to sand flies or develop the disease, but do become seropositive after sand fly bite. Such animals include seropositive, non-infectious, asymptomatic dogs from endemic areas that are able to maintain an effective cellular immune response against the parasite. Alvar and others demonstrated that naturally infected asymptomatic seropositive dogs (resistant or latent) are infectious to sand flies (Alvar et al., 1994). Dye also refers to a previous cohort study that used xenodiagnosis to show that infected dogs became infectious to sand flies after a median period of approximately 200 days; although several dogs died of clinical VL, the data indicate that infectiousness was unrelated to the severity of symptoms (Dye, 1996). This model demonstrated that targeting control measures at infectious dogs has a potential impact to reducing transmission. Conversely, infectiousness to sand flies has been shown to be positively associated with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) and to the intensity of skin disease (dermatitis, alopecia, and chancres) (Courtenay et al., 2002).

In the natural history of L. chagasi infection in dog, following transmission the parasites initially multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can disseminate via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis et al., 2006). In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to Leishmania parasites (Cardoso et al., 1998; De Luna et al., 2000; Martinez-Moreno et al., 1995; Pinelli et al., 1994; Pinelli et al., 1999; Santos-Gomes et al., 2002; Solano-Gallego et al., 2000). After infection and before seroconversion, dogs infected with L. chagasi present with enlarged lymph nodes and dermatitis, without signs of visceral leishmaniasis or changes in behavior. This phase is followed by dissemination of the infection and clinical findings, including loss of appetite, fever, weight loss, alopecia, skin ulceration, onychogryphosis, keratoconjunctivitis, uveitis, bleeding, diarrhea, neuralgia, polyarthritis, interdigital ulceration, and kidney insufficiency (Abranches et al., 1991; Bettini et al., 1986; Molina et al., 1994).

**INITIAL IMMUNE RESPONSE**

L. chagasi infection initiates when the parasite is inoculated into the skin by a female phlebotominae that probes the skin for blood (Rogers et al., 2004). Incoming and resident phagocytes exit the blood vessels and become infected with the parasites (Moll et al., 1993; Santos-Gomes et al., 2000; Wilson et al., 1987). Based on *in vitro* and *in vivo* animal models of visceral leishmaniasis, it is widely accepted that macrophages play a central role in the control of Leishmania infection. Most of these studies have involved human or murine
monocytes/macrophages (Bodman-Smith et al., 2002; Gomes et al., 2000; Murray, 2001), and only a few in vitro studies used canine macrophages and *L. chagasi* (Bueno et al., 2005; Gonçalves et al., 2005; Sampaio et al., 2007). Previously, Gonçalves and collaborators (2005) demonstrated that the frequency of peritoneal macrophages from naturally infected dogs expressing the monocyte surface molecules CD11b or CD18 significantly drops upon interaction with *L. chagasi*. More recently, Sampaio and collaborators (2007) showed that monocytes from naturally-infected animals compared to those from experimentally-infected ones are significantly more capable of binding to *Leishmania* promastigotes. Using peripheral monocytes from these naturally *L. chagasi*-infected dogs, the authors demonstrated that these cells display a higher frequency of CD11b-positive monocytes when obtained from peripheral blood. Similar to the previous study performed by Gonçalves and collaborators (2005) the frequency of macrophages expressing CD11b or CD18 has been shown to drop significantly upon interaction with *Leishmania*, and this decrease is more accentuated when *Leishmania* is incubated with exogenous serum (Sampaio et al., 2007). The authors propose that downregulation of these receptors may be related to two mechanisms, they can be occupied by *Leishmania*, or the receptor complexes can be internalized after *Leishmania*-macrophage interaction (Sampaio et al., 2007).

After phagocytosis, *Leishmania* promastigotes transform into amastigotes, which can survive inside macrophages. Some genetic alterations have been related to this event. The Slc11a1 (NRAMP) protein acts as a proton/divalent cation antiporter, which controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome (Gruenheid et al., 1997). The *Slc11a1* gene also regulates macrophage function – including upregulation of chemokine and cytokine genes such as TNF and interleukin-1β and increased expression of inducible nitric oxide synthase (iNOS) (Blackwell et al., 2001). Polymorphisms in the *Slc11a1* gene have been associated with CVL in dogs of different breeds (Sanchez-Robert et al., 2005). In 164 dogs, 24 polymorphisms were found in the *Slc11a1* gene and 3 polymorphisms were associated with an increased risk for CVL (Sanchez-Robert et al., 2008). Among these, two were single nucleotide polymorphisms (SNP) in the *Slc11a1* promoter region that disrupted putative transcription factor binding sites. These types of SNPs in the canine *Slc11a1* gene promoter suggest a possible role of differential *Slc11a1* gene expression that can interfere with Slc11a1 function and/or its interaction with many other genes, contributing to CVL susceptibility (Sanchez-Robert et al., 2005; Sanchez-Robert et al., 2008).

**COMPARTMENTAL IMMUNE RESPONSE**

**Skin**

From the original site of infection in the skin, amastigotes disseminate throughout the body, causing lesions in different tissues such as the lymph node, liver, spleen, gut, bone marrow and, in dogs, mainly other sites of the skin (Barrouin-Melo et al., 2004; dos-Santos et al., 2004; Reis et al., 2006). In the last few years, a growing number of systematic works provided important contributions to our understanding of the histopathological alterations that occur in these target organs (Brachelente et al., 2005; Giunchetti et al., 2007; Giunchetti et
Some studies evaluated histological alterations that occur in dog skin in response to *Leishmania* infection (dos-Santos *et al.*, 2004; Brachelente *et al.*, 2005; Solano-Gallego *et al.*, 2007). In a previous study, the histological pattern and parasite load were investigated in clinically normal skin of *Leishmania*-infected dogs (Solano-Gallego *et al.*, 2004). Two groups of *Leishmania*-infected dogs, symptomless animals that, although seronegative or only mildly seropositive, provided positive PCR results for *Leishmania* in the skin and a group of clinically affected dogs that were highly seropositive and PCR-positive were compared. The muzzle skin of symptomless dogs had no demonstrable microscopic lesions or amastigotes. This, together with the positive PCR results for *Leishmania*, indicates that the number of parasites in skin samples from the muzzle must have been very low. The most severe lesions and the greatest parasite loads were located around hair follicles, mainly around the isthmus, associated with the middle vascular plexus of the dermis. This finding suggested hematogenous dissemination of the parasite and tropism for the skin (Solano-Gallego *et al.*, 2007). In conclusion, the results of this study cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniasis (Solano-Gallego *et al.*, 2007).

In another recent study (Brachelente *et al.*, 2005), the question of whether a correlation exists between the number of parasites, the histological response, and the expression of cytokines produced by CD4⁺ Th (Thelper)-2 and Th-1 lymphocytes in lesional skin of naturally infected dogs was assessed. To achieve this objective, the authors evaluated the mRNA expression of canine cytokines such as IL-4, IL-13, TNF-α, and IFN-γ by real-time RT-PCR (qRT-PCR) to determine the cellular immune response in lesional skin biopsies from naturally infected dogs. qPCR was used to determine the number of *Leishmania* in paraffin-embedded tissue sections, and this was compared with cytokine expression in tissue lesions. In *Leishmania*-infected dogs, IL-4, TNF-α, and IFN-γ mRNA production were significantly higher than in controls. Furthermore, dogs with a high *Leishmania* burden demonstrated significantly higher IL-4 expression, whereas no difference was noted with regard to expression of other cytokines. By comparing the pattern of inflammation and cytokine expression, a clear trend became evident, that levels of IL-4, TNF-α, and IFN-γ were elevated both in biopsies with a periannexal nodular pattern and in biopsies where the severity of the periannexal infiltrate was equivalent to that of the perivascular interstitial infiltrate. Expression of IL-4, IL-13, and TNF-α was slightly increased in biopsies in which plasma cells prevailed over lymphocytes, whereas expression of IFN-γ was moderately higher when lymphocytes were predominant. In summary, this study demonstrates that the local immune response in naturally occurring leishmaniasis includes Th-1 as well as Th-2 cytokine subsets. Furthermore, the increased expression of the Th2-type cytokine IL-4 associated with both severe clinical signs and a high parasite burden in skin lesions connects severity of the disease to a Th-2-type of immune response (Brachelente *et al.*, 2005).

**From Skin to other Organs**

A variety of adhesion molecules are involved in phagocyte adherence to the extracellular matrix and cells of the connective tissue (Carlos and Harlan, 1994). Using adhesion blocking assays, our group observed that adherence of non-infected mononuclear phagocytes to the...
inflamed connective tissue is mediated by beta-1 and beta-2 integrins (Carvalhal et al., 2004). Flow cytometry experiments showed no consistent changes in the expression of several integrins on the surface of infected murine phagocytes (Carvalhal et al., 2004; Pinheiro et al., 2006). These data suggest that infected and non-infected phagocytes expressed similar amounts of integrins, even though adherence of the former cells to the connective tissue was diminished (Carvalhal et al., 2004). CCR1 and CCR7 have been shown to be upregulated about two-fold compared to the control group (Steigerwald and Moll, 2005) and, after infection, there is a decrease in CCR4 and CCR5 expression on phagocytes infected with L. amazonensis (Pinheiro et al., 2006). Another factor that may differentially interfere with the migration capability of infected cells is parasite burden. There is an inverse relationship between the degree of infection and the adhesive capability of infected cells. Although infection with small numbers of Leishmania does not reduce phagocyte adhesion to connective tissue, connective-tissue adhesion by infected phagocytic cells reaches levels of 20-30% that observed for non-infected cells under conditions of high parasite burden (Pinheiro et al., 2006). These data are in accordance with evidence suggesting that heavily Leishmania-infected phagocytes present a wide spectrum of suppressive changes, including changes in B7 expression, impaired integrin function, and inhibition of the expression of a variety of genes in mononuclear phagocytes (Buates and Matlashewski, 2001).

We still have a long way to go to understand the mechanisms that control parasite dissemination in dogs. It appears, however, that animals exhibiting more strict control of parasite burden develop a more limited pattern of differential dissemination of infected cells. As recently shown by Reis and collaborators (Reis et al., 2006), higher parasite burden is found in the skin and spleen than in the bone marrow, liver, and lymph nodes of infected healthy animals (Reis et al., 2006). The parasite distribution tends to be more uniform among tissues of unhealthy animals, and parasitism tends to be more intense in the spleen of animals with more severe disease.

In some visceral leishmaniasis-endemic areas of Brazil, the skin of dogs is prone to be in an almost permanent inflammatory state, not always related to Leishmania infection. In a series based on the study of stray dogs from the streets of Jacobina (Bahia state, Brazil), 81% of the dogs without evidence of Leishmania infection had inflammatory infiltrates in the ear skin (dos-Santos et al., 2004). Pucheu-Haston and collaborators (Pucheu-Haston et al., 2006) showed that inflammation causes dog skin to function as a source of chemoattractants and favors phagocyte adherence (Carvalhal et al., 2004) to the connective tissue. This may, therefore, constitute an important incentive to infected phagocytes remaining or disseminating to the skin of dogs during different phases of visceral leishmaniasis. A pattern of parasite dissemination to inflammatory sites has been confirmed in an experimental model of cutaneous leishmaniasis (Bertho et al., 1994).

In fact, it is not well established how Leishmania parasites are transported from the original infection site in the skin to other organs. Transport of amastigotes by cutaneous Langerhans cells from the skin to the draining lymph nodes was demonstrated by Moll and collaborators (1993) in murine models of infection, and Fiorini and collaborators (2002) detected myeloid cells containing Leishmania in human blood (Fiorini et al., 2002). In all lesions caused by Leishmania, the parasite is found inside mononuclear phagocytes, which maintain close contact with the extracellular matrix and cells of the connective tissue (Abreu-Silva et al., 2004). In only a few instances have parasites been found free in the tissues (Santos-Gomes et al., 2000; Wilson et al., 1987). These data suggest that the main interface
between *Leishmania* and the host may be the mononuclear phagocyte cell surface. It also suggests that the relevant information for *Leishmania* to remain within or to leave tissues may be expressed on the surface of these phagocytes.

Another interesting aspect of cell migration that affects the tissue in leishmaniasis is related to the loss of lymphoid tissue structure in the spleen, as reported by some authors for visceral leishmaniasis. Such alterations have been described in human beings by Veress and collaborators in 1977 (Veress *et al.*, 1977), and have recently been re-examined in a number of important studies performed by the group led by Paul Kaye. These studies have shown that the observed changes in the lymphoid tissue of the spleen are due to impaired leukocyte migration into the white pulp induced by TNF (Engwerda *et al.*, 2002) and IL10 (Ato *et al.*, 2002). Basically, a number of interactions between lymphocytes and mononuclear phagocytes may be disrupted in the marginal zone of the spleen, disturbing the entry of cells into the white pulp and follicle organization (Ato *et al.*, 2002; Engwerda *et al.*, 2002). We recently observed a similar pattern of lymphoid tissue disorganization in the spleens of dogs with visceral leishmaniasis. Such changes were more prominent in animals with a susceptibility pattern in response to *L. chagasi* infection (negative leishmanin skin test and positive spleen culture for *Leishmania*) than in non-infected animals or in animals with a positive leishmanin skin test (Santana *et al.*, 2008). These alterations of the white pulp appear to be associated with the disappearance of a population of marginal zone macrophages defined by an HI1 monoclonal antibody staining (Aguiar *et al.*, 2004). Whether this represents a cause or consequence of tissue disorganization remains unclear. Such a loss of lymphoid tissue structure may, however, underlie the increased susceptibility of these animals to bacterial infection and enhanced dissemination of *Leishmania* during late stages of the disease.

Recently, the cellular response in spleen was investigated (Lages *et al.*, 2008; Strauss-Ayali *et al.*, 2007). In both works, the mRNA expression levels for a wide panel of cytokines, transcription factors, and chemokines were examined. Both studies clearly show that Th1-1 and Th-2 immune responses occur simultaneously in the spleen during canine *L. infantum* infection (Lage *et al.*, 2007; Strauss-Ayali *et al.*, 2007). The frequency of IL-12 and IFN-γ expression within symptomatic dogs was significantly different from that of the uninfected group, although there were no significant differences between the symptomatic groups with respect to the expression of these cytokines (Lage *et al.*, 2007). In accordance, the other study identified higher IFN-γ, T-bet, IP-10, and RANTES mRNA levels in infected dogs during both oligosymptomatic and polysymptomatic stages of the disease (Strauss-Ayali *et al.*, 2007). These results agree with those reported by Quinnell and collaborators (Quinnell *et al.*, 2001b), who suggested that IFN-γ expression is not an appropriate indicator of resistance since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues, as is the case in humans, mice, and hamsters (Lage *et al.*, 2007). On the other hand, the Th-2 immune response in dogs was differentially described by these works. In one study, positive correlations between the levels of IL-10 expression with respect to the progression of the disease were observed. The other authors identified increased IL-4 and IL-5 expression during oligosymptomatic disease instead of enhanced expression levels of the Treg (T regulatory)-associated cytokines, IL-10 and TGF-β (Strauss-Ayali *et al.*, 2007).
Blood Compartment

The results of the serological analysis of VL-infected dogs also identified mixed Th-1 and Th-2 responses in the serum of infected dogs, with detectable expression levels of IFN-γ, TNF-α, and IL-12 together with IL-4 and IL-10. However, when clinical indications are considered alongside the biochemical data, the Th-2 response appears to be predominant, since the expression of IL-4 increased within the symptomatic group while the expression of IL-12 increased within the asymptomatic group (Santos-Gomes et al., 2002).

The nature of the dog’s PBMC responses to Leishmania is not completely understood. Asymptomatic dogs show protective immunity, which has generally been associated with a strong proliferative response of peripheral blood lymphocytes to leishmanial antigens (Cabral et al., 2019; Pinelli et al., 2015; Pinelli et al., 2013). However, development of a Th-1 and Th-2 mixed response by antigen-stimulated PBMCs from asymptomatic dogs expressing IL-2, IFN-γ, and IL-10 mRNA transcripts has also been reported. Although, in these studies, IL-2 and IFN-γ predominated in asymptomatic dogs, the development of symptomatic infections could not be related to IL-10 expression (Carvalho et al., 1994; Chamizo et al., 2005). Thus, in contrast to what occurs in human visceral leishmaniasis, the role played by PBMC-expressed IL-10 in L. chagasi-infected dogs is not well established (Carvalho et al., 1994).

Dogs with symptomatic CVL (Berrahal et al., 1996) present with depressed T cell-mediated functions and high levels of specific antibodies (Abranches et al., 2001; Barbieri, 2006; Killick-Kendrick et al., 1994; Oliva et al., 2004; Santos-Gomes et al., 2002). These animals present immunological changes involving T cells, including absence of delayed type hypersensitivity (DTH) to Leishmania antigens (Berrahal et al., 1996; Oliva et al., 2004; Quinnell et al., 2001b), decreased T cell numbers in the peripheral blood (Cabral et al., 1998; Killick-Kendrick et al., 1994; Oliva et al., 2004), and absence of IFN-γ and IL-2 production by PBMCs in vitro (Alvar et al., 2004; Oliva et al., 2004; Pinelli et al., 1994). Interestingly, the Th-1 cytokine profile in bone marrow aspirates positively correlates with humoral, but not with lymphoproliferative responses to Leishmania antigen. It is noteworthy that increased accumulation of IL-4, IL-10, and IL-18 mRNA was not observed in infected dogs, and the mRNA for these cytokines did not correlate with antibody or proliferative responses. However, infected dogs with detectable IL-4 mRNA display significantly more severe symptoms (Quinnell et al., 2001b). These data suggest that clinical symptoms are not due to a deficiency in IFN-γ production.

It is well established that early events are considered to be a determinant of infection outcome in humans and mice (Gomes et al., 2000; Rogers and Titus, 2004; Shankar and Titus, 1993; Veras et al., 2006). Prediction of dog immune responses in vivo early after exposure to L. chagasi is a difficult task (human models). We have established an in vitro priming system (PIV) using naïve canine PBMCs in order to assess dog PIV immune response to L. chagasi (Rodrigues et al., 2008). We co-cultivated PBMCs primarily stimulated with L. chagasi in vitro with autologous infected macrophages and found that IFN-γ mRNA is upregulated in these cells compared to control unstimulated cells. IL-4 and IL-10 mRNA expression in L. chagasi-stimulated PBMCs was similar to control unstimulated PBMCs when incubated with infected macrophages. Surprisingly, correlation studies showed that a lower IFN-γ/IL-4 expression ratio correlates with a lower percentage of infection. We proposed that the direct correlation between the IFN-γ/IL-4 ratio and parasite load is
dependent on the positive correlation of both IFN-\(\gamma\) and IL-4 expression with lower parasite infection. This PIV system was shown to be useful in evaluating the dog immune response to \(L.\ chagasi\), and the results indicate that a balanced expression of IFN-\(\gamma\) and IL-4 by these naïve cells is associated with control of parasite infection \textit{in vitro} (Rodrigues \textit{et al}., 2008).

In experimental infections, intradermal inoculation of promastigotes triggers asymptomatic infections, and PBMCs from these dogs stimulated with soluble leishmanial antigens (SLA) \textit{in vitro} express both Th-1 cytokines, such as IL-12, IFN-\(\gamma\), TNF-\(\alpha\), and IL-18, and Th-2 cytokines, such as IL-4, IL-6, and IL-10. Despite the fact that PBMCs from these asymptomatic dogs present such apparently mixed Th-1 and Th-2 responses, they predominantly produce IL-12 and IFN-\(\gamma\). In accordance with a previous observation (Pinelli \textit{et al}., 1994), these data support the protective immune response observed in these animals (Chamizo \textit{et al}., 2005). We recently observed that PBMCs from immunized dogs and than subcutaneously challenged with \(L.\ chagasi\) promastigotes are still asymptomatic. PBMCs from these apparently protected dogs liberate IFN-\(\gamma\) into the cell supernatant upon \(L.\ chagasi\) stimulation \textit{in vitro} (Rodrigues \textit{et al}., 2007). Moreover these cells express IFN-\(\gamma\) but not IL-4 mRNA (Rodrigues \textit{et al}., 2007), showing that these dogs display a predominant Th-1 type of immune response. On the other hand, dogs experimentally infected by intravenous inoculation of amastigotes develop progressive symptomatic infections. PBMCs from these dogs produce reduced levels of both Th-1 and Th-2 cytokines (IFN-\(\gamma\), IL-2, IL-12, IL-6, and IL-10) during the active phase of the disease (Santos-Gomes \textit{et al}., 2002).

In murine models, it is well established that macrophages participate in parasite killing via reactive oxygen and nitrogen intermediate-dependent mechanisms. However, mechanisms involved in \textit{Leishmania} killing by canine macrophages have not been as thoroughly investigated. There are cumulative data implicating canine macrophages in parasite killing by a NO-dependent mechanism. NO produced by macrophages has been found to be the principal effector molecule responsible for mediating intracellular killing of \textit{Leishmania} (Holzmuller \textit{et al}., 2006; Panaro \textit{et al}., 2001; Pinelli \textit{et al}., 2000). A canine macrophage cell line incubated with supernatant (containing IFN-\(\gamma\), IL-2, and TNF-\(\alpha\)) produced significant amounts of NO, sufficient to mediate \(L.\ infantum\)-killing (Pinelli \textit{et al}., 2000). PBMCs from vaccinated dogs were also able to reduce macrophage infection via an NO-dependent mechanism upon \textit{in vitro} stimulation with both \textit{Leishmania} promastigotes and concanavalin A (ConA). This effect was potentiated by the addition of LPS (Panaro \textit{et al}., 2001). Using a macrophage cell line, Pinelli and collaborators (Pinelli \textit{et al}., 2000) showed that parasite burden is reduced upon activation of cells with cytokine-rich supernatants. These supernatants were obtained from a \textit{Leishmania}-specific T cell-line generated from dogs immunized with soluble Ag (Panaro \textit{et al}., 2001). Infected canine macrophages incubated with autologous lymphocytes of immunized dogs also induced IFN-\(\gamma\) with increased NO production (Holzmuller \textit{et al}., 2005). The increased IFN-\(\gamma\) production and NO release by macrophages suggest a role for this cytokine in iNOS induction. We recently established an \textit{in vitro} model to test whether PBMC supernatants from asymptomatic dogs immunized with promastigote lysates and infected with \(L.\ chagasi\) promastigotes were able to stimulate PBMC-derived macrophages from healthy dogs to control parasite infection (Rodrigues \textit{et al}., 2007). Using our system, we demonstrated for the first time that PBMCs from these asymptomatic dogs stimulated exclusively with \(L.\ chagasi\) \textit{in vitro} reduce macrophage infection by the parasite (Rodrigues \textit{et al}., 2007). Moreover this effect is associated with high IFN-\(\gamma\), but not IL-4,
mRNA expression and release of this Th1 cytokine into the PBMC supernatant via an NO-dependent mechanism, as AMG reversed this effect (Rodrigues et al., 2007). In contrast to other works (Panaro et al., 2001), PBMCs in our system were exclusively stimulated with *L. chagasi* in vitro. Additionally, the protective response of these dogs to *L. chagasi* was demonstrated by the positive proliferative response to *Leishmania* antigens exhibited by PBMCs from these dogs in vitro (Rodrigues et al., 2007). In addition, PBMCs from the majority of these immunized and experimentally infected dogs expressed IFN-γ mRNA and released IFN-γ upon LSA stimulation. These data suggest that lymphocytes from apparently protected dogs produce cytokines related to a protective immune response (Rodrigues et al., 2007). A recent study demonstrated that, although Th-1 and Th-2 cytokines are produced in asymptomatic *Leishmania*-infected dogs, there is a prevalent Th-1 cytokine response that confers immunity against the parasite (Chamizo et al., 2005). Finally, our data reinforce the notion that the leishmanicidal effect of canine macrophages is NO-dependent (Rodrigues et al., 2007).

It was recently demonstrated that there are some differences in iNOS expression in lesion macrophages in situ. iNOS-negative dermal and splenic macrophages contain numerous *Leishmania* amastigotes. In contrast, dermal and splenic macrophages, which present high iNOS expression, contain few or no amastigotes, suggesting that iNOS-positive activated macrophages are able to destroy and/or do not allow multiplication of intracellular amastigotes (Zafra et al., 2008). PBMC-derived macrophages infected with *L. infantum* produce a significantly higher amount of NO than uninfected macrophages in vitro (Panaro et al., 2008; Rodrigues et al., 2007). In a comparison between infected dogs, the levels of NO in supernatants of *Leishmania*-infected macrophages were significantly higher in symptomatic than in asymptomatic animals. However, four months after diagnosis, the addition of autologous lymphocytes significantly decreased NO production only in symptomatic dogs, while NO production by macrophages co-cultured with autologous lymphocytes was significantly reduced eight months after diagnosis in *Leishmania*-infected macrophages from both asymptomatic and symptomatic dogs (Panaro et al., 2008). These higher levels of NO observed during follow-up of symptom-free (only 8 months) animals may suggest a protective role for this molecule in long-term asymptomatic parasitism.

**CONCLUSION**

The nature of the dog’s cellular immune response is not completely understood. Evidence points that although CVL dogs develop a mixed Th-1 and Th-2 cellular immune response, asymptomatic dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection, as well as predominance of Th-1 cytokines. On the other hand, as the disease progresses in susceptible dogs, the protective responses diminish with involvement of either IL-4 or IL-10 in uncontrolled infection. Disease progression occurs together with parasite dissemination. *Leishmania*-infected mononuclear phagocyte may stay or leave inflammatory sites disseminating the parasite through the host tissues. The migration of these cells depends upon the leukocyte phenotype and is modulated by parasite burden that results in changes in integrin function and in the expression of chemokine receptors.
ACKNOWLEDGMENTS

We thank all of our collaborators from the Immunology and Bio-Intervention Laboratory at the Gonçalo Moniz Research Center, FIOCRUZ, Bahia, Brazil. This work was supported by the Bahia State Research Supporting Agency - FAPESB and The Conselho Nacional de Desenvolvimento Científico e Tecnológico (The Brazilian National Research Council).

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ABSTRACTS PRESENTED IN - IV INTERNATIONAL SYMPOSIUM ON DEVELOPMENTAL BIOLOGY AND III STUDENT MEETING ON DEVELOPMENTAL BIOLOGY

ORGANIZING COMMITTEE:
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Maiaro Cabral Rosa Machado (USP)
Marcio Ribeiro Fontenele (UFRJ)
Ricardo Moraes Borges (USP)
Nadia Monesi (USP)
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<td>Ethan Bier</td>
<td>Dorsal-ventral patterning in the <em>Drosophila</em> embryo: from gradients</td>
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<td>Maria Luiza Paço</td>
<td>Ataxin-2 and A2bp1/Fox1 of Drosophila in stress granules and</td>
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<td>Peter Lwigale Rice University</td>
<td>The role of Semaphorin3A/neuropilin-1 signaling during vertebrate eye development.</td>
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**November 18th**

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<td>Richard Behringer University of Texas</td>
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Novel Regulators of the HIF Pathway in Drosophila

Agustina Bertolin', Andres Dekanty and Nuria Romero
Fundación Instituto Leloir, Buenos Aires, Argentina

The hypoxia-inducible factors (HIFs) are a highly conserved family of transcription factors that constitute the major regulators of cellular oxygen homeostasis throughout the animal kingdom.

These factors target genes that are crucial for systemic hypoxia responses, such as angiogenesis and erythropoiesis, and cellular hypoxia responses involving metabolism, proliferation, motility and autophagy. HIF is a heterodimeric DNA-binding complex composed of a constitutively expressed HIF-beta subunit and a HIF-alpha subunit that is tightly regulated through multiple mechanisms. We have previously defined a hypoxia-responsive system in Drosophila which is homologous to HIF, being the proteins Sima and Tango the functional homologues of HIF-1 alpha and beta, respectively. Although the core HIF pathway and a few canonical regulators have been extensively characterized in the past decade, a systematic loss of function screen has not been performed so far.

Here we report an RNAi screen aimed to identify novel regulators of the hypoxic response. We used a Drosophila S2 cell line bearing a stably transfected luciferase reporter, which is strongly up-regulated upon exposure to hypoxia. We considered as potential regulators of the hypoxic response those genes whose interference led to a reduction of reporter activity, without affecting cell viability. The screen has allowed the identification of

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approximately 70 novel positive regulators of the HIF/Sima pathway. These included several members of the PI3K signalling pathway, genes of the Brahma chromatin-remodelling complex, and members of the AAA+ family of DNA helicases. Interestingly, we have also identified dATF-4 which is considered a central component in many cellular stress-response pathways. Finally, various members of the microRNA machinery scored as positives in the screen. We focused our studies on this last group of genes, and our results show for the first time that miRNAs may act as regulators of the hypoxic response.

**Morphological and Functional Study of Cholesterol-Enriched Membrane Microdomains During Xenopus Laevis Early Development**

*Alice Helena dos Reis², Leonardo Andrade, Karla Almeida, Mariana Louza, and Mirna Abreu e José Garcia Abreu*

Programa de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, RJ

During vertebrate development, cell interactions are extremely important for correct formation of the anteroposterior and dorso-ventral axis. Although many different signaling mechanisms have been studied during early embryonic development, it is unclear how cells segregate these signals in order to achieve correct fate. In this respect cell membrane machinery play pivotal role in capturing and transducing cell signaling. Lipid rafts are membrane microdomains (MM) highly dynamic enriched for glycosylphosphatidylinositol-linked (GPI-linked) proteins, glycosphingolipids such as GM1 ganglioside and cholesterol. These structures are known for their ability to compartmentalize cellular processes and form large platforms for cell signaling. In order to study the MM function and organization during early crucial stages of embryonic development, Methyl-beta-cyclodextrin (MβCD) was injected into the blastocoele at blastula stage embryos to disrupt the MM. Phenotype analysis by Scanning electron microscopy showed that cholesterol depletion by MβCD caused anterior defects in Xenopus embryo: optic vesicles, cement gland, gills, oral cavity and forebrain were malformed. Despite this phenotype, transmission electron microscopy of the embryonic cells showed that the overall cell architecture was not affected, but some organelles such as lipid bodies and mitochondria were smaller. The caveolin and flotillin transcripts were identified during embryogenesis since early development until tadpole stages. Immunoassaying and dot blot analysis showed that lipid raft markers such as GM1 ganglioside, caveolin1 and flotillin1 were affected upon MβCD microinjection. Negative staining showed that lipid raft fraction obtained from MβCD injected embryos was not able to form organized and associated membranes, compared to those ones observed in non injected embryos. These results support that cholesterol-enriched membrane microdomains play crucial role during Xenopus anterior development and that cholesterol depletion by MβCD specifically affect the MM components and their ultrastructure, leading to head defects in Xenopus embryos.

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DIFFERENTIAL DISTRIBUTION OF SOG FRAGMENTS REGULATES DPP SIGNALLING IN POLARIZED EPITHElia OF DROSOPHILA MELANOGASTER

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How morphogen concentration gradients are established are fundamental issues in developmental biology of vertebrates and invertebrates. It is important because these gradients are responsible for determining territories from which cells differentiate into many tissues. Dpp (Decapentaplegic) protein is an extracellular morphogen and it has important roles during Drosophila melanogaster development. The generation of Dpp gradients depends on interactions with extracellular modulators such as the BMP-binding protein, Sog (Short gastrulation). Sog activity can be modulated by metalloproteases which cleaves it giving rise to different fragments: N-terminal and C-terminal. In addition, Integrin receptors can regulate the Sog activity/mobility and consequently Dpp signaling in the pupal wing epithelium, impacting on the formation of wing veins.

In this study, we seek to understand how the distribution of Sog protein is regulated in polarized epithelia during follicular and pupal wing epithelia development, impacting on Dpp activity to pattern the dorsoventral axis of the embryo and vein formation, respectively. Immunostaining analysis show that Sog fragments have differential extracellular distribution during pupal wing development. Furthermore, subcellular distribution of Sog fragments is distinct at the apical and basolateral levels of wing cells. Subcellular Sog distribution in the follicular epithelium is also compartmentalized and it is dynamic according to different stages of follicular maturation. In addition, clonal analysis in follicular epithelium shows that the distribution of Sog fragments in the perivitelline space is regulated by Integrins, as it was previously reported in the wing epithelium. These results point to a conserved mechanism for the regulation of Sog distribution in the follicular and wing epithelia, based on the action of metalloproteases and Integrin receptors. This mechanism which controls the diffusible proprieties of Sog protein may be fundamental to spatially restrict Dpp morphogenetic activity in growing epithelia.

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ULTRABITHORAX EXPRESSION AND BRISTLE PATTERNING DURING POST-EMBRYONIC DEVELOPMENT OF HIND LEGS OF APIS MELLIFERA QUEENS AND WORKERS

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The queen-worker diphenism in the honey bee Apis mellifera results from differential feeding of female larvae. Adult workers have a structure on their hind legs that is used for carrying pollen and propolis, the corbicula (a central region of the tibia that is free of bristles), while queens lack this structure. Using scanning electronic microscopy we found that these leg structures are already formed in brown-eyed pupae in both castes. Oligonucleotide microarray analyses performed on prepupal hind legs showed that 68 genes are overexpressed in workers and 103 in queens, including genes related to juvenile hormone metabolism and the insulin pathway. Using qRT-PCR we assessed the transcription pattern of some of these differentially expressed genes, as well as of others with already known participation in leg development: abdominal-A, ataxin-2, cryptocephal, dachshund, distal-less, grunge, Retinoic and fat acid Binding Protein (RfaBP) and ultrabithorax. ultrabithorax and abdominal-A had higher expression levels in prepupae and early pupae of workers than in queens. RfaBP expression was higher in queen than in worker prepupae. Using immunohistochemistry, we localized Ultrabithorax protein in the prepupal stage in the tibia and basitarsus of worker hind legs, as well as in the basitarsus of queens during prepupal and white-eyed pupal stages. We found that groups of cells in the basitarsus of white eyed queens pupae and workers prepupae were anti-Ultrabithorax negative. These cells, evidenced by DAPI, were arranged in a similar pattern to that observed in adult leg bristles, suggesting that the transient absence of Ultrabithorax is crucial in bristle morphology and patterning of the hind legs of A. mellifera females. We conclude that, based on their differential expression, abdominal-A and RfaBP are involved in leg structure development during caste differentiation in the honeybee, and we also evidence a specific role of ultrabithorax in this process.


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SUBCELLULAR LOCALIZATION OF JAZIGO IN DYING LARVAL SALIVARY GLANDS OF DROSOPHILA MELANOGASTER

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In a previous “forward genetics” screen we have identified a mutation that presents a drastic defective phenotype: the persistency of larval salivary gland (LSG). In wild type animals, LSG are fated to dye in an autophagy-like programmed cell death (PCD) process early into the pupal stage of Drosophila’s development. This mutation was named jazigo (jaz). We mapped the affected gene and using bioinformatics a great homology between JAZ encoded protein and components of the family of proteins called formins was seen. Formins are proteins involved in the active remodeling of actin-tubulin cytoskeleton in many different biological processes such as the establishment of cell polarity and cytokinesis. Formin’s signature is the presence of FH domains, most formins exhibit both FH1 and FH2 domains. The FH1 domains are proline rich domains and can serve as binding sites for SH3- and WW-motif containing proteins, as well as profilin. The FH2 domain is the most conserved domain and it has been related to the dynamics of actin polymerization. Due to the mutant phenotype and biological significance of formins, we have initiated the functional characterization of JAZ by generating polyclonal anti-JAZ antibodies in order to address its sub-cellular localization in dying LSG.

Immunoprecipitation assays revealed that JAZ is cleaved in dying LSG into two polypeptides (80kDa and 60kDa) that together add up to the expected size of the JAZ protein (~140kDa). Using confocal microscopy we could see that JAZ is initially scattered throughout the cytoplasm of dying LSG cells and at later stages of the process it migrates to the nucleus localizing in between politene chromosomes. In persistent SGs of jaz mutants, the -JAZ staining revealed a “mosaic” pattern in which, those cells arrested at later stages of PCD, we could still see nuclear labeling, even though less intense, while cells arrested at initial stages of cell death, if present, we could see labeling only in the cytoplasm. These results are consistent with previous findings in HeLa cells that show FHOD1, a formin sharing the highest similarity to JAZ. FHOD1 has been shown to be cleaved and migrate to the nucleus. However its function, as well as JAZ functions into the nucleus, is still uncertain. The most obvious hypothesis would be that formins might play a role in disassembling the nuclear structure in such type of cell death. We are currently studying JAZ functionaly in order to address this question.

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ESTABLISHMENT OF A MODEL SYSTEM TO STUDY THE VASCULOGENIC POTENTIAL OF PROEPICARDIA IN ADULT TISSUES

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The Proepicardium (PE) is a transient extracardiac structure giving rise to all components of the coronary vessels. To evaluate the vasculogenic potential of the PE in an adult site, we transplanted a neonatal heart into the subcutis of an adult ear. Later, two PE from eGFP-transgenic mice were transferred to the surface of this heart. In another group, we transferred the PE directly into the ear pinna.

To evaluate the incorporation of eGFP cells derived from the PE, and to investigate their possible differentiation, we performed immunofluorescence for eGFP in combination with other markers: Cardiac Troponin-1 (cTn-1), Smooth Muscle alpha Actin (SMαA), PECAM/CD31 and endothelial Nitric Oxide Synthase (eNOS/NOSIII).

In the group that received the heart and the PE transplantations, double immunofluorescence against eGFP and cTn-1 showed extensive incorporation of eGFP+ cells in the periphery of the grafted heart. Double labeling with anti-SMαA revealed that eGFP+ cells are found internal to the medial smooth muscle layer and into the lumen, suggesting the incorporation of PE cells in the endothelial layer and in blood island hemangioblasts.

The endothelial contribution of PE cells was confirmed in the two groups by double staining with eGFP and PECAM, which is an early marker of endothelial and blood differentiation. Double staining with eGFP and eNOS, a terminal endothelial marker, was present only in the group that received heart transplantation with PE. Double eGFP/PECAM immunofluorescence revealed expression in intraluminal cells, consistent with the blood differentiation that is observed in coronary vessels precursors before overt endothelial differentiation.

Therefore, PE cells, can differentiate and likely participate in the process of neovascularization when transplanted to adult sites. These findings demonstrate that the vasculogenic potential of the PE cells is conserved in an adult site and our model is adequate to study the mechanisms involved in the development and regeneration of vasculature.

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INACTIVATION OF THREE SUBFAMILIES OF TNT1, A TOBACCO’S RETROTRANSPOSON

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Transposable elements (TEs) are genetic units which can move within the host genome. They are present in large numbers within the genome of many multicellular organisms, and can constitute up to 70% of the genome in some plants. Tnt1 is a retrotransposon superfamily found in high copy number in the genome of tobacco and related Nicotiana spp. It is composed of three subfamilies which differ in their regulatory sequences, the “U3” region. The main goal of this work is to evaluate the biological role of Tnt1 in two Nicotiana species: N. tabacum and N. benthamiana. Genetic constructs designed to inactivate the retroelement through RNA interference were inserted in the genome of two species of Nicotiana by Agrobacterium transformation. The phenotypes of the regenerants, as well as in the filial plants, were compared to the wild type. Two generations of transgenic plants showed leaves with dead cell spots, reduced (or even absent) roots and seeds that lost viability more quickly than those of the wild type. Currently, more specific constructs are being designed in order to inactivate each Tnt1 subfamily individually based on the U3 region. It is known that each Tnt1 subfamily is preferentially expressed in different plant tissues and it is therefore hoped that inactivation of each Tnt1 subfamily will identify specific regulatory networks in the N. tabacum and N. benthamiana genomes. In parallel, histological root sections are being made in order to find possible differences in cell division between the transgenics and the wild type. Preliminary results showing that silencing generates aberrant plant phenotypes indicates that Tnt1 retrotransposons may play an important role of the development of the two Nicotiana species.

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Dapper1: A Partner of the Wnt Signaling Pathway Generates Two Isoforms During Vertebrates Development

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Most of the developmental processes depend on the action of a few main families of paracrine factors, such as the Wnt proteins, that coordinate the organogenesis of metazoan embryos and will lead to the formation of complex organisms. The study of the molecules that interact with these paracrine factors is of extreme importance in order to understand their means of action. Dapper1 (Dpr1) is an adaptor protein that modulates the Wnt signaling during cephalic morphogenesis and mesoderm specification, through the interaction with Disheveled, a key protein of the Wnt pathway. Considering that adaptor proteins often use the alternative splicing machinery to increase proteomic variability, in the present study we used bioinformatic tools to search for Dpr1 isoforms in the vertebrate genome using the UCSC genome browser and databases. Initially, we aligned 127 mRNA and EST sequences of the mouse Dpr1 transcript with the provided RefSeq. The analyses showed that 10 of the sequences present an additional 111 bp residue, resulting in two putative isoforms, one longer and one shorter, which were named α and β isoforms, respectively. The inclusion of this segment to the Dpr1 mRNA adds 37 aa to its predicted protein sequence without disrupting the open reading frame, and is located in a non-conserved region of exon 4, known to be necessary for the interaction with the transcriptional factor Tcf3. In the next step we analyzed the genome of the other vertebrates and noted the presence of the two putative acceptor splice sites in the exon 4 of human, chicken and Xenopus Dpr1. In zebrafish, however, only a single acceptor splice site was identified. The existence of these two isoforms were confirmed by RT-PCR assays, suggesting that the balance between them may play an important role in the development of these organisms.

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GENE ACTIVITY IN SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA, SCiaridae): THE SECOND GROUP OF AMPLIFIED GENES

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In the salivary gland polytene chromosomes of Diptera larvae, the RNA puffs are sites of intense transcription. Besides those puffs, the Scaridae also exhibit the DNA puffs, which are sites where gene amplification and transcription, developmentally controlled, occur. The hormone 20-0H ecdysone (20E) is involved in these controls. In B.hygida, two groups of amplified genes are present in two distinct groups of DNA puffs. The transcriptional activity of the first group demands high levels of 20E, while the second group needs low levels. Some genes of the first group are already cloned, but any of the second group. The DNA puffs A14, B3d, C6 and X4 (second group) start to expand and remain transcriptionally active in larvae from the age E7+12 hours to E7+24h, near to the pupal molt. Here, we are trying to produce and clone cDNA fragments of some genes of the second group. Poly(+)+RNA was isolated from salivary glands of larvae at age E7+20h, when the second group of DNA puffs are active. The double strand cDNA was fractionated by electrophoresis in 1% agarose gel. The fractions were treated with Taq DNA polymerase and cloned in the vector pGEM-T Easy. PCRs, using the vector primers, were run with some colonies. From one of the samples, a small fragment was produced. This fragment was separated by electrophoresis, Southern blot was prepared. The fragment hybridized with a BhSGAMP-1 probe labeled with AlkPhos (GE). As the new fragment is longer than the original, it is possible that an isoform was cloned. BhSGAMP-1 encodes an antimicrobial peptide, which is expressed exclusively in the salivary glands and is developmentally regulated. We are waiting for the results of the fragment sequencing.

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TRIFLUOPERAZINE AND CHLORPROMAZINE, TWO PHENOTHIAZINES, REVERT MULTIDRUG RESISTANCE PHENOTYPE IN EMBRYONIC CELLS OF THE SEA URCHIN - ECHINOMETRA LUCENTER – (LINNAEUS 1758)

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Phenothiazines are antipsychotic drugs used in the treatment of psychosis. These drugs exhibit a lot of pharmacological properties such as antagonism of dopamine D2 receptors, inhibition of calmodulin-dependent events and modulations of ABC superfamily proteins. ABC proteins are a large family of membrane proteins involved in the phenomenon of multidrug resistance (MDR). Previous works have shown a relevant activity of ABC proteins in embryonic cells of sea urchins. This study aimed to analyze the effect of two phenothiazines, trifluoperazine (TFP) and chlorpromazine (CPZ), in the resistance of embryonic development of the sea urchins Echinometra lucunter to the antimitotic drug vinblastine (VBL). Animals were collected at Cabo Branco beach (João Pessoa, PB - Brazil) and maintained in filtered sea water under constant air flow. Gametes were collected by intracoelomic injection of KCl (0.5 M). Embryos (1 x 104 embryos/mL) were cultured in 24-well plates at 26 °C ± 2 °C. After fertilization phenothiazines were added in concentrations ranging from 5 to 10 µM. Ten minutes later, VBL, a well-known ABC proteins substrate, was added at different concentrations (50 to 300 nM). At different time intervals, samples were collected and fixed in 4% paraformaldehyde. TFP and CPZ increased the sensitivity of embryos to VBL cytotoxic effect. MDR modulation was obtained with concentrations that did not block the early embryonic development. Furthermore, TFP was two times more efficient than CPZ. These results were similar to TFP and CPZ effects observed in MDR human tumor cell lines by other authors. Our work shows that TFP and CPZ were able of reverting MDR phenotype in the embryonic cell of E. lucunter, underlining the importance of ABC proteins in the maintenance of a biochemical barrier in sea urchin embryos.

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SILENCING OF TNT1 RETROTRANSPOSON RESULTS IN THE MODIFICATION OF THE EXPRESSION OF A WRKY53-LIKE GENE IN TOBACCO PLANTS

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Tnt1 retrotransposons are present in solanaceae genomes in hundreds of copies. It becomes active when the plant is under specific stress conditions, such as treatment with methyl jasmonate or onozuka cellulase, and can be mutagenic. High copy number and activation under specific conditions suggests that Tnt1 may potentially be a key-element in the formation of gene regulatory networks. Its activation may impact the expression of cellular genes associated with host genome responses to specific stimulus. The silencing of Tnt1 through RNA interference resulted in tobacco plants with necrotic wound spots on leaves and delay in the development of roots. Based on the hypothesis that Tnt1 may be involved in pathways related to defense or stress response, as well as developmental processes, we analyzed the expression of some genes involved in these processes, comparing tobacco wild type, Tnt1 antisense and Tnt1 silenced plants. Here we report the differential expression of a WRKY53 transcription factor-like gene in Tnt1 antisense and silenced plants. These results suggest a biological role for Tnt1, putatively involved in a network that includes the regulation of WRKY53. In Arabidopsis thaliana the WRKY53 transcription factor plays a central regulatory role in an early stage of leaf senescense, the final step of leaf development. Further large scale expression assays are necessary to identify other genes correlated with Tnt1 expression, in order to elucidate the mechanisms through this retrotransposon may impact the tobacco genome and developmental processes.

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CRUSTACEAN EMBRYOS AS MODELS FOR RESPONSES TO ULTRAVIOLET-B RAYS

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Exposure to ultraviolet-B radiation (UV-B) leads to increased cell damages in embryos that develop in shallow aquatic systems. Thus, we investigated responses of embryonic cells to UV-B rays using freshwater prawn embryos as models. Embryos at germinal disk stage (E3) were irradiated for 30min with 6W UVB lamp (dose 310 mW.cm-2). After, embryos were kept in the dark for 4 days, when they were analyzed at the initial morphogenesis stage (E7), which corresponds to embryos that still did not develop the eyes. Non-irradiated embryos at E7 were used as controls. The responses studied were: mitosis activity, apoptosis occurrence and the synthesis of the heat shock protein (hsp70), which may be produced as response to different environmental stressors. Immunohistochemistry with anti-phospho histone H3 revealed that the mitotic index (MI) shown by UV-B irradiated embryos (MI = 0.04, P < 0.0001) differed significantly from the controls (MI = 0.17). In addition, we observed that the embryonic structures visibly affected were the optic lobes and the naupliar appendages, i.e., the anterior part of the embryo. The TUNEL method showed that UV-B irradiated embryos presented an increased number of apoptotic cells also in the optic lobes and naupliar appendages. Immunohistochemistry using antibody anti-active caspase 3 demonstrated higher expression of this protein, specifically which be in the prospective nervous system cells at the anterior region of some UV-B irradiated embryos. Complementary immunohistochemical reactions revealed that UV-B irradiated embryos which did not express anti-caspase 3 showed an intense reaction with inducible anti-hsp70 in the prospective nervous system, which were identified with the Bp102 anti-axons antibody. Our results suggested that the UV-B rays changed the mitotic activity and induced apoptosis in the embryonic cells. Additionally, the UV-B effects were more evident in the nervous system cells, probably due to the required early differentiation of this system during the embryonic development.

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CHICKEN SCRATCH2 IS EXPRESSED DURING EMBRYONIC NEUROGENESIS

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In invertebrates, the Scratch (Scrt) genes encode transcription factors that promote neurogenesis during development. The Scrt function in vertebrates is unknown, but in mice Scrt1 and Scrt2 are specifically expressed in post-mitotic neurons in the embryo and in the adult central nervous system. In this work, we have cloned the coding sequence of chicken Scrt2 (cScrt2) and characterized its expression pattern in the embryo with quantitative PCR and in situ hybridization.

The complete coding sequence was cloned in the expression vector pMES-GFP and the predicted translation product is a 276-aminoacids protein. The aminoacid sequence shares identities of 70% with rat Scrt2 and 58% with zebrafish Sert. cScrt2 transcripts are firstly detected in the periphery of the neural tube in the hindbrain by HH 15 and in the spinal cord by HH 17, coinciding with the places where some of the first neurons differentiate during embryogenesis. Between HH 19-23, the expression in the motor domain of the spinal cord is progressively concentrated in the interface between the ventricular and mantle zones. Furthermore, cScrt2 expression is also observed in the dorsal root ganglia after HH22-23, particularly in the dorsomedial domain. The expression pattern of cScrt2 in the neural tube is complementary to that of Notch1, which is expressed in neural stem cells, and SCG10, a marker for differentiated neurons. Our results suggest that during embryogenesis cScrt2 is specifically expressed in post-mitotic undifferentiated neurons.

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VITELLOGENIN KNOCKDOWN ENHANCES CASTE-SPECIFIC TRANSCRIPTIONAL DIFFERENCES IN HONEYBEE (APIS MELLIFERA) FAT BODIES

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Honeybees (Apis mellifera) exhibit remarkable caste-specific morphophysiological characteristics. Workers are short-lived, functionally sterile, performing all non-reproductive tasks in the colony. Queens are engaged in laying eggs and are long-lived. This dimorphism represents the phenotypic manifestation of the differential gene expression and developmental programs originated from the same genome. Thus, the proteome status appears to be a major determinant of honeybee reproduction and longevity, and the vitellogenin gene seems to play a crucial role. We used microarray approach to compare global gene expression differences between queen and worker abdominal fat bodies, where Vitellogenin is preferentially synthesized. The same comparison was conducted using orally-treated females with 500ng of double-stranded RNA for vitellogenin (dsVg) mixed with larval food during larval phase. Larvae were maintained in the colony until adult emergence. Queens were collected at emergence. Newly-emerged workers were maintained in an incubator at 34°C and a relative humidity of 80%, and were provided with water, pollen and sucrose syrup for seven days. Total RNA from abdominal fat bodies was extracted and used to synthesize aRNA probes for microarray slides hybridization. Results from ‘RNAi-treated’ group were subtracted from ‘untreated’ one. Changes in gene expression greater than 5 fold-change are reported. Sixteen genes were found to be overexpressed in dsVg-workers. From them, the main finding was the over-expression of two senescence markers, the α1- and α2-glucosidase genes. These data confirm our previous observation that vitellogenin disruption anticipates a specific age status, the foraging behavior. In queens, 27 genes were up-regulated, including three from odorant binding gene family, one methyltransferase, and the vitellogenin gene itself. In fact, we previously observed that vitellogenin silencing is less effective in dsVg-queens (~70% disruption) than in dsVg-workers (~90% disruption). A noteworthy observation was the reactivation of hexamerin 70c expression in dsVg-queen fat bodies. Usually, the expression of this storage protein gene ceases during larval phase. Taken together, our data show that the effects of the vitellogenin knockdown are more pronounced in dsVg-queens, thus contributing to the understanding of the molecular differences that distinguish honeybee castes.

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DESCRIBING A MECHANISM FOR THE INTERPRETATION OF THE POSITIONAL INFORMATION ESTABLISHED BY THE MORPHOGENETIC PROTEIN BICOID IN DROSOPHILA DEVELOPMENT

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During Drosophila embryonic development the concentration gradients of maternal factors, like the Bicoid protein, establish positional information along the embryo. A cascade of developmental genes reads out this positional information by exhibiting different activation levels according to their position along these gradients. One of the first genes of this cascade, the gap gene hunchback (hb) has a strong anterior expression and a sharp on-off boundary in the mid-embryo. It has been shown that Bcd binds to hb promoter cooperatively and that hb activates its own regulation. The role of Bcd cooperative binding for Hb pattern positioning has been demonstrated already, but the mechanism that allows the shallow Bicoid gradient to regulate the sharp Hb border remains an unsolved problem. We used a predictive kinetic model for hb transcriptional regulation, describing ligand binding/unbinding; combined with a series of experimental data altering Bcd cooperative binding and hb self-regulation ability [1]. We found that bistability stemming from hb self-regulation produces the sharp Hb border; the loss of sharpness for the hb14F self-regulating mutant and the shallow pattern of a Bcd-dependent lacZ artificial construct support this result. In addition to that, our results indicate that Bcd cooperative binding determines the position of Hb pattern by determining the position where bistability occurs; our Bcd cooperative mutant data supports this conclusion. The ability to produce sharp borders is a central step for the expression of developmental genes in Drosophila and other organisms, and we show that spatial bistability can play a central role in this process.

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BHSGAMP-1, A REITERATED GENE DEVELOPMENTALLY REGULATED IN THE SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA, SCIARIDAE), ENCODES AN ANTIMICROBIAL PePTIDE AND IS UNDER THE DIRECT CONTROL OF 20-OH ECDSYONE

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Animals and plants present as a first barrier, to resist infection by microorganisms, a sophisticated system of innate defenses. In insects, these systems include the production of large amounts of antimicrobial peptides (AMPs). Plants have also developed a wide variety of defense systems for protection against pathogens.

Recently we have shown that BhSGAMP-1 is a developmentally-regulated reiterated gene that encodes an antimicrobial peptide and is expressed exclusively in the salivary glands, at the end of the larval stage.

We are committed to characterize gene BhSGAMP-1 (for Bradysia hygida salivary gland antimicrobial peptide) and to investigate and demonstrate the role of 20-OH ecdysone (20E) in controlling BhSGAMP-1 activity.

In this study, we show, for the first time, that a gene for an antimicrobial peptide is directly activated by 20E. This control probably involves the participation of short-lived repressor(s). We also found that the promoter of BhSGAMP-1 is not equipped with elements that respond to infection, provoked by the injection of microorganisms, in the salivary glands or in the fat body. We produced polyclonal antibodies against the synthetic peptide and found that the BhSGAMP-1 peptide is secreted in the saliva. The BhSGAMP-1 gene was also activated during the third larval molt. These facts confirm our hypothesis that this preventive system of defense was selected to produce an environment free of harmful microorganisms in the insect’s immediate vicinity, during molts.

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There is a sequence of events on ventral prostate (VP) development involving epithelial outgrowth, branching and canalization in parallel with epithelial cell differentiation and apoptosis (Anat Rec. 290:1223, 2007). During this process some enzymes acts on the extracellular matrix (ECM) remodeling. Matrix metalloproteinase-2 and -9 (MMP-2 and 9) are important in those events, either by degrading ECM or releasing cryptic components such as bioactive peptides and growth factors (Histochem Cell Biol 129:805, 2008). Heparanase (Hpse) is an endoglycosidase that degrades heparan sulfate, an important component of the basal membrane, which acts as a reservoir of growth factors regulating different aspects of cell physiology. We have hypothesized that heparanase-1 activity would have a relevant role in coordinating the dynamics of the epithelium and stromal remodeling during the early postnatal development of the rat VP. We have used In Situ Hybridization (ISH) at days 0 (date of birth), 3 and 6, and determined that heparanase-1 mRNA is concentrated in the epithelium, with no difference between proximal and distal part of the prostate ducts. The localization of the enzyme has been also demonstrated by immunohistochemistry The enzyme is located at the surface of the epithelial cell and also adjacent to it including the basal membrane. Within these results we have knocked-down Hpse in organ culture, with siRNA, to analyze the effects of this gene silencing on total area and epithelium area of the VP. These findings show that this enzyme might have an important role during VP development, especially in those events related to epithelial growth and differentiation.

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THE TRANSPONSON INSERTION IN DROSOPHILA LINE KG00562 INCREASES LETHALITY AND INTERFERES IN GENE EXPRESSION

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The transposon insertion site in Drosophila line KG00562 was mapped between loci CG8709 and kermit. Although studies of these evolutionarily conserved genes in different organisms indicate important roles in the control of lipid metabolism (CG879) and development (kermit), virtually no information exist on their functions in Drosophila. The CG8709, herein denominated DmLpin, encodes different isoforms. Previous studies have shown that a probe derived from the untranslated first exon of B isoform of DmLpin did not detected DmLpinB but rather a smaller 2.3Kb mRNA in the KG00562 mutant. It was also shown that the KG00562 mutant line presented high levels of lethality that were increased over the wild type background indicating a gain of function. Here we investigated the molecular bases of the mutation associated with the high lethality of the KG00562 line by sequencing the 2.3Kb mRNA and analysing the expression levels of DmLpinB and kermit in different genetic backgrounds. The sequence analyses, together with data on the expression profile of this transcript, have shown that the 2.3 kb mRNA is the result of an aberrant splicing between the first exon of DmLpinB and the second exon of the mini-white gene present in the KG construct. The expression of the chimeric mRNA seems not be deleterious since rescue of the normal lethality levels was observed in animals carrying KG00562 over a deficiency that covers the insertion region (Def7860). This result indicates instead that KG00562 mortality could be related with alterations in genes located in the vicinity of the insertion site. In agreement with this hypothesis qPCR analysis confirmed the absence of DmLpinB transcripts in all tissues analyzed and indicated overexpression of kermit in the intestinal tract (283%) and in Malpighian tubules (325%) of L3 larvae of line KG00562. The lack of DmLpinB expression does not contribute to the lethality of the KG00562 line since the animals carrying KG00562 over Def7860 present indices of mortality similar to the wild type. On the other hand, overexpression of kermit seems to be associated with the lethality of KG00562. Significantly higher levels of this gene transcript were detected in Malpighian tubules of animals carrying KG00562 over wild type background that present high mortality indices, but are reduced to levels similar to the wild type in the KG00562 over Def7860, which presents the mortality levels rescued to the normal levels.

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Retinoic acid (RA) signaling is crucial for correct embryonic development and Raldh2 is the major enzyme involved in RA synthesis during early development. Using bioinformatics, mice transgenesis, chicken electroporation and Xenopus injection we detected an intronic enhancer that activates raldh2 expression in spinal cord’s roof plate and dorsal interneuron. Via site-directed mutagenesis we demonstrated that enhancer activities are established through a combination of dorsal stimulation via predicted double Tcf/vertebrate caudal-type, Cdx, homeobox binding sites, plus inhibition via predicted TGIF homeobox and ventral Lim-homeodomain repressor sites. Comparative studies this enhancer prompted description of a novel RA signaling domain in the dorsal spinal cord (SC). Double in situ hybridization with raldh2 and math-1, or cath-1, indicates the existence of a transient and novel endogenous domain of raldh2 expression in dI1 interneurons, which give rise to ascending circuits and intraspinal commissural interneurons. This suggests roles for RA signaling in the ontogeny of spinocerebellar and intraspinal proprioceptive circuits. To establish the phylogenetic representation of this novel RA signaling domain in vertebrates we performed raldh2 in situ hybridization in agnathans and teleosts. We found that raldh2 is expressed in dorsal interneurons throughout the agnathan SC, consistent with the transient expression of the gene in dorsal interneurons of tetrapods. This suggests an ancestral role of RA signaling in the ontogenesis of vertebrate intraspinal proprioception. Conversely, lack of raldh2 expression in the SC roof plate of agnathans, which do not posses paired fins, and of raldh2 expression throughout the SC in teleosts, which underwent reduction of their paired fins, suggests that roof plate RA signaling went missing with secondary simplifications, or loss of vertebrate paired fins/limbs.

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CALCIUM INFLUX IS REQUIRED FOR EARLY EMBRYONIC DEVELOPMENT OF THE SEA URCHIN ECHINOMETRA LUCUNTER

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A transitory rise in cytosolic Ca2+ concentration in sea urchin eggs begins at sperm-egg fusion site and travels through the egg as a wave. Elevation of intracellular Ca2+ concentration is a vital signal for resumption of the cell cycle. It was shown that in most species studied, calcium influx is not essential for the initiation of Ca2+ wave and, therefore, it is not critical for embryonic cell division. In the present work, we evaluated the need of extracellular calcium in early embryonic development of the sea urchin Echinometra lucunter. Animals were collected at Cabo Branco beach, João Pessoa, northeast of Brazil. Eggs and spermatozoa were extracted with intracoelomic injection of KCl (0.5 M). Eggs were washed two times before fertilization to remove the jelly coat. Fertilization was induced by the addition of activated sperm suspension to eggs suspension. Two calcium chelators, EDTA and EGTA, were added to 1 x 10⁴ embryos/mL five minutes after fertilization at different concentrations. The progression to the first and second cleavage was monitored under optical microscopy and 100 embryos were analyzed to each treatment. Both chelators inhibited development at all stages monitored. However, EGTA was more effective than EDTA in blocking the development. EGTA is a specific Ca2+ ion chelator, while EDTA is chelator for divalent cations. Considering that these chelators were able to block the early embryonic development, our data suggest that calcium influx is required for early embryogenesis of E. lucunter.

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EFFECT OF THE CALCIUM IONOPHORE IONOMYCIN IN THE SEA URCHIN ECHINOMETRA LUCUNTER EGGS ACTIVATION

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An increase in cytosolic calcium ion concentration at fertilization plays a key role in egg’s activation. It was shown that in the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus fertilization membrane elevation was induced by treatment with calcium ionophore A23187. This fertilization-like change did not require calcium in the

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surrounding sea water, demonstrating that ionophore was releasing calcium from internal stores. In the present work we investigated the effect of the calcium ionophore ionomycin in *Echinometra lucunter* eggs activation in the presence or absence of external calcium ion. Sea urchins were collected at Cabo Branco beach, João Pessoa, northeast of Brazil. Eggs were collected with intracoelomic injection of 0.5 M KCl. Eggs were washed twice – ASW (with calcium) or ASW Ca²⁺ free (without calcium) - before activation to remove the jelly coat. Ionomycin was added to 1 x 10⁴ embryos/mL at different concentrations. Eggs were monitored in optical microscopy at 1 and 5 minutes after ionophore addition. Ionomycin induced fertilization membrane elevation in ASW and ASW Ca²⁺ free. The elevation of fertilization membrane in ASW was evident since from 1 µM. Five times more ionomycin was needed to induce the same response when eggs were cultured in ASW Ca²⁺ free. These data show that ionomycin was able to induce calcium ions mobilization from internal stores and from the surrounding sea water. Our data also suggest that the calcium concentration present in intracellular stores is sufficient to promote egg activation in *Echinometra lucunter*.

**INDUCING OF A “ZONE OF POLARIZING ACTIVITY (ZPA)-LIKE” IN THE FIRST BRANCHIAL ARCH BY SONIC HEDGEHOG**

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Recently we have shown in chick embryos that Sonic hedgehog (Shh) derived from ventral foregut endoderm plays a critical role in the development of the first branchial arch (BA1) derivatives by two distinct ways: first by ensuring the survival of mesenchymal cells derived from the neural crest (NC), second, by patternning the oral ectoderm.

To further explore the role of Shh on BA1 development, we performed grafts of quail fibroblasts transfected with a *Shh* construct (QT6-Shh cells) into the presumptive BA1 territory in 5-6 somites stages (ss) chick embryos. At E3-4 the experimental side was hyperplastic and showed an expanded domain of *Fgf8* and *Bmp4* expression in BA1 ectoderm and an ectopic expression of Shh. This effect was also observed in the mesenchyme in which the transcription of *dHand*, *Dlx5*, *Sox9* and *Pitx1* was increased. Later on, at E11 supernumerary lower jaws developed in the side where QT6-Shh cells were grafted with a mirror image polarity. Moreover, the growth of supernumerary skeletal elements derived from BA1 (eg. Meckel’s cartilage) was preceded by the ectopic expression of *Bmp4* in the distal part of BA1 ectoderm.

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Altogether our results confirm that Shh is important in patterning of BA1 ectomesenchyme. We have evidence that the effect of Bmp4 on NC cells consists in insuring their survival. On the other hand Shh seems responsible for the induction of Bmp4 expression in BA1 ectoderm. Bmp4 itself controls the growth of supernumerary skeletal pieces.

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IDENTIFICATION OF RING GLAND TRANSCRIPTIONAL ACTIVATORS

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The BhC4-1 gene is expressed in Bradysia hygida salivary glands at the end of fourth larval instar. The mechanisms that control the BhC4-1 salivary gland expression are conserved in transgenic Drosophila. The functional characterization of the DNA puff BhC4-1 promoter revealed that the proximal promoter contains both a 129 bp (-186/-58) salivary gland enhancer and a 67 bp (-253/-187) ring gland enhancer that drive tissue specific patterns of developmentally regulated gene expression. In transgenic Drosophila, BhC4-1-lacZ expression in the ring gland is initially detected in late embryos, continues during the larval stage and prepupal stage, and is no longer detected in 24h pupae. The BhC4-1 ring gland enhancer is, to our knowledge, the only identified enhancer that is able to drive developmentally regulated gene expression exclusively in the ring gland of late embryos. The ring gland is the main larval endocrine organ and the mechanisms that control gene expression in this organ are largely unknown. In order to characterize transcriptional activators that act in the ring gland, we have employed the yeast one-hybrid system to identify proteins that are able to bind the 67pb BhC4-1 ring gland enhancer. The initial screen was performed in the presence of 40mM 3-AT, and 257 positive clones were identified. In order to eliminate false-positive clones we used the lacZ gene as a second reporter gene which reduced the number of initial 257 positive clones to 190 clones. Ninety two percent of the positive clones that are able to activate both reporter genes in the yeast one-hybrid system were sequenced. Preliminary results reveal that 112 clones constitute unique sequences associated with annotated genes (CGs) of Drosophila melanogaster. The 112 sequences were classified according to the category Biological Process of Gene Ontology as follows: cell cycle and DNA replication (7.2%), cell transport (7.2%), cytoskeleton organization (10%), extracellular matrix and morphogenesis (4.5%), metabolic process (14%), other functions (4.5%), signal transduction (4.5%), transcription (8%), translation (24.1%) and unknown functions (16%). The functional validation of the sequences identified as transcriptional factors is currently being performed.

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EFFECTS OF FOLIC ACID AND HOMOCYSTEINE ON CELL PROLIFERATION, APOPTOSIS AND NCAM EXPRESSION IN THE SPINAL CORD OF THE CHICKEN EMBRYO

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Neural tube defects (NTD) comprise disorders of primary neurulation and are among the most distressing congenital anomalies. These anomalies originate from failure of neural tube closure or from abnormalities of the axial mesenchyme development. Maternal ingestion of folic acid (FA) reduces NTD, which are associated with high homocysteine (Hcy) levels. This study evaluated the effects of FA and Hcy on cell proliferation, apoptosis and cell adhesion during development of the spinal cord and mesenchyme. Fertile eggs of Gallus domesticus were incubated (38°C) and after 30 h, embryos were divided into 3 groups (n = 10 per group) and submitted to treatments by the injection into the yolk sac: group I - 25 µl saline with 0.5 µg FA; group II - 25 µl saline with 10 µmol D,L-Hcy; group III - 25 µl saline with 0.5 µg FA and 10 µmol D,L-Hcy. Control embryos were injected with 25 µl saline. After 96 h of incubation, embryos were anesthetized at 4°C, removed from the egg membranes and fixed in formaldehyde. TUNEL staining was used to identify apoptotic cells and immunohistochemistry was performed to identify the proliferating cells (anti-phospho histone H3) and NCAM (anti-NCAM) expression. Stereological analysis was performed using the M-42 test system (Weibel Nº 2) to evaluate whether the treatments induced apoptosis or affected cell proliferation. FA-treated embryos showed the highest numerical density of apoptotic cells in spinal cord (68.76 ± 9.43 mm2). FA reversed the decrease of proliferating cells in the spinal cord induced by Hcy (45.84 ± 4.71 mm2). Hcy treatment reduced NCAM expression and FA prevents this effect. The results demonstrate in situ that the imbalance between FA and Hcy levels can lead to disruptions in spinal cord and mesenchyme development, changing proliferation, apoptosis, and cell adhesion and consequently changing the arrangement of the spinal cord layers.

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LIMBLESNESS AND HOXA-13 EXPRESSION IN PYTHON MOLURUS

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The origin of Snake clade in squamates coincided with some morphological changes, such as limb loss, body elongation and the appearance of the cloacal scent glands. The evolution of this body form in Squamates, was likely associated to modifications in parameters related to embryonic development, for example in the expression pattern of homeotic genes. In limbless organisms, the homeotic genes involved in limb development might be maintained in the genome due to pleiotropic effects on other traits that are of selective value to this organisms. One example would be Hoxa-13, a transcription factor involved in autopodium development. In snakes, a possible scenario would be that, after limbs were lost, the selective pressures were relaxed on specific sites of the sequence of Hoxa-13 associated to limb development. However, a recent study (Kohlsdorf et al, 2009) has detected a consistent signal in Hoxa-13 sequences associated with origin and loss of limbs in several species of Gnathostomata. This pattern of molecular evolution suggests that, even if selective pressures were relaxed immediately after limbs were lost in snakes, positive and directional selection began to influence the evolution of this gene at some point of the evolutionary history of the clade. Moreover, such evidence of directional selection in Hoxa-13 might suggest that the appearance of evolutionary innovations became possible after a specific selective pressure (related to autopodium development) was relaxed when limbs were lost. In this context, the expression of Hoxa-13 in snakes could be associated to the appearance of a new trait, for example, the cloacal scent glands. This study aims to analyze the Hoxa-13 expression in different developmental stages of Python molurus embryos, with a subsequently comparison to expression patterns in the limbed species Gallus gallus and Iguana iguana. Hoxa-13 expression is observed in specific regions of the embryo (pectoral girdle, anterior and posterior limbs in Gallus gallus e Iguana iguana case, post cloacal region, and restant region of tail) using specific primers in a Reverse transcriptase polymerase chain reaction (RT-PCR). This study represents a landmark in Evo-Devo for combining information from bioinformatics with morphological and molecular data in a comparative setup.

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Honeybee female larva has the capacity to develop into two different phenotypes: queens or workers. Differential nutrition triggers the development of caste-specific characteristics, such as body size and mushroom bodies/brain volume which are greater in workers than in queens. In adults, mushroom bodies are related to memory and learning, and are connected to tasks outside the colony led by workers. They are present in both sides of the protocerebrum and are composed of two pedunculi with a calyx in each one. These pedunculi and calyces are formed by dendrites and axons of Kenyon cells. Despite our understanding of adult brain structures, little is known about its development during early larval instars. To investigate whether differences could be detected in brain development in the context of caste differentiation, we performed high-resolution neuroanatomical studies of whole mount preparations of queens and workers larval brains. We used actin phalloidin/rhodamin to visualize axons and DAPI to visualize nuclei. In third instar larvae the most pronounced features are axons from optic tubercles, antennal lobes and inter tubercle tract, and also clusters of neuroblasts nuclei in each side of the brain. No morphological differences between queens and workers were detected at this stage. In fourth instar, phalloidin/rhodamin staining revealed the appearance of the pedunculus in the queen’s brain whereas it is still absent in workers. In the fifth instar, pedunculus appears in worker brain and the formation of calyces of neuropils started in the queen’s brain. Our results revealed that during larval development a delay is present in the formation of worker brain structures compared to queen. These caste-specific neurogenic events result from differential nutrition offered to larvae during early stages.

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TRANSCRIPTIONAL SILENCING OF JAZIGO USING IN VIVO RNA INTERFERENCE

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During metamorphosis of Drosophila two pulses of the steroid hormone 20-hydroxyecdysone regulate stage and tissue-specific responses such as cell death and differentiation. While the first pulse triggers the larval midgut cell death and differentiation of CNS the second pulse is responsible for triggering larval salivary gland autophagic cell death. Trying to address how a systemic signal is responsible for the different cell responses during metamorphosis, we have assumed that several uncovered genes would participate in modulating such distinct responses. In order to answer that we performed a “forward genetics” screen looking for mutations affecting the salivary gland cell death process. Screening the collection of 1475 enhancer trap lines (P-element insertions) we found that one of these insertions (mutation) drastically affects the programmed cell death (PCD) of larval salivary glands during metamorphosis. The gene flanking the P-element insertion (affected gene) was named jazigo (jaz) and encodes a protein which shares high homology to FH proteins (“formin homology”) usually called formins. Our transcriptional analysis revealed that jaz is expressed in response to the second pulse of ecdysone in a similar fashion to other cell death genes. Formins had been related to the actin and tubulin cytoskeleton remodeling in different biological processes. Based on the phenotype, transcriptional pattern and jaz regulation by ecdysone we are interested in understanding how such protein functions in the cell death of larval salivary glands. To address that we have generated modular transgenic RNAi fly lines. The induction of transgene expression resulted in increased embryonic and larval lethality when compared with control animals. Furthermore, the dsRNAi expression during metamorphosis using controlled heat-shock produced salivary glands that failed to die (persistent salivary glands). The cytoskeleton analysis of these persistent salivary glands showed a distinct aspect of disassembling of the actin cytoskeleton when compared to other jazigo mutant alleles and dying wild-type salivary glands.

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DPR1 AND DPR2 HAVE A DYNAMIC EXPRESSION PATERN DURING LIMB DEVELOPMENT IN CHICKEN (GALLUS GALLUS)

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The Dapper proteins (Dpr) are multifunctional molecules coded by a family of genes that are present only in the genome of vertebrates. These proteins are required for key processes of development, such as morphogenetic movements, mesoderm and neural tissue specification, heart and eye morphogenesis. In chicken, two Dpr genes (Dpr1 and Dpr2) were identified and their expression patterns were established during early development. The limb bud was one of the structures of the chicken embryo identified as having the expression of both Dpr genes. This fact suggested that at later stages of development these molecules could also be expressed in the limbs. To explore this hypothesis, we performed whole-mount in situ hybridization of chicken embryos from HH21 to HH32.

The expression of Dpr1 and Dpr2 was clearly evident in limb buds from HH21 onwards. Between HH21 and HH25 transcripts were associated with the limb mesenchyme however avoiding the central core that forms the cartilage/bone elements. A strong downregulation of Dpr1 expression was identified at later stages of development where Dpr1 mRNAs were restricted to a narrow region around the growing bones. In comparison to Dpr1, Dpr2 displayed a more dynamic expression pattern. At HH25, Dpr2 transcripts were specifically identified in the cartilage rudiments that form the limb bones. At later stages, Dpr2 was expressed at the joints, at the tips of the digits and in tendons. Overall, these results revealed that Dpr1 and Dpr2 are expressed in a dynamic fashion during limb development in chicken, indicating that the activity of these genes may be important for the ontogeny of these structures. Further analyses are being conducted to identify the specific tissues expressing the Dpr genes in the limbs environment.

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CONTRIBUTION OF THE PRECHORDAL PLATE MESODERM PRODUCING SHH TO THE ANTERIOR FOREGUT ENDODERM FORMATION

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The prechordal plate (PcP) is a transitory embryological structure formed by migrating cells derived from Hensen’s node and localized between the neuroectoderm and the endoderm. Later on, PcP cells will interact with the endoderm and may be called “mesoendoderm”. PcP is a organizing center for the forebrain development and is the first source of SHH in the most anterior region of the embryo. Using the chick embryo as a model, we have demonstrated that SHH produced by the anterior foregut endoderm is critical for lower jaw development. In the chick, Shh expression in the foregut endoderm can be detected at 4-5ss, in its most anterior part, in close contact with the prechordal plate (PcP). Furthermore, we showed that the forehead ablation including the anterior foregut endoderm and the PcP hampers Shh expression in the first branchial arch (BA1) endoderm and the lower jaw development.

Our previous works showed that, during encephalic neural crest cell migration, only the anterior ventral endoderm, which contacts PcP, expresses Shh. However, it’s not known whether the anterior endoderm is capable of secreting Shh or it depends on the PcP induction. In this study, we analyze the contribution of the PcP for the anterior foregut endoderm. We describe the kinetics of Shh expression on histological sections from HH4 to HH9 as a way of detecting molecular limits of the prechordal plate. We applied the Schiff procedure to see if the localized expression of Shh was due to the presence of a basal lamina, which characterize tissue distinctiveness. We also show scanning electron micrographs of the PcP in the search for morphological differences between the tissues composing it and the precise timing of the mesoendoderm formation. In this work, we did a descriptive approach to better understand the interaction between the PcP and the anterior foregut endoderm.

In the present work, we show preliminary results pointed that the PcP cells become integrated to the anterior endoderm at HH5. These data suggest that the PcP cells rather than the endoderm will be responsible for SHH production in the anterior ventral endoderm.

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A STUDY OF THE TRANSCRIPTIONAL REGULATION OF THE ROUGHEST GENE DURING THE DEVELOPMENT OF DROSOPHILA MELANOGASTER COMPOUND EYE AND THE MOLECULAR CHARACTERIZATION OF ROUGHEST DOMINANT (RST\textsuperscript{D}) REVERSIONS

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Previous phenotypic characterization of \(rst^D\), a dominant regulatory \(rst\) allele, showed that the correct expression of Rst protein is essential for pigment cell specification during the final stages of compound eye development. \(rst^D\) also shows a high reversion rate, either to rough or wild type–like eyes. Here we report additional molecular and transcriptional characterization of this mutant and its revertants using Southern blotting and quantitative real time PCR. Although Southern Blot analyses detected high molecular weight bands in \(rst^D\) and nearly all of its revertants that are absent from wild type genomic DNA, no significant difference between wild type and wild type-like revertant \(rst^{DRWT\#6}\) could be seen. \(rst^{DRWT\#6}\) sequence analysis showed only single nucleotide alterations, besides a small 10-12 nucleotide deletion inside the genomic region where \(rst^D\) regulatory mutation was previously mapped. Additionally \(rst\) transcriptional dynamics of retinas from wild type, \(rst^D\) and \(rst^{DRWT\#6}\) at 0, 25 and 35\% pupal development (pd) were examined by Real Time PCR. The preliminary data obtained so far indicates that \(rst\) mRNA levels in \(rst^D\) mutants are slight higher than wild type at the time of cell sorting (25\% pd) while in \(rst^{DRWT\#6}\) revertants it is at basal levels in all three stages. These results lend support to earlier data from our laboratory indicating that the timing of expression, rather than the absolute amount of Rst, is critical for its function in the pupal retina.

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STUDY OF PUTATIVE MALE FERTILITY GENES LOCATED ON THE Y CHROMOSOME OF D. MELANOGASTER USING DOUBLE-STRANDED RNA INTERFERENCE TECHNOLOGY (RNAI)

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In Drosophila, sex not is determined by the Y chromosome. However, the Y is necessary for male fertility since males lacking a Y chromosome are viable but completely sterile (BRIDGES 1916). The Drosophila Y chromosome was the first heterochromatic chromosome to be characterized genetically and cytologically. 6 chromosomal regions have been characterized, each harboring at least one gene responsible for male fertility. (BROSSEAU, 1959). Because of its heterochromatic character, the discovery of new genes has been slow. Carvalho et al, 2001 and 2008 described 3 genes expressed exclusively in males that may be responsible for male fertility. They are coined ORY (Ocluddin Related Y), CCY (Coiled-Coils Y) and WDY (WD-40 Y), that map to regions ks-1, ks-2 and kl-1 respectively. Classic studies of gene function involve the generation of loss-of-function alleles and the evaluation of mutant phenotypes. However, due to its heterochromatic nature, the generation of mutations in loci located in the Y is very complicated. For this reason, we have used the technique of interference by double-stranded (ds) RNA to knockdown the expression of the loci cited above. We have generated Drosophila strains bearing insertions of a pWIZ vector containing repeated and inverted sequences of the genes of interest under the control of a UAS promoter. For generation of the dsRNA, expression was directed by use of a GAL4 driver under the control of a testicular promotor (nos-GAL4VP16, expressed in the germline). Effects on male fertility were evaluated by counting the number of viable embryos generated from crosses between wild type females and nosGal4>UAS-(gene of interest) males, and compared to appropriate controls (wild-type and nosGAL4). Our initial experiments indicate that all 3 genes tested reduce the number of viable embryos and thus may be involved in male fertility. These essays are being repeated with the same and additional GAL4 drivers. The expression pattern of the genes tested will be presented by in situ hybridization as well as alterations in the levels of expression of ORY, CCY and WDY by Real Time-PCR.

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MATERNAL DPP/BMP PATHWAY REGULATES CALPAIN A ACTIVITY TO PATTERN THE EMBRYONIC DORSAL-VENTRAL AXIS IN DROSOPHILA MELANOGASTER

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Dorsoventral (DV) patterning in Drosophila melanogaster is regulated by the Toll pathway by modulating Cactus/IkB protein degradation, and thus nuclear Dorsal/NFkB protein levels in the early embryo. In addition to the Toll pathway, previous genetic studies have shown that the maternal Dpp/BMP pathway regulates nuclear Dorsal levels by controlling signal-independent degradation of Cactus. In this work, we show that the maternal Dpp pathway requires Calpain A, a calcium-activated cysteine protease, to target Cactus degradation. We find that Calpain A can regulate Dorsal levels by targeting Cactus protein. In addition, the Cactus PEST domain, which is needed for signal-independent degradation, is also required for Calpain A to exert an effect on Cactus. Using Real-time qPCR and biochemical approaches we show that the maternal Dpp pathway regulates the activity and/or mRNA stability/translation of Calpain A in early embryos. These results suggest that maternal Dpp functions through a non-canonical pathway, since the effects of Dpp blockage are observed before the onset of zygotic transcription. We also search to define the functional role of maternal Dpp on DV patterning. in situ hybridization and immunofluorescence analysis in embryos with disrupted Dpp pathway suggests that Dpp signals regulate discrete nuclear Dorsal levels. This raises the possibility that Dpp provides robustness to DV patterning.

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LIPIDOMIC ANALYSIS IN *XENOPUS LAEVIS* DEVELOPMENT

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The cell membrane has an important role in cell signaling, and there are specific regions that act as platforms for aggregating signaling molecules, the cholesterol-rich membrane microdomains (MM) or lipid rafts. Our group has recently shown that cholesterol depletion by Methyl-beta-cyclodextrin (MβCD) affects anterior development of *Xenopus* embryos, suggesting a correlation between MM and signaling pathway necessary for head formation. This work aimed to analyze lipid composition during the development of *Xenopus* embryos, and specifically how they appear in the MM.

Ventral and dorsal halves of *Xenopus* embryos at blastula and gastrula stages were analyzed by Thin Layer Chromatography (TLC). We observed a major lipid concentration at the ventral side, at the gastrula stage, but at blastula this difference was not so evident. In order to isolate MM fractions, embryos at four cells, blastula, gastrula and early neurula stages were lyzated and submitted to a sucrose density gradient. Cholesterol measurement analysis demonstrated that this fraction has a peak of this lipid at blastula and gastrula stages, suggesting that MM organization might occur in a specific time point. TLC revealed that MM are mostly composed of cholesterol esters, tryacylglycerols, phosphatidylethanolamines and phosphatidylcholines. Stage 9 *Xenopus* embryos (blastula) were microinjected or not with 40 mM MβCD and allowed to develop until gastrula stage, and the embryo extracts were then passed through a sucrose density gradient. Analysis of protein concentration and SDS-page revealed no drastic differences between MM fractions obtained from injected and uninjected control embryos. However, analysis of neutral lipids by TLC showed that MβCD microinjection increased the fatty-acids content, while decreased the triacylglycerol.

This work analyzed for the first time the lipid content during early *Xenopus* development. Our results suggest that there is a qualitative distribution of lipids along dorsal and ventral sides of the embryo. We also investigated the lipid content of the *Xenopus* MM during embryonic development and observed that cholesterol depletion affected neutral lipid composition. Since we observed a high content of phospholipids in MM, we also suggest that these structures may be involved in cell signaling during *Xenopus laevis* development.

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SUBCELLULAR LOCALIZATION OF DFOX-1 IN THE VISUAL SYSTEM OF DROSOPHILA MELANOGASTER DURING DEVELOPMENT

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A2BP1/FOX-1 is a member of a new family of RNA-binding proteins conserved in evolution. In Drosophila melanogaster the ortholog of FOX-1 is annotated as CG32062, which encodes five different isoforms generated from alternative splicing, according to in silico analysis based on a complete cDNA and EST data. We have examined by confocal microscopy the pattern of dFox-1 immunolocalization in the visual system of Drosophila during development. In the eye imaginal discs (ID) of third instar larvae, the label is mainly posterior to the morphogenetic furrow, which is a region of cell determination, proliferation and differentiation. In pupal retina the label follows a cell specific pattern. Label is restricted to the border of interommatidia cells of retina at 16% of pupal development (p.d.), and is no longer detected at 25% p.d. In the cone cells at 16-29 % p.d. the label seems to be on the cell border. In these cells at 33% p.d. labeling is intense and dispersed throughout the cytoplasm, but no longer detected at 37,5% p.d. In primary pigmentary cells the label in the cytoplasm is strong at 16-29 % p.d., diminishes at 33% p.d. and is absent at 37,5% p.d.. Markedly, heterogeneity occurs in the label pattern of primary pigmentary and cone cells; in the same ommatidia, strong labeled, weakly labeled and non-labeled cells can be observed. In the bristles, the label is first detected at 25% p.d., and is very weak at 37,5% p.d. In the cells of the ID or retina, dFOX-1 is concentrated in cytoplasmatic foci. Incubation of the ID in the presence of protein synthesis inhibitors, such as cycloheximide or puromycin, interferes in this pattern of dFox-1 sub-cellular localization in a way similar to that reported for protein components of RNA processing bodies (P-bodies). The localization of dFox-1 in cytoplasmatic foci was also disrupted in IDs treated with RNAase. Altogether, these data suggest that dFox-1 is involved in cellular process during the development of the compound eye of D. melanogaster, most probably acting in the control of translation.

Financial Support: FAPESP, CNPq, FAEP-AHCRP

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**MOLECULAR EVOLUTION OF THE GENE HOXA-13 IN SNAKES: WHAT IS THE FUNCTIONAL RELEVANCE OF CHANGES IN THE NUCLEOTIDE SEQUENCE?**

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Developmental genes are involved in multiple functions during embryo development, and present a clear fenotipic relationship as they determine the presence and position of several structures. Some of these genes are extremely conserved among species of vertebrates, as the gene family HOX. Although a relationship between the Hoxa-13 nucleotide sequence and autopodial anatomy is evident in a comparative analysis with more than 40 species of vertebrates, the functional relevance of mutations in the exon-1 region of this gene remains obscure in the context of the genetic network involved in limb formation in tetrapods. In this context, we aim to test the hypothesis that changes in the nucleotide sequence of the Hoxa-13 exon-1 that were identified in snakes as evolving under positive and directional pressure have a functional relevance and influence the expression of specific proteins, as Bone-Morphogenetic-Proteins (BMPs). We first need to standardize in our lab a system already developed for testing interactions between Hoxa-13 and BMP2 in mammals. Then, we construct recombinant plasmids with sequences of Hoxa-13 mutated in the five sites detected as evolving under directional selection in snakes. NG108-15 cells are transfected with these constructs, and interactions between Hoxa-13 and BMP2 are detected by Luciferase activity. The present study innovates for elucidating some of the possible functional effects of changes in nucleotide sequences in the cascade of molecular interactions involving Hoxa-13, focusing specifically on the expression Bone-Morphogenetic-Proteins.

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IMMUNOCHARACTERIZATION OF DFOX-1 IN THE D.MELANOGASTER OVARY

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FOX-1 is an evolutionarily conserved protein with orthologs in metazoans. This family of proteins is characterized by the presence of a conserved RRM domain, indicating a functional relation with RNA metabolism. In agreement with this hypothesis, in vitro experiments have shown that in mouse and zebrafish these proteins can control tissue-specific alternative splicing. In Drosophila, the ortholog of FOX-1 (named here dFox-1) was annotated as CG32062 for which there are five isoforms predicted by in silico analyses and EST data. Results from our laboratory indicate dFox-1 as a possible component of mRNA processing bodies in S2R+ cells and in the eye-antenna imaginal discs and retina. Given the importance of proteins of mRNP complexes in the localization of maternal mRNAs in the oogenesis and initial embryogenesis, we have initiated a study on dFox-1 in the ovary of Drosophila. Here we present results on dFox-1 immunodetection using an antibody produced in our laboratory against a region that is common to all the predicted isoforms. Western Blot of protein extracts of Drosophila ovaries probed with -dFox-1 revealed three bands with molecular masses of about 82, 66 and 50 kDa, which are in the mass range of the isoforms predicted for dFox-1. Immunolocalization analysis by confocal microscopy showed that dFox-1 is concentrated at the posterior end of the developing oocyte in stages 3-7. In stage 8, it is detected along the oocyte cortex in the posterior, dorsal and ventral regions. In the nurse cells, the label is concentrated in the perinuclear region in stages 5 and 6, and accumulates in cytoplasmic foci in stage 7. In stages 8-10 dFox-1 label is very intense in the follicle cell layer of the egg chamber anterior region. Our data indicate the expression of at least three isoforms of dFox-1 in the Drosophila ovary. More interesting, some aspects of the sub-cellular localization of dFox-1 in the Drosophila egg chamber are similar to proteins such as dDCP1 and MeB31-2, which have a role in P-bodies and are also involved in mRNA localization in the oocyte.

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TRANSCRIPTIONAL ANALYSES OF CANDIDATE GENES RELATED TO CASTE DIFFERENTIATION IN APIS MELLIFERA

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Social organization among bees is well characterized in Apis mellifera mainly with respect to caste differentiation which is induced by differential feeding in the beginning of third larval instar. In this process a cascade of endogenous reactions is triggered that influences genetic expression during development. Genome sequencing of Apis mellifera enabled the identification and annotation of genes associated to caste differentiation and two functional groups involved in regulation of metabolism and developmental processes were found in ESTs (Expressed Sequence Tags) clusters. Bioinformatics studies in experimental databases generated inferences about groups of genes co-expressed and their putative interaction. Five genes of these groups with the highest values of divergence regarding differential expression between queens and workers, during certain phases of development, and number of motives potentially regulated in upstream regions (5’-URL) were studied by transcriptional analyses. We aimed to validate the expression by Real Time PCR (qRT-PCR) of five genes inserted in these gene networks. As reference, larvae of workers and queens in two developmental stages were collected. The results obtained by qRT-OCR were analyzed by REST (Relative Expression Software Tool 2008, V2.0.7) software using queen phenotype as reference. In fourth larval instar of workers a significant higher expression of 13,5 fold was detected when compared to control for the predicted gene GB19006 (protein architecture was similar to domain DM9 in Drosophila). The sesB gene (stress sensitive B) presented 3,5 fold higher expression in fifth larval instar of workers. However, the gene zeelin did not present significant results. These data suggest that specially one predicted gene (GB 19006), functionally unknown, may be involved in caste differentiation.

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The evolutionary success of insects is to a large extent due to the structural and mechanical properties of the integument, which is formed by the cuticle (or exoskeleton) and the subjacent epidermis. Building and maturation of the adult exoskeleton include complex biochemical pathways where the enzymes Laccases (E.C. 1.10.3.2) may have a key role. The function of these enzymes has been particularly linked to cuticle tanning (pigmentation and sclerotization). It was our aim to investigate the function and regulation of the gene encoding Laccase 2, Amlac 2, during development of the adult integument in the honeybee, Apis mellifera. RT-PCR analyses evidenced that Amlac 2 is highly expressed in the integument of pharate adults in correlation with cuticle tanning. Consistent with the fact that exoskeleton differentiation occurs from the anterior to the posterior body axis, the increase in the levels of Amlac 2 transcripts occurs earlier in the thoracic than in the abdominal integument. Post-transcriptional Amlac 2 gene knock down resulted in abnormalities in cuticle structure, as revealed by histological analyses, and drastically affected the adult molt. Abdominal ligature in early pupae temporarily prevented the abdominal increase in the levels of ecdysteroids and Amlac 2 transcripts, and severely impaired abdominal exoskeleton differentiation. Such results indicate that Amlac 2 expression is controlled by ecdysteroids and has a critical role in the differentiation of the adult exoskeleton.
IMMUNODETECTION OF LIPINS IN DROSOPHILA

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Lipin belongs to a new family of evolutionarily conserved proteins that present phosphatidic acid phosphatase Mg\(^{2+}\) dependent activity, acting in biosynthesis of storage lipids and membrane phospholipids, and as a transcriptional co-activator of lipogenesis genes in mammals. In the present work we developed antibodies against two fragments of the Drosophila melanogaster lipin and characterized its sub-cellular localization in S2R+ cells. In immunoblots of protein extracts from S2 cells both antibodies detected a major band of ~130 kDa, which is in agreement with the masses of the polypeptides deduced from the lipin cDNAs. Immunostaining of lipin in S2R+ cells reveals a cytoplasmic dispersed localization; with a small percentage of cells (~5%) presenting the label apparently concentrated in the periphery of vesicles with maximum diameter of 3 μm, and devoid of neutral lipid. Biochemical experiments showed that most of the lipin proteins are soluble in S2 cells, although a small fraction co-sediment with heavy membranes. This observation, and the fact that only a small percentage of cells presented lipin concentrated in vesicles raise the possibility that the lipin-positive vesicles might reflect a dynamic process potentially related to the biogenesis of membranes needed for growth before the cell division.

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EXPRESSION PATTERN OF GENES RELATED TO LIPID RAFTS FORMATION DURING XENOPUS LAEVIS DEVELOPMENT

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Lipid rafts are membrane microdomains (MM) with high content of cholesterol and sphingolipids. It has been shown that these structures play a role in intracellular trafficking and can act as platforms for signaling complex. Recently we showed that cholesterol depletion within plasma membrane disrupted MM assembly, affecting anterior development of Xenopus embryos. These data suggest a correlation between cholesterol-rich MM and the signaling pathways required for head formation. Two genes previously identified and related with cholesterol biosynthesis (Xen 2) and organization of lipid rafts (Xen 1) are here investigated.

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Since cholesterol and MM seems to play a role during Xenopus development, we decided to investigate the expression pattern of these genes during embryonic development of Xenopus laevis.

The cDNAs containing ESTs (expression sequence tag) of these genes were obtained at gene database. These sequences showed high degree of identity with human, zebra-fish, rat and mouse, and are therefore possible candidates for orthologues in Xenopus. In situ hybridization was performed in different stages of development and showed these genes are expressed at the animal pole, branchial arches, optical vesicles, olfactory placodes, pronephric area and neural plate. At late neurula stage Xen2 expression was restricted in neural crest tissue. At tailbud and tadpole stages all of them showed expression at dorsal region, along the anteroposterior axis and somites.

The PCR analysis revealed Xen1 and 2 are maternally expressed and can also be detected at latter stages. Interestingly, Xen1 expression was dramatically reduced at stages 20, 25 and 30, suggesting that this gene need to be finely regulated during Xenopus development.

These results showed that Xen1 and Xen2 are expressed at restricted areas and specific embryonic stages, suggesting that these ortologues can play a significant role during Xenopus development. Overexpression and functional studies are under investigation.

Functional Validation of Potential Ring Gland Enhancers Identified in the Drosophila Melanogaster Genome

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The BhC4-1 gene is expressed in the salivary glands of Bradysia hygida at end of the fourth larval instar. The mechanisms that regulate the BhC4-1 salivary gland expression are conserved in transgenic Drosophila. Functional studies revealed that the BhC4-1 expression also occurs in the ring gland of transgenic Drosophila and led to the identification of a 67 bp ring gland enhancer. A bioinformatics analysis identified 67 sequences in the D. melanogaster genome that are similar to sequences contained in the ring gland enhancer. Further bioinformatics analysis revealed that 10 out of the 67 sequences are localized in potential regulatory regions, are conserved in at least 7 Drosophila species. In addition, for these 10 sequences, the available data for the nearest downstream open reading frame is inconclusive with respect to embryonic expression.

In order to functionally validate part of these sequences, the genomic sequences localized in the regulatory regions of CG12433, CG13711, CG15306 and CG10609 were cloned into the pBSKS+ vector. The regulatory regions of CG12433, CG13711 and CG15306 were transferred to the Drosophila P element vector, pBP. The constructs present the following structure: P element / white / regulatory region to be tested / Fbp1 basal promoter / lacZ / P element. The constructs CG12433-pBP and CG13711-pBP were both injected into D.

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melanogaster embryos and transgenic lines were obtained. The pattern of expression of the reporter gene has already been analyzed in five transgenic lines transformed with the CG12433-pBP construct. The results indicate that the tested fragment does not drive ring gland gene expression. These results are not totally unexpected since data derived from a bioinformatics analysis, designed to identify genes that are potentially regulated by the transcription factor Dorsal in D. melanogaster, led to the demonstration that only about one third of the identified Dorsal binding sites present functional relevance. In order to extend the functional validation of part of the sequences identified through the bioinformatics analysis, in the near future we plan to investigate the pattern of expression driven by the cloned genomic fragments localized in the regulatory regions of the CG13711, CG15306 and CG10609.

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DROSOPHILA MELANOGASTER LIPIN PRESENTS DISTINCT TISSUE AND DEVELOPMENTAL EXPRESSION PATTERNS

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Lipins encompass a novel family of evolutionarily conserved proteins recently characterized as Mg2+-dependent phosphatidate phosphatases (PAP1), with a role in the biosynthesis of membranes and lipid metabolism. While a single lipin gene is detected in less complex organisms, such as yeast and worm, in mammals there are three genes that express different lipin paralogs in a tissue-regulated manner. We show that the unique ortholog of lipin identified in the Drosophila genome (CG8709) expresses at least three isoforms, generated by alternative splicing and transcription initiation, nominated here as DmLpinA, DmLpinB and DmLpinC. Their expression is regulated at the mRNA and protein levels during development and in the different tissues. The amounts of lipin mRNAs are higher in embryos and male adults than in larvae, while lipin proteins are more abundant in third instar larvae. Moreover, while the level of DmLpinA mRNA is higher in extracts from the intestinal tract/Malpighi tubules, the amount of lipin detected on immunoblots of protein extracts is relatively low. On the other hand, although lipin proteins are present in elevated amounts in protein extracts of the fatty body, the levels of DmLpinA and B mRNAs detected in this tissue are lower than in the other analysed tissues. DmLpinC isoform is exclusively expressed in testis. In addition to the DXDXT motif characteristic of PAP1, the Drosophila lipins present a conserved nuclear localization signal and the LXXIL coactivator motif that in mammals are essential for the nuclear activity of these proteins, suggesting that Drosophila lipins might have both a cytoplasmatic and nuclear function. In agreement with this hypothesis, confocal microscopy immunolocalization analysis, using an antibody that

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recognizes all Drosophila lipin isoforms, detected label in the cytoplasm and nuclei of third instar larvae fat cells. In whole mount preparations of intestinal tract/Malpighi tubules, lipin was detected exclusively in the cytoplasm of midgut longitudinal muscle cell layer; similarly, in the testis it was also restricted to the muscle layer. In both cases the label presented a sarcomeric pattern, indicating that lipin is concentrated in the A band. Also in the ovaries, the pattern of lipin subcellular label was peculiar, being heavily concentrated in the ring channels, which make connections between the cytoplasm of nurse cells and the oocyte.

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**RHO-ROCK SIGNALING IS NECESSARY FOR LENS PLACODE INVAGINATION IN CHICK EMBRYOS**

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Vertebrate lens is formed by invagination of the placode that overlies the optic vesicle. Here, we investigated the contribution of acto-myosin cytoskeleton and the Rho-ROCK pathway to the lens placode initial invagination. Actin filaments and myosin II were detected at the apical region of chick lens placode prior to invagination whereas no such polarization was detected in the surrounding non-lens ectoderm. Inhibition of actin polymerization and myosin II ATPase activity with Cytochalasin D or Blebbistatin treatments, respectively, completely abolished lens placode invagination, indicating the relevance of actin-myosin contraction to the initial lens placode bending step. RhoA, an acto-myosin regulator is also apically enriched in the lens placode prior to its bending. Inhibiting Rho signaling by C3 exoenzyme electroporation or its downstream effector Rock by Y27632 treatment also arrested lens placode invagination. Moreover, we show that inhibition of Rock also reduced the concentration of actin filaments and myosin II at the lens apical side. Taken together, these data suggest that contraction of the apical acto-myosin in the lens placode might initiate its invagination and that the Rho-Rock pathway is necessary for the correct localization of acto-myosin cytoskeleton at the lens placode apical portion.

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HYPOXIA PATHWAY AS A PUTATIVE NOVEL ELEMENT IN THE HONEY BEE CASTE DETEMINATION PROCESS

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Honey bees (Apis mellifera) provide a prime example of diphenic development. Females develop into either of two castes, queen and workers, that differ not only in size and several structural features but also in individual fitness and life history. Developmental fate is initially determined environmentally, by the larval diet, which then acts on two important regulators of caste determination, juvenile hormone and the insulin/TOR pathway. Recent studies on Drosophila melanogaster suggest a third possible mechanism that may play a role in this process, the hypoxia pathway which is controlled by transcription factors of the helix-loop-helix-PAS (HLH-PAS) family. In order to obtain information on this pathway in honey bees we used, as a first approach, available genomic information from Drosophila melanogaster to find the respective ortholog genes in Apis mellifera. BLAST searches on the honey bee genome database revealed three genes which showed best matches with the Drosophila hypoxia genes (sima, fatiga and tango), and these were named amsima, amfatiga, amtango, respectively. The three orthologs were sequenced, annotated by Artemis v9.0 and their expression profiles were checked by RT-PCR in postembryonic stages of worker bees. Our analysis showed that amsima is expressed almost without variation from the fourth larval instar until the end of the prepupal stage, while amtango and amfatiga showed a higher expression in the cocoon-spinning stage. Taken together, this focus on the hypoxia pathway rekindles interest in yet little understood studies performed in the 1980s which had suggested differences in respiratory metabolism of queen and worker larvae. The genomic information and quantification of expression levels of hypoxia genes now allows to follow these earlier clues and to investigate a novel element in the complex process of diphenic development in this social insect.

DEVELOPMENTAL MECHANISMS UNDERLYING CARDIAC ANTERO-POTERIOR PATTERNING BY THE RALDH2 CAUDOROSTRAL WAVE

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Early establishment of anteroposterior (AP) orientation of cardiac precursors is a key step in heart organogenesis because anterior and posterior segments give rise to cardiac ventricles and atria, respectively. Previous findings from the laboratory indicated that a wave of Raldh2 promotes asymmetric synthesis of retinoic acid (RA) to the cardiac field in the AP axis. This
Caudorostral wave endows the posterior cardiac mesoderm with the capacity to synthesize its own RA, which instructs this progenitor area to differentiate as sinus and atrial tissues.

We cultured HH5-6 chicken embryos on albumin plates overnight at 37º and observed that they were able to develop to HH7-8, expressing the normal raldh2 caudorostral wave invading the cardiac field. Afterwards, we cultured isolated cardiac mesoderm fragments (CMF) in the same conditions and found that most of these fragments were unable to express raldh2 (93%). We then cultured CMF from chicken with its corresponding posterior region from quail to try and restore the raldh2 caudorostral wave. We performed a QcPN Immunohistochemistry in toto to differentiate quail from chicken tissue. In these culture conditions, we restored the raldh2 expression in 35% of the CMF, i.e., five times more than in isolated fragments, without significant migratory contribution of quail cells to the anterior domain of raldh2 expression. Surprisingly, quail axial tissue, normally unable to induce raldh2 expression, restored in 51% the raldh2 caudorostral wave when juxtaposed to the chicken CMF, suggesting a non-specific reestablishment of raldh2 expression in these culture conditions. To test the development of raldh2 caudorostral wave in the cardiac field, disassociated from the posterior mesoderm, in the whole embryo; we are currently cultivating, for 3, 6 or 12 hours, HH5-6 embryos where the left-side posterior and cardiac mesoderms are separated by a slide of glass. Longer times of incubation brought morphological disturbances, sometimes including cardia bifida. It remains to be confirmed if raldh2 expression in these experiments is perturbed and in which manner to further explore the possible candidates as regulators of Raldh2 transcription.

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FORMATION OF THE GERMINAL EPITHELIUM DURING THE GONADAL MORPHOGENESIS IN MALE OF THE CYPRINUS CARPIO (TELEOSTEI: OSTARIOPHYSI: CYPRINIFORMES)

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During the gonadal morphogenesis in Cyprinus carpio the proliferation and differentiation of germ and somatic cells from the gonadal primordium result in the formation of testicular structures and the constitution of germinal epithelium that borders seminiferous tubules. Thus, to study these events, gonadal tissues of juveniles of Cyprinus carpio were prepared for observation under transmission electron microscopy and high resolution light microscopy, including Reticulin’s Method.

The gonadal primordium is an elongated structure with individual primordial germ cells (PGCs) scattered among somatic cells. In the undifferentiated gonad, somatic cells wrap around and individualize the PGCs. PGGs divide and form continuous cords delimited by a

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basement membrane. Reorganization from PGCs results in the loss of the cord organization. Each PGC, now differentiated in spermagonia, is wrapped by the now pre-Sertoli cells giving rise to a cyst. The cysts join one another forming acinar-clusters, the first signal of the male gonad differentiation. A basement membrane is synthesized around each cluster, being incomplete between adjacent clusters. Pre-Sertoli cells rest upon the forming basement membrane.

In the center of the clusters a space is created when Sertoli cells move away from one another. In the region where the basement membrane is absence, nearby clusters fuse to one another becoming connected by the same luminal space. The progressive fusion of the clusters gives rise to the seminiferous tubules that are bordered by the newly formed germinal epithelium constituted by the cysts that rest upon the basement membrane. Mesenchymal cells surround the seminiferous tubules give rise to the cellular components of the interstitial compartment.

Inside the cysts spermatogenesis starts. As spermatocytes are releasing, the anastomosis of the testicular tubules occurs forming the spermatic duct on the dorsal region of the testis. During all gonadal development the basement membrane separates the germinal epithelium from the interstitial compartment.

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MYOSIN VA EXPRESSION DURING EARLY SENSORY NEUROGENESIS

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Dorsal root ganglia (DRG) are components of the peripheral nervous system that harbor the cell bodies of sensory neurons. During normal development, the establishment of the appropriate connections through neuritogenesis is an essential step in sensory neurogenesis. In this process, regulation of growth cone directionality and speed contribute towards the guidance and control of filopodial extension. Myosin Va, an evolutionarily conserved molecular motor involved in organelle transport, has been implicated in filopodial extension. Myosin Va is widely expressed in the vertebrate developing nervous system. The inactivation of Myosin Va in growth cones of chick dorsal root ganglion neurons in culture significantly decreases the rate of filopodial extension. Moreover, mouse mutants for Myosin Va present severe neurological defects, which phenocopy human Griscelli’s Syndrome. Taken together, those findings suggest that Myosin Va plays an important role in neural development. However, since previous studies on its role in neuritogenesis have been carried out in vitro, we aim to assess the role of Myosin Va in sensory neuritogenesis in vivo. Thus, we first characterized the expression of Myosin Va at different stages of DRG development: at the

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start of its condensation (HH19), at the onset of neuronal production (HH25) and after the appearance of the ventral root (HH30). Furthermore, we correlated the expression of Myosin Va with that of Islet-1. Islet-1 is a transcription factor expressed in post-mitotic DRG neuronal cells and a known marker of early neuronal differentiation. Our results show that Myosin Va is expressed exclusively in Islet-1-positive cells in the DRG of all stages investigated. Islet-1 positive cells in the HH19 DRG do not present discernible neurites. Thus, the expression of Myosin Va in cell bodies at this stage suggests that Myosin Va might contribute towards other events in early neurogenesis and/or is required for the initiation of neurite extension. As DRG development progresses, Islet-1-positive cells emit neurites that label positively for Myosin Va, thus confirming the previous reports on its role in sensory neuritogenesis. Taken together, the data presented here suggest that Myosin Va might contribute not only towards sensory neuritogenesis but also towards other events in early neurogenesis.

Financial support: FAPESP and CNPq

**Organization and Larval Transcription Profile of Genes Associated to Neurogenesis in *Apis Mellifera* Castes**

*Vanessa Bonatti*⁴⁸¹; *Ana D. Bomtorin*²; *Zilá L.P. Simões*³; *and Angel R. Barchuk*¹

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Contrary to the intuitive thinking, dietary restriction has been increasingly shown as a key trigger of neurogenic events in several model systems. In the honeybee *Apis mellifera*, females that receive low amount of food during larval development become workers, bees with a more developed adult brain than that of the higher fed queens. A more developed brain allows workers to accomplish the variety of duties needed to maintain a healthy colony: foraging, nursing, nest cleaning and protecting, etc. During most part of post-embryonic development, however, queen’s brain shows a bigger area of neuroblasts than that of workers, what may reinforce the intuitive idea of the importance of a high nutritional input as responsible for a high nervous system output. Extensive cell death events during the second part of pupal development turn queen’s brain proportionally less developed than that of worker’s. Due to the differential feeding on presumptive queen and worker larvae, the early larval differential neurogenesis in honeybees is a natural experiment and good model to be used for the identification of genes linking differential nutrition to differential nervous system development. Here, we report the organization and larval transcription profile in queens’ and workers’ honeybee brain of seven genes known as associated to neurogenesis in different animal models and previously found as differentially expressed between castes in the whole body of fourth instar larva honeybees (Barchuk et al., BMC Dev Biol, 2007):

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tetraspanin 5D (tsp5D), dachshund (dac), failed axon connection (fax), cryptocephal (crc),
ataxin-2 (atx-2), Ephrin Receptor (ephR) and short stop (shot). Using qPCR we analyzed the
expression profile of these genes in brains of third to fifth larval stages. Our results show
three groups of expression patterns: One including profiles with expression levels increasing
progressively, represented by the expression of dac and fax; one with profiles peaking in
fourth instar larvae, represented by atx-2, EphR and shot; and the last one represented by
tsp5D, showing a decreasing profile. Shot, whose expression is required for the axons
extension and cell proliferation in Drosophila, was found more expressed in fourth stage
queen larvae. This may help explain the existence of a larger area of neuroblasts in brains of
fifth instar queen larvae when compared to workers and indicates shot as a pivotal player in
the scene of the gene expression cascade induced by differential feeding in animals,
particularly in the honeybee A. mellifera.

Financial Support: CNPq Processo No 473748/2008-8

**GENERATION OF MUTANTS FOR THE CALPAIN A LOCUS IN**
**DROSOPHILA MELANOGASTER USING P ELEMENT**
**AS MUTAGENIC AGENT**

*Viviane Vieira*49 and *Helena Araujo*
Institute for Biomedical Sciences – UFRJ, Rio de Janeiro, RJ – Brazil

In early embryonic development, a gradient of nuclear protein NFκB / Dorsal is required
to establish the ectoderm and mesodermal domains. Maternally expressed genes encode
components of a proteolytic cascade that culminates in activation of the Toll transmembrane
receptor, inducing degradation of the inhibitor IκB / Cactus and allowing nuclear
translocation of Dorsal protein.. Parallel to the Toll pathway, signaling through maternal
BMP4/Dpp inhibits the degradation of Cactus, regardless of the signal from Toll. Studies in
our laboratory (Fontenele, M et al, 2009) describe a new component in this pathway, the
protease Calpain A (CalpA). CalpA alters Cactus levels, while mutant loss-of-function for
cact alter the activity of CalpA. To investigate in more detail the interaction between these
two genes and the effects of CalpA on embryogenesis, we aimed to generate mutants for the
CalpA locus. The strategy is based on the use of a transposing element located in the
promoter region of the gene CalpA, that when mobilized may delete some sequences of this
gene and compromise its expression. With this methodology we established 20 homozygous
lethal lines wich showed excisions of the transposing element. All lines presented similar
phenotypes: anterior holes in the cuticle and lowered staining with CalpA antisera. These
phenotypes are consistent with decreased zygotic expression of CalpA and suggest that this
locus contributes to the establishment of anterior structures of the larva. Complementation
tests show that almost all lines do not complement and thus represent alleles of a same locus.
However, lethality was complemented by two deficiencies that cover the CalpA locus. In
order to understand these contradicting results we are characterizing in more detail these
deficiencies and their resulting embryonic phenotypes.

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CHARACTERIZATION OF MADS-BOX GENE FAMILY IN COFFEA ARABICA

Raphael Ricon de Oliveira and Marcelo Carnier Dornelas
UNICAMP – Instituto de Biologia, Dep. Biologia Vegetal, Rua Charles Darwin s/no

The MADS-box gene family encodes transcription factors that act as key regulators in many steps in the development of various organisms. In plants these genes are involved in determining the identity of reproductive meristems and floral organs, in the control of flowering time, in fruit development, among other process. This study aims to identify genes of MADS-box family related to the determination of floral organ identity in Coffea arabica. Searches were conducted by similarity between MADS sequences deposited in GenBank and expressed sequence tags (EST) database arising from The Brazilian Coffee Genome Project. Bioinformatics programs were used for assembly the sequences in EST-contigs, that after resequencing their representative clones, could be confirmed. In this phase, polymorphisms between alleles could be observed, besides differences in splicing expression. A phylogenetic analysis was made indicating putative orthologs of genes described and studied in Arabidopsis thaliana, allowing us to establish a starting point to test this hypothesis by comparing the expression profiles in different tissues and stages of reproductive development.

CHARACTERIZATION OF EXPRESSED GENES DURING EMBRYOGENESIS IN PASSIFLORA EDULIS

Juliana Lacorte Cazoto and Marcelo Carnier Dornelas
Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária “Zeferino Vaz”, Barão Geraldo, CEP: 13418-970, Campinas, SP

Different from animal development, that has its organs formed during the embryo development, plant structures are formed by the shoot and root apical meristems (SAM and RAM, respectively), which are established during plant embryogenesis. This process is organized by the concomitant activity of specific transcription factors that are expressed in a hierarchical way, determining cellular domains that are responsible for the installation and maintenance of these meristems. The activity of those transcription factors is under control of environmental information like light, hormonal concentrations among others. With the aim of characterizing the embryo development in the commercial passion fruit, Passiflora edulis Deg, we identified and characterized genes expressed during the zygotic and somatic

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embryogenesis. Among the identified genes, members of NAC/CUC, YABBY, WOX, SCARECROW, AUX/IAA and ARF families were found. These genes were identified by bioinformatics analysis in the data bank from PASSIOMA project. The expressed sequence tags (ESTs) were clustered and the predicted protein sequences compared by sequence alignment with their potential homologs in Arabidopsis. The gene expression pattern will be studied by in situ hybridization during different stages of P. edulis embryo development.

Funding Agency: FAPESP (Proc. 08/57588-1)

**ANATOMICAL CHARACTERIZATION OF THE ANDROGYNOPHORE MOVEMENT IN PASSIFLORA SPP. SECT. XEROGONA I. PASSIFLORA CITRINA**

*Livìa Scorza*52 and *Marcelo Carnier Dornelas*

Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Biologia Vegetal. Cidade Universitária “Zeferino Vaz”. Campinas, SP

Some species of Passiflora Subgen. Decaloba, sect. Xerogona (Raf.) Killip exhibit a touch-induced movement of the androgynophore. This reaction is a relatively rapid response in a direction determined by the placement or direction of the stimulus. The androgynophore movement in Passiflora is not yet described in the literature and remains as an anecdotal observation of collectors of ornamental species from this gender. This androgynophore particular behavior could be related to pollination habits of these species. Thus, the aim of this work is to characterize the androgynophore movement through an anatomical study of one of the species from section Xerogona, Passiflora citrina. The samples were collected after being stimulated or not by touch, fixed in 4% paraphormaldehyde, dehydrated in an ethanol series and embedded in plastic resin glycol methacrylate. The materials were cut in a rotative microtome, stained with toluidine blue and the permanent slides were prepared for optical microscope observation. Our partial results show that the androgynophore movement is probably caused by changes in the cortical cells volume, on the base of the androgynophore column, in the stimulated side. In transverse sections it was observed that, in approximately one third of the circumference, stronger stained extracellular content and more plasmolized cells as compared to the cells of other regions. This means that the decrease in the cells volume is due to water loss and of other still uncharacterized compounds. We hope to provide a better understanding of pollination systems evolution in Passiflora and of the floral morphology dynamics of these species and their pollinators.

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CHARACTERIZATION OF MADS-BOX GENES FROM CITRUS INVOLVED IN FRUIT DEVELOPMENT

Pedro Araújo53 e Marcelo Carnier Dornelas
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Some molecular elements involved in fruit development are extensively studied in Arabidopsis thaliana – a model plant, which produce dry fruits called siliquas. The FRUITFULL (FUL) and SHATTERPROOF (SHP), that belong to multigenic family MADS-box, are related to ontogenesis and ripening of Arabidopsis dry fruit. Recent studies suggest that these genes, FUL and SHP, are conserved in dry and fleshy fruits. Thus, we decided to characterize, minutely, the development of Citrus sinensis e Citrus reticulata and identify possible homologies FUL e SHP in Citrus spp. Furthermore, the aim of this study was to evaluate the expression pattern of these homologies during fruit development of C. sinensis. The data bank of project CitEST contains 36 genes homologous to MADS-box genes, six of which correspond to FUL and SHP in four species of Citrus. These sequences were characterized by bioinformatic tools and phylogenetic analysis. The use of light and scanning electron microscopy (SEM) techniques allowed the identification of important anatomic differences in mesocarp and exocarp during fruit development in oranges and tangerines. RT-PCR and in situ hybridization results showed the expression pattern of FUL and SHP genes through fruit development in Citrus.

5-BROMO-2’-DEOXYURIDINE INHIBITS THE ACTIVITY OF THE AMPLIFIED GENES, BHC5-2, BHC4-1 AND BHB10-1, IN THE SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA, SCIARIDAE)

Juliana Conti54 Viana e Jorge Cury de Almeida
Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto, SP

Among the several effects of the 5-bromo-2’-deoxyuridine (BrdUrd), an analog of thymidine (dThd), the inhibition of cell differentiation is the most interesting. The injection of BrdUrd into Bradysia hygida larvae, during the period of gene amplification, leads to a notable morphological result – the DNA puffs do not expand, the chromatin, in these chromosomal sites, remains very compacted during all the rest of the 4th larval instar and transcription process seems to be severely decreased in the DNA puffs.

Here we show, by morphological and molecular techniques, the effect of BrdUrd on cell differentiation, studying its role on the activity control of three specific amplified genes: BhC5-2, BhC4-1 and BhB10-1.

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In the experiments, 1 μl of 4 mM solutions, of dThd (control) or BrdUrd, was injected into larvae, during the first part of the gene amplification period. We performed Total RNA extraction, RT-PCR or Northern blotting followed by nucleic acid hybridization. Protein synthesis was studied by SDS-PAGE, Western blotting and immuno-detection.

The treatment with BrdUrd had a very pronounced inhibitory effect on mRNA production, demonstrated by RT-PCR (for gene BhC5-2) and Northern blot (for genes BhC4-1 and BhC5-2). The same occurred in relation to the synthesis of proteins BhC4-1 and BhB10-1. These experiments show an interesting correlation, between the morphological effect of BrdUrd on the DNA puff anlages, and the activity of specific amplified genes. Next step, comparison with the activity of non-amplified genes will be established.

Financial Support: CAPES, FAPESP e FAEP.
ABSTRACTS PRESENTED IN - X BRAZILIAN SYMPOSIUM ON EXTRACELLULAR MATRIX AND V INTERNATIONAL SYMPOSIUM ON EXTRACELLULAR MATRIX

PROGRAMA AT A GLANCE

<table>
<thead>
<tr>
<th>Nov 1</th>
<th>18h00</th>
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<tr>
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<td>18h30</td>
<td>Conference: The metastatic niche: adapting the foreign soil</td>
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<td>Nov 2</td>
<td>8h30</td>
<td>Symposium 1: ECM and maternal-fetal interface</td>
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<td>Symposium 2: ECM interplay with soluble proteins: role in cell migration</td>
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<td>Course: 3D model for cell culture</td>
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<td>Symposium 3: ECM and cancer cell biology</td>
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<td>Conference: Cell communication in tumoral microenvironment</td>
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<td>Poster Session I</td>
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| Nov 3  | 8h30         | Symposium 4: Glycosaminoglycans: a whole world in ECM biology |
|        | 10h30        | Coffee break             |
|        | 11h00        | Symposium 5: ECM in tissue differentiation/dedifferentiation and pathology |
|        | 13h00        | Lunch/free time          |
|        | 14h15        | Course BD Conference: 3D model for cell culture Aplicações das matrizes extracelulares: MatrigelTM e o novo PuraMatrixTM para Cultura 3D |
PROGRAMA AT A GLANCE (CONTINUED)

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<tr>
<td>16h00</td>
<td>Symposium 6: ECM in chronic/degenerative diseases</td>
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<tr>
<td>17h30</td>
<td>Conference: Biomechanical and signaling roles of extracellular matrix in regulating early morphogenesis</td>
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<td>Poster Session II</td>
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<td>Nov 4</td>
<td>8h30 Symposium 7: ECM and stem cell biology and therapy</td>
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<td>Coffee break</td>
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<tr>
<td>10h30</td>
<td>Conference: Signaling pathways involved in the effect of heparin on endothelial cells</td>
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<tr>
<td>11h30</td>
<td>Closing ceremony</td>
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DETAILED PROGRAM

November 1st

18h00 Opening Ceremony

18h30 Opening Conference
Chairperson: Roger Chammas
*The metastatic niche: adapting the foreign soil*
David Lyden (Cornell University, USA)

November 2nd

8h30- 10h30 Symposium 1: ECM and maternal-fetal interface
Chairperson: Telma Zorn
08h30- 09h20 Regulation of trophoblast cell behavior by extracellular matrix
Ann Sutherland (Univ. Virginia Health System, USA)
09h20- 09h50 Extracellular matrix remodeling at the maternal-fetal interface
Telma M.T. Zorn (USP, Brazil)
09h50-10h15 Hormone-regulated expression and distribution of versican in mouse uterine tissues
Renato de Mayrinck Salgado (USP, Brazil)
10h15- 10h30 Selected abstract: Expression of decorin and biglycan in human normal term placenta and in invasiveness- changed trophoblast pathologies
Alexandre Urban Borbely (USP, Brazil)
10h30 coffee break
11h00- 13h00 Symposium 2: ECM interplay with soluble proteins: role in cell migration
Chairperson: Cecília Hedin Pereira
11h00- 11h45 The Extracellular Matrix as a Modulator of Tumor Invasion and Metastasis
William G. Stetler-Stevenson (NIH, USA)
11h45- 12h30 ECM and soluble proteins control neuronal cell migration
Cecilia Hedin Pereira (UFRJ, Brazil)
12h30-12h45 Selected abstract: April modulates in vitro thymocyte migration and cell adhesion by interacting with HSPG
Cecília Rocha (FIOCRUZ, Brazil)
12h45-13h00 Selected abstract: The role of laminin and CXCL12 in T-cell migration during cardiac allogeneic graft rejection
Ingo Riederer (FIOCRUZ, Brazil)

13h00 15h00 lunch/free time

15h00 -16h00 Course: 3D Model for Cell Culture
Vanessa M Freitas (USP, Brazil)

16h00- 17h30 Symposium 3: ECM and cancer cell biology
Chairperson: Ruy Jaeger
16h00-16h30 Laminin peptides influencing carcinoma behavior
Ruy Jaeger (USP, Brazil)
16h30- 17h00 Platelet activation as a novel target for galectin-1-glycan interactions: implications in inflammation, thrombosis and metastasis
Mirta Schattner (ANM, Argentina)
17h00-17h15 Selected abstract: RECK-mediated inhibition of glioma migration and invasion
Renato Ramos Massaro (USP, Brazil)
17h15-17h30 Selected abstract: The RECK B isoform is downregulated by simvastatin in human melanoma cells
Fernanda Augusta de Lima Barbosa (UFPR, Brazil)

17h30- 18h30 Conference
Chairperson: Mauro Pavão
Cell communication in tumoral microenvironment
Lubor Borsig (Univ. Zurich, Switzerland)

18h30 19h00 coffee break

19h00- 21h00
Poster Session I

November 3rd

8h30- 10h30 Symposium 4: Glycosaminoglycans: a whole world in ECM biology
Chairperson: Paulo Mourão

08h30-09h15 Defining the heparan sulfate code that increases FGFR function during branching morphogenesis
Matt Hoffman (NIH, USA)

09h15-10h00 Sulfated polysaccharides ensures a carbohydrate-based mechanism for species recognition during sea urchin fertilization
Paulo Mourão (UFRJ, Brazil)

10h00-10h15 Selected abstract: Structural characterization and anticoagulant properties of a unique heparan sulfate from a mollusk bivalve
Angélica Maciel Gomes (UFRJ, Brazil)

10h15-10h30 Selected abstract: Fucan and the stimulus in the synthesis of antithrombotic endothelial heparan sulfate: relationship between structure and biological activity
Valquíria P Medeiros (UNIFESP, UFRN, Brazil)

10h30-11h00 coffee break

11h00-13h00 Symposium 5: ECM in tissue differentiation/dedifferentiation and pathology
Chairperson: Eva Burger

11h00-11h45 Vascular Extracellular matrix and Vessel Wall Development
Robert Mecham (Washington Univ, USA)

11h45-12h30 ECM in experimental paracoccidioisis infection
Eva Burger (USP, Brazil)

12h30-12h45 Selected abstract: Laminin alpha5 expression after human myoblast transplantation into Rag-/- Gammac-/- mice
Ingo Riederer (FIOCRUZ, Brazil)

12h45-13h00 Selected abstract: Schwann cells transdifferentiation: possible role of myofibroblast in neural fibrosis during leprosy
Rafael Petito (FIOCRUZ, Brazil)

13h00-15h00 lunch/free time

14h15-16h00 Course 3D Model for Cell Culture
Vanessa M Freitas (USP, Brazil)

Aplicações das matrizes extracelulares: MatrigelTM e o novo PuraMatrixTM para Cultura 3D
Aline Fukuzawa - Especialista de Produto BD Biosciences

16h00-17h30 Symposium 6: ECM in chronic/degenerative diseases
Chairperson: Vilma Martins

16h00-16h45 Heparin Cofactor II Modulates the Response to Arterial Injury
Douglas Tollefsen (Washington Univ, USA)

16h45-17h15 Prion protein binding to laminin: neurotrophic properties to be explored in degenerative diseases
Vilma Martins (Inst. Ludwig, Brazil)
17h15-17h30 Selected abstract: Glycosaminoglycan anticoagulant properties from atherosclerotic lesion
Lisandra A C Teixeira (UFRJ, Brazil)

17h30- 18h30 Conference
Chairperson: Estela Bevilacqua
Biomechanical and signaling roles of extracellular matrix in regulating early morphogenesis
Raymond Keller (Univ. Virginia, USA)

18h30- 19h00 coffee break

19h00- 21h00 Poster Session II

November 4th

8h30 Symposium 7: ECM and stem cell biology and therapy
Chairperson: Tatiana Coelho-Sampaio
08h30- 09h00 Laminin and stem cell therapy to treat spinal cord injury
Tatiana Coelho-Sampaio (UFRJ, Brazil)
09h00- 09h30 The use of xeno-free, defined extracellular matrices in the derivation of human embryonic stem cells for clinical applications
Adriana Bos-Mikich (UFRGS, Brazil)
09h30- 09h45 Selected abstract: Effect of dermatan sulfate in the migration and proliferation of endothelial progenitor cells after arterial injury in mice
Juliana A P Godoy (UNICAMP, Brazil)
09h45-10h00 Selected abstract: Quail neural crest cell differentiation: the effects of FGF2 and microenvironment
Denise A Bittencourt (UFSC, Brazil)

10h00- 10h30 coffee break

10h30-11h30 Closing Conference
Chairperson: Marimélia Porcionatto
Multivectorial model for thymocyte migration: a Voyage from physiology to pathology
Wilson Savino (FIOCRUZ, Brazil)
Closing Ceremony

ABSTRACTS

C1. The Metastatic Niche: Adapting the Foreign Soil

David Lyden (Cornell University, USA), dcl2001@med.cornell.edu
C2. Cell-Cell Interactions Defines the Metastatic Microenvironment of Tumor Cells

*Lubor Borsig*
Institute of Physiology, University of Zürich, Switzerland. lborsig@access

Abstract: Recent evidence strongly suggests that cancer progression is dependent on the microenvironment consisting of stromal and inflammatory cells. Interactions of tumor cells with endothelium in a microvasculature of distant organs determine the outcome of metastasis. Here we provide evidence for the molecular mechanism involved in the tumor cell-mediated activation of endothelial cells leading to formation of a metastatic niche. Selectin-mediated cell-cell interactions of tumor cells with platelets and leukocytes induce endothelial production of the inflammatory chemokine, which is the key chemoattractant for monocytic cells. Inhibition of monocyte recruitment strongly reduced survival of tumor cell and metastasis. Similarly, L-selectin deficiency reduced the recruitment of monocytic cells, and attenuated metastasis. Our findings demonstrate that the selectin-dependent endothelial expression of chemokines contributes to the formation of a permissive metastatic microenvironment.

C3. Biomechanical and Signaling Roles of Extracellular Matrix in Regulating Early Morphogenesis

University of Virginia, Departments of Biology¹ and Cell Biology³, University of Pittsburgh, Department of Engineering², USA, ICORP Organ Regeneration Project, Japan Science and Technology Agency, Tokyo, Japan⁴ rekek@virginia.edu

Live imaging with time-lapse confocal microscopy reveals the dynamics of the assembly and remodeling of fibrillar fibronectin matrix at tissue interfaces during early *Xenopus* (frog) development and suggests both mechanical and signaling roles for this matrix. Disrupting the non-canonical Wnt/planar cell polarity (PCP) pathway reveals a role for this pathway in normal assembly of fibronectin at planar tissue interfaces, and a second role in the mediolateral polarization of cell protrusive activity (mediolateral intercalation behavior: MIB) that is essential for the convergent extension movements of gastrulation. Blocking integrin alpha5beta1 binding to fibronectin increases the frequency and randomizes the orientation of protrusive activity, suggesting that fibronectin-integrin signaling normally functions in polarizing protrusive activity by suppressing background, random activity. Fibrillin, the extracellular matrix molecule implicated in the human diseases Marfan syndrome and congenital contractural arachnodactyly, is expressed in the dorsal mesoderm of *Xenopus*, and disruption of the fibrillin matrix by three methods results in failure of MIB. Regionally targeted disruptions of the fibrillin matrix result in failure of the mechanical integrity of the notochord and notochordal-somitic boundary. Ectopic expression of fibrillin sub-domains on
the ventral side of the gastrula results in second, dorsal axes, suggesting that fibrillin may have organizer activity.

**C4. Signaling Pathways Involved in the Effect of Heparin on Endothelial Cells**

*H.B. Nader, hbnader.bioq@epm.br*

Disciplina de Biologia Molecular, Departamento de Bioquímica, UNIFESP, São Paulo, São Paulo, SP, 04044-020

Heparins (Hep), low molecular weight heparins (LMWH) and sulfated fucans (SF) from brown seaweed are antithrombotic drugs that specifically up-regulate the synthesis of an antithrombotic heparan sulfate (HS) when exposed to endothelial cells. The effect is specific for endothelial cells. The binding is fast and saturable with an apparent $K_D$ of 83 nM to adherent cells and 44 nM to the extracellular matrix (ECM) in the absence of cells. By confocal and electron microscopy, the compounds bound only to the ECM, co-localizing with fibronectin. This was confirmed by flow cytometry using syndecan-4 monoclonal antibody as well as wheat germ agglutinin that label cell surface; on the other hand using both Hep and SF as well as a fibronectin polyclonal antibody no binding was observed. Furthermore, the stimulus in HS synthesis is not elicited by the drugs in the absence of ECM. When the cells were exposed to the compounds at 37°C, they were internalized and with time localized in lysosomes. However, endocytosis of the drugs was not required for the stimulation of HS synthesis. The effect is not dependent of lysosomal enzymes activity, since when the cells were incubated with chloroquine, a lysosomotropic amine that raises the lysosomal pH thus inhibiting enzymatic degradation of internalized compounds, the stimulus on HS synthesis was still observed. Signal transduction pathways were then investigated. Endothelial cells stimulated by these antithrombotic drugs showed activation of phosphotyrosine kinases. Both western blotting and confocal microscopy analyses revealed activation of the focal adhesion complex proteins as FAK, Src and paxillin as well the MAP kinase protein like ERK 1/2. The involvement of protein tyrosine kinases and Ras-MAP kinase pathway was confirmed by the use of specific inhibitors as well as ras-transfected endothelial cells. Other experiments clearly demonstrated the role of phospholipase C, intracellular Ca$^{2+}$ mobilization and the nitric oxide pathways the synthesis of HS. To our knowledge this is the first study clearly showing the cell signaling pathways related to the effect of heparin and heparinomimetics on endothelial cells. *Supported by Fapesp, CNPq, CAPES, FINEP.*

**Course: 3D Model for Cell Culture**

*Vanessa M Freitas (USP, Brazil), vmfreitas@usp.br*

Cell culture provides a defined assay to study cell biology and pathology *in vitro*. Two-dimensional culture has been the paradigm for a typical cell culture system. However, the seminal work of Bissell and coworkers demonstrated that human breast epithelial cells recapitulate glandular phenotype when cultured in 3D analogs of their native microenvironment. As a result, biologists and bioengineers have investigated different three-dimensional scaffolds that recapitulate aspects of the native cellular microenvironment for in
vivo cell culture. Basement membranes serve as key components in 3D culture. These thin continuous sheets of specialized extracellular matrix separate epithelial and endothelial cells from underlying stroma and play important roles in cellular homeostasis and tissue architecture. This course will introduce the properties of three-dimensional cell culture, main substrates and biological scaffolds used. Specific methodologies will focus on both qualitative and quantitative analysis of three-dimensional cell culture.

S1.1. Regulation of Trophoblast Cell Behavior by Extracellular Matrix

Ann E. Sutherland, Erin K. Klaffky, Isabel M. Gonzalez, and Bradley Phillips
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Extracellular matrix has specific effects on cell behavior, which affect many aspects of early development. In the early postimplantation mouse embryo the laminin isoforms laminin-111 and laminin-521 have distinct effects on trophoblast cell behavior, which influence the process of implantation. Laminin-111 promotes migration and decreases spreading and proliferation, while laminin-521 decreases cell movement and promote spreading and proliferation. When presented as adjacent substrates however, laminin 1 acts as a non-permissive substrate for migration and invasion, causing cells to form a boundary at the junction between substrates lacking laminin-111 and those containing any laminin-111. Laminin-111 also affects cell-cell adhesion through changes in the localization of vascular endothelial (VE) cadherin. Ectoplacental cone explants become single cells or very small groups on laminin-111 and VE-cadherin is lost from regions of cell-cell contact. In contrast, trophoblast cells maintain strong cell-cell contacts on substrates of laminin-521, and VE-cadherin is maintained in all regions of cell-cell contact. Embryonic laminin-111 is essential for formation of Reichert’s membrane, while uterine laminin-521 is required for trophoblast invasion and proper morphogenesis of the yolk sac placenta. Trophoblast cells interact with laminin-111 using integrin alpha7beta1, whereas they bind to laminin-521 using both integrins alpha7beta1 and alphaVbeta3. On laminin-521 these two receptors mediate different behavior; alpha7beta1 promotes migration while alphaVbeta3 promotes spreading. These results show that laminin isoforms influence the direction and quality of invasion of trophoblast cells during implantation, and provide epigenetic cues that drive the morphogenesis of the yolk sac placenta.

S1.2. Extracellular Matrix Remodeling at the Maternal-Fetal Interface

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In mammals, pregnancy leads to extensive adaptations of the uterine microenvironment to receive and implant the embryo. Uterine adaptation includes the formation of the decidua and consequent remodeling of the endometrial extracellular matrix (ECM) components. This is specially so in the mouse uterus where remarkable modifications of fibrilar and non fibrilar
ECM occur, particularly in the periimplantation period. Very thick collagen fibrils appear around mouse decidual cells as soon as decidualization is triggered. Incorporation of tritiated proline by decidual cells and posterior radiolabeled detection of thick fibrils with irregular profile have been observed. However, there are evidences that, at least in part, fibril thickening results from the lateral aggregation of previously existent thin collagen fibrils. It is remarkable that collagen fibrils of the interimplantation sites and those of the nondecidualized stroma of the implantation sites remain unmodified. These thick fibrils are composed by, at least, collagen types I, III and V. In addition, an unusual homotrimeric form of collagen type V was identified in the decidualized endometrium of implantation sites but not in the nondecidualized stroma of the interimplantation sites. In mice, besides being degradated by metalloproteinases, collagen fibrils are also phagocitosed by both fibroblasts and decidual cells. At the maternal-fetal interface, collagen type III is the predominant type of collagen suggesting this collagen type favor the embryo-maternal interactions. Besides collagen, adhesives glycoproteins, such as fibrillin 1 and particularly glycosaminoglycans and proteoglycans, are deeply affected by embryo implantation and decidualization. Hyaluronan, which is present in the whole endometrium before decidualization, nearly disappears from the decidualized estroma. In parallel, the aggregating proteoglycan versican increases in the decidualized areas. Moreover, the SLRPs decorin and biglycan also show a differential expression in the endometrium during pregnancy. Before embryo implantation, decorin is present in the endometrium, disappearing as soon as the decidua is formed. Contrarily, biglycan is highly expressed in the decidualized areas. Experimental studies demonstrated the important role of the ovarian steroids hormones on ECM remodeling. All these results together indicate a key role of ECM at the maternal fetal-interface.

S1.3. Hormone-Regulated Expression and Distribution of Versican in Mouse Uterine Tissues

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Remodeling of the extracellular matrix is one of the most striking features observed in the uterus during the estrous cycle and after hormone replacement. Versican (VER) is a hyaluronan-binding proteoglycan that undergoes RNA alternative splicing, generating four distinct isoforms. This study analyzed the synthesis and distribution of VER in mouse uterine tissues during the estrous cycle, in ovariectomized (OVX) animals and after 17beta-estradiol (E2) and medroxyprogesterone (MPA) treatments, either alone or in combination. Uteri from mice in all phases of the estrous cycle, and animals subjected to ovariectomy and hormone replacement were collected for immunoperoxidase staining for versican, as well as PCR and quantitative Real Time PCR. In diestrus and proestrus, VER was exclusively expressed in the endometrial stroma. In estrus and metaestrus, VER was present in both endometrial stroma and myometrium. In OVX mice, VER immunoreaction was abolished in all uterine tissues. VER expression was restored by E2, MPA and E2+MPA treatments. Real Time PCR analysis showed that VER expression increases considerably in the MPA-treated group. Analysis of mRNA identified isoforms V0, V1 and V3 in the mouse uterus. These results show that the expression of versican in uterine tissues is modulated by ovarian steroid hormones, in a tissue-specific manner. VER is induced in the myometrium exclusively by E2, whereas MPA
induces VER deposition only in the endometrial stroma. The intricate modulation of this proteoglycan by ovarian sex hormones strongly suggests the relevance of the ECM to promote a receptive uterine microenvironment for embryo implantation and development.

*Selected abstract:* Expression of decorin and biglycan in human normal term placenta and in invasiveness-changed trophoblast pathologies
Alexandre Urban Borbely (USP, Brazil) See Poster Session Abstracts pg 23

**S2.1. The Extracellular Matrix as a Modulator of Tumor Invasion and Metastasis**

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The critical role of the tumor microenvironment and the inefficiency of the metastatic process implies that organ specific homeostatic mechanisms, that maintain normal tissue integrity, are robust and inimical to invasive tumor cells. This concept was elegantly demonstrated by Mintz and Illmensee who showed that injection of malignant murine teratocarcinoma cells into a mouse blastocysts resulted in normal, non-malignant tissue formation in genetically mosaic mice. Recently, Hendrix and colleagues demonstrated that a human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of malignant melanoma. Thus the microenvironment of normal tissues posses conserved barriers that must be overcome during formation of the tumor microenvironment at the primary tumor site, during tumor cell invasion, as well as in the subsequent establishment of metastatic foci. Formation of a tumor microenvironment that facilitates tumor progression is complex and requires the removal of intrinsic physical, biological and chemical barriers. This process is initiated by proteolytic remodeling of the ECM and in particular the basement membrane. These barriers have been described as selective pressures for the metastatic phenotype. It is now well recognized that both the malignant tumor cells as well as a variety of host responses, such as angiogenesis and inflammation, are responsible for the evolution of the tumor microenvironment. Furthermore, specific genes play dual roles in that they both antagonize and promote tumor progression. TIMP-2 has multiple roles in the extracellular matrix, in this presentation we will identify, and describe the mechanisms and inter-relationships of the multiple functions of TIMPs in tissue homeostasis, and the tumor microenvironment.

**S2.2. ECM and Soluble Proteins Control Neuronal Cell Migration**

*Cecilia Hedin Pereira (UFRJ, Brazil), hedin@biof.ufrj.br*

*Selected abstract:* April modulates in vitro thymocyte migration and cell adhesion by interacting with HSPG
Cecilia Rocha (FIOCRUZ, Brazil) See Poster Session Abstracts pg 16
Selected abstract: The role of laminin and CXCL12 in T-cell migration during cardiac allogeneic graft rejection

Ariany Oliveira Santos (FIOCRUZ, Brazil) See Poster Session Abstracts pg 17

S3.1. Laminin Peptides Influencing Carcinoma Behavior

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Extracellular matrix (ECM), a three-dimensional network of macromolecules including collagens, laminins, fibronectin, nidogen, and proteoglycans, not only represent a solid support to cells, but also contains cryptic domains, which are exposed by proteolysis and elicit biological responses distinct from intact molecules. Cryptic sites are fragments and bioactive peptides, probably present in all ECM molecules. Laminin present multiple cryptic sites related to adhesion, migration, differentiation, angiogenesis and protease secretion. Our results demonstrated that laminin is cleaved in human carcinoma, generating cryptic sites with relevant biological roles (poster by Pinheiro JJV et al). This prompted us to study effects of laminin-derived peptides in cancer biology. Our initial findings were related to salivary cancer. Adenoid cystic carcinoma, a salivary cancer with recurrence and metastasis, expresses prominent basement membrane. We showed that basement membrane of adenoid cystic carcinoma is 40-fold thicker compared to basement membrane at dermal-epidermal junction. It is obvious from the basement membrane thickness that an overproduction of laminin occurs in vivo in adenoid cystic carcinoma cells. We then decided to study role played by laminin peptides in cells (CAC2) from human adenoid cystic carcinoma. Laminin peptides were SIKVAV, YIGSR, AG73, and C16. Peptides regulate morphology, migration, invasion and protease activity of CAC2 cells. We have also unveiled molecular mechanisms underlying regulatory activities of these peptides. SIKVAV interacts with integrins alpha3beta1 and alpha6beta1, and increases protease activity of CAC2 cells through ERK pathway. Signal generated by AG73 is transduced by syndecan-1 and beta1 integrin, regulating adhesion and protease activity of CAC2 cells. Effects of peptide C16 are similar to AG73. Peptide YIGSR and its receptor 67 kDa combine forces to modulate migratory activities of CAC2 cells. We are currently extending our observations, by studying effects of laminin peptides in different malignant tumors. AG73 regulates invasive biology of human oral squamous cell carcinoma cells (poster by Siqueira AS et al), and C16 regulates genes involved in malignant phenotype of breast cells (poster by Santos E et al). Our results strongly suggest that laminin peptides are expressed in vivo and influence carcinoma behavior. Support: FAPESP (06/57079-4, 08/57103-8), CNPq (470622/2007-5, 304868/2006-0)
S3.2. Platelet Activation as a Novel Target for Galectin-1-Glycan Interactions: Implications in Inflammation, Thrombosis and Metastasis

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Platelets are anucleated blood cells derived from megakaryocytes that are essential for proper hemostasis and thrombosis and also play critical roles in inflammatory processes, tumor metastasis and host defense. When platelets perceive activating signals through their cell surface receptors, they undergo dramatic structural and chemical changes, involving a complex interplay of cell adhesion and signaling molecules. Activated platelets rapidly bind circulating platelets, via membrane glycoprotein complex $\alpha_{IIb}\beta_3$ and fibrinogen, to form a thrombus or a plug for preventing bleeding at sites of vascular injury. However, platelet aggregation can also occlude atherosclerotic arteries causing cardiac and cerebrovascular diseases. Galectins, an evolutionarily conserved family of animal lectins, have recently emerged as novel regulators of immune cell homeostasis, inflammation and cancer. Extracellularly, galectins can bind to N-Acetyllactosamine-containing glycoconjugates present on the cell surface and extracellular matrix and trigger a cascade of transmembrane signaling events leading to apoptosis, cytokine secretion, cell adhesion and migration. In addition, galectins are engaged in intracellular processes that are essential for basic cellular functions, such as pre-mRNA splicing, regulation of cell growth and cell cycle progression. Galectin-1 (Gal-1), a 14.5 kDa member of this family, preferentially recognizes multiple Gal$\beta_1,4$GlcNAc (LacNAc) units, which may be presented on the branches of $N$- or $O$-linked glycans on cell surface glycoproteins. We have recently characterized Gal-1 as a new mediator of platelet activation. Gal-1 binds to human platelets in a carbohydrate-dependent manner and trigger outside-in (platelet spreading) and inside-out (expression of neoepitopes in $\alpha_{IIb}\beta_3$ complex as well as fibrinogen binding) signaling. It also promotes platelet aggregation, secretion of dense and alpha granule content as well as P-selectin and shedding of microvesicles. In addition, Gal-1 favors the generation of leukocyte-platelet aggregates. A further mechanistic analysis revealed the involvement of Ca$^{2+}$, cyclic nucleotide, MAPKinase and IP3/AKT pathways in Gal-1-mediated control of platelet activation. Moreover, endogenous Gal-1 appears to be a mediator of platelet aggregation induced by classical platelet agonists. In conclusion, the control of platelet physiology by Gal-1 is a novel unrecognized role for this lectin with potential implications at the crossroad of thrombosis, inflammation and cancer.

Selected abstract: RECK-mediated inhibition of glioma migration and invasion
Renato Ramos Massaro (USP, Brazil) See Poster Session Abstracts pg 35

Selected abstract: The RECK B isoform is downregulated by simvastatin in human melanoma cells
Fernanda Augusta de Lima Barbosa (UFPR, Brazil) See Poster Session Abstracts pg 34
S4.1. Defining the Heparan Sulfate Code that Increases FGFR Function During Branching Morphogenesis

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Heparan sulfate (HS) mediates high affinity binding of FGFs to their receptors and FGF10/FGFR2b signaling is critical for the development of multiple organs, including the salivary glands. The developmental activities of FGFs are controlled by a number of factors: 1. The gradients that they form by binding HS in the extracellular matrix (ECM), and 2. The size and sulfate modifications present on the HS that bind to both the FGF and the FGFR. We hypothesize that the different binding affinities of FGFs for HS impacts the strength/duration of FGF signaling and the localization of FGF within the ECM. Reducing HS binding affinity of FGF10 by point mutation in the HS binding pocket converted it into a functional mimic of FGF7. In contrast, reducing the affinity of FGF10 for its receptor, did not alter its HS-binding properties, but affected the extent of the response. These studies provide a framework to explain how HS regulates diffusion and gradient formation of HS-binding FGFs through the ECM. We also hypothesize that the location of FGF10-induced proliferation is determined by an epithelial heparan sulfate proteoglycan (HSPG) with specific patterns of sulfation. The 3-O-sulfotransferases (3-OST), which are the largest family of sulfotransferases involved in HS biosynthesis, form the most rare HS modifications. 3-O-sulfated HS binds to antithrombin and the herpes simplex virus. We have identified a novel role for 3-O-sulfated HS, which binds FGFR2b and increases FGF10-dependent epithelial proliferation and morphogenesis. To study the function of 3-O-sulfation, under-3-O-sulfated kidney HS was modified with 3-OST enzymes. The 3-O-sulfated HS increases epithelial proliferation and branching morphogenesis. There was increased FGF10 signaling and cellular differentiation. Reducing Hs3st expression decreased morphogenesis and was rescued by exogenous 3-O-sulfated HS. Further, 3-O-sulfated HS increased the amount of FGF10 bound to FGFR2b in pull-down assays, suggesting it stabilizes the FGF10-FGFR2b complex. Thus, 3-O-sulfated HS localized at the end buds increases FGFR2b signaling resulting in end bud proliferation, differentiation, and branching morphogenesis.

S4.2. Sulfated Polysaccharides Ensures a Carbohydrate-Based Mechanism for Species Recognition During Sea Urchin Fertilization

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The evolution of barriers to inter-specific hybridization is a crucial step in the fertilization of free spawning marine invertebrates. In sea urchins, molecular recognition between sperm and egg ensures species recognition. The jelly surrounding sea urchin eggs is not a simple
accessory structure; it is molecularly complex and intimately involved in gamete recognition. It contains sulfated polysaccharides, sialoglycans and peptides. The sulfated polysaccharides have unique structures, composed of repetitive units of α-L-fucose, β-D-galactose or α-L-galactose, which differ among species in the sulfation pattern and/or the position of the glycosidic linkage. The egg jelly sulfated polysaccharides show species specificity in inducing the sperm acrosome reaction, which is regulated by the structure of the saccharide chain and its sulfation pattern. Other components of the egg jelly do not possess acrosome reaction inducing activity, but sialoglycans act in synergy with the sulfated polysaccharide, potentiating its activity. The system we describe establishes a new view of cell-cell interaction in the sea urchin model system. Here, structural changes in egg jelly polysaccharides modulate cell-cell recognition and species specificity leading to exocytosis of the acrosome. Therefore, sulfated polysaccharides, in addition to their known functions as growth factors, coagulation factors and selectin binding partners, also function in fertilization. The differentiation of these molecules may play a role in sea urchin speciation.

Selected abstract: Structural characterization and anticoagulant properties of a unique heparan sulfate from a mollusk bivalve. Angélica Maciel Gomes (UFRJ, Brazil) See Poster Session Abstracts pg 1

Selected abstract: Fucan and the stimulus in the synthesis of antithrombotic endothelial heparan sulfate: relationship between structure and biological activity. Valquíria P Medeiros (UNIFESP, UFRN, Brazil) See Poster Session Abstracts pg 3

S5.1. Vascular Extracellular Matrix and Vessel Wall Development

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An important factor in the transition from an open to a closed circulatory system was a change in vessel wall structure and composition that enabled the large arteries to store and release energy during the cardiac cycle. The component of the arterial wall in vertebrates that accounts for these properties is the elastic fiber network organized by medial smooth muscle. In most animals with a closed circulatory system, pulse pressure and overall blood pressure increase as the cardiovascular system matures during fetal development and early postnatal life. As blood pressure increases during this period, the smooth muscle cells (SMC) change the types and amounts of extracellular matrix (ECM) they produce in order to strengthen the vascular wall and maintain the appropriate mechanical properties. By changing the amount of elastin in the vessel wall through elastin gene deletion or elastin transgene expression, we show that cardiac and vascular development are physiologically coupled, and we provide evidence for a universal elastic modulus that controls the parameters of ECM deposition in the vessel wall. The major changes associated with elastin insufficiency are an increased number of smooth muscle cell layers in the vessel wall and a substantial increase in blood pressure. Interestingly, these animals live a normal life span with no evidence of cardiac hypertrophy. The unique cardiovascular remodeling seen in elastin insufficient mice indicates that the developing vascular cell can adapt its building process to accommodate
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environmental changes and produce an altered overall wall structure that operates at different physiologic setpoints.

S5.2. ECM in Experimental Paracoccidioisis Infection

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Paracoccidioidomycosis (PCM) is a systemic mycosis, caused by the fungus Paracoccidioides brasiliensis (Pb), that affects healthy individuals living in rural areas in Latin America. There are many clinical forms of the disease, but severe forms are characterized by the presence of numerous disseminated granulomatous lesions, anergy in cellular immunity and high levels of specific antibodies, in contrast, mild forms have with few localized granulomatous lesions, preserved cellular immunity and low levels of specific antibodies. Granuloma formation can be interpreted as a host defense mechanism to destroy or contain this fungus and avoid its dissemination. We used susceptible (S) and resistant (R) mice to Pb infection to study the granulomas and evaluated the effect of drugs that interfere with fibrosis, which causes severe sequelae to PCM patients. We analysed the architecture of the granulomas and associated with the presence of morphologically preserved or destroyed Pb, deposition of some extracellular matrix (ECM) components (collagen fibers types I, II, IV, osteopontin, laminin, biglycan, decorin), presence of relevant cytokines to granuloma formation (γ-IFN, TGF-β, TNF-α) and of matrix metalloproteinases (MMP). We detected all the above mentioned ECM elements and could propose some roles for each one: the thick fibers of collagen type I, more abundant in the R mice may be associated with Pb infection containment; the thin reticular fibers of collagen type III may promote the microenvironment for Pb cell ECM interactions; the marker of newly formed vessels collagen type IV may promote Pb dissemination and favor the influx of inflammatory cells and the proteoglycans biglycan and decorin, both more abundant in R mice may promote fungal containment. The cytokines TNF-α and γ-IFN, this later conspicuously more observed in R mice may promote macrophage activation, enhancing Pb killing by these cells and the control fungal dissemination; TGF-β, more observed in S mice may promote deactivation and inhibition of Pb killing by macrophages, favoring fungal dissemination and osteopontin may favor infection at its onset and promote protection later, because it is more prominent respectively in S and in R mice at these time points. MMP-9 was detected in both S and R mice with active infection. We treated S mice with drugs that interfere with fibrosis. The best indicators of successful local P. brasiliensis lysis were the presence of compact granulomas, required to contain P. brasiliensis, of a continuous deposit of collagen type I arranged in concentric orientation and the production of high concentration of cytokines IL-12 and γ-IFN as well as of NO. Based on these parameters, we can conclude that therapy with γ-IFN and / or Tetracycline seems promising, reducing the fungal load and altering the granulomas architecture to provide P. brasiliensis containment without excessive fibrosis. Grants: FAPESP 06-60091-6, 07/56745-3 e CNPq 307492/2006-0
Selected abstract: Laminin alpha5 expression after human myoblast transplantation into Rag-/- Gammac-/- mice

Ariany O Santos (FIOCRUZ, Brazil) See Poster Session Abstracts pg 42

Selected abstract: Schwann cells transdifferentiation: possible role of myofibroblast in neural fibrosis during leprosy

Rafael Petito (FIOCRUZ, Brazil) See Poster Session Abstracts pg 45,46

S6.1. Heparin Cofactor II Modulates the Response to Arterial Injury

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Thrombin is a key participant in blood coagulation, wound healing, and inflammation. Heparin cofactor II (HCII) circulates in the blood and inhibits thrombin in the presence of dermatan sulfate (DS) or heparin. HCII-null mice do not develop spontaneous thrombosis, but they form thrombi more rapidly than wild-type mice after disruption of the carotid arterial endothelium. IV administration of DS prolongs the thrombosis time of wild-type but not of HCII-null mice. Injection of either wild-type recombinant HCII or a variant with low affinity for heparin corrects the abnormally short thrombosis time of HCII-null mice, while a variant with low affinity for DS has no effect. HCII is undetectable in the wall of the uninjured carotid artery. After endothelial disruption, it becomes concentrated in the adventitia, where it binds specifically to DS. These results suggest that HCII interacts with DS in the vessel wall after disruption of the endothelium and that this interaction regulates thrombus formation. Human studies indicate that plasma HCII levels are inversely correlated with in-stent restenosis (neointimal smooth muscle cell accumulation) and atherosclerosis. Atherogenesis is induced in apoE-null mice by feeding them a high-fat diet. After 12 weeks, mice that are also HCII-null have ~64% larger plaque areas in the aortic arch than HCII wild-type mice. Neointima formation is induced by mechanical dilation of the common carotid artery. Three weeks after injury, the neointimal area is 2-3-fold greater in HCII-null than in wild-type mice. DS administered intravenously within 48 h after injury inhibits neointima formation in wild-type but not in HCII-null mice. Thus, HCII deficiency promotes atherogenesis and neointima formation, and treatment with dermatan sulfate reduces neointima formation in an HCII-dependent manner.
S6.2. Prion Protein Binding to Laminin: Neurotrophic Properties to be Explored in Degenerative Diseases

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Flavio Beraldo1, Camila Arantes1,2, Kill Lee1 Cleiton Machado1,4, Fabiana Caetano5, Nicole Queiroz1,4, Marilene Lopes1 Margareth Magdesian6, Tatiana Américo7, Rafael Linden7, Marco Prado5,8 and Vilma Martins1.

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The prion protein (PrPc) is a highly conserved cell surface glycoprotein expressed in the Central Nervous System. Its conformationally modified isoform, PrPsc, is responsible for prion diseases. Multiple and diverse functions of PrPc have been reported which strongly suggests that loss-of-function have a participation in the pathogenesis of prion diseases. PrPc is a specific ligand for a peptide at the carboxi-terminal of the laminin γ-1 chain (RNIAEIIKDI) promoting neuritogenesis and modulating neuronal plasticity. In fact, this interaction promotes short and long-term memory formation. Using an intracellular Ca2+ probe, Fluo-3 AM, we verified that the γ-1 peptide, in particular its KDI domain, increased intracellular Ca2+ in wild type neurons whereas no effect was observed in PrPc-null neurons. Neuritogenesis as well as Ca2+ signaling were abrogated by U73122, a specific inhibitor of the phospholipase C (PLC) and by 2APB, a specific inhibitor of inositol 3-phosphate receptor at the endoplasmatic reticulum. Remarkably, inhibition of a group 1 metabotropic glutamate receptor (mGluR1) with LY367385 decreased the effect of PrPc-γ-1 interaction upon mobilization of intracellular Ca2+, and blocked the activation of PKC. These data are consistent with the hypothesis that PrPc interacts with extracellular ligands and a variety of transmembrane proteins can be recruited to a multi-component cell surface complex. They also support the idea that PrPc has neurotrophic properties that can be explored not only in prion disease but also in other neurodegenerative illnesses. Supported by – FAPESP, CNPq, FAPERJ, Institutos do Milenio, FINEP, PrioNet Canada, and HHMI.

Selected abstract: Glycosaminoglycan anticoagulant properties from atherosclerotic lesion

Lisandra A C Teixeira (UFRJ, Brazil) See Poster Session Abstracts pg 2
S7.1. Laminin and Stem Cell Therapy to Treat Spinal Cord Injury

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Spinal cord injury (SCI) involves a complex pathophysiology, in which the primary injury is followed by a secondary reaction, where inflammation plays a major role. Research efforts to treat SCI have focused on developing strategies to either decelerate secondary damage or rebuilding the nervous tissue, overcoming the presence of growth-inhibitory molecules and the poor regenerative potential of adult central nervous system. Here we report the positive outcomes of treating acute SCI either with a biomimetical polymer of the extracellular matrix protein laminin or with the injection of mesenchymal stem cells isolated from human adipose tissue (hAT-MSC). Laminin has been largely implicated in neural development and regeneration in both peripheral and central nervous system, but in spite of that, there were no reports of exogenous laminin contributing to improve the outcome of experimental SCI. We had previously described that laminin underwent fast self-polymerization upon pH acidification, giving rise to a biomimetical polymer, morphologically identical to natural laminin matrices. Local injection of such polymers largely improved motor function after thoracic compression or transection. Polymerized laminin presented both anti-inflammatory and regenerative effects, increasing tissue preservation, reducing macrophage and neutrophile infiltration and the levels of systemic inflammatory markers such as TNF-alpha, IL-1beta and C reactive protein. Fluorogold-labeled neurons were detected in the spinal cord, brain stem and motor cortex, indicating re-growth of short and long fibers across transection. In a parallel study we tested the potential of hAT-MSC in improving recovery from cord transection in rats. Human MSC are known to negatively modulate the immune system, which permits cross species studies. Acute local injection led to an increase of 200% of the score registered in the open-field locomotor test BBB at the eighth week after lesion. Such improvement was only observed after 4 weeks, which is compatible with a regenerative effect. This was confirmed by the enhanced expression of the neuronal regeneration marker GAP-43 and of the number of fluorogold-labeled fibers. We propose that acid-polymerized laminin and hAT-MSC are promising therapeutic agents to treat human SCI.

S7.2. The Use of Xeno-Free, Defined Extracellular Matrices in the Derivation of Human Embryonic Stem Cells for Clinical Applications

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The therapeutic application of most human embryonic stem [hES] cell derivatives has been hindered by the exposure of the lines to culture components of animal or human origin. The vast majority of the existing hES cell lines have been derived and continued to be maintained with mouse or human fibroblasts as feeder layers and matrices which represent a
complex mixture or isolated proteins of animal and human origin. Products of animal origin are of concern for infection with recognized or as yet unrecognized infectious agents and diseases such as HIV-1 or HIV-2, hepatitis B or C among others can be transmitted from human donor cells to a recipient in organ or tissues allotransplantations. In this context, it is clear that the derivation and establishment of new human embryonic stem cell lines for therapeutic applications should be performed in defined conditions, ideally in the total absence of components of animal or human origin. Feeder-independent, defined hES cell culture media has been successfully used for the culture of hES cells (Ludwig et al., 2006) for some time. However, the extra-cellular matrix remains a problem as the purified human components of the matrix are expensive to produce and represent a potential source of contamination. More recently, a xeno-free, defined extracellular matrix substitute was launched [CELLStart, Invitrogen™] and proved to be highly effective for the culture of established hES cell lines in a feeders-free system using a defined medium[Stem Pro, Invitrogen™]. One of the areas that needs further research and improvements is the initial derivation process, which seems to depend on unknown components of the medium or of the extracellular matrix and has proven to represent a major challenge for scientists to achieve good repetitive results in a totally defined culture system.

**Selected abstract:** Effect of dermatan sulfate in the migration and proliferation of endothelial progenitor cells after arterial injury in mice

*Juliana A P Godoy (UNICAMP, Brazil)* See Poster Session Abstracts pg 40

**Selected abstract:** Quail neural crest cell differentiation: the effects of FGF2 and microenvironment

*Denise A Bittencourt (UFSC, Brazil)* See Poster Session

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**01. BIOCHEMISTRY OF ECM**

**01.1- Structural Characterization and Anticoagulant Properties of a Unique Heparan Sulfate from a Mollusk Bivalve**

*Angélica Maciel Gomes, Eliene Oliveira Kozlowski, Vitor Hugo Pomin, Cristiano Lacerda Reis, Mauro Sergio Gonçalves Pavão*

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Heparin-like glycosaminoglycans are an important subset of complex acidic polysaccharides that represent the third major class of biopolymer. These molecules are very heterogeneous in terms of molecular weight, charge density, physicochemical properties and biological activities. Heparin-like structures appeared very early in metazoan evolution and have been preserved in complex organisms. The heparin is used as an exogenous anticoagulant compound and its effect is due the formation of a ternary complex with antithrombin and the different serine proteases of the coagulation cascade. However, heparin
has a large hemorrhagic effect and its use is very restricted. The present study focuses on the structural and anticoagulant characterization of a heparin-like polymer isolated from the tissues of a bivalve mollusk. Interestingly, although the polymer resists heparinases I and III, it is just partially cleaved by nitrous acid, as observed by gel filtration chromatography, where two peaks, denominated P1 (~30-40 KDa, nitrous acid-resistant fraction) and P2. In vivo assays demonstrated that at the dose of 1 mg/Kg, the HS-like glycan was able to inhibit thrombus growth in photochemically injured arteries without any bleeding effect. In the model of venous thrombosis this compound was capable to decrease the thrombus size. In conclusion, our results suggest that this molecule constitute potential therapeutic compound as alternative to heparin.

**Keywords:** heparan sulfate, glycosaminoglycans, anticoagulant, antithrombotic

01.2- Effects of Oversulfated and Fucosylated Chondroitin Sulfates on Coagulation: Challenges for the Study of Anticoagulant Polysaccharides.

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We report the effects of a chemically oversulfated chondroitin sulfate and a naturally fucosylated chondroitin sulfate on the coagulation system. The former has been recently identified as a contaminant of heparin preparations and the latter is a glycosaminoglycan extracted from extracellular matrix of sea cucumber. The mechanism of action of these polymers on coagulation is complex and target different components of the coagulation system. They have serpin-independent anticoagulant activity, which preponderates in plasma. They also have serpin-dependent anticoagulant activity but differ significantly in the target coagulation protease and preferential serpin. Their anticoagulant effects differ even more markedly when tested as inhibitors of coagulation proteases using plasma as a source of serpins. It is possible that the difference is due to the high availability of fucosylated chondroitin sulfate whereas oversulfated chondroitin sulfate has strong unspecific binding to plasma protein and low availability for the binding to serpins. When tested using a venous thrombosis experimental model, oversulfated chondroitin sulfate is less potent as an antithrombotic agent than fucosylated chondroitin sulfate. These highly sulfated chondroitin sulfates activate factor XII in vitro assays, based on kallikrein release. However, only fucosylated chondroitin sulfate induces hypotension when intravenously injected into rats. In conclusion, the complexity of the regulatory mechanisms involved in the action of highly sulfated polysaccharides in coagulation requires their analysis by a combination of in vitro and in vivo assays. Our results are relevant due to the urgent need for new anticoagulant drugs or alternative sources of heparin.
Keywords: antithrombotic drugs, heparin, sulfated polysaccharides

01.3- Anticoagulant Activity of aHeparin from Ascidian Phallusia Nigra (Chordata: Tunicata)

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Heparin is a well-known glycosaminoglycan for its ability to prevent blood coagulation. Although heparin from mammalian origin is clinically used as an anticoagulant drug, the appearance of bovine prion diseases and the haemorrhagic effect of high doses of heparin have limited its use. Therefore to look for new sources of heparin-like compounds is an important goal of glycobiologists. In this work, a heparin-like glycan was extracted from ascidian Phallusia nigra and purified using anion-exchange chromatography on a DEAE-cellulose and MONO-Q/FPLC columns. The ascidian heparin has a low molecular weight glycan (~12 KDa), as determined by PAGE in barbital buffer. The anticoagulant activity was measured by the aPTT assay, in which it showed a ~60-fold lower anticoagulant activity (3 units/mg) than bovine heparin (180 units/mg). Ascidian heparin is able to inhibit factor Xa via antithrombin (IC50 = 3.14 μg/mL), but thrombin inhibition was very lower (IC50 = 0.74 μg/mL), as comparing with mammalian heparin (IC50 = 0.143 μg/mL and IC50 = 0.90 ng/mL, respectively). Taken together, these results point to a selective activity of the ascidian heparin as a factor Xa inhibitor. A detailed study about the structure of this heparin is being performed to establish a correlation among the low molecular weight, structural features and anticoagulant activity.

Keywords: ascidian, anticoagulant, glycosaminoglycan, heparin

01.4- Age-Related Changes in Glycobiology of Lumbar Intervertebral Discs in Mice

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Low back pain (LBP) is a serious public health problem, and the main cause of early retirement in many developed countries. Approximately 80% of the world’s individuals will suffer, at least once, from a back pain episode, during the lifetime, and a few percentage will evolve to a chronic pathology. Most of the cases involve back lifting muscles and movement dysfunction. The Lumbar Intervertebral Discs (LID) seen to be responsible for 40% of the cases and is the most damaged structure of the condition. The main pathologic feature of the discs is the decrease of glycosaminoglycans (GAGs) concentration and the length of their chains. Studies about models of degeneration are important for the establishment of new
therapies or treatment for LBP. In this work, we performed a glycobiology study of LIDs in mice. LIDs from C57 black-6 of 6, 12 and 24 weeks were dissected. The GAGs were extracted and purified. The pure samples were analyzed by agarose and polyacrylamide gel electrophoresis, gel-filtration and ion exchange chromatography. The samples exhibit molecules of chondroitin sulfate and dermatan sulfate. The concentration of GAGs decreases with aging and there are a change in the proportions of the disaccharide units, with decrease of UA-GalNAc (6S) and increase of UA-Gal NAc (2,4 S). The results can show how aging affects the progression of disc degeneration, which could became a good model to study LBP.

**Keywords:** Aging, Disc Degeneration, Glycosaminoglycans, Intervertebral Disc

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### 01.5- Glycosaminoglycan Expression In Co-Cultures of Endothelial Cells and Smooth Muscle Cells. Endothelial Cells Have a Vectorial Secretion Phenotype

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It is known that glycosaminoglycans (GAG) play important roles in several cellular processes. Heparan sulfate (HS) could bind and modulate proteins related to hemostasis, and the exposure of endothelial cells (EC) to heparin (hep) stimulates the synthesis of an antithrombotic HS. Since co-cultures of EC and smooth muscle cells (SMC) are one of the most similar *in vitro* model to represent the vessel wall, co-culture experiments using a permeable polyethylene terephthalate (PET) support that isolates the apical (luminal) and basal compartments were performed to study GAGs expression of co-cultured ECs with SMCs (EC/SMC; SMC/EC). Another goal of this study was to analyze the vectorial secretion of GAGs by ECs cultured alone (EC/-) since a characteristic heparan sulfate proteoglycan (HSPG) of ECs (syndecan-4) is shedded to the culture medium. Scanning electron microscopy (SEM) analysis of EC/SMC co-cultures showed minor alterations in EC and SMC morphology, with preservation of the polygonal shape. PET cultured ECs showed a major adhesion phenotype when compared to ECs cultured in regular glass coverslip. GAG biosynthesis was measured by metabolic labeling with 150 μCi [35S]-sodium sulfate/mL for 18h in EC/-, SMC/EC and EC/SMC and SMC/- cultures. It was shown that EC/- cultures have a vectorial GAG secretion pattern, in which over 95% of all secreted GAG is addressed to the luminal environment, and only residual GAG amounts were observed in the basal compartment. In constrast SMC/- showed significant amounts of GAGs secreted to the basal compartment, corroborating the vectorial secretion phenotype observed in EC/- cultures. In the co-culture studies (SMC/EC), the expression of HS present in the cellular fraction by EC was reduced 50%, while the other co-cultures studies showed no significant differences regarding GAGs expression. The treatment of EC cultures with heparin both at the basal compartment and/or at the apical compartment stimulates the synthesis of HS secreted only for apical culture medium. This treatment had no influence in the vectorial GAGs secretion.
phenotype. These combined results indicate that the GAGs secretion is vectorial in EC cultures, and the heparin treatment suggests the relevance of the GAG secretion mainly to the vessel lumen in the modulation hemostasis.

**Keywords:** glycosaminoglycans, heparan sulfate, vectorial secretion, co-culture, vascular biology

01.6- Glycosaminoglycan Anticoagulant Properties from Atherosclerotic Lesions

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In order to investigate if the concentration and/or the anticoagulant activity of arterial wall glycosaminoglycans (GAG) are affected in atherosclerosis we compared normal segments from human aorta (78-year-old woman) with segments exhibiting different grades of atherosclerotic lesions, fibrous plaque (atheroma) or calcified ulcerated lesions. Atheroma and complicated lesions presented significant lower glycosaminoglycan concentrations, 1.52 ± 1.60 (n=15) and 0.97 ± 0.53 (n=18), respectively, when compared with normal segments, 2.41 ± 1.08 µg hexuronic acid / mg tissue dry weight (n=11). The anticoagulant activity of aortic GAGs was evaluated by a thrombin amidolytic assay using plasma as a source of thrombin inhibitors or purified antithrombin (AT) or heparin cofactor II (HCII). As indicated by the concentration of GAG necessary to inhibit by 50% the thrombin activity (IC50), no significant difference was observed in the anticoagulant properties of the GAGs from normal and lesionated areas. Thus, the IC50 was 1.40±0.83, 1.11±0.83 and 1.83±0.86 µg/ mL for normal, atheroma and complicated lesions areas, respectively. Similar results was observed when purified AT or HCII was used instead of plasma. Fractions enriched in heparan sulfate (HS) or chondroitin sulfate plus dermatan sulfate (CS+DS) from the three groups were obtained by anion exchange chromatography. All HS fractions exhibit the same anticoagulant properties. Surprisingly, CS+DS fractions from lesionated areas present a higher anticoagulant potential in the presence of AT. This activity was not altered by chondroitinase ABC treatment, indicating the presence of heparin-like chains. Our results show that atherosclerotic lesions present lower GAG content, however this anticoagulant property is not affected.

**Keywords:** atherosclerosis, anticoagulant, glycosaminoglycan, heparan sulfate
01.7- Integument of Adult Lithobates Catesbeianus: Glicoconjugates and Glycosaminoglycans Identification and Localization

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The distribution of specific carbohydrate moieties in the amphibian integument can be relevant due to its functions since the integument has been involved in water and ion movement. Glycosaminoglycans (GAGs) are long unbranched polysaccharides of a repeating disaccharide unit, and are important to water retention and electrolyte control. Lectins as non-immune proteins or glycoproteins are widely used as probes to detect different type glycoconjugates. The main purpose of this work was to identify GAGs and localize glycoconjugates in the adult Lithobates catesbeianus integument. Adult males were obtained from a commercial farm in Rio de Janeiro State (ethics committed DAHEICB-021). Integument fragments were processed according to standard histologic techniques. Slices were stained with hematoxylin-eosin (HE), periodic acid-Schiff method (PAS), Alcian blue (AB) staining at pH 1.0 and pH 2.5 and toluidine blue O. For biochemical assay, crude GAGs were applied to Mono-Q FPLC column and after agarose-gel analysis. Previously investigation by thin-layer chromatography (TLC) was employed to drive the lectin choose. Therefore, the sections were incubated with following biotinylated lectins: AAA (fucose), WGA and STL (N-acetyl-glucosamine), PNA (galactose) and Jacalin (acetyl-galactosamine). In the spongious dermis, the mucous glands exhibited AB and PAS positive reaction, while the serous and granular glands showed no reaction to both methods; however, some cells of the small serous glands revealed alcianophilic reaction at apical cytoplasm, suggesting other gland type constituted by mucous and serous cells, being classified as mixed gland. The Eberth-Katschenko (EK) layer that occurs between the spongious dermis and the compact dermis demonstrated weak alcianophilic reaction as well as the hypodermis. Through lectin histochemistry, N-acetyl-glucosamine was observed between keratinocytes of the corneum and spinosum strata as well as in the mucous gland besides the subepidermal connective tissue and the EK layer. Galactose was identified only in the mucous gland, but the acetyl-galactosamine is visualized in the mucous and small serous gland and in the EK layer. The biochemical results indicated dermatan sulfate and hyaluronan in the integument. This work indicate that some glycoconjugates are specific for epithelial secretory products while others can participate in GAGs structure, important to structural extracellular matrix maintenance.

Keywords: amphibians, integument, lectin, glycoconjugate, glycosaminoglycans
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01.8- Nanoparticles of Heparins of Marine Invertebrates: Anti-Inflammatory Effect on Model of Inflammatory Bowel Disease.

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Heparin is a polysaccharide of animal origin, mainly used as an anticoagulant in the prevention and treatment of deep vein thrombosis. Recent pre-clinical studies have also shown a potent anti-inflammatory effect of this glycosaminoglycan. Despite highly used in clinical practice, heparin has a high-risk of bleeding and its pharmacological effect is only observed after parenteral administration. Therefore, the search for heparin analogues with lower side effects and better oral activity it is extremely important. In this context, several studies have shown promising results. For example, several works indicate the presence in marine invertebrates of heparin-like glycans containing low anticoagulant activity, potent anti-inflammatory effect and no bleeding tendency. It has been shown recently that nanoparticles increase the oral bioavailability of macromolecular drugs, including heparin. The present work describes the effect of nanoparticles of heparin analogues from marine invertebrates in an experimental model of inflammatory bowel disease (IBD) in rats. Preliminary results showed efficient formation of nanoparticles of heparin, with a good range of distribution and size. Performance and efficiency assays of encapsulated heparin, as well as releasing tests in different pH ranges were satisfactory. In IBD, increased production of TNF-α is associated with tissue damage mediated by immune response of the cellular infiltrate. Rectal administration of TNBS induced significant increase in the levels of TNF-α in inflamed colon. The evaluation of the anti-inflammatory action of the heparin nanoparticles in animals revealed a great reduction in cellular infiltrate and other pro-inflammatory parameters, in addition to a drastic reduction in the levels of TNF-α. These results indicate that heparin nanoparticles possess anti-inflammatory effect in an animal model of IBD. Financial support: CNPq, FAPERJ.

**Keywords:** Glicosaminoglicanos, Matriz extracelular, Doença inflamatória intestinal, Doença de Crohn
01.9- Fucan A and (The Stimulus in the Synthesis of Antithrombotic Endothelial Heparan Sulfate: Relationship Between Structure and Biological Activity

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Fucans is the term used to define a family of α-L-fucose sulfate. The high density of negative charges of fucans may be related to biological activities shown by these polysaccharides. Studies show that fucans of seaweed have pharmacological actions as potential drugs anticoagulant, antithrombotic, antitumor, antiinflammatory, among others. In this work will be investigated the relationship between structure and activity anticoagulant and antithrombotic of the fucan A extracted from marine brown algae Spatoglossum schröederi. The fucan A was submitted to carboxyirreduction and dessulfatation. The fucan A native (nFucA), carbozirreduced (cFucA), partially (dFucA2h) and totally (dFucA8h) dessulfated were evaluated by chemical and physical-chemical methods. The anticoagulant effect in vitro was investigated by the method of USP, and antithrombotic activity in vivo by venous thrombosis induced by obstruction of the inferior vena cava in Wistar rats. The able to stimulate of the synthesis of antithrombotic heparan sulfate (HS) in rabbit aorta endothelial cells (RAEC) was analyzed in the presence or absence of native or modified fucan A. This same effect was investigated when the cells were exposed to the polysaccharides together with cloroquin or when they were exposed to the fucan at 4ºC. The result suggests that the fucan A may stimulate HS synthesis indirectly through their interaction with the extracellular matrix (ECM). To confirm this hypothesis, fucan A was labeled with biotin. This probe was used to quantify the number of sites of fucan A on endothelial cells by an ELISA-like assay by using streptavidin conjugated with europium. By confocal microscopy fucan A was detected bound on the ECM and was co-localized with fibronectin. Our results suggest that the fucan A from S. schröederi is a potent antithrombotic agent, which gives more prolonged effect than heparin in a model of venous thrombosis in rats. This effect is evidenced by the ability of fucan A to increase the synthesis of HS by RAEC. As heparin, the effect is mediated by binding of fucan A to ECM of endothelial cells, possibly by interaction as fibronectin and integrin signaling pathway. The results also suggest that sulfate is essential for the biological activity of fucan A since that 50% reduction in the content of sulfate abolishes the effect of cellular and venous thrombosis. CNPq, CAPES e FAPESP

Keywords: Antithrombotic activity, Fucan, Rabbit aorta endothelial cells, Structure conformation of fucans, Extracellular matrix
01.10- Non-Invasive Marker for Detection of Hepatitis C Virus Infection: Increased Serum Hyaluronic Acid in Brazilian Asymptomatic Blood Donors

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Background: Infection of Hepatitis C virus (HCV), a public health problem worldwide and can be asymptomatic for years. The main injury caused by HCV is the hepatic fibrosis, as a result of a chronic inflammatory process in the liver characterized by the deposit of components from the extracellular matrix. The gold standard method to evaluate the liver damage associated with HCV is the biopsy and searching for non-invasive marker tests proposed a series of them like platelet count, serum levels of gamma-glutamyl transferase (G-GT), alanine aminotransferase (ALT), hyaluronic acid (HA) among others. Accordingly hepatic fibrosis progression, serum Hyaluronan (HA) level is increasing. Objective: The purpose of this study is to determine the HCV viral load in asymptomatic infected blood donors and correlate to the content of serum HA, G-GT, ALT and total proteins. Methods: Samples of Brazilian blood donors were screened by two ELISA anti-HCV methods, showed 308 anti-HCV positive samples. HCV-RNA was detected by Nested RT-PCR method followed by HCV genotype characterization by RFLP method; HCV viral load was determined by real-time PCR. Serum determinations of HA, G-GT, ALT and total proteins were performed biochemically; to comparisons, where it was utilized serum determinations of 308 samples of health donors and gender group. Results: Among initial 308 anti-HCV positive samples, they were confirmed 99 HCV-RNA positive samples (32.14%) and most prevalent HCV genotype is 1 (82.83%), followed type 3 (13.13%). The average viral load was 122,371±35,932 copies/mL; no difference was showed between the viral load and HCV genotypes. Increase in serum ALT (9.1%), G-GT (41.4%), and proteins content (8.2%) do not correlated positively to the HCV viral load; in contrast, HA serum determinations showed a positive correlation to HCV viral load. HA content was statistically increased in positive RNA-HCV samples (37.2±3.5 ng/mL) than in only positive anti-HCV one (14.7±1.2 ng/mL) and male HCV infected blood donors showed significant increase in HA (34.0±2 ng/mL) serum content than the females (16.5±1.9 ng/mL).Conclusion: In asymptomatic HCV infected blood donors, serum HA content correlates to the presence of HCV, no genotype type observed difference, indicating that HA could be used as non-invasive marker of the HCV infection. The subjects are asymptomatic, but serum levels of HA indicate a potential histological liver alteration.

Keywords: ácido hialuronico, carga viral, G-GT, transaminase, vírus da hepatite C
01.11- The Effect of Arrabidaea Chica Extract on the Healing of Partially Transected Achilles Tendon of Rats

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The Arrabidaea chica extract has been used for treatment of epithelial lesions in skin and gastric ulceration, however it is unknown how is its effect on the healing of a fibrous tissue like the Achilles tendon. In this work we have analyzed the partially transected Achilles tendons of rats with 60 days. The partial transection was 4 mm from the insertion of the tendon in the calcaneus. Immediately after the transection a concentrated extract of A. chica was applied on the wound before the skin suture and during the consecutive 7 days after the transection. The rats were euthanized and their tendons removed for analysis at 7, 14 and 21 days after the injury. Extracellular matrix (ECM) components of the injured region were extracted with 4M guanidinium chloride in 0.05M acetate buffer pH 5.8. The extract was used for protein dosage, SDS-PAGE and western-blotting. For hydroxyproline (HO-Pro) quantification the tissue was hydrolyzed in 6N HCl and then treated with perchloric acid/cloramine T. A higher concentration of HO-Pro (mg/g dried tissue) was found in tendons treated with the plant extract, 7 (50.21±9.88) and 21 (44.77±4.83) days after injury, compared with their respective controls (without A. chica), where we found 39.33±3.62 and 34.91±1.95 mg/g dried tissue, respectively. Regard non-collagenous protein concentration, a lower concentration was detected in rats with 7 days after injury and treated with A. chica. Western blotting analysis indicated a remarkable presence of type III collagen in the tendon 7 days after transection and treated with the plant extract. No differences were observed at 14 days, but at 21 days more type III collagen was found in injured tendon that received saline in comparison to the transected tendon treated with A. chica extract, probably because the repair process has already been completed. Our results suggest the A. chica extract has a determinant effect on the synthesis of collagen and also on the repairing process. If the treatment improves the organization of the ECM still will be clarified.

Keywords: Achilles tendon, Arrabidaea chica, collagen, extracellular matrix, healing
01.12- Analysis of the MMP-2 and 9 Activities in Achilles Tendon of Rats Submitted to Exhaustive Acute Exercise with Different Resting Periods

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The main function of tendons is transmitting the force arisen from muscles to the bone. The tendon adapts itself to different mechanical stimulus, through a remodeling process involving metalloproteases (MMPs) and collagen synthesis. Although the mechanisms of these processes are not completely known, previous works have shown that the exercise causes alterations in the collagen content and MMPs activities. However, it is missing a study on the effect of exhaustive acute exercise intercalated with different periods of resting on the collagen content and MMPs activity. Male rats were submitted to 3 daily sessions of exhaustive acute exercise with resting periods of 1 and 3h between the sessions. The animals were euthanized at days 1, 3 and 6. For quantification of HO-Pro the tendons were hydrolyzed in 6N HCl, and treated with perchloric acid/chloramine T, and the absorbance read at 550 nm. For detection of MMP-2 and 9 isoforms activities, gelatin zymography techniques were employed, and the densitometry (pixels x 105) of bands was used to evaluate the alterations in the quantity of the latent (72 kDa) and active (62 kDa) MMPs isoforms. Analysis of our results showed a larger presence of the latent isoform at day 1 (65.4±8.4) and of the active isoform at day 6 (35.2±5.5), both with 3h of resting, in relation to the control (30.9±7.9 and 4.6±1.2 respectively for latent and active isoforms). No differences were detected for MMP-9. As regard the HO-Pro content expressed as mg/g dried tissue, all groups exhibited larger concentration (around 111.82±10.45) in relation to the control (85.48±10.12), with 1 and 3h of resting, except at day 3 (96.83±13.2) with 3h of resting. Interestingly at day 6 with 1h of resting, we found more HO-Pro (137.49±13.44) compared with 3h (118.34±9.08), which is according to the smaller presence of the active isoform of MMP-2 found in the 1h resting group. Our results suggest that the 3h pause between the exercise sessions leads to a larger production of the latent and active isoforms of MMP-2, with consequent reduction of collagen.

Key words: Achilles tendon, acute exercise, collagen, metalloprotease
01.13- Sulfated Polysaccharides from Marine Sponges (Porifera): An Ancestor Cell-Cell Adhesion Event Based on Carbohydrate-Carbohydrate Interaction

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Marine sponges (Porifera) are ancient and simple eumetazoans. They constitute key organisms in the evolution from unicellular to multicellular animals. We now demonstrated that pure sulfated polysaccharides from marine sponges are responsible for species-specific cell-cell interaction in these invertebrates. This conclusion was based on the following observations: 1-) Each species of marine sponge has a single population of sulfated polysaccharide, which differ among the species in their sugar composition and sulfate content; 2-) Sulfated polysaccharides from sponge interact with each other on a species-specific way, as indicated by an affinity chromatography assay. This interaction requires calcium; 3-) Homologous, but not heterologous sulfated polysaccharide inhibits aggregation of dissociated sponge cells; 4-) We also observed a parallel between synthesis of sulfated polysaccharide and formation of large aggregates of sponge cells, known as primmorphs. Once aggregation reached a plateau, the demand for de novo synthesis of sulfated polysaccharides ceased. Heparin can mimic homologous sulfated polysaccharide on the in vitro interaction and also as an inhibitor of aggregation of the dissociated sponge cells. However, this observation is not relevant for the biology of the sponge, since heparin is not found in the invertebrate. In conclusion, marine sponges display an ancestor event of cell-cell adhesion, based on calcium-dependent carbohydrate-carbohydrate interaction.

**Keywords:** aggregation factor, proteoglycans, cell aggregation, primmorphs, heparin

01.14- Extracellular Matrix Remodeling Is Improved after Treatment with a Synthetic Peptide Derived From Lopap

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Defective content and remodeling of extracellular matrix (ECM) is a pathological state for some diseases and a hindrance factor for tissue repair. An important event for tissue repairing consists in the synthesis of ECM proteins by fibroblasts in a well-organized and regulated process. This study was designed to investigate the effect of a synthetic peptide, which has been previously characterized as antiapoptotic peptide (AP), on the synthesis of collagen in vivo and its wound healing property in a rat skin lesion-induced model.
Preliminary results demonstrated that this peptide was able to induce the synthesis of ECM proteins such as fibronectin, tenascin and collagen in human fibroblast culture. To evaluate this effect in vivo, mice were treated with local injections of AP (i.d.), and the collagen content was quantified in the dermis by histological analysis and Picrossirius red coloration. The area in the dermis which received AP treatment showed a local increase of collagen content in comparison with a non-treated area, which was injected with saline. This effect was observed one week after treatment and sustained for more than three months. In the rat skin wound healing model, AP or saline was applied locally in the lesions, and the wound closure was observed macroscopic and microscopically. The regenerating tissue was subjected to histological analysis, and to the extraction and quantification of collagen, matrix metalloproteinases (MMPs) and glycosaminoglycans. Results obtained showed a slight improvement of wound contraction, a better tissue remodeling and organization of collagen fibers. The collagen content was substantially increased in the AP-treated lesions. On the other hand, the amount of glycosaminoglycans did not change. A significant increase in MMPs was observed only in the third day after the induction of lesions. Zymographic analysis revealed a band of 62kDa, which corresponds to the active form of MMP-2. This band of activity was most intense in the lesions treated with AP. These data altogether demonstrates that AP is able to stimulate fibroblasts and can improve the process of tissue repair in vivo with greater deposition of collagen and better tissue remodeling. Financial support: Fapesp, Finep and CNPq.

**Key words:** collagen, fibroblasts, Lopap, synthetic peptide, tissue repair

01.15- Unusual Architecture of Collagen V Fiber Related with the Collagen V Alpha-2 Biochemical Profile in Cutaneous Fibroblasts from Systemic Sclerosis

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Background: The collagen V (COLV) is involved in mutations of collagen diseases, such as systemic sclerosis (SSc), which was observed an accumulation of unusual collagen, also demonstrated through SSc developed by immunization of collagen V in health rabbits. Purpose: Our purpose was to analyze the tridimensional reconstruction (3D) and biochemical profile of COLV alpha-1 and alpha-2 chains in skin fibroblasts culture from patients in SSc. Patients and Methods: Skin biopsies of 5 patients according American Collagen of Rheumatology Criteria (mean age: 45.5±11.) and 5 healthy control skin fibroblasts were also obtained from thorax region during mamoplasty (ACR criteria; mean age: 40.6±16.2y). COLV 3D reconstruction was performed in the immunofluorescence confocal microscopy and the COL V expression was analyzed by SDS-PAGE electrophoresis and immunoblotting.
methods. Results: The structure of COL V fiber in 3D reconstruction presented thickness in SSc fibroblasts compared with pattern thin fibers in the healthy controls. We have observed the biochemical profile of the collagen V with the increase of expression of the alpha-2 chain related with controls, besides the alteration of molecular weight of the quoted chain. Conclusion: The overexpression and the unusual organization of COLV fibers, besides the biochemical changes, suggest an interference with the fibrillogenesis process in SSc.

**Keywords:** collagen V, Sistemic Sclerosis, fibroblasts culture, biochemistry

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**02. MOLECULAR AND CELLULAR BIOLOGY OF ECM**

**02.1- Senescence Influences on Igf-1, Androgen Receptor, Matrix Metalloproteinase and Dystroglycan Features in the Prostatic Lesions**

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Senescence is a determining factor for the occurrence of morphological changes in the prostate, considering hormonal imbalance and paracrine signaling disturbance between the epithelium and the stroma. Thus, the objective of this work was to characterize and associate the α and β-dystroglycans (α-DG, β-DG), androgen receptor (AR), matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) and insulin-like growth factor receptor (IGFR-1) immulocalization in the peripheral zone of elderly men's prostate showing benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostate cancer (PC). Sixty prostate samples from post mortem patients, aged 60-90 years were divided into 4 groups: Normal; BPH; PIN and PC. The diagnosis of the prostatic lesions was based on architectural disturbance according to the criteria of Mostofi and Price. The results showed intense IGFR-1, MMP-2 and MMP-9 immunoreactivities in the CP and PIN groups than those found in the other groups. The α-DG and β-DG immunolocalization were weaker in the CP and PIN than those found in the BPH and Normal groups. Intense AR immunoreactivity was characterized in the four groups. Thus, it could be concluded that senescence led to epithelial-stromal imbalance, shown by means of decreased α-DG and β-DG levels and increased IGFR-1, MMP-2 and MMP-9 levels, which are primordial elements for the maintenance of glandular paracrine signaling, contributing to the disease progression. Also, these finding indicated that there were direct correlation between IGF-1 and MMPs, pointing towards IGF-1 as a molecule target in the prostate therapy as well as a possible factor for MMPs positive signalization.

**Keywords:** Dystroglycans, Matrix Metalloproteinases, Prostate, Senescence, Steroid Hormones
02.2- Activity of MMP-2 A Related to the Eruption Rate and Periodontal Ligament Resistance in Rodent Incisors Submitted to Hypofunction

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In the rodent teeth have been demonstrated that the hypofunction condition increases the rate of tooth eruption. The constant movement that occurs in rat incisor eruption is accompanied by intense activity in periodontal ligament remodeling. The ligament extracellular matrix remodeling occurs by direct action of enzymes known as metalloproteinases (MMPs). In this sense, this study investigated the relationship among: 1) the eruption rate of rat incisors, 2) the periodontal ligament resistance strength and 3) the MMP-2 activity in periodontal ligament.

Ten male Wistar rats had their lower left incisors cut, every two days, at the interdental papilla level, using high-drill rotation, after anesthesia by halothane, producing hypofunction condition (HP). Other ten rats were kept in normal condition of eruption (N) and were used as control. The eruption rate was measured with an ocular millimeter from the gingival margin to the top of the tooth in group HP, and even from a mark made in the tooth, previously, in the N. After 1, 7 and 14 days the animals were killed by cervical dislocation under anesthesia, and the tensile strength of the periodontal ligament was measured using an algometry. After extracting, the periodontal ligament of the animals was scraping with a periodontal curette, collected in microtubes containing culture medium DMEM and used to assess activity of MMP-2. It was applied 0.1 and 0.05µg of samples protein total in zymogram (policrylamide 10% gel added with gelatin 5%). In the hypofunctional group (HP) the eruption rate was higher (p<0.01) and the resistance strength of the periodontal ligament was lower (p<0.01) when compared with normal condition eruption group (N). The MMP-2 activity in hypofunctional group was greater at 1 and 7 days (p <0.01). At 14 days there was a tendency for the collagenase activity return to normal. Thus, we conclude that there was a direct relationship between increasing of eruption rate and MMP-2 activity, and decreasing of the force of ligament resistance in hypofunctional group. It may suggest that MMP-2 has a role in the extracellular matrix remodeling process on periodontal ligament of incisor rat.

Keywords: incisor rat, eruption rate, metalloproteinases, hypofunction
02.3- Doxycycline Effects on Eruption Rate, Activity and Expression of MMP-2, MT1-MMP And TIMP-2 In Odontogenic Region of the Rat Incisor Tooth in an Accelerated Eruption Condition.

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Doxycycline is an antibiotic that inhibit the metalloproteinases (MMPs) activity in pathological process but its effects on tissues of the rat incisor tooth were not evaluated yet. Thus, doxycycline effects on the eruption, activity and expression of MMP-2, MT1-MMP and TIMP-2 present in odontogenic region of rat incisor submitted to shortened treatment after 14 days were evaluated. Wistar rats were divided in four groups according to eruption condition. Normofuncional (NF), Doxycycline normofunctional (DNF), Hypofunctional (HP) and Doxycycline hypofunctional (DHP). DNF and DHP groups received daily 80mg/w.b. of doxycycline by gavages during 14 days. The lower left incisor teeth of HP and DHP groups were shortened using a diamond high-speed rotating instrument every two days. The eruption rates were measured using a calibrated grid under a microscope eyepiece every two days where distances (mm) from the gingival margin to the marks made on the teeth (NF and DNF groups) and up to the end of incisor (HP and DHP groups) were recorded after all rats had been anaesthetized with halothane. After cervical dislocation the hemi-mandibles were removed and immersed in paraformaldehyde 4%. After demineralization in EDTA 4,12% the samples were included in paraplast to obtain sections with 5µm. For zymogram and Western Blot methods a window was open in bone that recover the odontogenic region to collect the tissues which were immersed in DMEN or in an extraction buffer. It was applied 0,2; 0,1 and 0,05µg of samples in zymogram (poliacrilamid 10% gels added with gelatin 5%) and 7µg to Western Blot gel. In each group 3 rats were used to immunohistochemical method, 4 rats were used to zymogram method and six rats were used to Western Blotting to MT1-MMP. Monoclonal antibodies were used 1:200 (MMP-2), 1:250 (TIMP-2), 1:1000 (MT1-MMP) and 1:100 to Western Blot. Immunohistochemical and immunoblot results were analyzed by Image J software and by ANOVA and Tukey post test, as well as the eruption rate at P< 0.05 level. The doxycycline did not change the eruption or either activity or expression of molecules studied however, the hypofunctional condition increased only the eruption rate, the expression of MT1-MMP and TIMP-2. It allows us to conclude that MT1-MMP and TIMP-2 but not MMP-2 present in odontogenic region can be involved in eruption process. Ethics Animal:1083-1 Environment:265/2007 UNICAMP.FAPESP

Keywords: eruption, incisor, rat, MMPs, doxycycline
02.4- Features of the Steroid Hormone Receptors on the Ventral Lobe of the Prostate of Rats (Sprague Dawley) During the Senescence and Hormone Replacement

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The senescence is responsible for hormonal imbalance and hormone replacement has been a therapy to restore these steroid levels. Thus, the main objective of this work was to immunolocalize the AR, ERalpha and ERbeta on the prostate ventral lobe in senile rats and to analyze these hormone receptors before and after hormone replacement, associating these results with morphological features. 30 male rats were divided into 6 groups: Senile Control group (SC) received 5 ml/Kg dose of peanut oil subcutaneously; Testosterone group (ST) received 5 mg/Kg dose of testosterone cypionate; Estrogen group (SE) received 0,025 mg/Kg dose of 17beta-estradiol; Castrate group (CA): the rats were castrated by surgery; Castrate-testosterone group (CT): the castrated rats received the same treatment of the testosterone group; Castrated-estrogen group (CE): the castrated rats received the same treatment of the estrogen group. All groups were treated for 20 days and submitted to immunological and morphological analysis. The results showed that there were no significant changes in the prostatic epithelium among the groups: SC, SE and ST. However, hypertrophied elements of extracellular matrix as reticular and collagen fibers were verified in the stroma of the SE and ST groups. Also, inflammatory foci were identified specially in the SE and CE groups. Moreover, intense AR immunoreactivity was characterized on the ventral lobe epithelium from animals of SC and ST groups, moderate in the CT group and weak in the other ones. Moderated AR localization was verified in the stroma of the SC and ST groups and weak in the SE and CT groups. Weak ERalpha immunolocalization was verified in the epithelium of SC, CA and CT groups, moderate in the CE group and absent in the ST group. Weak stromal ERalpha immunolocalization was verified in SC and ST groups, moderate in the CT group and intense in the other ones. Intense ERbeta immunolocalization was characterized in the epithelium of SC group; moderate in ST, SE and CT groups, weak in CA group and absent in the CE one. In the stroma, ERbeta was seen weakly only in the ST group. Thus, the differential distribution of the steroid receptors in both epithelium and stroma compartments of the prostate as the result of hormone alterations, signaled a specific activity in each one of them, pointing to the essential interactions among these steroid hormone receptors in the maintenance of prostatic homeostasis and the promotion of prostatic diseases.

Keywords: Immunohistochemistry, Morphology, Prostate, Senescence, Steroid hormones
02.5- Further Investigation of Crotamine Uptake Mechanisms

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Introduction: Cell penetrating peptides (CPPs) hold great potential as delivery vectors for use in research and medicine. The current use of these CPPs is limited by insufficient knowledge of their uptake mechanism. Previously, we isolated and characterized crotamine, a rattlesnake venom compound capable to penetrate into the cytoplasm and the nuclei. The interaction of crotamine with centrioles and chromosomes during cell proliferation and division suggests that its uptake depends on the cell cycle. Previously, our group demonstrated that crotamine penetrates into cells via endocytosis and interaction with heparan sulfate. We aim to verify: a) the crotamine effect in cell proliferation; b) its uptake in different cell cycle phases; c) its interaction with different syndecans (1 to 4); and d) clathrin and/or caveolin endocytosis involved on crotamine uptake. Methods: For this study, we performed MTT assay, imunofluorescence and flow cytometry using B16-F10, CHO-K1 and mouse peritoneal cells. Crotamine was conjugated with FITC or Cy3 for its visualization into the cell. For the proliferation assay, the crotamine concentration was 0.01-10 µM. To analyze crotamine kinetic into cell cycle, we synchronized the cells in G0/G1 phases and the uptake was verified each four hours, during 28 hours. To verify the colocalization of crotamine with syndecans and the proteins related to endocytosis, anti-syndecans 1-4, anti-clathrin and caveolin antibodies were used. Results: We observed that on the concentration up to 1 µM, crotamine induced higher cell proliferation (up to 30%). After cell synchronization, we analyzed crotamine fluorescence in different cell cycle phases. This CPP did not reveal any difference in treated cultures, it suggests that crotamine uptake is independent of cell cycle. Crotamine did not present specificity for any syndecans tested, the colocalization of these molecules was observed on the plasmatic membrane and in citoplasmatic vesicles. We also observed crotamine colocalization with clathrin and caveolin. Conclusion: Our data indicated that crotamine: a) in low concentration increased cell proliferation; b) uptake did not depend on phase of cell cycle; c) interacted with four syndecans types; and d) can be internalized concomitant by different mecanisms: clathrin and caveolin endocytosis pathways. These data showed that crotamine behaves like others CPPs - Tat and penetratin. Financial support by Fapesp.

Key words: crotamine, syndecan, endocytosis, celular uptake, proteoglycan
02.6- Arginine Supplementation Prevents the Angiogenic Damage in Health Tissues Caused by Radiotherapy

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Introduction: Radiotherapy is used to treat cancer patients but side effects are an inevitable consequence. One of the organs that use to be affected by pelvic irradiation is the urinary bladder because frequently it is included in the irradiated field of a wide variety of pelvic tumors. Some authors show that diet supplementation is a good and inexpensive option to reduce the radiation side effects. Objective: The goal of this study is to evaluate whether L-arginine supplementation can prevent the long-term bladder complications after radiation. Material and methods: Adult Wistar rats were separated into 3 groups: control group (C) – receiving food and water ad libitum; irradiated group (I) – received a unique pelvic radiation dose of 1164cGy; and irradiated group supplemented with L-arginine for 7 days before and 15 days after the irradiation. All animals were killed 15 days after the irradiation. Hematoxilin and Eosin was used to quantify the vascular density and Weigert Resorcin Fucsin to evaluate arterial wall thickness. RT-PCR was used to determine the VEGF and FGF genes expression. One way ANOVA followed by Newman-Keuls test was used to analyze the results. Results: The irradiated group presented a significant reduction in the expression of VEGF (C=0.5±0.1; I=0.1±0.09; I+A=0.5±0.2, p=0.0018) and FGF genes (C=1.2±0.5; I=0.5±0.2; I+A=0.8±0.3, p=0.02 ), in the vessels number (C=15.3±11.25; I=11.19±6.3; I+A=16.19±9.3, p=0.0003) and in the arterial wall thickness (C=9.71±3.9; I=6.8±3.7; I+A=10±3.3, p<0.0001). The arginine supplementation prevented all these alterations. Conclusion: The supplementation with L arginine seems to prevent the bladder wall damage in relation to angiogenesis. These results suggest that arginine supplementation could be efficient in protecting health tissues from radiotherapy side effects. Instituição de fomento: CAPES

Key words: Angiogenesis, Bladder, L Arginine, Radiotherapy

02.7. Glutamine Supplementation Prevents the Collagen Expression and Extracellular Matrix Damage in Health Tissues Caused by Radiotherapy

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Patients who have had pelvic radiotherapy as part of their cancer therapy may experience urinary bladder late effects such as hyperactive bladder, incontinence and dysuria. Some papers show that diet supplementation is a good inexpensive option to reduce the radiation side effects. The nutritional supplementation with L-glutamine amino acid has been shown...
effective in maintaining the colon wall mucosa structure in irradiated rats. The goal of this study is to evaluate whether glutamine supplementation can prevent the long-term bladder complications after radiation. Adult wistar rats were separated into 3 groups: control group (C) – receiving food and water ad libitum; irradiated group (I) – received a unique pelvic radiation dose of 1164cGy; and irradiated group supplemented with L-glutamine for 7 days before and 15 days after irradiation (0.65 g/kg body weight) – (I+G). All animals were killed 15 days after the irradiation. RT-PCR was used to determine the collagen I and III genes expression. Stereology method was used to evaluate the extracellular matrix and the muscle volumetric density with the Image J software. One way ANOVA followed by Newman-Keuls test was used to analyze the results. The extracellular matrix volumetric density was significantly reduced in the irradiated group, while glutamine supplementation prevented the decrement (C=36.84±4.37; I=31.64±5.00; I+G=35.53±2.60, p<0.0001). The expression of collagen I and III genes was also significantly reduced in the irradiated group, while glutamine supplementation prevented the decrement (C=1.067±0.31; I=0.579±0.17; I+G=1.816±0.66, p=0.0009; C=0.99±0.28; I=0.54±0.13; I+G=1.07±0.28, p=0.0080 respectively). On the other hand, the volumetric density of muscle was significantly reduced in the irradiated group, but glutamine supplementation did not prevent this alteration (C=36.43±6.15; I=29.39±7.08; I+G=31.38±3.14, p=0.0001). The supplementation with L glutamine seems to prevent the bladder wall damage in relation to the extracellular matrix volumetric density and collagen expression. These results suggest that glutamine supplementation could be efficient in protecting health tissues from radiotherapy side effects. Instituição de Fomento: CAPES, CNPQ e FAPERJ.

Keywords: bladder, collagen, glutamine, radiotherapy

02.8- Estrogen Imprinting Causes Inhibition of Heparanase-1 Expression in the Adult Rat Ventral Prostate

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Heparanase is an endoglycosidase that degrades heparan sulfate on the cell surface and extracellular matrix. The physiological functions of heparanase include heparan sulfate turnover, embryo development, hair growth, and wound healing. It is implicated in a variety of pathologies, such as tumor growth, angiogenesis, metastasis and inflammation. Heparanase overexpression in a variety of malignant tumors suggests that it could be a target for anticancer therapy. Heparanase activity can be regulated by androgens in the rat ventral prostate (VP) (Augusto et al., Cell Tissue Res 332:307, 2008) and by estrogens (Xu et al., Hum Reprod. 22(4):927, 2007) in human endometrium. The promoter region of heparanase gene have estrogen responsive elements (EREs), suggesting that the gene is highly regulated by estrogens. In this work, we investigated the expression of heparanase-1 in the VP of 90-day-old rat after neonatal exposure to high dose estrogen. Western blotting and RT-PCR revealed a marked reduction in the content of heparanase-1 and its mRNA. Immunohistochemistry showed that heparanase-1 could be found in the stroma but not in the epithelium. To
determine whether epithelial absence of heparanase-1 was due to pre- or post-transcriptional regulation we isolated epithelial cells through centrifugation in Percoll gradient and used RT-PCR to investigated the presence of heparanase-1 mRNA. The results showed that epithelial cells did not express heparanase-1. Considering that heparanase-1 transcription is affected by the level of cytosine-methylation in the promoter region, we used the methylation sensitive restriction enzyme HpaII and RT-PCR to show that a single CCGG site at +162 appears methylated in the VP epithelium of estrogenized animals. These results suggest that heparanase-1 expression is blocked in the epithelial cells of the VP by estrogen imprinting by a pre-transcriptional mechanism. In parallel, we are also studying whether the levels of EGR-1 transcription factor, which is known to regulate heparanase-1 expression is also affected by estrogen imprinting. TMA is recipient of a FAPESP DR1 fellowship.

**Keywords:** estrogen, prostate, heparanase-1, heparan sulfate

### 02.9- Urinary Bladder Collagen Expression is Programmed by Maternal Malnutrition During Lactation In Rat

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Some authors have shown that lactation could be a critical period in determining the future endocrine status of the progeny. Previous papers show that the urogenital system can be programmed by maternal malnutrition. However, at this time, the effects of maternal malnutrition on the urinary bladder are unknown. The goal of this study was to evaluate the collagen expression at the urinary bladder of adult animals whose mothers were malnourished during lactation. At parturition, dams were randomly assigned into control group (C), with free access to a standard laboratory diet containing 23% protein; protein-energy-restricted group (PER), with free access to an iso-energy and protein-restricted diet containing 8% protein and energy-restricted group (ER), receiving restricted amount of the 23% protein diet. After weaning, all male pups had free access to the diet containing 23% protein until 90 days old when they were killed. Real Time PCR and immunohistochemistry were used to determine the collagen I expression. Despite a significant reduction in the food consumption and body weight gain in the malnourished groups during the lactation time, at the end of the experiment there was no difference in the body weight (C=370.8±3.5; PER=281.7±3.3; ER=368±3.5) or food intake (C=318.8±59.7; PER=281.3±40.8; ER=276.7±114.6) among the groups. The collagen I expression was significantly decreased when evaluated by Real Time PCR (C=3.2±0.9; PER=0.7±0.6; ER=0.9±0.3; p<0.03) and immunohistochemistry. We can surmise that the collagen expression of the urinary bladder was programmed by maternal malnutrition during lactation.

**Keywords:** Bladder, Collagen, Q-PCR real time, Malnutrition, Rats
02.10- Mechanical, Biochemical and Morphometric Analysis of the Femur of MDX Mice

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Introduction: In mdx mice, muscle degeneration starts to become severe in the third week after birth, with the onset of degeneration/regeneration cycles that decrease the emission of mechanical stimuli to bone. Thus, the bone remodeling process provokes the loss of bone mass throughout the growth of the animal. On the basis of previous findings showing an altered metabolism in mdx tissue, we tested the hypothesis that the alterations in mdx bone might not only be due to muscle weakness, but also to factors inherent to the genetic alterations of the animal. Objective: To identify alterations in the femur of 21-day old mdx mice, an age when the bone has not yet been under the influence of muscle weakness. Material and Methods: Femurs from male animals were used. Thirty animals were divided into two groups: mdx and control (C57Bl/10-ScCr). Mechanical testing (3-point bending test), hydroxyproline quantification, thermogravimetric analysis (TGA) and morphometry of the diaphysis were carried out and the results were compared between the two groups. The mechanical properties analyzed were maximum stress ($\sigma_m$), yield stress ($\sigma_y$) and elastic modulus (E). Diaphyseal cortical bone area and thickness were calculated. The results were analyzed statistically by the Student t-test at a level of significance of 5%. Results: The mechanical properties were higher in the control group ($\sigma_m = 209 \pm 18$ MPa, $\sigma_y = 136 \pm 16$ MPa, $E = 3114 \pm 404$ MPa) than in mdx mice ($\sigma_m = 121 \pm 13$ MPa, $\sigma_y = 94 \pm 9$ MPa, $E = 1891 \pm 862$ MPa). Hydroxyproline content was similar in the two groups (control = 21.05 ± 2.88 mg/g and mdx = 21.09 ± 2.52 mg/g). TGA showed that inorganic material weights at temperatures of 600, 700 and 1000$^\circ$C were always lower in the mdx group. Diaphyseal cortical layer area and thickness were two times higher in the control group (0.204 ± 0.02 mm² and 0.065 ± 0.004 mm) compared to the mdx group (0.114 ± 0.01 mm² and 0.037 ± 0.005 mm). Conclusion: The results demonstrated a lower quantity of inorganic bone matrix and lower mechanical strength of the femoral diaphysis in mdx mice compared to the control group. Thus, in mdx femurs osteopenia and weakness develop prior to the manifestation of muscle alterations. Supported by CAPES/PROEX and FAPESP.

Key words: mdx, bone, mechanical test, hydroxyproline, inorganic bone matrix
02.11- Hyperglycemia and Insulin Replacement Impact in the Rat Prostates MMP-2 and MMP-9 Activities During Pubertal Growth

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The matrix metalloproteinases (MMPs) are a family of molecules that are associated with the breakdown of constituents of the extracellular matrix (ECM). Both MMPs and their tissue inhibitors (TIMPs) are involved in the regulation of the ECM metabolism. The most common extracellular pathology in diabetes is the thickening of the basement membrane as a result of the deposition of ECM proteins. The expression and activity of MMPs in diabetes thus far have been reported predominantly in relation to macrovascular and microvascular complications. Here, we investigated if the STZ-induced diabetes interferes with MMP-2 and MMP-9 activities during prostate pubertal growth and the effect of a simultaneous or late insulin replacement. Prepubertal male Wistar rats (40 days-old) were used. Diabetes was induced through administration of a single dose of streptozotocin (STZ; 40mg/kg body weight) at 40-days old. Three (simultaneous) or twenty days (late) after STZ-administration, insulin was replaced (3U/100g) for 17 and 20 days, respectively. Diabetic, diabetic plus insulin (simultaneous and late) and age-matched controls animals were killed by overdoses of pentobarbital after 20 days and 40 days of beginning of the experimental period. Prostatic lobes, ventral, dorsal and anterior, were dissected out, weighted and processed for biochemical analysis of gelatin-zymography. The zymography analysis showed clear bands of MMP-2 and MMP-9 in control, diabetic and diabetic plus insulin replacement. The densitometric analysis of the bands showed that prostatic lobes from diabetic animals presented reduced activities of MMP-2 and -9. Simultaneous and late replacement of insulin restores the MMP-2 and -9 activities even over the control levels. In conclusion, diabetes, directly or indirectly (testicular damage and androgen deprivation), disturbs the structure of the prostate gland growth and led to an effective reduction in the MMPs activities. The insulin replacement, even delayed, restores the glandular structure and the MMPs activities, showing that adverse effects of this disease in the prostate are reversible and MMPs play a role in the glandular structure restoration.

Keywords: diabetes, extracellular matrix, metalloproteinases, prostate, zymography
Could the Association of Heteropterys Aphrodisiaca (O.Mach) Infusion and Endurance Exercise Modify the Collagen Content and the Biomechanical Properties of Rat Achilles Tendon?

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Heteropterys aphrodisiaca, also known as “nó-de-cachorro”, “nó-de-porco” and “cordão-de-São-Francisco”, is used as an aphrodisiac, a tonic or stimulant agent and for the treatment of nervous debility, nervous breakdown and for muscle and bone weakness. Previous studies with this species suggested that the root extract could increase corporal and testicular weight, as well as Leydig cell volume. Since sexual organ weights in animals treated with H. aphrodisiaca infusion showed no alterations, it was suggested that this plant could exert an anabolic effect in rats. The purpose of this study was to evaluate the effect of H. aphrodisiaca infusion on tendon properties under endurance exercise. Wistar rats were grouped as follows: CS- control sedentary, HS- H. aphrodisiaca sedentary, CT- control trained, HT- H. aphrodisiaca trained. The training protocol consisted in running on a motorized treadmill, five times a week, with weekly increase in treadmill velocity and duration. Water or H. aphrodisiaca (dose: 35 mg/animal/day) was administered daily by gavage for 8 weeks. The rat’s Achilles tendons were either frozen for hydroxyproline dosages and biomechanical analysis or preserved in Karnovsky fixative and processed for histomorphological analysis by polarized light microscopy. The hydroxyproline content increased significantly in the HT group. Biomechanical analysis showed significant increase in maximum stress and modulus of elasticity in tendons of the HT animals. The most intense birefringence was observed as in compression as in tension regions of the tendon of HT animals, which may indicate a higher organizational level of the collagen bundles. Therefore, the association of endurance exercise with H. aphrodisiaca resulted in stronger and better-prepared tendons to support high loads from intense muscle contraction. This association could avoid the marked degradation of collagen and others proteins occurring during endurance exercise or stimulate tendon remodeling by promoting higher collagen content and probably increased cross-links. Despite the clear anabolic effects of Heteropterys aphrodisiaca and the endurance exercise association, no side effects were observed, like those provided by synthetic anabolic androgenic steroids. FAPESP, CAPES/PROEX

Keywords: Achilles tendon, Biomechanical, collagen, endurance exercise, Heteropterys aphrodisiaca
02.13- Study of the Interaction between Anastellin and the Tenth Repeat of Fibronectin Type III by Molecular Docking: Insights into Fibronectin Fibrillogenesis

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Fibronectin (FN) is a large glycoprotein composed of several globular domains (type I, II and III) that appears in a soluble dimeric form in plasma that polymerizes into insoluble fibrils when it incorporates into extracellular matrix (ECM). Through a process that is not fully characterized, fibril formation appears to require cell traction forces, which leads to partial unfolding of FN molecules. FN type III modules (FNIII) fold into seven $\beta$–strands (named A−G) divided between two $\beta$–sheets. Under tension, these strands gradually peel apart from the globular core. FNIII modules, which have been proposed to mediate the fibrillogenesis process, share a structural motif that enables an elastic response to mechanical stress given the absence of intra–module disulfide bonds to stabilize the fold. Anastellin, a C–terminal fragment from the first type III domain (1FNIII), induces fibril formation in vitro when incubated with soluble FN. The resulting polymer, termed superfibronectin (SFN), resembles the natural form of FN found in ECM, and exhibits enhanced adhesive properties.

The process of SFN formation is also unknown, and it is unclear whether the mechanism behind its assembly is similar to the one used during natural matrix formation. Despite evidence that anastellin binds to FNIII modules, the molecular interactions that mediate this interaction remain unknown. In an attempt to better define the interacting interface between FNIII modules that may occur during FN fibrillogenesis, we carried out molecular docking and molecular dynamics simulations (using AUTODOCK and GROMACS software packages) between the anastellin-like folding intermediate of 1FNIII and 10FNIII, since both modules have known unfolding intermediates that were previously suggested to be involved in FN fibrillogenesis. Our simulation results suggests the E strands from each module may bind in an antiparallel manner, which excludes solvent from the previously exposed hydrophobic cores, thereby promoting molecular assembly. If experimentally confirmed, this model may contribute to understanding of a key step in the fibrillogenesis process that is critical for formation of FN fibrils in the context of both SFN formation, and natural ECM assembly. The knowledge generated by these studies may open new perspectives in biotechnology, guiding the development of self–assembling nanobiopolymers and nanofibrils for clinical use.

Keywords: Anastellin, Fibronectin Fibrillogenesis, Molecular Docking, Self-assembling biopolymers, Superfibronectin
02.14- Disturbed of Purinergics Receptors Activation in GAG-Deficient CHO-745 Cells

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Proteoglycans and integrins mediate cell attachment to various extracellular ligands. These molecules physically bridge the ECM and cytoskeleton, and act as transducers of “outside-in” and “inside-out” signaling. Due to this, a myriad of cellular functions such as differentiation, proliferation, migration, apoptosis, and inflammatory response can be affected. It has been shown that the purinergic P2X7 receptors binds integrin-laminin complex and its activation not only promote a cytosolic influx of Ca²⁺, but it also results in rearrangement of cytoskeleton. Here, we report on the effects of glycosaminoglycans (GAGs) deficiency on purinergic activation in CHO cells. CHO-K1 and its defective mutant on the biosynthesis of GAGs, CHO-745 cells, were examined for their cellular responses to the purinergics receptor agonists. Differences in proliferation and cell death were found between CHO-K1 and CHO-745 when stimulated by ATP and analogs (BzATP, ADP, UTP, UDP). In addition, intracellular Ca²⁺ response was also altered. We verified that in CHO-745 cells, ATP and BzATP (P2X7 selective agonist) were less potent to mobilize intracellular Ca²⁺. Similar expression at protein level of P2X7 receptor was observed in GAG-deficient CHO-745 cells and CHO-K1 cells, this result suggested that the differences observed is not associated with its expression. Thus, both CHO lineage cells express the P2X7 receptor and its activation can be altered in GAG-deficient CHO-745. Also, aberrant Ca²⁺ steady-state levels were observed in CHO-745 cells, in non-stimulated CHO cells the basal levels of cytosolic Ca²⁺ in CHO-745 cells was 30% smaller than the basal levels of CHO-K1 cells. The results suggested the involvement of GAGs in the control of purinergic receptor activation in CHO cells.

Keywords: Ca²⁺, P2X7 receptors, purinergic receptors, CHO cells

02.15- Laminin Expression in the Anuran Integument of Two Bufonids of The Rhinella Genus

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Laminin is a large noncollagenous glycoprotein found in the extracellular matrix, composed of combinations of five known alpha, four beta and three gamma chains in mammals. It represents the major component of basement membrane produced by the majority of epithelial cells, including the endothelial cells. Through specific interactions with integrins, dystroglycan, and other receptors, laminin contribute to cell adhesion, growth, migration, proliferation and differentiation, maintenance of tissue phenotypes and survival, having enormous clinical significance. Since amphibian integument has a large structural complexity that reflects its physiological properties, performing several functions, the
amphibian can survives in different environments. In order to increase the knowledge about anuran integument, the purpose of this study was to identify the laminin expression in the integument of two bufonids. Adult males of *Rhinella ornata* collected in São Conrado and *Rhinella icterica* collected in Teresopolis, Rio de Janeiro (license 12164-1 – IBAMA/MMA; ethics committed DAHEICB 017) were used. Integument fragments from dorsal and ventral regions were fixed with formaldehyde and processed according to the paraffin embedding standard histological technique. Five micrometers slices were stained with hematoxilin-eosin (HE), Periodic Acid-Schiff reaction (PAS) and used for immunolocalization of laminin at 1:60 dilution (Sigma, Rabbit Anti-laminin, L9393). The results showed that the laminin occurs just below the epidermis in the basement membrane, which also was evidenced by PAS method, indicating the presence of neutral glycoptroteins. The laminin was also visualized surrounding the secretory portion of the mixed and granular glands as well as around the blood vessels. The laminin expression in anuran integument indicates its participation in the normal structure of the basal lamina, since basal laminae are important sheets of specialized extracellular matrix that underlie and surround groups of cells, such as epithelia or endothelia, enabling the cells to orientate their basal/apical polarity and creating a microenvironment for them. Moreover, the laminin is a component of the hemidesmosome that links the basal keratinocytes to the adjacent connective tissue. Then, the laminin contribute to the maintenance of the anuran integument integrity and its functionality. Support: CAPES, FUJB/UFRJ.

**Keywords:** basement membrane, integument, laminin, *Rhinella icterica*, *Rhinella ornata*

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**02.16- Organization of Collagen Bundles During Tendon Healing in Rats Treated with L-Name**

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The Achilles tendon can support high tension forces and may experience lesions. The damaged tissue does not regenerate completely; the organization and mechanical properties of the repaired tendon are inferior to those of a healthy tendon. Previous reports have demonstrated that nitric oxide (NO) plays important role in the wound repair, but a few studies have discussed about its effect on the reorganization of ECM components. In this work we examined the structural reorganization and repair in Achilles tendon after injury in rats treated with the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME). The right Achilles tendon of male Wistar rats was partially transected. One group of rats was treated with L-NAME (~300 mg/kg/day, given in the drinking water) for four days prior to sectioning of the tendon and throughout the post-operative period. Control rats received water without L-NAME. The tendons were excised 7, 14 and 21 days post-injury and used to quantify hydroxyproline and for mechanical tests. Tendons were also processed for
histomorphological analysis by polarized light microscopy. Polarized light microscopy showed that the collagen fibers were disorganized by day 7 in non-treated and L-NAME-treated rats. In non-treated rats, the organization of extracellular matrix was more homogeneous by days 14 and 21, compared to day 7, although this homogeneity was still less than in normal tendon. In contrast, in injured tendons from L-NAME-treated rats, the collagen fibers were still disorganized on day 21. Tendons from L-NAME-treated rats had more hydroxyproline but lower mechanical properties compared to those from non-treated rats. These results indicate that NO modulates tendon healing, with a reduction in NO biosynthesis, delaying reorganization of the extracellular matrix, especially collagen. Supported by Capes

Keywords: collagen, tendon, biomechanics, nitric oxide, L-NAME

02.17- Increased Col5alpha1 and Col5alpha2 Chains in Fibroblast Culture from Skin and Lung Oo Systemic Sclerosis (Ssc)

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Background: Systemic sclerosis (SSc) is a fibrotic disease characterized by an increased extracellular matrix deposition, vasculopathy and autoimmune dysfunction. We have found an excessive and distorted type V collagen expression in fibroblasts from lung and skin in SSc patients. Purpose: To evaluate the morphological pattern of type V collagen and COL5α1 and COL5α2 gene expression in fibroblasts of lung and skin from SSc patients. Patients and Methods: Lung biopsies of 7 patients and 3 controls as well as skin specimens of 6 patients and 6 controls were obtained from SSc patients according American Collagen of Rheumatology Criteria (ACR). For fibroblast culture skin and lung evaluation we used semiquantitative score: intense expression (1-4), fibroblast number/field (1-2) and collagen fibers architecture (1-3). The total evaluations were: mild (3-5), moderate (6-7) and sever (8-9). Molecular analyzes COL5α1 and COL5α2 gene expression in fibroblasts of skin and lung was performed in Real Time PCR-RT. Results: Fibroblasts of lung and skin from SSc patients showed increased thickening and irregular bundles of COL V distributed in parallel and perpendicular arrangements resulting in a dense network pattern. The semiquantitative analyze in the cutaneous fibroblast in SSc and controls showed changes and an increase of COLV fiber structure expression (82,50 ± 9,5% vs 47,5 ± 9,5% p= 0,002) and in lung fibroblast presented increase and same morphological pattern in COLV fibers ( 38,87 ± 2,99% vs 20,33 ± 7,50% p=0,002). The molecular evaluation demonstrated an increased of COL5α1 and COL5α2 mRNA expression in SSc fibroblast skin when compared to control (1,375 ± 0,373au vs 0,0047 ± 0,0013au, p= 0.05). Similar results were observed in lung: (1,61 ± 0,654 vs 0,99 ± 0,51 au; p= 0,05). The proportion COL5α1/COL5α2 mRNA in fibroblast lung and skin was higher in SSc patients than in controls being the chains ratio 1:2. Conclusion: Intense expression of abnormal COLV was demonstrated in skin and lung...
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fibroblast culture of SSc patients. This unusual COLV structural and molecular profile can justify the morphological pattern, and maybe, cutaneous thickening as well pulmonary fibrosis viewed in SSc patients. These results provide new insights in the pathogenesis, and treatment of this severe disease. Funded: FAPESP and Federico Fundation.

**Key words:** systemic sclerosis, collagen V, fibroblasts culture

02.18- Estradiol Modulates the Synthesis and the Organization of ECM Components in Thyroid Cells

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The higher prevalence of thyroid disease in women suggests that estrogen (E2) might be involved in the pathophysiology of thyroid dysfunction. TS7 thyroid stromal cells, previously isolated and characterized in our laboratory, synthesize basement membrane components and express the cytokine transforming growth factor beta 1 (TGF-β1). Under E2 stimulus, the secretion and activation of TGF-β1 was increased. So, we hypothesized that the effects of E2 via TGF-β1 synthesis and secretion on follicular thyroid cells could be mediated by extracellular matrix (ECM) secreted by stromal cells (paracrine action). Here, we investigated the effect of E2 on ECM secretion and assembly in co-culture assays of PCCL3 thyroid follicular cells and TS7 cells. We first demonstrated an increase of PCCL3 cell proliferation and a decrease of iodide uptake when the cells were cultivated with TS7 cells, suggesting the involvement of the stroma in thyroid function. Furthermore, we observed that TGF-β1 was able to decrease PCCL3 cell iodide uptake. On the other hand, when the cells were co-cultivated with TS7 cells, E2 and TGF-β1 together stimulated the iodide uptake. Once is reported that TGF-β1 enhance ECM remodeling, we analyzed the ECM components fibronectin (FN), laminin (LN), tenascin C (TNC), type IV collagen (COLIV), decorin (DEC) and chondroitin sulfate (CS) secreted by these cells stimulated with E2. Our results showed that E2 was able to modulate the synthesis and the organization of ECM components: CS and TNC in TS7 cells; FN, LN and CS in PCCL3 cells; DEC and CS in TS7/PCCL3 cell co-culture. These effects of E2 on thyroid cells could be related probably to TGF-β1 pathway, suggesting a novel mechanism by which stromal-follicular interactions modulate thyroid function.

**Keywords:** estrogen, extracellular matrix, stromal cells, TGF-β1, thyroid
02.19- Heparin Activates M3 Receptor to Promote Rat Aortic Ring Relaxation through Integrin Interaction

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Heparin is an established anticoagulant drug that has been widely used as antithrombotic agent since the 1940s. Furthermore, other pharmacological actions have been described for this glycosaminoglycan, including reduction of blood pressure in hypertensive humans, induction of nitric oxide production, and reduction vasoconstrictor endothelin-1 expression. In this study, the biochemical and pharmacological characteristics of heparins from different sources to induce vascular relaxation in rat aortic rings was investigated. The results indicated that these heparins are able to promote endothelium-dependent relaxation; however, with low affinity. Among the different heparins tested, unfractionated heparin extracted from bovine intestinal mucosa exhibited the maximal efficacy. Heparin extracted from bovine intestinal mucosa induces vascular relaxation and nitric oxide production by activation of the M3 receptor in rat aortic rings. Even though, no direct binding of heparin to endothelium was observed, fluorescent resonance energy transfer indicate an interaction between M3 receptor and integrin. In addition, pre-treatment of samples with the Src inhibitor (PP1) blocked heparin-dependent relaxation. Moreover, this effect is not related to either the molecular weight or the degree of sulfation of heparin molecules. On the other hand, the chemical reduction of carboxyl groups abolishes the relaxation effect of heparin. These results provide an insight into the molecular requirements and potential pharmacological actions of heparin in inducing vascular relaxation, opening a novel promising field of investigation on the potential use of heparin in medicine.

Keywords: Heparin, Integrin, M3 receptor, Vascular smooth muscle
02.20- Increased Cutaneous Collagen Gene Expression in Experimental Systemic Sclerosis

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Background: Systemic sclerosis (SSc) is a rheumatic disease of unknown etiology characterized by vascular involvement, autoimmunity and skin and internal organs fibrosis. Skin thickening is the hallmark of the disease and the extent of cutaneous involvement has been shown to interfere in patient’s abilities, activities, and outcome. PURPOSE: To evaluate collagen I, III and V distribution and gene expression in skin during the early and the late stage of scleroderma in the experimental model. Methods: To induce scleroderma, New Zealand females rabbits (n=12) were subcutaneously immunized with 1mg/ml of collagen V in complete Freund’s adjuvant. At 30th day it was administrated an identical dose. Sequentially, at 45th and 60th day the animals were boostered intramuscularly with type V collagen in incomplete Freund's adjuvant. Skin biopsies were collected at 7 and 120 days after the first immunization and stained with hematoxilin-eosin, Masson's trichrome and Sirius red for morphological and morphometric analysis. The mRNA was isolated from fibroblasts culture and collagen V gene expression was evaluated through RT-PCR Real time. Results: An increase in collagen fibers was found in the dermis of animals corresponding to the immunized group at 7 days compared to controls (9,72± 3,41 vs 6,54±0,94; p=0.05) and 120 days (65,47±0,94 vs 20,69±5,60 p=0,01). The analysis by RT-PCR Real time showed an increase in the mRNA expression of collagen V alpha-1 chain. The quantification was performed at 7(p=0,021) and 120 days (p=0,03). Conclusion: The results obtained using this experimental model might be very important to a better understanding of the pathogenic mechanisms involved in the early stage of human SSc. Therapeutic protocols may lead in the future to promising treatments for SSc to avoid early remodeling of the skin.

Keywords: collagen V, sistemic sclerosis, collagen gene expression, skin
02.21- Real Time PCR: A New Excel–Based Data Sheet to Optimize Calculation of Reaction Preps and Automatically Analysis Efficiency Curves, Intra-Group Variation of Gene Expression for Selection of Endogenous Control and RQ Results by ΔΔct Method

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Real Time PCR (qRT-PCR) has been extremely useful as a tool for gene expression analysis, especially due to the sensitivity of the method. The necessity of using correct dilutions and preparation of the reaction components make difference in final results, and altering the preparation of the reaction among experiments or replicates may alter the final analysis. Aiming to minimize the time to calculate volumes and order of compounds to prepare reaction mixes, as well as to calculate the data, an Excel data sheet was developed. For planning the experiment, one informs the concentration of the sample in the reaction and in stock solution, the number of replicates, the number of genes tested and the number of variations in experimental model. The output is that the total number of reactions is informed, allowing the researcher to plan how many plates will be consumed in each experiment. Also, the volume of mixes and pre-mixes and the components needed, the volumes to dilute samples, primers and probes are calculated. After the run is completed, by copying and pasting the Cts from de csv file of run’s exported results into the document, the intragroup variation of genes expression, the efficiency of the reaction between endogenous control and target gene, and gene expression by ΔΔCt method using one internal control are calculated. The document is freely distributed by the authors.

Keywords: real time PCR, endogenous control, gene expression, reaction calculation, data analysis

02.22- Investigation of the Signaling Pathways Involved in the Cellular Response to Polymerized Laminin

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Laminin (LM), an extracellular matrix glycoprotein, plays a pivotal role in central nervous system (CNS) development. The laminin molecule, which is arranged in polymers in vivo and which can form artificial polymers in vitro, regulates several cellular functions in CNS, such as survival, proliferation, migration and differentiation. We have previously shown that neural cells can recognize and specificity respond to polymerized LM. The aim of this work was to investigate the intracellular pathways that mediate the effects induced by LM polymers. We produced artificial LM matrices polymerized at pH 4.0 (LM4) or at pH 7.0 (LM7), and analyzed retinal cells behavior in vitro. The rat retinal cell suspension was plated
on the substrates LM4, LM7 or poly-L-ornithine (PLO). After 24 hours, the cells were fixed, immunolabeled for α-tubulin III and analyzed using phase-contrast or fluorescence microscopy. In order to investigate signal transduction, inhibitors of the kinases MAPK (PD98059), PKA (H89) and PKC (queleritrin chloride) were used, as well as Rolipram and Forskolin, substances that increase cAMP levels. These inhibitors were also used in cell proliferation assays. Our results show that on LM4 cells were arranged in monolayers where neurons emitted long processes evenly distributed over the substrate. On the other hand, cultures established on LM7 showed the formation of cell clusters, where cellular extensions were apparently confined to the clusters. Quantitative analysis revealed an increase of ~100% in the incorporation of thymidine when the cells were plated on LM4 when compared to LM7. Treatment with H89, which blocks activation of PKA, decreased cell proliferation only on LM4. In contrast, inhibitors of PKC and MAPK did not affect proliferation on LM4. In addition, we showed that inhibition of PKA suppressed neurite outgrowth in LM4, while outgrowth was increased by Forskolin or Rolipram. Our data suggest that LM4 signaling in retinal cells involve activation of PKA. Supported by CNPq and FAPERJ.

Keywords: differentiation, laminin, proliferation, signaling

02.23- Folic Acid and Homocysteine Regulate the Microenvironment of the Murine Neural Crest Cells in Vitro.

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The neural crest NC is a transient structure of vertebrates embryos composed of highly pluripotent cells. The NC cells originate an extraordinary variety of cell types including neurons and glial cells of the peripheral nervous system, many endocrine cell types, and melanocytes. In addition, the NC of the cephalic region produces the greatest part of connective and skeleton tissues of the head and neck. The NC cells are highly responsive to the microenvironment. Folic acid (FA) deficiencies are associated with increased levels of homocysteine (HC) and implicated in many congenital abnormalities, such as neural tube closure defects and neurocristopathies which involves abnormal NC development. However, the effects of FA and HC on the morphogenetic processes of the NC are not well understood. Primary cell cultures were performed by mechanical dissection of 8.5 days mouse embryos neural tubes at the mesencephalic region. Explants were cultured on plastic dishes coated with fibronectin. After 48 hours the neural tubes were removed and the remaining emmigrated NC cells were then trypsinized, and subcultured in the same culture condition for additional 10 days in the complex media containing HC (0, 75 to 300 uM) or FA (22.5, to 90uM). Cell phenotypes were analyzed by immunofluorescence using the lineage-specific markers to glial cells (GFAP), neurons (β-III-Tubulin), smooth muscle cells (αSMA) and undifferentiated NC cells (p75). It was also analyzed the NC cell migration area, cell proliferation (by BrdU incorporation) and the proportion of apoptotic nuclei. The results demonstrated that HC significantly reduces the proportion of smooth muscle cells in a dose-dependent manner. This effect is prevented by the addition of AF. The proportion of neural and glial cells were not
affected by the treatments. In addition, HC induced NC cell proliferation in a dose-dependent manner, effect prevented by the addition of FA. The proportion of apoptotic nuclei was insignificant in all tested condition. Taken together, our results demonstrate an important effect of FA and HC in NC differentiation and proliferation which may be involved in congenital anomalies, such as neurocristopathies.

**Key words:** Neural crest, Homocysteine, Folic acid, Neurocristopathies

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03. ECM AND CELL GROWTH AND DIFFERENTIATION

03.1- Insulin-Like Growth Factor-I (Igf-I) Stimulate Migration of Murine Endothelial Cell Line in Vitro

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Insulin-like growth factors (IGFs) belong to a family of growth factors with structural homology to proinsulin. IGF-I is an important regulator of growth, survival, and differentiation in many tissues and can influence extracellular matrix (ECM) deposition. In addition, IGF-I stimulates repair of endothelial injury in cases such as atherosclerosis and may act as a stimulator of angiogenesis. The aim of this study was to investigate the effects of IGF-I on growth, production of ECM proteins and migration of endothelial thymic cells line (tEnd.1) in vitro. For the growth assay the endothelial cells were treated or not with IGF-I at different concentration (5, 10, 50 and 100 ng/mL) for 8 hours. A significant increase was observed in tEnd.1 growing depending on the concentration of IGF-I, when compared with control. Immunocytochemistry assay was carried up using anti-laminin, anti-fibronectin and anti-collagen IV antibodies. After washing cells were incubated with a second antibody. It was noticed an enhanced of fibronectin, laminin and collagen IV production by tEnd.1 cells treated with 100 ng/mL for 8 hours when compared to controls. Wound healing assay was performed using tEnd.1 confluent in six well plates. Wounds were generated with a sterile pipette tip. Cells were washed with PBS and treated or not with IGF-I (100 ng/mL). Photographs were taken at 0, 6, 12 and 24 hours. Analysis showed that tEnd.1 treated with IGF-I migrates faster during the time of the experiment, as compared to untreated controls. Cell migration was also evaluated using transwell culture inserts (8.0 mm pore size). Endothelial cells (1.0 x 106) were added to the upper chamber of the insert in 200 ml RPMI with or without IGF-I (100 ng/mL). Cells were allowed to migrate for 2, 4, 6 and 8 hours. When the endothelial cells were treated with IGF-I (100 ng/mL for 4, 6 and 8 hours), it was observed a significant increase in the number of migrating cells, compared to control. Our results show that IGF-I acts on endothelial thymic cell line (tEnd.1), improving growth, production of ECM and migration in vitro.
Keywords: Cell Migration, Endothelial Cell, IGF-I, Angiogenesis

03.2- WNT/B-Catenin Pathway Activation and Myogenic Differentiation are Induced by Cholesterol Depletion

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Skeletal muscle differentiation is a multi-step process that begins with the commitment of mononucleated precursors that withdraw from cell cycle. These myoblasts elongate while aligning to each other. This step is followed by cell fusion and the formation of long and striated multinucleated myotubes. Myogenic differentiation is influenced by a number of growth factors secreted for extracellular matrix, such as Wnt proteins, which are required for the induction of embryonic myogenesis. Palmitoylation appears to be required for the secretion of Wnt. In the absence of the palmitoylation site, Wnt protein accumulates in the cells that synthesize it. Palmitoylated proteins are frequently targeted to cholesterol-enriched membrane domains. Recently, our group has shown that cholesterol depletion by methyl-β-cyclodextrin (MβCD) induces skeletal muscle differentiation and proliferation. The main interest of the present work was to study the effects of cholesterol depletion in the Wnt/β-catenin signaling during muscle differentiation and whether this depletion could alter the Wnt availability. We used primary cultures prepared from breast muscles of 11-day-old chick embryos, and after 24 hours we treated it with MβCD. Cholesterol depletion increased the expression of β-catenin, its translocation to the nuclei, and activation of Wnt pathway. Frizb-1 enriched medium blocked the effects of MβCD. Moreover, myogenic cells grown with MβCD-conditioned medium and Wnt3a-enriched medium showed an enhancement in myogenic differentiation, as seen by the fusion index of myotubes. In addition, we analyzed the presence of Wnt3 in conditioned media. We found a more than 3-fold increase in the levels of Wnt3 in MβCD-conditioned medium when compared to control-conditioned medium. Our results show that Wnt/β-catenin pathway is activated in the early steps of myogenic differentiation and can be regulated by membrane cholesterol. The addition of MβCD-conditioned medium led to an increase in fusion index of myogenic cultures in a similar way as MβCD by itself does, suggesting that the effects induced by MβCD could be caused by soluble factors present in the culture medium, such as Wnt molecules.

Keywords: cholesterol, myogenesis, Wnt/β-catenin
03.3- Changes in the Expression of Adhesion Proteins after Membrane Cholesterol Depletion

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The formation of a skeletal muscle fiber begins with the withdrawal of committed mononucleated precursors from the cell cycle. These myoblasts elongate while aligning with each other, guided by recognition between their membranes. This step is followed by cell adhesion, fusion and the formation of long striated multinucleated myotubes. Interactions between myoblasts and the extracellular matrix (ECM) are very important since it can regulate both cell migration and adhesion of myoblasts. Actin, integrin, vinculin and paxillin are proteins involved in these interactions. Integrins are transmembrane glycoproteins that link the actin cytoskeleton to the ECM, and vinculin and paxillin are actin-associated proteins. Many plasma membrane molecules have been implicated in myoblast adhesion and fusion. One such molecule is cholesterol, a ubiquitous and abundant component of the membrane that plays pleiotropic roles in plasma membrane function; it is involved in maintaining membrane fluidity and impermeability and the structure of lipid microdomains. Methyl-beta-cyclodextrin (MCD) can selectively removes cholesterol from cell membranes allowing the investigation of cellular and molecular mechanisms associated with cholesterol depletion. Recently, our group has shown that cholesterol depletion by MCD induces the proliferation and differentiation of chick-cultured myogenic cells (Mermelstein et al.,2005), and that Wnt/beta-catenin pathway is involved in these events (Mermelstein et al.,2006,Portilho et al.,2007). The main interest of the present work was to study the effects of cholesterol depletion to ECM during myogenesis. We used myogenic primary cultures prepared from breast muscles of 11-day-old chick embryos, and after 24 hours we treated it with MCD at 4 mM for 30 minutes. Cell extracts with 28 and 48 hours were prepared for SDS-PAGE and immunoblotting. We also analyzed MCD treated-cells by indirect immunofluorescence to study the distribution of cell-ECM adhesion proteins. Our preliminary results show that MCD treatment induces a decrease in 30% in paxillin expression when compared to control cells. However, MCD treatment increases in approximately 20% the expression of vinculin. Our results shows that disorganization of microdomains induces changes in the expression of ECM adhesion proteins, suggesting that rafts could be involved in the adhesion, migration and fusion processes during the initial steps of skeletal muscle differentiation.

Keywords: skeletal muscle, myogenesis, adhesion, Methyl-beta-cyclodextrin, membrane
03.4- Bone Regeneration Using Biocomposite of Carbon Nanotube and Collagen Type I

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The structural properties of carbon nanotubes provide them with characteristics that may be advantageous for numerous applications in biology and medicine. Biocompatible nanotubes have the potential to be used as molecular carriers capable of delivering drugs and other reagents to the inside of a cell, as physical support for artificial biological tissues, and possibly as stimulators of tissue regeneration. However, one major drawback which precludes the use of nanotubes is the difficulty in maintaining them in a desired, specific location. Therefore, we propose to include nanotubes in a gel consisting of native type I collagen. This biocomposite should provide a matrix which will restrict nanotubes in the desired location, plus also serve as a three-dimensional support for cells and tissues. The goal of the present project was to test the positive or negative effects of different concentrations of nanotubes associated with gels of type I collagen on the regeneration of alveolar bone. For these experiments, male Wistar rats were anesthetized and one upper molar on each side was extracted. The alveolar cavities were filled with a soluble mixture of collagen alone or collagen plus carbon nanotubes, both of which form a solidified gel in situ within minutes, and then the rate of bone regeneration was compared to that seen in untreated control cavities. The animals were allowed to recover for 3, 7 and 14 days, after which the jaws were removed and fixed for histological analysis. The samples were examined both macroscopically and microscopically in order determine the effects of nanotubes on bone regeneration. Macroscopic analysis evaluated the overall process of tissue regeneration, while microscopic analysis examined the morphology of the calcified area and the formation of new bone tissue. Immunohistochemical analysis showed that the nanotube-collagen biocomposites stimulate the expression of markers of osteogenesis, osteopontina and BMP-2, during the 3 and 7 day periods. However, after 14 days neither macroscopic nor morphological results showed any significant variations as compared to the controls. These results indicate that nanotube-collagen biocomposites do not interfere in the process of bone regeneration and provide a useful support for future studies.

Keywords: Biocomposite, Carbon nanotube, Collagen type I, Extracellular matrix
03.5- The Role Played by Collagen-I in the Compatibility of CP-Titanium Screws to Human Bone Cells

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Due to their mechanical properties and corrosion resistance commercially pure (cp) Ti have been utilized for implants fabrication. In despite of the wide use of Ti for fabrication of dental implants, little is still known on the metal biocompatibility. Biocompatibility in turn, is a material property related to the acceptance of an artificial implant to the surrounding tissue and the body as a whole. It means, therefore, material biocompatibility is greatly dependent to what occurs at the cells-material interface and the mechanobehavior of this environment. Since the human bone extracellular matrix is a well known collagen rich one, we here explore the ability of human bone osteoblastic (HOB) cells to both associate and spread onto each one of uncoated and collagen I (COL)-coated dental mini-screws made of cp-Ti grade 4. Taken also in account that COL mechanics might influence the cytocompatibility of COL-coated Ti screws we used nested networks (3D) at 3mg/ml. HOB cells from confluent cultures were sequentially collected by centrifugation, rinsed with serum-depleted medium, counted (10^6 cells/ ml), and then they were allowed to interact with the Ti screws. Such cell-Ti interaction process was followed by scanning electron microscopy and zimography. The morphology of cells in common contact with the screws (2D environment) was compared with cells soaked in collagen matrix (3D environment) both in contact with the screws using SEM. Images by SEM turns evident that cells have been involved by collagen matrix, and the cells morphology in contact with titanium do not differ from 2D and 3D environment. But comparing zimography results, a different band were found in the 3D condition. Tridimensional ambient seems to change the secretion of proteases by HOB cells, so, other studies should be designed with the perspective to elucidate cells behavior when in interaction with different biomaterials respecting the mechanical view. Mechanotransduction shows it importance in HOB cells behavior. As an in vitro biocompatibility assays are an important way to found possible future damage to organisms tissues caused by biomaterials, the manner to work with cells should be as closer as possible than in nature. Supported by CNPq (a scholarship to LMG) and INPeTAm-Instituto Nacional de Pesquisa Translacional Integrando Saúde e Ambiente na Região Amazônica.

Keywords: collagen, titanium, human osteoblastic cells, biocompatibility, 3D environment
03.6- Extracellular Matrix Molecules Influence the Differentiation of Human Dental Pulp Putative Stem Cells

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In the human development, the origin of the dental pulp interactions between oral epithelium and ectomesenchymals tissues derivatives of cranial neural crest (NC). The NC consists of multipotent cells that differentiate into a wide variety of cells types including neurons, glial cells, melanocytes. In the head, the NC originates the mesectodermal derivatives including cartilage, bone and connective tissues and vascular smooth muscle cells (SMCs). The NC is influenced for different microenvironmental factors of migratory pathway, such as local growth factors and extracellular matrix (ECM). The ECM molecules, type I collagen (COL I) and fibronectin (FN), affect the migration of mesenchymal cells. These molecules have been identified in human dental development and in the NC cell differentiation. In this study, we established methods for cultivate human dental pulp cells. Our results demonstrate that the cellular adhesion was significantly enhanced on COL I or FN than on plastic culture dishes. COL I and FN displayed similar values of cell adhesion although FN promoted increased cell dispersion. We identify in these cultures population of cells with NC characteristics. RT- PCR and immunohistochemistry revealed the presence of the NC stem cell markers p75 and nestin as well as markers of neurons (β-tubulin 3) and SMCs (α-smooth muscle actin, αSMA). This study provides evidence that human dental pulp cultures produce NC-like cells, suggesting a recapitulation of their embryonic state. These human dental structures revealed itself as a viable alternative source of primitive precursors to be used in stem-cell therapies. Supported by: PIBIC/UFSC, CNPq, CAPES, REUNI, MCT/INFRA, CNPq/PRONEX, FAPESC.

Keywords: human dental pulp, Mesenchymal stem cell, extracellular matrix, putative stem cells, collagen I and fibronectina

03.7- Extracellular Matrix Molecules Favours the Phenotypic Conversion of Neural Crest Derivatives

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In the vertebrate embryo, the neural crest cells (NCCs) that migrate out from the neural primordium yield multiple phenotypes, including peripheral neurones and glia in the peripheral nervous system; cartilage, bone and connective cells in the head and vascular smooth muscle cells (SMCs) in cardiac outflow tract. Since NC cells are a migratory cell
population, the differentiation of pluripotent NCCs is strongly directed by microenvironmental factors. A number of ECM molecules with permissive influences on NC cell migration, such as type I (CLI), type IV collagen (CLIV) and fibronectin (FN) have been identified in NC cell migratory pathways. In addition, ECM proteins have also been suggested to influence NC cell differentiation. It was previously demonstrated in the quail NC, some examples of phenotypic conversion. Pigment cells and glial Schwann cells can convert into each other in vitro when exposed to the mitogenic signal of endotelin-3 and, Schwann cells can be converted into smooth muscle cells both in vitro and in vivo. In the present work, we provide an in vitro analysis of the influence of ECM molecules on the developmental potential of avian SMCs. We have focused our attention on α-smooth muscle actin (αSMA)-positive cells of NC origin. Thoracic aortic-smooth muscle cells expressing αSMA, isolated from quail embryos, were plated in plastic culture dishes coated with FN (50µg/ml) or CLI (50µg/ml). The 24 hours-primary-culture was composed essentially of αSMA+ cells. These cells progressively lose their αSMA marker and progressively express β-III-Tubulina (β-III-Tub) and Schwann myelin protein (SMP) markers. On cells were cultivated on CLI, significantly higher proportions of neurons (β-III-Tub+ cells) as well as glia (SMP+ cells) were observed. Taken together our results show evidences for the acquisition of a neuronal and glial phenotypes by NC-derived-smooth muscle cells and suggest that the differentiation state of NC-derived cells is unstable and capable of reprogramming. Supported by: MCT/CNPq, CAPES, FAPESC.

Keywords: differentiation, glia, Neural crest, Neuron, smooth muscle cells

03.8- Quail Neural Crest Cell Differentiation: The Effects of Fgf2 and Microenvironment

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The neural crest (NC) is an embryonic structure originated at origin from the edges between the neural plate and the epidermis during the neurulation process. The cells of the NC (CNC) are composed of populations of precursors already determinate and pluripotent
cells capable to originate a variety of derivatives, including neurons and glial cells of the peripheral nervous system, melanocytes, endocrine and glandular cells, skeletal and connective tissues of the head and neck. The development of the CNC involves the segregation of some multipotent progenitors of an initial population of migratory cells. The microenvironment in which NC cells migrate and differentiate have a greatly influence in the phenotype that they express. In this work, we investigate the effects of the FGF2 in the progenitors differentiation/potentialities of the CNT in cultures coated with collagen type I (COL I) or on a monolayer of 3T3-NIH mouse fibroblasts. The neural tubes of quail embryos were dissected at the trunk level (15-16 HH stage) and cultivated in a medium containing α-mem medium enriched with, bovine fetal serum and chicken embryonic extract. After 24 h, the explants were removed and the remaining cells were subcultured with FGF2 on COL I-coated dishes or on 3T3NIH. Cells were treated with FGF2 during 6 days and additional 4 days in a complex medium of the differentiation medium. Cultures were analyzed in the presence of cell phenotypic markers: myelin anti-protein (Schwann cells); anti-βIII-Tubulin and tyrosine hydroxylase (neurons); anti-α-smooth muscle actin (smooth muscle cells); melanocyte/melanoblast early marker (melanocytes). Our results demonstrate that FGF2 stimulates the proliferation of the NC cells of the CNT in cultures cultured on COL I and increases the expression of the phenotypic markers, except for the melanocytes. NC cells cultures on 3T3-NIH fibroblasts displayed increased proportions of neurons and glial cells. Taken together, our results demonstrate that the removal of FGF2 treatment followed by NC cell culture in conditions of cellular differentiation on 3T3-NIH fibroblasts in differentiation medium increases the NC differentiation to glial and neuronal phenotypes at the expenses of the melanocytes and smooth muscle phenotypes. Therefore, FGF2 has a central role in NC differentiation programs that is dependent of the microenvironment.

Key words: Neural Crest, Differentiation, FGF2, Microenvironment, Quail

03.9- Morphological Behavior of IEC-6 Epithelial Cells Grown in 2D and 3D Matrices Made of Type I Collagen

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The extracellular matrix (ECM) is a proteinaceous meshwork which in turn, may serve as a scaffold for cell growth, proliferation, survival and differentiation. Collagen (COL) is the most abundant component of the animal ECM. It is formed by an association of COL fibrils, which results from the polymerization of monomeric COL units. Depending on the organization and the resulting supramolecular structure of such fibers, the ECM can create specific microenvironments within animal tissues. The two-dimensional (2D) arrangement of the fibrils formed by COL I (monomeric COL I) on plastic surfaces provides a more rigid substrate for cells, while the three-dimensional (3D) one (fibrillar COL I) provides a more flexible, porous and irregular substrate. Much of mammalian cells respond differently to each one of 2D and 3D COL I, being that such cell responsiveness may be followed by structural
and biochemical tools. Here, we did try to characterize some properties concerning IEC-6 (rat small intestinal epithelial) cells grown in a twenty-four multiwell plate which have been coated or not with COL I: biofilms (2D) and fibers (3D). 10^5, 5.10^5 or 10^6 IEC cells/ml were allowed to interact for 24, 48, and 72h at 37°C with uncoated and COL I-coated surfaces which in turn, have been constituted at 2D or 3D. Cell morphology was followed by both light and scanning electron microscopies. As expected, cells exhibited different morphologies in 2D or 3D environments. At 2D cultures, cells appeared smaller than at 3D. After 24h of incubation in 2D (in absence or presence of COL biofilm) we did not observed any remarkable difference among cells grown onto uncoated and COL I-coated surfaces. By contrast, cells cultured on 3D appeared stretched and most of them appeared to be aligned with the COL fibers. The cells exhibited large cytoplasmic expansions after 48h of incubation, and they also tended to associate themselves, not forming monolayers. The molecular mechanisms that underlie such differential cell behavior are currently under investigation. Supported by CNPq-PIBIC, CNPq-UNIVERSAL, and INPeTAm.

**Keywords:** IEC-6, morphology, three-dimensional matrix, type I collagen

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03.10- Characterization of Hair Follicle Stem Cells Cultured on Type I Collagen Matrix

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The adult mammalian hair follicle (HF) contains a population of epidermal stem cells in a specific niche, the bulge area. The HF stem cells are multipotent and act in the support, grown and development of HF, epidermis and sebaceous gland. However, it has been recently identified in the bulge of HF, the presence of cell with neural crest (NC) characteristics. The NC is a transient embryonic structure that originates in the neural folds. NC cells invade the embryo, and differentiate into a wide array of cell types and tissues. NC derivatives include neurons and glial cells of peripheral nervous system, endocrine cells, myofibroblasts, pigment cells, as well as bone and cartilage. In the present study, we established a protocol to cultivate HF cells from 60 day-mice whiskers. HFs were dissected and cultured directly in the plastic dishes or on type I collagen (Col-I) coated dishes during 21 days in the presence of Alfa-MEM supplemented with chicken embryo extract, fetal calf serum and growth factors and hormones (T3, insulin, glucagon, hydrocortison, transferring, EGF and FGF 2). In order to perform a molecular characterization of these cell cultures, we analyzed by RT-PCR the mRNA expression of the following phenotypic markers: NC stem cells or their derivatives (PAX-3, FoxD3, Sox10, P0, SMA), epidermal phenotypes markers (cytokeratins 5, 14, 15, 19) embryonic stem cell (OCT-4) and HF cells (CD 34, CD 200 and). We used HF dissected of adult mouses (6-8 weeks old) from vibrissae region and put in 35mm wells culture dishes. With we observed that Col-I matrix were more efficient in promoting HF attachment, cell adhesion, migration and proliferation than the plastic. Furthermore, the addition of growth factors and hormones induced the expression of the NC markers. In conclusion, we
established a cell culture protocol that was effective to isolate NC-like cells from the adult mouse whisker follicle. The addition of growth factors and hormones was effective in stimulating the expression of NC-derived cell-markers, and was also favorable to the maintenance of NC-like stem cells. HF stem cells are promising candidates for cell therapy due to their high plasticity and accessibility in the adult mammalian skin.

**Keywords:** Hair follicle, Cell culture, Collagen type I

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**03.11- Experimental Study of the Fracture Components in Bone under Dynamics Loads**

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Basic scientific and clinical investigations show bone tissue responds to certain patterns of loading by increasing matrix synthesis and, in many instances, by changing the composition, organization, and mechanical properties of their matrix. This study is a proportional application of dynamic loads in callus mending the structure components of the extracellular matrix, the cell proliferation index and the differentiation process accelerating the regeneration of bone tissue, considering the direction, the magnitude and frequency of the load. Eighty male Wistar rats, age 3 months and average weight of 270 Kg were used. Tritiated thymidine was applied one hour before the fracture. The animals were divided for each time of fracture, respectively, 12hs, 1, 2, 3, 4, 6, 8 and 10 days after the fracture. The fracture in the right tibia were held with a standardized instrument for closed fracture and controlled by X-ray. the protocol was approved by the Ethics Committee in accordance with the principles adopted by the COBEA. The sample were prepared and processed by histological, immunohistochemical and autoradiography technique. The results of the radiography confirmed the effectiveness of standardized fracture equipment developed to maintain the fracture alignment. the hematoxilin-eosin stained images, show large areas of cartilaginous cells migrating to form the callus two days after the fracture. Also, the Weigert resorcin-funchsin histochemical technique with prior oxidation, revealed the presence of a vast network of oxytalan fober in the pre-cartilage in the areas of cell proliferation two days after the fracture. there was no immune-staining for PCNA in animals with 12 hs after fracture. However, there was reasonable immune-staining from one day after the fracture and a strong immune-staining starting 2 days until the 10 days after the fracture, suggesting intense cell proliferation. The results of the sections stained with Picro Syrius red technique and observed through a polarization microscope did not show collagen fibers after 12 hs of fracture. However, 2 days after the fracture there was moderate and strong staining of collagen fibers with picro Syrius Red technique. The results of the immune-staining technique for TGF-α demonstrated strong distribution in the hematoma area 12 hours after the fracture until 10 days after fracture in the callus.

**Keywords:** Bone Fracture, Dynamics Loads, Extracellular Matrix, Cell Proliferation
04. ECM AND INFLAMMATION AND IMMUNOLOGY

04.1- Cardiac Inflammatory Response in Experimental Duchenne Dystrophy

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Duchenne muscular dystrophy (DMD), a severe X-linked inflammatory myopathy caused by mutations in the dystrophin gene, is characterized by progressive degeneration of skeletal and cardiac muscles. The mdx mouse, a strain presenting spontaneously mutant dystrophin gene, has become widely used as a model for DMD and presents distinct and sequential phases of the disease, namely, myonecrosis, regeneration and fibrosis. This work aimed to evaluate, in the cardiac muscle, the migration of cellular subpopulations with possible effector/regulatory roles that might correlate to the progression of the disease and reveal possible important regulatory pathways. Tissues were collected from mdx mice at stages of myonecrosis (6 weeks after birth) and regeneration (12 weeks after birth). Histopathological analysis showed mostly macrophages and fibroblasts composing the inflammatory cardiac foci, and mast cells, which are mostly immature and present in pericardium by the age of 2 weeks. However, these cells apparently migrate to endocardium while acquiring staining-based mature characteristics, peaking at 6 weeks after birth. FACS analysis of collagenase-dissociated tissue, from mice at the myonecrosis stage showed a lymphocyte population comprised mostly of CD3+CD4+ cells, but also with CD3+CD8+ cells and a progenitor population of Sca1+Mac1-CD4-CD8- cells. Further phenotyping of this population revealed them as Sca+B220+CD117- cells. However, at the regeneration phase they were depicted as Sca+B220+CD117+ cells. We also found about 12% of γσ+ T cells within the lymphocyte population, although in the blood they were less than 1%. Interestingly, in 12-week old mice, lymphocytes were barely found in cardiac tissue, and this coincides with strong down-regulation of CD62L on blood lymphocytes. In conclusion, we observed the infiltration of different inflammatory cells into the heart at different stages of the disease, and ongoing functional in vitro assays aim to analyze migratory capability of these cells.

Key words: Duchenne muscular dystrophy, inflammation, CD62L

04.2- The Role of Galectin-3 In Homeostasis of B Lymphocytes and in the Structure of Lymphoid Organs

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Introduction: Galectin-3 is a beta-galactoside binding protein involved with several physiological and pathological processes, such as cell activation, proliferation, differentiation, migration, apoptosis and tumoral progression. The role of this lectin is unclear in the
lymphohematopoietic cell biology, although it was demonstrated that its absence interferes with B lymphocyte compartment, accelerating the plasma cell generation during Schistosoma mansoni infection (Oliveira et al., 2007). Objectives: In this study, we evaluated B lymphocytes in their different compartments, as well as analyzed the lymphoid organs involved in homeostasis of these cells, like spleen and bone marrow (BM), in galectin-3 deficient mice (Gal-3 -/-). Methodology: Male C57/bl6 and Gal-3 -/- mice were sacrificed 60-90 days after birth. For histological analysis, formalin-fixed tissue sections were deparaffinized and stained with hematoxilin and eosin. For immunohistochemistry, sections were processed with standard reagents, followed by incubation of anti-B220 or anti-CD138 antibodies, detected with biotin/avidin-peroxidase reaction. By flow cytometry, B cells were defined by anti-B220 FITC and anti-IgM Biotinylated antibodies. Results: The histological analysis revealed an alteration of spleen structure in gal-3-/- mice, presenting larger disorganized lymphoid follicules and heterogeneous sizes. B220 staining was increased in gal-3-/- mice, mainly in red pulp. The same profile was observed for CD138 staining, showing higher numbers of plasma cells in these animals. In the BM, we observed significant differences such as reduced number of granulocytes and mononuclear cells and atypical distribution of megacariocytes compared with wild type mice. In addition, we observed an increase in B220+ IgM+ cells (immature B cells) in peripheral blood of gal-3-/- mice. Conclusions: Gal-3 interferes with histological aspects of lymphoid tissues, modulating their hematopoietic functions. The increase of B220+ cells in the spleen could indicate an accelerated efflux of these cells from the BM to complete the terminal differentiation into plasma cells (CD138+). The large number of B220+ IgM+ cells in the peripheral blood, as well as the decreased number of total BM cells suggested that gal-3-/- regulates the organization of microenvironments related with B cell development. Support: CNPq.

Keywords: Spleen, B Lymphocyte, Plasma Cell, Galectin-3

04.3- Effect Of Exogenous Galectin-1 on Leukocyte Migration: Modulation of Cytokine Levels and Adhesion Molecules in Experimental Inflammation

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Galectins are a conserved family of glycan-binding proteins, usually found in the extracellular matrix, that have emerged as pleiotropic regulators of innate and adaptive immune responses. Some members of the galectin family, including galectin-1 (Gal-1), can regulate immune cell trafficking, activation, cytokine secretion, and apoptosis by triggering multivalent interactions with cell surface glycoconjugates. In this study we focus the effect of galectin-1 (Gal-1) on the expression of integrin (CD11b) and L-selectin (CD62L) during leukocyte migration. C57BL6 mice received i.p. injection of zymosan (1.0 mg) in 0.5 ml of sterile saline, whereas control animals were injected with an equal volume of saline. Another experimental group was treated with human recombinant Gal-1 (hrGal-1; 0.3 µg per animal)
before zymosan administration. At 4 and 24h later, animals were sacrificed and peritoneal wash, blood and mesentery were processed for light and electron microscopy analysis. Cytokine levels of peritoneal fluid were analyzed by ELISA and expressions of CD62L and CD11b were monitored in blood neutrophils (PMN) by flow cytometry and mesenteric PMN by immunocytochemical analysis. Zymosan peritonitis provoked expected signs of inflammation at 4h, including significant increase in extravasated PMN in mesentery and peritoneal wash, mirrored by blood neutrophilia. These changes subsided after 24h. Pharmacological treatment with hrGal-1 produced a significant reduction of PMN recruitment at 4h in all tissues analyzed compared to non-treated animals. In this early phase, the anti-inflammatory effects of hrGal-1 were associated with inhibition of the TNF-α and IL-1β levels in the peritoneal fluid. Immunocytochemical analysis showed a co-localization with Gal-1 and adhesion molecules CD11b and CD62L in plasma membrane of mesenteric PMN. The addition of hrGal-1 resulted in an increased expression of CD62L in blood and mesenteric PMN, but diminished the expression of CD11b in PMN during the transmigration process. Our results indicate an important mechanism of anti-inflammatory action of Gal-1 through modulation of pro-inflammatory cytokines and firm adhesion induced by CD11b on PMN recruitment, and might have an impact on the development of new strategies for inflammatory diseases.

**Keywords:** galectin, integrin, neutrophil, selectin, zymosan peritonitis

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**04.4- Induced Asthma by Oxidative Stress in Balb/C Mice**

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Most of the factors that result in oxidative stress are known. However, the correlation between these mechanisms and the pathophysiology of asthma is still unclear. The present study investigated the involvement of oxidative stress in asthma. BALB/c male mice were used and divided into two groups (n = 40 each) that received either 10% of ovalbumin by subcutaneous injection (OVA group) or phosphate buffer (Control group) for 4 intercalated days. After seven days, the groups were challenged with 1% of nebulized ovalbumin for 20 minutes during 5 consecutive days. On the 21st day, animals were euthanatized. Homogenized lungs were prepared for biochemical dosages for protein, enzymatic assays for Catalase (CAT), superoxide dismutase (SOD) and Glutathione peroxidase (GPx), neutrophil elastase, metalloproteinase (MPO) and malondialdehyde forms (MDA) dosages were done. Also, TIMP-2, MMP-9 and 12 expressions were measured (n=20 each). The content of protein increased 55% in the OVA group (P<0.01). The CAT activity 26% was decreased in the OVA group (P<0.05). SOD activity was also decreased in the OVA group (36% P<0.05).
However, GPx activity was increased 142% in the OVA group (P<0.001). The OVA group showed a 21% reduction in the activity of MPO (P<0.05). The level of MDA increased 47% in the OVA group (P<0.001). The levels of nitrite were 21% reduced in OVA group (P<0.05). Although there was an increase of HMGB-1 expression in the OVA group, the expression of neutrophil elastase was lower. The expression of TIMP-2 was similar in both groups, however, the gelatinolytic activity of MMP-2 and -9 increased in the OVA. These data indicated that oxidative stress is an initial factor of the inflammatory process of asthma, promoting the growth of proteolytic activation. This study helps understanding the potential involvement of redox unbalance is the precursor of changes in asthma.

**Keywords:** asthma, allergy, Balb/c mice, inflammation, oxidative stress

**04.5- Collagen V-Induced Nasal Tolerance in Experimental Systemic Sclerosis Downregulates Cutaneous Remodelling**

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**Introduction:** Systemic sclerosis (SSc) is an autoimmune disease of the conjunctive tissue characterized by vascular damage, autoantibodies presence, collagen deposit and fibrosis in the skin and internal organs. Collagen V (Col V) has been described as potential allo/auto-antigen in animal models. Recently, our group described an SSc experimental model in health rabbit induced by Col V immunization. **Objective:** Our aim was to study skin remodeling and autoantibody production in the experimental model of Scleroderma (SSc), following nasal tolerance with human Col V. **Methods:** Female New Zealand rabbits (N=12) were immunized with two doses of 1mg/ml of Col V in complete Freund’s adjuvant and additional two boosters in incomplete Freund’s adjuvant to induce SSc. After 150 days, half of from these immunized rabbits were submitted to type V collagen-induced tolerance receiving a daily nasal administration of 25mg of Col V. Control animals (N=6) were only submitted to type V collagen-induced tolerance. Serial skin biopsies were performed at 0, 150 and 210 days, and stained with HandE, Masson’s trichrome and Picrosírius for morphological and morphometric analysis. Types I, III and V collagen, TGFβ and PDGF were identified by immunofluorescence. The animal’s sera samples were collected in the same times to determine anti types I, III, IV and V collagen, anti-Scl-70 and antinuclear antibodies (ANA). **Results:** Skin biopsies from immunized animals confirmed SSc morphology as previously described, such as progressive decrease of papillary dermis, appendages atrophy, increased type I, III and V collagen deposition and augmented expression of TGFβ and PDGF. Rabbits with Col V-induced nasal tolerance showed reduction of skin involvement, with significant decrease of total collagen amount (p=0.002), and types I (p<0.01), III (p<0.01) and V (p<0.01) and lower TGFβ and PDGF expressions. Humoral immune response did not change with nasal tolerance. **Conclusions:** Col V nasal tolerance promotes regression of skin remodeling process in the experimental model of SSc. We suggest that nasal tolerance with
Marimellia Porcionato

Type V collagen can be a promising therapeutic option to treatment scleroderma patients. Funded by FAPESP, CNPq, Frederico Foundation, LIMs 17 and 05

Keywords: Systemic Sclerosis, Nasal Tolerance, Collagen V, cutaneous remodelling, autoantibodies

04.6- April Modulates in Vitro Thymocyte Migration and Cell Adhesion by Interacting with Hspg

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Proteins of the tumor necrosis factor (TNF) family play an important role in many biological processes like cell proliferation, differentiation, survival and death. APRIL (A Proliferation-Inducing Ligand) is a member of this cytokine family, promotes tumor proliferation and survival, and modulates B cell activities, enhancing cell survival and antibody production. However, whether APRIL exerts any effect on T cells remains unclear. APRIL acts by interacting with two receptors, BCMA and TACI, both expressed on lymphocytes. Additionally, APRIL binds to cell surface heparan sulphate proteoglycans (HSPG). Transgenic mice overexpressing APRIL develop a B-1 cell neoplasia. In preliminary analysis, we observed by immunohistochemistry, an increase in B220+ cells in the thymus from APRIL transgenic mice compared to the controls. The thymus is a central lymphoid organ in which T cell differentiation occurs. This process is deeply dependent on thymocyte migration throughout the organ, and for that the thymocytes interact with the thymic epithelial cells (TEC) and extracellular matrix molecules such as fibronectin and laminin. APRIL effects in the thymus remain unknown. This work aimed to study the effect of APRIL on thymocyte migration of normal mice. Firstly, we investigated whether APRIL could modulate thymocyte survival and proliferation. We did not find any alterations in response to in vitro treatment with APRIL recombinant protein. Even though, the ex vivo migration assays revealed a chemorrepulsive effect probably owning to a decrease on thymocyte adhesion as showed by co-culture with TEC. Interestingly, these results did not correlate with any difference in the membrane expression of fibronectin or laminin receptors (respectively VLA-4 / VLA-5, and VLA-6) on thymocyte subpopulations. APRIL chemorrepulsive effect was enhanced by CXCL12 and laminin (but not by fibronectin). Besides, differing from TEC, thymocytes did not express typical APRIL receptors. Nevertheless, pre-treatment with heparin partially blocks migration, suggesting that the effect of APRIL on thymocyte migration is mediated by HSPG interaction. In conclusion, APRIL seems to stimulate
thymocyte migration, and downregulates cell adhesion, by interacting with HSPG. Financial support: CNPq, FIOCRUZ/INSERM.

Keywords: APRIL, HSPG, MIGRATION, THYMOCYTE

04.7- TGF Beta and Collagen I and V Alfa-1 Chains Mrna Decrease by Collagen V-Induced Nasal Tolerance in Lung of Systemic Sclerosis Model

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Purpose: Systemic sclerosis (SSc), or scleroderma, is a rheumatic disease characterized by autoimmunity, vasculopathy and interstitial fibrosis in skin and several internal organs. Pulmonary manifestations of this disease normally are causes of disease-related morbidity and mortality. To evaluate the inflammatory process, collagen deposition, mRNA collagen synthesis, TGF-β expression in the pulmonary tissue in model of SSc after COLV-induced nasal tolerance. Methods: Female New Zealand rabbits (N=12) were immunized with 1mg/ml of Col V emulsified in Freund’s complete adjuvant, followed by two extra immunizations with Freund’s incomplete adjuvant (IM). Nasal tolerance was induced in a group of six IM animals, 150 days after the beginning of immunization and was treated for 60 days, receiving a daily nasal administration of 25mg of Col V (IM-TOL). Control animals (N=6) were only submitted to type Col V-induced tolerance (CT). The inflammatory cells and TGF-β expression in vessel and septal interstitium were evaluated by point counting method. For evaluation of collagen amount was used software Image Pro-plus 6.0 in immunofluorescence microscopy and types I, III and V collagen alfa-1 mRNA expression by Real-time PCR. Results: IM-TOL, when compared to IM, presented decrease lymphocytes (4.33±1.71 vs. 11.45±2.52, p<0.05), macrophages (5.74±2.27 vs. 7.66±1.57, p<0.05) and monocytes (1.92±0.73 vs. 27.67±3.72, p<0.05) as well as significant reduction in collagen content around the small vessels (0.37±0.12 vs. 0.87±0.28, p<0.001) and bronchioles (0.29±0.14 vs. 0.65±0.17, p<0.001). The lung tissue of IM-TOL, when compared to IM, showed decrease immunostaining of types I, III and V collagen, reduced alfa-1 mRNA expression to types I (0.10±0.07 vs. 1.0±0.53, p<0.002) and V (1.12±0.42 vs. 4.74±2.25, p<0.009) collagen. The TGF-β expression in lung vessel endothelium cells (10.77±4.3 vs. 43.5±5,7, p<0.0001; IM vs. IM-TOL) and pulmonary fibroblasts (9.93±3.77 vs. 53.68±3.77, p<0.0001; IM vs. IM-TOL) decreased in IM-TOL. Conclusions: COL V-induced nasal tolerance in the experimental model of SSc decreased the inflammatory process and regulated the pulmonary remodeling process, inhibiting TGF-β expression, collagen deposition and collagen I and V gene synthesis. Additionally, the tolerance with COL V can be a promising therapeutic option for...
human scleroderma treatment. Funded by FAPESP, CNPq, Federico Foundation, LIMs 17 and 05

**Keywords:** COLLAGEN V, NASAL TOLERANCE, SYSTEMIC SCLEROSIS MODEL, PULMONARY, TGF BETA

**04.8- The Role of Laminin and CXCL12 in T-Cell Migration During Cardiac Allogeneic Graft Rejection**

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Rejection is an immune process caused mainly by Major Histocompatibility Complex (MHC) differences between donor and recipient. During rejection, recipient T cells are activated in secondary lymphoid organs and migrate to the graft in order to destroy it. Cell and extracellular matrix (ECM) contact causes many important biological events, and ECM proteins are involved in the rejection process. Using a non-vascularized model of allogeneic heart transplantation, where a heart from a neonatal is transplanted subcutaneously in the ear of an adult mouse, we have previously demonstrated a correlation between the cellular infiltrate, and the ECM. Moreover, the treatment of the heart graft with an anti-laminin (LN) antibody (a major ECM protein), reduced the cellular infiltration to the graft. It’s also known the importance of chemokines driving T cell migration during immune response, specifically the chemokine CXCL12 influencing naive T cell migration. In the same model, we decided to study the relationship of the T cell population with LN and CXCL12 in the graft draining lymph nodes (cervical draining lymph nodes - CDLN) and the graft. We observed an enhancement of cell numbers, mainly CD8+, expressing VLA-6 (LN receptor) in high densities, in CDLN from transplanted animals. We also observed the presence of CXCL12 in CDLN, both in transplanted and control. However, an increased deposition of LN was detected in transplanted CDLN. Moreover, we verified the migratory ability of T cells obtained from CDLN from transplanted and control animals through functional analyzes, based on a transmigration assay using LN as a substrate. This experiment revealed that T cells of transplanted animals had an increased migratory ability towards LN when compared with controls. However, a higher migration was observed when we placed cells to migrate towards LN combined to CXCL12. Our study unravels the importance of LN and CXCL12 in the circuit graft/draining lymph nodes, and reinforce the importance of T cell migration in the rejection process.

**Keywords:** Laminin, CXCL12, Migration, Graft rejection, Lymph nodes
04.9- In Vivo Mast Cells Regulation by Stem Cell Factor and Death Receptors after Trypanosoma Cruzi Infection

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Although mast cells (MC) are pivotal in inflammatory and fibrotic etiologies, their role in Trypanosoma cruzi-induced cardiomyopathy is unexplored. Most studies are restricted to MC quantification in the heart and association with fibrosis. In this work, we studied cardiac and peritoneal MC in T. cruzi infected mice and observed a reduction of 60% in the number of MC in both compartments after infection. The reduction of cardiac MC numbers was mainly due to apoptosis of pericardial mucosal MC (MMC) and this death is possibly associated with a primary decreased in the local stem cell factor (SCF) production. Through RT-PCR we observed that the infection induced the expression of P2X7 and Fas in cardiac MC, two molecules reported to be involved in cell death and inflammatory regulation. Using gld/gld mice we observed that Fas/Fas-L interaction can be responsible for MC apoptosis induced by the infection in the heart, but not in peritoneal cavity. Conversely, infected P2X7-/- mice indicated that this purinergic receptor induces MC death in peritoneum. These data illustrate the complexity of molecular interactions controlling inflammatory cell function and indicates that MC not only influence the inflammatory response, but are also under the constraint of the inflammatory milieu in different compartments. Financial support: CNPq, Fundação Oswaldo Cruz, PRONEX FAPERJ.

Keywords: Mast cells, P2X7, Fas, Chagas Disease, Inflammation

04.10- Collagen Type V Distribution in Rheumatoid Arthritis (RA) Induced by Methylated Bovine Serum Albumin (Mbsa)

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Collagen type V Distribution in Rheumatoid Arthritis (RA) induced by methylated Bovine Serum Albumin (mBSA) Background: RA is an autoimmune disease, characterized by chronic sinusitis in peripheric articulations. Inflammatory infiltrate and pro-fibrotic cytokines induce the synthesis of extracellular matrix components, especially collagen. We development the autoimmune disease model that present over expression and remodeling of type I, III and V collagen (COL V) in synovial tissue. Purpose: To examine the (COL V) distribution in the synovial tissue in experimental RA after 45 days after (RA) induced by methylated bovine serum albumin (mBSA) Methods: Arthritis was elicited in males Lewis
rats (n=6), weighting 250g, by subcutaneous injection of methylated bovine serum albumin (mBSA) emulsified on Freund complete adjuvant into the right knee. After 7 days it was administrated an identical doses and at 14th day the animals were boostered with 500 μg of mBSA diluted in saline solution (RA). As control, NaCl 0,9% was injected in the left knee of the animals (CT). After 45 days the synovial tissue were collected, maintained in 10% formaldehyde and used HandE, Masson’s thricromic and Picrosirius staining to morphological analysis, and COL V expression by immunofluorescence. Results: After 45 days of RA, evaluation the rat’s right knee articulation was characterized by pronounced inflammatory cells in the synovial tissue, pannus formation and destruction of cartilage and bone in relation to rat’s control articulation. In RA it was observed a synovial membrane and vessel wall thickness, besides substitution of subsynovial adipose tissue by collagen fibers with a fine and heterogenic pattern, characterizing a chronic disease phase. Immunostaining demonstrated COL V higher expression around the vessels as well in the subsynovial reparation tissue. Conclusions: The increase of subsynovial tissue COL V expression in artritogenic rats suggests that this protein can be present during the inflammatory process evolution in human disease.

Keywords: Collagen type V, Rheumatoid Arthritis, autoimmune disease model, synovial tissue, inflammatory process

04.11- Characterization of the Novel Systemic Sclerosis Model Induced by Rabbits Type V Collagen Immunization

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BACKGROUND: Systemic sclerosis (SSc) is a rheumatic disease of unknown pathogenesis, characterized by endothelium lesion, autoantibodies production, collagen deposition in skin and internal organs. Experimental models have been used to understand SSc pathogenesis, but none reproduce all manifestation found in this disease. Recently, it was developed a rabbit autoimmunity model by immunization with type V collagen (COL V), a peculiar fibril with high antigenicity. PURPOSE: To characterize the morphological and immunological changes and organs involvement in rabbits immunized with COL V and to compare with the features found in human SSc. METHODS: New Zealand female rabbits (n=20) were subcutaneously immunized with 1mg/ml of collagen V in complete Freund’s adjuvant. At 30th day it was administrated an identical dose. Sequentially, at 45th and 60th day the animals were boostered intramuscularly with type V collagen in incomplete Freund’s adjuvant. Control groups (n=20) were immunized with Freund’s adjuvant in the same way.
To morphologic study, the serial skin biopsies were collected at 0, 7th, 15th, 30th, 75th and 120th day, and samples of the internal organs at 75th (n=10) and 120th (n=10) day. Collagen mRNA expression was evaluated by immunostaining and RT-PCR-RealTime. Sera were analyzed by ELISA, immunoblotting and immunofluorescence. RESULTS: Morphologic analysis demonstrated progressive skin fibrosis with increased collagen expression in cutaneous fibroblasts; pulmonary vasculitis with cellular infiltration, increased collagen deposition and vessels thickness leading to vessel obliteration; and systemic involvement with collagen deposit in esophagus, heart, kidney and synovia. It was found endothelial damage and vascular lesion in skin and internal organs. Immunologic analysis showed presence of autoantibodies, such as anti-Scl-70, anti-endothelin, antinuclear antibodies (ANA) and anti-collagen I, III and IV antibodies. A peculiar founding was the expression of unusual COL V fibrils pattern in the analyzed tissues. CONCLUSION: The same organs normally affected in patients with SSc are compromised in COL V immunized rabbits. The development of progressive scleroderma-like lesions, such as vascular compromising, fibrosis and presence of autoantibodies, like Scl-70, a specific antibody, demonstrated that it is a multifactorial SSc model, necessary to understand the pathology of the disease.

**Keywords:** Collagen V, Systemic sclerosis, autoimmunity, experimental model

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**04.12- Endothelial Dysfunction in Experimental Model of Systemic Sclerosis**

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Purpose: The aim of the present study was to investigate endothelial dysfunction in an experimental model of SSc induced by collagen V (COLV). Methods: Female rabbits from New Zealand lineage were immunized with COLV plus Freund’s adjuvant as previously described (Pathol Res Pract 200:681, 2004). Animals immunized only with Freund’s adjuvant were used as controls. The animals were sacrificed two hundred and 10 days after the first immunization. Endothelial dysfunction was evaluated by endothelin-1 (ET-1) immunoexpression in lung. Results: Endothelial dysfunction evaluated by ET-1+ endothelial cells was strongly increased in COLV (87.66 ± 2.11%; p=0.001) compared to controls (11.20 ± 2.39%; p=0.001) in lung sections, respectively. Conclusion: Endothelial dysfunction was found in a rabbit model of SSc induced by collagen V, emerging as a promissory model for future investigations of vascular remodeling. Support: Federico Foundation, FAPESP 07/59791-6, FAPESP 07/59792-2

**Keywords:** Systemic sclerosis, Collagen V, Experimental model, endothelium

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In spinal cord injury the primary damage is followed by events that expand the initial injury. The exacerbated inflammatory response is responsible for demyelinization and neuronal apoptosis, which contributes to the formation of a microenvironment non-permissive to axonal regeneration. In previous studies, we verified that laminin polymerized in acidic buffer was capable of increasing neuritogenesis in vitro. In addition, the application of these polymers to the injured cord led to a significant functional recovery as early as 24 hours post-lesion, which suggests containment of the inflammatory damage. In order to confirm such anti-inflammatory effect of the polymers Wistar rats were subjected to partial transection and received 10µl injections of acidic buffer or acidic laminin immediately after the lesion. The animals that received acidic laminin showed macrophage infiltrates only at the lesion epicenter, while the control group showed macrophage positive staining all over the spinal cord extension. Furthermore, the serum levels of C reactive protein, an acute inflammatory process marker, was reduced in 50% in the first 24 hours. In the same period, the number of neutrophils in the lesion epicenter was 61% smaller in the treated group (27,3+-4,5 vs. 10,6 +-1,7). Our data suggest that laminin polymerized in acidic buffer showed an immunomodulatory effect, helping to preserve the nervous parenchyma and facilitating the regenerative process.

Keywords: Inflammation, Laminin, Spinal Cord Injury

04.14- Loxosceles Intermedia Sphingomyelinase D Induces the Activation of Proteases on Human Erythrocyte Membrane, Able to Hidrolise Fluorescence Substrates

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Introduction: Spiders of the Loxosceles genus, also known as “brown spiders”, are distributed worldwide. Accidents caused by these spiders are associated with severe clinical symptoms, including dermonecrotic lesions, thrombosis, vascular leakage and, eventually, intravascular hemolysis and renal failure, that can be lethal. In Brazil, three major species, implicated with human envenoming, are responsible for more than 6.000 cases of envenomation each year. The main toxic component present in the venom is a sphingomyelinase D (SMase D). Loxosceles SMase D promotes the activation of membrane bound proteinases on erythrocytes and nucleated cells (Tambourgi et al, 2000; van den Berg
et al, 2002). In the case of erythrocytes, this led to an increased susceptibility to activation of complement (C), via the alternative pathway, because of proteinase-induced cleavage of glycophorins, which are important regulators of C-activation (Tambourgi et al, 2000).

Objectives: The aim of the present study was to further investigate the activation mechanism of the membrane-bound proteinase(s), responsible for the cleavage of the erythrocyte glycophorins, induced by SMases D. Methods: We have used the fluorescence resonance energy transfer (FRET) substrate Abz-FRSSRQ- EDDnp as a tool to explore the proteolytic activity of human erythrocyte membranes. Membranes were incubated with 5 mM of the substrate, in the presence or absence of a recombinant SMase D from *L. intermedia* (named as P2 - 5 µg) at 37°C, in a 96 well plate. The hydrolysis reaction, of the fluorogenic peptide, was monitored during 40 minutes in a spectrofluorimeter. In addition, inhibition assays were performed in order to determine the class of the activated proteinase(s). Results: Data obtained show that the erythrocyte membrane preparation have a basal proteolytic activity upon the fluorogenic peptide. However, treatment with SMase P2 induces the increase of this enzymatic activity in approximately 20%. Moreover, the use of elastase inhibitor was able to reduce the membrane proteolytic activity, induced by treatment with P2, in about 40%. Conclusion: Our results suggest that SMase P2 is able to activate proteinase(s) on erythrocytes membrane preparations and that elastase maybe involved in the process.

**Keywords:** enzymatic activity, erythrocyte membrane, fluorogenic substrate, proteases, sphingomyelinase D

**04.15- Galectin-3 Regulates Thymocyte Homeostasis**

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Galectin-3 belongs to a family of beta-galactoside-binding proteins highly conserved throughout animal evolution. It is found at different subcellular compartments, modulating biological processes such as cell adhesion, migration, proliferation and apoptosis. In the thymus, this endogenous lectin is found in the cortex and the medulla, being produced by thymic epithelial cells, phagocytes and thymocytes. Galectin-3 interacts with glycoproteins on the cell surface and in extracellular matrix proteins (ECM), such as fibronectin and laminin. We showed that galectin-3 plays a de-adhesive role in the thymus, modulating interactions between thymocytes and the thymic microenvironment. Galectin-3 null mice represent a useful tool for the study of the role of this lectin in thymus physiology and pathology. Accordingly, we used these animals to further approach the influence of galectin-3 in thymocyte homeostasis. We initially observed that thymus weight and cellularity were significantly decreased in galectin-3 null (gal-3-/-) mice when compared to strain/age/sex-matched wild type animals. This led us to investigate spontaneous thymocyte proliferation, evaluated by 3H-thymidine incorporation. Gal-3-/- thymocytes showed a decreased proliferative ratio when compared to wild type thymocytes. Moreover, cytofluorometric analysis of thymocyte subpopulations in gal-3-/- mice showed a decrease in the percentage
and absolute numbers of CD4+CD8+ cells. We also observed a decrease in the expression of extracellular matrix receptors (VLA-4, VLA-5 and VLA-6), as well as the CXCR4 chemokine receptor in gal-3-/- thymocytes, which are important molecules for thymocyte migration during the process of intrathymic differentiation. Our data suggest that galectin-3 is a relevant regulator of thymocyte homeostasis, with likely implications in thymopoiesis.

**Keywords:** extracellular matrix, galectin-3, thymus

**04.16- Galectin-3 is Probably Related to the Thymic Alterations Observed in Nod Mice**

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Galectin-3 belongs to a family of endogenous lectins which bind to beta-galactosides presented on the cell surface and extracellular matrix glycoproteins. It is involved in biological functions such as cell growth, adhesion, proliferation and apoptosis. Moreover, galectin-3 is found in several tissues and organs, being highly conserved among animal species. Recent papers related galectin-3 to autoimmune diseases such as insulin-dependent diabetes mellitus. This disease is characterized by pancreatic beta-cell destruction by T cells and auto-antibodies. NOD (non-obese diabetic) mice represent a suitable model to study diabetes, as they develop diabetes in a similar way as humans. Previous data from our group showed important thymic alterations in these mice with the development of giant perivascular spaces, intermingled with extracellular matrix network, suggesting a deficiency in the exit of mature thymocytes from the organ. This defect is associated with low expression of the fibronectin receptor VLA-5. Considering that galectin-3 is a de-adhesive protein recently related to the pathogenesis of type I diabetes, the aim of this study is to evaluate the participation of this lectin in the alterations observed in NOD thymus during the development of diabetes. In order to attend this objective, we evaluated galectin-3 expression in the thymus by immunohistochemistry; transwell thymocyte migration in the presence of galectin-3 towards fibronectin, laminin and CXCL12; and adhesion of galectin-3-treated thymocytes onto fibronectin and laminin. We showed an apparent increase in galectin-3 expression in the thymic medulla of NOD mice. In addition, preliminary data on NOD thymocytes treated with galectin-3 showed a decreased migratory and adhesive capacity towards fibronectin when compared to control thymocytes. However, under laminin and CXCL12 stimuli they presented an increased migratory response. Our data suggest that galectin-3 is able to modulate thymocyte interactions with the thymic microenvironment, probably contributing to the arrest of thymocytes in giant perivascular spaces as found in the thymus of NOD mice.

**Keywords:** extracellular matrix, galectin-3, insulin-dependent diabetes mellitus, thymus
05. ECM AND HOST-PARASITE INTERACTIONS

05.1- Effect of a Fucosylated Chondroitin Sulfate in Experimental Cerebral Malaria and in Parasite Cytoadherence

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Severe malaria is characterized by the sequestration of *Plasmodium falciparum*–infected erythrocytes (IEs) in vital organs. Adhesion of IEs to endothelial cells has a key role in the pathogenesis of life-threatening malaria and could be targeted by antiadhesion therapy. Sulfated polysaccharides, like heparin, have been shown to inhibit cytoadherence of parasitized erythrocytes to host receptors to different extents. Heparin was previously used in the treatment of severe malaria, but it was discontinued due to the occurrence of serious side effects such as intracranial bleedings. We showed that fucosylated chondroitin sulfate (FucCS), a polysaccharide isolated from sea cucumber, *Ludwigothurea grisea*, which is similar to heparin, composed of a chondroitin sulfate backbone substituted at the 3-position of the β-D-glucuronic acid residues with 2,4-disulfated α-L-fucopyranosyl branches, is a potent inhibitor of the IEs cytoadhesion to human lung endothelial cells (HLEC). Inhibition seems to be nonspecific and occurs in a concentration-dependent-manner. Furthermore, treatment with FucCS at 1 mg/Kg/animal/day showed to improve survival of C57BL/6 mice infected with *Plasmodium berghei* ANKA, an experimental model for cerebral malaria and characterized by a potent inflammatory process. Of note, treated mice did not exhibit visible side effects during therapy. Thus, we suggest FucCS as a promising candidate for adjunct therapy to prevent severe malaria outcomes. This project is supported by CNPq.

**Keywords:** Fucosylated Chondroitin Sulfate, Cerebral malaria, Severe malaria, parasite cytoadherence
05.2- Regulation of Extracellular Matrix Expression and Distribution in Trypanosoma Cruzi -Infected Cardiomyocytes

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Extracellular matrix (ECM) alterations have been demonstrated in Chagasic cardiomyopathy. ECM enhancement in the heart is detected together with inflammatory infiltrates and elevated TGF-β and TNF-α levels in plasma, which may indicate that inflammation process participates actively in this event. However, the mechanisms underlying ECM alterations still need to be investigated, and poor attention has been given to the specific response of infected cardiomyocytes concerning ECM expression. Cardiomyocyte primary cultures obtained from mouse embryos were infected with T. cruzi, Y strain. After 24h of infection, the cultures were treated with recombinant TGF-β (1, 2, 5, 10 and 15 ng/ml) and TNF-α (50 and 100 ng/ml) for 48h. The localization of fibronectin (FN) and laminin (LM) was visualized after 72h of infection by indirect immunofluorescence. For quantitative analysis of ECM expression, total protein extract from cytokine treated cultures was separated by electrophoresis, transferred to nitrocellulose membrane, and ECM expression was detected through western blot procedure. Confocal laser microscopy showed that treatment of cardiomyocytes cultures with TGF-β and TNF-α leads to an enhancement of FN matrix only in uninfected cardiomyocytes, while infected myocytes displayed low FN expression. Digital image analysis also revealed low superposition of FN signal with parasite nests in cytokine treated and untreated cultures. Cytochalasin D treatment demonstrated that microfilaments disarray induced a disturbance in the FN network of cardiomyocytes, suggesting that cytoskeleton disruption caused by T. cruzi infection disorganizes FN matrix. Additionally, western blotting analysis revealed a 2-fold increase in the FN expression in cardiomyocyte cultures after cytokines treatment, whereas T. cruzi infection significantly reduced FN levels in all conditions. In contrast, no change in the LM expression was detected after cytokines treatment by western blotting. LM distribution was altered in T. cruzi-infected cardiomyocytes, showing intense LM labeling only at the cells periphery even after cytokine treatment. Therefore, our data demonstrate that TGF-β and TNF-α stimulates FN expression only in uninfected cells of the T. cruzi-infected cultures, whereas the cells harboring the parasites presents low or no FN fibrils. Supported by FIOCRUZ, FAPERJ, CNPq, PAPES

Keywords: Cardiomyocyte, Fibronectin, TGF-beta, TNF-alpha, Trypanosoma cruzi
05.3- Differential Effect of TGF-β on Extracellular Matrix Remodeling of Trypanosoma Cruzi-infected Cultures

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Extracellular matrix (ECM) components are involved in the pathogenesis of the chronic phase of Chagas’ disease, TGF-β, a cytokine related to ECM stimulation, is implicated with chagasic fibrosis. Based on the fact that high levels of TGF-β are detectable in the plasma of chronic chagasic individuals [1] and the low responsiveness of cardiac cells to TGF-β stimulus in vitro [2], we evaluated the effect of TGF-β treatment on the ECM remodeling after infection of different cell types with T. cruzi. Mouse embryo cardiomyocyte primary cultures (CM), fibroblasts from cardiac muscle (FMC) and a skeletal myoblast lineage (L6E9) were infected with T. cruzi (Y strain) and treated for 48h with recombinant TGF-β (1, 5, 10 and 15 ng/ml). The spatial distribution and expression of fibronectin (FN) was analyzed by confocal laser scanning microscopy and western blot assay after 72h of infection, respectively. Confocal microscopy analysis revealed enhancement of FN signal in CMs stimulated with doses higher than 10 ng/ml of TGF-β, as previously demonstrated [2]. Curiously, a dose 10 folds lower of TGF-β (1 ng/ml) mediated augment of FN fibril formation in L6E9 cells and FMC. In contrast, T. cruzi infection induced the lack of fibrillar FN matrix in CM and L6E9, while uninfected cells in the T. cruzi-infected culture presented staining profile of FN similar to control. Remarkably, FN disorganization in highly infected cells was visualized even after stimulation of the cultures with high doses of TGF-β (10-15 ng/ml). Preliminary data from protein expression revealed a raise of 2.39 and 3.35 folds in FN levels in L6E9 stimulated with 1 ng/ml and 10 ng/ml of TGF-β, respectively. In CMs, only an elevated dose of TGF-β (15ng/ml) induced a maximum raise of 2 fold in FN expression, suggesting that CM are less responsive to TGF-β treatment than skeletal mioblasts and cardiac fibroblasts concerning FN expression. This way, our data open new perspectives to evaluate alterations in TGF-β signaling and the mechanisms that result in ECM reduction in T. cruzi infected cells, including cytoskeleton alterations and receptors modulation. [1] Araújo-Jorge et al. 2002, J Infect Dis, 186(12):1823 [2] Calvet et al, 2009, Int J Med Microbiol, 299(4):301. Supported by CNPq, FAPERJ, FIOCRUZ, PAPES V.

Keywords: Fibronectin, TGF-β, Trypanosoma cruzi
05.4- Analysis of Ecm Elements in Experimental Paracoccidioidomycosis Wound Healing Induced by Hene Laser

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Introduction: Paracoccidioidomycosis is a systemic mycoses highly prevalent in Brazil caused by a thermally dimorphic fungus, Paracoccidioides brasiliensis. Its clinical forms vary from cutaneous to systemic forms. Conventional methods of treatment are uncomfortable and last long time. Previous studies in our group have demonstrated the positive capacity of the HeNe laser to accelerate the wound healing process and to slow down the fungi dissemination. Here we have demonstrated the alterations in laminin and fibrinectin induced by treatment with HeNe laser. Material and methods: 8 week-old male mice foot pad were inoculated with Paracoccidioides brasiliensis. After one week, the foot pad were treated with 3J/cm² HeNe laser and then collected and submitted for immunofluorescences staining with specific antibodies to laminin and fibronectin. Results: HeNe Laser treatment has an influence on the density of ECM elements analyzed with quantitative and qualitative differences between treated and non-treated lesions. Statistics analysis show the following data to laminin non-treated 71.93 (±16.08) and treated 60.26 (± 16.26) and, to fibronectin non-treated 54.72 (±7.80) and treated 35.75 (±10.00). When compared to treated lesions, non-treated lesions have a major density of both laminin and fibronectin ECM elements. Conclusions: The slower density of ECM elements in the treated lesions shows that the wound healing process has already been finished when comparing to non-treated lesions which enhances the capacity of HeNe laser to accelerate the wound healing process.

Keywords: Paracoccidioidomycosis, Laser HeNe, ECM, Wound Healing

05.5- Role of P38 Mapk and Mlk in the Transendothelial Migration of the Pathogenic Fungus Sporothrix Schenckii

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Sporotrichosis is a subcutaneous mycosis caused by Sporothrix schenckii. In immunocompromised hosts, this fungus may invade the bloodstream and disseminate to tissues such as lungs and bones. We demonstrated that TGF-beta 1 induces transendothelial migration of Sporothrix schenckii by a paracellular route. Here, we investigated the role of a few signaling pathways related to cell contraction in S. schenckii transendothelial migration. We analyzed the effect of genistein (tyrosine kinase inhibitor), SB 203580 (a p38 MAPK
inhibitor) and W7 (MLCK inhibitor) in this process. Endothelial cells (EC) grown on 3 µm-porous inserts were pretreated with the inhibitors, before their 6-hour interaction with yeast cells. Genistein significantly inhibited (63%) transendothelial migration of yeast cells. We further verified that the activation of p38 MAPK and MLCK is required for fungus transmigration across EC since this event was inhibited (71% and 60% respectively) by SB 203580 and W7. Since in previous works we observed that \textit{S. schenckii} leads to an increase in tyrosine phosphorylation of a 125 kDa EC protein, we investigated the role of integrin-dependent FAK (focal adhesion kinase) phosphorylation in \textit{S. schenckii} transendothelial migration, by immunoprecipitation and western blotting analysis. We verified that FAK phosphorylation is not altered by the interaction yeasts with EC. We concluded that \textit{S. schenckii} transendothelial migration seems to be modulated by tyrosine and serine/threonine-phosphorylation events. The passage of the fungus across EC triggers a signaling transduction cascade that involves p38 MAPK and MLCK activation. We also showed that FAK activation is not associated with this process. Supported by CNPq, CAPES and FAPERJ.

**Keywords:** Adhesion, Cell Signaling, Endothelial Cells, Migration, \textit{Sporothrix schenckii}

**05.6- The Mechanochemical Interaction between the Parasitic Protozoan Entamoeba Histolytica and 3D Collagen I Matrices**

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Trophozoites of the human parasite \textit{Entamoeba histolytica} recognize and bind to extracellular matrix (ECM) components including collagens, fibronectin and laminin-1. Data underlie the occurrence of a bidirectional signal of mechanochemical nature exchanging from ECM to parasites and from parasites to ECM. Both chemistry and mechanics concerning the meshwork formed by ECM triggers indeed the activation of many cell functions, including invasiveness. We explore herein the mechanochemical interaction between trophozoites of \textit{E. histolytica} and tridimensional (3D) matrices made of collagen I (COL). \textit{E. histolytica} trophozoites (strain HM1:IMSS) were cultured in each one of 2D (immobilized at 50 and mg.ml$^{-1}$ COL) and 3D collagen. Cultivation of the protozoa at 3D was performed at the following COL densities (mg/ml): 1.5 (low), 3.0 (medium), and 5.0 (high) mg.ml$^{-1}$, being that COL was polymerized for 1 h at 37°C. Just after, the resulting COL matrices were rinsed, and allowed to interact with parasites for 24h and 48h (invasion assay). Alternatively, parasites were associated to COL during its polymerization (interaction assay). Followed by scanning electron microscopy (SEM) we observed that parasites induced a profound architectural rearrangement in the networks formed by COL. Also at SEM (field emission mode) we observed thin cytoplasmic projections emerging from trophozoites to COL surface at 2D. Such cytoplasmic expansions were not observed among parasites during interaction with COL fiber at 3D. Zymograms were carried out in order to investigate proteases releasing by parasites during their cultivation at 3D. The resulted zymograms clearly showed an indirect correlation between COL density and amount of proteases release by the parasites.
Interestingly, results obtained from invasion assays showed that parasite exhibited a protease release higher than that found among the parasites collected from interaction assays. Furthermore, by using video microscopy we followed the parasite behavior during migration in 3D and 2D. Under interaction with COL at 2D, most of parasites presented a migration rate higher than that observed among those at 3D. Altogether, the results here presented demonstrated that the supramolecular structure of the environment where *E. histolytica* is found greatly influence each one of morphology, adhesion, migration rates, and protease activity of the parasite. Supported by CNPq, FAPERJ, FAPEMIG, and INPeTaM.

**Keywords:** collagen, 3D matrix, entamoeba histolytica

### 05.7- Early Events Related yo the Interaction between Leishmania Amazonensis and 3D Collagen-I Matrix

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Leishmaniasis is a widely world spread disease in which Leishmania leishmania amazonensis is the causative agent of the related cutaneous form. Recent published data also demonstrated that the parasite alters the extracellular matrix (ECM) organization on the infection site on mice footpad. The ECM has indeed a key role in the pathogenesis associated to Leishmania. It seems to us that ECM may determine parasites preferred niche as well as to serve as molecular roadmaps into hosts. Data available on the interaction between parasitic protozoa and ECM underlie the existence of a bidirectional signal exchanging from ECM to parasites and from parasites to ECM. Both chemistry and mechanics concerning networks formed by ECM may trigger the activation of cell and tissue invasiveness by parasites. The present study explores the mechanochemical interaction between *L. amazonensis* promastigotes and each one of 2D and 3D matrices, all made of Collagen-I (COL). Promastigote forms of *L. amazonensis* were here allowed to interact with each one of glass slides, 50g ml-1 COL-coated glass slides (2D), and 3.0 mg.ml-1 COL (immobilized or unattached both at 3D) which in turn, was leaded to polymerize for 1 h at 370C. In order to follow the ability of the parasite to remodel COL matrix it was performed an contraction assay were COL gel were unattached from the bottom of the plastic surface. The resulting alterations of the total unattached COL gel area were analised by digital scanning of the gels and resulting images were analyzed by using the Image Pro Plus 4.0 software. The total area of the 3D COL unattached gel was 1.2 times smaller after 24h of interaction with promastigotes. Following such interactive process at light microscopy we found that most promastigotes remained migratory even when associated to COL at 3D. Otherwise, after 24h of Leishmania-COL interaction many parasites seemed to scape from the both forms of 3D COL matrix (immobilized and unattached). It seems that promatigotes forms can pocess the migration habity on their host tissue and that migration could start an remodelling process of the ECM. SDS-PAGE analysis was carried out in order to investigate the proteins realesed by the parasites during their cultivation in each one of 2D and 3D environments. Intrestingly, three different bands of high proteins mass could only be observed on samples from *L. amazonensis* cultivated on 3D COL matrices. Supported by CNPq and INPeTaM.
**Keywords:** Collagen I, Leishmania, 3D matrix

05.8- A Protein Analysis of the Protozoan Acanthamoeba Polyphaga before and After its Binding to Immobilized Laminin 1

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*Acanthamoeba polyphaga* is a free-living amoeba that cause human opportunistic infections such as granulomatous encephalitis, osteomielitis and keratitis. The last which has been mostly observed among contact lens users seems to be initiated by the binding of *A. polyphaga* trophozoites to Laminin 1 (LMN). In in vitro conditions such trophozoites are also able to recognize others extracellular matrix (ECM) components including fibronectin, collagen I and elastin, all in a cation-dependent manner. Otherwise, previous contact of amoeba trophozoites to ECM molecules seems to become the protozoan highly adherent and toxic to mammalian cultured cells. Thus, it seems to us that the *A. polyphaga*-ECM interaction may play important roles in the human host tissues invasion exhibited by the protozoan. The LMN recognition by *A. polyphaga* has been reported to result in both morphological and physiological alterations in the protozoan. Here, we investigated the binding of *A. polyphaga* trophozoites to immobilized LMN by focusing the proteins released by the protozoan during its interaction with this ECM macromolecule. For the experiments, twenty-four-multiwell plates were incubated for two hours at 37°C with 20 µg/ml LMN-I in two different pHs (4.4 and 6.6). After coating, wells were washed gently once with PBS in order to remove unadsorbed LMN. Trophozoites grown in PYG medium at 25°C were detached and washed twice in PBS, and suspended in a buffer containing divalent cations (Ca²⁺, Mg²⁺, Mn²⁺). This suspension was leaded to interact with each one of uncoated or LMN-coated surfaces (400000 trophozoites/cm²) for until 120 min, at 25°C. Following the incubation, the supernatants were collected and the secreted proteins profiling was revealed by SDS-PAGE using silver staining. Protein profiles showed in SDS-PAGE revealed that among the bands observed, two bands of medium molecular weight (between the 60 and 35 kDa markers) were only detected in the presence of LMN-I. However, any remarkable difference was not found in terms of protein bands when amoebae interacted for 20 min on LMN. Collectively, our data suggest that trophozoite binding to LMN-I possibly induce modifications on the amoeba cell signaling, leading to the release of differential proteins, in a time-dependent manner. Supported by PIBIC (to KLI), CNPq (scholarship to DBP), and INPeTAm-Instituto Nacional de Pesquisa Translacional Integrando Saúde e Ambiente na Região Amazônica.

**Keywords:** Acanthamoeba, Laminin, SDS-PAGE, Cell signaling, Silver staining
06. ECM AND PATHOLOGICAL PROCESSES

06.1- Anti-angiogenic effect of a Heparin-Like Glycosaminoglycan Isolated From a Marine Shrimp

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It has been shown that a heparin-like glycosaminoglycan obtained from shrimp cephalotorax (Litopenaeus vannamei) is capable to modulate inflammatory responses without interfering on hemostasis. Besides it reduced bleeding potential and anticoagulant activity in vitro, the shrimp heparin-like compound is able to reduce the activity of matrix metalloproteinases. Thus the objective of this study was to evaluate the anti-angiogenic effects of this new compound on an “in vitro” and “in vivo” models. The ability of endothelial cells form capillary-like structures “in vitro” when plated on top of a reconstituted basement membrane extracellular matrix (Matrigel) was investigated in cells treated with the heparin-like compound (0.09, 0.9 and 9 ìg/mL). The cytotoxicity of this compound was evaluated by MTT test in retinal pigmented epithelial cells (ARPE-19) and endothelial cell cultures. The “in vivo” model to evaluate the anti-angiogenic effect of this compound was the laser-induced choroidal neovascularization (CNV) in rats. The CNV was induced using argon laser, and at the end of laser session, saline or heparin-like glycosaminoglycan were injected intravitreously in the 4 groups of animals (laser, and laser with 0.09, 0.9 and 9 ìg/mL of heparin-like glycosaminoglycan). After three weeks, the eyes were enucleated and immunofluorescence was performed (flatmount) using anti-von Willebrand factor, a marker for endothelial cells. All three tested doses of the heparin-like glycosaminoglycan induced a significant decrease (p<0.05) in total length of tubes formed by endothelial cells in Matrigel. Immunofluorescence analysis showed significant reduction in CNV lesion area of all groups treated with heparin-like glycosaminoglycan. The regression observed was 28%, 53% and 41% in 0.09, 0.9 and 9 ìg/mL treated groups, respectively (P<0.0001). No cytotoxic effect was detected in ARPE-19 or endothelial cell culture. Intravitreal injection of the the heparin-like glycosaminoglycan from a marine shrimp significantly reduces the CNV lesion area. The optimal dosage to reduce angiogenesis was 0.9 ìg/mL and it was no cytotoxic effect in ARPE-19 cells or endothelial cells. These results demonstrated a new useful antiangiogenic compound that can be used as a therapy to control neovascularization. Supported by FAPESP, CNPq and CAPES.

Keywords: extracellular matrix, inflammation, glycosaminoglycans, neovascularization
06.2- Gene Expression Profile of Matrix Metalloproteinases and their Inhibitors in Human Cholesteatomas

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Cholesteatoma is a middle-ear cyst formed by epidermal keratinized tissue with the capacity of migration and erosion of adjacent structures. Cholesteatoma may compromise the ossicular chain, favor middle ear infections and cause irreversible damage, leading to permanent hearing disability. The matrix metalloproteinases (MMPs) are enzymes involved in degradation of extracellular matrix (ECM) and are regulated by specific tissue inhibitors (TIMPs). The balance between MMPs and TIMPs is critical to determine the integrity and function of ECM, and thus variations in the presence and activity of these proteins may contribute to many of the tissue events observed in cholesteatoma. The purpose of this study is to determine the gene expression profiles of MMP and TIMP isoforms in human cholesteatomas and to evaluate the influence of such profile to the aggressiveness of the disease. For analysis of gene expression, samples from patients with clinical and radiological diagnosis of chronic otitis media with cholesteatoma were surgically removed, immediately frozen in liquid nitrogen and stored at -70o C. Total RNA extraction was carried out by guanidinium extraction and ethanol precipitation or silica column affinity. Amplification of MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and the positive control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by RT-PCR. Eleven cholesteatomas were processed, all expressed GAPDH. Preliminary analysis of six samples showed expression of MMP-2, MMP-3 and TIMP-1 in one case, and MMP-2, MMP-13 and TIMP-1 in another sample. The other four cholesteatomas did not express any of these genes. MMP-9 expression was negative for all six samples. The presence of MMP-2, MMP-9, MMP-13 and MMP-3 in cholesteatoma has been reported previously in the literature respectively by immunohistochemistry and zymogram. This is the first time that TIMP-1 expression is observed in cholesteatoma. Preliminary results showed different expression of MMPs and TIMPs in cholesteatomas, so this analysis could have value for classification of lesions and prognosis. Financial Support: PIBIC/CNPq and NEPAS

Keywords: Cholesteatomas, Gene expression, Matrix metalloproteinases, RT-PCR
Mice lacking the extracellular matrix protein Microfibril Associated GlycoProtein-1 (MAGP1) display delayed thrombotic occlusion of the carotid artery following injury by a photochemical assay as well as prolonged bleeding time from a tail vein incision. Normal occlusion times were restored when recombinant MAGP1 was infused into deficient animals prior to vessel wounding. Blood coagulation was normal in these animals as assessed by activated partial thromboplastin time (aPTT) and prothrombin time (PT). Platelet number was lower in MAGP1-deficient mice, but the platelets showed normal aggregation properties in response to various agonists. MAGP1 was not found in normal platelets or in the plasma wild-type mice. Looking for a possible partner for MAGP1 by ligand blot assays, MAGP1 binds to fibronectin, fibrinogen, and von Willebrand factor, but von Willebrand factor was the only protein of the three that binds to MAGP1 in surface plasmon resonance studies. Mapping MAGP1 domains important for occlusion time rescue suggest that RVYVVNK peptide is playing a role in that. Taking into consideration that TGF-beta is released from platelets during thrombus formation we decided investigate if Losartan, a putative antagonist of angiotensin II receptor AT1, that decreases TGF-beta expression and activation, has any effect in this process. MAGP1 deficient mice Losartan treated had their occlusion time decreased to wild type mice levels. Treatments used were effective in recovering the normal occlusion time in Mapg1-ko mice suggesting that TGF-beta, may be a important player in this model of hemostasis. Financial support: FAPESP, CNPq and CAPES.

Keywords: thrombosis, elastic fiber, MAGP1, microfibril
06.4- Involvement of TGF-Beta and VEGF in Bone Regeneration in Critical Defects in Rats Calvaria

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Introduction: Periodontal bone defects are highly prevalent in adults worldwide and are still a challenge in dental regeneration procedures. Therefore the use of biomaterials and growth factors are already clinically employed, however further researches are required to better fulfillment in critical bone defects. Objectives: In this study, we evaluated bone regeneration with different biomaterials in critical bone defects by histological and histomorphometrical techniques associated to immunohistochemical localization of TGF-beta, VEGF, and macrophages. Methodology: Calvaria bone defects (5 mm) were performed in Wistar rats fulfilled with autogenous bone, bioglass, hydroxyapatite, and bioOss. All animals were sacrificed after 15 and 45 days post surgery and calvarias were fixed in 4% buffered formaldehde, and decalcificated in Morse solution. Paraffin sections were stained with hematoxylin-eosine, Masson´s trichrome, and picro Sirius red. For Immunohistochemistry, antibodies against rat ED1 antigen, and VEGF were applied on paraffin sections and revealed with the β pan TGF-LSAB®-HRP kit for use in rat tissue specimens (DAKO) and the chromogen was diaminobenzidine. Results: The histological analysis revealed that after 45 days new bone formation almost fulfilled the bottom of the defects, mainly with autogenous and bioOss groups. Morphometrically a significant amount of neoformed bone was only achieved in animals that received autogenous bone after 45 days (p<0.05) while diminution of connective tissue formation was statistically significant after 45 days in autogenous bone group. Different subpopulations of ED1+ macrophages were present in the different groups. In neoformed bone, periosteal and endosteal surfaces were partially covered with ED1+ cells. TGF-beta reactivity was present in inflammatory cells, mainly macrophages and in osteoblasts, aspect predominant in all 15 days groups, diminishing in the 45 days groups. VEGF also was present in inflammatory cells and mesenchymal osteoprogenitors and osteoblastic cells covering periosteal and endosteal surfaces both at 15 and 45 days after surgery. Conclusions: It seems that regeneration of calvaria bone defects requires the presence of various macrophagic population and that both TGF-beta and VEGF are involved in the signal transduction leading to osteoprogenitor/osteoblast activation

Keywords: bone defect, regeneration, TGF beta, VEGF
06.5- Expression of Decorin and Biglycan in Human Normal Term Placenta and in Invasiveness-Changed Trophoblast Pathologies

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Decorin and biglycan are members of the small leucine-rich proteoglycans (SLRPs) family, and are constituents of both extracellular matrix and cell surface. In the extracellular matrix, decorin and biglycan have been pointed as important factors in the control of trophoblast proliferation, migration and invasion. There are some pathologies that present an increased invasive activity of the trophoblast. These pathologies can be developed during or after gestation, and could be fatal for both mother and fetus, like the placenta accreta, invasive mole and choriocarcinoma. The aim of this study was to characterize, throughout immunohistochemistry analyses, the differential expression and the localization of proteoglycans, decorin and biglycan, in normal term placenta, placenta accreta, invasive mole and choriocarcinoma. In normal term placenta, decidual cells presented positivity for decorin whereas the extravillous cytotrophoblast was negative. Decorin was weakly stained in endometrial matrix, however, the matrix-type fibrinoid was not reactive. In placenta accreta and invasive mole, extravillous cytotrophoblast cells were positive for decorin whereas endometrial matrix was negative. In choriocarcinoma samples, only cytotrophoblast cells and metastatic cells were immunoreactive for decorin, including mithotic figures of cytotrophoblast cells. Immunohistochemistry for biglycan showed similar results to decorin immunohistochemistry in almost all cases, with exception of the matrix-type fibrinoid in normal term placenta and placenta accreta that presented strong staining for biglycan. These results demonstrated that decorin and biglycan are differentially expressed in normal term placenta and in placental pathologies. These results further suggest that the expression patterns of both proteoglycans in placenta accreta, invasive mole and choriocarcinoma might play a possible role in modulating trophoblast migration and invasion.

Keywords: Biglycan, Decorin, Human Term Placenta, Placenta Pathologies, Trophoblast Invasion
06.6- High Affinity Binding with Collagen Determines the Vascular Damage Induced by Hemorrhagic Snake Venom Metalloproteinases

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Local hemorrhage is an important symptom of viper venom envenoming correlated to the action of Snake Venom Metalloproteinases (SVMPs). This action depends on SVMPs catalytic action on basement membrane components. However, although all SVMPs are catalytic, only class P-III SVMPs induce strong hemorrhage while P-I SVMPs exhibit lower activity. This evidence suggests that hemorrhage is modulated by adhesive mechanisms involving the non-catalytic domains, present only in P-III SVMPs. In order to test this hypothesis, we compared hemorrhagic lesions induced by jararhagin (highly hemorrhagic P-III) and BnP1 (weakly hemorrhagic P-I) using the mouse skin as experimental model. As previously reported, 15 minutes after injection of equimolar doses of toxins, only jararhagin induced strong skin hemorrhage, located mainly in hypodermis. Tissue localization of Alexa488-labeled toxins revealed an accumulation of jararhagin around venules and capillary vessels labeled by anti CD-31, and a co-localization with basement membrane collagen IV. The same distribution pattern was detected with jararhagin-C, which has only disintegrin-like and cysteine-rich domains of jararhagin. In contrast, BnP1 did not accumulate in the vicinity of blood vessels and did not co-localize with collagen IV. After tissue accumulation of jararhagin, a strong disorganization and degradation of collagen fibers was observed in hypodermis stained by picrossirius-hematoxilin. Also, a marked degradation of collagen IV in the basement membrane was observed by immunofluorescence using anti-collagen IV. Similar effects were not detected using BnP1. Thus, we suggest that binding to collagen IV through the non-catalytic domains leads to the accumulation of hemorrhagic SVMPs on blood vessel walls, and consequent damage of its basement membrane. This effect causes weakening of the capillary vessel and results in the strong local hemorrhage induced by P-III SVMPs. Supported by INCTTOX Program, FAPESP and CNPq

Keywords: collagen, metalloproteinases, basement membrane, hemorrhage, snake venom
06.7- A Selective Cyclooxygenase-2 Inhibitor Suppresses the Growth of Endometriosis with an Anti-Angiogenic Effect in a Rat Model

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Endometriosis is believed to be the result of implantation of retrograde shed endometrium during menstruation, causing chronic pelvic pain, dysmenorrhea and infertility. The survival of ectopic endometrium is related to the acquisition of an adequate blood supply, and the use of angiostatic agents promises a new therapeutic option. Cyclooxygenase-2, a rate-limiting enzyme in the biosynthesis of prostaglandin E2 (PGE2), is highly expressed in the endometriotic tissue and results in an increase of the peritoneal PGE2 concentration. Although the inhibition of COX-2 prevents the establishment and growth of endometriosis lesions in different animal models, the mechanisms underlying this regression remain to be further determined. In this study, we investigated the antiangiogenic effects of the selective COX-2 inhibitor Parecoxib on the growth of endometrial implants in a peritoneal endometriosis rat model. We analyzed the vascular density, the expression of vascular endothelial growth factor (VEGF) and its receptor Flk-1, the distribution of activated macrophages, the expression of COX-2 and the prostaglandin concentration in the endometriotic lesions treated with Parecoxib for 30 days. The treatment significantly decreased the implant size, and the histological examination indicated mostly atrophy and regression. It was also observed a reduction in the microvessel density and in the number of macrophages, associated to a decrease of VEGF and FLK-1 expression. Finally, the treatment group showed low concentration of PGE2. These results suggest that the use of COX-2 selective inhibitors could be effective to suppress the establishment and growth of endometriosis, partially by their antiangiogenic activity. Studies of this agent in humans as a potential therapy are indicated.

Keywords: Angiogenesis, Cyclooxygenase-2 inhibitor, Endometriosis, Prostaglandin, Vascular Endothelial Growth Factor
06.8- Cathepsin B Activity as a Viral Infection Marker in Asymptomatic Blood Donors

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Cathepsin B is a lysosomal cysteine protease widely distributed in tissues that can degrade extracellular matrix (ECM) molecules. This enzyme that can be released in serum is involved in various physiological roles like antigen presentation, apoptosis and inflammatory processes, and in pathological events as tumor progression, metastasis and viral infections. This work suggests the utilization of cathepsin B level as marker of viral infection in asymptomatic blood donors. Two hundred three serum samples of healthy blood donors (control group, CT) and forty one serum samples of blood donors that had blood bag excluded after donation because serological viral positive results (SVPR) to: HIV (N=5); HTLV (N=5); HCV (N=16) and HBV (N=15) have the cathepsin B activity measured spectrofluorometrically, using the fluorogenic substrate Z-FR-MCA at 37°C in 50 mM sodium phosphate buffer, pH 6.3, containing 200 mM NaCl and 2 mM EDTA. Fluorescence intensity was monitored in a microplate reader, with excitation and emission wavelengths set as 365 and 420 nm, respectively. The assay was performed by preincubating the serum samples with the enzyme activator 2 mM DTT for 20 minutes and then adding the irreversible inhibitor E-64. Cathepsin B data are expressed in UAF/μg protein as mean ± SE. Statistical analysis performed by ANOVA and Receiver Operating Characteristic (ROC) curve tests Cathepsin B level in CT group was 24.97 ± 4.08 while in SVPR groups it was observed a remarkable increase: HIV - 873.7 ± 497.8; HTLV - 1,850.0 ± 987; HCV - 838.6 ± 228.6; HBV - 338.9 ± 91.1. Statistical studies of cathepsin B diagnostic properties, show sensibility 96.2%, specificity 91% and cut points between healthy, serological positive retroviruses (HIV and HTLV) and other viruses (HCV and HBV). The results suggests cathepsin B as a new diagnostic marker for viral infections, being possible to establish different values to act like biomarker for different virus type, cathepsin B showed to be a stable marker without epidemiology interferences. (Supported by CAPES, CNPq and FAPESP).

Keywords: Blood Donors, Cathepsin B, Diagnostic Marker, Viral Infections
06.9- Extracellular Matrix and its Relationship With Palatopharingeal Muscle in the Sleep Apnea Syndrome

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Obstructive Sleep Apnea Syndrome (OSAS) is a complex disease characterized by continuous and intermittent obstructive events of the airways. This can evolve into systemic complications of great importance for the patient’s metabolism and quality of life. OSAS physiopathology is multifactor condition, being the airways compliance a very important component. The aim of this study was to evaluate the tissue changes in the palatopharyngeal muscle by morphometric-stereological and immunohistochemical analysis, with special attention to extracellular matrix components associated with this muscle at structural and ultrastructural levels. Thirty patients with OSAS were divided in groups of 10 according to disease severity: mild (Mi), moderate (Md) and serious (S) OSAS. In addition, the control group (Ct) consisted of 10 patients. Fragments of palatopharyngeal muscle removed from patients with OSAS, and tonsillectomies from patients in the Ct group were submitted to light and transmission electron microscopy. The results showed important differences in analyzed groups, such as reduction of the muscle fibers diameter in patients with OSAS (mayor diameter = Ct: 112.4 ± 30.9 µm, Mi-OSAS: 80.1 ± 16.9 µm, Md-OSAS: 80.7 ± 13.3 µm, S-OSAS: 81.6 ± 17.1 µm). The stereological data showed a progressive increase in the relative frequency of collagen and elastic fibers, when comparing the patients in the Ct group (collagen: 11.3 ± 3.6%; elastic fiber: 9 ± 2.8%) those patients with OSAS (collagen = Mi-OSAS: 16.5 ± 4%, Md-OSAS: 14.9 ± 3.2%, S-OSAS: 19.4 ± 6.4%; elastic fibers = Mi-OSAS: 15.3 ± 4%, Md-OSAS: 14.9 ± 3.5%, S-OSAS: 16.6 ± 3.3%; p ≤ 0.05). The immunostaining for MMP-2 and MMP-9 demonstrated weak reaction in the connective tissue and in the muscle fibers cytoplasm from Ct patients. These enzymes were increased in both muscle fibers cytoplasm and connective tissue in the samples from the patients with different levels of OSAS. The ultrastructural analysis showed that palatopharyngeal muscle fibers of OSAS group presented early cellular aging, characterized by the presence of autophagic vesicles and heterogeneous residual bodies. We can conclude that the increase of tissue compliance in individuals with OSAS may be, among other factors, a consequence of the muscle fiber contractile reduction, which exhibited clear signs of early senescence. Moreover, extracellular matrix components changes may contribute to the muscle myopathy during OSAS progression.

Keywords: collagen fibers, elastic system, MMPs, sleep apnea syndrome, palatopharyngeal muscle
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06.10- Composition of Sulfated Glycosaminoglycans and Immunodistribution of Chondroitin Sulfate in Deeply Infiltrating Endometriosis Affecting the Rectosigmoid

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Endometriosis is a prevalent condition in which endometrium-like glandular and stromal tissue exists at ectopic sites outside the uterus. Ectopic lesions exhibit recurrent menstruation-like bleeding that result in blood-filled cysts, local inflammatory reactions and fibrosis. Endometriosis affects approximately 10% of the female population in their reproductive years, and women with endometriosis present characteristic signs and symptoms: dysmenorrhea, dyspareunia, chronic pelvic pain or sub-fertility. Deeply infiltrating endometriosis is defined as pelvic endometriosis infiltrating deeper than 5 mm below the peritoneum, and the major component of the lesions is fibromuscular tissue. Bowel endometriosis is one of the greatest concerns of specialists treating deep infiltrative endometriosis because of the severity of its symptoms, concomitant infiltration of pelvic organs, the possibility of bowel obstruction resulting from the progression of the disease, and the technical difficulties in surgical removal. In the present study, we investigated the composition of sulfated GAGs and the tissue distribution of chondroitin sulfate (CS) in deeply infiltrating endometriosis (DIE), using histochemical, biochemical and immunohistochemical analyses. The sulfated GAGs were characterized as dermatan sulfate (DS), heparan sulfate (HS) and CS; and DS strongly predominated compared to HS and CS. Immunostaining procedures showed that CS was concentrated in the endometriosis foci, distributed throughout the stroma around the glands. This is the first report describing the composition of sulfated GAGs and the tissue location of CS in DIE by means of histochemical, biochemical and immunohistochemical analyses. These results confirmed that in DIE of rectosigmoid, as in eutopic endometrium, CS was the dominant sulfated GAG in stroma of the lesion foci.

Keywords: Chondroitin sulfate, Deeply infiltrating endometriosis, Glycosaminoglycan, Human endometrium
06.11- Extracellular Matrix Changes of Cartilage and Sinovial Tissue in Diabetic Rats

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Background: Changes in extracellular matrix components of tendons and ligaments are frequently observed in diabetic patients. It can result in angiogenesis, healing, inflammatory response and cells proliferation delays, what may be involved in limited joint mobility. The cartilage and synovial tissue disease can have be relationship with limited joint mobility in diabetic patients. Purpouse: To evaluate the collagen and proteoglican cartilage amount and synovial tissue architecture after the development of diabetes by streptozotocin inoculation in rats. Methods: We used a 10 weeks-old Wistar rats (N=30), weighting 200-250g divided into two groups: diabetic group (DG=15) and control group (CG=15). Diabetes was induced by streptozotocin caudal injection (35mg/kg) and the CG received physiological solution. The animals were monitored to weight and blood glucose during 10 weeks. The femorotibial joints were isolated, immersed in formalin 10%, decalcificated, included in paraffin and stained with Hematoxilin-Eosin (HandE), Picrosirius and Safranin-0 with “Fast-green”. The quantitative analysis of cartilage collagen and proteoglycan was evaluated in Olympus BX-51 microscopy by software Image Pro-Plus 6.0 in different randomly selected fields using Picrosirius and Safranin-0. The synovial tissue histoarchiteture was evaluated in HandE, Masson’s tricromic and Picrosirius. The collagen II and XI cartilage expression was evaluated by immunofluorescence. Results: It was found higher blood glucose levels in the DG when compared to CG (426.2±65.4 vs 97.46 ± 6.7 ml/dl; p<0.01). The weight of DG was significantly lower than in CG (263 ± 19.97vs 460.46 ± 65.43g; p<0.01). The morphometric evaluation showed an collagen thin fibers increase (4,79±7,62vs23,06±16,07; p=0,01), an decrease of thick fibers collagen (33,05±8,53 vs28,64±8,21;p=0,04) and proteoglycan expression (343,53±156,66 vs186,07±146,35 p=0,03) in DG. Was observed collagen XI deposition in DG cartilage. In addiction the synovial analysis by HandE showed inflammatory infiltrate absence and the morphological analysis of collagen shows adipose tissue substitution for fibrous tissue thickness collagen fibers in DG. Conclusions: This cartilage and synovial remodeling can be responsible for articular dysfunction in diabetic rats, suggesting that this process is the result of pathophysiological mechanisms in this illness.

Keywords: joint, experimental diabetes, cartilage, collagen, remodeling
06.12- Fibrinolytic Activities are Associated with Smooth Muscle Cells in Aneurysms of the Ascending Aorta

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Background: Human aneurysms of the thoracic ascending aorta (TAA) are characterized histologically by fibrillar extracellular matrix breakdown and mucoid degeneration within the aortic media associated with progressive smooth muscle cell (SMC) rarefaction. The TGF-β1/Smad2 signaling pathway is assumed to play a role in this process. Since the fibrinolytic system is able to activate MMPs, to induce SMC disappearance, and to increase the bio-availability of TGF-β1, we explored the plasminergic system within the aortic wall in TAA.

Material and Methods: Ascending aortas from 19 controls and 23 patients with TAA were analyzed by immunohistochemical, biochemical and molecular approaches. Results: Immunohistochemical approaches showed that the serine-proteases (tPA, uPA and plasmin) accumulated in close association with the remaining SMCs in the TAA media but were absent from the mucoid material. The over-expression of t-PA, and u-PA in the lesions was confirmed at the transcript level by RT-PCR and at the protein level by immunoblotting on tissue extracts and tissue-conditioned media. Plasminogen was also associated with the remaining SMCs, at their surface and inside cytoplasmic vesicles, but plasminogen synthesis (mRNA) was undetectable in the aortic media. Activation of serine proteases was assessed by zymography, showing low molecular weight u-PA and by immunoblotting for plasmin and proteases-antiproteases complexes. Plasmin-antiplasmin complexes were detected in tissue-conditioned media. Activation of the fibrinolytic system was associated with an increased production of fibronectin-degradation products released in tissue-conditionned media. Within tissue, fibronectin-related material appeared in dense clumps around SMCs and co-localized with latent TGF-β Binding Protein-1 (LTBP-1). Conclusion: Together, these observations indicate that serine proteases of the fibrinolytic pathway could play a critical role in the progression of TAA, via proteolysis of extracellular matrix molecules and consecutive modulation of TGF-β1 bio-availability.
07. ECM AND DEVELOPMENT

07.1 - Differential Expression of Heparanase During Neonatal Ventral Prostate Development in Wistar Rats

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There are a number of enzymes involved in the development of ventral prostate (VP) in the first week after birth. Matrix Metalloproteinase-2 and 9 have been implicated in extracellular matrix (ECM) remodeling and signaling by degrading collagen, proteoglycans, and other components of ECM and allowing epithelial growth towards the stroma (Bruni-Cardoso et al., Histochem Cell Biol 129: 805, 2008). Heparanase is an endoglycosidase that degrades heparan sulfate, an important component of the basal membrane, which acts as a reservoir of growth factors regulating different aspects of cell physiology. It has been reported that this enzyme is relevant in tumor invasiveness and metastasis (Ilan et al., Int J Biochem Cell Biol 38: 2018-2039, 2006), angiogenesis, development (Patel et al., Development 134, 4177-4186, 2007), and also ECM remodeling in adult VP after castration (Augusto et al., Cell Tissue Res 332: 307, 2008). We hypothesized that heparanase-1 activity would have a relevant role in coordinating the dynamics of the epithelium and stromal remodeling during the early postnatal development of the rat VP, and used Western blotting, RT-PCR and qRT-PCR to compare heparanase-1 protein and mRNA content at days 0 (day of birth), 3, 6, and 91. Heparanase was found during VP development and the protein level increased after birth, reaching a plateau after day 3. The amount of the pro-enzyme showed a similar behavior. Activation (the ratio between the active form and the pro-enzyme) was constant during this period. The level of heparanase-1 mRNA also increased from day 0 to day 6. These results showed that heparanase is present during VP development and may play an important role in ECM remodeling and cell signaling by degrading heparan sulfate proteoglycans and releasing bioactive molecules.

Keywords: Heparanase, Development, Ventral prostate, Neonatal

07.2 - MMP-2 Modulates the Development of the Rodent Ventral Prostate

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We have shown before that matrix metalloproteinases (MMP) exhibit localized distribution during the early postnatal development of the rat ventral prostate (VP) (Histochem Cell Biol 129: 805, 2008). We then hypothesized that epithelial growth, branching, and canalization in the VP would require matrix remodeling, and hence MMP activity. Thus, we decided to evaluate the role of MMP-2 in the ventral prostate development either blocking MMP-2 using siRNA in whole organ culture and analysing morphological aspects of this gland in MMP-2 null mice. Inhibition of MMP-2 by siRNA compromised VP morphogenesis. MMP-2 silencing reduced organ size (on day 6) and epithelial area (on days 4
and 6), resulting in decreased number of epithelial tips. Histology and second harmonic generation (SHG) revealed that MMP-2 silencing affected VP architecture by interfering with lumen formation and cellular organization in both epithelium and stroma, paralleling intense accumulation of collagen fibers. The ventral prostate of adult MMP-2 null mice showed smaller relative weight, epithelial and smooth muscle cell volume, besides accumulating of reticular and collagen fibers. MMP-2 null neonate mice (day 6) showed lower cell proliferation rate and and also reticulin fiber accumulation in their mesenchymal/stromal compartment. No difference in cell proliferation was noted between adult MMP-2 null and wild type (C57BL/6) mice. In conclusion, MMP-2 plays an important role in ventral prostatic growth, being directly involved with epithelial morphogenesis and acquisition of normal adult histology.

**Keywords:** development, MMP-2, ventral prostate

**07.3- Type I and Type III Collagen and Elastic Fibers Appearing During Early Post-Natal Rat Prostate Development**

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The process of branching morphogenesis in rats and mice will give rise to three distinct bilaterally symmetrical prostatic lobes: the anterior prostate (AP), the dorsolateral prostate (DLP), and the ventral prostate (VP). Concurrent with the process of ductal branching morphogenesis, epithelial and mesenchymal/stromal differentiation occurs in the first 2–3 weeks after birth in rats and mice. Herein, we investigated the dynamic of extracellular matrix fibers deposition, such as type I and type III collagen fibers and elastic system fibers, in the rat prostate stroma during the first 4 weeks of the postnatal development. Wistar rats (n=16) were killed by overdose of pentobarbital (30mg/kg) after 7, 14, 21 and 28 postnatal days. The prostate complex together with the bladder and urethra were excised, fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr, washed, dehydrated, cleared, and embedded in Paraplast Plus embedding medium. Sections of 5 µm were stained by Weigert's Resorcin-Fucsin and some sections were processed for immunohistochemistry for type I collagen (Sigma, C2456, Clone COL-1) and type III collagen (Sigma, C7805, Clone FH-7A). Type I collagen fibers appeared around prostatic epithelial cords and in the interstitium at the first week and a major amount were identify in the next weeks, with thicker fibers appearing organized around the ducts in the fourth week. Delicate type III collagen fibers were identified at the second week filling the stroma in a disorganized distribution and after 21 and 28 days of the postnatal development were organized restricted surrounding the ducts. Very thin elastin containing fibers were observed in the prostate stroma only around the ducts and in the interstitial stroma at the second week. At the third and fourth weeks these fibers became thicker and organized in a bigger amount around the ducts. In conclusion, this
study showed that distinct extracellular matrix fibers deposition occurs simultaneously with the mesenchyme cytodifferentiation in fibroblasts and smooth muscle cells and at beginning of the glandular secretory function in the rat prostates.

**Keywords:** collagen fibers, elastic fibers, post-natal development, prostate

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**07.4- Fetal Programming Impairs Glandular Maturation and Decreases Matrix Metalloproteinases 2 and 9 Activity in Rat Ventral Prostate**

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Fetal programming has been associated with impairment of fetal growth and development. Although the majority of studies of developmental programming have investigated the cardiovascular system, recently studies focusing the reproductive system demonstrated changes in some reproductive parameters such as: reduction in serum testosterone, in testicular weight and delay of testicular descent. Thus, the aim of this study was to investigate the effects of fetal programming induced by in utero protein restriction on rat ventral prostate morphophysiology. Male Wistar rats with underwent in utero fetal programming by protein restriction (normal diet=17% protein; protein in restricted diet=6%) was killed at age of 16 week and the ventral prostate was excised, weighted and processed for histology and gelatin-zymography analyzes. The programmed male offspring presented decrease in both absolute and relative ventral prostate weight compared to matched control. Histologically, the prostate showed ducts with reduced luminal area with few prostatic secretion accumulated into the ducts. Moreover, the interstitial stroma appears thinner compared to the control groups. The analyses of gelatin-zymography revealed decrease in both MMP-2 and MMP-9 activity in programmed rats, which can be correlated with stromal condensation. In conclusion, the fetal programming induced by protein restriction appears to delay the ventral prostate maturation and alters activity of enzymes involved with tissue remodeling.

**Keywords:** fetal programming, MMPs, stroma, protein restriction, ventral prostate
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07.5- Intrauterine Estrogenization by Ethinylestradiol Disrupt the Development of Male and Female Adult Mongolian Gerbil Prostate

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Estrogens play a physiological role during prostate development with regard to programming stromal cells and directing early morphogenic events. However, the estrogenic exposures are abnormally high during the critical developmental period, a process referred to as developmental estrogenization. Thus, the present study aimed to analyze the stromal aspects of male ventral prostate and female prostate of adult gerbil, both in normal condition and under the influence estrogenic agents. In addition, we verified the postnatal androgenic effects in these glands exposed to estrogenization during the development. Pregnant females received daily injections of ethinylestradiol (EE, 10 μg/Kg) from the 17th until 19th day of pregnancy. The offspring, consisting of intrauterine estrogenized gerbils, received subcutaneous doses testosterone cipionate (1mg/Kg/ 48h), for 14 days, starting at the 105th postnatal day (EE/T group). When animals of EE and EE/T groups completed 120 days of age, they were sacrificed and male and female prostate fragments were processed for light microscopy and stained with Gömöri’s reticulin. Immunohistochemistry for smooth muscle (SM) α-actin was also performed. Male and female prostates exhibited acini lined by a simple epithelium varying from cubic to tall prismatic profile and inserted into fibromuscular stroma. In the EE and EE/T groups of male and female it was observed increase of collagen fibrils, mainly around lesions. This compared to control group of both prostates present accumulation of collagen fibrils in adjacent region to the epithelium. Furthermore, the data showed a disorder and increase of reticular fibers of treated groups of both prostates in relation to control group. The immunohistochemical study of SM α-actin revealed a decrease in muscle layer that surrounded the alveolus prostatic of male and female EE and EE/T groups compared to control. In other hand, the treated groups verified an increase of muscle layer in regions with lesions in epithelium of both prostates. In male and female EE/T group an absence of muscle layer was observed where epithelial buds are forming. The results showed alteration and apparent increase of stroma in regions with epithelial lesions of treated prostates, mainly with EE/T. Thus the findings of this study demonstrate the importance of estrogen action in the prostatic development of males and females and the additional action of androgens in the adult life.

Keywords: Estradiol, Prostate, Estrogen, Intrauterine, Development
07.6- Differential Matrix Metalloproteinases Activity (Mmps -2, -9 And -14) During Mice Endochondral Ossification

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MMPs are zinc-dependent endopeptidases that, collectively, degrade all components of the ECM. They are able to remodel the ECM during normal processes such as embryogenesis and organogenesis, as well as in pathological processes such as tumor invasion. Our goal was to evaluate the temporal-spatial enzymatic activity of MMP-2, -9 and -14 in mice embryos and newborns during endochondral ossification. Femurs (n=5/period) were collected from fetuses (E13-E20) and 1 day postnatal (PN1), cryosectioned (8 µm) and stored at -80ºC. For in situ zymography, three FITC-conjugated substrates were used (DQ-gelatin, DQ-collagen type I and IV – 1/10 dilution, Molecular Probes) diluted in reactive buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, 0.2 mM NaN3, 0.5% low melting agarose, 0.03% Triton-X 100, 1% DAPI, pH 7.6), for 24-26 hours at 37°C. Negative controls were performed using general inhibitor (100 mM EDTA), MMP inhibitor (10 mM phenantroline) and synthetic MMP inhibitor (SB-3CT, 16 nM, 180 nM for MMPs -2 and -9, and 900 nM for MMP-14, respectively). Our results demonstrate general gelatinolytic activity more intense than general collagenolytic activity in all periods evaluated. During chondrocyte differentiation (E13), fluorescent areas were observed at chondrocytes and perichondrium. At cartilaginous anlagen (E14), enzymatic activity was restricted to bone core and proliferative chondrocytes. After vascular and cellular invasion of cartilaginous anlagen (E15) enzymatic activity was also detected at the center of cartilaginous anlagen in hyperthrophic chondrocytes. From E16 to PN1, both gelatinolytic and collagenolytic activities were found in the growth plate. Using synthetic MMP inhibitor, the differential MMP activity was observed in all periods evaluated and in all substrates used. Taken together, our findings showed, for the first time, the differential MMP activity, and that MMP-2, -9 and -14 play an important role during endochondral ossification, mainly at early stages and at the primary bone formation. Financial Support: FAPESP

Keywords: MMP, in situ zymography, Endochondral Ossification
08. ECM AND NEUROBIOLOGY

08.1- Astrocytes Treated by Lysophosphatidic Acid Induce Axonal Outgrowth by Modulating the Amount of Extracellular Matrix Proteins

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Introduction: Lysophosphatidic acid (LPA) is a potent lipid mediator that evokes a variety of biological responses in many cell types via its specific G protein-coupled receptors. LPA plays important roles in many biological processes such as brain development, oncogenesis, wound healing, and immune functions. We have previously demonstrated that astrocytes primed by LPA induce neuronal commitment of cerebral cortical progenitors in vitro (de Sampaio e Spohr et al., J.Biol.Chem., 2008). Objective: In the present work, by using a neuron-astrocyte coculture model, we examined possible mechanisms behind the neurite outgrowth induced by LPA-treated astrocytes. Results: We showed that LPA, through astrocytes, increases neuronal differentiation, arborization and neurite outgrowth of developing cortical neurons. Treatment of astrocytes with LPA or with conditioned medium derived from LPA-treated astrocytes (LPA-CM) yielded similar results, suggesting the involvement of an astrocyte-derived soluble factor induced by LPA. Further, LPA-CM or LPA astrocyte treatment led to an increase in the extracellular matrix (ECM) proteins laminin and fibronectin. Such increase in ECM protein levels as well as astrocyte permissiveness to neurite outgrowth was reversed by inhibitors of the mitogen-activated protein (MAP) kinase (MAPK) or protein kinase A (PKA) signaling pathways. Conclusions: We provided evidence that LPA induces neuronal fate commitment and increases the complexity of neuronal arborization by modulating the content of ECM proteins in the surface of astrocytes. Our data reveal an important role of astrocytes as mediators of bioactive lipids action in brain development and implicate ECM and MAPK and PKA pathways in this process. Financial Support: FAPERJ, CNPq, CAPES

Keywords: lysophosphatidic acid, neuron-glia interaction, extracellular matrix proteins, axonal outgrowth

08.2- TGF-Beta 1 Promotes Cortical Synaptogenesis in Vitro

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Neuron-astrocyte interactions play critical roles in several events during central nervous system (CNS) development such as neuronal survival and differentiation, cell fate specification, modulation of formation and efficacy of synapses. One of the first identified
The synaptogenic glia-derived molecule was the extracellular matrix protein, thrombospondin. However, the mechanism underlying this event remains unknown. Recently, the transforming growth factor beta 1 (TGF-beta1), a cytokine that binds and is activated by thrombospondin, has been shown to promote synaptogenesis in the peripheral nervous system. Here, we investigated the role of TGF-beta1 as a mediator of thrombospondin effects in synapse formation in cerebral cortex neurons in vitro. Cerebral cortical neurons from 14 days embryonic mice were treated with TGF-beta1 (10ng/ml) and maintained for 12 days in culture. Synapse formation was evaluated by immunocytochemistry labeling against the pre-synaptic protein, synaptophysin and the post-synaptic protein, PSD-95. TGF-beta1 dramatically increased the levels of either synaptophysin (100% increase) and PSD-95 (122%). These data were supported by western blot assays that revealed 60-70% increment in the levels of both proteins in TGF-beta1-treated neurons. Together, our results show a new role for TGF-beta1 as a synaptogenic molecule in CNS and suggest a mechanism underlying thrombospondin induced-synaptogenesis. This work was supported by CAPES, CNPq and FAPERJ.

**Keywords:** TGF-beta1, D-serine, synaptogenesis

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**08.3- Role of MAPK and PI-3K Pathways Activated by TGF-Beta 1 on Radial Glia-Astrocyte Transformation in Vitro**

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Objective: The major progenitor of the developing cerebral cortex is the radial glial (RG) cell, which originates neurons and glial cells. We previously described that the transforming growth factor beta 1 (TGF-beta1) promotes RG-astrocyte transformation in vitro. TGF-beta1 actions are mainly triggered by its receptors, TGFRI and TGFRII, followed by phosphorylation and nuclear translocation of the transcription factors, SMADs 2 and 3. Alternatively, TGF-beta1 might activate non-canonic pathways such as MAPK (mitogen activated kinase protein) and PI3K (phostatidilinositol-3-kinase), although the contribution of these signaling pathways to astrocyte differentiation is less known. Here, we analyzed the role of MAPK and PI3K on astrocyte differentiation induced by TGF-beta1 in vitro. Methods and Results: Primary RG cell cultures derived from cerebral cortex of 14 days (E14) embryos were maintained for 24h with DMEM-F12 medium supplemented with TGF-beta1 (10ng/mL) and/or the inhibitors of TGFRI (SB431542, 10uM), PI3K (LY294002, 5uM) and MAPK (PD98059, 50uM). Cell differentiation was analyzed by immunocitochemistry for the markers: BLBP, (RG cell), GFAP (astrocyte), Nestin (neural progenitors) and betaTubulinIII (neuron). The immunocitochemistry analysis revealed a 43% increase in the number of GFAP+ cell in response to TGF-beta1 treatment. This event was reversed by the MAPK inhibitor, PD98059. These data were supported by western blotting of RG cell cultures: whereas treatment of RG cultures with TGF-beta1 increased the levels of phosphorylated MAPK, simultaneously addition of TGF-beta1 and PD98059 inhibited Erk phosphorylation. The number of Nestin+/BLBP+ was decreased by 36%, and this event was reversed by the
PI3K inhibitor, LY294002. TGF-beta1 treatment decreased the number of betaTubulinIII by 18%, and this effect was reversed by both inhibitors. Conclusões: These data suggest that TGF-beta1 might act through distinct pathways to determine cell fate: through MAPK, on astrocyte generation, through PI3K, on the maintenance of RG progenitor phenotype, and through both pathways, on neuronal generation. Financial support: FAPERJ, CNPq, CAPES.

**Keywords:** Astrocyte, Radial glia, TGF-beta1

**08.4- TGF-Beta 1 Promotes Differentiation of Radial Glia Into Astrocyte and Disrupts Basal Membrane in Cerebral Cortex in Vivo**

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The major cerebral cortex stem cell population present in the developing brain is the radial glial cell (RG), which originates neurons and glial cells. RG cells present a bipolar morphology, which is maintained by anchoring its pial process in the basal membrane structure that covers the brain surface. The mechanisms that modulate RG progenitor morphology maintenance or its differentiation into specific cell types are not completely known. Recently, we showed that the transforming growth factor beta 1 (TGF-β1), which is a regulator of extracellular matrix (ECM) protein synthesis and deposition, promotes RG into astrocyte differentiation in vitro. Here, we analyzed the potential of TGF-β1 in RG-astrocyte transformation in vivo and the involvement of basal membrane in this event. Pregnant Swiss female mice (14 days) were anesthetized and the embryos were subjected to intraventricular injections of TGF-â1 (100ng) followed by an intraperitoneal injection of BrdU solution (10mg/mL) 1h later. After 48h, the female was sacrificed, the E16 embryos removed and the brains collected and fixed in 4% paraformaldehyde. Brains were vibratome-sectioned and processed for immunohistochemistry with antibodies against RG markers (BLBP and Nestin), astrocytes (GFAP) and the pial surface basal membrane of the brain (Laminin). The TGF-β1-injected cortex presented huge changes in RG cell morphology, characterized by the loss of the typical process bearing morphology of RG in 60% of BLBP+ cells and a 90% increase in the number of GFAP+ cells. Number of GFAP/BrdU cells was not affected by TGF-β1. This event was followed by a robust disruption of basal membrane, characterized by ectopic Laminin+ cells throughout the cerebral cortex. Conclusions: Our data show that RG differentiation into astrocytes induced by TGF-β1 might be associated to reorganization of the ECM protein, Laminin, suggesting a novel role for this molecule in cerebral cortex development. Financial support: FAPERJ, CNPq, CAPES.

**Keywords:** astrocytes, cerebral cortex, radial glia, neural stem cell, TGF-beta 1
08.5- Astrocytes as Mediators of Thyroid Hormones Actions in Neuronal Differentiation and Neurite Outgrowth: Role of Chondroitin Sulfate

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Introduction: Thyroid hormones, thyroxin (T4) and 3, 5, 3’-triiodothyronine (T3), are essential for a wide range of processes of mammalian brain development, such as neuronal migration, neural cells differentiation and myelination. Some of THs actions are mediated by astrocytes, though the mechanism of action underlying this event is still controversial. Here we investigated the interplay between THs action and glycosaminoglycans (GAGs) in neuron-astrocyte interactions in vitro. Experimental Procedures: Astrocytes primary cultures were prepared from cerebral cortex derived from newborns Swiss mice, in DMEM/F12 supplemented with 10% of fetal bovine serum. After reaching confluence, the cultures were incubated an additional day in medium without serum followed by three days-treatment with 50nM of T3 or T4. Progenitor cells derived from 14 days embryonic (E14) cerebral cortex were cultured either onto treated astrocyte monolayers or in the presence of their conditioned medium (CM). To analyze a possible influence of GAGs, cultures were enzymatically digested with chondroitinase AC 2h prior addition of progenitor cells. After 24h of coculture, neuronal differentiation was analyzed by immunocytochemistry assays against the neuronal marker, ã-tubulin III, and morphometrical analysis. Results: T3- and T4-treated astrocytes increased neuronal population by 36% and 50%, respectively. In addition, THs astrocyte treatment enhanced neurite length by 73% and increased neuronal arborization as revealed by doubling the number of neurons with two and three neurites. Surprisingly, treatment of astrocyte monolayers with chondroitinase AC completely inhibited these effects. Similarly, culture of E14 progenitor cells in THs treated-astrocyte CM yielded a 48% increase in neurite length, in both conditions; although only T4-CM enhanced neuronal population. Conclusions: Our results show that cerebral cortex astrocytes treated by THs increase neuronal differentiation and maturation through modulation of extracellular cellular components. Our data clearly suggest the involvement of chondroitin sulfate glycosaminoglycans as key mediators of THs actions during neuron-astrocyte interactions in the cerebral cortex. Financial Support: FAPERJ, CNPq and CAPES.

Keywords: astrocytes, glycosaminoglycans, neuronal differentiation, neuron-glial interactions, thyroid hormones
08.6- Flavonoids and Astrocytes Crosstalking: Implications for Brain Development and Pathology

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Introduction: Flavonoids are naturally occurring polyphenolic compounds present in a variety of fruits, vegetables, cereals, tea and wine. Evidences have suggested that these phytochemicals might have an impact in brain pathology and aging, however, either their mechanisms of action are not completely known. We previously demonstrated that astrocytes treated by the flavonoid castacin increased cerebral cortex neurons by modulating the pool of neuronal progenitors (Spohr et al., J.Neurosci.Res., 2009). Objective: In the present work, we performed a screening of flavonoid actions by analyzing the effects of these substances (hesperidin, quercetin and rutin) on murine cerebral cortex astrocytes and neural progenitors. Methodology: Neural progenitor cultures from 14 days embryonic mice (E14) were treated with flavonoids (10μM) and maintained for 2 days in culture. The indirect effect of flavonoids, via astrocytes, was evaluated by culturing E14 progenitors for 48 hours in the presence of conditioned medium (CM) from flavonoid-treated astrocyte. In both cases, direct and indirect assays, neuronal, astrocytic and oligodendrocytic differentiation was evaluated by immunocytochemistry against specific cellular markers. Cell proliferation and cell death were analyzed by Ki67 and caspase 3-immunolabelling, respectively. Results: Treatment of neural progenitors with hesperidin enhanced neuronal population as revealed by 75% increase in the number of beta-tubulin III cells. This effect was mainly due to modulation of neuronal progenitor survival by decreasing cell death. Pools of astrocyte and oligodendrocyte progenitors were not affected by hesperidin. Culture of neural progenitors with CM from hesperidin treated-astrocyte yielded similar results, suggesting the involvement of an astrocyte-derived soluble factor. Quercetin and rutin had no effect on neuronal population. Cellular differentiation, proliferation and neuronal complexity were not affected by any of the flavonoids used. None of the flavonoids affected astrocyte morphology and proliferation. Conclusions: Together, our data suggest that hesperidin influences neuronal population by two mechanisms: 1) directly, by decreasing neuronal death; 2) indirectly, via astrocytes, by modulating the pool of neuronal progenitors. Grant Support: FAPERJ, CNPq, CAPES.

Keywords: Astrocytes, Flavonoids, Neural progenitors
08.7- Retinal Vascular Abnormalities in PRPC Knockout Mice

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Prion scrapie (PrPsc), the altered form of cellular prion protein (PrPc), is the infectious particle of a class of diseases named spongiform encephalopathies. However, the physiological function of PrPc has not been established so far. In this work, we prepared retina whole mounts of newborn PrPc knockout and wild-type mice and labeled them with DAPI for nuclei labeling, anti-laminin, anti-type IV collagen and anti-PECAM-1 antibodies in order to investigate possible structural alterations associated to the lack of PrPc expression in the eye. Laminin polymers observed in wild-type mice displayed polygonal patterns, comparable to those previously described in embryonic mice astrocyte in culture [Freire et al. (2004) J. Cell Sci. 117: 4067-4076]. This polygonal pattern was not observed in knock-out mice, which is compatible with PrPc as being a laminin receptor. In addition, several morphological changes were observed in knockout mice, namely: 1) decrease in vessel diameter, 2) reduction in capillary branching, 3) loss of pericyte adherence and 4) an increase in the incidence of retinal detachment. We are currently examining ocular fundus in knockout and wild-type animals in order to analyze the vascular network in vivo. Our findings suggest that molecular, cellular, histological and functional particularities in mice retina may be correlated to PrPc function in vasculogenesis and vessel maturation in mammals. Supported by CAPES, CNPq and FAPERJ

Keywords: PrPc, retinal vasculogenesis, knockout, visual function, laminin

08.8- Interaction between a Specific Ganglioside and Integrins During Neuronal Migration in Rat Developing Cerebellum

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During development of nervous system there are some important steps occurring such as cell proliferation and migration. In concerning to neuronal migration during cerebellum development, a number of molecules have been studied to understand the possible signaling pathways involved in this event. One of these molecules is the ganglioside 9-O-acetyl GD3 which immunoblockade by monoclonal antibody Jones leads to a significant decrease in neuronal migration either in cerebral cortex and cerebellum. One of our hypotheses is the interaction of this ganglioside to integrins. Based on this idea, the ganglioside may be a possible candidate as a modulator of integrins related signaling pathways. To address these questions we performed rat cerebellar microexplants cultures and antibody perturbation
experiments to assess the influence of integrins on neuronal migration. We also performed immunoprecipitation assays to ensure the link between beta 1 integrin and 9-O-ac GD3 in our model as well as confocal microscopy analysis of immunocytochemical experiments. Sucrose gradient was performed to identify the possible location of 9-O-acetyl GD3 within membrane microdomains. We observed that in our model, the blocking peptide against alpha 6 integrin is capable to arrest the migration. These data was analyzed by T test comparing control cultures to treated ones. Alpha 6 and beta 1 integrins subunits co-localize with 9-O-acetyl GD3 in culture explants and the ganglioside immunoprecipitates the beta 1 integrin subunit in our cultures. In sucrose fractionating experiments we find 9-O-acetyl GD3 in low density fractions indicating its possible participation in specific membrane microdomains. We can partially conclude that 9-O-acetyl GD3 ganglioside is linked to the beta 1 integrin in our model suggesting a modulation of its activity, maybe altering integrin affinity for laminin. Once in cerebellum the beta 1 subunit can be associated to alpha 6 subunit, we showed that blocking alpha 6 subunit with blocking peptides have led to inhibition of migration. These molecules can be associated to each other within specific membrane microdomains facilitating the triggering of signaling pathways. Further experiments will be necessary to evaluate the involvement of specific molecules such as FAK and src-c, key molecules in integrin signaling pathway.

**Keywords:** gangliosides, migração neuronal, integrinas

### 08.9- Effects of Epidermal Growth Factor on Cerebellar Glia

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Two glial cell population play pivotal role during cerebellar development: Bergmann glia (BG) cells, responsible for guiding neuronal migration and astrocytes, the main source of trophic factors for granular neurons. Here, we analyzed the effect of epidermal growth factor (EGF) in cerebellar glial development and neuronal migration in vitro and the signalling cascades involved in these events. Cerebellar astrocytes from newborn rats were cultured for 10 days in vitro in DMEM/F12 medium, supplemented with 10% fetal bovine serum. After reaching confluence, cells were washed and maintained for additional 2 days in the presence of 10 ng/ml of EGF. For BG studies, cultures from 7 days old rats cerebella were maintained for 3 days in vitro in the previous medium, followed by 2 days in the presence of 10 ng/ml of EGF. Morphometric analyses of BG were performed by immunocytochemistry for the glial marker glial fibrillary acidic protein (GFAP). Levels of extracellular matrix proteins (ECM) of astrocytes were evaluated by immunocytochemistry for laminin and fibronectin. Signaling pathways were analyzed by using different signaling cascade inhibitors: PD98059 (MAPK; mitogen-activated protein kinase), KT5720 (PKA; protein kinase A), Staurosporin (PKC; protein kinase C) and LY294002 (PI3K; phostatidilinositol-3-kinase). Treatment of cerebellar astrocytes with EGF strongly induced synthesis and secretion of ECM proteins, fibronectin and laminin. Similarly, EGF greatly induced BG elongation (increase by 50%), an essential
condition for neuronal migration. Both effects, in BG and astrocytes, were impaired by the MAPK inhibitor. To address neuronal migration, explants from 7 days old rats cerebellum were kept on laminin in the presence of EGF (10 ng/ml), and cell migration was analyzed after 48hr. After this period, EGF increased by 30% the distance of cell migration. Time lapse microscopy assays suggested that the effect of EGF in neuronal migration is delayed since EGF did not alter either neuronal velocity or mobility after short-term (2hr) treatment. Neuronal migration induced by EGF was inhibited by the MAPK inhibitor, PD98059. Our results suggest that EGF through MAPK pathway is an important modulator of glial biology in the cerebellum: 1) EGF induces synthesis and secretion of ECM proteins by astrocytes; 2) EGF induces neuronal migration and BG elongation. Grant Support: FAPERJ, CAPES and CNPq.

**Keywords:** Bergmann glia, Cerebellum development, Epidermal Growth Factor

### 08.10- Prion Protein Transduces Signals after Binding to Laminin Gama1 Chain Via Metabotropic Glutamate Receptors

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The prion protein (PrP C) is a cell surface glycoprotein that is highly expressed in the nervous system whose conformationally modified isoform is responsible for prion diseases. Multiple and diverse functions of PrP C have been reported. PrP C interacts with a peptide at the carboxi-terminal of the laminin γ1 chain (RNIAEIIKDI) promoting neuronal survival and neuritogenesis. Using an intracellular Ca²⁺ probe, Fluo-3 AM, we verified that the laminin γ1 peptide, in particular its KDI domain, increased intracellular Ca²⁺ in wild-type neurons whereas no effect was observed in PrP C-null neurons. Neuritogenesis as well as Ca²⁺ signaling were abrogated by U73122, a specific inhibitor of the phospholipase C (PLC) and by 2APB, a specific inhibitor of inositol 3-phosphate receptor at the endoplasmatic reticulum. Remarkably, inhibition of a group 1 metabotropic glutamate receptor (mGluR1) with LY367385 decreased the effect of PrP C-laminin γ1 peptide interaction upon mobilization of intracellular Ca²⁺, and blocked the activation of PKC. The expression of mGluR1 or mGluR5 receptors in HER293 cells reconstituted the signaling pathways mediated by PrP C-laminin γ1 peptide interaction. These data established that group I mGLuRs are key molecules promoting PrP C-laminin neurotrophic properties. Moreover, our data suggest that Ca²⁺ is the first
intracellular signal activated by PrP$^C$- laminin $\gamma$-1 peptide interaction which is followed by consecutive PKC, AKT and ERK activation which finally promotes neuritogenesis.

**Keywords:** calcium, glutamate receptors, laminin, prion protein

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**08.11- Impairement in Ca2+ Signaling Mediated by Laminin in Neurons Expressing Mutant Prion Proteins Associated with Genetic Prion Diseases**

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Prion protein (PrPC) is a GPI anchored protein mostly expressed at the surface of neuronal and glial cells. Mutations in the PrPC gene are involved in neurodegenerative disorders called Transmissible Spongiform Encephalopathies (TSEs) or genetic prion diseases. It is still under debate if PrPC loss-of-function is, at least for a part, responsible of the pathogenic process of these diseases. Some PrPC functions have been associated with its ability to bind specific cellular proteins. We previously demonstrated that the region between aminoacids 173 to 182 of PrPC binds to a peptide (RNIAEIIKDI) at the laminin $\gamma$1 chain promoting neuritogenesis and memory formation. A 3 fold increase in the intracellular calcium concentrations was observed in primary neuronal cultures derived from wild-type mice upon treatment with laminin $\gamma$1 peptide while any effect was observed in PrPC-null neurons. The expression of PrPC in an immortalized cell line derived from PrPC-null neurons (CF10) rescued calcium signaling responses mediated by PrPC-Laminin $\gamma$1 peptide interaction. Three PrPC mutations associated with genetic Creutzfeldt-Jakob disease and Fatal Familial Insomnia map within the laminin $\gamma$1 binding site. In order to evaluate PrPC-loss-of-function, cDNAs coding mouse PrPC molecules with the equivalent human mutations were inserted into pCDNA3 and expressed in CF10 cells. The expression of PrPC mutants 177N, 179I and 182A as well as the wild-type protein was confirmed by flow cytometry. Cells expressing the wild-type or mutant proteins were labeled with the intracellular calcium probe Fluo 3 AM, treated with laminin $\gamma$1 peptide in the presence of extracellular calcium. Data acquisition for intracellular calcium was performed with a confocal microscope Bio-Rad Radiance 2100/Nikon. Cells expressing the mutations 177N or 182A exhibited a similar increase in intracellular calcium when compared to the wild-type PrPC. Conversely, the PrPC mutation 179I, presented 30.5 ± 1.1 % lower intracellular calcium levels upon laminin $\gamma$1 peptide interaction compared to wild-type PrPC. These data suggest that PrPC mutation at codon 179 causes a partial impairment in calcium signaling mediated by its association with laminin. The impact of this impairment on neuronal plasticity and its possible participation in the disease pathogenesis is under investigation.

**Keywords:** Prion Protein, Laminin, Calcium
08.12- Flavonoids from Croton Betulaster Induces Glial and Neuronal Differentiation of Human Glioblastoma Multiform Cells and Changes the Pattern of Fibronectin Expression.


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Glioblastomas are rapidly proliferating malignant tumours, particularly resistant to classic antitumor treatment. Flavonoids derived from plants are active compounds responsible for various biological effects including antitumoral activity. Our previous study demonstrated that flavonoids isolated from the Brazilian plant *Croton betulaster* (acacetin, casticin, apigenin and pendulitin) can inhibit the growth of human glioblastoma multiform cell line GL-15, and down regulated TGFbeta secretion. In this study we investigated the effect of apigenin and pendulitin, the most active flavonoids from C. betulaster, on morphogenesis, and expression of ECM component fibronectin in human glioblastoma cells. Synchronized GL-15 and U251 cell lines, derived from human multiform glioblastomas, were cultured in supplemented DMEM on a ECM support of laminin and treated with flavonoids (50µM) for 48 h, or with the vehicle DMSO (0.1%). Immunocytochemistry assays show that glioblastoma cells in control conditions present a bipolar phenotype and express constitutively nestin, the marker of precursors cells, and low levels of GFAP (astrocyte marker) and betaIII-tubulin (neuronal marker). We observed that in both glioblastoma lineages nestin levels were down regulated after flavonoids exposure. On the other hand a subpopulation of U-251 and Gl-15 cells presented a multipolar phenotype with over expression of GFAP, characterizing astroglial differentiation, and another subpopulation presented a filamentar phenotype with long cellular process expressing betaIII-tubulin, characterizing neuronal differentiation. Moreover, beside neuroglial differentiation, we observed that flavonoids induced over expression of fibronectin in glioblastoma Gl-15 and U-251 cells. These results indicates that flavonoids extracted from *C. betulaster* present antitumoral and morphogenetic activity to glioblastoma cells, and suggests these molecules as promising supplementary drugs for glioblastoma treatment.

**Keywords:** Glioblastoma, Flavonoids, Differentiation, Fibronectin, ECM
08.13- Effects of A Spinal Cord Lesion on the Proliferation of Subventricular Zone Precursors of Adult Rats

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Spinal cord injuries (SCI) promote apoptosis on sensorimotor cortex of adult rats. Apoptosis induced by specific lesion of neurons on cortical layer V stimulates the formation of new neurons by the subventricular zone of the lateral ventricles (SVZ). Also, a SCI promotes extensive sensorimotor cortex reorganization. On the present study, we investigated if a model of SCI that induces cortical apoptosis and plastic reorganization also modulates SVZ proliferation. Adult Wistar rats were subjected to laminectomy at T9 and compression with a vascular clip (30g 2 min). We analyzed two groups: injured (n=4) and sham (n=3) and 2 survival times: 1 and 5d. This model of compression causes a moderate injury and the animals showed hindlimb paralysis. As a control of the injury, the animals were analyzed for their motor behavior (BBB test). 2h after the surgery, all animals received an injection of proliferation marker BrdU. The animals sacrificed 1d after injury received 3 BrdU injections. For the 5 days survival group, the BrdU injections were daily. All animals were sacrificed 2h after the last injection. The brains and spinal cords were dissected and prepared for immunohistochemistry or stained for HE or Kluver-Barrera. The brains were analyzed as anterior (1.20-1.00; 0.70-0.48; 0.20mm) or posterior SVZ (-0.26; -0.30-0.40; -0.80-0.92mm) relative to Bregma. For each ventricle, all BrdU+ cells on the lateral wall were counted under fluorescence microscope. We found that 5d after injury, experimental animals had more BrdU+ cells in both SVZ regions when compared to control animals (unp t test; antSVZ: control 137.5±4.66; exp 160.9±4.94, P

**Keywords:** neurogenesis, spinal cord lesion, cortical plasticity

08.14- Changes in Heparan Sulfate Proteoglycan Expression Pattern During Early Cerebellar Development

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The correct development of the CNS relays on accurate processes controlled by intrinsic programs as well as on extracellular cues. Glial cells play important roles in the development of the CNS although it is not completely understood how those cells influence neuronal precursors during brain development. Heparan sulfate proteoglycans are components of cell surface and extracellular matrix that participate in a wide range of events, including axonal guidance, synapse formation, and modulation of growth factor and morphogen signaling. We
were interested in knowing if there are changes in the expression of the two major brain heparan sulfate proteoglycans, glypican and syndecan, during early postnatal development and if their expression is modulated by glial cells. Initially, we investigated the expression of four members of the syndecan family and six members of the glypican family, as well as heparan sulfate sulfotransferases, and extracellular sulfatases by cerebellar neuronal precursors using quantitative real time RT-PCR. Our data shows that, in isolated granule cell precursors, syndecans 2, 3 and 4 are upregulated overtime, and that glypicans 2, 3, 5 and 6 are downregulated from postnatal day 3 to 9. Also, the expression of the heparan sulfate sulfotransferases changed during early postnatal development, being up- or downregulated depending on the sulfotransferase analyzed. Both extracellular sulfatases evaluated are downregulated in the early postnatal period. In order to look at the influence of glial cells on neuronal precursors, we compared the expression of syndecans and glypicans by neuronal precursors treated with glial conditioned media. Our data show that glial cells release soluble factors that stimulate axonal outgrowth by cerebellar neuronal precursors in an age-dependent manner. Three-day old neuronal precursors treated with glial conditioned media upregulate the expression of syndecans and downregulate the expression of glypicans. The data presented here indicate that heparan sulfate proteoglycan expression is controlled during CNS development and can be modulated by interactions between neuronal precursors and glial cells. Support: FAPESP, CNPq.

**Keywords:** Cerebellum, Development, Glial Cell, Glypican, Syndecan

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**08.15- Physical Activity Triggers Neuroplasticity Events in Mouse Cerebellum**

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**Purpose:** Physical activity stimulates neuroplasticity in CNS. The cerebellum is involved with motor learning and control presenting a high neuroplasticity. In order to better understand the molecular and cellular mechanisms involved in the process, gene expression was evaluated using real time RT-PCR and protein expression was analyzed by immunofluorescence. The genes investigated in this study were the heparan sulfate proteoglycans syndecans 1-4, the axonal growth associated protein 43 (GAP43) and CD133, a marker for neural stem cell. Methods: Adult male C57/BL6 mice were randomly assigned to sedentary and trained groups. Physical training consisted of two 1.5-h daily sessions of swimming training, 5 days/wk, for 4 wk. Sedentary mice were placed in the swimming apparatus for 5 min twice/wk during the experimental protocol. Two animals from each group received BrdU injections at training days 3, 7, 14, 21 and 28. After the training period, animals were anesthetized and euthanized followed by removal of cerebella. Animals that received BrdU injections were perfused transcardially and the sectioned cerebella were submitted to immunofluorescence labeling using anti-BrdU and anti-CD133 antibodies. Results: No changes were found for GAP43 and CD133 gene expression of cerebella after
training. Physical activity regime did not alter the expression of syndecan 1 and 4. However, the expression of syndecan 2 was three times higher in trained group compared to sedentary (p

**Keywords:** neuroplasticity, physical activity, stem cell, syndecans

### 09. ECM AND CANCER

#### 09.1- The Mechanism of Lymphocyes Heparanase Activation

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Syndecan family corresponds to cell surface heparan sulfate proteoglycans and is related with cell adhesion, migration and proliferation. Heparanase-1 (HPA1) is an endo-beta-glucuronidase encoded by 4q21.3 gene that specific degrades heparan sulfate (HS) chains from proteoglycans. HPA1 increases syndecan-1 shedding promoting angiogenesis, tumor growth and metastasis (Sanderson et al., 2007). The heparanase-2 (HPA2) isoform presents no enzymatic activity and the gene is located in the 10q23.24 chromosome. Previous studies have shown that serum or plasma from breast cancer patients enhances both heparanase isoforms expression (HPA1 and HPA2) in the healthy women lymphocytes (Theodoro et al., 2007). The aim of this study is to elucidate the mechanism of lymphocyte activation by tumor cells. In vitro assays demonstrated that co-culture medium obtained from a human breast cancer cell line (MCF-7) and healthy woman lymphocytes were also able to stimulate both isoforms of heparanases (HPA1 and HPA2), while MCF-7 conditioned medium had no effect. Using specific monoclonal antibody target to heparan sulfate proteoglycan F69-3G10 (Seikagaku Corporation, JAPAN) and digesting conditioned co-culture medium with heparitinases I and II from Flavobacterium heparinum it was elucidated that HS should be involved in the stimulatory effect upon lymphocytes. This data was confirmed using a monoclonal antibody against syndecan-1, MCA681-CD138 (Serotec Accurate Scientific, USA). Labeling lymphocytes with [35S]-sulfate it was confirmed that activated lymphocytes synthesized chondroitin sulfate and secrete dermatan sulfate and heparan sulfate. The results confirmed that lymphocytes heparanase isoforms expression may be stimulated by the presence of the tumor and be used as breast cancer marker. The mechanism of lymphocytes activation involves heparan sulfate signaling that is secreted by the tumor cells.

**Keywords:** heparan sulfates, heparanase, heparitinases, lymphocytes, tumor
The incidence of kidney cell carcinoma has been rising around 4% each year. In the United States, the incidence reached 54390 new cases with 13010 deaths in 2008 (Jemal et al. 2008). Molecular biology and extracellular matrix (ECM) remodelling has an important role in the understanding of the structural alterations of kidney tumor cells, as well as in the precocious diagnosis. Increased expression of heparanase (HPA) is involved with tumor development and metastasis (Hullet et al. 1999; Vlodavsky et al. 2000; Staquicini et al., 1990). HPA is an endo-beta-glucuronidase that cleavages intradisaccharide heparan sulfate chains from proteoglycans and has been involved with tumor development and metastasis (Cohen et al. 1994). The main objective of this study was to determine HPA expression and the sulfated glycosaminoglycans (GAG) profile in the kidney cell carcinoma, compared to the non tumoral and transition tissues obtained from surgery. Real time PCR was performed to quantified both HPA isoforms, HPA1 and HPA2, using two housekeeping gene, the ribosomal protein RPL13a and GAPDH. Immunohistochemistry (IHC) was quantified by digital software ImageLab® using four specific antibodies to HPA1 and HPA2 isoforms (Santa Cruz). GAG was identified and quantified by agarose gel electrophoresis and densitometry. HPA1 was significantly higher expressed in kidney cell carcinoma tissues compared to the non tumoral tissues. HPA1 and HPA2 isoforms presented the same IHC labeling intensity. However, the active isoform of HPA1 (HPA1 50 kDa) has shown higher index of positive cell number indicating that possibly this isoform could be related to carcinogenesis. GAG quantification revealed a decreased in heparan sulfate (3.6 ± 3.5), (17.6 ± 20.5) and dermatan sulfate (16.9 ± 21.7), (44.2 ± 28.5), respectively, for cell kidney carcinoma and non tumoral tissues, while chondroitin sulfate was increased in cell kidney carcinomas (50.6 ± 37.2), compared to the non tumoral tissues (10.0 ± 9.4). The alterations in the HPA isoforms expression and GAG profile could be useful to detect cell kidney carcinomas and potentially target future therapeutic molecules.

Keywords: carcinoma, glycosaminoglycans, heparanase, isoforms, kidney
09.3- Role of the Microenvironment in the Treatment of Melanoma Cells with Doxorubicin

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In the past 50 years, melanoma incidence in developing countries grew faster than any other type of cancer, mainly due to the sun exposure. During tumor progression, the tumor cells are in contact with macromolecules of extracellular matrix (ECM), which mediates the adherence, migration, proliferation and gene expression of these cells. The melanoma is characterized by its high capacity of invasion, metastasis and chemoresistance to drugs. The tumor invasion and metastasis occurs when there are proteolytic degradation of basement membrane and ECM components by matrix metalloproteinases (MMPs). This work establishes the role of the microenvironment in the treatment of melanoma cells with doxorubicin, an antibiotic used as chemotherapy in solid tumors. The cytotoxicity, MMP-2 activity and p53 protein expression were evaluated when metastatic melanoma were cultured on type I collagen containing human dermal fibroblast (dermal equivalent culture), simulating in vitro the environment of tumor invasion. Fibroblast of human dermis (FF-287) were cultured in type I collagen, forming the dermal equivalent that serves as substrate for human melanoma cell line (SK-Mel-103) growth. The toxicity of Doxorubicin was tested by the Trypan blue exclusion assay. Zymography was performed to analyze MMP-2 activity, and Western Blotting to evaluate p53 protein expression. Cytotoxicity of Doxorubicin (0.7 mg/ml) is lower in the presence of the dermal equivalent (17%), when compared to the other conditions: uncoated substrate (75%) or seeded on type I collagen (67%) after 24 and 48 hours of treatment. In addition, inhibition of p53 protein activation and MMP-2 activity by doxorubicin are seen when melanomas are grown on the dermal equivalent compared to the other conditions. From these results we conclude that the dermal equivalent culture offers a protection to the melanoma cell when treated with doxorubicin, when compared to uncoated substrate, showing the importance of the microenvironment in drugs screening tests. Support: FAPESP

Keywords: doxorubicin, melanoma, microenvironment
09.4- Heparanase Expression and Glycosaminoglycans Profile in Different Breast Cancer Cell Lines

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The fact that heparan sulfate proteoglycans are so widely distributed is testament to the vital importance of these molecules in cell development and function. Remodeling of extracellular matrix following heparanase cleavage of heparin sulfate chains results both in liberation of glycosaminoglycan–anchored bioactive molecules fragments that modulate growth factor binding to their receptors (Sanderson et al., 2004). The amount of heparanase required for heparin sulfate remodelling appears to be of key importance as low levels of the enzyme enhances FGF2 binding and subsequent downstream activation of ERK/FAK signalling in human metastatic cells, whereas very high levels are inhibitory (Reiland et al., 2006). Heparanase−1 (HPA1) gene is formed by 40 kb localized at human chromosome 4 (4q21.3) (Vlodavsky I., et al. 1999). A second cDNA had recently been encoded for a new human isoform, named heparanase−2 or HPA2, encoded at chromosome 10q23−24 (Mackenzie et al. 2000). The main objective of this study was to correlate heparanase isoforms expression, glycosaminoglycans profile and breast tumor development. Heparanase isoforms expression were analyzed by RT–PCR and sulfated glycosaminoglycans were identified and quantified by agarose gel electrophoresis and densitometry using different breast cancer cell lines (MCF−7, MDA–MB−231 e SKBR−3) and a non tumoral breast cell line (MCF−10A). Confocal microscopy assay has shown that HPA1 cloned from MCF−7 presented a lysosomal localization. The results demonstrated that stable transfected HPA1 MCF−7 cells, that over expressed HPA1 not only changed GAG profile, but also decreased syndecan−1 proteoglycan synthesis and enhanced HPA2 expression. The data elucidated that there is a correlation between heparanase expression, sulfated glycosaminoglycans and proteoglycan synthesis in the breast cancer cell lines that possibly could explain different patterns of tumor development. Supported by FAPESP, CNPq and CAPES.

Keywords: heparanase, heparan sulfate, proteoglycans, glycosaminoglycans, breast cancer
09.5- Microarray Analysis of Differentially Expressed Genes in Tumor Breast Cells Treated with Laminin Derived Peptide C16

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Human breast cancer constitutes worldwide health care problem and represents 10% of all human malignant tumors. A complex pattern of genetic and epigenetic alterations is involved in the process of tumorigenesis, which often confers to transformed cells a higher proliferative potential, evasion to apoptosis, sustained angiogenesis, and capacity to invade the surrounding tissue and metastasize. Extracellular matrix molecules play important role influencing malignant behavior. Our Laboratory has been studying the involvement of laminin and its bioactive peptides in regulation of normal and neoplastic glandular cells. We have found that C16 (KAFDITYVRLKF), a peptide derived from laminin-111 gamma-1 chain, induces migration, invasion, and protease activity in salivary gland cells. This prompted us to study whether this peptide would regulate gene expression in breast gland cells. In this study, cells derived from breast tumors (MCF-7 and MDA-MB-231) were treated with C16. Cells treated with scrambled peptide (FKLRVYTIDFAK) served as control. After treatment for 24 hours, cells were collected for total RNA extraction, followed by cDNA and cRNA synthesis, and hybridization with microarray membranes. GEArray Expression Analysis Suite software was used to normalize and analyze the microarray data. Normalization was done using housekeeping genes (GAPDH, B2M, HSP90AB1, and ACTB). Preliminary results show that C16 regulates genes involved in cell cycle control, cell adhesion, signal transduction, cell death, invasion and metastasis in both MCF-7 and MDA-MB-231. Among these genes HTATIP2, a metastasis supressor, was downregulated in both cell lines upon C16 treatment, while CDK4 (cyclin-dependent kinase 4) expression was increased in both cells. MMP2 (matrix metalloproteinase-2) expression, on the other hand, was upregulated in MDA-MB-231 but not in MCF-7 cells. Our data suggest that C16 induces differential expression of cancer-related genes in breast tumors. These genes may be associated with relevant biological effects induced by C16.

Keywords: C16, LAMININ, BREAST, CANCER, MICROARRAY
09.6- The Role of Anti-Inflammatory Protein Annexin A1 And Soluble Paracrine Factors on the Interactions Between Stromal and Tumor Cells

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Several evidences have showed that the development and progression of cancer depends on the interactions between stromal and tumor cells. Recent studies have demonstrated that the anti-inflammatory protein annexin A1 (ANXA1) is related to the control of tumor cell growth. In the present study we investigated the growth curve of Hep-2 cells (derived from human larynx epidermoid carcinoma) and fibroblasts (derived from tumor stromal) after treatment with the peptide Ac2-26 (N-terminal sequence of ANXA1) and soluble paracrine factors, produced by stromal and neoplastic cells. Besides, we analysed the ANXA1 expression in the Hep-2 cells and fibroblasts, by immunocytochemistry and PCR real time. In order to investigate the effect of soluble factors produced by cells, Hep-2 cells received the conditioned medium from tumor stromal fibroblasts and fibroblasts received the conditioned medium from Hep-2 cell. Hep-2 cells and fibroblasts were maintained in control medium (C), in control medium treated with the peptide (C+P), in conditioned medium (CM) and in conditioned medium treated with the peptide (CM+P), at 6, 24, 72, 120 and 168 hours. Both CM and peptide treatments resulted in decreased on Hep2 and fibroblast proliferation, suggesting that factors produced by both cells and the ANXA1 interfere in cell growth. The decreased of Hep-2 growth was observed at 72 and 120h after the treatments (C+P), (CM) and (CM+P). Fibroblast growth was inhibited by CM and peptide treatments during all 168 hours. The ANXA1 protein expression, analysed by immunocytochemistry, was increased in Hep-2 at 72h after CM treatment. Fibroblast showed high ANXA protein expression at 72h after treatment with CM+P. The ANXA1 gene expression, analysed by PCR, was increased in Hep-2 cells at 6h after peptide and CM treatments. In contrast, fibroblasts showed reduced ANXA1 gene expression at 6h under all conditions. These data stress the important role of ANXA1 and soluble paracrine factors on the regulation of stromal and tumor cell proliferation. The importance of the ANXA1 is emerging and is likely to contribute to our understanding of the link among inflammation, hyperproliferation and carcinogenesis. Their roles in molecular pathways as well as their clinical implications, are just beginning to emerge in the literature. However, more information on the subject and prospective studies which deal with diagnosis, prognosis and treatment are still needed.

Keywords: Annexin A1, cancer, fibroblast, Hep-2 cell, soluble paracrine factors
09.7- Structural and Molecular Disorganization of Collagen V is Associated with Tumoral Growth in Non-Small Cell Lung Cancer

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Collagen V shows promise as an inducer of the death response via caspases. Remodeling of the microenvironment by collagen V, tumoral/vascular apoptosis and the immune response were evaluated, based on the prognosis of 65 patients with surgically excised non-small cell lung cancer. Immunofluorescence, immunohistochemistry, morphometry, tridimensional reconstruction and a real-time polymerase chain reaction were used to evaluate the amount, structure and molecular chains of collagen V, tumoral and vascular apoptosis, immune cells and microvessel density. The impact of these markers was tested on follow-up until death from recurrent lung cancer occurred. A decreased and abnormal synthesis of collagen V was found to lead to increased angiogenesis due to a low endothelial death rate and a low immune response. A Cox model analysis, controlled for the lymph node stage, demonstrated that only collagen V and vascular apoptosis variables were significantly associated with survival time. A point at the median for collagen V and vascular apoptosis divided patients into two groups, each with a distinctive prognosis. Those with a collagen V higher than 9.40 % and vascular apoptosis higher than 1.09 % had a low risk of death (0.27 and 0.41, respectively), compared to those with a collagen V lower than 9.40 % and vascular apoptosis lower than 1.09 %. Collagen V and vascular apoptosis in resected non-small cell lung cancer was strongly related to the prognosis, suggesting that strategies aimed at preventing low collagen V synthesis, or local responses to low vascular apoptosis, may have a greater impact in lung cancer treatment.

Keywords: Apoptosis, Caspase 9, Collagen V, Lung Cancer, Survival

09.8- Study of Galectins and Mast Cells in the Experimental Gastric Carcinogenesis in Rats

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Galectins are a family of proteins defined by a conserved carbohydrate-recognition domain with affinity for beta-galactosides. In cancer biology, galectin-1 (Gal-1) and -3 (Gal-3) can play crucial roles in tumor cell-cell or cell-matrix interactions through their binding activities to the tumor cell surface carbohydrate. The mast cells (MCs) represent one of main immune cell types implicated in tumor-associated inflammation and present Gal-1 and -3 expressions in their cytoplasmic granules that can be modulated during inflammatory
response. In this study, MC distribution and Gal-1 and -3 expressions were evaluated in an experimental gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats with chemical denervation of the stomach with benzalkonium chloride. Four experimental groups were evaluated: non denervated (group I) and denervated (group II) without lesions; non denervated (group III) and denervated (group IV) with lesions. Histological sections of the pyloric region were stained with Toluidine Blue for histopathological analysis and with Alcian Blue-Safranin (AB-SAF) for heterogeneity and distribution of MCs. For immunohistochemical study of galectins, sections of the stomach were incubated with monoclonal mouse anti-Gal-1 and polyclonal rabbit anti–Gal-3. MNNG treatment provoked the development of benign and malignant gastric lesions with stromal tissue enriched by inflammatory cells (MCs, neutrophils, plasm cells and lymphocytes). Mucosal MCs (AB positives) were observed mainly in animals of groups I and II, whereas the phenotypes AB and AB-SAF positives were detected in the gastric lesions of animals from groups III and IV. The increase in the number of MCs in the gastric tissues was associated with the development of lesions induced by MNNG treatment, compared to groups I and II. Gal-1 expression was detected in MCs of all experimental groups and in ECM of gastric lesions. Gal-3 was strongly positive in MCs, leukocytes and ECM of peritumoral lesions. These results showed a strong association among the density of MCs, Gal-1 and -3 expressions and the development of gastric neoplasms induced by MNNG in the stomach of denervated or not denervated rats. Then, the study of MCs and galectins as a target of new therapies in the process of tumorigenesis and their future clinical application must be considered. Supported by FAPESP.

**Keywords:** galectin-1, galectin-3, gastric lesions, myenteric denervation, immunohistochemistry

**09.9- Sulfated Polysaccharides Isolated from Marine Invertebrates Inhibit Prostate Cancer Cell Proliferation and Interaction with Endothelial Cells in Vitro**

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To form metastases tumor cells must complete a series of steps, including the invasion of normal tissues and intravasation through endothelial cells in direction of the bloodstream. Once in the circulation, tumor cells must be able to adhere and migrate across the endothelium (extravasation). After adherence to endothelial cells and subsequent endothelial retraction, metastatic tumor cells must adhere to elements of the subendothelial basement membrane, and migrate into the subendothelial stroma to grow at this new site. Interactions between endothelial selectins and selectin ligands expressed on tumor cells have been implicated in the binding of circulating metastatic cancer cells to the vascular endothelium.
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during extravasation. Cancer cells frequently exhibit abnormal glycosylation patterns, resulting in the synthesis and expression of altered carbohydrate determinants including sialyl Lewis a and sialyl Lewis x, that are recognized by endothelial selectins. Recent studies suggest that sulfated polysaccharides could inhibit the metastasis and proliferation of tumor cells by binding to growth factors and cell adhesion molecules. In this work, we studied the effect of the sulfated polysaccharides isolated from sea urchin (Lytechinus variegatus, Strongylocentrotus pallidus, Strongylocentrotus franciscanus, Glyptocidaris crenularis and Echinometra lucunter) and from sea cucumber (Ludwigothurea grisea) in the proliferation and interaction of prostate cancer cell with endothelial cells (HUVECs) and subendothelial extracellular matrix. We observed that the sulfated polysaccharides (100 µg/ml) inhibited prostate tumor cells adhesion and transmigration through TNF-α activated endothelial cells. However, only the polysaccharide isolated from L. variegatus was able to inhibit the adhesion of tumor cells to the native subendothelial extracellular matrix, by binding directly to this substrate. All polysaccharides, with exception of that isolated from G. crenularis, inhibited prostate tumor cell proliferation after 72 hours. The polysaccharide isolated from L. variegatus was the most effective growth inhibitor since the effect was visible in the first 24 hours (58% of inhibition) and was maintained until 72 hours (50% of inhibition). These results suggest that these polysaccharides, mainly that isolated from L. variegatus, may be potential candidates for an anti-metastatic drug. Supported by CNPq, Capes and Faperj.

Keywords: Adhesion, Endothelial Cells, Extracellular Matrix, Polysaccharides, Tumor Cells

09.10- The RECK B Isoform is Downregulated by Simvastatin in Human Melanoma Cells

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RECK encodes a membrane-anchored protein which suppresses invasion/metastasis by negatively regulating matrix metalloproteinases, particularly, MMP-2, MMP-9 and MT1-MMP. A positive correlation has been found between the relative abundance of RECK expression in tumor samples and better prognosis for patients with gastric, lung, pancreatic and colorectal cancers. In addition to downregulating cholesterol levels, statins exert other effects, which include anti-inflammatory and anti-proliferative actions. In particular, it has been described that Simvastatin (SIM) is able to inhibit melanoma cells growth and invasion. However, the molecular mechanisms underlying these delayed drug-induced effects are still unclear. Here, we evaluated the viability of human melanoma cell lines (SK-Mel28 and MeWo), through crystal violet staining, upon treatment with SIM at different concentrations (0.1, 0.5, 1, 5 and 10 µmol.L-1) for 48 and 72h. For SK-Mel28 cells, we observed a 20% reduction in viability upon treatment with SIM for 48h. After 72h, we observed 45 and 51% reduction in cell viability when these cells were treated with SIM at 5 and 10µmol.L-1, respectively. On the other hand, SIM had no effect in viability of MeWo cells upon treatment
Marimellia Porcionato

for 48h. Only after 72h, we observed 20 and 28% reduction in viability when MeWo cells were treated with SIM at 5 and 10µmol.L-1, respectively. In order to evaluate the molecular mechanism involved in the anti-proliferative effects of SIM in melanoma cells, we analyzed the mRNA expression levels of the RECK gene (canonical form and alternative isoforms, namely: RECK B, RECK C, RECK D and RECK I) in melanoma cells treated with SIM at 1 and 5 µmol.L-1 for 72h. The expression profiles of these genes were investigated through quantitative real time RT-PCR assays. Preliminary results indicate that the mRNA levels of the RECK B isoform is significantly reduced in SK-Mel28 cells upon treatment with SIM in a dose-dependent manner. The RECK B isoform showed a tendency to be downregulated also in MeWo cells treated with SIM. On the other hand, the mRNA levels of the RECK canonical form and of other isoforms (RECK C, D and I) were not altered upon treatment with this drug. Our results suggest that differently from the canonical form, the RECK B isoform may play an important role in the molecular signaling induced by SIM in melanoma cells. Support: FAPESP, CAPES, CNPq, FINEP, Redoxoma.

Keywords: Melanoma, MMPs, RECK

09.11- Laminin-Derived Peptide Ag73 Increases Invadopodia Activity in a Cell Line Derived from Adenoid Cystic Carcinoma

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Our laboratory studies effects of laminin and its derived peptides in tumor biology. We demonstrated that the peptide AG73 (RKRLQVQLSIRT, LG4 domain of laminin alpha1 chain) is involved in migration, invasion and protease of a cell line (CAC2) derived from adenoid cystic carcinoma. This tumor is a malignant salivary gland neoplasm with recurrence and metastasis long time after initial therapy. Metastatic tumor cells that actively migrate and invade surrounding tissues rely on invadopodia to degrade extracellular matrix (ECM) barriers. Invadopodia are actin-rich membrane protrusions that localize enzymes required for ECM degradation. These protrusions contain actin, cortactin and membrane type 1 matrix metalloproteinase (MT1-MMP) superimposed to areas of digested matrix. We have already characterized invadopodia in CAC2 cells (Nascimento et al., 2009). Here we studied whether the peptide AG73 would increase invadopodia activity of CAC2 cells. Cells were treated with AG73. Control cells were treated with scrambled peptide (AG73SX). Treated and control samples were subjected to fluorescent-substrate degradation assay, to assess in situ protease activity of CAC2 cells. Cells were cultured overnight on gelatin-FITC, followed by fixation and labeling to actin. In this assay, digestion spots in fluorescent substrate appear as black areas in green background. On the other hand, actin staining outlines cell shape and identifies protrusion (invadopodia) superimposed to digested areas. Samples were studied by fluorescence microscopy. Invadopodia counting showed that AG73 significantly increased in situ protease activity of CAC2 cells compared to controls. Our preliminary results suggested that laminin-derived peptide AG73 enhanced invadopodia formation and protease activity of
09.12- Laminin-Derived Peptide Ag73 Regulates Migration, Invasion and Protease Activity of Human Oral Squamous Cell Carcinoma Cells Through Syndecan-1 and Betal Integrin

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Oral squamous cell carcinoma is a prevalent head and neck tumor, related to high mortality rates. Its growth and invasiveness is stimulated by interactions with extracellular matrix and basement membrane, which prominently expresses laminin. This molecule regulates tumor biology and harbors cryptic sites with important roles upon release by proteolytic enzymes such as matrix metalloproteinases (MMPs). MMP-mediated breakdown of basement membrane is a critical step in tumor progression that involves laminin cleavage and release of peptides which may influence carcinoma’s behavior. Our Laboratory studies the effects of laminin-derived peptides in tumor biology. Here we analyzed the role played by AG73 (RKRLQVQLSIRT, alpha1 chain) on migration, invasion and protease activity of a cell line (OSCC) from human oral squamous cell carcinoma. Laminin alpha 1 chain and MMP9 were immunolocalized in human oral squamous cell carcinoma cells in vivo and in vitro. AG73 increased migratory activity of OSCC cells, as shown by monolayer wound assay and migration assay. This peptide also stimulated cell invasion in chemotaxis chambers coated with Matrigel. Invasion depends on protease activity. OSCC cells cultured on AG73 showed a dose-dependent increase of MMP9 secretion. We searched for AG73 receptors regulating activities in this cell line. OSCC cells grown on AG73 exhibited colocalization of syndecan-1 and betal integrin. siRNA knockdown of syndecan-1 and betal integrin decreased AG73-dependent migration and invasion of OSCC cells. Our results suggest that syndecan-1 and betal integrin signaling downstream of AG73 regulate migration, invasion and MMP production by OSCC cells. Support: FAPESP (06/57079-4, 07/51950-8), CNPq (470622/2007-5).

Keywords: Oral squamous cell carcinoma, Laminin, Matrix metalloproteinases, Syndecan-1, Betal integrin
09.13- Regulation of Mmps Expression by HPV16 Oncoproteins in Primary Epithelial Cultures

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Cervical cancer is etiologically associated to high-risk human papillomavirus (HPV) infection. Matrix metalloproteinases (MMPs) -2, -9, and MT1-MMP are required for cervical carcinoma progression, but is still unclear whether the macrophages rather than keratinocytes contribute to their secretion. Moreover, a counterbalancing among MMPs and their regulators, such as TIMPs and RECK, is necessary to prevent invasion. In order to study the effect of HPV oncogenes on MMPs expression, primary human keratinocytes (PHKs) were infected with recombinant retroviruses expressing wild-type HPV16 E6 and/or E7 oncogenes and were used to seed epithelial cultures. Quantitative real-time PCR (Q-PCR), western blot, zimography and gelatinolytic assay were used to determine the expression level and activity of MMP-2, MMP-9, MT1-MMP and their inhibitors RECK and TIMP-2. We observed that, when individually expressed, HPV16 oncoproteins exert a differential effect on MMP-9. While MMP-9 activity was down-regulated in cultures expressing HPV16 E7 oncogene, its activity was up-regulated in HPV16 E6-expressing cells. On the other hand, cultures expressing both E6 and E7 exhibited MMP-9 levels similar to those observed in PHKs infected with the empty vector (pLXSN). Interestingly, these cells expressed lower RECK levels than PHKs transduced with the empty vector or with HPV16 E6 or E7. Furthermore, we observed that expression of HPV16 E6 and/or E7 proteins do not affect MMP-2, MT1-MMP and TIMP-2 levels and/or activity. However, the gelatinolytic assay showed that cells expressing HPV16 E6 exhibit a different degradation pattern when compared to the others cultures. Finally, we show that the gelatinolytic degradation depends on MMPs activity since it is decreased by a MMPs pan inhibitor (Ilostatic). Taken together, our results demonstrate that HPV16E6E7 presence down-regulates RECK protein levels, leading us to suggest that the both E6 and E7 oncoproteins contribute to modulate the matrix degradation in cervical carcinoma. Financial Support: Fapesp, CNPq, FINEP, PRP-USP and LICR.

Keywords: Metalloproteinases, RECK gene, HPV, Cervical carcinoma
09.14- RECK-Mediated Inhibition of Glioma Migration and Invasion

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RECK is an important tumor suppressor gene in which anti-tumoral activity has traditionally been associated to its inhibitory effects on MMPs, regulating at least MMP-2, MMP-9 and MT1-MMP. Elevated MMP activity due to lack of functional RECK compromises tissue integrity. RECK levels have been shown to be decreased in progression of glioblastoma from less invasive grade II gliomas to very invasive human glioblastoma multiforms (GBMs). Due to the inverse correlation between RECK expression and glioma invasiveness, we hypothesized that RECK expression would compromise the invasive behavior of these gliomas. We therefore conducted this study to explore forced RECK expression in the highly invasive T98G human GBM cell line, which displays high levels of both MMP-2 and -9. Expression levels as well as protein levels of RECK, MMP-2, MMP-9 and MT1-MMP were assessed by qPCR and immunoblotting in T98G/RECK+ cells (stable transfection of T98G overexpressing RECK). The invasion and migration capacity of this GBM cell line expressing high levels of RECK was shown to be inhibited in transwell and wound assays. Dramatic cytoskeleton modifications were observed in the T98G/RECK+ cells, when compared to control cells, such as the abundance of stress fibers (contractile actin-myosin II bundles) and reduction of lamellipodia. T98G/RECK+ cells also displayed phosphorylated focal adhesion kinase (P-FAK) in mature focal adhesions associated with stress fibers; whereas P-FAK in control cells was mostly associated with immature focal complexes. Interestingly, the RECK protein was predominantly localized to the leading edge of migrating cells, associated with membrane ruffles. Surprisingly, in this glioma model, the overexpression of RECK had no detectable effects on MMP-2 and -9 as predicted; no alterations were observed at the mRNA level (qPCR), at the protein level (immunoblotting) nor in protein activity (gelatin zymography). Unexpectedly, introduced expression of RECK effectively inhibited the invasive process through rearrangement of actin filaments, promoting a decrease in migratory ability. This work has associated RECK tumor suppressor activity with the inhibition of motility and invasion in this GBM model, which are two glioma characteristics responsible for the inefficiency of current available treatments.

Keywords: RECK, migration, invasion, glioma
09.15- Apoptotic Effect of a Plant Kallikrein Inhibitor on Prostate Cell Lines

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Prostate cancer occurs with high rates of incidence, mortality and invasion from the primary tumor. Tissue kallikrein hK3 (PSA) and integrins are directly related to prostate neoplasia, interfering in cell adhesion, differentiation and cellular proliferation. Compounds that interfere in these processes are targeted for investigation. In this work, using prostate cancer cellular lines PC3 and DU145, we studied the effect of recombinant kallikrein inhibitor (rBbKI) and its modified form (rBbKm), in which the sequence containing the signal motif sequence RGD of the inhibitor BrTI from Baumina rufa was inserted. rBbKIm inhibited cell proliferation of PC3 (70%, 50 andμM, 48 h) and of DU145 (60%, 100 andμM, 48 h) but did not affect the proliferation of fibroblast normal cell line (100 andμM, 48h). rBbKIm affects cell cycle of PC3, increasing apoptosis and decreasing cell G2 fase (mitosis). In cell adhesion, rBbKm (25 andμM) containing the adhesion motif RGD, inhibited approximately 30% of the PC3 adhesion on fibronectin, whereas rBbKI inhibition was 20%. The confocal microscopy analysis showed that both rBbKI and rBbKIm interact with cell membrane. Previous studies showed that rBbKIm induces nitric oxide (NO) release upon inducible nitric oxide synthase (iNOS) activation. This property is under investigation in order to clarify if the apoptotic effect of rBbKIm is correlated to its mechanism of action. Supported by CNPq, CAPES, FAPESP, FADA/FAP and UNIFESP.

Keywords: kallikrein inhibitor, Prostate, Cancer, PC3, DU145

09.16- Head and Neck Cancer Progression to Metastatic State Involves Cathepsin B and Heparan Sulfate Glycosaminoglycans Levels Modifications

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Epithelial to mesenchymal transformation is the first manifestations occurring during the transformation of epithelial cells into malignant carcinoma. As cells lose cell-cell contact and cell-extracellular matrix interaction, new growth, motility, ECM degradation are generated during metastasis. Close correlations have been observed between Cathepsin B and Heparan Sulfate levels at the cell surface of squamous cell carcinoma. Oral cancer is one of the 10 most frequently occurring cancers worldwide. The aim of this study is to investigate the
involvement of Cathepsin B and Heparan Sulfate glycosaminoglycans during the cell differentiation process. Cathepsin B and Heparan Sulfate amounts, localization, activity and gene expression between a primary human hypopharynx carcinoma cells, line UMSCC–22a, and its neck metastatic cells, clone UMSCC-22b has been analyzed. The results obtained by qPCR analysis showed that the metastatic clone UMSCC-22b have an increased expression of Cathepsin B mRNA comparing with the primary tumor cell line. Gene expression levels of Heparan Sulfate proteoglycan (Syndecan) and Chondroitin Sulfate proteoglycan (Versican) were also analyzed by qPCR showing significant differences between both cell lines. On direct glycosaminoglycans analyzes Heparan Sulfate levels of metastatic cells decrease 5.6-fold and Chondroitin Sulfate was increased 8-fold in relation of primary tumor cells at cell surface following the data obtained on proteoglycans mRNA study. Also, both immunocytochemistry analysis by flow cytometry and confocal microscopy images showed that the intracellular amount of cathepsin B protein was increased in the metastatic cells. More, the enzymatic determination of cathepsin B by using the fluorogenic Z-FR-MCA substrate showed that the metastatic clone UMSCC–22b exhibited 6-fold more cathepsin B activity than its primary tumor cells UMSSC–22a. Take together, these results show that the primary tumor cells UMSSC-22a undergo a epithelial to mesenchymal transformation conversion to the migratory metastatic UMSSC-22b phenotype. Supported By: FAPESP, CNPq and CAPES

**Keywords:** Cancer, Cathepsin B, Glycosaminoglycans, Heparan Sulfate, Chondroitin Sulfate

**09.17- Laminin Chains Alpha 1 and Gamma 2 are Cleaved in Oral Human Squamous Cell Carcinoma, Generating Fragments with Relevant Biological Role**

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Squamous cell carcinoma represents 95% of malignant oral neoplasms and constitutes an important health problem. Microenvironment of carcinoma is formed not only by neoplastic cells, but also by surrounding stroma. Therefore, its progression requires tumor cell interactions with extracellular matrix (ECM). In epithelia, cells form a specialized ECM sheet-like structure, the basement membrane, composed by laminin, type IV collagen, nidogen and perlecan. Laminins are heterotrimeric glycoproteins prominently expressed in basement membranes that promote cell adhesion, migration, growth and differentiation. Here we studied expression of different laminin chains, such as α1, α3, β1, and γ2 in human oral squamous cell carcinoma. Fifty-five cases of this neoplasm were studied by immunohistochemistry. Sections from formalin-fixed paraffin-embedded tissues were subjected to EnVision System. Antigen retrieval was carried out with citrate buffer (pH 6.0) in Pascal chamber for 30 seconds. Laminin chains α1, α3, β1, and γ2 were immune localized
in human oral squamous cell carcinoma in vivo. It is known that laminin undergoes MMP
processing, generating small fragments that can influence cell behavior. To address whether
laminin from oral squamous cell carcinoma would release such bioactive fragments, tumor
lysates were electrophoresed and immunoblotted with antibodies against different laminin
chains. Samples from oral squamous cell carcinoma were compared to tissue extracts from
human oral mucosa. Purified laminins 111 (former laminin-1) and 332 (former laminin-5) run
in the same gels served as positive controls. Immunoblot showed laminin chains α1, α3, β1
and γ2 in both oral squamous cell carcinoma and oral mucosa. Furthermore, carcinoma
samples showed cleavage of α1 and γ2 chains. Molecular weight of α1 fragment (~50kDa)
was consistent with laminin-111 E3 domain. Molecular weight of γ2 product (~80kDa)
strongly suggested laminin γ2x fragment from laminin-332. It is widely known that both E3
and γ2x exhibit important biological effects in neoplastic cells. Our preliminary results
indicate that laminin chains α1 and γ2 undergo cleavage in human squamous cell carcinoma,
generating fragments with relevant biological role.

**Keywords:** Extracellular matrix, Laminin, Oral human squamous cell carcinoma

09.18- Peptide A3-10, Derived From Laminin-332 Modulates Migration
of Human Adenoid Cystic Carcinoma Cells

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Adenoid cystic carcinoma is a frequently occurring malignant salivary gland neoplasm,
with high level of recurrence and distant metastasis. This tumor prominently expresses
extracellular matrix proteins. One of these proteins is laminin, a glycoprotein with important
biological roles, including cellular adhesion, migration, differentiation, proliferation, tumor
metastasis, and protease secretion. Our laboratory studies the roles of laminins and their
bioactive peptides in tumor biology. The aim of this study was to analyze the effects of A3-
10, a peptide derived from laminin-332 (also known as laminin-5), on migration and invasion
in an adenoid cystic carcinoma cell line (CAC2 cells). A3-10 migration assays were carried
out using a Neuro Probe chemotaxis chamber with porous membrane (8μm pore size),
containing different A3-10 concentrations (10-100 μg/well). Same concentrations of
scrambled peptide (A3-10SX) were used as controls. Results showed that A3-10 promoted a
dose-dependent increase in migration rate of CAC2 cells. Invasion activity was assessed in
Boyden chamber with membranes coated with Matrigel, using different peptide
concentrations. No significant differences were found between invasion rates in A3-10 group
compared to control A3-10SX. Our data suggest that A3-10 peptide promote migration, but
not invasion, of human adenoid cystic carcinoma cells.

**Keywords:** Adenoid Cystic Carcinoma, Extracellular Matrix, laminin-332
09.19- The Role Played by Adamts-1 on Migration and Invasion of Malignant Mammary Gland Cells

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ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin motifs) is a member of the ADAMTS family of metalloproteases, and related to cancer progression. In spite of ADAMTS-1 biological relevance, mechanisms underlying its involvement in tumor biology remain elusive. ADAMTS-1 is downregulated in human breast carcinoma. Here we studied role played by ADAMTS-1 in migration and invasion of a malignant mammary cell line (MDA-MB-231). Cells had ADAMTS-1 silenced by either shRNA-GFP or siRNA. MDA-MB-231 cells transfected with scrambled oligos served as controls. Immunoblot confirmed silencing efficiency. Migration was investigated by time-lapse microscopy. Images (5 min interval) were recorded sequentially (4 hours). Tracking measurements yielded length (µm) and speed (µm/hour) of single cells. Silencing of ADAMTS-1 significantly increased migration of MDA-MB-231 cells. Knockdown of ADAMTS-1 also increased invasion of MDA-MB-231 cells in Boyden chambers coated with Matrigel. Silencing of ADAMTS-1 enhanced protease activity, as shown by zymography. Migration, invasion and protease activity are essential for tumor progression and metastasis. Metastatic cells rely on invadopodia to degrade and invade extracellular matrix (ECM). Invadopodia are finger-like membrane protrusions with enzymes required for ECM degradation. These protrusions contain cortactin and MT1-MMP superimposed to areas of digested matrix. Invadopodia proteins were studied by immunoblot on MDA-MB-231 cells with reduced expression of ADAMTS-1. Silencing of ADAMTS-1 increased cortactin and MT1-MMP expression. We investigated role played by ADAMTS-1 on invadopodia formation and protease activity of MDA-MB-231 cells. These cells were transfected with an shRNA-GFP vector to knockdown ADAMTS-1. Control cells expressed non-silencing GFP vector. Treated and control cells were grown on fluorescent gelatin (gelatin-Alexa 647) overnight. In this assay, digested gelatin appears as black spots on fluorescent matrix. After growing overnight on fluorescent gelatin, MDA-MB-231 cells were fixed and stained to cortactin. Cells with silenced ADAMTS-1 exhibited expression of cortactin and increased number of digestion spots compared to controls. Our results suggested that ADAMTS-1 plays a role in migration, invasion and protease activity of MDA-MB-231 cells. Support: FAPESP (2006/54963-0), and CNPq (470779/2007-1).

Keywords: ADAMTS-1, breast cancer cell lines, invasion, migration, proteases
09.20- Collagen Type I Treatment Inhibits Grow of Tumor in Bearing Mice Melanoma and Breast Adenocarcinoma Activating Caspase-3.

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Introduction: Collagen type I is a prevalent component of the stromal extracellular matrix; its expression being spatially and temporally regulated during mammary ductal formation and dermal differentiation. The primary function of collagen fibers is to add strength to the connective tissue can have a stimulatory or inhibitory effect on cell proliferation and organized fibrillar structure inhibits normal and malignant cell proliferation.

Objective: Evaluated the effects potential inhibits proliferative response and apoptosis by type I collagen in mice bearing B16F10 melanoma and adenocarcinoma of Ehrlich’s tumor.

Methods and Results: Were determined the inhibitory concentrations (IC50%) of collagen type I in B16F10 melanoma, breast adenocarcinoma of the Ehrlich and normal fibroblasts by the colorimetric method - MTT. Activity of collagen in induced cell death pathway caspase-3 was tested by enzymatic-fluorogenic method. The inhibitory concentrations (IC50%) were 18.6 mg for B16F10 melanoma and 49.9 mg for breast adenocarcinoma. Microscopic analysis of tumor cells showed multicellular aggregation and lost adhesion in extracellular matrix. The treatment by collagen showed cytotoxic effects and morphologic changes but proliferation cellular and sites of collagen by fibroblasts dermal. The collagen type I had significant activity as a substrate for active site specific of proteolytic cleavage of caspase 3. The mice C57BL/6J and Balb-c were implanted with 5x104 of B16F10 melanoma cells and 5x104 cells breast adenocarcinoma. The treatment was performed with the collagen in different concentrations, administered by intraperitoneal during 40 days. Animals bearing the B16F10 melanoma and adenocarcinoma of Ehrlich treated with Collagen showed significant reduction of tumor mass and area respectively 79% and 68%. The survival rate calculated by the Kaplan-Meier test showed that treatment with the samples of collagen induced a significant reduction in mortality rate and reduction in number internal metastases. Conclusion: Collagen significantly reduced tumor growth, active caspase-3 and formation of metastases in experimental models of melanoma and breast adenocarcinoma. Collagen Type I and products hydrolysates peptides with small molecular mass is a promising new weapon for cancer treatment. Financial Support: CNPq

Keywords: Collagen Type I, Melanoma, Breast Cancer, Caspase-3, Tumor
09.21- Characterization of Three Novel Splice Variants of the Reck Tumor and Metastasis Supressor Gene: Correlation with Glioma Progression

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REversion-inducing Cysteine-rich protein with Kazal motifs (RECK) encodes a membrane-anchored protein that suppresses both invasion and metastasis by negatively regulating at least three MMP’s, namely: MMP-9, MMP-2 and MT1-MMP. Matrix metalloproteinase (MMP) family members are directly involved in tumor invasiveness and metastasis. In general, relative levels of MMPs increase with tumor progression, with a positive correlation being observed between the abundance of RECK expression in tumor samples and better prognosis for patients with several types of cancers. Recent studies showed mutations in splicing cis regulatory elements and alterations in the cellular splicing regulatory machinery, leading to changes in the splicing pattern of several cancer related genes, such as CD44, BRCA1, APC, p53 and FHT. In the present study, three novel alternative isoforms of the RECK tumor suppressor gene, namely RECK B (1,548bp), RECK D (1,737b) and RECK I (1,101bp) were isolated by RT-PCR and their expression profiles were investigated using quantitative real time RT-PCR assays in a normal tissue RNA panel and, also, during glioma progression. Our results show that RECK isoforms display independent expression patterns when compared to the canonical form, indicating a more complex role of both the canonical and alternative RECK isoforms in glioma progression. Support: FAPESP, CNPq, FINEP

Keywords: Alternative-splicing, RECK, Tumor-suppressor-gene

09.22- Cadmium (Cd++) Inhibits the Gelatinolytic Activities Of MMP-2 and -9: A Possible Role in Cadmium-Induced Prostate Carcinogenesis

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Matrix Metalloproteinases are a set of neutral endopeptidases calcium and zinc-dependent. This family of enzymes plays a major role in the extracellular matrix components degradation and turnover in the most normal physiologic conditions and also in many diseases, such cancer. In this work, we evaluated the effect of cadmium, a divalent metal with known carcinogenic potential on prostate gland, on the MMP-2 and MMP-9 gelatinolytic
activity. For such, MMP-2 and MMP-9 extracted from normal rat ventral prostate were processed for gelatin zymography. The cadmium effect was tested during incubations step at different concentration (5mM, 2mM, 50uM and 1uM of Cadmium chloride) with or without 5mM of calcium chloride. The gels were incubated for 20 hours at 37°C in a solution of 50mM pH 8.4 Tris-HCl containing the different concentration of cadmium and calcium described above. The presence of cadmium chloride in the incubation buffer inhibited the gelatinolytic activity of both MMP-2 and MMP-9 even in the calcium chloride presence. This result suggests that cadmium, such as magnesium, manganese and cobalt, has an inhibitory effect on MMP-2 and MMP-9 activity, in vitro. Although these metals not are essential for normal physiology, such as calcium and zinc, the prostate gland is the main organ where cadmium accumulates. Thus, the inhibitory action of cadmium on MMPs activity may, at long term, participates in the process of prostate carcinogenesis induced by cadmium. Further studies will address if prostates from cadmium-treated rats also present changes in MMP-2 and MMP-9 expression and gelatinolytic activity.

**Keywords:** cadmium, prostate, cancer, metalloproteinases

### 09.23- Treatment with Phosphoetanolamine Synthetic Induced Apoptosis Arrest in Cell Cycle Phases and Collagen Matrix Remodeling in Melanoma


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Introduction: Phosphoetanolamine is involved in the metabolism of phospholipids and responsible for maintaining the potential Deltapsi-m mitochondrial acting cell death signaling pathways. Objective: Evaluated effects antitumor Phosphoetanolamine Synthetic (PHO-S) in model B16F10 melanoma. Materials and Methods: The cytotoxic activity of PHO-S was tested in vitro in murine and human tumor lines (Skmel-28, Mel-85; Mewo and B16F10) by MTT colorimetric method. The model of implantation of the tumor cells were used 40 mice of strain C57BL-6J receiving 5x104 cells B16F10 after 4 days treatment with (0.0117 and 0.0468 uM) from PHO-S (ip). The tumor volume was evaluated by scintigraphic with radioactive complex [[99mTc] (V) (DMSA) 2]). Part of lesions of tumor and metastasis internal were used for histological analysis (HE) of irrigation and endothelial vessels (Verhoeff), synthesis and content of collagen (Picrosirius), and determine the phases of the cell cycle (apoptosis, G0/G1, S and G2/M), by flow cytometry. Results: Showed selective cytotoxicity with IC50% inhibitory concentration for cells of melanoma Skme-28 0.20mg/mL; 1.82mg/mL of Mel-85; Mewo of 2.39mg/mL and the murine melanoma B16F10 of 1.44mg/mL. Treatment of animals bearing a dorsal melanoma of PHO-S induced significant (p = 0.0001) reduction in tumor burden (2.9 ± 1.4 mm3), compared to the control group (13.8 ± 6.6 mm3), inhibiting the number of internal metastases. The cell cycle, induces PHO-S increase the proportion of cells in apoptosis in the concentration of 0.0117uM (17.5% ± 2.3),
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at a dose of 0.0468 uM decreased the proportion of quiescent cells (G0/G1) (12.7% ± 4.06). The ability of synthesis (S phase and G2/M) decreased significantly (P Conclusion: The PHO-S reduced volume of dorsal tumors, inhibition of tumor spread, and inhibition of proliferative and increased production of fibrillar matrix. During the cell cycle by flow cytometry of the treated tumors induced PHO-S "arrest" in the G0/G1 phase, non-proliferating and increasing the population of cells killed by apoptosis. Financial Support: FAPESP

Keywords: Phosphoethanolamine, Apoptosis, Cell Cycle, Melanoma, Matrix

09.24- Structural Analysis of the N-Terminal Fragment of Endostatin by Molecular Dynamics

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Endostatin is a potent antiangiogenic factor whose mechanism of action is not fully characterized. It was suggested that the activity of murine endostatin was retained on its 27 amino acids N-terminal fragment. This peptide coordinates a zinc ion by three histidine residues. When this metal is removed from the peptide, the antiangiogenic activity is abolished. In this work, we studied by Molecular Dynamics the N-terminal fragments of human and murine endostatin (N27hES and N27mES, respectively) to characterize key elements, like the zinc ion, that may have significant structural role on their biological function. Models built with PyMol, based on the crystallographic structures (1BNL and 1DY1), were simulated with and without zinc ion using GROMACS. An energy minimization protocol and three steps of molecular dynamics were used: (i) 500ps with positions restraint for peptide, (ii) 5000ps with all atoms free and (iii) 120ns for data gathering. Two additional N-terminal peptides of human endostatin were simulated: N25hES, without the last two residues, and N27hES/R4Q, with the mutation R4Q. We show that N27mES, when bound to the zinc ion, assumed well-defined secondary and tertiary structures in approximately 80ns, while the peptide without zinc collapsed in a compact fold without secondary structures. The conformation assumed by the N27mES has similarity with the functional β–hairpin of canstatin, a known anti–angiogenic protein derived from collagen IV. N27hES did not achieve a stable structure in the same amount of time in both simulations, with or without zinc. We postulated that this behavior was due to internal electrostatic repulsion between arginines 4 and 27. Confirming our hypothesis, simulations of N27hES/R4Q and N25hES presented stable β–hairpin conformations. Our study may help the development of new endostatin–based anti–angiogenic drugs.

Keywords: Anti-angiogenesis, Endostatin, Cancer, Molecular Dynamics, Rational Drug-design
09.25- Increased Expression of Hyaluronan Synthases by the Tumoral Microenvironment is Associated to Low Grade Non-Small Cell Lung Carcinomas

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Introduction/Objective: Hyaluronan, a tumor promoting extracellular matrix polysaccharide, is elevated in tumors including lung cancer, and associates with an unfavorable prognosis. The heterogeneity of HAS expression has been identified in tumors and could be related to the histological types and degree of differentiation. To explore possible contributors to the accumulation of hyaluronan, we examined the imunoexpression of hyaluronan synthases (HAS1, HAS2 and HAS3) in frozen tissue obtained from non-small cell lung carcinomas (NSCLC). Material and Methods: We examined HAS1, HAS2 and HAS3 in tumor tissues from 12 patients with surgically excised NSCLC. We used frozen section immunohistochemistry and histomorphometry to evaluate the amount of tumor areas staining for HAS1, HAS2 and HAS3. Tumor tissue from 3 patients submitted to surgical resection by non tumoral causes was used as control. Impact of these markers was tested on histological types and degree of tumor differentiation. Results/Conclusion: A significant direct association was found between HAS1 and HAS2 expression (p=0.04). HAS1 and HAS2 expression was significantly increased in adenocarcinoma compared to control and other histological types (p=0.02). HAS2 expression was lower in neuroendocrine carcinomas than in control and non neuroendocrine tumors, but this difference didn’t achieve statistical significance (p=0.42). HAS expression was tested against histological degree, and an association was found between low grade tumors (well and moderate differentiation) with HAS2. We concluded that tumor staining for HAS2 may be related to prognosis of patients with NSCLC. Financial Support: FAPESP.

Keywords: hyaluronan, lung cancer, Glycosaminoglycans, Hyaluronidase
09.26- Inhibition of Cell Migration and Endothelial Cell Adhesion and Tubulogenesis by DM43, a Glycoprotein from Didelphis Aurita Serum

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Several animals, including the opossum Didelphis aurita, are resistant to snake venoms due to the presence of neutralizing factors in their blood. An antihemorrhagic protein named DM43, isolated from D. aurita serum, inhibits snake venom metalloproteinases through the formation of a noncovalent complex with these enzymes. DM43 is also a potential inhibitor of matrix metalloproteinase (MMPs). The MMPs are a family of extracellular endopeptidases that selectively degrade components of the extracellular matrix, and have been implicated in many physiologic and pathologic processes. Angiogenesis is an invasive process that requires proteolysis of the extracellular matrix, migration and proliferation of endothelial cells. The angiogenic response is critical for progression of wound healing, rheumatoid arthritis and is also a prerequisite for tumor growth and metastasis formation. In this study, we investigated the effect of DM43 in cell adhesion, proliferation, migration, invasion and differentiation. Using a prostate cancer cell line (DU-145), two breast cancer cell lines (MCF-7 and MDA-MB-231), and human umbilical vein endothelial cells (HUVEC), we first tested whether DM43 could be a potential inhibitor of cell migration. We observed that DM43 (4000 ng/ml) inhibits chemotaxis of all cancer cell lines (MDA-MB-231- 45% , MCF-7 -58% and DU-145 - 70%), as well as in human umbilical cells (HUVEC- 66%). DM43 also significantly decreased the adheriveness of HUVEC cells to gelatin in a dose-dependent manner (250-4000ng/ml), with the maximal effect at 4000ng/ml (29 % of inhibition), as demonstrated by the MTT method. Finally, DM43 also inhibited significantly the formation of capillary-like structures by HUVEC, on a 3D-Matrigel matrix. This study suggests that DM43 has a potential as a therapeutic agent in pathological situations where angiogenesis is stimulated, such as tumor growth and progression. Supported by: CNPq, FAPERJ, CAPES.

Keywords: Angiogenesis, Endothelial cells, Extracellular matrix, Matrix metalloproteinase, Tumor cells
09.27- Screening of Flavonoids Effects of Growth and Aggregation of Human Glioblastoma Initiating Stem Cells: Inhibitory Effect of Quercetin on Cellular Adhesion and Migration

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Gliomas are the most frequent and malignant among human intracranial tumors. Despite radical surgery, the prognosis with radiation therapy and conventional chemotherapy remains low due to invasion of surrounding brain tissues. The isolation from human biopsies of malignant cells that express markers of neural stem cells support the existence of tumour stem cells within gliomas. As our previous study have been suggested plant derived flavonoids as inhibitors of growth of human and murine glioblastoma cell lines, in this study we investigated the effect of fifteen commercially disponible flavonoids on vitality and adhesion on a human cell line of tumour initiating cells (TG1) and in viability and invasion of the human glioblastoma cell line U251. As revealed by WST and MTT test after 72 h exposure to 1-100µM flavonoids. Depending on the degree of methylation and hydroxylation flavonoids inhibited dose-dependently the growth and viability of both glioblastoma cell lines, and between them one of the most active was flavonoid quercetin. Then we tested by time lapse microscopy the effect of quercetin on adhesion of spheroids of TG1 cells and by phase microscopy the migration of synchronized U251 cells on different ECM support (laminin, collagen, collagen plus fibronectin) through the use of a standard in vitro monolayer wound assay. Compared with cells in control conditions (vehicle DMSO 0.1%) 50µM quercetin reduced the aggregation of TG1 cells spheroids since 15h after exposure. Moreover 50µM quercetin also reduced significantly the closure of the wounded area indicating inhibition on U251 glioblastoma cells migration. These results suggest flavonoids quercetin as inhibitor of growth of glioblastoma tumor initiation cells and may find and application to treatments for chemotherapy resistant malignant gliomas. Supported by CNPq, CAPES and INSERM U-894.

Keywords: Glioblastoma, Flavonoid, ECM, adhesion, migration

09.28- Polyhydroxylated Flavonoids Regulate Mmps Activity, MEC Expression, and Invasion of Human Glioblastoma Cells

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Malignant gliomas are the most common primary brain tumors in adults and its incidence has increased dramatically over the past several decades. Due to its invasive character, and
despite advances in standard therapy, surgery is unable to remove all tumor tissue, and the prognosis for patients with gliomas remains poor. Matrix metalloproteinases (MMPs) have been implicated as important factors in the control of the invasive capability of gliomas. Flavonoids are polyphenolic compounds present in a large variety of plants, and have been presented antitumoral activities. In this study we investigated the effect of polyhydroxylated flavonoids chrisyn (5,7- dihydroxiflavone), 3’,4’-dihydroxiflavone, apigenin (4’,5,7-tetrahydroflavone), and kaempferol (3,4’,5,7-tetrahydroflavone), on MMPs activity, ECM component fibronectin expression, invasion, and viability of GL-15 human glioblastoma cells. Cells were cultured in serum free supplemented DMEM and after 24 h exposure to 50µM flavonoids were analyzed by Immunocytochemistry for fibronectin expression and by zymography to determine MMPs activity. Cell viability was determined by MTT test. Synchronized GL-15 cells were cultured on different ECM supports (laminin, collagen, collagen plus fibronectin), treated with flavonoids (50µM) and migration of glioblastoma cells was investigated through the use of a standard in vitro monolayer wound assay, and closure of the wounded cell free area was followed after 1-5 days. Compared with cells in control conditions (vehicle DMSO 0.1%) flavonoids reduced viability of GL-15 cells and induced changes on pattern of extracellular fibronectin expression. Phase contrast microscopy and Rosenfeld staining showed that the cell-free wound gap of GL-15 control monolayer healed faster, and more cells appeared in the wounded gap on collagen plus fibronectin support, which represented enhanced migratory activity towards the wounded area. However, in the flavonoids treated groups, the closure of the wounded area was significantly slower indicating inhibition on glioblastoma cells migration. Moreover, pro-MMP2 and MMP-2 activity were efficiently inhibited in the presence of polyhydroxylated flavonoids. Taken together these results suggest Polyhydroxylated flavonoids as inhibitor of glioblastoma cells growth and invasion and may find and application to traditional chemotherapeutic treatments for malignant gliomas. Supported by CNPq, CAPES and INSERM U894.

Key words: glioblastoma, flavonoids, MMPs, fibronectina, invasion

09.29- Laminin Peptide Sikvav Regulates Migration, Invasion and Protease Activity of Adenoid Cystic Carcinoma Cells Through Integrins, Fak and ERK

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Adenoid cystic carcinoma is a malignant salivary gland which presents prominent expression of extracellular matrix molecules. The extracellular matrix and basement membrane play an important role regulating phenotypic characteristics of neoplasms. Laminin is a glycoprotein predominantly expressed in basement membrane, which influences tumor behaviour. This molecule also contains cryptic sites with biological activity, which can be avaible for cells through proteolytic cleavage. During invasion, neoplastic cells produce proteolytic enzymes, as matrix metalloproteinases (MMPs) that degrade laminin and other
extracellular matrix components. This process promotes release of fragments and bioactive peptides of laminin that can influence tumor biology. One of these peptides is SIKVAV (carboxy terminal domain of the laminin-alpha1 chain) which is involved in malignant transformation, cell proliferation, angiogenesis and protease activity in a number of cell types. In this study we investigated regulation of migration, invasion and protease activity by the peptide SIKVAV in a cell line (CAC2) derived from adenoid cystic carcinoma. Monolayer wound assays and Transwell migration assays showed that SIKVAV increased migration of CAC2 cells compared to controls. This peptide also stimulated invasion in Boyden chambers coated with Matrigel. Invasion depends on protease activity. SIKVAV increased secretion of MMPs 2 and 9 compared to controls. We also studied putative receptors involved in SIKVAV activities. siRNA knockdown of alpha3 and beta1 integrins inhibited SIKVAV-induced migration and protease activity. Different signaling pathways could be related to SIKVAV effects in CAC2 cells. This peptide increased both phospho-ERK and phospho-FAK compared to controls. We propose that SIKVAV increases migration, invasion and protease activity of CAC2 cells through integrin heterodimer alpha3beta1. Signal generated by SIKVAV is transduced by FAK and ERK1/2 signaling pathways. Supported: FAPESP(2006/57079-4, 2008/57103-8 and 2009/06299-2) and CNPq (304868/2006-0 and 470622/2007-5).

**Key words:** adenoid cystic carcinoma, extracellular matrix, invasion, laminin, matrix metalloproteinases

09.30- BRAF- Mutation and Expression of Hyaluronan and Hyaluronidase in Differentiated Thyroid Carcinoma

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Differentiated thyroid cancers (papillary - PTC and follicular - FTC) are the most common endocrine malignancies. In PTC, about 70% of cases has a known genetic event, specially RET/PTC rearrangement and BRAF mutation. Point mutations of BRAF are found in about 40-50% of PTC and have been implicated with poorer outcome. The development and progression of human tumors is accompanied by various cellular, biochemical and genetic alterations which include tumor cells interaction with extracellular matrix molecules including hyaluronan (HA). HA is a high molecular weight linear glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. Its implication in malignant transformation, tumor progression and with the degree of differentiation in various invasive tumors has been reported and well accepted. There are evidences that tumors cells from different origins can degraded HA by specific enzymes, Hyaluronidases (Hyase), generating low molecular weight fragments. These fragments have distinct functions in tumor biology, including angiogenesis, which have been implicated in
tumor invasion and progression. In this study, we investigate if there is differential expression of HA and Hyase in two PTC cell lines, one positive for BRAF mutation (NPA) and the other with RET/PTC rearrangement (TPC-1). Cell lines were cultured in F12 medium supplemented with 10% bovine fetal serum until 90% confluence. Afterwards, culture medium and cellular extract were analyzed separately. HA was analyzed by an ELISA-like fluoroassay using a specific biotinilated HA-binding protein as probe. Hyase activities from culture media were determined by an ELISA-like fluoroassay and compared with standard testis hyaluronidase in pH 4.0 and 7.0. TPC-1 synthesized significantly more HA if compared to NPA (213.2 ± 9.4 versus 10.3 ± 1.1 ng/mg protein). Interestingly, a distinct profile of Hyase activities was observed when the two cell lines were compared. TPC-1 showed an increased enzymatic activity in pH 7.0 when compared to NPA (14.3 versus 1.2 mUI/mL) while in pH 4.0 the enzymatic activity was higher in NPA (25.6 versus 1.1 mUI/mL). Although preliminary, our results provide evidence for a process that represents a probable activation mechanism, related to BRAF mutation, which leads to the promotion and maintenance of the higher malignant phenotype of thyroid carcinoma bearing this mutation. Supported by FAPESP and CNPq

**Keywords:** thyroid cancer, BRAF mutation, hyaluronan, hyaluronidase, glycosaminoglycan

10. ECM AND CELL THERAPY

10.1- Effect of Dermatan Sulfate in the Migration and Proliferation of Endothelial Progenitor Cells after Arterial Injury in Mice

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Coronary artery disease is the leading cause of mortality in the western world. Some of the principal treatments for this disease include angioplasty, stenting and arterial bypass. The success of these interventions remains limited by negative remodeling resulted in lumen restenosis; approximately 50% of the patients presents restenosis after 6 months of the vascular injury. Neointima formation is characterized by endothelial dysfunction that is mainly associated with elevated smooth muscle cells (SMC) proliferation. Endothelial progenitor cells are able to migrate to the site of endothelial lesions and differentiate into mature endothelial cells helping the recovery of the injured endothelium; they are characterized by markers like CD34 and CD133. Vascular repair by circulating progenitor cells of ongoing vascular injury may be essential for vascular integrity and function. Bone marrow is the main source of these cells. It has been described that transfusion of bone marrow cells (BMC) to mice after arterial injury can help restore the endothelium, avoiding
the neointima formation and restenosis. The mechanisms of migration and differentiation of these cells to the site of injury are not well established. We studied the relationship between the administration of dermatan sulfate (DS) and injection of bone marrow cells (BMC) in neointima formation. For this we injured the left common carotid of C57BL06 mice using a wire guided probe that mimics angioplasty. We studied 4 groups, control (1), DS injected (2), BMC injected (3) and BMC + DS injected (4). We analyzed the neointimal area, cell proliferation in media and intima, and migration of inflammatory cells in different days after lesion. We observed that the number of cells in medial and intimal area in mice that received only the BMC were higher than that in the other groups in all analyzed times. The percentage of neointima formation was 100%; in group 1, 34,45% in group 2; 46,02% in 3, and 26,63% in 4. The BMC injected contained 0,5 % of cells CD34/CD133 positive, what is according to many authors to identity the progenitor cells presents in the BM. We can conclude that the DS inhibits the initial inflammatory process after arterial injury and, when administrated together with BMC, it decreases neointimal area avoiding SMC proliferation. These results suggest that DS can help in the migration and proliferation of the BMC, by inhibiting macrophages migration and SMC proliferation in the site of injury.

**Key words:** bone marrow cells, angioplasty, neointima, smooth muscle cells, CD34

10.2- Cartilage Progenitor Cells and Alginate: A Tissue Engineering Approach


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Cartilage is a tissue with low regeneration capacity. After a wound, it is generally substituted by a non-functional fibrous tissue, the fibrocartilage. Chondrocytes may lose their chondrogenic phenotype *in vitro*, so that progenitors cells have been investigated as alternative sources of cells for cartilage engineering. The universal strategy of tissue engineering is to seed cells into three-dimensional scaffolds. The aim of this study is to isolate and characterize human cartilage progenitor cells and to associate them with scaffolds that could be used in cartilage bioengineering protocols. Cartilage samples were obtained from human nasal biopsies during aesthetic surgery procedures. Cells were isolated after collagenase digestion (2mg/ml for 2 hours), expanded *in vitro* at 37°C with 5% humidified CO2 and characterized by morphological analysis, expression of mesenchymal stem cells surface markers, proliferation and differentiation assays. Finally, they were encapsulated in 2% alginate beads, and incubated in 0,1M CaCl2 in order to form a hidrogel. Cartilage progenitor cells were negative for CD34 and CD145 and positive for CD44, CD73 and CD105. They showed osteogenic, chondrogenic and high proliferation capacity. Notably, they also revealed a spontaneous chondrogenic differentiation, different from other progenitor cells
described in the literature. When encapsulated in alginate hidrogels, they acquired a rounded morphology, typical of chondrogenic lineage cells \textit{in vivo}. This work showed that cells isolated from human hyaline cartilage have a high chondrogenic capacity and could be successfully associated with alginate. In parallel, our laboratory has developed a fiber mesh scaffold based on silk fibroin with interesting physical properties and already associated it with alginate. In the future, cartilage progenitor cells previously encapsulated in alginate will be associated to our fiber mesh in order to generate a cartilagenous tissue \textit{in vitro}.

**Key words:** alginate, cartilage, progenitor cells, tissue engineering

10.3- Mononuclear Bone Marrow Cell Transplantation Decreases Collagen Accumulation in The Liver of Fibrotic Rats

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Hepatic fibrosis is a pathogenic process that results from a chronic lesion to the hepatic parenchyma, generally due to intoxication, congenital biliary atresia or hepatitis. The main feature of hepatic fibrosis is the process of collagen accumulation either in the hepatic parenchyma and/or portal areas, with massive hepatocyte death and substitution by a fibrous extracellular matrix. Chronic fibrosis often leads to loss of hepatic function and development of cirrhosis and hepatic failure. Liver transplantation is the most effective treatment to hepatic failure, but it is limited by organ shortage. Recently, transplantation of bone marrow stem cells has emerged as an alternative to treat subjects with moderate hepatic fibrosis and may help to slow the progression of the disease and improve hepatic parameters [1]. In this study, bone marrow mononuclear (BMMN) cells were injected in rats with hepatic fibrosis induced by bile duct ligation (BDL), and collagen levels were analyzed. Rat bone marrow cells from tibias and femurs were purified using a Ficoll-gradient-centrifugation, labeled with Cell Tracker and injected in the jugular vein of fibrotic rats obtained 14 days after BDL. After 7 days, rats were sacrificed, livers were collected and then paraffin sections were stained with Picro Sirius. To quantify liver fibrosis, 15 random fields per animal were captured in a light microscope with a CCD camera and then analyzed with the software Image Pro. The percents of fibrotic areas were then compared with the control groups (normal animals, 14-day and 21-day liver fibrosis) using ANOVA and Kruskal-Wallis post-test. In this study, liver fibrosis resulted in abnormal collagen accumulation in portal areas and in the formation of collagen-rich septa between hepatic lobules. These features were evident 14 days after BDL, and continued to progress in the 21st day. However, fibrotic rats treated with BMMN cells had fewer septa in the parenchyma, displaying less collagen (3.31%±1.85) than their controls (rats with 21-day fibrosis, 5.3%±1.05, and rats with 14-day fibrosis, 4.7%±1.27). Therefore, BMMN cells transplantation decreased collagen accumulation in fibrotic rats after BDL. We hypothesize that BMMN cells may help restore the normal balance of the extracellular matrix.

**Keywords:** mononuclear bone marrow cells, liver fibrosis, bone marrow stem cells, collagen

### 10.4- Reevaluating the Number of Colony-Forming Units-Fibroblast in the Adult Human Bone Marrow

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In the bone marrow, stromal cells play an important role in microenvironment formation and hematopoietic stem cells self-renewal, proliferation, and differentiation. The frequency of these progenitors in healthy patient's bone marrow aspirates is 30 +/- 12 per 106 cells. CFU-Fs can also be found in umbilical cord blood and peripheral blood, and adipose tissue in much lower and higher frequencies, respectively. In the present study we isolated the mesenchymal population from the acetabular reaming bone marrow fragments. Acetabular reaming were obtained from patients with ages range between 20 and 60 years, mal and female, with no osteometabolic diseases, undergoing total hip arthroplasty. Fragments were suspended and homogenized in saline solution (5 times), supernatant was recovered and centrifuged. Pellet was resuspended, cells were counted, and 200,000 cells were plated per well. Three days later, supernatant was discarded and the adherent population was incubated in fresh medium for extra 10 days. Colonies were fixed, stained, and counted. Colony forming efficiency (CFE) was presented as colonies per million of nucleated cells. Phenotypic analyses were performed by a hemogram, RT-PCR and FACS analyses. All cells isolated expressed higher levels of CD105, CD44, and CD13. Less then 5% of cells expressed both CD45 and CD34. RT-PCR analyses indicated that mesenchymal population is heterogeneous, expressing osteogenic, adipogenic, and chondrogenic markers. The average of CFE was 233 (30 – 720), almost seven fold higher than that regularly described in the literature. Our data shows that cells isolated from the acetabular reaming (surgical waste) present similar phenotype to that described in the literature, but indicates that skeletal progenitors frequency is much higher than usually believed. CFE values may provide useful insights into reevaluating the way mesenchymal cells are obtained.

**Key words:** mesenchymal cells, skeletal progenitors, Bone marrow
10.5- Effect of Leucurogin, A Recombinant Disintegrin, Upon Fibroblast Adhesion

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Disintegrins show specifics sequences that can bind with integrins, inhibiting cellular processes like adhesion, migration and proliferation in normal and tumor cells. Leucurogin is one recombinant disintegrin produced from a salivary gland of Bothrops leucurus snake. This work was carried out to characterize the action of leucurogin upon murine and human adhesion in fibroblasts cell lines, and verify the competition of the recombinant protein with collagen type I in the adhesion process. To analyze the adhesion process were used human (hFb) and murine (3T3) fibroblasts. Cells were cultivated in plates containing 10% fetal calf serum (FCS) in humid atmosphere with 5% CO2 at 37ºC. After trypsinization and verification of cells viabilities, cells were added (40 x 103 cells/well) in plates of 96 wells, previously incubated with collagen type I (40 μg/mL), leucurogin (10 μM) or 100% FCS. For negative control were used wells without previous incubation. The positive control for human fibroblast was used collagen type I and for murine, FCS. In the competition test, wells were covered with extracellular matrix components (ECM). Then, the cells, previously incubated with leucurogin at concentrations in the range 0.1-10 μM for 1h, 37ºC were added to the wells. The unspecific interaction was blocked by incubating the wells with inactive 2% BSA for 1h at 37ºC. The cellular adhesion was analyzed by the absorbance in 595 nm after labeling the adhered cells with violet crystal (0.5%). Low level of hFb adhesion was observed mediated by leucurogin (OD 595 = 0.13), when compared with the collagen, positive control (OD 595 = 0.28). However for the 3T3, the result of adhesion mediated by leucurogin (OD 595 = 0.1) was similar to that observed to the control, FCS (OD 595 = 0.09). In the competition assay between leucurogin and collagen, recombinant disintegrin inhibited, in a dose-dependent manner, the adhesion of collagen with both fibroblasts cell lines. At 10 μM leucurogin was able to reduce 50% adhesion in hFb and 90% in 3T3. The results indicate that leucurogin is capable to inhibit cellular processes dependent of collagen I-integrin interaction. Financial support: Fapesp, CNPq, Capes, Finep, Faep and DAAD.

Keywords: disintegrin-like, integrins, collagen type I, cell adhesion
10.6- Mesenchymal Stem Cells from Human Adipose Tissue Promotes Regeneration of SCI

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Spinal cord injury causes profound changes in the nervous microenvironment that culminate in axonal degeneration and demyelization. In the acute phase, bleeding causes local hypoxia, which, combined with the production of inhibitory molecules and inflammatory mediators, lead to the destruction of neuronal tissue. Mesenchymal stem cells from adipose tissue (AT-MSC) have been shown to differentiate into different cell types and to participate in the angiogenic process. Here we investigate the effect of human AT-MSC in SCI. Acute local injection of hAT-MSC was performed after thoracic compression or transection (105 cells diluted in 10 ul of DMEM). In both models there was a significant improvement in locomotor function, and the final BBB score increased from 17.75 + -0.75 to 19.75 + -0.25 in compression and from 4.12 + -0.23 to 9.6 + -1.14 in complete transection. Morphological analyses of the experimental animals showed that treatment reduced the area of the cystic cavity and increased the number of cell bodies and fibers labeled with the neuronal regeneration marker GAP-43. It was also verified an increase in the amount of small blood vessels along the central canal, as well as in white and gray matters. Neovascularization of the spinal parenchyma could explain the beneficial effects of hAT-MSC, since it would increase the oxygen supply to the cells, thus preventing the progression of ischemic necrosis. Finally, we observed no sign of rejection of grafted human cells, which confirms the immunomodulatory character previously proposed for MSCs.

Keywords: Mesenchymal stem cells, spinal cord injury, axonal regeneration

10.7- Characterization of Bone Marrow Stromal Cells as a Source for Tendon Graft Recellularization

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It is well established that bone marrow stromal cells (BMSC) may differentiate into bone, cartilage, smooth muscle, adipose and tendon tissues, being an important tool for tissue bioengineering and regenerative surgery. Tendon grafts are currently used to replace injured tissue, but their application is limited by the morbidity, in patients using autografts, or
immunologic response against allografts. Over the past few years, protocols using tendon allografts have risen as an alternative source for tendon and ligament replacement. These tissues are decellularized, removing almost all allogeneic antigens. Then cell injection into acellularized tendon scaffolds may maintain tissue homeostasis and biomechanical properties by expression of both the fibrillar and non-fibrillar matrix. In the present work, we intend to characterize BMSC cultures obtained from acetabular reaming fragments in order to improve tendon allografts. Bone marrow was washed in CMF solution and cell pellet was resuspended in IMEM SBF 10% during three days. After this step, supernatant was discarded and cells cultivated during more 10 days. Flow cytometry showed an intense staining for CD105 and CD146, two markers present on mesenchymal cells. RNA extraction was performed for osteogenic, chondrogenic, adipogenic, tenogenic, myogenic and stemness markers. Sex is a transcription factor that induces the expression a tenocyte proliferative protein, Tenomodulin, while Fibromodulin and Biglycan are proteoglycans present in tendon stem cell niche. These Cells express tenogenic markers scx, tenomodulin and Fibromodulin but not Biglycan. Moreover, they were positive for all lineages studied and the embryonic stem cell marker Oct-4. These data point to a potential BMSC population more efficient for tendon repair, since it is described on literature that BMSC differentiate in tenocytes, expressing tenomodulin, under stimulation with BMP-12. In the near future, we intend to optimize protocols for acellularization and recellularization for better designing therapeutical strategies, including understanding on BMSC behavior in situ.

Keywords: BMSC, scx, Tenomodulin, Tendon

10.8- Laminin Alpha5 Expression after Human Myoblast Transplantation Into RAG-/-/Gammac-/- Mice

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Myoblast transplant therapy (MTT) can be envisioned as a clinical alternative in the treatment of several myopathies. One of the problems that remain to be solved to improve the efficiency of MTT is the massive early cell death, low proliferation and limited migration of transplanted cells. Cell contact with proteins of extracellular matrix (ECM) can protect cells from death, and increase cell proliferation and migration. Laminin (LN) is an ECM protein and the major component of muscle basal membrane. Different LN isoforms are involved during muscle development and regeneration. In this work, we decide to study LN expression during human myoblast differentiation. In vitro, the isoform LN-alpha5 is expressed only from 3 days after differentiation induction and LN-alpha2 is expressed later, from 7 days. Interestingly, LN-5 was strongly detected in fused myoblasts (myotubes) but weaker in not
fused cells, linking this isoform with the differentiation program. However, when we used a polyclonal antibody to LN, a positive staining was always detected, in proliferation medium as well in differentiation medium, indicating the expression of other LN isoforms. We recently showed that the immunodeficient mice RAG-/-gammaC-/- is an attractive and efficient recipient to support engraftment of human myoblasts. In this model, 5x105 human myoblasts were injected into the Tibialis Anterior (TA) muscles of RAG-/-gammaC-/- recipients. Mice were sacrificed after 0h, 1h, 3h, 6h, 24h, 12h, 3 days, 5 days and TA muscles were analyzed by immunofluorescence. After cell injection we observed a progressive LN deposition, with a polyclonal antibody, in the area of transplanted cells. During this kinetics, no LN-alpha2 was detected. On the other hand, using the antibody against LN-alpha5, we clearly detected this isoform from day 3, time point where we detected the differentiation of the human injected myoblasts. This data relate the expression of this isoform with the differentiation program and corroborate the in vitro results. We have recently demonstrated a precocious differentiation of the transplanted human myoblasts, phenomena which could impairs the efficacy of MTT. The relation of the LN-alpha5 isoform expression with the differentiation of human myoblasts emphasize the importance of ECM components in muscle biology and will certainly be important to design new strategies for cell mediated therapy.

**Keywords:** Cell Therapy, Laminin, Myoblast differentiation, Myoblast Transplantation, Skeletal Muscle

**11. ECM AND HISTOLOGY AND HISTOPATHOLOGY**

**11.1- HMG-Coa Reductase Inhibitors Differently Affect Rat Cutaneous Wound Repair**

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Statins are HMG-CoA reductase inhibitors that have immunomodulatory activities and pleiotropic effects but their role on cutaneous wound healing is unclear. This study investigated the effects of three statins on cutaneous wound repair. Rats were separated into control (received water only), simvastatin, pravastatin and atorvastatin groups (20mg/kg/day). Statins were administrated by gavage 10 days preoperatively until the end of the experiment. Eleven days after the beginning of the treatment, an excisional wound was performed. The wound area was measured to evaluate wound contraction and re-epithelialization and animals were sacrificed 7 (n=20) or 14 (n=20) days after wounding. Hematoxylin-eosin, picrosirius red, immunohistochemistry to alpha-smooth muscle actin, CD-68, and 4-hydroxynonenal (marker of lipid peroxidation), hydroxyproline analysis and determination of NO2- in serum were done. All statins (simvastatin, pravastatin and atorvastatin) accelerated wound contraction 7 days after wounding, but 14 days after wounding, the wound contraction was higher in pravastatin, and atorvastatin showed delayed wound contraction. The re-
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epithelialization was higher only in simvastatin and pravastatin groups. Seven days after wounding the number of CD68+ cells was higher in the pravastatin and simvastatin groups; the number of neutrophils was lower in the pravastatin and atorvastatin groups compared to the control group, and in pravastatin than simvastatin group. In the deep area of the granulation tissue, both simvastatin and pravastatin groups presented a higher density of thickened red collagen fibers compared to the other groups in 7 days after wounding. Fourteen days post wounding, hydroxyproline concentration was lower in the simvastatin and atorvastatin than control group. The volume density of myofibroblasts (Vv[myof]), 7 days after wounding was lower in the simvastatin than pravastatin group; 14 days post wounding, statins treatment reduced the Vv[myof], mainly in the pravastatin and atorvastatin; and the simvastatin increased the Vv of blood vessels. The statin treatment also reduced the number of 4-HNE positive cells, mainly in the simvastatin 7 days after wounding, and in the pravastatin and simvastatin 14 days post wounding. Data suggest that statins present different effects on rat cutaneous wound repair. Supported by CAPES and CNPq.

**Keywords:** Rat, Skin, Statins, Wound Healing

11.2- Healing of Ischemic Wounds in Young and Mature Rats

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Cutaneous wound repair is an interactive process, in which several complementary events occur. When one of these events is prolonged or delayed, the next stage of the repair is disturbed and the healing will be impaired, developing chronic wounds. Considering that wound repair in young and mature animals presents differences, the aim of this study was to compare the progression of impaired ischemic wound repair in young and mature rats. Young and mature male Wistar rats (3 and 7 months old, respectively) were separate in control and ischemic groups. For ischemia, two parallel incisions were done, and skin was separated from the underlying tissue; incisions were sutured, and an excisional wound was performed between incisions. Wound contraction and re-epithelialization were measured. Wounds were collected 14 days after wounding, and tissue sections were stained with hematoxylin-eosin, sirius red or toluidine blue and immunohistochemistry to alpha-SMactin and PCNA was performed. Wound contraction and re-epithelialization did not present difference between control groups, however the wound area was higher in the ischemic groups compared to control groups. In the ischemic groups, wound area was smaller in young animals when compared to mature animals. Re-epithelialization was higher in control groups compared to ischemic groups 12 and 14 days after the injury. All groups presented great amount of inflammatory cells. In control group and ischemic groups, younger animals presented thick red-yellowish collagen fibers parallel to surface, while the mature animals presented thin and disorganized red collagen fibers. Ischemic groups presented an increase in amount of blood vessels compared to control groups. Data demonstrated that this model of chronic wound
Marimellia Porcionato

represents a good model for the study of impaired wound repair, being more evident in mature rats.

**Keywords:** Aging, Ischemia, Skin, Wound healing

### 11.3- MMP2 and MMP9 Expression in Gerbil Female Prostate During the Estrous Cycle


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Hormonal oscillations that occur during the gerbil estrous cycle affect both the structure and function of the female prostate. These alterations include increased prostatic growth during the proestrus and estrus phases and decrease of glandular development in the diestrus I and II. These repeated cycles of glandular development and involution can cause extensive reconstruction and stromal remodeling in the female prostate. Thus, this study evaluated the matrix metalloproteinases (MMPs 2 and 9) expression in the gerbil female prostate during the estrous cycle. Daily vaginal smears were collected at 10:00 am to determine estrous cycle phases. Females were divided into 4 groups (n = 5): proestrus (P), estrus (E), diestrus I (DI), and diestrus II (DII). The female urethras were isolated for dissection of prostate tissue and the blood samples were collected for serological analysis of testosterone, estradiol and progesterone. Histological sections were submitted to immunohistochemistry for the detection of MMP 2 and 9. Serum estradiol levels remained constant through P, DI and DII (33–39 pg/ml) before reaching their maximum concentration peak during the E phase (49.6 ± 15 pg/ml). The progesterone levels were higher during the DII phase (19.08 ± 10.77 ng/ml), and remained constant during the other phases (10.4–12.8 ng/ml). The serum testosterone levels were similar during P, DI and DII (0.31–0.41 ng/ml), and slightly higher in the E (0.48 ± 0.09 ng/ml). The immunohistochemical analysis showed that higher expression of MMP 2 and 9 occurs during the more hormonal fluctuation of estradiol and progesterone (E and DII, respectively). The MMP2 showed high epithelial and stromal expression during the DI and DII. Conversely, MMP9 showed high epithelial and stromal expression during E, which is the stage of highest prostate development. MMP2 and 9 are the most studied enzymes because of their ability in degrading various elements of the extracellular matrix such as collagens I, III and IV, fibronectin and elastin. In E, the estradiol peak appears to be related to the glandular development and to MMP9 activity in the stroma reshape expansion. Moreover, high
expression of MMP2 is mainly in DII, which is a stage marked by high progesterone levels and intense stromal remodeling that occurs during female prostate involution. Thus, these results support a role of these enzymes in modulating the structure and function of gerbil female prostate during estrous cycle.

**Keywords:** estrous cycle, female prostate, immunohistochemistry, matrix metalloproteinases, steroids hormones

**11.4- Changes in Bladder of Rats Pre-Puberty as a Result of High Doses of Corticosterone Simulating Estrsse Chronic. Stereological and Morphometric Analysis**

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**Introduction:** Stress causes the activation of hipotalamo-hipofise-adrenal axis (HHA), which interferes in hipotalamo-hipofise-gonadal axis (HHG). Clinical trials and experiments involving humans showed the side effects of glucocorticoids (GC) excess in testis production of testosterone. Prolonged treatment with high doses of glucocorticoids simulates in organism a chronic stress condition and a long exposition to any stressing factor causes additional reactions such as increase of GC releasing. **Objective:** To evaluate stress effect in pre pubertal Wistar rats bladder caused by chronic treatment with corticoid. **Material and methods:** Ten new born Wistar male rats divided into 2 groups, control and treated which was given a daily corticosterone intraperitoneal injection (schedule: 9-10 am) (2 mg/100g body weight), since the 7th day of life till sexual maturity, marked by testis descending (25th day). Animals were killed in the 65th day and the bladder was removed, fixed in formalin buffered, processed and embedded in paraffin. Sections of 5 µm were stained with: Masson´s Trichromic, to evaluate vessels and to measure the epithelium, Weigert´s resorcin fucsin, to analyze elastic system fibers and Picro Sirius red for qualitative analysis of collagen types. Stereology method was used to evaluate all the parameters with the Image J software. A biochemical hidroxiprolin assay was performed to quantify the total collagen. Statistical analyses were done using test t student (significance p<0.05). **Results:** There was a decrease of elastic system fibers (p<0.0008) and blood vessels (p<0.0006) in treated group. Picro Sirius red under polarized light suggests predominance of collagen III in treated group and collagen I in control group. The other parameters didn’t show statistical difference between the groups. **Conclusion:** Stereological analysis showed that stress caused by chronic treatment with high doses of corticosterone in pre pubertal phase decrease elastic system fibers and number of vessels per area in rats bladder, however it didn’t show any alteration in total collagen.

**Keywords:** Bladder, Glucocorticoids, Stress
11.5- Collagen I and III Location in the Normal and Doxazosin-Treated Rat Prostate

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Doxazosin, an alpha-adrenoceptor antagonist, induces the relaxation of smooth muscle cell tonus and reduces the clinical symptoms of the benign prostatic hyperplasia (BPH). However, the doxazosin effects in the prostate stromal microenvironment are not fully known. In a previous study, we showed that doxazosin treatment increases the elastic fibers deposition in the three rat prostatic lobes. Herein, we investigated the effects of doxazosin on types I and III collagen fibers deposition in the stroma compartment from ventral, dorsal and anterior rat prostatic lobes, comparing the results with the control group. Intact adult Wistar rats (n=5) or adult Wistar rats (n=5) treated with doxazosin (25mg/kg/day) during 30 days were killed by an overdose of pentobarbital and the ventral, dorsal and anterior prostates were excised. The prostates samples were frozen in liquid nitrogen and processed for frozen section immunohistochemistry for type I collagen (Genetex, GTX 26308, lot. 16425) and type III collagen (Abcam, ab 6310-100, lot. 424315). The immunohistochemistry showed that types III collagen fibers are widely distributed in the prostate stroma, mainly close to the epithelium and epithelial infoldings, organized bordering the smooth muscle cells and in the interstitium, while type I collagen predominated in the interstitium in the three prostatic lobes. Doxazosin-treatment increased the amount of both collagen fibers types in the three rat prostatic lobes, with a major collagen deposition in the anterior prostate than the other lobes. Morphologically, both fibers became denser and waved, comparing with the fibers found in the control prostatic lobes. In conclusion, the smooth muscle cells contraction blockade by doxazosin may promote changes in prostatic tissue mechanical demand, which is counterbalanced by collagen fibers deposition around cells. This stromal remodeling should be considered in the BPH long-term treatment by alpha-blockers and further studies are need to investigate if this changes can interfere on the prostate epithelium-stroma interactions.

Keywords: Collagen Fibers, Doxazosin, Immunohistochemistry, Prostate, Stroma
11.6- The Decrease Proteoglicans and Increase Elastin and Fibrilin Content in Low-Intensity Physical Activity in Aorta from Wistar-Kyoto and Spontaneously Hypertensive Rats

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Studies of spontaneous hypertension in rats have focused on the presence of sympathetic hyperactivity and the mechanisms by which this alteration contributes for changing mean blood pressure and the structure and function of arterioles. Nowadays, hypertension is mainly considered as a cardiovascular risk factor, and more attention has been focused on the structure and function of hypertensive arteries, which greatly influence the development of complications in hypertensive vascular disease. Different proportions of elastin and microfibrils promote variable functional characteristics adaptable to the tissue local needs. The ECM is critical for tissue remodeling after an injury or in a disease. It is well established that elastin and collagen content is increased in hypertensive patients and in genetic or experimentally induced hypertension in animals. It is well known that exercise training can reduce blood pressure, with simultaneous improvement in endothelial-dependent vasodilatation. Thus, our aim is to investigate with more accurate tools if exercise training alters the behavior of fibers of the elastic system in the aorta of genetically hypertensive rats.

32 male rats with two-month-old were allocated into 4 groups: sedentary WKY (WKY-SED), exercised WKY (WKY-EX), sedentary SHR (SHR-SED), and exercised SHR (SHR-EX). Sedentary rats were restricted to cage-bound activity, while exercised ones underwent a 1 h/day, 5 days/week exercise training; the experiment last 20 weeks. Desired final training intensity and duration (16m/min, 0% grade, 60 min/day). Exercise training was characterized as low-training since O2 consumption did not exceed 55% of O2 max throughout experiment. Blood pressure was measured weekly (BP). Ultrastructural appearance and elastic fiber system were investigated. Results: BP increased progressively in SED-SHRs reaching 189±4 mmHg, but exercise training impaired this process. Final BP in EX-SHR was similar to WKY rats (118±4 vs. 114±4 mmHg, respectively). Exercised groups had lower proteoglicans expression than respective sedentary groups. Percentage of elastic fibers was lower in SED-SHR compared to SED-WKY, but exercise training increased them. Elastin and fibrilin immunostain were seldom seen in SED-SHR, but both EX-WKY and EX-SHR groups showed higher amount of stain. Conclusion: Low-intensity exercise training increases elastin and fibrilin and decrease de proteoglicans content in aorta wall from WKY and SHR rats.

Keywords: aorta, elastin, fibrilin, physical activity, spontaneously hypertensive rats
11.7- Remodeling of Extracellular Matrix in the Carotid Body of Hypertensive Rats Induced By L-Name

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The high prevalence of hypertension and subsequent increased risk of cardiovascular diseases including acute myocardial infarction, the stroke and chronic renal failure, high blood pressure poses a principal risk factor for mortality worldwide. Among non-pharmacological therapies for hypertension, the changes in lifestyle, such as reduced body weight, restriction of salt in the diet, restriction of alcohol consumption and physical activity. In this context, evidence about that the reduction of respiratory rate by use of electronic devices that interact with the patient, advising him to breathe in more slowly, decreases blood pressure in patients with mild and moderate hypertension and those with resistant hypertension and without changes in the use of medication. In front of the bifurcation of common carotid artery is the carotid body (CC), structure sensitive to changes in arterial blood gases and concentration of hydrogen ions. The parenchymal cells of the CC detect changes of these gases. They are activated when the PO2 decreases and when the PCO2 and / or [H +] increase, and responds with increased releasing neurotransmitters (especially catecholamines), causing a hyperventilation to restore the concentration of these gases in the blood. The present work aims to study the remodeling of the extracellular matrix of carotid body of animals with induced hypertension (induced by L-NAME, a nitric oxide synthase inhibitor) and compared to the carotid body of animals with normal. 14 male rats (250-300g) were divided into two groups (control and L-NAME). The L-NAME group, was administrated 40mg/kg/dia in drink water for 45 days. The body mass and blood pressure were followed. The carotid body was removed along with the bifurcation of the carotid artery and processed for optical microscopy. Were analysed the cell nucleus, proteoglycans and collagen 1 and 3. In L-name group, there was an increase of proteoglycans between the cell glomus regarding control group. The carotid body appears less preserved, decrease the number of type 1 collagen fibers and increase the expression of type 3 collagen. Moreover, there is a discontinuity of the capsule around the body. In conclusion, the hypertension induced by L-NAME increased the expression of proteoglycans and collagen type 3, suggesting that hypertension causes a remodeling of the extracellular matrix in the carotid body.

Keywords: carotid body, hypertension, L-name, remodeling
11.8- Mycobacterium Leprae Modulates TGF-Beta1 Expression in Schwann Cell Line

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Neural fibrosis is one of the causes of deformities during leprosy, which remains a major global health problem. In the progress of neural degeneration, as suggested by others, the Schwann cells (SC) are involved in the degeneration and regeneration of the nerve. Some studies have shown that *Mycobacterium leprae* (ML) induces TGFβ1 production, and its high levels were detected on leprosy. Since TGFβ1 is important in fibrosis development, the aim of this study was to evaluate the possible role of ML and TGFβ1 on the Schwannoma cell line (ST88-14) in leprosy neural lesion context. ST88-14 cells were stimulated with or without ML for 1, 3, 6, 24, 72 hours and 7 days to evaluate TGFβ1 levels, its mRNA (PCR), its intracellular protein and its receptor (TGFRII) expression (flow cytometry). This cytokine levels were measured (ELISA) in these supernatants cultures. Our results showed that ML stimulation induced a rapid increase in TGFβ1 mRNA expression, followed by a decline that maintained until 6 hours. This cytokine secretion seems to accompany mRNA expression. However, after 6 hours, TGFβ1 secretion seems to return to baseline levels and to rise again after 7 days. TGFβ1 intracellular expression also seems to accompany cytokine secretion. However, after 6 hours, TGFβ1 intracellular levels seems to return to increase and reach its maximum at 48 hours and, after 72 hours, to return to baseline levels again after 7 days. ML also up regulated the TGFRII expression. These partial results suggest the involvement of ML in Schwann cell line (ST88-14) answer modulation to TGFβ1, and their contribution in neural fibrosis. Supported by CNPq, CAPES, FAPERJ, FIOCRUZ.

**Keywords:** LEPROMY, Mycobacterium leprae, SCHWANN CELL, TGF-BETA1

11.9- An In Situ Study of the Effect of Metallo and Serine Proteinases Inhibitors on the Birefringence of the Secretory-Stage Enamel Organic Extracellular Matrix

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Dental enamel formation occurs extracellularly and the establishment of an ordered enamel organic extracellular matrix (ECM) seems to be a crucial step for proper construction of the enamel mineral phase. Polarizing microscopy shows that the ordered supramolecular structure of the secretory-stage enamel organic ECM exhibits strong birefringence. We have
previously reported that this birefringence is lost in unfixed specimens, probably due to extensive proteolytic cleavage of enamel proteins. Therefore, the present work aimed at investigating the association between enamel proteinases’ activities by analyzing the effects of metallo and serine proteinases inhibitors in situ on the birefringence of the secretory-stage enamel organic ECM. Male Wistar rats weighing ≈ 200 g were used in the present study. After sacrifice by cervical displacement, distal 10 mm fragments of upper incisors were immediately removed and immersed for 15 h under continuous shaking, at 37º C, in one of the following solutions: 1. 10 mM Tris pH 8.0, 150 mM NaCl (NEGATIVE CONTROL, n=8); 2. 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2 (POSITIVE CONTROL, n=5); 3. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM 1,10-phenanthroline (n=9); 4. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM phenylmethylsulphonyl fluoride (PMSF) (n=8); 5. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM 1,10-phenanthroline, 2 mM PMSF (n=9). Samples were then immersed in proper fixative solution for 24 h and submitted to laboratorial procedures which allowed obtaining 5-µm-thick longitudinal sections of the secretory-stage enamel organic ECM. These sections were immersed in 80% glycerin for 30 min and analyzed by transmitted polarizing light microscopy. 1,10-phenanthroline (inhibitor of metalloproteinases) and 1,10-phenanthroline + PMSF (inhibitor of serine proteinases) clearly prevented decrease in the optical retardation of birefringent brightness from the tissue referred to. PMSF alone promoted a slight preservation of the birefringence exhibited by the secretory-stage enamel organic ECM. Concluding, rapid loss of birefringence in not immediately fixed secretory-stage enamel organic ECM is caused by the activity of enamel proteinases and the activity of metalloproteinases seems to lead to a preliminary degradation of the enamel organic ECM, which in turns probably facilitates subsequent serine proteinases activity. Support: Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP.

**Keywords:** Enamel, Birefringence, Enamel proteinases, PMSF, Phenanthroline

### 11.10- Low-Dose of Celecoxib Accelerates Cutaneous Wound HealinD Delayed by High-Fat Diet in Obesity Prone and Resistant Rats

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Obesity is a problem of epidemic proportions and is associated with increased incidence of chronic wounds. Nonsteroidal anti-inflammatory drugs have been widely used to diminish the inflammatory reaction, through the inhibition of cyclooxygenase enzyme. Our aim was to study the effects of high-fat diet on cutaneous wound healing of obesity prone and resistant rats and to investigate the effects of two doses of celecoxib about these conditions. Wistar rats were fed either with a high-fat (n=39) or a standard diet (n=18, control, C) for 20 weeks, when the diet group was subdivided in obesity prone (n=20, OP) and resistant (n=19, OR) groups, an excisional lesion was done, and the animals were killed 7 days later. Treated groups received a daily dose of celecoxib 5 or 10 mg/kg/day from two days before wounding until euthanasia. Wound contraction, re-epithelialization, body mass and retroperitoneal fat...
were evaluated. After euthanasia, lesion and adjacent normal skin were formalin-fixed and paraffin-embedded. Granulation tissue was analyzed and hydroxyproline assay were realized. Body mass was higher in OP (p<0.001) than other groups and retroperitoneal fat was different between all groups, being higher in OP (p<0.001) and smaller in C group (p<0.01 versus OR). Animals fed high-fat diet presented delayed wound contraction (p<0.001). OP and OR groups presented higher amount of inflammatory cells than C group, being mast cells in OP (p<0.01 versus C; p<0.05 versus OR) and polymorphonuclears in OR (p<0.05). The density of myofibroblasts in OP and OR groups was reduced compared with C group (p<0.01 and p<0.001, respectively). Collagen fibers density and hydroxyproline levels were higher in OP group (p<0.05) compared with other groups. In groups treated with celecoxib we observed a reduction in body mass in OP treated with 5 mg (p<0.001) and 10 mg (p<0.01), without alteration in percentage of retroperitoneal fat. Wound contraction was delayed in C group treated with 10 mg of celecoxib (p<0.001) and this dose didn’t alter the wound contraction in OP and OR. However, 5 mg of celecoxib accelerates wound contraction in OP (p<0.001) and OR (p<0.01) groups to the level of control group. This study showed that high-fat diet exerts negative effects on rats cutaneous wound healing, in obesity prone as well as in obesity resistant animals, due mainly to prolongation of the inflammatory phase and that a low-dose of celecoxib accelerates cutaneous repair in these conditions.

**Keywords:** Celecoxib, High-fat diet, Skin, Wound healing

### 11.11- Effects of Androgen and Estrogen Suppression on the Prostatic Stroma of Old Gerbils

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The aim of this study was to evaluate changes in prostatic stroma of old gerbils (18 months) after surgical castration associated or not to steroidal blockade. The animals were divided in 6 groups and all of them were surgically castrated, excluding the control composed by intact animals. The other two groups were formed by castrated animals and castrated that received the drug vehicle. In the experimental groups doses of flutamide (antiandrogen, 10mg/kg/day) and/or tamoxifen (antiestrogen, 1mg/kg/48h) during 1, 3, 7 (short-term) and 30 (long-term) days post-castration were applied. The methodologies involved: morphological (HE, Gömöri reticulin, Gömöri trichrome), immunohistochemical analysis (chondroitin sulphate, type IV collagen) and quantitative and statistic evaluation. Proteoglycans were identified at transmission electron microscopy after fixation with Cuprolinic Blue. The castration associated or not to anti-steroidal drugs by short or long-term promoted alterations in the distribution of stromal cells and of extracellular matrix elements of the prostate.
Gradually, the epithelial compartment, more representatives in intact animals, was significantly exceeded in volume by the stromal compartment, characterizing a gland atrophy process. The smooth muscle cell (SMC) frequency increased significantly after 30 days of therapy in experimental and control vehicle groups, and this component had an effective participation to the increase of the stroma in the experiment end. Large collagen I and chondroitin sulphate deposits in the subepithelial region was a hallmark of prostatic acini in the experimental groups up to 7 days. Additionally, in regions of epithelial projections into the acinar lumen was also observed a large of type IV collagen deposition. Two populations of prostatic stromal proteoglycans were identified associated with basement membrane based on their morphology and dimensions and designated P1 (31-54 nm) and P4 (83-179 nm). These elements appeared in the form of filaments and were modulated by treatments performed. The response of the prostatic stroma front of hormonal ablation suggested that the gland architecture is often related to changes in extracellular matrix and SMC, and this showed specific reactions to short-and long-term therapy. Furthermore, this study seems to be a pioneer in the use of steroid blockers in old animals and that is looking closer to the period in which humans have higher prostate cancer rates. Support: FAPESP

Keywords: castration, old gerbil, prostatic stroma, proteoglycans, smooth muscle cell

11.12- Remodeling of Extracellular Matrix in the Left Atrioventricular Valve in Hypertensive Rats

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The heart valves are designed to maintain the unidirectional blood flow and for this reason they have an anisotropic architecture of the extracellular matrix that ensures and maintains proper function under conditions of high and low pressure. The valve disease is a disorder that can affect any of the heart valves, marked by any change in its structure that prevents its proper operation. Studies show that the mechanical stress caused by blood flow may interfere with the organizational structure of cells and extracellular matrix of the valve. This study aimed to investigate the possible structural and ultrastructural changes that hypertension, induced by L-NAME, can cause to the left atrioventricular valve (mitral) from hearts of rats. The animals received L-NAME (40mg/kg/dia), previously diluted in water, for five weeks. At the end of the fifth week euthanasia of animals was performed and the parietal leaflet fragments were collected and processed for light microscopy and electron microscopy. It was analyzed the morphological structure of the valves at both light microscopy and electron microscopy, as well as the thickness of the valve and quantification (% / area) of the elastic and collagen fibers through the ImagePro-Plus 4.5. The animals treated with L-NAME developed hypertension from the second week of administration of the drug. Structurally, it was possible to identify a disorganization of the elastic and collagen fibers with regions of fragmentation. The animals with hypertension showed thicker valve regions than control, plus a smaller amount of collagen fibers. Ultrastructurally, it was identified changes of the
endothelium in animals treated with L-NAME, where it presented irregular contours and accumulations of vesicles inside. Furthermore, it was also observed fragmentation of basal lamina, disorientation of collagen fibers, accumulation of oxytalan fiber near the endothelium and fragmentation of elastic fibers of the atrial layer. This study concluded that increased blood pressure, characterized by hypertension, causes changes in the morphological structure of the valve, which may be linked to the synergistic inhibition of nitric oxide synthesis, and may thus undermine its perfect operation.

**Keywords:** Extracellular Matrix, Hipertension, L-NAME, Valve

### 11.13- Microfibril-Elastin Fiber System Distribution in the Rat Mitral Valve: A Structural and Ultrastructural Study

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With increasing valve disease tissue-engineered heart valves are the ultimate solution for treating these conditions. Healthy heart valves maintain unidirectional blood flow and the anisotropic matrix architecture assures sustained and adequate functioning under high-pressure conditions that remodel in response to changes in local mechanical forces. A major challenge in tissue engineering is determining the tissue structures similar to heart valve anatomy and function. The essential extracellular mitral valve components include glycoproteins, collagen and elastin. However, little is known about the elaunin and oxytalan fibers which together with the elastic fibers form a system with elastic characteristics associated with a common and sequential ontogenetic development. The microfibril-elastin fiber system was studied to investigate the interrelationship of oxytalan, elaunin and elastic fibers in mitral valve morphology. The valves were stained with orcinol-neo fuchsin and Weigert’s resorcin-fuchsin with or without prior oxidation and observed under light microscopy. Routine electron microscope was used to study valve samples for elastic, elaunin and oxytalan fibers which were fixed in 2.5 % glutaraldehyde. For elastin and fibrillin immunostaining for electron microscopy, the tissues were gradually infiltrated in LR WHITE resin and the ultrathin sections were incubated in primary antibody to highlight fibrilin-1 and elastin. In the valve, the existence of the fibers of different diameters was differentiated by staining affinities and in transmission electron microscope was observed that the microfibril-elastin fiber system was distributed in different proportions in the mitral valve layers. In atrial layer, elastic fibers predominated, characterized by large amounts of elastin. The spongiosa layer was composed mainly by elaunin fibers formed by microfibrils interspersed with elongated patches of amorphous material, and oxytalan fibers characterized by large bundles of microfibrils without elastin. In ventricular layer there were elaunin fibers arranged in continuous bundles oriented parallel to endothelium. The present study suggesting that the microfibril-elastin fibers play a role in the mechanical protection and maintenance of the integrity of the mitral valve.
11.14- Schwann Cell Transdifferentiation: Possible Role of Myofibroblast in Neural Fibrosis During Leprosy

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The development of deformities during leprosy remains a major global health problem. Fibrosis has been seen in leprosy nerve biopsies of patients and Schwann cell (SC) seems to be involved in nerve degeneration and regeneration. Our group showed, in a human Schwannoma lineage (ST88-14), increased mRNA levels of TGFβ1 and its receptors after *Mycobacterium leprae* (ML) stimulus. Since TGFβ1 is important to fibrosis development, and promotes transdifferentiation of SC into myofibroblast, the aim of this study was: a) to analyze αactin smooth muscle expression (αSMA, myofibroblastic marker) in patients nerve biopsies without pathological changes or with fibrosis, and b) to evaluate *in vitro* SC-myofibroblastic transdifferentiation and extracellular matrix (ECM) secretion/deposition. αSMA expression was analyzed in patients’ nerve biopsies by immunohistochemistry and immunofluorescence. In order to evaluate, *in vitro*, SC-myofibroblastic transdifferentiation (immunofluorescence and flow cytometry to αSMA) and ECM (collagen IV, laminin, fibronectin and tenascin) secretion/deposition and proteins structural form (ELISA and immunofluorescence, respectively), human primary Schwann cells and ST88-14 cultures were stimulated with recombinant human TGFβ1 (10 ng/ml) for 24 hours, 4 or 7 days. Our results showed, for the first time, αSMA expression in perineurium of normal nerves tissues and in some endoneurial cells of nerve tissues with fibrosis. After 7 days of stimulation, an increased expression of collagen IV, laminin, fibronectin and tenascin, and, in some cases, the structural form modulation of these proteins were also observed. Moreover, TGFβ1 induced transdifferentiation of SCs into myofibroblasts. The αSMA intracellular increased after 24 hours, 4 and 7 days of stimulation. These results suggest a TGFβ1 involvement in SC-myofibroblastic transdifferentiation, which can contribute to fibrosis and, consequently, to neural degeneration. Supported by CNPq, CAPES, FAPERJ, FIOCRUZ.

**Keywords:** Leprosy, Schwann cell, Myofibroblast, Fibrosis, Extracellular matrix

11.15- Effect of Oral Supplementation of L-Glutamine on the Rats Ventral Prostate Subjected to Abdominal Irradiation

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PURPOSE: To analyze the effects of L-glutamine on the rats ventral prostate submitted to radiotherapy. METHODS: Wistar rats, approximately 90 days and 350g, were subjected to pelvic irradiation with or without nutritional supplementation of the amino acid L-glutamine,
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divided into three groups: (1) Control (n=5), (2) group subjected to irradiation abdominal (n=5), without amino acid supplementation, (3) group submitted to abdominal irradiation with supplementation of L-glutamine (n=5). Supplementation of L-glutamine (0.65g/kg body weight) by gavage occurred during the 21 days of experiment. Pelvic irradiation was used for the single dose of 1000cGy. on the 8th day of experimentation. Euthanasia of the three groups occurred in the 22 th day of the experiment. After the ventral prostate was removed and fixed by drying. The morphometric analysis was conducted for the area of the prostate acini and height of the acinar epithelium. According to the area, the acini were classified into three groups: small (with area up to 10,000 ìm2), average (with area greater than 10,000 ìm2 and ìm2 less than 100,000) and large (with an area exceeding 100,000 ìm2). Statistical analysis: Kruskal-Wallis followed by Mann-Whitney for non-parametric analysis of data; chi-square for the analysis of proportions and percentages. In all cases the differences were considered statistically significant when p<0.05. RESULTS:In the supplemented group had a significant weight gain of weight (+14.1g) when compared to the control (+20.8 g) and irradiated (3.1 g). There was significant reduction in acini classified as small in the supplemented group (6.52%) compared to control (35.23%), the Spent (12.84%). In contrast there was an increase of acini average (76.94%) in the group receiving supplementation, as compared to the control (43.24%) to irradiated (55.23). Referring to large acini, the proportion was higher in the irradiated group (31.93). There was a significant decrease in the height of the acinar epithelium in groups 2 (44.28 nm) and 3 (31.88 nm) compared to 1 (66.55 nm). The distribution of collagen was higher in group 3 (9.13%) than in group 1 (8.14%) and 2 (7.0%). CONCLUSION:Irradiation changed the distribution of acini, the acinar epithelium of the characteristics and distribution of collagen per field. And so far it is believed that L-glutamine has a role in protection/restoration of the ventral prostate. Keywords: L-glutamine, Radiotherapy, Rat Financial Support: FAPERJ, CNPq

Keywords: L-glutamine, Radiotherapy, Rat

11.16- Elastogenesis in the Development of the Interpubic Ligament During Mouse Pregnancy

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The organization of elastic fibers involves the synthesis and the deposition of molecules in a high regulated sequence to ensure the elastic characteristics of each organ in the early stages of development. Pelvic tissues, which are extremely rich in elastic fibers, remodel during pregnancy as does the birth channel in mammals. This remodeling is essential to the vaginal delivery and it occurs mainly in the uterus, cervix and vagina. In mouse and guinea pig, the pubic symphysis articulation also remodels in a controlled hormonal process which...
allows safe delivery. This phenomenon comprises the “transformation” of the fibrocartilage into an interpubic ligament (IpL) followed by its relaxation before parturition. After the first parturition, morphological aspects of the interpubic articulation become similar to that which is observed in nulliparous mouse. Recently, prolapse and pelvic organ support impairment had been described in genetically modified mouse for the proteins involved in the elastogenesis as lisil oxidase-like 1 and fibulin-5 at postpartum and fibulin-3 in aged mice. Since, ligaments are the main supportive structure of pelvic organs, the initial aim of this work was to evaluate the elastogenesis in the development of the IpL during mouse pregnancy, due to its association with pathologies in the mouse pelvic organ support. Thus, virgin and pregnant C57Bl/06 female mice groups were studied using light and transmission electron microscopy. In virgin and 12th day of pregnancy (D12), the fibrocartilaginous tissue had slender bundles of microfibrils randomly distributed among the fibrochondrocytes. Weigert”s Resorcin-Fucsin stain and ultrastructural studies revealed the presence of immature elastic fibers (IEF), which consist of small conglomerates of amorphous material, distributed among the bundles of microfibrils in the development of the IpL at D15. In the D18, the IpL showed IEF and all tissue compounds aligned to the opening axis of the articulation. So, the induction of the elastin deposition on the microfibrils of the developing ligament could contribute to the elasticity necessary to the pelvic girdle in the period of preparation for delivery. This process that occurred during the adult life has unique characteristics of a model that could be used to understand the fiber elastic formation. Still, further analyses will be done using a fibrillin-1 deficient mouse that recapitulates the vascular aspect of Marfan syndrome. Fapesp: 08/56492-0

**Keywords:** Pubic symphysis, Biology of reproduction, Elastogenesis, Extracellular matrix, Mouse

11.17- Matrix Metalloproteinases, Timps and Growth Factors Regulating Ameloblastoma Behaviour

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Ameloblastoma is an odontogenic neoplasm with local invasiveness and recurrence. We previously suggested that growth factors and matrix metalloproteinases (MMPs) influence ameloblastoma local invasiveness (Pinheiro et al, 2004). Here we studied expression of MMPs, TIMPs, and growth factors in ameloblastoma. Correlation analysis assessed relationships among these molecules. Furthermore, we searched for cell signalling markers in ameloblastoma. Immunohistochemistry detected MMPs, TIMPs, and growth factors in formalin-fixed paraffin-embedded samples of human ameloblastoma. Immunohistochemical staining was quantitatively assessed by three independent observers (A.S.S., M.R.D.C., A.C.D.M.), with a minimal inter observer variability (>5%). Sections were analysed using
light microscopy, at a final magnification of 400x. Both neoplastic and stromal cells were evaluated. Expression of different antigens was scored according to number of cells with positive staining, without considering differences in staining intensity. Labelling index (LI = positive cells/total cells x 100) for MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, EGFR, EGF and TGF-alpha was determined by observing 1000 cells. All ameloblastoma samples presented MMPs 1, 2 and 9; TIMPs 1 and 2; and EGF, TGF-alpha and EGFR. LI of MMP-9 and TIMP-2 was equally distributed in neoplastic cells and stroma. In ameloblastoma cells MMP-1 and MMP-2 expression was significantly higher compared to stroma. EGF and TGF-alpha exhibited no differences between neoplastic cells and stroma. EGFR LI was increased in ameloblastoma cells compared to stroma. We found positive correlation between EGF and TIMP-1; and between TGF-alpha and TIMP-2. Immunohistochemistry detected ERK 1/2 and phospho-ERK in ameloblastoma. Phospho-ERK was mostly detected in stroma. Results suggest an interplay involving growth factors, MMPs and TIMPs, which may contribute to ameloblastoma behaviour. Signals generated by this molecular network would be transduced by ERK 1/2 pathway.

**Keywords:** Ameloblastoma, Extracellular Matrix, Growth Factors, Metalloproteinases, TIMPs

### 11.18- Effect of Low Intensity Laser Therapy Irradiation on Skin Tissue Repair

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Low intensity laser therapy is a form of phototherapy used to promote wound healing in different clinical conditions. Laser therapy at an adequate wavelength, intensity, and dose can accelerate tissue repair. However, there is still conflicting information about the effect of multiple irradiations on the cellular responses of wounded cells. To better understand the use of low intensity laser therapy in cutaneous wound healing, clinical studies that correlate cellular effects and biologic processes are needed. The aim of this study was evaluated the effect of low intensity laser therapy on the wound healing process in rats' skin. Five Wistar rats were subjected to a surgical incision measuring 6cm in length, located to right side to the spinal column and sutured in U-shape. A surgical incision on the left side was done, sutured and used to control. The treated incisions were irradiated with a 658 nm laser, with the following parameters: 2.5 Hz, 3 min, and 4 J/cm2. Skin biopsy was performed on the 7, 14 and 21 days after wounding, for morphological evaluation of the cicatricial process. Morphological analysis was performed with an optical microscope (Olympus, EUA) adapted to a computerized picture analysis system to capture image (Image Proplus 6). Results indicated that effect of lower doses (4 J/cm2) determines the stimulatory effect, showed complete re-epithelization around 14 days after surgical incision; non-irradiated incision was
Marimellia Porcionato

re-epithelized around 21 days. In addition, fibroblasts and inflammatory cells were observed in treated and non-treated incisions after 7 days of the surgical procedure, decreased in 14 and 21 days. We concluded that low intensity laser therapy can accelerate skin wound healing.  

**Key words:** laser, skin, wound healing

11.19- Effects of Metoclopramide-Induced Hyperprolactinemia on the Gene Expression of Hyaluronan Synthases I, II and III in Mouse Uterine Along the Different Phases of the Estrous Cycle

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**OBJECTIVE:** To evaluate effects of metoclopramide-induced hyperprolactinemia on the gene expression of hyaluronan synthases I, II and III in mouse uterine along the different phases of the estrous cycle. **DESIGN:** Experimental study using an semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) method for examined the expression pattern of hyaluronan synthase (HAS I, II and III) mRNAs in the mouse uterine. **ANIMAL(S):** 80 adult (100 days old) female virgin mice. **METHODS:** The animals were randomly divided into two groups of 10 animals each: control (Ctr), which received the vehicle (0.2 ml NaCl 0.9% in distilled water) and experimental (HPrl), which received 200 µg metoclopramide dissolved in 0.2 ml NaCl 0.9% in distilled water. The medications and the vehicle were administered subcutaneously once daily at 10 a.m. for 50 consecutive days. In the 50th day, one hour after the last drug or vehicle injection, a vaginal smears evaluation was performed in order to verify the estrous cycle phase. **INTERVENTION(S):** Soon thereafter the animals were sacrificed and properly separated into 8 subgroups: proestrus (Ctr) e (HPrl), estrus (Ctr) e (HPrl), metaestrus (Ctr) e (HPrl) and diestrus (Ctr) e (HPrl). Following euthanasia, the uterine horns were removed, sectioned and immediately frozen in liquid nitrogen for RNA extraction to detect tissue (HAS I, II and III) by Polymerase Chain Reaction (RT-PCR). Blood was collected for the dosage of prolactin and serum estrogen and progesterone using ELISA-like assay. The results were expressed as mean ± SD and were analyzed by the Student’s t-test. Significance level was set at P<0.05. **RESULTS:** The HAS I, II and III were gene expressed of the metoclopramide-treated group was superior to those of experimental group during the proestrous, estrous and diestrous. Hormonal serum levels were as follows: estradiol (pg/mL), Ctr = 21 ± 0.816 vs. HPrl = 30 ± 0.923 (P<0.001); progesterone (ng/mL), Ctr = 9.67 ± 0.077 vs. HPrl = 7.01 ± 0.031 (P<0.001); and prolactin (ng/mL), Ctr = 4.1 ± 0.082 vs. HPrl = 6.8 ± 0.134 (P<0.001). **CONCLUSION:** Our results suggest that metoclopramide-induced hyperprolactinemia may affect the gene expressed on female mice uterus during all estrous phases. Possibly, this change may help to understand the infertility problems related to high levels of prolactin. Peripherally, the serum levels of progesterone were reduced and those of estradiol were increased.
Keywords: Hyaluronan Synthase, Hyperprolactinemia, Prolactin, Uterus

11.20- L-Glutamine and L-Arginine Protective Effect in Corpus Cavernosus of Rats Submitted to Pelvic Irradiation

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Introduction: Radiotherapy is commonly used in cancer treatment affecting pelvic organs next to target organ. Many alterations can be observed in pelvic radiation treatment, such as: erectile dysfunctions and urinary morbidity. To prevent this problem different radioprotectors drugs have been studied. Objective: To evaluate the protector action of L- arginine and L-glutamine in erectile tissues submitted to pelvic radiation.Material and methods: It was used 35 Wistar rats penis, the rats aged between 3 and 4 months. Animals of control group (C) were maintained under standard laboratory conditions, while animals belonging to the other groups, IA and IG, were submitted to pelvic radiotherapy on the 8th day of the experiment. Animals were irradiated with a single dose of 1,164 cGy applied to the pelvis, been killed 7 (I-7, IA-7 e IG-7) and 15 (I-15, IA-15 e IG15) days after irradiation. The amino acids were administered once a day at the dose of 0.65 g/kg per day, starting 7 days before the irradiation until the last day of experiment. Animals from control and irradiated groups received 5 ml of water similarly administered once a day throughout all the experiment. Histochemical techniques were performed to stereological analysis of smooth muscle, collagen and elastic system fibers of corpus cavernosum. Image J software was used to quantify the corpus cavernosum area with 100 points grid. For qualitative analysis different types of collagen it was used Picro Sirius red under polarized light. Statistical analysis was performed by one way ANOVA and by Bonferroni post-test. The level of significance was set at (P<0.05). Results: I-15 group showed a significant increase in collagen amount (p <0.004). Smooth muscle increased in I-7, IG-7, IA-7, IA-15 and IG-15 groups (p<0.05). Elastic system fibers increased in I-15 and IG-15 (p<0.05) when compared to control and IA-15 groups. Qualitative analysis of collagen, showed modifications in different types of collagen disposition, in I-7 group prevalence of green color propose a bigger disposition of type III collagen, while I-15 group has a bigger incidence of red color, when compared to I-7 group. Similar alterations happened between IG-15 and IA-15.Conclusion: L-arginine seems to protect the corpus cavernosum. L-glutamine prevent collagen alteration, but didn't act in elastic system fibers recovery. Both amino acids seem do not present a protective effect in relation to smooth muscle.

Keywords: Radiation, L-glutamine, L-arginine, Rat
11.21- Maternal Protein Restriction Induces Fibrosis in Rat Ventral Prostate of Male Offspring

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Fetal programming has been associated with impairment of fetal development and cardiovascular disease. Recent studies have associated fetal programming by in utero protein restriction with delay in sexual maturation in male rats. Thus, the aim of this study was to investigate the effects of fetal programming induced by in utero protein restriction on the collagen fibers content in the rat ventral prostate. Male Wistar rats with underwent in utero fetal programming by protein restriction (normal diet=17% protein; protein in restricted diet=6%) was killed at age of 16 week and the ventral prostate was excised, weighted and processed for histochemistry and immuhistochemistry for type I and III collagens. The programmed male offspring presented decrease in both absolute and relative ventral prostate weight compared to matched control. The histochemistry reaction showed that programmed prostates presented increased stromal space with stronger immunostaining for both type I and III collagens, mainly in the interstitial space, than control prostates. The programmed prostates also exhibited a decrease in parenchymal volume fraction compared to age matched control. In conclusion, fetal programming induced by in utero protein restriction lead to a prostatic fibrosis associated with impairment in parenchyma compartment maturation.

Keywords: collagen fibers, fetal programming, prostate, protein restriction

11.22- Effect of Testosterone on the MMP-2 and Timp-2 Expression in the Gerbil Male and Female Prostates

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Matrix Metalloproteinases (MMPs) are enzymes that participate in the process of Extracellular Matrix (ECM) remodeling and are regulated by a set of glycoproteins called Tissue Inhibitors of Metalloproteinases (TIMPs). MMPs and TIMPs are known to play a role in normal physiological processes and pathological states. An imbalance between TIMPs and MMPs rate may participate in tumor progress in several tissues, including the prostate. Morphological evidences point to a similarity between the ventral lobe of the male prostate
and the female prostate of the gerbil Meriones unguiculatus. Thus, this work presents the distribution of MMP-2 and TIMP-2 in both gerbil female prostate and ventral lobe of male prostate, and the effect of Testosterone (T) over these enzymes. 5 intact males and 5 intact females in the proestrus phase were kept as control groups (Con). Experimental groups consisted of 10 males submitted to orchietomy for 7 and 21 days (groups 7C and 21C respectively, n=5) and 10 females injected with Testosterone Cipionate (5mg/kg/48h) during 7 and 21 days (groups 7T and 21T respectively, n=5). Histological sections of prostate tissues were submitted to immunohistochemistry for the detection of MMP-2 and TIMP-2. The male ventral prostate of Con showed marked expression of active MMP-2, mainly in the blood plasma, and its expression seemed to increase with the ablation of T, being more intense in 21C blood vessels and fibroblasts. Conversely, immunostaining for TIMP-2 occurred in the epithelial cells of control males and decreased with castration, being positive mainly in blood plasma in the 21C group. In the intact female prostates, expression of active form of MMP-2 occurred in epithelial cells, secretion, blood plasma and stromal fibroblasts. In 7T group there was a reduction in the activity of these enzymes, showing weak stain in the secretion, fibroblasts and few secretory cells. On contrary, 21T group showed intense expression of MMP-2, especially in basal membrane and prostatic secretion. Epithelial cells of control female prostates were TIMP-2 positive but in the 7T and 21T groups its expression decreased, with positive reaction only in border of apical cells. The results demonstrate that either the lack of androgen in males or the excess of T in females promote an imbalance in MMP/TIMP rate, increasing the MMP-2 expression, which is related to the process of ECM remodeling that occur in the development of pathologies. Proc. FAPESP 2006/06985-5

**Keywords:** Female prostate, Gerbil, MMP, TIMP, Ventral prostate

### 11.23- Doxazosin Decreases MMP-2 and MMP-9 Activity in Rat Ventral Prostate

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Doxazosin (Dox), a quinazoline-derived α1-adrenoceptor antagonists has been found to induce apoptosis in prostate epithelia cells and to suppress the formation of prostate tumors and the metastasis of prostate cancer in the TRAMP model. Recently, new studies have demonstrated that Dox also downregulate expression of AR and PSA in vitro. Here, we investigated the effect of Dox on activity of MMP-2 and MMP-9, enzymes related to the extracellular matrix remodeling and prostate cancer invasiveness. Adult male Wistar rats were treated with daily doses of doxazosin (25mg/kg), dissolved in corn oil and subcutaneous injected. Age matched animals, receiving only the vehicle, were used as control. After 3 and 30 days of treatment, a group of six animals were killed with sodium pentobarbital overdoses. The ventral prostatic (VP) lobe was excised and immediately weighed and processed to histology or snapped frozen in liquid nitrogen and processed to gelatin-zymography. Picrossirius analyzes demonstrated that Dox treatment increased collagen volume fraction in
the prostatic estroma. The activity of both MMP-2 and MMP-9 was reduced after 3 and 30 days of Dox treatment. These results demonstrate that Dox treatment results in fibrosis and changes activity of extracellular matrix-remodeling enzymes.

**Keywords:** collagen, doxazosin, MMPs, prostate

11.24- Stromal Remodelation and Metalloproteinases (MMP2 And MMP9) Activity in Prostatic Chronic Inflammation from Adult Rats Exposed to Di-N-Butyl-Phthalate (DBP) During Gestation and Lactation

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People are constantly exposed to environmental contaminants from industrial processes, through air, food, water or contact with a variety of consumer products. Di-ç-butyl-phthalate (DBP) is widely used as a plasticizer in many products including medical devices, flexible plastics and some cosmetic formulations. In the present study we evaluated the toxic effects on the male adult rat prostate of DBP exposure during fetal and lactational periods, because although many studies have addressed the influence of phthalates on the male reproductive system, only a few have discussed their possible effects on prostate development. Pregnant females were distributed into two experimental groups: Control (C) and Treated (T). The females of the T group received DBP (100 mg/Kg, by gavage) from gestation day 12 to postnatal day 21, while C rats received the vehicle (corn oil). In adulthood (90 days old), the animals were euthanized. The serum and testicular testosterone levels were measured. Ventral prostate was removed and weighed. Distal segment fragments of the ventral prostate were fixed and processed for histochemistry (HandE and Gömöri reticulin). Protein extraction from ventral prostate fragments was performed for Gelatin zymography for MMP-2 and MMP-9 (MMP, metalloproteinase). Stereological and histopathological analyses were also performed. Serum and testicular testosterone levels and prostate weight were comparable between groups. In the T group the relative proportions (%) of epithelial (C=32.86; T=42.04*) and stromal (C=21.61; T=27.88*) compartments were increased, while the luminal compartment was decreased (C=45.54;T=30.08*), *p<0.05. Increase of collagen fibers among the acini, which enlarged the space among the glandular portions, was found in the T group. This aspect corroborated the stereological analyses that showed elevation in the relative stromal volume in these animals. Furthermore, in T animals, mononuclear inflammatory infiltrated areas were observed and, a fibrillar rearrangement occurred with formation of collagen bunches
dispersed among the inflammatory cells while in some areas the collagen and reticular fibers appeared to support the infiltrate under the epithelium. The gellanotytic activity of MMP9 was more intense in the T than the C group, while the MMP2 activity was similar between the groups. Furthermore, the elevated MMP9 activity observed was involved in the fibrillar degradation and rearrangement caused by inflammatory processes.

**Keywords:** prostate, metalloproteinases, collagen fibers, reticular fibers, inflammation

11.25- Rotational Stress Delays Cutaneous Wound Healing

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Objective: To evaluate macro and microscopically the effects of the rotational stress on cutaneous wound healing in mice. Method and Results: Male Swiss mice aged 8-12 weeks (25-30 g) were housed five per plastic cage and chronically submitted to stress environment. Rotational stress was applied to the animals by spinning the cages at 45 rpm for 15 minutes every hour three days before wounding and was continued daily until euthanasia. Control animals (n=5) were not submitted to rotational stress. A full-thickness excisional lesion (1 cm²) was created. Wound area was measured soon after wounding and 3 and 7 days after and the animals were killed 7 days after wounding. Lesions and adjacent skin were formalin-fixed and paraffin-embedded. Sections were stained with hematoxylin-eosin and toluidine blue, and immunostained for F4/80, alpha-smooth muscle actin and proliferating cell nuclear antigen (PCNA). Furthermore, the myeloperoxidase (MPO) activity was measured on wound area. Data are reported as mean ± standard error of mean. The wound area was smaller in the control group than in the stress group 3 and 7 days after wounding (p<0.05 and p<0.01, respectively). Seven days after wounding, the number of PCNA-positive connective tissue cells was greater in the control group than in the stress group (p<0.001). Furthermore, the MPO activity and the number of F4/80-positive macrophages and mast cells were greater in the control group than in the stress group 7 days after wounding (p<0.0001, p<0.05 and p<0.0001, respectively). Nevertheless, the number of blood vessels and myofibroblast density were smaller in the control group than in the stress group 7 days after wounding. Conclusions: Rotational stress impairs mice cutaneous wound healing by reducing the wound closure, cellular proliferation and inflammatory cells migration and increasing the angiogenesis and myofibroblastic differentiation. Financial support: Centro de Produção da Universidade do Estado do Rio de Janeiro (CEPUERJ) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

**Keywords:** cutaneous wound healing, psychological stress, collagen fibers, inflammatory cells, myofibroblasts
11.26- Intrauterine Androgen Exposure Promotes Epithelial and Stromal Disorders in the Adult Mongolian Gerbil Male and Female Prostate

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Some researches have demonstrated that exposure to endocrine-disrupting chemicals, like androgens, during embryonic phase is associated with the appearance of benign and malignant prostatic lesions on adult and aging life. Moreover, alterations on the normal development of female phenotype like the malformation of external genitalia, appearance of seminal vesicle and early death may happen. In this manner, the aim of this work was to study the effect of the intra-uterine exposure to testosterone on prostatic embryogenesis of gerbil (Meriones unguiculatus) females and males. Thus, the prostatic glands of the adult animals including normal and those treated in gestation (day 18º) with testosterone cypionate (1mg) were processed for light microscopy. The total blood of them was collected for serologic analyses of testosterone, estrogen and progesterone levels. Histological sections were stained with hematoxylin-eosin and Gömöri reticulin for general studies. Immunohistochemistry was performed for α-actin. The results showed that female exposed to androgens on gestation have high hormonal levels for all hormones, while in the treated males only progesterone presented elevated values when comparing with control animals. The morphological analyses showed that the both male and female controls have prostatic glands of typical standard, with acini lined in simple cylindrical epithelial, a lumen rich in secretion and stroma composed mainly by fibroblasts and smooth muscle cells. However, the animals exposed to androgens during embryonic period showed many alterations in prostatic gland. In both male and female was possible identify some inflammation foci in association with prostatic intraepithelial neoplasia (PIN). Throughout immunohistochemistry for α-actin it was possible to identify some areas of smooth muscle layer interruption caused by epithelial cells proliferation into glandular stroma. These results showed severe alterations in the prostatic glands of the animals exposed to testosterone during prostatic embryogenesis, showing the potential that endocrine-disrupting agents may have on imprint of some reproductive tract organs. Taking into account it is important to know the action mechanisms of these disruptors and understand the factors that determinate the different process of prostatic morphogenesis in males and females, as well as those that can predispose this gland to develop malignant lesions during adult and aging life.

Keywords: Androgen exposure, Intrauterine, Mongolian Gerbil, Prostate, Prostatic embryogenesis
11.27- Morphological Analysis of Wound Healing in the Skin of Wistar Rats Treated with Marigold

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Calendula officinalis (marigold) has been widely used in homeopathic medicine for the treatment of many diseases. It has been reported to possess many pharmacological activities, which include antioxidant, anti-inflammatory, antibacterial, antifungal, and antiviral. It also possesses cytotoxic as well as tumor reducing potential. The aim of this study was to evaluate the scarring capability of marigold on experimental skin wounds in Wistar rats. Each animal was subjected to a surgical incision measuring 6cm in length, located to right side to the spinal column and sutured in U-shape. Marigold extract (10 mg) was placed on the incisions for 7, 14 and 21 days, and no treatment was provided to the left side (control). Skin biopsy was performed on 7, 14 and 21 days after wounding, for morphological evaluation of the cicatricial process. Morphological analysis was performed with an optical microscope (Olympus, EUA) adapted to a computerized picture analysis system to capture image (Image Proplus 6). Results showed that marigold allowed the highest growth in cells directly involved in the cicatricial process, as fibroblasts and inflammatory cells, when compared to control animals. Control animals showed complete re-epithelization around 21 days after surgical incision while treated animals had reduced this period of time to 14 days. Our finds suggest that marigold possesses a potent wound healing activity. Biological activity could be related to pharmacological activities of this product.

Keywords: calendula officinalis, skin, wound healing

11.28- Immunolocalization of SLRPS in the Pregnant Endometrium of Decorin-Deficient Mice

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Changes in collagen fibrillogenesis have been previously detected in the pregnant mouse endometrium, where very thick collagen fibrils are present exclusively in the decidualized stroma. Fibrillogenesis is a complex process that involves several steps, including association with other extracellular molecules, such as proteoglycans. The small leucine-rich proteoglycans decorin, biglycan and lumican are known to play an important role in this process. The present study is an investigation into the distribution of biglycan and lumican in the pregnant endometrium of wild-type (Dcn+/+) and decorin-deficient mice (Dcn-/-). Methods: Immunoperoxidase staining was performed on days 3 and 7 of pregnancy in the
decorin-null (Dcn−/−) and wild-type (Dcn+/+) mice. The samples were fixed in 4% paraformaldehyde, incubated with rabbit polyclonal antibodies raised against murine biglycan (LF-106) (Fisher et al. 1995) and lumican (Chakravarti 2000). Peroxidase was visualized using 3, 3’-diaminobenzidine (Sigma) in PBS with 0.03% H2O2. The sections were counterstained with Mayer’s hematoxylin. Results: Our results demonstrate that on day 3 of pregnancy, biglycan was expressed exclusively around bundles of smooth muscle cells of the myometrial external layer in Dcn+/+ animals. In contrast, in Dcn−/− animals biglycan was detected in the uterine stroma and glandular epithelium, and was maintained in the external layer of the myometrium. Lumican immunostaining, however, was intense in the decidualized and non decidualized stroma from Dcn−/− animals on day 7 of pregnancy being absent in the Dcn+/+ animals, lumican in either decidualized or nondecidualized stroma. Together with previous results from our group, these results suggest that decorin, biglycan and lumican play an interactive role on collagen fibrillogenesis in the mouse endometrium, which is modulated according to the stage of pregnancy.

Key words: decidua, collagen, decorin-deficient

11.29- Evaluation of Markers of Oxidative Stress in Ischemic and Non-Ischemic Skin Lesions

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A large proportion of the population is affected by problems of skin healing, but the mechanisms that cause these disorders in wound healing are not well known. Studies suggest oxidative stress as one of the possible causes of these problems. The objective of this project is to evaluate the redox balance in Wistar rats comparing ischemic and non-ischemic wounds. Wistar rats were divided into two groups: control (n = 7) and ischemia (n = 7). Under anesthesia, in d0, an excisional non-ischemic wound (1 cm2) was performed in the control group. In ischemic group, two bilateral incisions measuring 7 cm separated by a distance of 3 cm were performed, the skin was separated from the subjacent tissue and the incisions were sutured; then an excisional wound (1 cm2) was performed between the incisions. Wound contraction was evaluated macroscopically. Euthanasia was performed seven days after wounding. The wound and normal skin were removed, homogenized and biochemical analyses to antioxidant enzyme activities and biomarkers of oxidative damage were evaluated. The activity of glutathione peroxidase was lower in ischemic wound when compared to non-ischemic wound (p <0.05). The catalase and superoxide dismutase activities were lower in the ischemic wound compared to normal skin (p <0.001). The glutathione/oxidized glutathione ratio, a marker of oxidative stress was reduced in the ischemic wound as compared to non-ischemic wound (p <0.01). The myeloperoxidase...
activity was increased in ischemic wound when compared to non ischemic wound (p<0.001). Nitrite level was higher in ischemic wound when compared to non-ischemic wound (p<0.01). Our result indicates a redox imbalance suggesting oxidative stress in non-ischemic wounds that may be delay the wound healing.

**Keywords:** skin, wound ischemic, wound non-ischemic, wound healing, oxidative stress

### 11.30- Effects of Cyclooxigenase Blockade on Cutaneous Wound Healing Depends on Gender

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A substantial body of work describes the contrasting influences of androgenic and estrogenic sex steroids on the healing of acute skin wounds. These endogenous sex steroid hormones profoundly influence the response to cutaneous injury. The aim of this study was to investigate if cyclooxigenase blockade affects cutaneous wound healing in the same way in both genders. Fourty adult male and female (20 males and 20 females) BALB/c mice (3 months; 20 – 25 g) were used and divided in two groups in each gender: one control and one treated. Treated group received a daily dose of aspirin 100 mg/kg/day dissolved in saline, i.p and the control group received the same volume of saline, i.p.. The treatment started two days before wounding and extended for fourteen days after wounding until euthanasia, when wounds with adjacent skin were removed. The wounds consisted in a full-thickness excisional wound (1 cm²) in the dorsal surface. Sections obtained were stained with hematoxylin-eosin and Sirius red. Imunohistochemistry against α-smooth muscle actin was also performed. In both male and female treated groups the wound contraction and the neo-epidermis formation were delayed when compared to the control group, but the female group presented a more expressive delay. The inflammatory infiltrate was abundant in both male and females treated groups, being also more pronounced in the female group. Concerning collagen density, both male and females treated groups presented smaller density of these fibers when compared to the control group. In female group collagen density was also smaller than the male group as well as myofibroblastic differentiation. In conclusion, it was showed that cyclooxygenase pathway inhibition, by high dose of aspirin, affected wound healing process delaying wound contraction, neoepidermis formation and granulation tissue formation in both male and female groups, the delay was more expressive in females.

**Keywords:** Aspirin, Cyclooxygenase, Gender, Skin, Wound healing
11.31- The Effects of Diabetes on the Structure and Cell Proliferation of the Myometrium in the Early Pregnancy of Mice

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The myometrium is a highly dynamic uterine compartment, which is deeply remodeled along the full course of pregnancy. Four sequential myometrial phenotypic phases were identified during rat pregnancy. The first phase is characterized by hyperplasia of the smooth muscle cells (SMCs). Afterwards, there is a transition phase, followed by a phase of hypertrophy of SMCs and extracellular matrix synthesis. Finally, at the end of pregnancy, myometrial SMCs acquire a contractile phenotype to perform labor. Type 1 diabetes mellitus is associated with elevated rates of preterm birth. However, the mechanisms underlying its causes are hampered by our limited knowledge of the impact of this pathology to the myometrium. The goal of this study was to investigate the effects of diabetes on the myometrium during the first adaptation phase of pregnancy. Diabetes was induced in female Swiss mice by intravenous injection of alloxan (40mg/Kg). Diabetic animals (>400 mg/dl) were mated between 50-70 and 100-120 days after diabetes induction. Pregnant animals were killed at 168 hours after the detection of the vaginal plug. The myometrium was analyzed at light and electron microscopy and evaluated by histomorphometry. Cell proliferation was determined by the percentage of PCNA-positive nuclei detected by immunohistochemistry. Diabetic mice showed high levels of hyperglycemia, hypoinsulinemia, glycosuria, increased food and water consumption and decreased body weight. No considerable alterations were found 50-70 days after diabetes induction. On the other hand, important alterations in the structural organization and cell proliferation were observed among the diabetic mice 100-120 days after diabetes induction. These alterations were divided in to subgroups, D1 and D2. Compared to the control group, the distance between muscle layers in D1 was augmented and showed signs of edema. Contrarily, in subgroup D2 the distance between layers showed a dramatic reduction. In both D1 and D2, the thickness of the muscle layers was reduced. Supporting the histomorphometric data, cell proliferation was significantly decreased in the internal and external layers of both D1 and D2. Thus, diabetes-induced myometrial alterations are duration-dependent. Variation among the animals may represent different stages of diabetic complications. We suggest that these alterations may be correlated with disorders occurring in pregnancy, such as preterm delivery. Financial Support: FAPESP

Keywords: myometrium, diabetes, cell proliferation
11.32- Ovarian Hormone-Regulated Distribution of Decorin and Biglycan in Mouse Uterine Tissues

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The extracellular matrix (ECM) is a complex structure of macromolecules capable of self-assembly, which exert essential biological functions in the tissues. The endometrial ECM plays important roles in the processes of decidualization, embryo implantation and trophoblast cell invasion. Our laboratory has previously demonstrated the differential distribution of four members of the small leucine-rich proteoglycan (SLRP) family during the estrous cycle and early pregnancy. Decorin and biglycan are class I SLRP and share structural homologies. The aim of this work is to analyze the influence of the ovarian sex hormones estrogen (E2) and progesterone (P4) on the distribution of decorin and biglycan in mouse uterine tissues. For that purpose, mice were ovariectomized and after 20 days they were either sacrificed or treated with E2, medroxiprogesterone acetate (MPA) or E2+MPA. After the surgery or last injection, the uteri were removed, fixed in Methacarn and embedded in Paraplast. Immunoperoxidase was performed using anti-decorin and anti-biglycan primary antibodies. After incubation with the secondary antibody and streptavidin-peroxidase, the reaction was visualized with DAB. After ovariectomy, decorin was found only in the deep stroma and external muscle layer, and biglycan was absent from all uterine tissues; in the E2 group strong immunoreaction for decorin was found in the whole stroma and myometrium, whereas biglycan was observed mainly in the region of basement membranes; in the MPA group decorin was present in the deep stroma and myometrium, and biglycan was present in all uterine tissues; finally, in the E2+MPA group immunoreaction for both proteoglycans was observed in the stroma and in the myometrium. In summary, these results show that the ovarian hormones modulate the distribution of decorin and biglycan in the mouse uterus and may be involved in the kinetics of synthesis, deposition and degradation of ECM molecules in reproductive tissues. Financial Support: FAPESP

Keywords: uterus, proteoglycans, estrogen, progesterone

11.33- Bioengineering of Blood Vessels Using Porcine Xenogenic Implants

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Large-diameter vascular grafts have been successfully developed from polymers such as Dacron or e-PTFE. However, it has been difficult to develop small-caliber grafts (inner diameter < 6 mm) because of biological reactions at the blood-material and tissue-material interface, such as acute thrombogenicity, intimal hyperplasia and formation of aneurisms. Autologous vein grafts require a removal of a vein from another part of the body, when
available. Synthetic grafts pose long-term health risks and are not successful for small diameter graft applications. Here we developed a mechanically stable vascular graft using intact iliac arteries of pig decellularized by using sodium dodecyl sulfate (SDS). Components of extracellular matrix involved with the mechanical support and elastic properties (elastin, collagen, chondroitin sulfate, laminin and fibronectin) were evaluated, before and after treatment, by biochemical and histochemical methods. Iliac arteries of pig rendered acellular with SDS and has well-preserved extracellular matrix and basement membrane structure. Our results indicate that we were able to produce a vascular graft with optimal cell seeding characteristics. These properties suggest proof of concept for its use as a scaffold for further vascular tissue engineering.

**Keywords:** Bioengenharia, Acelularização, SDS, PróteseVascular

### 11.34- Chronic Stress Affects Collagen and Muscle Fibers of Penile Corpus Cavernosum of Rats

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Chronic stressful stimuli can challenge tissue homeostasis, due to adverse effects of glucocorticoids. Early exposure to glucocorticoids can accelerate or delay the functional organic maturation, depending on the dose and exposure time. Intense stress has been associated with reduced serum testosterone levels and reduced genital blood flow, but there is no knowledge about its effect on penile tissue. This work aiming to analyze quantitatively the collagen and muscle fibers in penile corpus cavernosum of chronically stressed rats. Eight Wistar rats were assigned into the stress group (SG) and were submitted to two hours of tube restraint daily, from the 4th to the 9th week of life. Other 7 rats were assigned as control group (CG) and maintained in standard conditions. After this period, animals were sacrificed by anesthetic overdose, blood was collected for testosterone dosage by radioimmunoassay, and penis was grossly dissected. The mid-shaft of the penis was separated and fixed in 10% formaldehyde, being then processed for paraffin embedding and sliced in 5µm. Twenty five fields of corpus cavernosum stained with Masson’s trichromic were captured under 200x of magnification from each. A grid of 100 points was overlayed in the figures with ImageJ software for point counting. Points hitting collagen fibers or muscle fibers were separately counted. Student t test was applied to mean comparisons, considering P<0.05 significant. Testosterone serum concentration decreased from 0.19(±0.04) ng/ml in the CG to 0.08(±0.01) ng/ml in the SG (P=0.02), showing that the stress protocol was effective in reducing testosterone as previously demonstrated. Muscle fibers also decreased from 14.07(±1.4)% (CG) to 8.98(±1.3)% (SG) (P=0.02) and Collagen fibers increased from 53.66(±3.7)% (CG) to 64.47(±0.9)% (SG) (P=0.01). Stress stimuli induced structural changes in the corpus cavernosum of rats suggestive of penile fibrosis, which may play a role in erection dysfunction.

**Keywords:** corpo cavernoso, estresse, rato wistar
11.35- Extract of Jateí-Kaá Affects Extracellular Matrix Components of the Rat Placenta

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In the state of Mato Grosso do Sul, Achyrocline alata, known as jatei-kaá, is a plant species popularly used in the treatment of uterine and prostatic infections, hernia, stomachache and bronchitis. However, there is no data available about its influence on pregnancy. The placenta is formed by different tissues, such as the labyrinth, which is formed by vascularized mesenchyme covered by trophoblast layers that interact directly with the maternal blood lacunae. The fetal vessels’ basal lamina contributes to the exchange of nutrients and metabolites between the mother and her fetus. Moreover, the placenta has been acknowledged as an important regulator of cell behavior, cell migration and cell-cell interactions. The objective of this work was to analyze the expression of the basement membrane molecules collagen IV and laminin in the placenta of rats treated with jateí-kaá extract and whether this component affects the morphology of normal placentas in vivo. For this purpose, twenty pregnant rats were divided into groups A and B, and received the hydroethanol extract of jateí-kaá, which was diluted in the water for consumption. Group A received the extract from day 6 to 15 of pregnancy, whereas Group B received it from day 1 to 20 of pregnancy. A control group ingested only filtrated water during the whole course of gestation. Two placentas were obtained from each animal in all groups, fixed in Methacarn and embedded in paraffin. Serial sections were submitted to immunoperoxidase technique for collagen IV and laminin. We observed an increase in the deposition of collagen IV and laminin in the labyrinth of placentas from group B, as well as a reduction in the maternal vascular spaces, when compared with the control group. However, the immunoreaction for both molecules was similar in the placentas from Group A and the control group. Thus, we suggest that the jatei-kaá extract presents time-dependent toxicity for the rat placenta, as the remodeling of extracellular matrix molecules was observed only in the group submitted to this component for a longer period during pregnancy. Further studies will be necessary to evaluate the consequences of these placental alterations for the fetuses.

Keywords: jatei-kaá, placenta, collagen, remodeling
Congenital malformations may occur when noxious substances cross the placental barrier. In the state of Mato Grosso do Sul, Achyrocline alata, known as jatei-kaá, is a plant species popularly used in the treatment of uterine and prostatic infections, hernia, stomachache and bronchitis. However, there is no data available about its effects on pregnancy. The process of normal placentation is clearly influenced by the composition and organization of the endometrial extracellular matrix (ECM). Moreover, collagens are ECM macromolecules that exert important structural and functional activities in the various tissues. The aim of the present study was to analyze the morphological characteristics and collagen distribution in the placenta of rats treated with jatei-kaá extract during different periods of pregnancy. For this purpose, twenty pregnant rats were divided into groups A and B, and received the hydro-ethanolic extract of jatei-kaá, which was diluted in the water for consumption. Group A received the extract from day 6 to 15 of pregnancy, whereas Group B received it from day 1 to 20 of pregnancy. A control group ingested only filtrated water during the whole course of gestation. Two placentas were obtained from each animal in all groups, fixed in Methacarn and embedded in paraffin. Serial sections were submitted to Haematoxilin-eosin and picrosirius staining techniques, as well as immunofluorescence for collagen I. In all placentas from group B and some from group A, there was a remarkable inflammatory process in the peripheral tissue, especially around the basal decidua and the Reichert’s membrane. The picrosirius-stained sections showed that in groups A and B, there was an increase in the general collagen content in the chorionic plate and the labyrinth. To confirm that, immunofluorescence for collagen I showed a strong immunoreaction in these same placental regions, being more intense in Group B. Thus, we suggest that the jatei-kaá extract presents time-dependent toxicity for the rat placenta, as the remodeling of collagens of the ECM was more conspicuous in the group submitted to this component for a longer period during pregnancy.

**Keywords:** Placenta, jatei-kaá, collagen type I, Inflammation, extracellular matrix
11.37- The Protective Effect of Sebastiana Híspidum Aqueous Extract and Low Level Laser Irradiation (LLLI) Against Local Effects of Bothrops Moojeni Snake Venom

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The leaves extract of Sebastiana hispidum (Euphorbiaceae) have been used in folk medicine. Low level laser irradiation (LLLI) has been shown to accelerate healing in a number of affected tissues. Here, we compared the phytotherapy with LLLI to neutralize the local muscle effects caused by i.m. gastrocnemius injection of Bothrops moojeni crude venom (40 μg/mL) in Wistar rats. Three groups of animals were used (n = 4 each): Control, saline-injected (SS); Venom-injected (i.p. 40 μg/mL) (V); Venom-injected + aqueous extract of S. hispidum (VSh); Venom-injected + 40 seg irradiation with Indium-Gallium-Aluminium Phosphide (InGaAlP), 660nm, 4 joules/cm² (VL) daily. At 3, 24 and 48h post-venom, blood samples were collected for evaluation of creatine kinase (CK) serum levels, used as marker of muscle fiber damage. After anaesthesia, the animals were killed, and gastrocnemius processed for evaluation of muscle fiber damage and measure of connective tissue deposition. Evaluation of the body weight gain indicated significant difference after 48 h post-envenoming between VL and VSh (353 ± 5.8 and 226.7 ± 5.8). The mean ± SD showed that plasma CK (U/L) levels were significantly different higher in V group compared to control. In all time intervals there was significant decrease in CK release in groups treated with LLLI or VSh compared to Venom group. The morphological damage was attenuated both in animals treated with the plant extract and lasertherapy, with laser irradiation giving better results than the S. hispidum (p<0.05). Bothropic venoms produce local muscle damage with fast evolution. Serum therapy is efficacious in treating systemic effects but is ineffeective to treat local damage. Alternative non-invasive therapeutic measures such as the described here can be useful in bothropic accidents. Apoio: Anhanguera/UNIDERP, CPP, CNPq, UNICAMP

Keywords: snake venom, Phytotherapy, Laser therapy, CK, histopatology
11.38- Metal-Substituted Cytochrome C And Microperoxidases Microinjection Promote the Lost of Cellular Adhesion and Induce Apoptosis in Vascular Smooth Muscle Cells

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Adhesion-mediated apoptosis resistance is an emerging concept that may explain the observed differences in survival cells. Tumor cells interacting either with a specific extracellular matrix protein substrate or with each other or with non-transformed cells, such as fibroblasts, exhibit increased resistance towards a wide variety of therapeutic approaches. Cytochrome c is a mitochondrial respiratory hemeprotein that contributes for apoptosis triggering when it is released from mitochondria as well as it is exogenously deliver to cells via microinjection or carriers. In the present study, apoptosis was induced by delivery to cells of exogenous metal-substituted cytochrome c and microperoxidases. The deliver of the agents of cell death was done by cellular microinjection in rabbit aortic smooth muscle cells. It was observed that only zinc-substituted cytochrome c that retains the native cytochrome c structural features was efficient to activate caspase-3 and consequently apoptosis with lost of cellular adhesion. The free base, Fe3+, Mn3+ microperoxidases-11 and free base- cytochrome c led to occurrence of typical events of the apoptosis program, such as: nuclear fragmentation, membrane blebbing and extracellular exposure of phosphatidylserine, independently of caspase-3 activation. We also observed that cellular treatment with zinc-substituted cytochrome c as well as with native cytochrome c lead to lose of cellular adhesion triggering cell death. These results show that the lost of cellular adhesion and consequently induction of cell death in vascular smooth muscle cells is independently of caspase-3 activation. Supported by FAPESP, CNPq and FAEP-UMC.

Keywords: cytochrome C, microperoxidases, cellular adhesion, apoptosis, smooth muscle cells