

GRACIELI PRADO ELIAS

EFEITO DA HIPERTENSÃO E DO ATENOLOL
SOBRE A ATIVIDADE SALIVAR E A
MICRODUREZA DENTAL: ESTUDO
EXPERIMENTAL EM FILHOTES DE RATAS
ESPONTANEAMENTE HIPERTENSAS (SHR)

Tese apresentada à Faculdade de Odontologia da
Universidade Estadual Paulista "Júlio de Mesquita
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obtenção do título de DOUTORA EM
ODONTOPEDIATRIA

Orientadora: Prof^a. Dr^a. Cristina Antoniali Silva

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Dados Curriculares

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Dedicatória

Dedicatória

Dedico este trabalho

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*Mas o que você dedicou a sua mente
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*"As pessoas não se importam realmente com o quanto você sabe
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Agradecimentos Especiais

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(Vinícius de Moraes)

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MINHA SINCERA GRATIDÃO...

Επίγραφε

Epígrafe

“Não pode existir conhecimento sem emoção.

*Podemos estar cientes da verdade,
mas até que tenhamos sentido sua força,
ela não é nossa.*

*A cognição do cérebro deve ser acrescentada
a experiência da alma.”*

(Arnold Bennett)

Sumário

Sumário

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Resumo

Resumo

ELIAS, G. P. **Efeito da hipertensão e do atenolol sobre a atividade salivar e a microdureza dental: estudo experimental em filhotes de ratas espontaneamente hipertensas (SHR)**. Araçatuba, 2006. 128 f. Tese (Doutorado em Odontopediatria) – Faculdade de Odontologia de Araçatuba, UNESP - Universidade Estadual Paulista.

O objetivo deste trabalho foi avaliar a atividade das glândulas salivares, a mineralização dental e a participação da metaloproteinase de matriz (MMP-9) nesta mineralização em filhotes de ratas espontaneamente hipertensas (SHR) tratadas ou não com atenolol. Ratas SHR e normotensas Wistar foram tratadas com atenolol (100mg/Kg/dia, via oral) durante os períodos de prenhez e lactação. Os grupos controle receberam o mesmo volume de água sem atenolol. O fluxo salivar, induzido por nitrato de pilocarpina, a concentração de proteínas (método de Lowry), a atividade da amilase (método cinético a 405 nm), o peso das glândulas salivares (parótidas, submandibulares e sublinguais), a microdureza do esmalte e da dentina de incisivos e molares e a expressão da MMP-9 (imunoperoxidase) no tecido dental foram comparados entre filhotes de ratas SHR e Wistar tratadas ou não com atenolol. Os resultados obtidos foram submetidos ao teste estatístico mais adequado, paramétrico (ANOVA ou test t de Student's) ou não paramétrico (Kruskal-Wallis), sendo consideradas significativas as diferenças quando $p < 0,05$. Filhotes SHR apresentaram menor fluxo salivar e concentração de proteínas do que filhotes Wistar, mas a atividade da amilase não foi diferente entre os grupos. O peso das glândulas salivares foi semelhante entre filhotes SHR e Wistar.

Resumo

A microdureza do esmalte e da dentina foi menor em filhotes SHR do que em Wistar, tanto em incisivos quanto em molares. Não houve diferença na expressão da MMP-9 na pré-dentina e em odontoblastos entre os grupos, porém maior marcação para MMP-9 foi observada em ameloblastos e na superfície externa de formação do esmalte (SEFE) dos incisivos de filhotes SHR. Após o tratamento das ratas com atenolol, os filhotes SHR e Wistar apresentaram uma redução no fluxo e na concentração de proteínas salivares, sem nenhuma alteração na atividade da amilase salivar. O tratamento reduziu o peso das glândulas submandibulares dos filhotes de ambos os grupos, sem interferir no peso das demais glândulas. Os dentes incisivos de filhotes SHR e Wistar de mães tratadas apresentaram microdureza de esmalte e dentina aumentada em comparação aos filhotes controles. Nos molares, o atenolol promoveu aumento na microdureza do esmalte de filhotes Wistar. O tratamento com atenolol não alterou a expressão da MMP-9 nas estruturas avaliadas em dentes de filhotes SHR, mas diferenças nas marcações desta proteína na SEFE e em odontoblastos foram detectadas em filhotes Wistar. A partir destes resultados conclui-se que: 1- A hipertensão materna, pelo menos em parte, está relacionada à redução da atividade salivar e da mineralização dental dos filhotes, 2- O tratamento das ratas com atenolol prejudica a atividade salivar, porém aumenta a mineralização dental dos filhotes, 3- A maior expressão da MMP-9 em ameloblastos e na SEFE dos dentes incisivos está associada à menor microdureza do esmalte destes dentes em filhotes SHR.

Palavras-chave: atenolol, saliva, dureza, SHR, MMP-9

Abstract

Abstract

ELIAS, G. P. Effects of hypertension and atenolol on salivary activity and dental mineralization: experimental study in pups of female spontaneously hypertensive rats (SHR). Araçatuba, 2006. 128 f. Tese (Doutorado em Odontopediatria) – Faculdade de Odontologia de Araçatuba, UNESP - Universidade Estadual Paulista.

The objective of the present study was analyzed the salivary activity, the dental mineralization and the role of matrix metalloproteinase-9 (MMP-9) on this mineralization, in pups (30 days) of spontaneously hypertensive rats (SHR) treated, or not treated, with atenolol. Female SHR and normotensive Wistar rats were treated during pregnancy and lactation periods with Atenolol 100mg/Kg/day by oral administration. For the control group, the animals received the same water volume without the drug. The salivary flow rate (stimulated by pilocarpine injection), the protein concentration (Lowry method), salivary amylase activity (kinetic method at 405 nm), the weight of salivary glands (parotid, submandibular and sublingual), the enamel and dentin microhardness of incisors and molars teeth and the matrix metalloproteinase-9 (MMP-9, gelatinase B) localization (imunoperoxidase) in dental tissue were compared between SHR and Wistar pups of female rats treated or not with atenolol. The results were analyzed by parametric (ANOVA or Student's tests) or non-parametric (Kruskal-Wallis) tests ($p < 0,05$). The salivary flow rate and salivary protein concentration were reduced in SHR pups. There was no alteration in amylase activity between groups. The salivary glands' weight was not different between SHR and Wistar pups either. Decreased enamel and dentin microhardness were observed in incisors and molar teeth of SHR pups. No alterations in MMP-9

Abstract

positive staining were observed in predentin and odontoblasts of both groups, however the density of stained ameloblasts cells and external enamel surface were higher in incisors teeth of SHR pups. Atenolol-treated SHR and Wistar rats' pups showed decrease in submandibular gland weight, in saliva's flow rate and protein concentration, but no alteration in amylase activity. Atenolol increased enamel and dentin microhardness of incisors teeth of SHR and Wistar pups. The higher efficacy of atenolol on dental microhardness was observed in SHR pups. In molars, atenolol increased the enamel microhardness only in Wistar pups. Atenolol treatment did not alter the MMP-9 immunoreactivity in SHR' teeth, however after atenolol' treatment, the density of stained external enamel surface and odontoblasts were typically altered in Wistar pups' teeth. In conclusion: 1- Maternal hypertension could be associated to the reduced salivary activity and dental mineralization, 2- Atenolol' treatment impaired the salivary activity, however it increased the dental microhardness of SHR and Wistar pups, 3- The higher MMP-9 immunoreactivity in ameloblasts and in external enamel surface of SHR could be associated to the reduced enamel microhardness observed in incisors teeth of SHR pups.

Key words: atenolol, saliva, hardness, SHR, gelatinase B

Introdução **G**eral

Introdução Geral

A hipertensão arterial é um distúrbio cardiovascular que afeta a população em geral, inclusive as gestantes. Diversas patologias têm sido diagnosticadas em filhos de mães hipertensas, entretanto os estudos que avaliaram as possíveis alterações na sua saúde oral não são conclusivos.

Em humanos, a hipertensão tem sido considerada uma condição sistêmica que altera a formação dental, após a observação de que o diâmetro da coroa de dentes decíduos e permanentes está reduzido em filhos de mães hipertensas (Garn et al., 1979). Algumas complicações durante a gravidez (Shulman, 2005) tais como diabetes, infecções e deficiências nutricionais também foram associadas com problemas na formação dental de crianças, uma vez que estas apresentaram hipoplasia de esmalte, um problema que pode tornar os dentes mais susceptíveis ao desenvolvimento de lesões cáries (Aoba, 2004). Investigações clínicas e experimentais provêm evidências para o aumento dos níveis de cárie associado à hipoplasia de esmalte e hipofunção de glândulas salivares em crianças e ratos com deficiências nutricionais pré e pós-natal (Psoter et al., 2005). Sabe-se que alterações na quantidade e qualidade da saliva secretada podem prejudicar a neutralização de ácidos, através da capacidade tampão (Bardow et al., 2001) e interferir na colonização e aderência das bactérias cariogênicas ao esmalte dental (Fejerskov, Kidd, 2005), o que contribui para o início do aparecimento da lesão de cárie.

Introdução Geral

Embora estas evidências indiretas sugiram uma associação entre a cárie na dentição decídua e eventos adversos durante a gestação como a hipertensão, esta hipótese não pode ser comprovada por dados demonstrados em recentes estudos epidemiológicos (Shulman, 2005), o que reafirma a necessidade de mais pesquisas sobre o assunto.

Também o uso de alguns anti-hipertensivos tem sido associado ao aparecimento de cárie. Um aumento na susceptibilidade à cárie em ratos durante o tratamento crônico com propranolol já foi demonstrado (Watson et al., 1990). No entanto, estudos desta natureza não foram desenvolvidos com o atenolol, um medicamento de primeira linha no tratamento da hipertensão durante a gravidez (Qasqas et al., 2004). O atenolol é um antagonista de receptores β_1 -adrenérgicos (beta-bloqueador) que atravessa a barreira placentária e é excretado no leite materno (Tabacova et al., 2003) chegando com facilidade até o feto de mães hipertensas tratadas e recém-nascidos amamentados. Embora, vários estudos em humanos e animais (Tabacova et al., 2003) tenham avaliado os efeitos tóxicos do atenolol no período pré-natal (alterações placentárias, retardo do crescimento intra-uterino, diminuição do peso fetal) e pós-natal (diminuição do ganho de peso), pouca atenção foi direcionada aos efeitos do atenolol sobre a atividade salivar e os tecidos dentais mineralizados, quando administrado durante a organogênese e no período pós-natal.

Introdução **G**eral

Em estudos humanos e animais os dados que avaliam o efeito do atenolol (Nederfors et al., 1994) e outros beta-bloqueadores sobre o fluxo e a composição salivar (Streckfus et al., 1991; Schneyer et al., 1985) são controversos, sendo que nenhuma pesquisa foi desenvolvida em filhotes ou filhos de mães hipertensas. A relação dos beta-bloqueadores com o tecido dental ainda não foi avaliada, sendo que estudos em humanos e animais apenas associam seu uso com o aumento da densidade de massa óssea (Pasco et al., 2004; Schlienger et al., 2004; Bonnet et al., 2006; Takeda, 2005).

Uma vez que os parâmetros bucais, saliva e dente, estão intimamente relacionados ao processo de cárie e poucos estudos foram direcionados à avaliação dos efeitos da hipertensão materna e do uso do atenolol durante a gravidez e lactação, na formação dental e na atividade salivar dos descendentes, nosso estudo foi conduzido utilizando para isso um modelo animal que mais se assemelha à hipertensão essencial humana, submetido a um tratamento comumente observado durante a gravidez humana e no pós-parto.

Capítulo 1

Dental mineralization and salivary activity
are reduced in offspring of spontaneously
hypertensive rats (SHR)

Dental mineralization and salivary activity are reduced in offspring of spontaneously hypertensive rats (SHR)

A mineralização dental e a atividade salivar estão reduzidas em filhotes de ratas espontaneamente hipertensas (SHR)

Abstract Several pathologies have been diagnosed in children of hypertensive mothers; however, some studies that evaluated the alterations in their oral health are not conclusive. This study analyzed the salivary gland activity and dental mineralization of offsprings of spontaneously hypertensive rats (SHR). Thirty-day-old SHR males and Wistar rats were studied. The salivary flow was evaluated by injection of pilocarpine, the protein concentration and salivary amylase activity, by the Lowry method and kinetic method at 405 nm, respectively. Enamel and dentin mineralization of the mandibular incisors was quantified with aid of the microhardness meter. The results were analyzed by the ANOVA or Student's t test ($p < 0.05$). It was noticed that the salivary flow rate ($0.026 \text{ mL/min/100 g} \pm 0.002$) and salivary protein concentration ($2.26 \text{ mg/mL} \pm 0.14$) of SHR offspring were reduced compared to Wistar normotensive offspring ($0.036 \text{ mL/min/100 g} \pm 0.003$ and $2.91 \text{ mg/mL} \pm 0.27$, respectively), yet there was no alteration in amylase activity (SHR: $242.4 \text{ U/mL} \pm 36.9$; Wistar: $163.8 \text{ U/mL} \pm 14.1$). Microhardness was lower both in enamel ($255.8 \text{ KHN} \pm 2.6$) and dentin ($59.9 \text{ KHN} \pm 0.8$) for the SHR teeth compared to the Wistar teeth (enamel: $328.7 \text{ KHN} \pm 3.3$ and dentin: $67.1 \text{ KHN} \pm 1.0$). These results suggest that the SHR offspring are more susceptible to development of pathologies impairing oral health, once they presented lesser flow and salivary protein concentration and lower dental mineralization.

Uniterms: Hypertension; Spontaneously hypertensive rat (SHR); Saliva; Mineralization; Tooth.

Resumo Diversas patologias têm sido diagnosticadas em filhos de mães hipertensas, entretanto alguns estudos que avaliaram as possíveis alterações na saúde oral não são conclusivos. Neste trabalho foram analisadas a atividade das glândulas salivares e a mineralização dental de filhotes de ratas espontaneamente hipertensas (SHR). Ratos machos, com 30 dias de vida, SHR e Wistar, foram estudados. O fluxo salivar foi avaliado pela injeção de pilocarpina e a concentração de proteínas e atividade da amilase na saliva, pelo método de Lowry e método cinético a 405 nm, respectivamente. A mineralização do esmalte e dentina de incisivos inferiores foi quantificada através da medida da microdureza. Os resultados foram analisados pelo teste ANOVA ou t de Student's não pareado ($p < 0,05$). Verificou-se que o fluxo salivar ($0,026 \text{ mL/min/100 g} \pm 0,002$) e a concentração de proteínas na saliva ($2,26 \text{ mg/mL} \pm 0,14$) de filhotes SHR estavam reduzidos em comparação com filhotes normotensos Wistar

($0,036 \text{ mL/min/100 g} \pm 0,003$ e $2,91 \text{ mg/mL} \pm 0,27$, respectivamente), porém sem nenhuma alteração da atividade da amilase (SHR: $242,4 \text{ U/mL} \pm 36,9$, Wistar: $163,8 \text{ U/mL} \pm 14,1$). A microdureza mostrou-se menor tanto no esmalte ($255,8 \text{ KHN} \pm 2,6$) quanto na dentina ($59,9 \text{ KHN} \pm 0,8$) dos dentes de SHR em comparação com os de Wistar (esmalte: $328,7 \text{ KHN} \pm 3,3$ e dentina: $67,1 \text{ KHN} \pm 1,0$). Estes resultados sugerem que filhotes SHR seriam mais susceptíveis ao desenvolvimento de patologias que prejudicam a saúde oral, uma vez que apresentaram fluxo e concentração proteica salivares reduzidos e menor mineralização dental.

Unitermos: Hipertensão; Rato espontaneamente hipertenso (SHR); Saliva; Mineralização; Dente.

Introduction

Chronic maternal hypertension, as well as hypertensive disease of pregnancy, may significantly alter the fetal growth and well-being. The incidence of stillbirths, neonatal mortality and perinatal mortality is five times more frequent in pregnant women with chronic hypertension than in normotensive pregnant women²⁴. Maternal mortality in the pregnancy-puerperium cycle secondary to hypertension is related to complications as eclampsia, brain hemorrhage, acute pulmonary edema, acute renal insufficiency and coagulopathies⁴.

Concerning the fetus and the newborn, several studies elucidate the complications that may occur because of maternal hypertension. Delayed intrauterine growth, oligohydramnios, premature birth and fetal suffering are common perinatal morbid disturbances. Neonatal morbidity is associated with a higher incidence of low birth weight and small newborns for the gestational age, in addition to frequent complications as hyaline membrane disease, polycythemia, leukopenia, hypoglycemia, infection and necrotizing enterocolitis¹. There may also be pathologies associated with calcium and magnesium disturbances and hormones related to maintenance of serum and tissue concentrations of these minerals, as parathormone (PTH) and calcitonin, in neonatal and newborns of hypertensive mothers¹³. Associated with calcium disturbances, there might be alterations in bone and dental formation in these children; however, few studies so far have evaluated the possible alterations and its effects on the oral health of children of hypertensive mothers.

Increased prevalence of opaque and hypoplastic enamel resulting from impaired tooth development in utero has been attributed to intrauterine under nutrition, hypoxia, deprivation of calcium and phosphate, as well as fluoride and vitamins A, C and D particularly from 12th and 16th gestational week when mineralization of primary central incisor begins^{11,26}. Enamel alterations in animal have also been attributed to intrauterine under nutrition of vitamin A, D and calcium¹⁶. A

reduced saliva secretion rate has been observed in rats after prolonged protein deficiency starting in utero⁸.

Clinical and experimental investigations provided evidences for increased caries levels associated with enamel hypoplasia and salivary gland hypofunction, in children and rats with pre- and postnatal nutritional deficiencies²². While these indirect evidences suggest the association between caries of the deciduous dentition and adverse events during gestation like hypertension, this hypothesis is not supported by data demonstrated in a recent epidemiological study²⁷.

Based in these not conclusive literature data, the objective of the present study was analyzed the enamel and dentin teeth microhardness and salivary gland function in thirty days aged, male spontaneously hypertensive rats (SHR). SHR is the experimental model most similar to human essential hypertension. As these animals develop hypertension spontaneously without exception at the age of 7-15 weeks³⁰, possible observed alterations in SHR offsprings' oral tissues could result from chronic maternal hypertension.

Materials and Methods

1- Animals

The Institutional Review Board on Animal Studies of FOA-UNESP approved this study (protocol n. 37/03). The experiments were conducted employing 30-day-old offsprings of spontaneously hypertensive (SHR) and normotensive Wistar rats. Male offsprings of female SHR and Wistar were evaluated for establishment of the oral parameters (saliva and dental mineralization). The pregnant and in lactation female rats, as well as their offsprings, were kept in controlled room temperature (22-24°C), light cycle (12 h/light and 12 h/darkness, lights on 08:00 a.m.) receiving standard food and water "ad libitum".

The spontaneously hypertensive rat (SHR) was used in this experiment because it is the animal model with the most similar essential arterial hypertension compared to that observed in human beings. Laboratorial studies have indicated that the hemodynamic characteristics of these animals are very similar to those of human essential hypertension³⁰. These animals are born normotense with average PA around 112mmHg and develop spontaneously or with no apparent or predetermining cause, an increase in PA from the 8th week after birth, which can reach values near 200mmHg at 12 weeks of age³⁰.

2- Establishment of physiological parameters

Blood pressure

The systolic blood pressure (SBP) of female rats was recorded during pregnancy and lactation, at predetermined periods (pregnancy:

days 0, 7, 14, 16, 18, 20; lactation: days 7, 14, 21, 28), by indirect tail-cuff plethysmography (Plethysmograph Physiograph® MK-III-S / NBS – Narco Bio-Systems / Houston, Texas, USA). Prior to the PA registration, the rats were warmed at 40°C for 10 minutes in a hot-house with controlled temperature, which made it possible to obtain pulsation through tail artery check. Next, the animals were placed in a respective device composed with resin and then pulsation was detected by making use of an electrosphygmomanometer and pneumatic pulse transducer.

The registrations were made in a specific laboratory where noises were controlled, once only the operator was present in the room, allowing a better control of the animals' stress. The room's luminosity was controlled in the same manner as in the bioterium, where the animals were being kept. The PA registrations were carried out in the morning, since the lowest stress levels in rats can be established through pilot studies, mainly SHR, in that period. The rats were not anesthetized for the procedure, once plethysmography is not an invasive method.

Female Wistar rats with SBP around 112 mmHg and SHR with SBP equal or higher than 150 mmHg, in the zero day of pregnancy, were used³⁰.

Weight of rats and offspring and number of offspring per breeding

The weight gain during pregnancy and lactation was established by weighing the rats at predetermined periods (pregnancy: days 0, 3, 7, 10, 14, 17, 20; lactation: days 7, 14, 21, 28) and comparison between the normotensive and hypertensive groups. Comparison was also performed for the number of offspring per breeding and their weight at 30 days of age.

The rat offsprings were not weighed at the moment of birth due to SHR rats' peculiar characteristics. Especially as regards this strain, mothers show a tendency to mutilate their offsprings when the operator gets in touch with them in the first weeks of life.

3- Establishment of oral parameters

Saliva collection and removal of teeth

Thirty-day old male rats, after 12-hour fasting, were anesthetized with xylazine (25 mg/kg body weight, by intramuscular (IM) injection, Copazine®, Coopers/10 mL) and ketamine (45 mg/kg BW, IM, Dopalen, Vetbrands/10mL). The salivary flow was stimulated by pilocarpine nitrate (Sigma, 5 mg/kg BW, IP). Saliva collection was performed according to the method of Bernarde⁵ (1956). The animals were placed in an inclined bed and saliva was collected in flasks kept in ice, for 15

minutes after the first drop. The saliva volume was calculated by the difference in weight of full and empty flasks, considering the density of saliva as being equal to 1 mg/mL. After collection, the salivary glands were removed and weighed. The animals were killed by excess anesthesia, and the mandibular incisors were individually removed, so that only the teeth were removed from the dental arch, without concomitant removal of bone tissue. Thereafter, the teeth were embedded in acrylic resin for evaluation of dental microhardness.

Salivary protein concentration and salivary amylase activity

Immediately after the saliva was collected, the protein concentration in the saliva was determined by the method of Lowry¹⁸ (1951) and salivary amylase activity was measured by the kinetic method at 405 nm, using 2-chloro-p-nitrophenyl- α -D-maltotrioxide (CNP-G3) as substrate (Kit Amilasa 405, Wiener lab., 2000 – Rosario, Argentina).

Enamel and dentin mineralization

The mandibular incisors were embedded in acrylic resin, and the specimens were ground and polished for microhardness analysis in longitudinal section (MHLS) using the microhardness meter SHIMADZU MICRO HARDNESS TESTER HMV-2,000 (Shimadzu Co.- Kyoto-Japan), with Knoop indenter (KHN) and a static load of 15 grams for 10 seconds, connected to the software for image analysis CAMS –WIN (New Age Industries, USA). Three sequences of indents were performed, being the former from 200 μ m of the incisal edge and the others at 500 μ m from each other. At each sequence, 3 indents were performed at 20, 40 and 60 μ m from the external enamel surface. For the dentin, the same sequence was followed, and the 3 indents were achieved from the dentinoenamel junction.

The Knoop hardness value at each point was achieved from the following equation: $KHN = 14.228 L/d^2$ Kg/mm², where L and d represent the load applied (Kg) and the length of the larger diagonal of indent (mm), respectively²⁰.

4- Statistical analysis

The results were analyzed with utilization of the graphic and statistic software Graph Pad Prism 3.0. Data were evaluated as to normality and homogeneity and expressed as means $\pm$ SEM. The significant differences in the SBP and weight gain of rats were analyzed by one-way analysis of variance for multiple comparisons, followed by the Tukey test. Unpaired Student's t test one-tailed was used for comparisons of two independent samples and evaluation of the saliva, dental microhardness, offspring's weight and number of offsprings per

breeding. The significance level adopted was 5% ($p < 0.05$).

Results

1- Physiological parameters of rats and offspring

The spontaneously hypertensive female rats presented a mean value of blood pressure (BP) (179 mmHg $\pm$ 3) higher than observed in Wistar rats (110 mmHg $\pm$ 2). During the days of pregnancy (0-20) and lactation (7-28), the BP values of SHR were always higher than those of Wistar rats; however, variations in the BP of these animals were observed at these periods (Figure 1). A significant reduction of BP was detected in SHR at days 16 (159 mmHg $\pm$ 4), 18 (153 mmHg $\pm$ 3) and 20 (145 mmHg $\pm$ 3) of pregnancy. However, the BP values measured in SHR at these days were higher ($p < 0.05$) than the values measured in Wistar rats. In the group of Wistar rats, there was a tendency towards a reduction of BP associated with the end of pregnancy (102 mmHg $\pm$ 2, day 20). After the birth of the offspring, during lactation, the BP values of SHR rats were gradually increased. At the seventh day after birth, the BP of SHR was still reduced (157 mmHg $\pm$ 2) when compared to the values measured on day zero, being reestablished at 14 days of lactation (169 mmHg $\pm$ 4). At the end of the lactation period (day 28), the BP measured in SHR was similar to that measured on day zero of pregnancy.

The weight of female rats during pregnancy and lactation were compared between the groups of normotensive and hypertensive animals (Table 1). At day zero, SHR presented lower mean weight than the Wistar rats. During the entire period of pregnancy (days 0-21), the weight gain of Wistar rats (100.1 g $\pm$ 6.57) was larger than that of SHR (57.6 g $\pm$ 3.25), and the largest increases occurred at the last week (days 14-21) for both groups. After birth and with the onset of lactation, the weight of Wistar rats and SHR rats was reduced. The weights of Wistar rat and SHR returned to the values observed at onset of pregnancy after 28 days of lactation. A smaller number of offspring was born from SHR when compared to the Wistar rats (Table 1).

Despite the smaller breeding (Table 1), the SHR offsprings did not achieve similar weight gain as the offspring of Wistar rats (Figure 2). At 30 days of life, the weight of SHR offspring (38.6 g $\pm$ 1.1) was lower than the weight of Wistar rats (58.1 g $\pm$ 2.0).

2- Oral parameters of the study groups

There were no differences in the weight of submandibular glands (SHR: 0.29 g $\pm$ 0.01; Wistar: 0.30 g $\pm$ 0.01), parotid glands (SHR: 0.36 g $\pm$ 0.02; Wistar: 0.40 g $\pm$ 0.03) and sublingual glands (SHR: 0.074 g $\pm$ 0.003; Wistar: 0.066 g $\pm$ 0.003) of hypertensive and normotensive offspring.

The stimulated salivary flow rate (Figure 3A) in SHR offspring ($0.026 \text{ mL/min/100 g} \pm 0.002$) was reduced ($p < 0.05$) compared to the control group ($0.036 \text{ mL/min/100 g} \pm 0.003$). In some tests, measurement was not possible in SHR offspring, due to the small volume of saliva produced (experiments not included). The salivary protein concentration (Figure 3B) of Wistar offspring ($2.91 \text{ mg/mL} \pm 0.27$) was significantly higher than that of SHR ($2.26 \text{ mg/mL} \pm 0.14$). The specific amylase activity (Figure 3C) in saliva (U/mL of saliva) was not different between SHR ($242.4 \text{ U/mL} \pm 36.9$) and Wistar ($163.8 \text{ U/mL} \pm 14.1$) offspring.

Microhardness analysis of the incisors teeth showed that enamel microhardness (Figure 4A) of SHR offspring ($255.8 \text{ KHN} \pm 2.6$) was lower ($p < 0.05$) than that of Wistar ($328.7 \text{ KHN} \pm 3.3$). This alteration was also observed in dentin (Figure 4B) of SHR offspring teeth ($59.9 \text{ KHN} \pm 0.8$), which have reduced hardness ($p < 0.05$) when compared to teeth of Wistar offspring ($67.1 \text{ KHN} \pm 1.0$).

Discussion

This study was the first to demonstrate that 30 days old male spontaneously hypertensive rat (SHR) present reduced dental mineralization and salivary gland activity when compared to Wistar rat at the same age. Besides the mechanisms involved in these alterations were not yet established, and more studies are necessary, the present findings suggest that these alterations would be secondary to the effect of maternal hypertension on the processes of new tissue formation during fetal and neonatal development.

In the present study, BP values higher than 150 mmHg were measured in SHR female rats during pregnancy; however, a significant reduction of these values was observed between days 16 and 20 of this period. These results confirm other data from the literature, which associate the end of pregnancy of hypertensive and normotensive rats to physiological processes that reduce the peripheral vascular resistance⁷. Despite this reduction in BP, hypertensive rats do not reach the normal blood pressure values observed in Wistar rats. Furthermore, the mean weight gain of female SHR during pregnancy and lactation were lower than observed for Wistar rats, the breeding of SHR was smaller, and the postpartum development of the SHR offspring did not follow the normal development of Wistar rats. These alterations could be associated to complications during pregnancy such hypertension. The lower weight of pregnant SHR rats would be directly associated to fetal development of offspring. As demonstrated previously, the fetal weight of SHR is lower than normotensive Wistar-Kyoto rats²⁹ yet the weight of placenta remains unaltered. All these results corroborate with others suggesting that SHR is a model of intrauterine growth restriction that arises

spontaneously without nutritional, surgical or endocrine intervention.

As observed in humans and animals, intrauterine growth restriction contributes significantly to perinatal morbidity and mortality as well as a predisposition to systemic diseases including hypertension and diabetes³, salivary gland hypofunction¹⁵ and increased prevalence of opaque and hypoplastic enamel²¹ latter in life.

Decreased saliva rate, decreased buffering capacity, and decreased salivary constituents, particular proteins, may define salivary gland hypofunction. Decrease in salivary secretion and alterations in the composition of saliva are often observed in individuals with type-1 diabetes mellitus and non-insulin-dependent¹⁹ and hypertensive¹⁰. Our results showed that SHR offsprings produce less saliva than offsprings of normotensive rats and the saliva has lower protein concentration. As no alteration was observed in amylase activity, more experiments were necessary to analyze the concentrations of others proteins like lysozyme, lactoperoxidase and immunoglobulins. These alterations would not be related to the weight of parotid, submandibular and sublingual glands, since there was no difference in relative weight between the two groups. The reduced salivary function could be observed during SHR life, as it was also observed in young and adult SHR¹⁷. A reduction in density or sensitivity of muscarinic receptors in the salivary glands of these animals might contribute to such altered responses. On the other hand, an increase in the sensitivity of the β -adrenoceptor / adenylate cyclase receptor, which participates in the excretion of small amount of viscous saliva from sympathetic stimulation, was observed in young SHR (6-8 weeks)²⁵.

In humans, hypertension has been considered a systemic condition that alters the dental formation, after observation that the crown diameter of deciduous and permanent teeth would be reduced in children of hypertensive mothers¹². Others complications²⁷ during pregnancy such as diabetes, infection and nutritional deficiencies were also associated with problems in dental formation and hypoplastic enamel in children. These alterations could make these teeth more liable to develop carious lesions². The results of the present study revealed that both enamel and dentin microhardness of mandibular incisors of SHR offsprings were lower compared to normotensive offsprings Wistar rats. The poor mineralization of these dental structures might favor the condition described as amelogenesis imperfecta observed in teeth of adults SHR-SP (stroke prone) rats¹⁴, due to the underdevelopment of dental ameloblasts, which are involved with the production, re-absorption and degradation of the enamel matrix and the transport of calcium for mineralization^{23,28}.

Clinical evidences and experimental studies have associated dental mineralization defects with variations in the serum calcium

concentrations. It has been demonstrated that, in SHR, hypertension is followed by a reduction in ionized serum calcium homeostasis⁹. Hypocalcemia may occur because of lower active calcium transportation from mother to fetus through the placenta, several perinatal disturbances or alterations in serum parathormone (PTH) concentration¹³. In SHR, it has been demonstrated that hypocalcemia leads to an increase in parathormone⁹. The mechanism involved in alteration of dental formation in SHR is not well explained, yet both hypocalcemia and the increase in PTH are directly involved with this alteration, since PTH receptors were found in odontoblasts, establishing the presence of hormonal control of odontogenesis through calcium transportation⁶.

Although the literature data are controversial and inconclusive^{22,27} the results of the present work reinforce the association between hypertension during pregnancy and buccal alterations, once the hypertensive (SHR) rats' offspring showed salivary hypofunction and dental hypomineralization. As these factors are a predisposition for caries development, specific studies on lesions (type/size of lesion and development speed) should be conducted in SHR offsprings in order to evaluate a higher or lower susceptibility of these animals to caries.

Conclusion

In conclusion, the present findings indicated that SHR offspring have lesser salivary flow, salivary protein concentration and dental microhardness. These observed alterations can make the buccal environment more favorable to caries development, since this is a multifactorial disease related not only to bacterial and food factors, but also to host factors (tooth, saliva and its components). Further investigations should be carried out in order to determine the mechanisms involved in the observed salivary and dental alterations.

The obtained results suggest that mothers' blood hypertension is related to buccal alterations observed in their offspring, and that these alterations, on their part, may interfere with their oral health.

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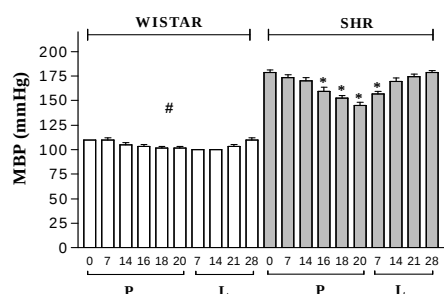


FIGURE 1- Mean blood pressure (MBP) of Wistar rats (n=8) and SHR (n=15) during the period of pregnancy (P) and lactation (L). The bars represent the mean $\pm$ SEM of the rats BP values measured on different days. * Significant difference ($p < 0,05$) between BP values of SHR in 0 day of pregnancy and others days of pregnancy and lactation, *significant difference between BP values of Wistar rats and SHR in every experiments performed ($p < 0.01$ by ANOVA)

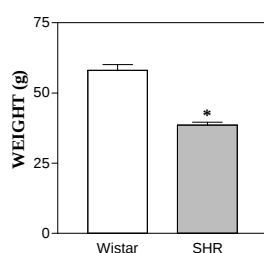


FIGURE 2- Weight of Wistar offspring and SHR offspring. The bars represent the mean $\pm$ SEM of the weight of Wistar offspring (n=74) and SHR offspring (n=94) at 30 days after birth. * Significant difference of weight between Wistar offspring and SHR offspring, at 30 days after birth ($p < 0.05$ by unpaired t test between both groups)

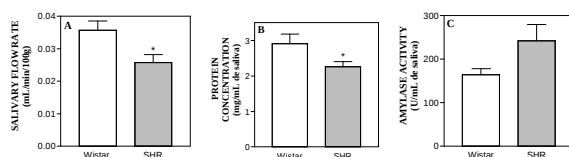


FIGURE 3- Salivary flow rate (A), salivary protein concentration (B) and salivary amylase activity (C) in the saliva of Wistar offspring (n=8) and SHR offspring (n=15). The bars represent the mean $\pm$ SEM of salivary flow rate, salivary protein concentration and salivary amylase activity in the saliva of evaluated offspring. *Significant difference of salivary flow and salivary protein concentration between Wistar offspring and SHR offspring ($p < 0,05$ by unpaired t test between both groups)

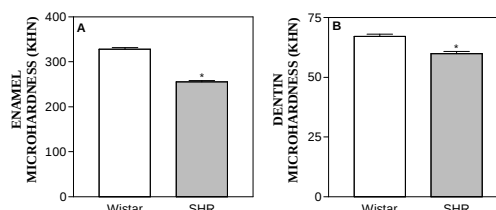


FIGURE 4- Microhardness of the mandibular incisors of Wistar and SHR offspring. The bars represent the mean $\pm$ SEM of (A) enamel microhardness of Wistar offspring (n=8) and SHR offspring (n=15) and (B) dentin microhardness of Wistar offspring and SHR offspring. * significant difference of enamel microhardness and dentin microhardness between Wistar offspring and SHR offspring ($p < 0,05$ by unpaired t test between both groups)

TABLE 1- Weight gain of Wistar rats and SHR rats during the days of pregnancy and lactation and number of offspring per breeding

WEIGHT OF RATS (g)			
PERIOD	DAY	WISTAR (n=8)	SHR (n=15)
Pregnancy (P)	0	247,5 $\pm$ 7,3 ^{a,f,g}	201,2 $\pm$ 4,1 ^d
	7	274,8 $\pm$ 10,1 ^{a,b,g}	217,5 $\pm$ 4,6 ^{d,e}
	14	293,9 $\pm$ 10,2 ^b	228,4 $\pm$ 3,1 ^{e,f}
	21	347,6 $\pm$ 11,1 ^c	258,8 $\pm$ 2,2 ^g
Lactation (L)	7	292,1 $\pm$ 8,9 ^A	213,1 $\pm$ 3,1 ^E
	14	289,0 $\pm$ 9,8 ^A	232,1 $\pm$ 4,2 ^{D,E}
	21	278,0 $\pm$ 9,7 ^{A,B}	226,6 $\pm$ 3,6 ^{D,E}
	28	252,3 $\pm$ 7,6 ^{B,D}	214,2 $\pm$ 4,1 ^E
NUMBER OF OFFSPRING PER BREEDING		9,3 $\pm$ 0,9	6,3 $\pm$ 0,6*

Capítulo 2

Atenolol increases dental mineralization

Atenolol increases dental mineralization

ABSTRACT

Objective: The objective of this study was to evaluate the effect of atenolol on dental mineralization in pups of spontaneously hypertensive rats (SHRp). *Design:* Female SHR (fSHR) and normotensive (fW) Wistar rats were treated with atenolol (100mg/Kg/day, orally) during pregnancy and lactation periods. The enamel (E) and dentin (D) microhardness (mh) were analyzed (tester Shimadzu HMV-2000®, 15g, 10s) in mandibular incisors (INCT) and molar (MT) teeth of male pups. Data were expressed as mean $\pm$ SEM and analyzed by ANOVA test ($p < 0,05$). *Results:* Decreased Emh and Dmh were observed in INCT and MT of SHRp. Atenolol reduced the arterial pressure (AP) in fSHR, did not change AP in fW rats, and increased Emh and Dmh of INCT in SHR and W pups. The higher efficacy of atenolol was observed in SHRp. Atenolol did not alter the width of E of INCT in both groups. In MT, atenolol increased only the Emh in W pups. *Conclusions:* Maternal hypertension reduced Emh and Dmh of INCT and MT in pup rats. Atenolol increased dental mineralization, and its effect was more evident on INCT in which growth and calcification are continuous. These results suggest that atenolol's effect on dental mineralization could not be directly associated with its pharmacological anti-hypertensive effect, once it was also observed in pups of fW rats that received the treatment. However, as the higher atenolol efficacy was observed in SHRp, it was

possible to suggest that atenolol's anti-hypertensive effect could prevent the impairment of high blood pressure on dental mineralization.

Keywords: Hardness, Atenolol, Spontaneously hypertensive rat (SHR)

1. Introduction

Complications¹ during pregnancy such as hypertension, diabetes, infection and nutritional deficiencies have been associated with problems in dental formation and hypoplastic enamel in children. In humans, the crown diameter of deciduous and permanent teeth would be reduced in children of hypertensive mothers.² In animals, it has been demonstrated recently³ that male pups (30 days old) of spontaneously hypertensive rats (SHR) have a decreased enamel and dentin microhardness of mandibular incisor teeth compared to normotensive Wistar rats. The poor mineralization of these dental structures might favor the condition described as amelogenesis imperfecta observed in teeth of adult SHR-SP (stroke prone) rats⁴, due to the underdevelopment of dental ameloblasts, which are involved with the production, re-absorption and degradation of the enamel matrix and the transport of calcium for mineralization.^{5,6} Besides, the mechanisms involved in these alterations have not yet been established, so that clinical evidences and experimental studies have associated dental mineralization defects with variations in serum calcium concentrations. It is well-known that, like hypertensive humans, SHR have abnormal calcium metabolism, such as low serum calcium and high serum parathyroid hormone (PTH) levels.⁷

Cardiovascular drugs, as anti-hypertensive, are often used in pregnancy for the treatment of maternal and fetal conditions. Mothers could also require continued postpartum drug therapy. Atenolol, an β -adrenergic blocker, is an effective agent for management of high blood pressure in pregnancy. Atenolol's pharmacokinetics has been reviewed, and the results demonstrated that atenolol rapidly crosses the placenta and accumulates in breast milk.^{8,9} Manifestations of atenolol's prenatal toxicity as placental changes, intrauterine growth retardation, decreased fetal/birth weight in the absence of congenital malformations, and postnatal effect as decreased weight gain, are similar in tested animal species (rats and rabbits) and humans.¹⁰ However, relatively little attention has been directed toward the examination of whether atenolol treatment during organogenesis time could affect mineralized tissues.

Recent studies suggested that β -adrenergic blockers therapy could promote an increase in density of bone mass in animals^{11,12} and in elderly human patients with hypertension.^{13,14} Although bones and teeth are tissues with peculiar characteristics, a few similarities between both tissues have been described as the presence of odontoclasts re-absorbing dentin's extracell matrix and cementum in a way similar to osteoclasts' activity, as well as the expression of molecules involved in bone re-absorption in odontoblasts, ameloblasts and pulp cells¹⁵ and also in early tooth primordia cells.¹⁶

The objective of this study was to evaluate the effect of atenolol treatment during pregnancy and lactation periods on dental mineralization in pups of spontaneously hypertensive rats (SHR).

2. Material and methods

2.1. Animals

The experiments were conducted employing 30-day-old male pup of spontaneously hypertensive rats (SHR) and normotensive Wistar rats (W). The pregnant and lactation female rats, as well as their pups after birth, were kept in controlled temperature environment (22-24°C), light cycle (12h/light and 12h/darkness) receiving standard food and water "*ad libitum*". Female rats were treated with Atenolol 100mg/Kg/day diluted in a predetermined water volume ingested by day. For the control group, the animals received the same water volume without the drug.

The experimental protocol was approved by Ethics Committee on Animal Experimentation, UNESP School of Dentistry, Araçatuba (protocol n. 37/03).

2.2. Arterial Blood pressure

The systolic blood pressure (SBP) of female SHR and W rats was recorded during pregnancy (days: 0, 7, 14, 16, 18, 20) and lactation (days: 7, 14, 21, 28) periods by

tail plethysmograph adapted for measurements in rats (plethysmograph Physiograph® MK-III-S - Narco Bio-Systems / Houston, Texas, USA). Only female W rats with SBP around 112mmHg and SHR with SBP equal or higher than 150mmHg, until treatment and in the zero day of pregnancy, were used in experiments. Pregnancy was determined by examination of vaginal smears; the day when spermatozoa were present in the vaginal smears was labeled zero day of pregnancy.

2.3. Weight of female rats and pups and number of puper per breeding

The weight of female rats during pregnancy and lactation was established by weighing the rats at predetermined periods (pregnancy: days 0, 3, 7, 10, 14, 17, 20; lactation: days 7, 14, 21, 28) and compared between the groups. Comparison was also performed for the number of pups per breeding and pups' weight at 30 days of age.

2.4. Removal of teeth

Male pups rats aged 30 days, after 12-hour fasting, were killed by excess anesthesia and the mandibular incisors and molar teeth were removed from the dental arch. Only the incisors were removed without concomitant removal of bone tissue.

2.5. Enamel and dentin mineralization

The mandibular incisors and molar teeth were embedded in acrylic resin, and the specimens were ground and polished for microhardness analysis in longitudinal section (MHLS) using the microhardness meter SHIMADZU MICRO HARDNESS TESTER HMV-2,000 (Shimadzu Co.- Kyoto-Japan), with Knoop indenter (KHN) and a static load of 15 grams for 10 seconds, connected to the software for image analysis CAMS –WIN (New Age Industries, USA). Three sequences of indents were performed in incisors, being the former from 200µm of the incisal edge and the others at 500µm from each other. At each sequence, 3 indents were performed at 20, 40 and 60µm from the external enamel surface. For the dentin, the same sequence was followed, and the 3 indents were achieved from the dentin-enamel junction.

In molar teeth, the same numbers of indents were performed in enamel and dentin between the first and the second molar. The three sequences of indents were performed at 45µm from each other and from the cusp edge.

The Knoop hardness value at each point was achieved from the following equation: $KHN = 14.228L/d^2$ Kg/mm², where L and d represent the load applied (Kg) and the length of the larger diagonal of indent (mm), respectively.¹⁷

2.6. Histomorphometrical analysis of enamel and dentin's thickness of incisal and medium thirds of incisor teeth

Ten blocks of resin (10 incisors) were selected from each experimental group. The images of the incisal and medium thirds of incisors' enamel were captured by means of a digital camera coupled to an optical microscope in a 50-time amplification and then saved into a computer. The accurate location of the medium third was determined by measuring all of the tooth length through a digital pachymeter and marking a dot corresponding to the half of the measured piece. Thickness analysis was made through the "ImageLab 2000" software (Diracon Bio Informática Ltda., Vargem Grande do Sul, SP, Brasil).

2.7. Statistical analysis

Through the GMC 2002 Version software, the results were evaluated as to normality and homogeneity and then submitted to variance analysis (ANOVA) with factorial scheme. The data that did not show normal distribution were submitted to Kruskal-Wallis non-parametrical test. The level of significance was taken as $p < 0,05$ and the results are expressed as mean $\pm$ standard error of the mean (SEM).

3. Results

As expected, the systolic blood pressure (SBP) was higher ($p < 0,05$) in female SHR on day zero and during pregnancy (0-20 days) and lactation (27-48) periods when compared to normotensive Wistar rats (Fig.1). SBP reduction was observed in SHR at days 16 ($159\text{mmHg} \pm 4$), 18 ($153\text{mmHg} \pm 3$) and 20 ($145\text{mmHg} \pm 3$) of pregnancy, but these BP values were still higher ($p < 0,05$) than the values measured in Wistar rats on the same days. In the Wistar rats group, there was the same tendency towards BP reduction associated with the end of pregnancy ($102\text{mmHg} \pm 2$, day 20); but these values were not different from those measured on day zero. Atenolol (100mg/Kg/day) treatment did not change SBP in female Wistar rats, but induced a significantly reduction ($p < 0,05$) of arterial pressure in female SHR, effect that could be observed since the 7th day of pregnancy to the end of the lactation period. Atenolol abolished the variations of arterial pressure observed during pregnancy and lactation in SHR. At atenolol's anti-hypertensive effect, SBP values in female SHR were closer than the normotensive values, however, these values were still different ($p < 0,05$) from the values measured in Wistar rats.

The weight of female rats during pregnancy and lactation was compared between the groups of normotensive and hypertensive animals, until and after atenolol treatment (Table 1). During the entire period of pregnancy (days 0-21), the weight of Wistar rats was larger than SHR ($p < 0,05$). Atenolol treatment promoted differences in weight between the experimental groups and analyzed periods. An increase ($p < 0,05$) in weight during pregnancy, but no alteration during lactation

was observed in female Wistar rats treated with atenolol. However, no weight alteration was observed in atenol-treated SHR during both periods. After birth and with the onset of lactation, the weight of Wistar rats and SHR rats was reduced gradually and the weight of both groups returned to the values observed at the onset of pregnancy after 28 days of lactation.

A smaller number of pups was born from SHR ($p<0.05$) when compared to the Wistar rats (Table 1). Despite the lower breeding, the SHR pups did not achieve similar weight as that of the Wistar rats' pups. At 30 days of life, the weight of SHR was lower ($p<0.05$) than the weight of Wistar rats. Atenolol did not change the number of pups per breeding in both groups and the weight of Wistar and SHR pups at 30 days of life.

Structural changes were also observed in incisors and in molar teeth in normotensive and hypertensive rat pups whose mothers had not been treated, or treated with atenolol (Table 2).

Enamel microhardness values of incisor teeth of SHR pups were lower than Wistar pups ($p<0.05$). The same alteration was observed in dentin of SHR teeth, which have reduced hardness when compared to the teeth of Wistar rats. Mother's treatment with atenolol increases ($p<0.05$) enamel and dentin microhardness in SHR and Wistar pups (Table 2). When atenolol effect on mineralized tissues was compared between groups, the higher efficacy of atenolol on enamel and dentin of incisor teeth of SHR was observed. The reduced enamel hardness observed in SHR

incisor teeth could be associated with the thin enamel thickness noticed in medium and incisal thirds (Table 2). Atenolol did not change thickness for medium and incisor thirds of SHR and Wistar pups' incisor teeth.

As observed in incisor teeth, a decreased microhardness of enamel and dentin of molar teeth was also observed in hypertensive pups (Table 2). However, in dental tissues of molar teeth, atenolol effects were not the same. Atenolol treatment increased the enamel microhardness of molar teeth only in normotensive pups. After atenolol treatment, no significant alterations were observed in enamel microhardness of SHR or in dentin microhardness of molar teeth in SHR and Wistar pups.

4. Discussion

The results of the present study show, for the first time, that atenolol treatment increased enamel and dentin microhardness of incisors and molar teeth in hypertensive and normotensive rats. As the dental microhardness was evaluated in pups of female rats that received atenolol treatment during pregnancy and lactation periods, it is possible to guarantee that the pups were treated with atenolol from their conception, via placental circulation, to the 30th day of life, via mother's milk or drinking water. So, the period encompasses tooth development from the earliest formation of the epithelial thickening to cytodifferentiation, and mineralization stages could be altered/influenced by atenolol.

The anti-hypertensive treatment with atenolol (100mg/kg/dia) by oral administration was effective as it could be observed since the onset of pregnancy until the end of lactation in female SHR. Atenolol reduced the high SBP values measured in female SHR during pregnancy and abolished the variations of arterial pressure observed during pregnancy and lactation periods. As previously reported as regards normotensive rats,¹⁸ no atenolol's effect was observed in arterial blood pressure of female Wistar rats.

Despite its anti-hypertensive effect, atenolol did not change the altered physiological parameters observed in female SHR or in their pups. Recently, it was reported³ that the mean weight of female SHR during pregnancy and lactation was lower than the observed as regards the Wistar rats, the breeding of SHR was lower, and the postpartum development of the SHR pups did not follow the normal development of Wistar rats. The lower weight of pregnant SHR rats would be directly associated with fetal development of their pups. Previous report showed that the fetal weight of SHR is lower than normotensive Wistar-Kyoto rats¹⁹ yet the weight of placenta remains unaltered, suggesting that SHR is a model of intrauterine growth restriction that arises spontaneously without nutritional, surgical or endocrine intervention. The prenatal or postnatal atenolol's toxicity effects on SHR could not be observed since maternal hypertension by itself can induce changes in utero-placental and fetal hemodynamic as high frequency of prematurity and growth restriction.^{20,21} However, in Wistar pup rats, atenolol effects on postnatal weight of normotensive pups were not observed either. Different results had been

observed in other animal studies, in which exposures limited to the period of major organogenesis, 6-15 and 6-18 days in rats and rabbits, respectively, produced signs of embryotoxicity and postnatal effect on both species at oral doses at or above 25mg/kg/day.¹⁰

Associated with embryotoxicity or postnatal effect, the present study demonstrated that atenolol increased the microhardness of enamel and dentin of incisor teeth of SHR and Wistar pups, without changing the width of enamel of the incisor teeth. In molar teeth, different effects of atenolol on dental mineralization were observed between hypertensive and normotensive rats. Atenolol increased microhardness only in enamel of molar teeth in Wistar pups, without any alteration on dentin of Wistar pups or on enamel or dentin of molar teeth in SHR pups. These findings show a predominant atenolol effect on enamel and dentin microhardness of incisor teeth in hypertensive or normotensive pups. The biological reaction of tissue depends upon the fact that while it is in the process of development, the formative cells can be affected by local or systemic factors and such disturbances are automatically reflected and recorded in the growing structure. In rats, incisors' enamel and dentin are continuously being apposed by cells that are constantly being derived from continuously proliferating odontogenic epithelium. In rats' molar, on the other hand, the odontogenic epithelium is limited in its activity and no new cells replace the old ones, and in that sense tooth growth is limited.²² Moreover, amelogenesis and dentinogenesis involve distinct mechanisms associated with cellular differentiation, ameloblasts and odontoblasts, deposition and resorption of

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extracell matrix and calcium deposition in incisor and molar teeth, and these mechanisms could be affected in different ways by atenolol. However, specific studies could be conducted in order to test this hypothesis.

These results also demonstrated that atenolol's effect on incisor teeth microhardness was potentiated in SHR pups.

The increased efficacy of atenolol on SHR dental microhardness, could be consequent from the differences in dental microhardness observed between the groups before treatment. As demonstrated,³ male SHR pups, at 30 days of life, showed marked reductions in enamel and dentin microhardness of mandibular incisor teeth. This lack of dental mineralization could be associated with the lower width of medium and incisal thirds of enamel in SHR's incisor teeth. Now, we also demonstrated a decreased microhardness on enamel and dentin of molar teeth in these animals. As noticed in humans and animals, intrauterine growth restriction contributes significantly to increased prevalence of opaque and hypoplastic enamel²³ later in life. The poor mineralization of enamel might be due to the underdevelopment of dental ameloblasts.^{5,6} Moreover, reduction in ionized serum calcium homeostasis⁷ consequent from lower active calcium transportation from mother to fetus through the placenta and several perinatal disturbances or alterations in serum parathormone (PTH) concentration²⁴ could affect dental mineralization. In SHR, it has been demonstrated that hypocalcemia leads to an increase in parathormone.⁷ It is well-known, that PTH secretion is also regulated by other factors such as adrenergic stimulus, vitamin D, phosphate and magnesium

ions, calcitonin and growth hormones. The mechanism involved in the alteration of dental formation in SHR could not be explained, yet both hypocalcemia and the increase in PTH are directly related to this alteration, since PTH receptors were found in odontoblasts establishing the presence of hormonal control of odontogenesis through calcium transportation.²⁵ Although a PTH receptor was not found in ameloblasts, it can alter ameloblast function and maturation.²⁵ An antagonist effect of atenolol on β -adrenergic receptors of parathyroid cells could inhibit the PTH secretion and promote an increase in dental mineralization of SHR teeth and Wistar teeth, although calcium and PTH disturbances were not related to normotensive rats.

Calcium plasma concentration or PTH levels cannot be suggested as a single mechanism involved with the atenolol effect on microhardness. Recent studies provided evidences for a new molecular mechanism involved with β -blockers' effect on mineralized tissues. The first evidences were provided by case-control clinical studies showing that the use of β -adrenergic blockers was associated with an 23-30% decrease in the risk of vertebral or non-vertebral (hand, forearm, foot) fractures.^{13,14} Despite controversial reported results²⁶ this effect has been correlated to many experimental evidences suggesting that bone turnover/remodeling may be subjected to central control, with the sympathetic nervous systems as the peripheral effector and leptin, a hormone produced in fat cells, as the regulator of neural pathways.^{27,28} For the molecular mechanism reported, activation of noradrenalin receptor causes osteoblasts to produce a RANK ligand (RANKL) that stimulates the

formation of osteoclasts, so β -adrenergic blockers will inhibit the formation of osteoclasts.

Rats' incisor teeth grow and calcify continuously, and the atenolol effect could be recorded relatively fast in this dental tissue. Experimental evidences suggest that sympathetic nervous system also plays an important role in mandibular bone metabolism and dental growth.²⁹ Dental pulp and odontoblast cell lines express osteoprotegerin (osteoclastogenesis inhibitory factor, OPG), RANKL and macrophage colony-stimulating factor (M-CSF), factors that are crucial for the regulation of osteoclast formation. *In vivo*, RANKL and OPG were located to odontoblasts, ameloblasts and pulp cells in developing mouse teeth¹⁵ as well as in thickening and bud epithelium, in both internal and external enamel epithelium and in papilla mesenchyme,¹⁶ suggesting a role for these factors on tooth development. PTH down-regulates OPG/OCIF (osteoclastogenesis inhibitory factor) mRNA expression in stromal cells, which could result in the stimulation of osteoclast formation. Similar to osteoclasts, odontoclasts that resorb dentin and cementum extracellular matrix have been described. All together, these results suggest a possible effect of atenolol on molecular mechanisms involved in tooth development.

In conclusion, the results of this study provide further insight on the effects of hypertension during pregnancy and lactation and its treatment with atenolol. Maternal hypertension has been associated with reduced enamel and dentin microhardness of mandibular incisor and molar teeth from 30-day-old rats. The treatment of female rats during pregnancy and lactation periods with atenolol, an β -

adrenergic blocker, increases microhardness of enamel and dentin of incisor teeth in their pups. These data demonstrated that atenolol increases dental mineralization, and that its effect is more important on teeth with continuous growth and calcification. Other studies are necessary in order to identify the exact mechanism involved in these effects.

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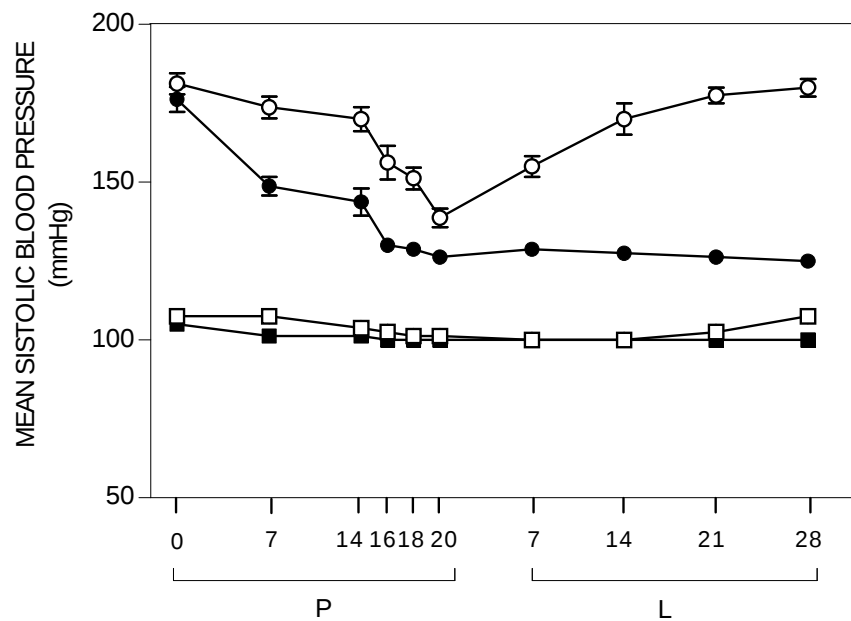


Fig. 1- Systolic blood pressure (mmHg) of Wistar rats (□, n=10), SHR (○, n=10), Wistar treated (■, n=10) and SHR (●, n=10) treated with atenolol during pregnancy (P) and lactation (L). Symbols represent mean $\pm$ SEM of systolic blood pressure (ANOVA, $p < 0.05$)

Table 1 - Body weight of Wistar and SHR female rats during pregnancy and lactation, number of pups per breeding, weight of 30- day-old male pup rats

Groups	Female (n=10) body weight during pregnancy	Female (n=10) body weight during lactation	Number of pups per breeding (n=10)	Pups' weight at 30 days of life
Wistar Control	277.5g $\pm$ 4.7 ^a	277.6g $\pm$ 4.4 ^a	9.4 $\pm$ 0.6 ^a	59.3g $\pm$ 5.5 ^a
Atenolol-treated Wistar rats	301.3g $\pm$ 8.2 ^b	262.3g $\pm$ 7.3 ^a	8.6 $\pm$ 1.1 ^{a,b}	49.8g $\pm$ 4.9 ^a
SHR Control	229.7g $\pm$ 3.6 ^c	223.3g $\pm$ 2.1 ^b	5.8 $\pm$ 0.8 ^b	32.8g $\pm$ 1.8 ^b
Atenolol-treated SHR	216.6g $\pm$ 4.3 ^c	206.1g $\pm$ 3.9 ^b	6.3 $\pm$ 0.6 ^{a,b}	38.1g $\pm$ 2.3 ^{a,b}

The results are expressed as means $\pm$ SEM. The different letters show significant differences between the groups of the same column (p<0,05 by ANOVA)

Table 2 – Dental Microhardness of incisor and inferior molar teeth and enamel's layer thickness of incisors in Wistar and SHR rats treated or not with atenolol

Groups	Microhardness (KHN)				Enamel Thickness	
	Incisors (n=10)		Molars (n=10)		Incisal Third (n=10)	Medium Third (n=10)
	Enamel	Dentin	Enamel	Dentin		
Wistar Control	^A 306.17 ± 6.795	^A 67.23 ± 1.555	^A 439.61 ± 4.058	^A 65.25 ± 1.493	^A 0.2565 ± 0.008	^A 0.0764 ± 0.0086
Atenolol-treated Wistar rats	^B 456.99 ± 4.667	^B 102.42 ± 1.510	^B 526.00 ± 4.425	^{A,B} 61.47 ± 0.745	^A 0.27970 ± 0.025	^A 0.0719 ± 0.0033
SHR Control	^C 259.52 ± 2.752	^C 60.23 ± 1.177	^C 364.16 ± 3.226	^B 59.91 ± 1.028	^B 0.20120 ± 0.008	^B 0,0514 ± 0.0018
Atenolol-treated SHR	^D 535.16 ± 3.062	^D 108.64 ± 0.8923	^C 370.51 ± 11.540	^{A,B} 62.35 ± 1.564	^B 0.17080 ± 0.005	^B 0,0614 ± 0.0071

The results are expressed as means ± SEM. The different letters show significant differences between the groups of the same column (p<0,05 by ANOVA and Kruskal-Wallis)

Capítulo 3

Atenolol's toxic effect on pups' salivary glands of spontaneously hypertensive (SHR) and normotensive rats treated during pregnancy and lactation

Atenolol's toxic effect on pups' salivary glands of spontaneously hypertensive (SHR) and normotensive rats treated during pregnancy and lactation

Abstract

In this study, pups' salivary activity of Atenolol-treated spontaneously hypertensive (SHR) and Wistar (W) rats treated during pregnancy and lactation was evaluated. Atenolol's anti-hypertensive effect on SHR was observed from the beginning of treatment. Atenolol-treated SHR and W rats' pups showed decrease in submandibular gland weight and in saliva's flow and protein concentration, but no alteration in amylase activity. Atenolol's toxicity on salivary glands can impair treated rats' pups' oral health, mainly SHR pups showing salivary gland hypofunction.

Keywords: Atenolol, salivary glands, spontaneously hypertensive rat (SHR)

Introduction

Intrauterine or postnatal growth restriction has been associated with salivary gland hypofunction in humans and animals (1). Pregnancy arterial hypertension and placental insufficiency are considered as pathogenic factors leading to growth restriction and intrauterine growth delay. Spontaneously hypertensive rats (SHR) are the model of intrauterine growth restriction spontaneously arising without

nutritional, surgical or endocrine intervention (2). Salivary gland hypofunction in 30-day-old pups of female SHR has been reported recently (3).

Pregnancy hypertension pharmacological treatment should be carried out not only for arterial pressure control, but also for cardiovascular diseases, brain accidents and kidney diseases in mothers (4) and for the prevention from the main cause of fetal morbimortality and new-born baby's death. Treatment favors non-premature removal of low-weight fetus with unsatisfactory vitality (5). One of the main medicines used for pregnancy hypertension control is Atenolol (6 – 7 – 8 – 9), a beta-blocker that crosses the placental barrier and is excreted in milk (10). In spite of its beneficial anti-hypertensive effect on mothers and fetus, comparative studies in animals and humans showed that Atenolol, used during pregnancy and lactation, can reduce fetus' and placenta's weight, birthtime weight and weight increase during postnatal development (11). No alteration related to blockage of these receptors in pups' central nervous system was noticed during the Atenolol-based treatment of pregnant rats (12). Among all of the experimental studies evaluating Atenolol's prenatal and postnatal effects, possible alterations associated with pups' oral health were not mentioned. Salivary glands showed beta-adrenergic innervation (13) and are involved in maintaining oral health balance through secretion and saliva action. Decrease in salivary activity has been associated with higher susceptibility to caries development due to the restriction of the protection effect saliva exerts on teeth (1).

This study aimed at evaluating if Atenolol-based treatment in rats during pregnancy and lactation periods would provoke alterations in pups' salivary gland activity. Salivary flow, total protein concentration and amylase activity in saliva, as well as parotid gland, submandibular gland and sublingual gland weight in pups of Atenolol-treated spontaneously hypertensive (SHR) and normotensive (Wistar) rats were evaluated. Parameters such as rat mothers' arterial pressure, rats' weight gain during pregnancy and lactation, the number of pups born per brood and 30-day-old pups' weight were also evaluated for possible correlation to the treatment carried out.

Methods

Animals

The experiments were conducted employing 30-day-old male pups of spontaneously hypertensive rats (SHR) and normotensive Wistar rats (W). The pregnant and lactation female rats, as well as their pups after birth, were kept in controlled temperature environment (22-24°C), light cycle (12h/light and 12h/darkness) receiving standard food and water "*ad libitum*". Female rats were treated during pregnancy and lactation periods with Atenolol 100mg/Kg/day diluted in a predetermined water volume ingested by day. For the control group, the animals received the same water volume without the drug. The experimental protocol was approved by Ethics Committee on Animal Experimentation, UNESP School of Dentistry, Araçatuba (protocol n. 37/03).

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Male and female rats were put in the same box for mating. In the morning after mating, the female rats' vaginal smears were collected and evaluated through an optical microscope. The day when spermatozoa were present in the vaginal smears was labeled day 0 of pregnancy. The female rats were separated from the male rats and placed in individual boxes for pregnancy and lactation follow-up.

Arterial Blood pressure

Systolic blood pressure (SBP) of female SHR and W rats was recorded during pregnancy (days: 0, 7, 14, 16, 18, 20) and lactation (days: 7, 14, 21, 28) periods by tail plethysmograph adapted for measurement in rats (plethysmograph Physiiograph® MK-III-S - Narco Bio-Systems / Houston, Texas, USA). Only female W rats with SBP around 112mmHg and SHR with SBP equal or higher than 150mmHg, until treatment and in the zero day of pregnancy were used in experiments. Pregnancy was determined by examination of vaginal smears; the day when spermatozoa were present in the vaginal smears was labeled zero day of pregnancy

Weight of female rats and pups and number of pups per breeding

The weight of female rats during pregnancy and lactation was established by weighing the rats at predetermined periods (pregnancy: days 0, 3, 7, 10, 14, 17, 20; lactation: days 7, 14, 21, 28) and compared between the groups. Comparison was also performed for the number of pups per breeding and pups' weight at 30 days of age.

Saliva collection and removal of submandibular, sublingual and parotid glands

Thirty-day-old male rats, after 12-hour fasting, were anesthetized with xylazine (25 mg/kg body weight, by intramuscular (IM) injection, Copazine[®], Coopers/10 mL) and ketamine (45 mg/kg BW, IM, Dopalen, Vetbrands/10mL). The salivary flow was stimulated by pilocarpine nitrate (Sigma, 5 mg/kg BW, IP). Saliva collection was performed according to Bernardes' method (14). The animals were placed in an inclined bed and saliva was collected in flasks kept in ice, for 15 minutes after the first drop. The saliva volume was calculated by the difference in weight of full and empty flasks, considering the density of saliva as being equal to 1 mg/mL. After collection, the salivary glands were removed and weighed. The animals were killed by excess of anesthesia.

Salivary protein concentration and salivary amylase activity

Immediately after the saliva was collected, the protein concentration in the saliva was determined by the method of Lowry (15) and salivary amylase activity was measured by the kinetic method at 405 nm, using 2-chloro-p-nitrophenyl- α -D-maltotrioside (CNP-G3) as substrate (Kit Amilasa 405, Wiener lab., 2000 – Rosario, Argentina).

Data Analysis and Statistics

Data were expressed as means $\pm$ standard error of the mean (SEM), evaluated as to normality and homogeneity and then submitted to variance analysis (ANOVA) with factorial scheme (GMC 2002 Version software). The level of significance was taken as $p < 0,05$.

Results

Female SHR showed SBP values higher than those of Wistar rats' during pregnancy and lactation periods (Figure 1). Female SHR rats' pregnancy ending (14^o – 20^oday) was associated with a significant reduction in SBP. After pups' birth and lactation start, a progressive increase in SBP up to the values observed in the beginning of pregnancy in SHR was noticed. In treated SHR, Atenolol's antihypertensive effect was observed from the beginning of treatment (Figure 1). On the 7th day of pregnancy, an important decrease in SBP (148 mmHg $\pm$ 3) was observed in Atenolol-treated SHR rats. At the end of pregnancy, the SBP values proved to be even more reduced for Atenolol-treated SHR rats (125 mmHg) and were kept in these levels until the end of treatment or lactation ending. In spite of significant SBP reduction in treated SHR rats, the values measured were kept higher ($p < 0.05$) than those of Wistar rats' SBP.

When evaluating normotensive Wistar rats' SBP, no significant differences were noticed ($p>0.05$) in SBP values between the treated and the non-treated groups (Figure 1).

Physiological parameters such as rats' weight during pregnancy and lactation, breeding size and 30-day-old pups' weight were compared between the groups of normotensive and hypertensive animals, treated or not with Atenolol (Table 1). Atenolol did not alter the SHR rats' average weight during pregnancy and did not interfere with the SHR rats' weight during the lactation period either. In normotensive Wistar rats after treatment, the rats' average weight during pregnancy was higher ($p<0.05$) than that of non-treated rats. During the lactation period, this treatment-related weight gain was not observed in the normotensive rats anymore, once the treated Wistar rats' average weight did not differ from that of non-treated Wistar rats'.

Comparing the groups (Table 1), it is noticed that the SHR rats' weight during pregnancy and lactation period was always lower ($p<0.05$) than that of Wistar rats', and that Atenolol did not alter this difference.

The number of pups born per breeding was significantly lower in female SHR rats (Table 1). After Atenolol treatment, no alteration was noticed. At 30 days of life, the SHR pups weighed less than Wistar rats' pups. Atenolol-treated rats' pups did not have any body weight alteration, and the treated SHR pups' weight was lower than that of treated Wistar rats'.

When evaluating the salivary flow (Figure 2) between the groups, flow reduction ($p < 0,05$) in SHR pups can be noticed ($0.019 \text{ mL/min/100g} \pm 0.001$) as compared to that of Wistar rats' pups ($0.041 \text{ mL/min/100g} \pm 0.001$). Atenolol treatment reduced ($p < 0.05$) the salivary flow in both SHR pups ($0.011 \text{ mL/min/100g} \pm 0.001$) and Wistar pups ($0.036 \text{ mL/min/100g} \pm 0.001$).

Total protein concentration in saliva (Figure 2) of Wistar pups ($3.46 \text{ mg/mL} \pm 0.085$) was significantly higher than that of SHR pups' ($2.32 \text{ mg/mL} \pm 0.065$) and Atenolol treatment in rats also reduced ($p < 0.05$) the concentration of proteins secreted in saliva of pups from both groups (Wistar: $1.80 \text{ mg/mL} \pm 0.125$; SHR: $0.69 \text{ mg/mL} \pm 0.050$).

The weight of salivary, submandibular, sublingual and parotid glands were not different between the groups of pups of hypertensive and normotensive rats (Table 2). Nevertheless, after mother rats were treated with Atenolol, a specific reduction ($p < 0.05$) in weight of submandibular glands was noticed in both female SHR rats' pups and Wistar rats' pups. The weight of sublingual and parotid glands did not show any considerable difference ($p > 0,05$) between the groups after Atenolol-based treatment.

Discussion

During pregnancy and lactation, certain physiological changes occur in the cardiovascular, renal, gastrointestinal and endocrine systems that could influence the pharmacotherapeutics of the drugs used for hypertension treatment, thereby

affecting drug transfer across the maternofetal unit. Most cardiovascular drugs taken by pregnant women/animal can cross the placenta and therefore expose the developing embryo and fetus to their pharmacologic and teratogenic effects. Many drugs are also transferred into milk and therefore can potentially have adverse effects on the nursing infant.

The anti-hypertensive effect of Atenolol is mediated by blocking β_1 -adrenergic receptors on cardiovascular system. To date, none of the β -blockers has been implicated as a causative agent of fetal malformation, despite Atenolol can cross the placenta and accumulates in breast milk in concentrations higher than those of maternal plasma (10).

The efficacy of Atenolol by oral administration on arterial blood pressure could be observed in female SHR but not in Wistar rats. The Atenolol effect on SBP of female SHR was observed from the onset of treatment to the ending of the long administration period (49 days between pregnancy and lactation periods). As atenolol has approximately 50 to 60% oral bioavailability (16), the lack of Atenolol effect on normotensive rats could not be associated with non-absorption neither to incomplete Atenolol absorption. As previously observed, after 8 weeks of Atenolol (30 mg/rat) oral administration, no anti-hypertensive effects were observed on normotensive rats, but a significant SBP reduction was observed in hypertensive rats (17-18).

Atenolol treatment had no significant effect on weight of female SHR rats during pregnancy or lactation periods suggesting no maternal toxicity by Atenolol

exposure. The same results were observed in other rat experimental studies (11) at oral doses at or above 25 mg/kg/day. Atenolol is also maternally non-toxic to humans at oral therapeutic dose range of 0,7-3 mg/kg/day.

Clinical and experimental evidences have associated Atenolol prenatal toxicity with reduced fetal growth (19), low birth weight (20) and postnatal toxicity, decreased weight gain, retarded growth and development, neurobehavioral deviations and mortality (11). In rats, controversial results have associated Atenolol's exposure limited to the period of major organogenesis, 6-15 days of pregnancy, to signs of embryo toxicity and postnatal effect as increased embryo/fetal loss, decreased litter size and decreased postnatal weight or weight gain (11). However, because of the very limited number of postnatal rat studies and controversial results, the reproducibility of these observations cannot be assessed.

Despite Atenolol's anti-hypertensive effect, in this present study, no changes were observed in litter size or postnatal weight of pups of SHR after Atenolol's treatment. Similar results were observed in normotensive Wistar rats' pups. No alteration was detected in pups' weight at 30 days after Atenolol's treatment. The reduced number of pups per breeding and lower weight of pups at 30 days of life of female hypertensive rats have been related to (2-3) and associated with mother rats' hypertension. Probably, the prenatal or postnatal Atenolol's toxicity effects on SHR could not be observed since maternal hypertension by itself can induce changes in utero-placental and fetal hemodynamic as high frequency of prematurity and growth restriction (21-22).

Our results showed that pups of female SHR or Wistar rats treated with Atenolol had no weight reduction, but had lower submandibular gland weight, impaired salivary response to pilocarpine stimulation and a decrease in salivary total protein concentration. All together, these results could suggest Atenolol's toxicity, embryo or postnatal effect, on salivary gland. In SHR pups, in which the salivary hypofunction was observed (3) until treatment, the effect of Atenolol was more evident. Since 70% of total saliva was from the submandibular gland (23), Atenolol's toxicity could explain the reduction in submandibular weight and the impaired salivary response to pilocarpine stimulation. Moreover, Atenolol, which blocks β_1 -adrenergic receptors, would be expected to interfere with secretions of proteins in acinar cells (24). The decrease in protein content in saliva of pups of female rats treated with Atenolol suggests that Atenolol at used dosage blocked salivary β -adrenergic receptors in pups. As previously observed, Atenolol treatment during ontogenesis, related to pregnancy periods, could affect the number and functionality of adrenergic receptors in rats during their lives (12).

Although the total protein concentration in saliva of Atenolol-treated pups was reduced, differences were not observed ($p>0.05$) in the amylase activity when compared with untreated animals. Probably, the concentrations of other proteins could be altered.

The effect of Atenolol on salivary activity was also observed in clinical studies. It has been established that intense 7-day Atenolol treatment in healthy young volunteer men did not alter the salivary flow (non-stimulated or stimulated by 3% citric acid

solution), nevertheless, it reduced the concentration of total proteins in saliva, amylase and peroxidase activities, and altered the calculated ratios of sialic acid/hexosamine and hexosamine/total protein, and the concentration of calcium and potassium (25-26).

However, new experiments shall be conducted in order to determine which proteins are associated with salivary activity reduction, specifically of submandibular glands, observed in pups of Atenolol-treated rats. Alterations in submandibular gland activity have been related to interferences in the dental remineralization process. (27).

In conclusion, Atenolol, an effective anti-hypertensive, selectively reduces submandibular gland weight in pups of hypertensive SHR rats and normotensive Wistar rats treated during pregnancy and lactation, with no alteration in parotid gland and sublingual gland weight. Reductions in salivary flow and total concentration of proteins, however with no change as regards salivary amylase activity, observed in pups were also associated with Atenolol-based treatment.

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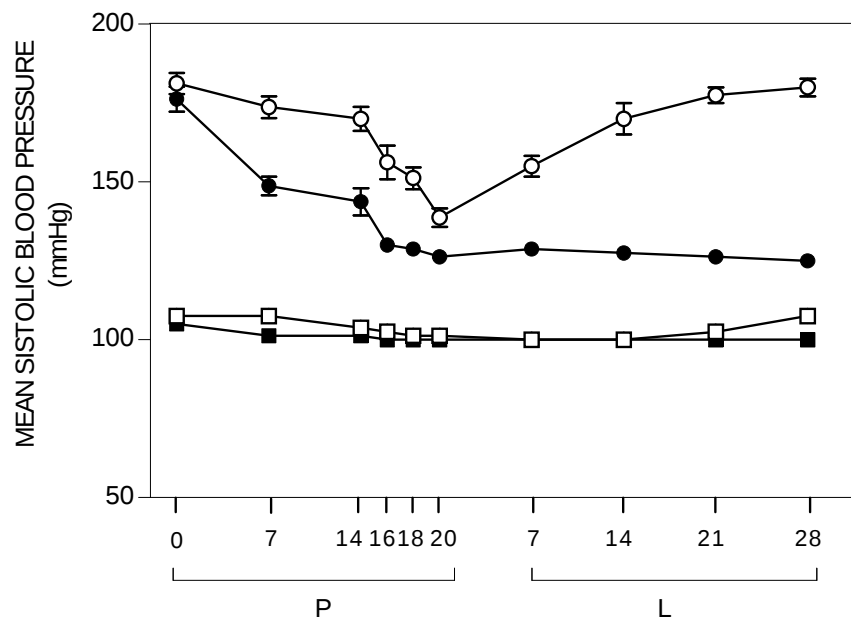


Figure 1- Systolic blood pressure (mmHg) of Wistar rats (□, n=10), SHR (○, n=10), Wistar treated (■, n=10) and SHR (●, n=10) treated with atenolol during pregnancy (P) and lactation (L). Symbols represent mean $\pm$ SEM of systolic blood pressure (ANOVA, $p < 0.05$)

Table 1

Body weight of wistar and SHR female rats during pregnancy and lactation, number of pups per breeding, weight of 30-day-old male pups

	Wistar Control	Atenolol-treated Wistar rats	SHR Control	Atenolol- treated SHR
Female (n=10) body weight during pregnancy	277.5g $\pm$ 4.73 ^a	301.3g $\pm$ 8.21 ^b	229.7g $\pm$ 3.57 ^c	216.5g $\pm$ 4.26 ^c
Female (n=10) body weight during lactation	277.6g $\pm$ 4.36 ^a	262.3g $\pm$ 7.35 ^a	223.3g $\pm$ 2.09 ^b	206.1g $\pm$ 3.97 ^b
Number of pups per breeding (n=10)	9.4 $\pm$ 0.68 ^a	8.6 $\pm$ 1.11 ^{a,b}	5.8 $\pm$ 0.82 ^b	6.3 $\pm$ 0.66 ^b
Pups' weight at 30 days of life	59.3g $\pm$ 5.6 ^a	49.8 g $\pm$ 4.9 ^a	32.8g $\pm$ 1.8 ^b	38.1g $\pm$ 2.3 ^b

The results are expressed as means $\pm$ SEM. The different letters show significant differences between the groups of the same line (p<0.05 by ANOVA)

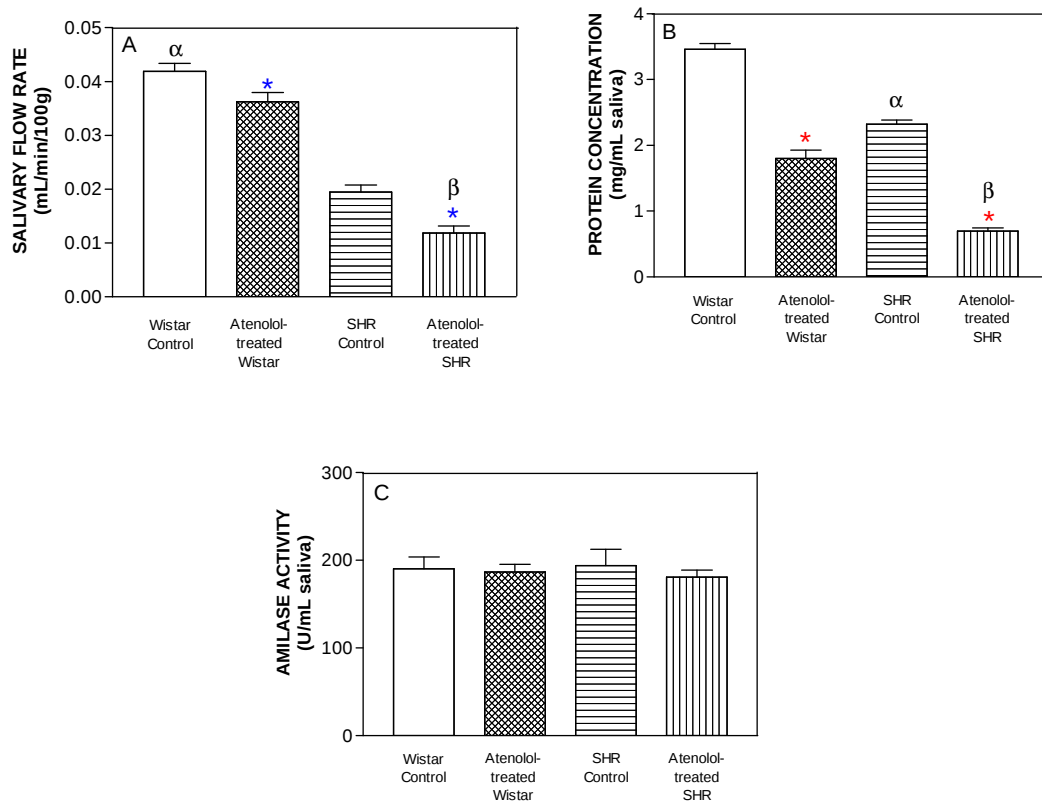


Figure 2. Salivary flow rate (A), salivary protein concentration (B) and salivary amylase activity (C) in the saliva of Wistar control (n=10) and SHR control (n=10), Atenolol-treated Wistar (n=10), Atenolol-treated SHR (n=10). The bars represent the mean $\pm$ SEM. * $p < 0,05$ (ANOVA) between Wistar control and Atenolol-treated wistar or SHR control and Atenolol-treated SHR, α $p < 0,05$ (ANOVA) between Wistar control and SHR control, β $p < 0,05$ (ANOVA) between Atenolol-treated wistar and Atenolol-treated SHR

Table 2

Weight of pups' submandibular, parotid and sublingual glands

GROUPS	Salivary Glands		
	Submandibular	Parotid	Sublingual
Wistar pups (n=10)	0.3208g $\pm$ 0.015 ^a	0.4372g $\pm$ 0.038 ^a	0.0708g $\pm$ 0.004 ^a
Atenolol-treated Wistar pups (n=10)	0.2239g $\pm$ 0.009 ^b	0.3376g $\pm$ 0.030 ^a	0.0717g $\pm$ 0.004 ^a
SHR pups (n=10)	0.3013g $\pm$ 0.012 ^a	0.4006g $\pm$ 0.018 ^a	0.0710g $\pm$ 0.004 ^a
Atenolol-treated SHR pups (n=10)	0.2059g $\pm$ 0.004 ^b	0.3736g $\pm$ 0.011 ^a	0.0811g $\pm$ 0.007 ^a

The results are expressed as means $\pm$ SEM. The different letters show significant differences between the groups of the same column ($p < 0,05$ by ANOVA)

Capítulo 4

Imunomarcacão da metaloproteinase de matriz (MMP-9) em dentes incisivos de filhotes de ratas espontaneamente hipertensas (SHR) tratadas ou não com atenolol

Imunomarcção da metaloproteinase de matriz (MMP-9) em dentes incisivos de filhotes de ratas espontaneamente hipertensas (SHR) tratadas ou não com atenolol

RESUMO

Objetivo: Neste trabalho foi avaliada a expressão da metaloproteinase de matriz (MMP-9) em dentes incisivos (DI) de filhotes de ratas espontaneamente hipertensas (fSHR) tratadas ou não com atenolol (At, 100mg/Kg/dia, v.o.) durante a prenhez e lactação. *Métodos:* Os filhotes (30 dias) foram sacrificados e nos DI a expressão da MMP-9 foi determinada através de reações imunohistoquímicas (imunoperoxidase). *Resultados:* MMP-9 foi observada em ameloblastos (AM), superfície externa de formação do esmalte (SEFE), odontoblastos (OD) e pré-dentina (PD) dos DI. A imunomarcção para MMP-9 em AM e SEFE foi maior em DI de fSHR do que em filhotes de ratas normotensas (fW). Nenhuma diferença entre os grupos foi observada em OD e PD. At não alterou a expressão da MMP-9 em AM, SEFE, PD e OD em DI de fSHR. Após o tratamento com atenolol, menor expressão foi observada em OD de fW do que de fSHR e uma diferença na expressão na SEFE foi observada dentro do grupo Wistar. Na PD, a imunomarcção para MMP-9 após o At não diferiu entre os grupos. *Conclusão:* Estes dados sugerem que a maior marcação para MMP-9 nos AM e SEFE de incisivos de fSHR poderia estar associada à reduzida microdureza do esmalte observada anteriormente nos dentes destes animais. No entanto, esta associação não poderia ser estendida à dentina. Como o At não interferiu na expressão da MMP-9 em AM, SEFE, PD e OD de fSHR, outros

mecanismos devem ser atribuídos ao aumento da microdureza observado após o tratamento com atenolol em DI de fSHR.

Palavras - chave: MMP-9, Dureza, Atenolol, SHR

1. Introdução

O processo de formação dental (odontogênese) e os fatores intrínsecos e extrínsecos que podem interferir neste processo em ratos SHR não têm sido alvo de estudo. Poucos trabalhos avaliando a estrutura dental de ratos hipertensos são descritos na literatura. Anormalidades morfológicas nos dentes de ratos SHR-SP (stroke prone), decorrentes do subdesenvolvimento de ameloblastos, sugerem um modelo de amelogênese imperfeita¹ e uma redução na microdureza do esmalte e dentina de incisivos de filhotes SHR foi observada em estudo recente.² Foi demonstrado que o tratamento de ratas SHR durante a prenhez e lactação com atenolol, um bloqueador β_1 -adrenérgico utilizado como anti-hipertensivo, aumenta a microdureza do esmalte e da dentina em incisivos e molares de filhotes.³ No entanto, os mecanismos envolvidos nestas alterações não foram totalmente explicitados.

A amelogênese em dentes de ratos envolve uma cascata de eventos que tem início com a secreção da matriz orgânica, rica em amelogenina, e termina com a mineralização do esmalte quando a matriz orgânica é fortemente reduzida.⁴ A amelogenina é a proteína mais abundante durante a formação do esmalte e é hidrolizada em poucas horas⁵ após sua secreção na matriz, juntamente com outras

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proteínas, pela ação das metaloproteinases da matriz (MMPs), que regulam o processo de mineralização e influenciam a formação dos tecidos dentais.⁶ As MMPs degradam uma série de componentes da matriz extracelular.⁷ Coletivamente, elas são capazes de degradar todas as proteínas da matriz.⁸ Estudos de proteinases presentes no desenvolvimento do esmalte sugerem que as MMPs são mais importantes durante a formação inicial do esmalte e as serinas proteases estariam envolvidas nos estágios finais da maturação.^{9,10,11,12}

As MMPs também estão ativas no início da mineralização da dentina, degradando as sialoproteínas dentinárias, que são as proteínas mais abundantes neste tecido.¹³ Quando a dentina e o esmalte estão em processo de formação, duas gelatinases (MMP-2 e MMP-9) e a estromelisina-1 (MMP-3) estão presentes, com distribuições específicas, sugerindo implicações funcionais para cada uma delas^{6,7,14} nestes processos.

Embora estudos recentes tenham determinado a localização e a expressão destas MMPs nos tecidos dentais de humanos e animais, a interferência de patologias sistêmicas como a hipertensão ou da terapia anti-hipertensiva sobre a expressão das metaloproteinases da matriz, ainda não foi determinada.

O objetivo deste trabalho foi avaliar, através de reações imunohistoquímicas, o envolvimento da MMP-9, que é expressa durante a amelogênese e a dentinogênese de ratos, na microdureza dental de filhotes de ratas SHR tratadas ou não com atenolol durante o período de prenhez e lactação. Parte-se do pressuposto

que a inibição das MMPs prejudica a formação e a mineralização dos tecidos dentais.

2. Material e Métodos

2.1. Animais

Os experimentos foram realizados utilizando filhotes machos de ratas espontaneamente hipertensas (SHR) e de ratas normotensas Wistar (NT), tratadas ou não com atenolol durante os períodos de prenhez e lactação. O rato espontaneamente hipertenso foi utilizado neste experimento, pois é o modelo animal com características hemodinâmicas que mais se aproxima da hipertensão arterial essencial observada em humanos.¹⁵ Estes animais nascem normotensos e desenvolvem espontaneamente, ou sem nenhuma causa aparente pré-determinante, um aumento da pressão arterial (PA) a partir da 8ª semana de nascimento, aumento este que pode chegar a valores próximos de 200 mmHg com 12 semanas de idade.^{16, 17, 18}

Os ratos SHR (machos e fêmeas) foram obtidos de uma cepa mantida pelo biotério do Departamento de Ciências Básicas da FOA-UNESP e os ratos Wistar (machos e fêmeas) foram fornecidos pelo biotério do Câmpus da Faculdade de Odontologia de Araçatuba (FOA-UNESP). Os animais foram mantidos em condições controladas de temperatura (22-24°C) com ciclo de luz (12 horas claro-escuro, luzes acesas: 08:00 a.m.) no biotério do referido departamento, recebendo ração padrão

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*"ad libitum"*. Os experimentos conduzidos neste trabalho foram aprovados pelo Comitê de Ética na Experimentação Animal da Faculdade de Odontologia Júlio de Mesquita Filho, FOA-UNESP, Araçatuba-SP (protocolo nº: 37/03) e conduzidos de acordo com as normas para o uso de animais da Federação das Sociedades de Biologia Experimental (FeSBE) e sociedades internacionais de fisiologia para pesquisa envolvendo animais. Os animais foram monitorados para que não sofressem nenhuma experiência de estresse ou desconforto.

## ***2.2. Tratamento com Atenolol***

Ratas fêmeas adultas SHR e Wistar foram colocadas para o acasalamento com os respectivos machos. O dia zero da prenhez foi detectado pela presença de espermatozóides no esfregaço vaginal das ratas. O tratamento das ratas com atenolol (100mg/kg/dia, v.o.) foi realizado durante todo o período de prenhez (21 dias) e lactação (30 dias). A massa de atenolol administrada foi determinada diariamente a partir do peso das ratas e diluída no volume de água pré-determinado. Os grupos não tratados receberam o mesmo volume de água (sem atenolol) das ratas tratadas.

## ***2.3. Pressão Arterial***

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A medida da pressão arterial sistólica (PAS) das ratas foi realizada pelo método indireto de pletismografia de cauda, com a utilização de pletismógrafo adaptado para medidas em ratos Physiograph® MK-III-S, NBS – Narco Bio-Systems (Houston, Texas, USA). Os registros foram realizados por um único operador, no período da manhã, em laboratório específico com controle de luminosidade e ruídos para ser evitado o estresse dos animais. Foram usadas ratas espontaneamente hipertensas (SHR) com pressão arterial sistólica igual ou superior a 150 mmHg e ratas normotensas Wistar com pressão arterial próxima a 112 mmHg. Os registros de pressão foram feitos em dias pré-determinados durante a prenhez (dia 0, 7, 14, 16, 18, 20) e a lactação (dia 7, 14, 21, 28).

### ***2.4. Remoção dos Dentes***

Filhotes machos foram sacrificados por excesso de anestesia, aos 30 dias de vida para a remoção das hemi-mandíbulas inferiores. Os dentes incisivos e molares de uma hemi-mandíbula de cada animal foram separados para a avaliação da microdureza dental. A outra hemi-mandíbula de cada filhote foi mantida íntegra e utilizada para a análise imunohistoquímica dos dentes incisivos.

### ***2.5. Avaliação Histológica e Imunohistoquímica dos tecidos dentais***

#### **2.5.1. Processamento Histológico**

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Imediatamente após a remoção das hemi-mandíbulas (n=5 por grupo), elas foram imersas em solução formalina 10% tamponada durante 48 horas para a fixação dos tecidos. Após este período as peças foram lavadas em água corrente por 8 horas e conservadas em álcool 70% até serem desidratadas totalmente e incluídas em parafina. Após a inclusão, as peças foram cortadas com uma espessura de 5 µm. A coloração das peças foi feita com Hematoxilina e Eosina.

### 2.5.2. Técnica Imunohistoquímica

A presença da proteína MMP-9 nos tecidos e células dentais de incisivos inferiores de filhotes SHR e Wistar foi determinada através de reações imunohistoquímicas, utilizando a imunoperoxidase como método de detecção. Após a inclusão em parafina, secções semi-seriadas de 5µm de hemi-mandíbulas foram colocadas sobre lâminas silanizadas (Erviegas) e desparafinizadas. O bloqueio da atividade da peroxidase endógena foi realizado com água oxigenada 3% (Perhidrol 30% H<sub>2</sub>O<sub>2</sub> - MERCK) por 40 minutos e as secções sofreram recuperação antigênica por tampão citrato (pH 6,0 – 95°C). Na seqüência, foi realizada a incubação do anticorpo primário na titulação de 1:50 em câmara úmida, a 4°C, por 18hs. Utilizou-se o anticorpo contra MMP-9, policlonal produzido em cabras (C-20: sc-6840, Santa Cruz Biotechnology, Inc.). Como anticorpo secundário foi utilizado o immunoPure® Rabbit Anti-Goat IgG, (H+L), biotin conjugated (PIERCE Biotechnology). Tanto o anticorpo primário quanto o secundário foram diluídos com solução de PBS + albumina

bovina. Em seguida, foi realizada a incubação com o complexo Avidina-Biotina (Strept ABComplex/HRP-Dako) por 45 min, com o intuito de amplificar o sinal das marcações. O cromógeno utilizado para revelar a reação foi a 3,3-diaminobenzidina-HCl / substrato (DAKO) e a contra-coloração nuclear foi realizada com Hematoxilina de Harri's.

## ***2.6. Análise Estatística***

As lâminas que sofreram o processamento imunohistoquímico foram analisadas em microscópio óptico e ao padrão de distribuição da imunocoloração foram atribuídos escores 0 (sem marcação), 1 (marcação fraca/difusa), 2 (marcação moderada) e 3 (marcação intensa), a fim de avaliar a presença da proteína MMP-9 nos dentes estudados. Os escores foram determinados por três observadores distintos e a média dos valores obtidos foi realizada para cada tecido (esmalte, pré-dentina e dentina), célula dental (ameloblastos, odontoblastos) ou região avaliada (superfície externa de formação do esmalte). Os resultados foram submetidos à análise estatística com o auxílio do programa estatístico GMC versão 2002 (teste não paramétrico de Kruskal-Wallis,  $p < 0.05$ ).

### 3. Resultados

Durante todo o experimento, as ratas SHR não tratadas apresentaram os maiores valores de PAS (Fig.1) A partir do início do tratamento com o atenolol, houve uma queda na PAS das ratas SHR, que pode ser bem visualizada ao 7º dia de prenhez, e a partir do 18º dia de prenhez quando a PAS se manteve baixa até o final da lactação. Pode-se constatar (Fig.1), a eficácia do efeito anti-hipertensivo do atenolol nas ratas SHR. Embora fosse observada uma queda significativa na PAS das ratas SHR tratadas, sua pressão não atingiu a normalidade observada em ratas Wistar, ou seja, os níveis pressóricos continuaram mais elevados ( $p < 0.05$ ) que os de ratas Wistar tratadas ou não com atenolol. Analisando as ratas Wistar normotensas, diferenças não foram observadas ( $p > 0.05$ ) nos valores de PAS entre os dois grupos, tratado e não tratado, demonstrando que o atenolol não possui efeito anti-hipertensivo nestes animais.

A análise histológica das lâminas proporcionou a avaliação de uma série de tecidos e células envolvidas na formação dental. Pode-se observar que os vasos sanguíneos da polpa seguiram um padrão de vascularização e a organização deste tecido que pode ser classificada como rudimentar nesta fase (30 dias de vida) em todos os grupos avaliados. Os ameloblastos apresentaram morfologia semelhante nos quatro grupos. Observou-se a formação de alças capilares na camada odontoblástica, que apresentou um aspecto de normalidade em suas células (odontoblastos) e nenhuma diferença evidente entre os grupos. Analisando as camadas de esmalte e dentina, com suas respectivas células (ameloblastos e

odontoblastos) e o tecido pulpar (inclusive os vasos sanguíneos) pode-se observar uma característica de homogeneidade e semelhança ao padrão de normalidade (Fig.2).

Quanto à presença da MMP-9 nas células e tecidos dos incisivos (Fig.2), foi observada maior imunomarcacão ( $p < 0.05$ ) em ameloblastos e na superfície externa de formação do esmalte (SEFE) de filhotes SHR em comparacão aos filhotes Wistar (Fig.2 e 3). Nenhuma diferenca nas marcações de MMP-9 em odontoblastos ou na pré-dentina foi detectada entre os grupos (Fig.2 e 3).

O tratamento com atenolol não alterou a expressão desta metaloproteinase nas células secretoras da matriz do esmalte, na SEFE, em odontoblastos e na pré-dentina dos incisivos de filhotes SHR (Fig.2 e 3). Porém, após o tratamento com atenolol, diferenças na expressão da MMP-9 na SEFE e em odontoblastos foram observadas entre filhotes Wistar e SHR. O tratamento com atenolol aumentou ( $p < 0,05$ ) a marcação da MMP-9 na SEFE dos dentes incisivos de filhotes Wistar, efeito que pôde ser visualizado ao serem comparados os resultados entre filhotes Wistar tratados e não tratados (Fig.3D). Em odontoblastos, menor imunomarcacão ( $p < 0.05$ ) foi detectada em filhotes de ratas Wistar tratadas quando comparados aos filhotes de ratas SHR tratadas (Fig.3B). Na pré-dentina, não houve diferenca na expressão desta proteína entre os quatro grupos (Fig.3C).

#### 4. Discussão

Ratos espontaneamente hipertensos são os modelos experimentais mais semelhantes à hipertensão essencial humana.<sup>15</sup> Estes animais desenvolvem espontaneamente um aumento da pressão arterial a partir da oitava semana de nascimento e os valores pressóricos podem alcançar 200 mmHg com doze semanas de idade.<sup>16,17,18</sup> Na Figura 1 é possível visualizar que o modelo de hipertensão utilizado é adequado para este estudo, uma vez que a PAS dos animais manteve níveis mais elevados do que os observados em ratos Wistar normotensos ( $p < 0.05$ ) durante todo o período experimental. Uma vez que a pressão arterial das ratas SHR se manteve elevada, as alterações observadas nos tecidos dentais dos filhotes SHR aos 30 dias de vida podem ser relacionadas com mais segurança ao quadro hipertensivo. Também pode ser observado o eficaz efeito do atenolol no controle da pressão arterial<sup>19,20,21</sup> de ratas hipertensas, embora estas ainda permanecessem com níveis pressóricos mais elevados do que as ratas normotensas. Nenhuma queda significativa foi encontrada na PAS de ratas Wistar após o tratamento com atenolol, indicando que a terapia com beta-bloqueador não altera a PAS de ratas normotensas.<sup>22</sup>

Como o atenolol é um beta-bloqueador que atravessa a barreira placentária e é excretado no leite materno,<sup>19,20,21</sup> os filhotes SHR e Wistar ficaram expostos ao seu efeito durante todo o experimento, nos períodos de prenhez e lactação quando as mães foram tratadas.

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Dentes incisivos de ratos SHR-SP apresentam alterações em sua estrutura, expressando uma coloração mais esbranquiçada e menor resistência ao desgaste.<sup>1</sup> Recentemente foi demonstrado que filhotes de ratas SHR apresentam redução na microdureza do esmalte e dentina em dentes incisivos.<sup>2</sup> Como estes modelos são modelos animais de hipertensão arterial, pode-se suspeitar que essa alteração sistêmica esteja relacionada ao aparecimento de defeitos dentais. Estudos em humanos demonstraram que o diâmetro da coroa dos dentes decíduos e permanentes de filhos de mães hipertensas é reduzido,<sup>23</sup> sugerindo mais uma alteração dental relacionada ao quadro hipertensivo. Evidências clínicas e estudos experimentais têm correlacionado defeitos de mineralização dental com variações nos níveis plasmáticos de cálcio. Foi demonstrado em SHR, que a hipertensão é acompanhada por uma redução na homeostase de cálcio sérico ionizado.<sup>24</sup> A hipocalcemia pode ser decorrente de um menor transporte ativo de cálcio da mãe para o feto através da placenta, de distúrbios perinatais diversos ou alterações na concentração de paratormônio (PTH) plasmático.<sup>25</sup> Em SHR, foi demonstrado que a hipocalcemia leva ao aumento do paratormônio.<sup>24</sup> O mecanismo envolvido na alteração da formação dental de SHR não está bem esclarecido, mas tanto a hipocalcemia quanto o aumento do PTH podem estar diretamente envolvidos com esta alteração, uma vez que receptores para o PTH foram encontrados em odontoblastos, determinando a existência de um controle hormonal na odontogênese.<sup>26,27</sup> Embora não tenha sido determinada a presença de receptores

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específicos para o PTH em ameloblastos, este hormônio é capaz de afetar a citodiferenciação dos ameloblastos alterando a função e a maturação desta célula.<sup>26</sup>

A análise histológica das lâminas dos dentes dos filhotes tratados ou não com atenolol proporcionou a avaliação de uma série de tecidos e células envolvidas na formação dental, contudo morfologia semelhante foi observada nos quatro grupos experimentais. Analisando as camadas de esmalte e dentina, com suas respectivas células, ameloblastos e odontoblastos, e o tecido pulpar (inclusive os vasos sanguíneos) pode-se observar uma característica de homogeneidade e semelhança ao padrão de normalidade.

As reações imunohistoquímicas permitiram observar que a presença da proteína MMP-9 nos tecidos e células dentais avaliados segue o padrão descrito na literatura.<sup>7</sup> Uma forte marcação positiva desta metaloproteinase foi observada em ameloblastos e nenhuma imunomarcação foi detectada em esmalte e dentina. Imunomarcação mais fraca foi observada em odontoblastos e pré-dentina, no entanto, uma forte marcação foi vista na parte externa do esmalte na grande maioria dos animais, fato que difere dos descritos por alguns autores.<sup>7</sup> Pode-se observar que as marcações positivas para MMP-9 em ameloblastos, odontoblastos, pré-dentina e na parte externa do esmalte, seguiram os dados da literatura,<sup>6,13,28</sup> embora variações na sua expressão pudessem ser detectadas.

Ao avaliar a presença da proteína MMP-9 em ameloblastos, pode ser evidenciada uma diferença significativa entre filhotes SHR e Wistar, sendo maior a marcação positiva em ameloblastos de filhotes SHR. Como esta metaloproteinase

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tem a função de degradar as proteínas do esmalte favorecendo a posterior mineralização,<sup>7</sup> era de esperar que o aumento na imunomarcção para esta proteína coincidisse com uma maior remoção de matriz orgânica e a melhor deposição mineral, favorecendo assim a mineralização. No entanto, foi observada<sup>2</sup> uma menor microdureza de esmalte em dentes incisivos de filhotes SHR. Estes resultados reforçam a hipótese de que um desequilíbrio no mecanismo de deposição de cálcio esteja ocorrendo. Uma grande remoção de matriz orgânica se processa em filhotes SHR, no entanto a deposição de minerais não estaria ocorrendo de maneira adequada e na mesma velocidade da degradação da matriz. As alterações nos níveis de PTH e na concentração de cálcio no organismo destes animais podem estar interferindo ativamente no processo de mineralização do esmalte de incisivos destes filhotes. Apesar do papel da superfície externa de formação do esmalte não ser bem definido, também é percebido um aumento na imunomarcção para MMP-9 em filhotes SHR.

Ao avaliar os odontoblastos e a pré-dentina dos incisivos nenhuma diferença na imunomarcção para MMP-9 foi observada entre filhotes Wistar e SHR. Entretanto, uma importante redução na microdureza da dentina foi observada em incisivos de filhotes SHR.<sup>2</sup> Provavelmente, esta alteração na microdureza poderia estar relacionada a distúrbios na regulação da deposição mineral, inclusive associados ao PTH.

Embora os resultados sejam sugestivos, é preciso ter em mente que nem sempre a presença da proteína no tecido significa atividade da mesma. Uma

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proteína pode estar expressa, mas em sua fase inativa. Para alguns autores, as MMPs são em sua maioria produzidas na forma latente, forma não-ativa, e sua ativação se dá posteriormente.<sup>29</sup> Ainda é preciso considerar que existem outras MMPs envolvidas na formação do esmalte e da dentina de incisivos de ratos e que estas também podem estar contribuindo, em maior ou menor grau, para as alterações observadas neste estudo. Estudos específicos para avaliações na expressão de outras MMPs, como a MMP-20 e a MMP-2 devem ser realizados neste modelo animal.

Avaliando os resultados da expressão da MMP-9 após o tratamento com atenolol pode-se perceber que este medicamento não alterou a expressão desta proteína em ameloblastos e na SEFE de filhotes SHR, não alterou a expressão em ameloblastos de filhotes Wistar, mas aumentou significativamente a expressão da MMP-9 na SEFE de filhotes Wistar. Apesar deste efeito do atenolol ser seletivo sobre a MMP-9 da SEFE de filhotes Wistar, este resultado sugere que o atenolol interfere na expressão de metaloproteínases. Provavelmente este efeito não foi observado em ameloblastos, pois nestas estruturas a marcação da MMP-9 é bastante intensa e alterações sutis não são significativas. No entanto, o tratamento de ratas com atenolol aumenta a microdureza do esmalte de dentes incisivos tanto em filhotes SHR como em filhotes Wistar.<sup>3</sup> Possivelmente, as alterações observadas são decorrentes do efeito indireto do atenolol sobre um dos mecanismos que regula a mineralização do esmalte. Como os filhotes SHR apresentaram maior imunomarcagem para MMP-9, isso sugere uma remoção maior de matriz orgânica do

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esmalte, criando uma área disponível para a mineralização. O atenolol, por sua vez, atuaria indiretamente disponibilizando maior concentração de minerais para o aumento observado na mineralização do esmalte. Estes dois fatores unidos poderiam ser responsáveis pela maior microdureza do esmalte observada em filhotes SHR. O mecanismo através do qual o atenolol promove o aumento da mineralização dental ainda não foi estabelecido, porém sabe-se que as paratireóides possuem inervação adrenérgica e a secreção de paratormônio pode ser inibida pelos beta-bloqueadores.<sup>30</sup> Estudos específicos devem ser conduzidos para se determinar com clareza os mecanismos moleculares alterados pelo atenolol, que estariam associados ao processo de mineralização dental.

Na pré-dentina dos dentes incisivos nenhuma diferença na imunomarcção para MMP-9 foi observada entre os quatro grupos, sugerindo que esta estrutura não poderia ser correlacionada com a diferença de microdureza da dentina observada entre os grupos antes e após o tratamento com atenolol.<sup>3</sup> Em odontoblastos, diferenças na marcação positiva para MMP-9 somente foram observadas ao serem comparados filhotes SHR e Wistar tratados. Não foram observadas diferenças entre filhotes tratados e não tratados no mesmo grupo. A partir destes resultados pode-se suspeitar que o atenolol também aumenta a imunomarcção para MMP-9 em odontoblastos de filhotes SHR. A degradação protéica ocorre em menor proporção na dentina do que no esmalte, no entanto alterações ocorridas em nível de odontoblastos podem ser expressas no grau de mineralização do tecido dentinário.

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Em estudo prospectivo foi observado que o atenolol aumenta a microdureza de dentina de dentes incisivos de filhotes de ratas SHR tratadas.<sup>3</sup>

No presente estudo, a presença da proteína MMP-9 nos tecidos dentais dos molares não foi avaliada, uma vez que o processo de descalcificação pelo qual o dente passa antes de ser incluído em parafina remove toda camada de esmalte presente. Outro fator levado em conta foi o estágio de formação dental dos molares no período do experimento. Como o molar não apresenta o processo de crescimento contínuo visto nos incisivos, aos 30 dias de vida, quando os dentes foram removidos, o esmalte já se encontrava totalmente formado e não mais era possível detectar a presença de ameloblastos secretores, uma das principais células avaliadas neste estudo e que expressam significativamente a MMP-9.<sup>7</sup> Assim, a menor microdureza do esmalte e dentina observada em dentes molares de filhotes SHR<sup>2</sup> não poderá ser correlacionada com a expressão de MMP-9 nas estruturas avaliadas.

Em conclusão, filhotes SHR apresentam maior expressão de MMP-9 nos ameloblastos e na superfície externa de formação do esmalte de incisivos do que filhotes Wistar, o que pode estar contribuindo para a reduzida microdureza dental observada no esmalte destes dentes. O atenolol interfere no processo de mineralização dental dos incisivos e aumenta a expressão da MMP-9 em odontoblastos de filhotes SHR em comparação aos filhotes de ratas Wistar tratadas. O mecanismo envolvido na menor mineralização dental de filhotes SHR e através do qual o atenolol promove as alterações minerais observadas na microdureza dental

tanto em filhotes Wistar como SHR ainda deve ser determinado, no entanto há uma forte tendência para o envolvimento do hormônio PTH na regulação deste processo.

### **Agradecimentos**

Este estudo recebeu apoio da CAPES, sendo o primeiro autor deste trabalho beneficiado com uma bolsa de estudos. Nós expressamos nosso especial agradecimento ao Prof Dr José Mauro Granjeiro e ao pós-graduando Flávio Amado pela colaboração no processamento das reações imunohistoquímicas.

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\* A formatação deste capítulo segue as normas da revista *Archives of Oral Biology*.

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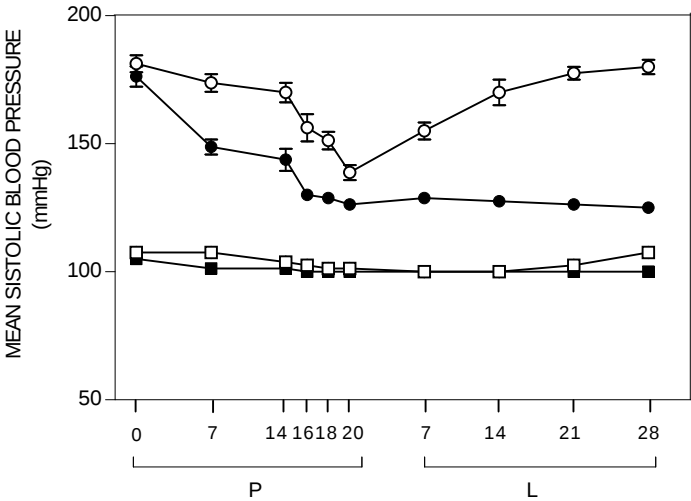


Fig. 1- Systolic blood pressure (mmHg) of Wistar rats ( $\square$ ,  $n=10$ ), SHR ( $\circ$ ,  $n=10$ ), Wistar treated ( $\blacksquare$ ,  $n=10$ ) and SHR ( $\bullet$ ,  $n=10$ ) treated with atenolol during pregnancy (P) and lactation (L). Symbols represent mean  $\pm$  SEM of systolic blood pressure (ANOVA,  $p<0.05$ ).

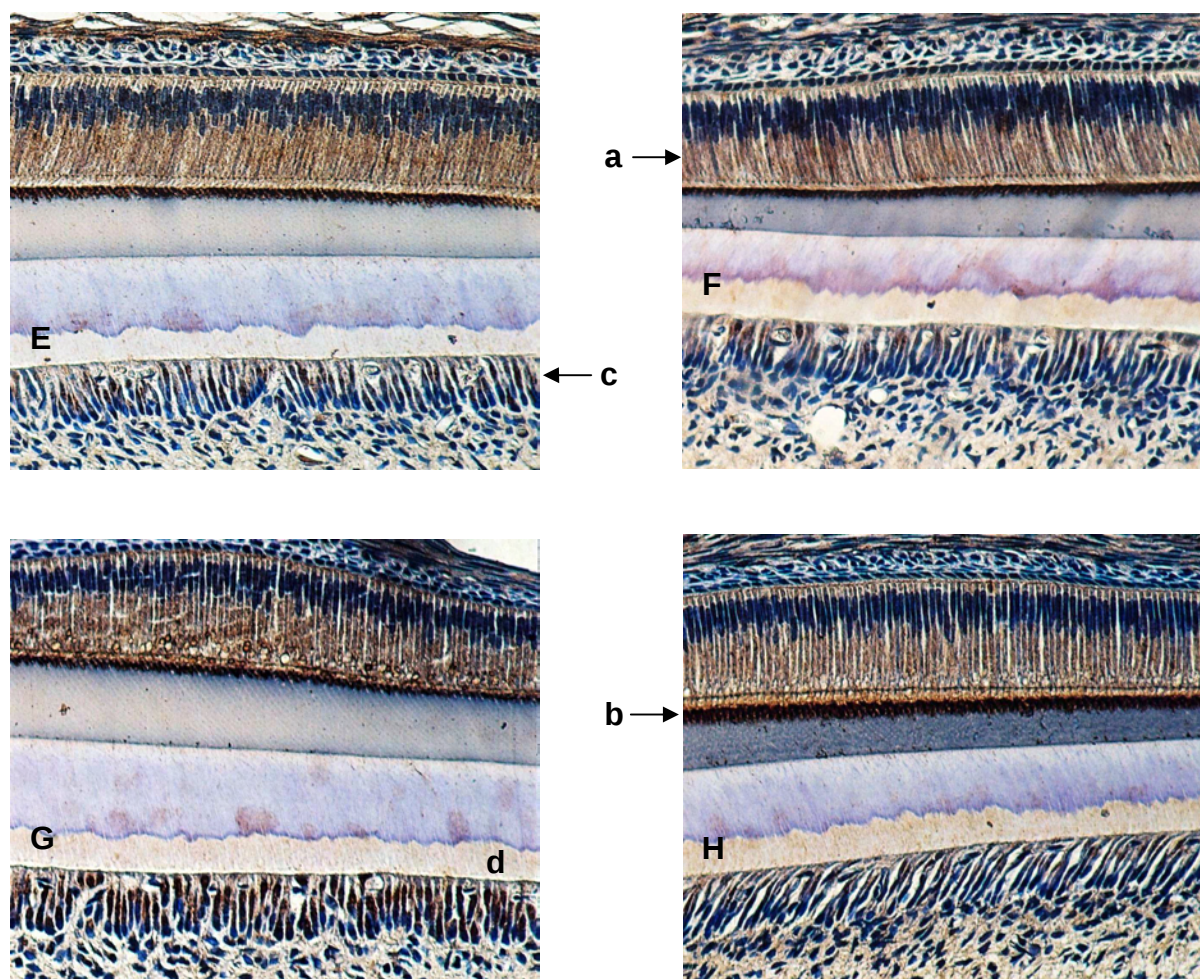
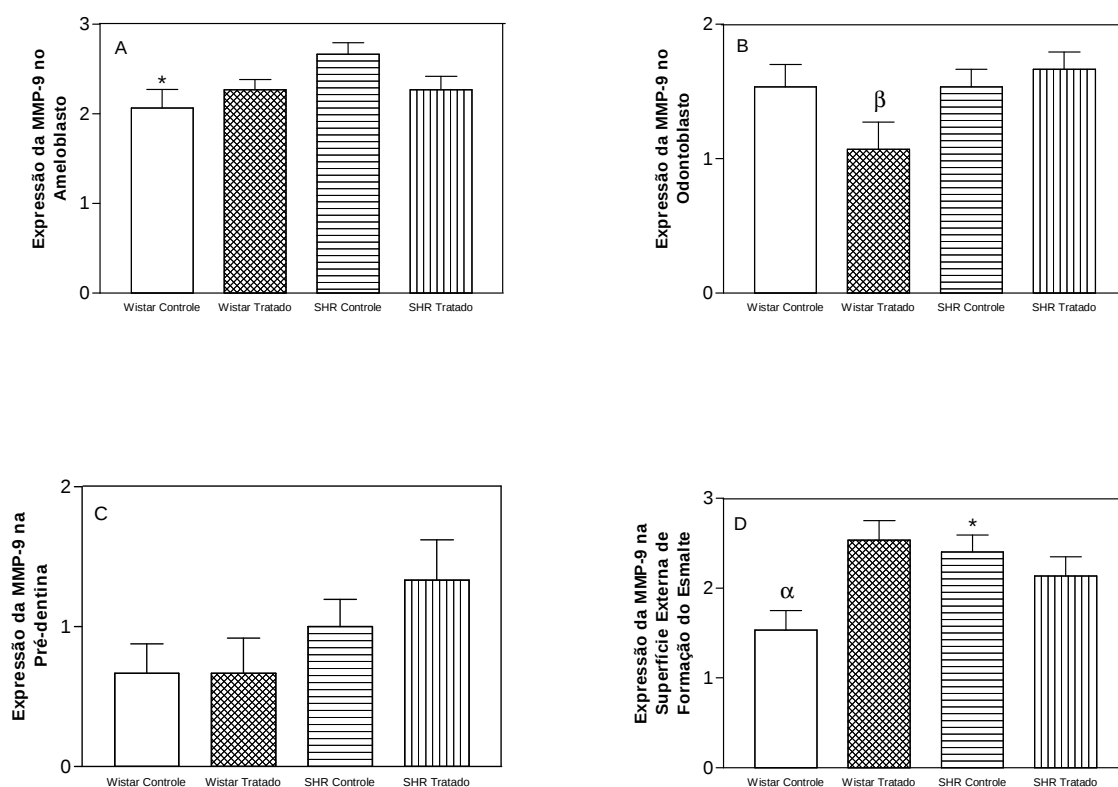


Fig. 2 – Imunomarcacão da metaloproteinase de matriz (MMP-9) em a) ameloblastos, b) superfície externa de formação do esmalte, c) odontoblastos e d) pré-dentina de incisivos de filhotes de ratas SHR (E) e Wistar (F) sem tratamento e filhotes de ratas SHR (G) e Wistar (H) tratadas com atenolol (160x)



**Fig. 3 - Expressão da MMP-9 nos ameloblastos (A), odontoblastos (B), pré-dentina (C) e superfície externa do esmalte (D) dos dentes incisivos de filhotes (n=5 por grupo) de ratas Wistar e SHR tratadas ou não com atenolol. As barras representam a média  $\pm$  EPM da expressão da MMP-9 nas diferentes áreas do dente. \* diferença estatística dos valores entre os grupos controle,  $\beta$  diferença estatística dos valores entre os grupos tratados,  $\alpha$  diferença estatística dos valores no grupo ( $p < 0,05$  – Kruskal-Wallis)**

# Conclusões

## Conclusões

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A partir do estudo realizado e dos resultados obtidos podemos concluir que:

- Filhotes SHR, aos 30 dias de vida, apresentam menor fluxo e concentração de proteínas salivares e menor microdureza de dentes incisivos e molares.
- A hipertensão materna estaria relacionada com as alterações bucais observadas nos filhotes SHR.
- O tratamento das ratas durante os períodos de prenhez e lactação com  $\beta$ -bloqueador atenolol (100 mg/Kg/dia, via oral) reduz os valores de pressão arterial sistólica de ratas SHR, porém não altera estes valores em ratas Wistar.
- O tratamento das ratas com atenolol durante os períodos de prenhez e lactação reduz o peso das glândulas submandibulares dos filhotes SHR e Wistar.
- O tratamento com atenolol durante os períodos de prenhez e lactação reduz o fluxo e a concentração de proteínas salivares em filhotes SHR.
- O tratamento com atenolol durante os períodos estudados aumenta a mineralização do esmalte e dentina de dentes incisivos de filhotes SHR e Wistar.

## Conclusões

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- Não foram observadas diferenças entre as estruturas morfológicas de dentes incisivos e molares de filhotes SHR e Wistar.
- Reações imunohistoquímicas para metaloproteinases demonstraram que filhotes SHR apresentam maior expressão da MMP-9 nos ameloblastos e na parte externa de formação do esmalte de dentes incisivos. Nenhuma alteração na expressão de MMP-9 foi observada em odontoblastos e na pré-dentina de dentes incisivos de filhotes SHR.
- Estudos complementares deverão ser realizados para determinar os mecanismos envolvidos nas alterações de atividade salivar e de microdureza dental observadas em filhotes SHR e a possível correlação destas alterações com a doença cárie.

**A**nexos

## Anexo 1 – Aprovação do Comitê de Ética

**unesp**  UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"  
CAMPUS DE ARAÇATUBA – FACULDADE DE ODONTOLOGIA  
CURSOS DE ODONTOLOGIA E DE MEDICINA VETERINÁRIA

**COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL**  
**(CEEa)**

**CERTIFICADO**

Certificamos que o Projeto "Efeito do atenolol sobre a saliva e a microdureza do esmalte de dentes de filhotes de ratas SHR tratadas" sob responsabilidade de **CRISTINA ANTONIALI SILVA e GRACIELI PRADO ELIAS** está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela CEEa em reunião de 07/07/2003, de acordo com o protocolo nº 37/03.

Araçatuba, 07 de julho de 2003.

  
Prof.ª Ass. Dra. Adelina Maria da Silva  
Presidente

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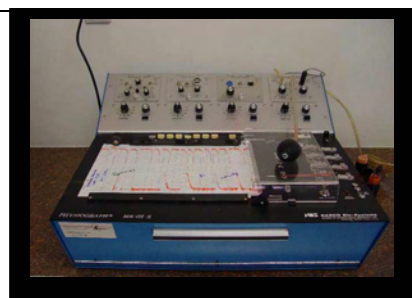
## Anexo 3 – Metodologia

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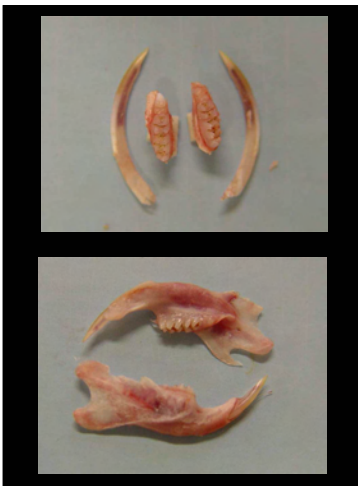
1. Microdurômetro Shimadzu Micro Hardness Tester HMV-2.000 (Shimadzu Corporation - Kyoto-Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMSWIN (NewAge Industries, USA).

2. Pletismógrafo (Plethysmograph Physiograph® MK-III-S / NBS – Narco Bio-Systems / Houston, Texas, USA).

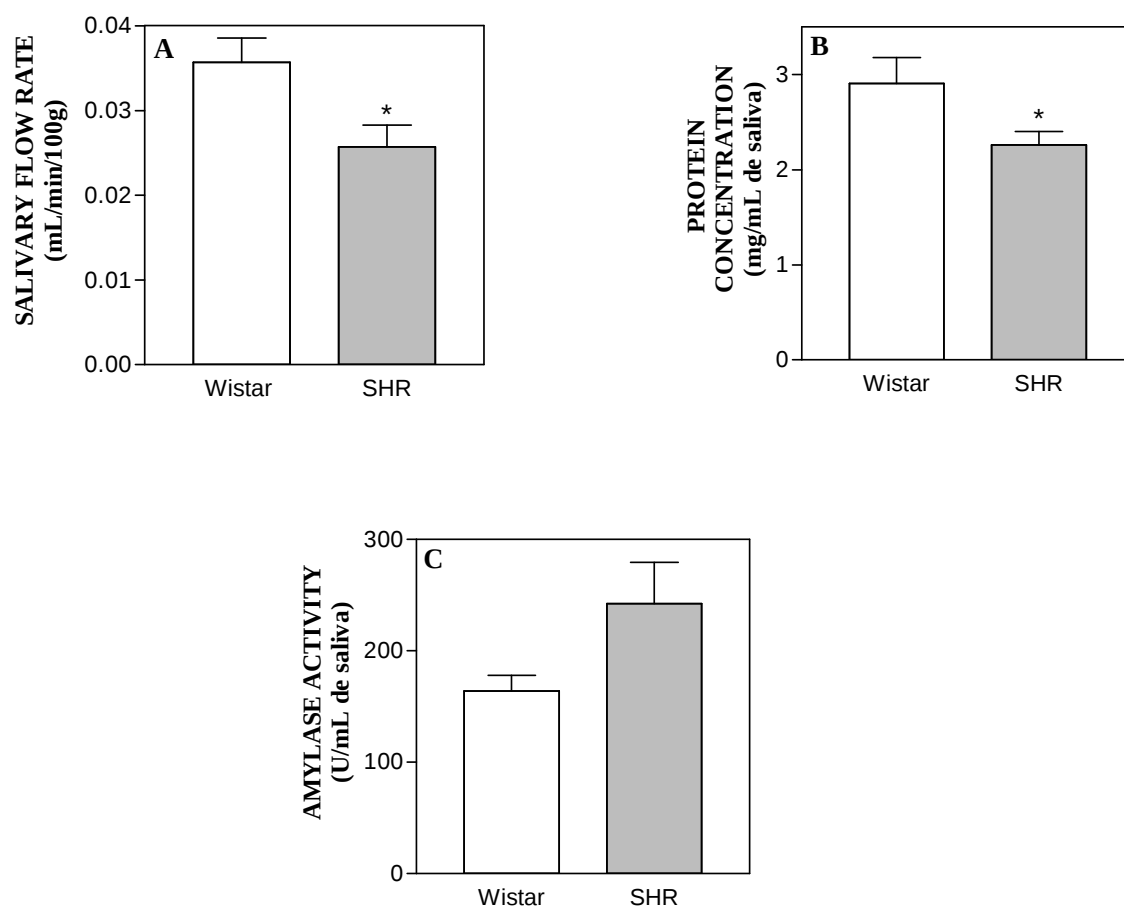


3. Espectrofotômetro (Hitachi® U-1100 Spectrophotometer)

## Anexo 3 – Metodologia

|                                                                                     |                                                                                                                           |
|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|
|    | <p>4. Coleta de saliva usando pranchas inclinadas</p>                                                                     |
| <p>5. Remoção das glândulas submandibulares, parótidas e sublinguais</p>            |                                        |
|  | <p>6. Remoção dos dentes incisivos, molares e hemi-mandíbulas para análise da microdureza dental e expressão da MMP-9</p> |

## Anexo 4 – Figura 3 do Capítulo 1



**FIGURE 3-** Salivary flow rate (A), salivary protein concentration (B) and salivary amylase activity (C) in the saliva of Wistar offspring (n=8) and SHR offspring (n=15). The bars represent the mean  $\pm$  SEM of salivary flow rate, salivary protein concentration and salivary amylase activity in the saliva of evaluated offspring. \*Significant difference of salivary flow and salivary protein concentration between Wistar offspring and SHR offspring ( $p < 0,05$  by unpaired t test between both groups)