UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

FRANCIELLY ANDRESSA FELIPETTI

AVALIAÇÃO DO EFEITO SISTÊMICO DO LÁTEX DA *HANCORNIA SPECIOSA* SOBRE A NEOFORMAÇÃO E A MINERALIZAÇÃO ÓSSEA

EVALUATION OF THE SYSTEMIC EFFECT OF *HANCORNIA SPECIOSA* LATEX ON BONE NEOFORMATION AND MINERALIZATION

PIRACICABA
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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Buco-Dental, área de Histologia e Embriologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Biology, in Histology and Embryology area.

Orientador: Prof. Dr. Pedro Duarte Novaes

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RESUMO

A *Hancornia speciosa* Gomes é uma árvore frutífera encontrada no Nordeste do Brasil que é utilizada popularmente para o tratamento de doenças. Alguns pesquisadores investigaram o benefício do uso do látex da *H. speciosa* e demonstraram que este possui propriedades anti-inflamatórias e antifúngicas. O grupo de pesquisa da FOP/Unicamp já confirmou que este látex possui também efeito osteogênico quando administrado topicamente sobre o defeito da calvária de ratos. Baseado nesses resultados, o presente projeto teve como proposta realizar novos experimentos *in vivo* para investigar o efeito sistêmico deste látex sobre a neoformação e a mineralização óssea de ratos. Para isso, o látex da *H. speciosa* foi coletado, diluído a 50% e analisado por colorimetria para verificar a presença de Ca e P. Em seguida, 25 ratos *Wistar* passaram por exodontia do incisivo inferior esquerdo e confecção de defeito de 2mm de diâmetro na calvária. Os ratos foram divididos em dois grupos (controle sistêmico – CS e látex sistêmico - XS) e, diariamente, após jejum de 5 horas, os grupos receberam, respectivamente, administrações orais em dose única de 1,5 mL de água e 1,5 mL de látex a 50% por gavagem. Ao longo do experimento, o peso corporal foi registrado. Após 15 dias, os ratos foram eutanasiados e as amostras foram coletadas. O sangue foi centrifugado e analisado por colorimetria para verificar a concentração plasmática de Ca e P. A calvária, a mandíbula e o estômago foram submetidos ao processamento histológico convencional e analisados por microscopia de luz para verificar o estágio de regeneração óssea, a área de osso neoformado e a morfologia estomacal. A mandíbula também foi analisada por MEV/EDX para verificar a composição elementar e a proporção mineral do osso basal e neoformado no alvéolo. Os dados obtidos foram comparados entre os grupos e submetidos ao *T-test* ou *Welch test* com $p<0.05$. Os resultados mostraram que o látex da *H. speciosa* a 50% apresentou cálcio em sua composição. Administrações orais e diárias deste látex durante 15 dias aumentou o conteúdo de Ca e P do osso basal e do osso neoformado no alvéolo mandibular de ratos sem, contudo, alterar o peso corporal, a morfologia estomacal e a concentração plasmática de Ca e P. Por outro lado, o produto não contribuiu para o aumento da área de osso neoformado nem na

calvária e nem no alvéolo. Este foi um estudo pioneiro que demonstrou a potencialidade do látex da *H. speciosa* em aumentar a mineralização óssea. Esses achados podem auxiliar na concepção e desenvolvimento de fármaco natural para prevenção de fraturas.

Palavras-chave: Apocynaceae. Hidroxiapatita. Histologia. Microscopia eletrônica de varredura. Terapias complementares.

ABSTRACT

Hancornia speciosa Gomes is a fruit northeastern Brazilian tree that is commonly used as an oral therapeutic for treatment of diseases. Researchers have investigated the benefit of using *H. speciosa* latex and demonstrated that it has anti-inflammatory and antifungal properties. The FOP/UNICAMP research group has already confirmed that this latex also has an osteogenic effect when administered topically on the calvarial defect of male rats. Therefore, this study aimed to evaluate the systemic effect of this latex on bone neoformation and mineralization in rats. For this, *H. speciosa* latex was first collected; the latex was then diluted at 50% and, finally analyzed by colorimetry to verify the presence of Ca and P. A total of 25 male *Wistar* rats underwent extraction of the lower left incisor and creation of defect in the left parietal bone (diameter, 2mm). The rats were divided into two groups (systemic control - CS and systemic latex - XS). Daily, the rats were fasted for 5h and were oral administered 1.5 mL of water or 50% latex solution in a single dose by gavage. Their body weight was measured every 5 days. Fifteen days after surgery, the rats were euthanized and their samples were collected. The blood was centrifuged and the plasma Ca and P concentration was analyzed by colorimetry. The calvaria, mandible and stomach were subjected to conventional histological processing, and then the stage of bone regeneration, newly formed bone area and stomach morphology were analyzed by light microscopy. The mandible was also analyzed by SEM-EDX to verify the elemental composition and the mineral proportion of basal and newly formed bone in the mandibular alveolus. The results were statistically analyzed using T-test or Welch test; the level of significance was set at 0.05. We showed that *H. speciosa* latex at 50% presented calcium in its composition. Oral and daily administrations of this latex for 15 days increased the Ca and P content of basal bone and newly formed bone in the mandibular alveolus of rats without, however, altering body weight, stomach morphology and plasma Ca and P concentration. On the other hand, the product did not contribute to the enlargement of the newly formed bone area neither in the calvaria nor in the mandibular alveolus. This was a pioneer study that demonstrated the

potential of *H. speciosa* latex in increasing bone mineralization. These findings may help in the development of a natural drug for fracture prevention.

Key words: Apocynaceae. Complementary therapies. Hydroxyapatites. Histology. Scanning electron microscopy.

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LISTA DE ABREVIATURAS E SIGLAS

CEUA - Comissão de Ética no Uso de Animais

CS - Grupo Controle Sistêmico

EDX - Espectroscopia de Energia Dispersiva

EDTA - Ácido etilenodiamino tetra-acético

ESALQ - Escola Superior da Agricultura Luiz de Queiroz

FOP – Faculdade de Odontologia de Piracicaba

HE – Hematoxilina e eosina

IL-6 - Interleucina-6

OPG - osteoprotegerina

PTH – paratormônio

RANKL - ligante do receptor do ativador do fator nuclear Kappa B

SEM - Microscopia Eletrônica de Varredura

TNF - Fator de necrose tumoral

UNICAMP - Universidade Estadual de Campinas

USP - Universidade de São Paulo

XS - Grupo Látex Sistêmico

LISTA DE SÍMBOLOS

Ca - cálcio

Mg - magnésio

Na - sódio

P – fósforo

SUMÁRIO

1 INTRODUÇÃO

A utilização de plantas para o tratamento de doenças é um método tão antigo quanto a história da humanidade. Desde a era paleolítica, os seres humanos utilizam plantas com propriedades medicinais como remédios para a sobrevivência. Existem relatos da utilização desses métodos datados há milhares de anos antes da civilização cristã em locais como a Índia, a China e o Egito. Na Antiguidade Grega, tais recursos também eram utilizados em associação com os métodos mágico-terapêuticos. Durante o Renascimento, a descoberta de outras terras permitiu que novas drogas e especiarias fossem levadas para a Europa, o que promoveu o aumento da utilização de plantas como disponibilidade terapêutica para essa região. E ainda hoje, na era contemporânea, observa-se uma expansão das pesquisas sobre a utilização das plantas medicinais como matérias-primas de recursos terapêuticos no mundo todo (Saad et al., 2009).

A *Hancornia speciosa* Gomes ou mangabeira, por exemplo, é uma árvore frutífera pertencente à família *Apocynaceae*, nativa do Brasil, e que tem alcançado interesse entre os pesquisadores em razão de seu uso popular e devido a sua possível eficácia no tratamento de doenças (Da Silva Jr e Lédo, 2006). O látex do tronco da mangabeira é popularmente utilizado para o tratamento de indivíduos que sofreram pancadas e quebraduras; que possuem úlceras, vermes, doenças pulmonares, herpes (Da Silva Jr e Lédo, 2006) e para o tratamento de doenças relacionadas com infecções fúngicas (Pott; Pott, 1994). Além disso, a casca desta planta é usada na medicina popular para o tratamento de hipertensão e doenças inflamatórias (Almeida et al., 1998). Baseado nessas crenças populares, alguns pesquisadores se empenharam em investigar, cientificamente, os benefícios causados com o uso da *H. speciosa* para o tratamento de enfermidades:

Moraes et al. (2008) investigaram o efeito do extrato hidroalcoólico da casca da *H. speciosa* em modelos experimentais de ratos e demonstraram que este extrato foi eficaz no combate e na cura de úlceras gástricas devido à sua habilidade em estimular a produção de muco.

Outras pesquisas revelaram que o extrato etanólico das folhas da mangabeira é capaz de causar hipotensão (Serra et al., 2005) e vasodilatação (Ferreira et al., 2007).

Marinho et al. (2011) avaliaram o efeito do látex obtido do tronco da mangabeira em ratos e relataram que a solução deste látex atenuou os sinais

inflamatórios, reduzindo a formação de edema, suprimindo o volume do exsudato e diminuindo a produção de interleucina-6 (IL-6) e fator de necrose tumoral (TNF). Outros pesquisadores relataram que o látex pode ter atividade antifúngica podendo agir contra *Candida albicans* (Da Silva et al., 2011). E, recentemente, o grupo de pesquisa do departamento de Morfologia da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas (FOP/UNICAMP) confirmou que este látex possui também efeitos osteogênicos. Após a aplicação tópica do látex sobre o defeito crítico realizado na calvária de ratos *Wistar* com broca trefina de 3mm, verificou-se aumento da área de osso neoformado no interior do defeito do grupo tratado em comparação ao controle (Dos Santos Neves et al., 2016).

O osso é um tecido conjuntivo mineralizado altamente dinâmico (Guyton et al., 2006; Raggatt and Partridge, 2010; Valenti et al., 2017) que pode servir como amplo reservatório de minerais. Cerca de 99% do cálcio e 85% do fósforo corporal se encontram armazenados nos ossos, sendo liberados em caso de queda da concentração e armazenados, em caso de excessos (Guyton et al., 2006).

As principais células ósseas (osteoblastos, osteoclastos, osteócitos e células de revestimento ósseo) desempenham papel fundamental na remodelação do tecido (Raggatt e Partridge, 2010; Valenti et al., 2017). A função das células de revestimento ósseo ainda não é bem compreendida, mas sabe-se que elas participam da diferenciação de osteoclastos produzindo osteoprotegerina (OPG) e ligante do receptor do ativador do fator nuclear Kappa B (RANKL). Os osteócitos funcionam como orquestradores da remodelação óssea, regulando a atividade dos osteoblastos e osteocastos e promovendo a manutenção da matriz óssea (Florencio-Silva et al., 2015). Os osteoclastos são capazes de secretar ácidos, colagenases e hidrolases responsáveis pela digestão da matriz óssea. E os osteoblastos depositam a matriz orgânica, liberam vesículas com estoque de cálcio e permitem a liberação do íon fosfato (Florencio-Silva et al., 2015; Valenti et al., 2017). O fosfato juntamente com o cálcio formam cristais de hidroxiapatita e mineralizam a matriz óssea, formando o osso novo (Raggatt e Partridge, 2010)(Raggatt and Partridge, 2010). Esse processo de remodelação óssea permite que o osso consiga regenerar-se espontaneamente após o estabelecimento de fraturas, lesões ou defeitos (Thurairajah et al., 2017)(Thurairajah et al., 2017). Porém, em defeitos ósseos extensos, muitas vezes, a regeneração fisiológica é incapaz de reestabelecer a integridade óssea. Nesses casos, outras

terapias complementares são necessárias para auxiliar no processo de reparo (Ereno et al., 2010; Walmsley et al., 2016).

A característica dinâmica e a capacidade de regeneração espontânea do tecido ósseo, a recente descoberta sobre o potencial osteogênico da aplicação local do látex da *Hancornia speciosa*, o intenso uso popular do “leite da mangaba” para o tratamento de fraturas e a falta de estudos sobre seus efeitos medicinais motivou-nos a investigar sua ação sistêmica sobre o reparo óssea.

Dessa forma, o objetivo geral desse trabalho foi avaliar o efeito sistêmico do látex da *H. speciosa* sobre a neoformação e a mineralização óssea de ratos *Wistar* através dos seguintes objetivos específicos:

- Verificar a presença de Ca e P no látex da *H. speciosa* através do método colorimétrico usando, respectivamente, Arsenazo III e Ácido Molíbdico;

- Examinar a concentração plasmática de Ca e P através do método colorimétrico usando, respectivamente, Cálcio Arsenazo III e Fósforo UV;

- Fazer o acompanhamento do peso corporal dos ratos a cada 5 dias de tratamento com látex da *H. speciosa*;

- Mensurar a área de osso novo formado na região do defeito de 2mm da calvária e na região do alvéolo mandibular;

- Descrever o estágio de regeneração óssea do defeito da calvária e do alvéolo mandibular bem como descrever a morfologia estomacal;

- Registrar a composição e a proporção elementar dos elementos que constituem a matriz óssea do osso basal e do osso novo formado no alvéolo dental usando Microscópio Eletrônica de Varredura (MEV) acoplado à Espectroscopia de Energia Dispersiva (*EDS*).

2 ARTIGO: *Hancornia speciosa* Gomes latex increases bone mineralization in rats

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Francielly Andressa Felipetti; Juliana dos Santos Neves; Ingrid Grazielle Sousa; Sônia Maria De Stefano Piedade; Pedro Duarte Novaes.

Abstract

Bone is a dynamic tissue capable of spontaneous regeneration. However, with extensive defects, physiological regeneration may be unable to reestablish bone integrity. Studies have been conducted to find an innovative biomaterial that can support bone repair process. Recently, researchers have suggested that latex from *Hancornia speciosa* Gomes exhibits this potential. Folk medicine practitioners have been using this product to treat fractures; however, no systemic studies have been conducted to confirm its efficacy. Therefore, this study aimed to evaluate the systemic effect of *H. speciosa* latex on bone neoformation and mineralization in rats. For that, *H. speciosa* latex was first collected and its composition was analyzed to verify the presence of calcium (Ca) and phosphorus (P) by colorimetry. A total of 25 male *Wistar* rats were used in this study; they simultaneously underwent two surgical procedures: extraction of the lower left incisor and creation of a 2-mm-diameter defect in the left parietal bone. The rats were divided into two groups (systemic control - CS and systemic latex - XS). After 5 h of fasting, the rats in respective groups were oral and daily administered 1.5 mL of water or 50% latex by gavage. Their body weight was measured every 5 days. After 15 days post treatment, the animals were euthanized, and their samples were collected. The blood was centrifuged and the plasma Ca and P concentrations were analyzed by colorimetry. The calvaria, mandible and stomach were subjected to conventional histological processing; then, the stage of bone regeneration, newly formed bone area and stomach morphology were analyzed by light microscopy. The mandible was also subjected to SEM-EDX method and, basal and newly formed bone in the mandibular alveolus was analyzed to verify their elemental composition and mineral proportion. The results were statistically analyzed using the t test or Welch test; the level of significance was set at 0.05. We showed that

H. speciosa latex contained Ca. Oral and daily administrations of this latex for 15 days increased Ca and P contents of basal bone and newly formed bone in the mandibular alveolus of rats without altering body weight, stomach morphology and plasma Ca and P concentration. On the other hand, the product did not contribute to the enlargement of the newly formed bone area neither in the calvaria nor in the mandibular alveolus. This was a pioneer study demonstrating the potential of *H. speciosa* latex in increasing bone mineralization. These findings may help in the development of a natural drug for fracture prevention.

Key words: Apocynaceae. Complementary therapies. Histology. Hydroxyapatites. Scanning electron microscopy.

1 Introduction

Bone is a highly dynamic mineralized connective tissue. The main cells of the bone, such as osteoblasts, osteoclasts, and osteocytes are essential for its remodeling (Raggatt and Partridge, 2010; Valenti et al., 2017). In the early stages of bone production, the osteoblasts deposit the organic matrix, release vesicles with Ca stores and induce pyrophosphate degradation, thus releasing phosphate ions (Florencio-Silva et al., 2015; Guyton et al., 2006; Valenti et al., 2017). Phosphate and Ca form hydroxyapatite crystals and mineralize the bone matrix, forming the new bone (Raggatt and Partridge, 2010). Through this process of bone remodeling, bone can spontaneously regenerate after the establishment of fractures, lesions or defects (Thurairajah et al., 2017). However, physiological regeneration is unable to restore bone integrity in extensive bone defects, where the repair process must be supported by some complementary therapies (Ereno et al., 2010; Walmsley et al., 2016).

The latex obtained from the trunk of *Hevea brasiliensis* (a native Brazilian tree known as Seringueira) can be an innovative biomaterial for bone repair. Some experiments that have been conducted with this product include the bone repair of dental sockets in rats (Balabanian et al., 2006), guided regeneration of calvaria rabbits (Ereno et al., 2010), dental implant osseointegration in dogs (Manfrin Arnez et al., 2012), and bone repair of a critical calvarial defect in rats (Issa et al., 2012a; Issa et al., 2012b).

Latex extracted from the trunk of *Hancornia speciosa* Gomes (another native Brazilian tree known as Mangabeira, belonging to the Apocynaceae family) also

demonstrated bone repair potential. Search group from Piracicaba Dental School (FOP/UNICAMP) demonstrated that topical application of *H. speciosa* latex on the calvarial defect of *Wistar* rats increased the area of newly formed bone on the borders of the defect (Dos Santos Neves et al., 2016).

Some communities located in northeastern Brazil have produced a milky juice called “leite da mangaba” from *H. speciosa* trunk latex. Ethnobotanical surveys have confirmed the use of this milky juice for therapeutic treatment of bone fractures (Silva Jr and Lédo, 2006). However, to date, no study has been conducted to confirm this effect.

In summary, these findings motivated us to investigate the therapeutic potential of *H. speciosa* latex. This study reports the systemic effect of *H. speciosa* latex on bone neoformation and mineralization in Wistar rats.

2 Materials and methods

2.1 Latex collection and analysis

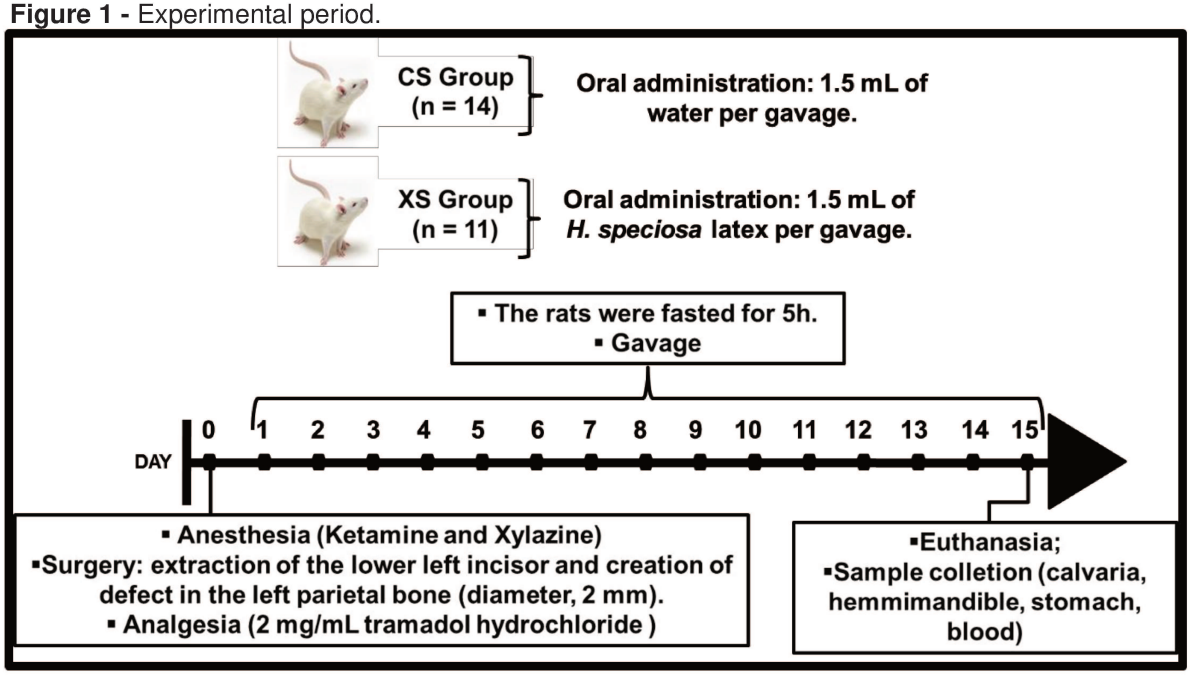
H. speciosa latex was collected from Mata de São João, Bahia, Brazil (12º27’42’’S 37º56’38’’O 69NE) in the summer. A voucher specimen was deposited at Luiz de Queiroz College of Agriculture (ESALQ) - University of Sao Paulo (USP) and received the number ESA 121,402. After drilling about ten tree trunk, 250 mL of the latex that dripped out was collected in a sterilized vessel and mixed with distilled water at a ratio of 1:1 to obtain a 50% latex solution. The solution was stored in syringes, which was first sealed, then protected from light and stocked in ice-cold styrofoam for transport. Finally, the syringes were stored at 4°C.

H. speciosa latex was diluted 11x and subjected to colorimetry for examining the presence of Ca and P. This method was based on the reaction of Ca and P with Arsenazo III (Sigma®, Sigma-Aldrich INC, St. Louis, MO, USA) and Molybdic acid (Sigma®, Sigma-Aldrich INC, St. Louis, MO, USA), respectively. The blue color intensity produced by the reaction was proportional to the amount of Ca or P in the sample. Microplate spectrophotometer reader (Multiskan, Thermo Scientific), at a wavelength of 650 nm, measured the blue color intensity and recorded the amount of Ca and P (mM).

2.2 Animals

A total of 25 male *Wistar* rats (average body weight, 390 g; age, 10 weeks) were used. The sample size was calculated from the data obtained with the pilot project (protocol number: 34271 – *Anexo 2*) with the formula: $n = (N[S]^2 [t]^2) / (N(Ex-)^2 + [S]^2 [t]^2$. The rats were housed in individual cages under standard conditions of temperature and light (12:12 h light/dark cycle). All animals received water and rodent feed *ad libitum.* Before surgery, the rats were anesthetized with an intraperitoneal injection of 80 mg/Kg ketamine (Dopalen®, Sespo Indústria e Comércio Ltda, Paulínia, SP, Brazil) and 8 mg/Kg xylazine (Rompun, Bayer SA, São Paulo, SP, Brazil). Two surgical procedures were simultaneously performed: the extraction of the lower left incisor and creation of defect in the left parietal bone (diameter, 2 mm). After surgery, the rats were intramuscularly administered 2 mg/mL tramadol hydrochloride (Tramal®, Grunenthal of Brazil Pharmaceutical Ltda, São Paulo, SP, Brazil) and rested for 24 h. Then, they were divided into two groups: systemic control (CS, n = 14) and systemic latex (XS, n = 11). Daily, the rats were fasted for 5 h and were administered 1.5 mL of water or 50% latex solution by gavage. Their body weight was measured every 5 days. Fifteen days after surgery (Figure 1), all rats were euthanized, and their calvaria, hemimandible, stomach and blood were collected. All the procedures were approved by the Ethics Committee on the Use of Animals of UNICAMP (CEUA/UNICAMP) with the protocol number 3790-1.

Figure 1 - Experimental period.



2.3 Blood analysis

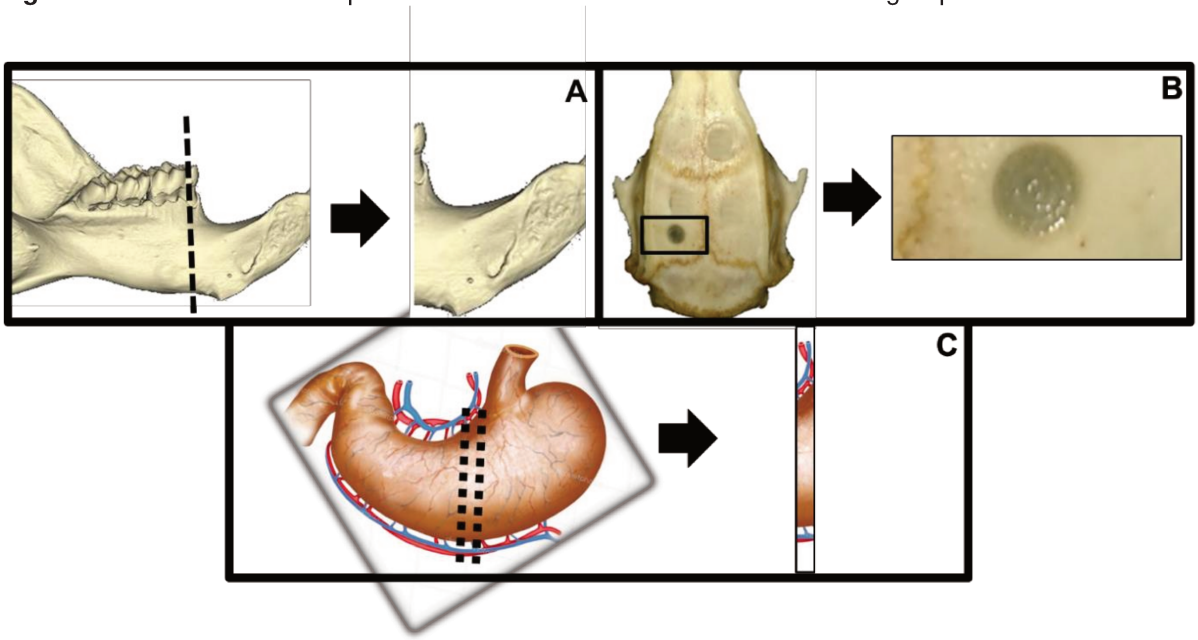
From each rat, a total of 1 mL of blood was collected in heparinized polypropylene tubes and centrifuged at 3000 g-force (or 5000 rpm) for 10 min at 4°C. After centrifugation, 450 µL of the supernatant containing plasma was collected using a micropipette and stored in another tube at -80ºC. The plasma was thawed prior to analysis. Ca and P concentrations were examined by colorimetric method using a Calcium Arsenazo III Kit (Bioclin®, Quibasa Basic Chemistry Ltda, Belo Horizonte MG, Brazil) and Phosphorus UV (Bioclin®) with BS 120-Mindray/Bioclin automation equipment. Ca and P concentrations were recorded in mg/dL and compared between the groups.

2.4 Histological analysis

2.4.1 Cleavage of the samples and histological processing

The stomach, hemimandible, and calvaria were fixed in Karnovski solution and subjected to conventional histological processing (Molinaro, 2010). First, the stomach was cut in halves, transverse to its greater curvature. Then, another incision was made in the body of the stomach and a ring-shaped portion was collected, which was reserved for further processing (Figure 2C). Second, the hemimandible was sectioned at the level of the mesial surface of the first molar and two fragments (anterior and posterior) were obtained (Figure 2A). Third, the calvariae were transversely sectioned and only the bone portion was obtained. Subsequently, the anterior fragment of the hemimandible and calvaria were decalcified with ethylene diamine tetra acetic acid (EDTA) solution at 4%, pH 7.4, for 1 month with two daily exchanges. After decalcification, the calvaria was cleaved and only the bone defect region was preserved for further processing (Figure 2B). Next, the ring-shaped portion of the stomach, bone defect of the calvaria, and the anterior fragment of the hemimandible were dehydrated, diaphanized, and embedded in paraffin to obtain the blocks (Molinaro, 2010).

Figure 2 - Section of the samples collected from the rats of the CS and XS groups.



2.4.2 Histological slides prepared

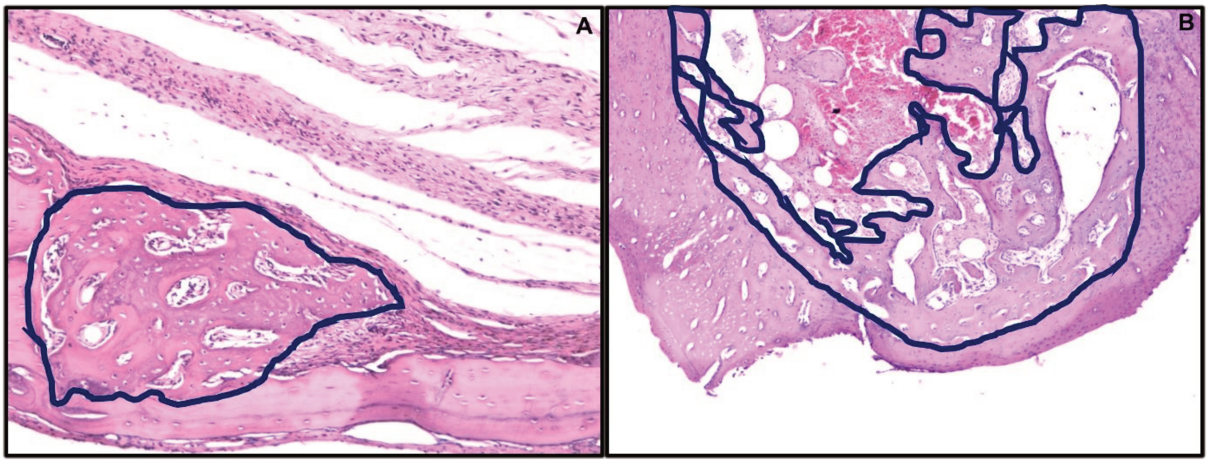
The transverse sections of stomach and mandibular alveolus blocks and coronal sections of calvaria blocks were then collected. All the sections were 6-µm thick. As a standard, eight consecutive sections of stomach blocks were collected and two histological slides were prepared. Also, 48 consecutive sections of mandibular alveolus blocks were collected and 12 histological slides were prepared. Similarly, eight consecutive sections of calvaria blocks were first collected; two histological slides were then prepared and 15 sections were finally discarded; these steps were repeatedly performed until 20 slides were obtained. All slides were stained using hematoxylin and eosin and observed under a light microscope.

2.4.3 Descriptive and histomorphometric analyzes

The slides were photographed using the Optica View 7 program and images were recorded at 50x magnification for the entire mandibular alveolus and at 100x for the stomach layers and entire calvarial bone defect. These images were analyzed using Image J program by a single examiner. The stomach morphology and mandibular alveolus and calvarial repair processes were demonstrated using descriptive analysis. The newly formed bone area within the entire mandibular alveolus and calvarial bone defect were examined using histomorphometric analysis (Figure 3 A and B). For this, a millimeter rules was first photographed at 50x and 100x magnification and then measured from end to end; the distance in pixels was recorded;

the known distance was established according to the size of the ruler (1 mm); and the unit of length was set in mm. This information was registered on each photo of mandibular alveolus and calvarial defect to set scale. Last, a line was drawn by marking all newly formed bone within the mandibular alveolus and calvarial defect, and finally the area of new bone was calculated. The quantified values were compared between the groups.

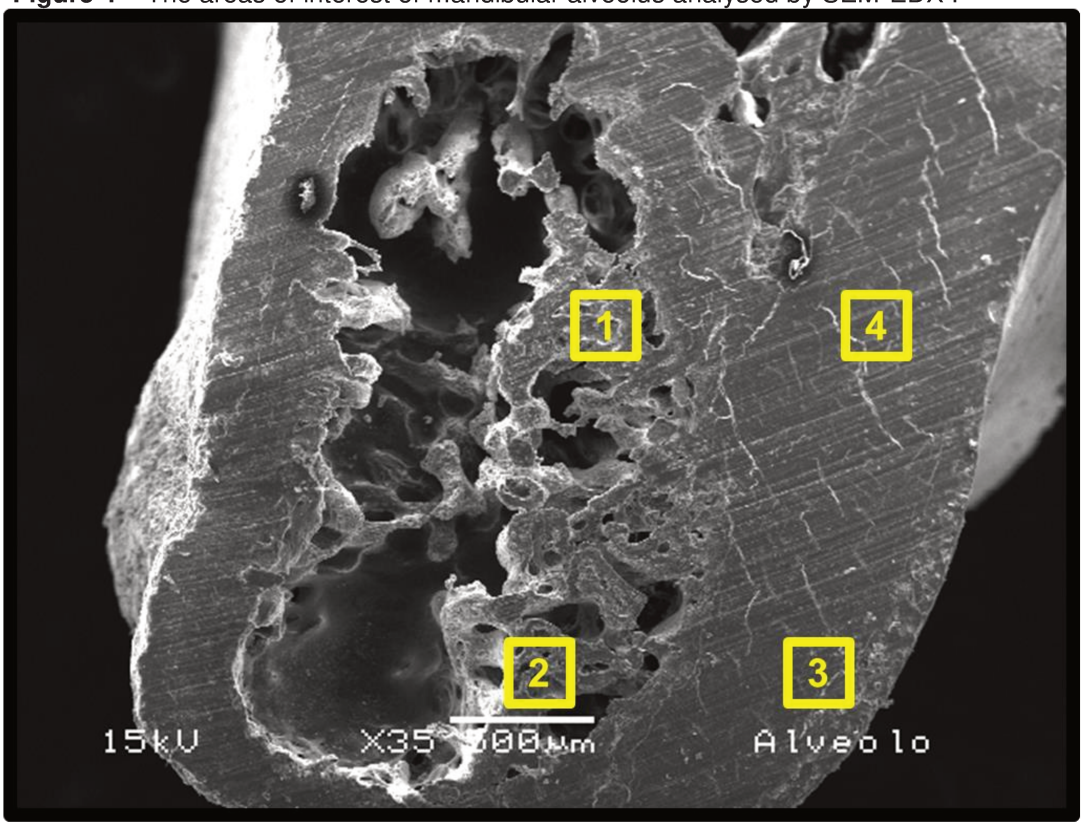
Figure 3 – Histomorphometric analysis of newly formed bone in calvarial defect and mandibular alveolus.



2.5 SEM-EDX analysis

For this analysis the posterior fragment of the hemimandible was used after washing with PBS. The fragment was dehydrated with an increasing ethanol series (50%, 70%, 80%, 95%, 100%), exposed to room temperature for drying, and adapted into stubs. After conductive carbon coverage, the mandibular alveolar region was analyzed SEM-EDX (De Souza, 2011). Once the sample image was acquired in the SEM, the areas of interest were selected: vestibular and proximal regions of the newly formed bone and the basal bone (Figure 3). These regions were examined by EDX at an acceleration voltage of 15 KV, working distance of 20 mm and acquisition time of 100 s. Easymicro program was used to decipher the EDX data. The atomic compositions were recorded, and the calcium (Ca)/phosphorus (P) ratio was calculated. The averages of the atomic content and proportions were compared between the groups.

Figure 4 – The areas of interest of mandibular alveolus analysed by SEM-EDX .



Note: This is a representative image of the CS and XS groups. The areas 1 and 2 indicate newly formed bone and the areas 3 and 4 indicate basal bone.

2.6 Statistical analysis

Data were analyzed using R software. The Bartlett and Shapiro-Wilk tests were used to verify the deviation of the assumptions. Once the homogeneity of variance and normal distribution were confirmed, either t test or Welch test was used to compare CS and XS groups. The animal weight data was evaluated using the mixed linear model. The significance level was set at 5% ($p < 0.05$).

3 Results

3.1 Latex analysis

H. speciosa latex was analyzed by the colorimetric method to verify the concentration of Ca and P. It was not possible to detect the presence of P with this method. But we observed that the *H. speciosa* latex diluted 11x contained 0.1780 mg/mL of Ca.

3.2 Body weight

The body weights of the rats were evaluated every 5 days to verify the influence of the treatment on this parameter. Table 1 compares the body weights between the CS and XS groups over time. We observed that the body weights of the rats in the XS group were equivalent to the body weights of rats in the CS group during the whole experimental period. In addition, rats in both the XS and the CS groups had similar body weights initially as well as after 5, 10 and 15 days. We suggested that neither the treatment nor the duration of the experiment influenced this parameter. In other words, the treatment and the experiment time did not alter the body weight.

Table 1 - Body weights of the rats over the time

Groups	1st day	5th days	10th days	15th days
CS	381.02 ± 8.25 A	361.70 ± 8.89 A	378.38 ± 8.78 A	384.02 ± 11.94 A
XS	404.48 ± 9.65 A	380.49 ± 10.51 A	392.15 ± 11.12 A	402.81 ± 13.39 A

Values are expressed as mean ± standard error. Equivalent letters indicate that there is no statistically significant difference between the groups, as calculated using mixed linear model with $p < 0.05$.

3.3 Plasma Ca and P analysis

In this study we used colorimetric method to analyze heparinized plasma and detected the total Ca and P. Table 2 shows that both groups (CS and XS) had an equal amount (mg/dl) of total Ca and P in blood plasma.

Table 2 – Plasma concentration of calcium and phosphorus in rats.

Groups	Total Calcium (mg/dl)	Total Phosphorus (mg/dl)
CS	8.79 ± 0.83 A	5.37 ± 1.32 A
XS	8.89 ± 0.52 A	5.05 ± 1.19 A

Values are expressed as mean ± standard deviation. Equivalent letters indicate that there is no statistically significant difference between the groups, as calculated using the t test with $p < 0.05$.

3.4 Elemental composition and bone mineralization of the mandibular alveolus

SEM-EDX semiquantitatively analyzes the atomic composition of the specimen (Del Rosario et al., 2015; Perdikouri et al., 2015). The results revealed that the elemental composition of all the evaluated samples and bone regions were similar. The main chemical elements were sodium (Na), magnesium (Mg), P and Ca.

3.4.1 Mineralization of the mandibular alveolus basal bone

We observed similar Na contents (%) in both the groups. However, the content of Mg (%) reduced by approximately 50%, whereas those of Ca (%) and P (%) increased by approximately 20% and 15%, respectively, in the basal bone of the XS group compared with that of the control group (Table 3). The Ca/P ratio in the XS group was high because the increase in Ca content was more than the increase in P content. An increase in the Ca and P contents indicates a higher degree of mineralization.

Table 3 - Semiquantitative chemical analysis (SEM-EDX) of the basal bone in the mandibular alveolus of rats.

Groups	Na (%)	Mg (%)	Ca (%)	P (%)	Ca/P (%)
CS	1.09 ± 0.09 A	0.82 ± 0.05 A	50.54 ± 3.52 A	23.36 ± 1.32 A	2.14 ± 0.03 A
XS	0.82 ± 0.09 A	0.42 ± 0.12 B	63.1 ± 1.93 B	27.33 ± 0.53 B	2.30 ± 0.03 B

Values are expressed as mean ± standard error. The percentages of Na, Ca, and P were evaluated using the t test, and that of Mg using the Welch test. Different letters indicate that the results are statistically different with $p < 0.05$.

3.4.2 Mineralization of the newly formed bone in the mandibular alveolus

Again, in the newly formed bone in the mandibular alveolus, we observed that the Na content was similar in both the groups. However, the content of Mg reduced by approximately 40%, whereas those of Ca and P increased by approximately 19% each in the newly formed bone of the XS group compared with that of the control group (Table 4). The Ca/P ratio was similar for both the groups because of an equal increase in the Ca and P contents. Nevertheless, the newly formed bone of the XS group was more mineralized than that of the CS group due to its higher mineral content.

Table 4 - Semiquantitative chemical analysis (SEM-EDX) of the newly formed bone in the mandibular alveolus of rats.

Groups	Na (%)	Mg (%)	Ca (%)	P (%)	Ca/P (%)
CS	0.98 ± 0.07 A	0.80 ± 0.02 A	51.98 ± 3.79 A	21.82 ± 1.37 A	2.36 ± 0.04 A
XS	0.75 ± 0.09 A	0.47 ± 0.12 B	63.86 ± 1.90 B	26.75 ± 0.53 B	2.38 ± 0.04 A

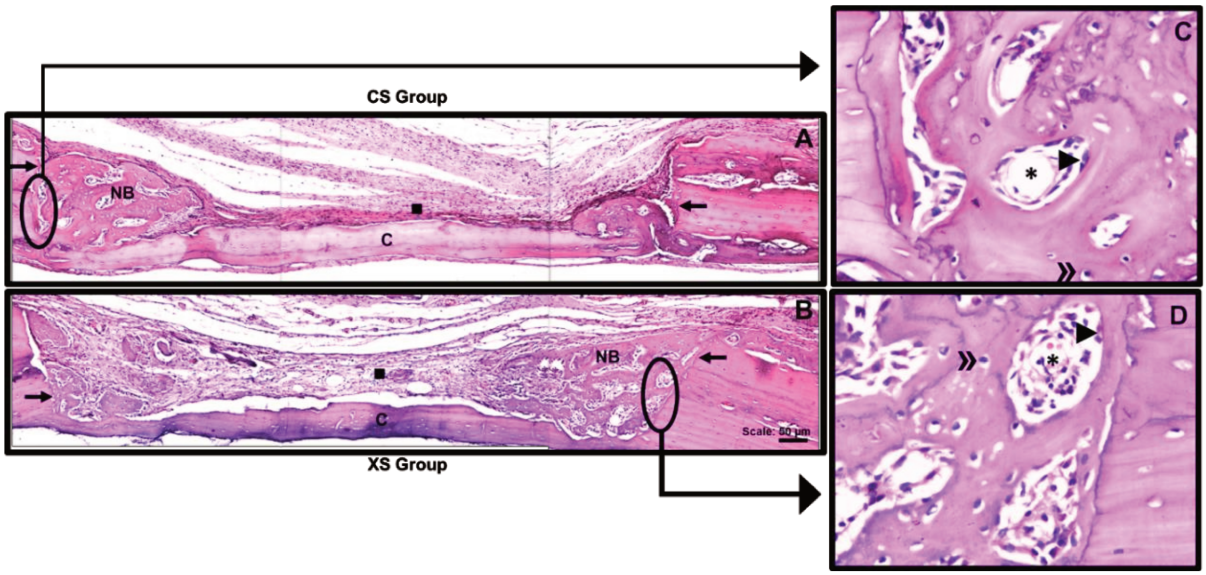
Values are expressed as mean ± standard error. The percentages of Na, Ca and P were evaluated using the t test, and that of Mg using the Welch test. Different letters indicate that the results are statistically different with $p < 0.05$.

3.5 Descriptive histological analysis: mandibular alveolus and calvarial bone defect

The conventional histological technique using light microscopy is widely employed for bone repair analysis. This technique allows a descriptive analysis of the tissue and the measurement of the area of the components of interest (histomorphometry) (Gomes and Fernandes, 2011). We used these analyses to evaluate the newly formed bone in the calvaria defect and in the mandibular alveolus.

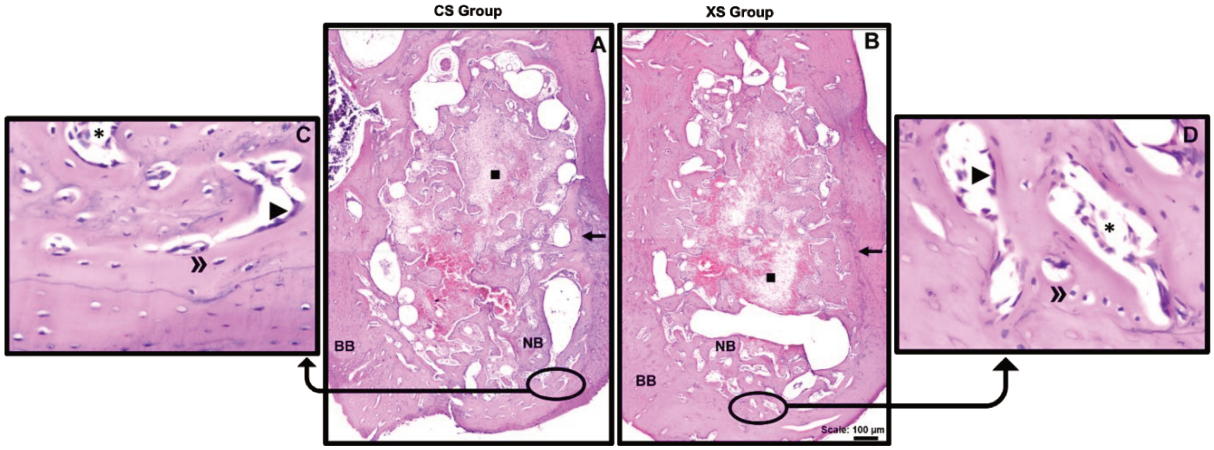
The descriptive analysis revealed a similar bone repair stage between the CS and XS groups. Mineralized trabeculae of the newly formed bone extended from the edges of the basal bone toward the center of the defect (Figure 5) and mandibular alveolus (Figure 6). Several osteocytes were trapped in the bone matrix, and there were marrow spaces filled with blood cells along the trabeculae. Numerous active osteoblasts were noted around the trabeculae. Abundant connective tissue that was widely vascularized filled the central portions of the defect and mandibular alveolus.

Figure 5 - Photomicrography of the calvarial defect in the CS and XS groups.



Note: This is a representative image of the CS and XS groups. Coronal section. Notice the newly formed bone extending from the edge of the defect toward the center. C, calvaria; NB, newly formed bone; →, edge of the defect; ▪, connective tissue; *, marrow spaces; ►, osteoblasts, «, osteocytes (hematoxylin and eosin; A and B, 100x; C and D, 200x).

Figure 6 - Photomicrography of the mandibular alveolus in rats (CS and XS groups).

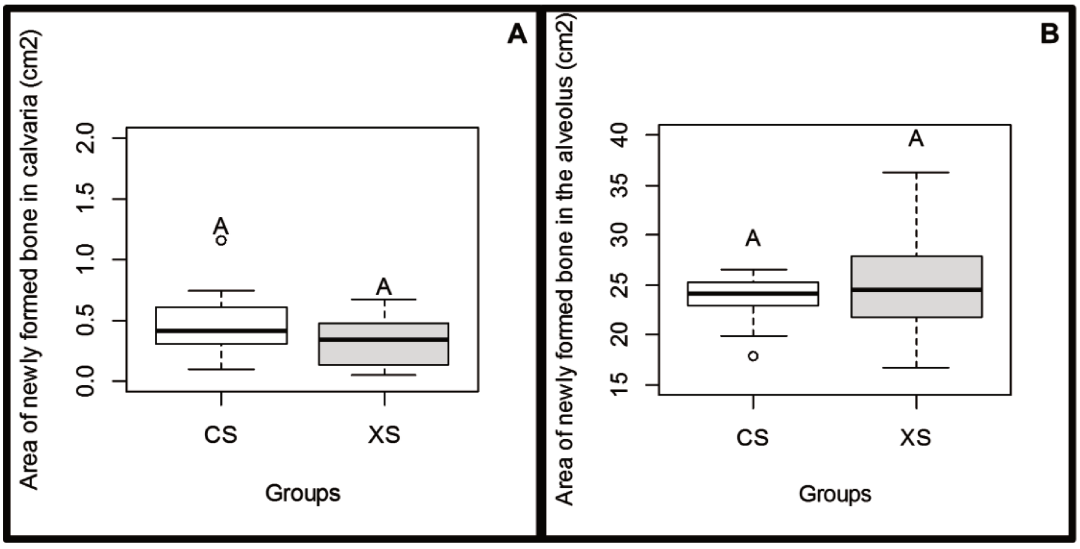


Note: This is a representative image of the CS and XS groups. Cross section. Notice the newly formed bone extending from the periphery of the basal bone toward the center of the mandibular alveolus. BB, basal bone; NB, newly formed bone; →, periphery of the basal bone; ▪, connective tissue; *, marrow spaces; ►, osteoblasts; «, osteocytes (hematoxylin and eosin; A and B, 50x; C and D, 400x).

3.6 Histomorphometric histological analysis: calvaria and mandibular alveolus

Calvaria and mandibular alveolus histomorphometry analysis is represented in Figure 7. We measured the area of the newly formed bone in each group. The results showed that the amount of new bone in the XS group was similar to that in the CS group, both in the defect of the calvaria (Figure 7A) and the mandibular alveolus (Figure 7B). These data agree with those of descriptive analysis.

Figure 7 - Area of newly formed bone in the calvaria defect and in the mandibular alveolus (cm^2) of rats in the CS and XS groups.

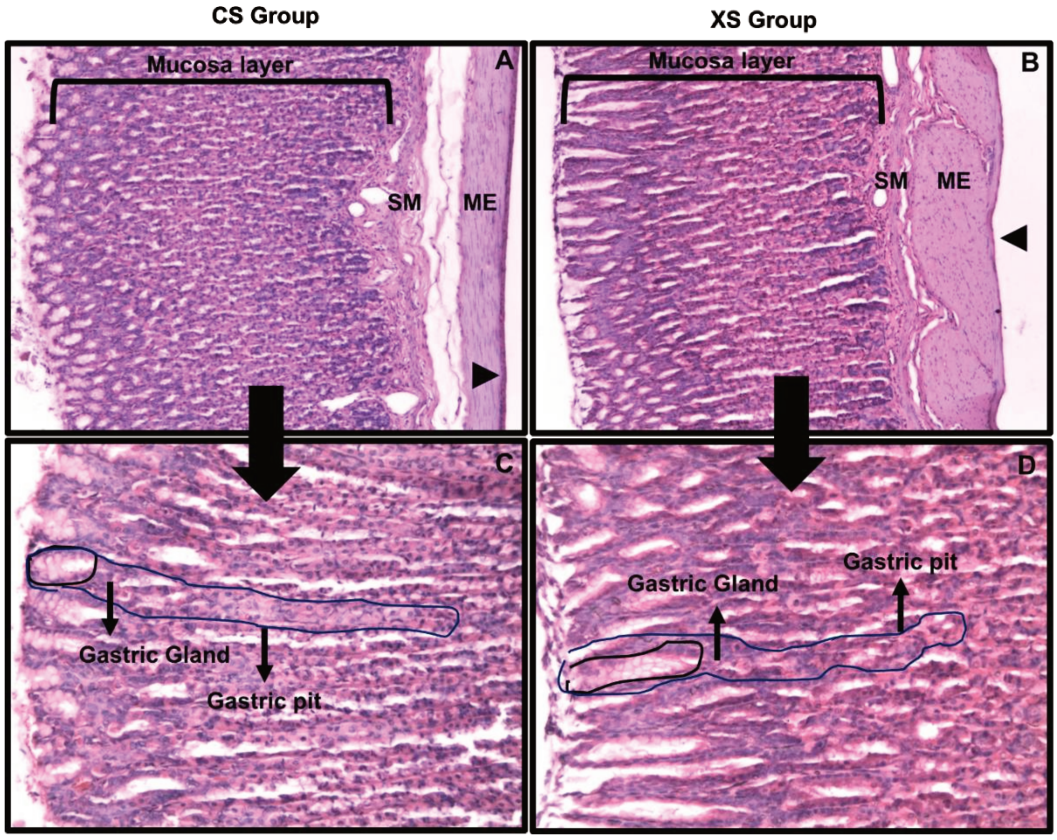


Note: Equivalent letters indicate that there is no statistically significant difference between the groups, as calculated using t test with $p < 0.05$.

3.7 Descriptive histological analysis: stomach morphology

There are three different histological regions in the stomach: peri-esophageal cardia, body and pylorus (Choi et al., 2014; Eroschenko, 2008). In this study we evaluated the morphology of only the body region of the stomach. We observed that, compared with the control group, the stomach of rats treated with *H. speciosa* latex presented normality along its four layers (Figure 8) The mucosal layer (innermost layer) exhibited regularity throughout the entire extension of the simple columnar epithelium. No damage was observed through the gastric pit and gastric glands located below the epithelium. Typically, the submucosal layer manifested large blood vessels and nerves intertwining with the dense connective tissue. No lesion was visualized along the muscularis externa and serous layer. In addition, there was no infiltration of the leukocytes, ulcers, erosions, perforations or gastric bleeding. Taken together, the latex administered systemically did not cause any damage to the stomach.

Figure 8 - Photomicrography of the body region of the stomach in rats (CS and XS).



Note: This is a representative image of the CS and XS groups. Cross section. Notice the aspect of normality in all the stomach layers of both groups. SM, submucosa; ME, muscularis externa; ► serosa (hematoxylin and eosin; A and B, 100x, C and D, 200x).

4 Discussion

This work investigated the systemic effect of *H. speciosa* latex on bone neoformation and mineralization in Wistar rats.

In summary, bone is a connective tissue (Raggatt and Partridge, 2010; Valenti et al., 2017) with large reservoir of minerals. Approximately 99% of the Ca and 85% of the P found in the body are stored in the bones. These elements can be released from the bone during reduced concentration and stored during an excess (Guyton et al., 2006).

After ingestion, Ca is absorbed into the intestinal mucosa with the help of vitamin D and then reaches the bloodstream. Increased blood Ca concentration above normal values causes immediate deposition of exchangeable salts in the bone. Exchangeable salts are amorphous calcium phosphate compounds (e.g., $CaHPO_4$) that are loosely bonded to the bone and can be readily mobilized. These salts are important in promoting rapid tamponade and in maintaining blood Ca concentration at normal levels. Through this defense mechanism, the Ca blood concentration can return to normal levels within 30-60 min (Guyton et al., 2006).

Simultaneous with the exchangeable salt deposition process, two hormone systems represented by the parathyroid hormone (PTH) and calcitonin also act to control the blood Ca concentration. Within 3-5 min after the acute increase in the Ca concentration, there is a decrease in PTH secretion and an increase in calcitonin secretion. Calcitonin causes rapid deposition of Ca in the bones and normalizes the high blood concentration of this ion (Guyton et al., 2006).

In this study, we found that *H. speciosa* latex is composed of Ca. This element was also found in the fruit (mangaba) of *H. speciosa* (De Oliveira Guilherme et al., 2007; Lima and Scariot, 2010) and *Hevea brasiliensis* latex (Gomes, 2013) by other researchers. Possibly, after the ingestion of latex, the blood Ca concentration increased in the XS group. However, we did not detect this change, possibly because of the rapid action of calcitonin and the process of deposition of exchangeable salts. These actions may have culminated to blood homeostasis and deposition of Ca and P in the bones. Therefore, we observed an increase in the relative Ca and P contents in the basal bone of the mandibular alveolus of the latex-treated group (XS) compared with the control group (CS).

Once deposited, the exchangeable salts can remain in the amorphous form for an infinite period of time and be quickly reabsorbed when extra Ca is needed in the

body. They can also be converted into hydroxyapatite crystals by the substitution and addition of atoms (Guyton et al., 2006). Our results showed that, in addition to the increasing of Ca and P contents, the treatment with latex also resulted in the reduction of the Mg content. We therefore suggest that the amorphous crystals present in the bones of the latex-treated rats were converted into hydroxyapatite crystals by the replacement and addition of atoms. In this way, the basal bone of the mandibular alveolus of the treated group became more mineralized.

In the presence of a fracture, the events related to healing result in the formation of a new bone (Lin et al., 2014). In the early stages of bone production, the osteoblasts deposit the organic matrix, forming a tissue known as osteoid. Then, osteoblasts release vesicles with Ca stores that are degraded by enzymes to release this ion onto the newly deposited matrix. Osteoblasts also secrete alkaline phosphatase, an enzyme that degrades pyrophosphate, thus releasing phosphate ions (Florencio-Silva et al., 2015; Guyton et al., 2006; Valenti et al., 2017). Phosphate and Ca form hydroxyapatite crystals and mineralize the bone matrix, forming the new bone (Raggatt and Partridge, 2010). The new mineralized bone can be analyzed by SEM-EDX method to calculate the Ca/P ratio and to identify the degree of bone mineralization (Lozano-Carrascal et al., 2017; Sotiropoulou et al., 2015). This pioneer study demonstrated that *H. speciosa* latex increased the content of Ca and P ions, thus leading to an increase in the degree of mineralization of the newly formed bone in the mandibular alveolus of Wistar rats after 15 days of treatment. This effect can be attributed not only to the presence of Ca in latex, as demonstrated in this study, but also to the presence of some phytochemicals. Dos Santos Neves et al.(2016) have identified that *H. speciosa* latex contains chlorogenic acid and naringenin-7-O-glucoside. There is evidence that these compounds stimulate osteoblastic activities (Li et al., 2014; Zhou et al., 2016). Active osteoblasts secrete a large amount of alkaline phosphatase and osteocalcin, which are important molecules for Ca deposition and bone mineralization (Florence et al., 2017). Thus, these phytochemicals may increase bone mineral density and improve bone microarchitecture (Li et al., 2014; Zhou et al., 2016). We still cannot state that the increase in the degree of mineralization of the newly formed bone is related to these compounds. However, other studies can be conducted to confirm these hypotheses.

Histological analysis showed trabeculae of the newly formed bone along the mandibular alveolus and calvarial defect. According to literature, in the presence of a

fracture, the events related to healing result in the formation of a bone callus that progresses to interconnect the two extremities (Lin et al., 2014). These concepts explain why the bone repair process started at the edges of the basal bone toward the center of the calvarial defect and the mandibular alveolus in both groups.

Histomorphometric analysis revealed that oral administration of latex did not increase the area of the newly formed bone in the mandibular alveolus and in the calvarial defect. Otherwise, the literature confirms that there is an increase in the area of the newly formed bone in both the calvaria (Dos Santos Neves et al., 2016) and mandibular alveolus (Balabanian et al., 2006) of rats treated with natural latex using local product applications. This difference between the results occurred due to administrations by different routes. To confirm the oral administration effect of latex on bone neoformation, other doses need to be evaluated in future studies. Although the oral treatment with 50% latex did not influence the amount of the newly formed bone, it increased the mineralization of this bone.

In this study, we demonstrated that the oral administration of latex did not cause any stomach injury. Marinho et al. (2011) also administered different doses of latex from *H. speciosa* by gavage and observed that the product did not lead to the development of any erosion, ulcer, perforation or bleeding in the stomach. These results suggest that latex causes beneficial effects in the body without damaging the gastric layers.

The body weight of the rats was evaluated every 5 days. We found that systemic latex treatment can be beneficial to bone without interfering with body weight. Zhou et al. (2016) also showed that systemic treatment with chlorogenic acid, one of the components of latex, did not cause any weight changes.

The model studied demonstrated that systemic latex did not contribute to the formation of new bone in the calvarial defect and mandibular alveolus. But, on the other hand, the product increased bone mineralization. These results support the popular belief about the benefit of consuming mangaba milk daily for the treatment of fractures (Silva Jr and Lédo, 2006).

In addition, our results can support future studies aimed at evaluating the systemic use of latex under conditions of osteoporosis and periodontal disease. Osteoporosis causes reduction in bone mass, whereas periodontal disease causes reabsorption of the alveolar bone (Penoni et al., 2017). Because *H. speciosa* latex contains Ca, we suggest that the systemic administration of this product could help in

building stronger bones and contribute to better quality of life for people with these diseases.

5 Conclusion

In this paper, we found that latex from *H. speciosa* is composed of Ca and we first demonstrated that daily oral administrations of the product over a period of 15 days increases the Ca and P contents and decreases Mg content of the basal and newly formed bone in the mandibular alveolus. An increase in the Ca and P contents indicates higher calcium phosphate deposition in the bone; and a decrease in the Mg content indicates that amorphous calcium phosphate present in the bones of the latex-treated rats were converted into hydroxyapatite crystals by the replacement and addition of atoms. Therefore, the basal and newly formed bone in the mandibular alveolus became more mineralized after latex treatment. Adding to that, the oral treatment with latex did not change body weight, stomach morphology and plasma Ca and P concentrations. On the other hand, we showed that *H. speciosa* latex did not contribute to the increase in the area of the newly formed bone in the calvarial defect and mandibular alveolus. However, as we have shown that latex increases mineralization, we may affirm that these results support the popular belief about the benefit of consuming *mangaba* milk daily for the treatment of fractures. Furthermore, our results may aid in the conception and development of a natural drug and favor the entire population that consumes the product.

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3 CONCLUSÃO

Neste trabalho, nós encontramos que o látex da *H. speciosa* contém cálcio em sua composição. Por isso, quando administrado oralmente e diariamente por um período de 15 dias, o produto aumentou os conteúdos de Ca e P e diminuiu o conteúdo de Mg no osso basal e no osso neoformado no interior do alvéolo mandibular de ratos. O aumento do conteúdo de Ca e P indica maior deposição de fosfato de cálcio nos ossos; a diminuição do conteúdo de Mg indica que o fosfato de converteu-se em cristais de hidroxiapatita por substituição ou adição de átomos. Portanto, o osso basal e o osso neoformado no alvéolo mandibular dos ratos tornaram-se mais mineralizados após o tratamento com látex. Somando-se a isso, o tratamento oral com látex não alterou o peso corporal, a morfologia estomacal e nem a concentração plasmática de Ca e P. Por outro lado, nós mostramos que o látex da *H. speciosa* não contribuiu para o aumento da área de osso neoformado nas amostras avaliadas. Todavia, como nós encontramos que o látex aumenta a mineralização, nós podemos afirmar que estes resultados sustentam a ideia da crença popular que acredita que o consumo diário do “leite de mangaba” pode ser benéfico para o tratamento de fraturas ósseas. Além disso, nossos resultados podem auxiliar na concepção e desenvolvimento de fármaco natural utilizando o látex e favorecer toda a população que consome o produto.

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ANEXOS

ANEXO 1: Certificado de aprovação do projeto pelo CEUA/UNICAMP

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Avaliação do Efeito da Administração Oral e Tópica de Látex da Mangabeira no Reparo Ósseo da Calvária e da Mandíbula de Ratos"** (protocolo nº **3790-1**), sob a responsabilidade de **Prof. Dr. Pedro Duarte Novaes / Francielly Andressa Felipetti**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **10 de abril de 2015**.

Campinas, 10 de abril de 2015.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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Caixa Postal 6109
13083-970 Campinas, SP – Brasil

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E-mail: comisib@unicamp.br
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ANEXO 2: Certificado de aprovação do estudo piloto pelo CEUA/UNICAMP

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
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C E R T I F I C A D O

Certificamos que o projeto **"Projeto Piloto: Avaliação do Efeito da Administração Oral de Látex da Mangabeira no Reparo Ósseo da Calvária de Ratos"** (protocolo nº **3427-1**), sob a responsabilidade de **Prof. Dr. Pedro Duarte Novaes / Francielly Andressa Felipetti**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **13 de junho de 2014**.

Campinas, 13 de junho de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
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ANEXO 3: Comprovante de submissão do artigo

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