UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

NÍVEL DOUTORADO

ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA/PERIODONTIA

Linha de pesquisa:

Epidemiologia, Etiopatogenia e Repercussão das Doenças da Cavidade Bucal e Estruturas Anexas

Tese:

EFEITO DA HEPARINA DE BAIXO PESO MOLECULAR NA PERDA ÓSSEA ALVEOLAR EM RATOS WISTAR MACHOS: ANÁLISES MORFOMÉTRICA E HISTOLÓGICA

Doutorando:

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PORTO ALEGRE 2017

Harry Juan Rivera Oballe

EFEITO DA HEPARINA DE BAIXO PESO MOLECULAR NA PERDA ÓSSEA ALVEOLAR EM RATOS WISTAR MACHOS: ANÁLISES MORFOMÉTRICA E HISTOLÓGICA

Tese apresentada ao Programa de Pós-Graduação em Odontologia, nível doutorado, da Universidade Federal do Rio Grande do Sul, como pré-requisito para obtenção do título de Doutor em Clínica Odontológica/Periodontia.

Orientador:

Prof. Dr. Cassiano Kuchenbecker Rösing

Porto Alegre

2017

CIP - Catalogação na Publicação

Rivera Oballe, Harry Juan
EFEITO DA HEPARINA DE BAIXO PESO MOLECULAR NA PERDA ÓSSEA ALVEOLAR EM RATOS WISTAR MACHOS: ANÁLISES MORFOMÉTRICA E HISTOLÓGICA / Harry Juan Rivera Oballe. -- 2017.
27 f.

Orientador: Cassiano Kuchenbecker Rösing.
Coorientador: Cassiano Kuchenbecker Rösing.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Programa de Pós-Graduação em Odontologia, Porto Alegre, BR-RS, 2017.

1. Heparina de baixo peso molecular. 2. perda óssea alveolar. 3. ratos wistar. 4. análise morfométrica. 5. análise histológica. I. Kuchenbecker Rösing, Cassiano, orient. II. Kuchenbecker Rösing, Cassiano, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Dedico este trabalho científico a meu amado pai Harry Rivera Azabache e a minha amada mãe Victoria Oballe de Rivera, pelo exemplo, pela fortaleza, pelo amor incondicional, pelo incansável apoio com meus projetos professionais e pessoais.

AGRADECIMENTOS

Dedico esta tese primeiramente a Deus por dar-me a imensa oportunidade de culminar este doutorado com sucesso e grande satisfação.

Um agradecimento eterno para a República Federativa do Brasil via CAPES que conseguiu outorgar a bolsa e fundos de estudos para poder desenvolver este trabalho de pesquisa durante todo este período de formação como doutorando.

Total agradecimento à Universidade Federal do Rio Grande do Sul pela oportunidade, pela infraestrutura, logística e gestão humana para o desenvolvimento e finalização de esta etapa de formação acadêmica.

Aos componentes de minha banca examinadora Prof. Dr. Profa. Dra. Marilene Issa Fernandes e Prof. Dr. Fabricio Batistin Zanatta.

A meu mentor, orientador, grande e querido amigo Cassiano Kuchenbecker Rösing pelo imenso apoio, pela plena confiança depositada em mim, pela imensa oportunidade de deixar-me ser seu aluno nesta etapa. Muito obrigado querido amigo Cassiano.

Agradeço ao Prof. Marcelo Lamers pela ajuda, parceria e conselhos para a realização de este trabalho.

Agradeço ao Prof. Dr. Eduardo Gaio e amigo (Duda) pelos grandes e excelentes conselhos durante toda minha etapa acadêmica.

Aos meus amados irmãos maravilhosos Giovanna Rivera Oballe e Gustavo Rivera Oballe pelo apoio e pela motivação se seguir nesta vida acadêmica que hoje culmina.

A Marco Fung e Magaly Sanabria e pela presença e apoio neste caminho que escolhi seguir.

À minha amada e linda esposa que desde o início foi, é e seguirá sendo meu porto seguro, minha paz, meu descanso e minha tranquilidade. Obrigado meu amor Alethea Komeroski Anusz, pela parceira, pela paciência, pela tolerância e amor incondicional nesta jornada.

A meu grande amigo e irmão que o Brasil me deu Tobias Rauber Spuldaro pelo carinho, pela parceria e pela amizade que nos uniu desde a época do mestrado e que segue sendo.

A Família Spuldaro pelo apoio, carinho e amor incondicional que recebi constantemente deles e que adotei como pais (César e Eliane) e irmãos (Douglas e Tobias). Muito obrigado.

O meu grandecíssimo amigo, colega, parceiro de pesquisa e de trabalho, a meu grande camarada Francisco Wilker Mustafa Gomes Muniz. Sem tua ajuda jamais teria conseguido terminar este trabalho.

A meus Professores da Periodontia UFRGS: Alex Haas, Tiago Fiorini, Eduardo Gaio, Juliano Cavagni, Sabrina Gomes, Marilene Fernandes e Fernando Daudt, obrigados pelos ensinamentos, pela confiança e os conhecimentos que hoje levo comigo e para toda a vida.

A meus colegas e grandes amigos do doutorado: Fernando Rios, Ricardo Costa, João Peixoto, Marina Mendez, Silvia Zanella, Tobias Spuldaro e Marcelo Ribas. Obrigado pela parceria.

A meus amigos e colegas do mestrado em Periodontia UFRGS pela camaradagem, amizade e apoio.

À Silvia Barbosa técnica do ICBS-UFRGS pelo apoio, pela parceria e pela paciência que teve comigo, por que graças a ela este trabalho hoje chegou a seu fim.

Si vous voulez changer le monde, a appris l'exemple, alors vous devriez faire ces changements et innovations dans votre intérieur.

Dali Lama

ÍNDICE

RESUMO

O objetivo da presente tese foi avaliar os efeitos da heparina de baixo peso molecular (HBPM) na perda óssea alveolar em ratos Wistar.

Para a melhor compreensão e entendimento dos efeitos da HBPM se elaborou um único artigo com 40 ratos machos da linhagem Wistar de 60 dias de nascidos, os quais foram dívidos em 4 grupos experimentais previamente randomizados: Grupo Controle (C), Grupos Doença Periodontal (DP), Grupo Heparina (Hp) e Grupo Heparina+Doença Periodontal (Hp+DP) com um período experimental de 60 dias. Um animal foi perdido no período de aclimação, dois animais foram perdidos na primeira de três coletas sanguíneas pré-programadas e um rato foi perdido na colocação da ligadura. Os resultados observados foram analisados são perda óssea alveolar induzida onde houve diferença significava entre os grupos (C) e (DP), entre o grupo (C) e (Hp+DP), entre o grupo (DP) e (Hp) e o grupo (Hp) e (Hp+DP). Foi avaliado perda óssea alveolar não induzida onde não existiu diferença entre os grupos. Foi avaliado o peso do início ao final do período experimental. Foram avaliados o consumo de ração e agua onde não houve diferença significativa entre os grupos. Foram avaliados o número de megacariócitos nos fémures, onde também não existiram diferenças estatísticas. Foram avaliados números de adipócitos no timo, não havendo diferença significativa entre os grupos. Foram avaliados as plaquetas e desvio padrão onde não existiu diferença significativa entre os grupos. Foram avaliados os leucócitos e desvio padrão onde não houve diferença significativa entre os grupos. Posteriormente foi avaliado a porcentagem de linfócitos onde se achou diferença estatisticamente significativa na segunda coleta sanguínea entre o grupo (C) e grupo (Hp+DP) e grupo (Hp) e grupo (Hp+DP). Foi assim que as conclusões deste trabalho foram que o presente estudo mostrou que a HBPM não foi capaz de produzir perda óssea alveolar nos ratos

Wistar, mas foi capaz de aumentar a quantidade de leucócitos e linfócitos, indicando a presença de um processo inflamatório.

Palavra Chave: Heparina de baixo peso molecular, Perda óssea alveolar, ratos wistar, análise Morfométrica, análise histológico

SUMMARY

The aim of the present thesis was to evaluate the effects of low molecular weight heparin (LMWH) on alveolar bone loss in Wistar rats.

In order to better understand and understand the effects of (LMWH), a single article was elaborated with 40 male rats of the 60 day old Wistar line, which were divided into four previously randomized experimental groups: Control Group (C), Groups Periodontal Disease (PD), Heparin Group (Hp) and Heparin Group + Periodontal Disease (Hp + PD) with an experimental period of 60 days. One animal was lost in the acclimation period, two animals were lost in the first of three preprogrammed blood collections and one mouse was lost in the ligation placement. The observed results were analyzed for induced alveolar bone loss where there was significant difference between groups (C) and (PD), between group (C) and (Hp + PD), between (PD) and (Hp) group and Group (Hp) and (Hp + PD). Uninduced alveolar bone loss was assessed where there was no difference between the groups. The weight of the onset at the end of the experimental period was evaluated. The ration and water consumption were evaluated where there was no significant difference between the groups. The number of megakaryocytes in the femurs was evaluated, in which there were also no statistical differences. Adipocyte numbers were evaluated in the thymus, with no significant difference between the groups. Platelets and standard deviation were evaluated where there was no significant difference between the groups. Leukocytes and standard deviation were evaluated where there was no significant difference between the groups. Later, the percentage of lymphocytes where a statistically significant difference was found in the second blood collection between group (C) and group (Hp + PD) and group (Hp) and group (Hp + PD) was evaluated. Thus the conclusions of this study were that the present study showed that LMWH was not able to produce alveolar bone loss in Wistar rats,

but was able to increase the amount of leukocytes and lymphocytes, indicating the presence of a process inflammatory.

Key words: Low molecular weight heparin, alveolar bone loss, wistar rats, morphometric analysis, histological analysis

ANTECEDENTES

1.1. Doença Periodontal

A gengivite é a primeira manifestação da doença periodontal, onde existe uma alta prevalência na etapa infantil e uma considerável diminuição na adolescência e um aumento considerável da periodontite na etapa adulta (Lopez, Smith, Gostemeyer, & Schwendicke, 2017). A periodontite é uma doença que, em geral, é gerada como consequência do acumulo de placa bacteriana devido a uma pobre higiene bucal e dando como resultado a proliferação de algumas bactérias específicas anaeróbias Gram-negativas que destroem as estruturas de suporte do dente, mais especificamento o ligamento periodontal e osso alveolar, e tendo como resultado eventual a perda dentária. (Tonetti et al., 2017). Fatores que interfiram no sistema imune, no acúmulo de placa dental e ou que promovam uma dieta inadequada, podem promover o desequilíbrio microbiano da placa dental e redução da proporção de microrganismos comensais (DuPont, 1997). Esta condição de desequilíbrio está associada ao aumento em proporção dos microrganismos patogênicos oportunistas envolvidos na patogenia das doenças periodontais, o acúmulo prolongado de placa nas margens gengivais causam inflamação gengival pela ação mais exacerbada do sistema imune local e favorece o crescimento de microrganismos anaeróbios estritos, incluindo bactérias proteolíticas que promovem destruição dos tecidos periodontais e ativam mecanismos imunológicos que agridem o próprio tecido periodontal (Marsh, 2003) É uma enfermidade progressiva e insidiosa com uma variedade de características clinicas como perda óssea, perda de inserção, sangramento à sondagem e presença de bolsas periodontais. (Su et al., 2017). A

doença periodontal inicia-se com uma inflamação da gengiva, e presença de cálculo supra gengival que geralmente é causada pela presença e acumulo de placa bacteriana (Q. Zhang et al., 2017). Estudos epidemiológicos revelam que as doenças periodontais representam entre 10% e 20% das doenças bucais mais comuns da população mundial (Ainamo et al., 1982).

1.2. Heparina de baixo peso molecular.

A Heparina de baixo peso molecular (HBPM), é uma das drogas mais utilizadas para a tromboprofilaxia (W. Zhang, Zhou, & Li, 2016), as HBPM são fragmentos de heparina não fracionada (HNF) obtidos por despolimerização química ou enzimática com peso molecular variando de 1.000 a 10.000 dáltons, com média de cerca de 5.000 dáltons (Hirsh, Anand, Halperin, & Fuster, 2001). HNF e HBPM exercem a sua ação anticoagulante através da ativação da antitrombina III, que tem a sua atividade acelerada em até 1.000 vezes no sentido de inibir os fatores da coagulação IIa e Xa e, em menor proporção, IXa, XIa e XIIa. A ligação da HNF e HBPM à antitrombina III depende da presença de uma única sequência de pentassacárides contida em cerca de um terço das moléculas de heparina. Os dois terços restantes têm mínima atividade anticoagulante nas concentrações terapêuticas usuais. Esta sequência de pentassacárides confere alta afinidade da HNF e HBPM pela antitrombina III (Chai-Adisaksopha et al., 2017). Qualquer molécula de heparina ou HBPM que contenha o pentassacáride pode inibir a ação Xa simplesmente pela ativação da AT III. Para inativar a trombina (IIa), a HNF ou HBPM tem que se ligar à antitrombina III e ao fator IIa

simultaneamente, formando um complexo ternário, que só ocorre com cadeias mais longas, com pelo menos 18 sacárides (Brandao, Shah, & Shah, 2014).

1.3. Doença periodontal e heparina de baixo peso molecular

Na literatura não existem estudos que relatam associação da doença periodontal induzida e espontânea com as HBPM. Embora existam alguns estudos que demostrem que as coagulopatias vêm sendo relacionadas como fatores de risco para doença periodontal e consequente perda óssea alveolar, os resultados são conflitantes, sendo os aspectos morfológicos, fisiológicos e etiopatogênicos dessa inter-relação não totalmente elucidados (Azhar, Yazdanie, & Muhammad, 2006). Em contra partida, estudos (Spolidorio et al., 2010) onde induziram trombocitopenia produzindo depleção plaquetária em curtos períodos de um, sete e treze dias usando soro de coelho normal antiplaquetário demonstraram um importante incremento da perda óssea alveolar. Em um estudo usado drogas antiplaquetárias como aspirina e clopidogrel (Coimbra et al., 2011) foi demonstrada a relação entre as desordens hemostáticas com a qualidade e velocidade de cicatrização periodontal. A mesma autora no 2013, avaliou os efeitos do Clopidogrel e da Aspirina como duas das drogas antiplaquetárias mais usadas na etapa inicial da ligadura em ratos onde chegou a conclusão que o Clopidogrel mas não a Aspirina preveem a perda óssea alveolar induzida. Assim, estudos recentes observaram também que as plaquetas têm um papel importante na resposta adaptativa ao desafio microbiano e a antígenos, representando a ligação entre a coagulação e a resposta imunológica (Ziebolz et al., 2011) na resposta adaptativa ao

desafio microbiano e a antígenos, representando a ligação entre a coagulação e a resposta imunológica (Weyrich & Zimmerman, 2004).

II. OBJETIVO

2.1. Objetivo Geral

Avaliar os efeitos da aplicação e uso da Heparina de Baixo Peso Molecular (HBPM) na perda óssea alveolar induzida e espontânea com análises morfométrica e histológica em ratos Wistar machos.

2.2. Objetivos específicos

- Avaliar os efeitos da heparina de baixo peso molecular (HBPM) na perda óssea alveolar induzida em ratos Wistar machos.
- Avaliar os efeitos da heparina de baixo peso molecular (HBPM) na perda óssea alveolar espontânea em ratos Wistar machos.
- Avaliar os efeitos da heparina de baixo peso molecular (HBPM) no número de megacariócitos apresentados no fêmur dos ratos Wistar Machos.
- Avaliar os efeitos da heparina de baixo peso molecular (HBPM) no número de adipócitos nos Timos dos ratos Wistar machos.

III. ARTIGO

EFFECTS OF LOW MOLECULAR WEIGHT HEPARIN ON ALVEOLAR BONE LOSS IN WISTAR RATS: MORPHOMETRIC AND HISTOLOGICAL ANALYSES

Article will be submitted to American Journal of Haematology

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ABSTRACT

Background and Objective: The aim of this study was to assess the effects of low molecular weight heparin on alveolar bone loss (ABL) in male Wistar rats. **Materials and methods:** Forty male 60-day Wistar rats were randomly divided into four groups: Control (C), Periodontal Disease (PD), Heparin (Hp) and Heparin + Periodontal Disease (Hp+PD). Low molecular weight heparin was applied for 60 days at doses of 1 ml/kg/day. Blood samples were collected at day 0, 30 and 60. On day 49, PD and Hp+PD groups were subjected to ligature-induced periodontitis around second upper right molar. The left side was assessed as spontaneous alveolar bone loss. **Results:** The mean ABL in the side with ligature showed significantly different between C (0.35±0.07) and Hp+DP (0.49±0.09) groups, between PD (0.55±0.11) and Hp (0.32±0.06) groups and between Hp and Hp+DP groups. No significant differences were found among groups for ABL in the side without ligature. Animal weight, food and water consumption showed no statistically significant difference among groups. The quantity of megakaryocytes of the femurs and adipocytes in the thymus were analyzed by optical microscopy and no statistically significant differences were found. There was an increase in the amount of lymphocytes and leukocytes. There was no decrease in the number of platelets in the three collection periods. **Conclusions:** Low molecular weight heparin was not able to modify spontaneous and ligature-induced ABL, but was able to increase the amount of lymphocytes and leukocytes in the experimental groups.

Key-words: Low molecular weight heparin, alveolar bone loss, wistar rats, morphometric analysis, histological analysis.

INTRODUCTION

Periodontal diseases are one of the most prevalent diseases of the oral cavity, which is generally originated by the presence of specific gram negative anaerobic bacteria in the subgingival biofilm, and results in destruction of the tooth support structures (Van Dyke, 2017). Periodontitis is characterized by clinical attachment loss, presence of periodontal pockets and bleeding upon probing. Its prevalence is increasing in industrialized countries (Q. Zhang et al., 2017), and it is well established that individuals with lower economic status show higher prevalence of periodontal disease in comparison to those in the higher socioeconomic level (Bastos, Boing, Peres, Antunes, & Peres, 2011).

Low molecular weight heparins (LMWH) are the most widely used drugs worldwide for the prophylactic treatment of thromboembolic disorders (Junqueira, Zorzela, & Perini, 2017). They are complex drugs that play the role of inhibiting the blood clotting cascade, acting on the inhibition of antithrombin (Mourier, Guichard, Herman, Sizun, & Viskov, 2017). LMWH are also used to prevent and treat thrombolytic diseases, such as venous thrombosis and pulmonary thromboembolism (Helms et al., 2017). These diseases are important causes of mobility and mortality in the adult population. In recent years, the levels of thromboembolism in patients have been reduced due to low molecular weight heparins (Spencer, Cawood, Frampton, & Jardine, 2014). In the last 30 years, studies have been demonstrated in more than 15,000 orthopedic surgeries, urological and general surgeries the incidence rate of pulmonary embolism formation has decreased from 3% to 1.7% and the rates of fatal incidences due to pulmonary embolism decreased from 0.9% to 0.3%

(Cyrkowicz, 2002). In contrast, there is little evidence of the adverse effects that heparins produce (Di Nisio et al., 2016).

In the literature, there is no study that associate the effects of LMWH and destructive periodontal disease. The null hypothesis of the present study was that there is no difference, in the alveolar bone loss, in the groups that received daily doses of heparin and the control groups. Therefore, this study aimed to evaluate the effects of low molecular weight heparin on alveolar bone loss in Male Wistar rats, using morphometric and histological analyses.

MATERIALS AND METHODS

Animals

For the present study, 40 male Wistar rats of 60 days of birth were used. The animals were provided by the Center for Reproduction of Animals of Laboratory Experimentation (CREAL-UFRGS). Three to four animals were housed per box. The boxes were of white plastic, measuring 25x25x15 cm and with shaving and clean grids. To all animals standard animal care to maintain their comfort and well-being in the best possible way was provided throughout the experimental period. This study was approved by the Animal Research Ethics Committee of the Federal University of Rio Grande do Sul (UFRGS) under protocol number 24470**.** Thirty-seven (37) 30-day Wistar rats were acclimated for four (4) weeks at the Animal Hospital of the Basic Health Science Institute–UFRGS. In this period, the animals were controlled in 12/12 hours light and dark cycles, humidity and temperature.

The experimental procedures performed in the present study are expressed in Figure 1.

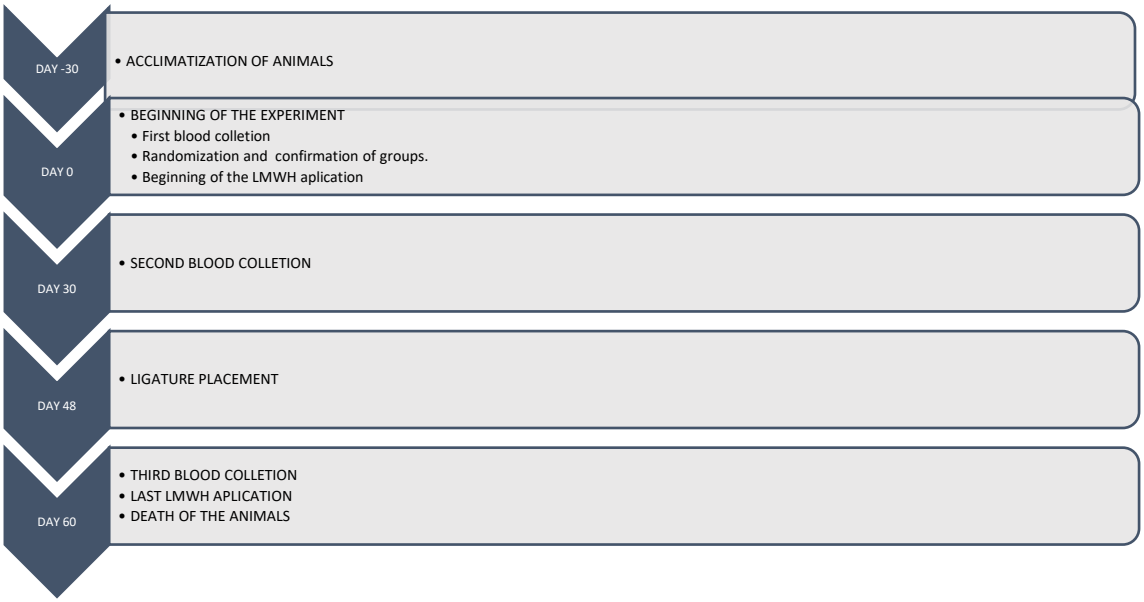

Figure 1. Flowchart of the experiment stages

Randomization

Forty (40) animals were weighed prior to the groups allocation. The weights were recorded and the animals were divided into tertiles. The four experimental groups were determined by weight-stratified randomization, using the webisite radomizer.org. The animals were identified with distinct color markers in their tails.

Distribution of study groups

The animals were randomly assigned to four experimental groups. To all groups, the experimental period lasted 60 days.

Control (C) group (N = 10).

The animals did not receive any dose or received any other procedure.

Periodontal Disease (PD) group (N = 10).

These animals received induction of alveolar bone loss by attaching a ligature with silk suture strand 0000/4 around the second right upper molar. No further substances was administered in those animals.

Heparin (Hp) group (N = 10).

The animals received low molecular weight heparin (CLEXANE® SANOFI AVENTIS, FRANCE,40mg 0.4ml) subcutaneously. No ligature-induced periodontitis was placed in those animals.

Heparin Group + Periodontal Disease (Hp+PD) group (N = 10)

The animals received the low molecular weight heparin and a ligature with silk suture strand 0000/4 were placed around the second right upper molar. Additionally, low molecular weight heparin (CLEXANE® SANOFI AVENTIS, FRANCE,40mg 0.4ml) was administered.

Application of Low Molecular Weight Heparin

All applications of LMWH were performed daily around 11:30 am for a period of 60 days. The animals in the Heparin and the Heparin + Periodontal Disease groups were manipulated daily, being previously weighed and then pre-programmed doses of LMWH applied subcutaneously to the nuchal region by an insulin syringe. The procedure consisted of lifting the coat of the animal and applying the substance. The dose administered was 1mg/ml/kg/day of animal weight.

To standardize the stress conditions among all animals, the rats in the Control and Periodontal Disease groups were also weighted daily. No medicine was applied in those animals, but they were chopped daily with an insulin syringe.

Blood collection

All blood collections (at days 0, 30 and 60 of the experiment) were performed at 08:00 am. ISOFLURANO BIOCHIMICO®, SÃO PAULO, SP, BRAZIL 100ml inhalation anesthetic was used. The anesthetic procedure and the blood collection were performed under the supervision of a veterinarian of the CREAL-UFRGS. The anesthetic was soaked in cotton and placed inside a chamber to place the rat snout inside the chamber and it breathed until it was completely anesthetized and asleep. To confirm that the rat was completely anesthetized the veterinarian tightened the animal's posterior paw to confirm whether the painful stimulus was positive or negative. The first blood collection was performed at the beginning of the study (day 0), for which the tail of the animal was previously and slightly warmed to create vasodilation so it was easier to find the caudal vein. With the help of a Scalp 25 and a microtubes for blood collection with EDTA, 1 ml of blood were collected. The tubes were stored in boxes and preserved in ice until the hematological analyses. At the first blood collection, two animals from the Control group were lost due to anesthetic dose. The second blood collection was performed at 30 days of experiment, repeating the same procedure performed in the first blood collection and no further loss occurred. The third blood collection, at day 60, was performed by cardiac puncture.

Ligature Placement

An experienced study collaborator (EJG) performed the ligature placement using a black silk strand 0000/4 (ETHICON® NEW JERSEY, USA) around the right upper second molar. Placement was performed on day 48 of the experiment in the groups Periodontal Disease and in the Heparin + Periodontal Disease. The animals were previously anesthetized with the use of ISOFLURANO BIOCHIMICO®, SÃO PAULO, SP, BRAZIL 100 ml under the care and supervision of the veterinarian. The black silk ligature was placed on the second maxillary right with the help of two Castro Viejo needle holders, light and scissors (Daudt et al., 2011). After the ligature was placed and the anesthetic was still low, the rats were placed in isolated boxes with clean, dry shavings that served as a box for their recovery. When the animals were awaked, they were placed in their original boxes. One study collaborator was permanently in the supervision and recovery of animals (HJRO). During this procedure, an animal from the Periodontal Disease group was lost. The death assigning was rupture of a posterior palatine artery.

Food and water measurements

During the experimental period, body weight measurements of all study groups were carried out. Every other day, 100 g of rat chow per animal was placed in each box. The food was weighted every other day by one the three researchers (HJRO, FWMGM or TRS) and the leftovers were discarded twice a week together with the grids. Regarding the consumption of water, 500 ml

bottles were used in each box. The water consumption was also measured every other day by one of the three researchers (HJRO, FWMGM or TRS) Once a week, the nozzles and bottles were replaced. The leftover water was measured and then discarded by putting fresh water in clean bottles and nozzles. The changes and replenishment of the food and water took place always between 11:30 am and 12:00.

Death of animals

On day 59 of the experiment, the last dose heparin was applied, and the animals were fasted, remaining only the bottles with water. At day 60 of the experiment, they were previously anesthetized with 100 ml ISOFLURANO BIOCHIMICO®, SÃO PAULO, SP, BRAZIL The veterinarian once again supervised the anesthetic procedure of the animals and performed the cardiac puncture for the third blood collection. Using 21G hypodermic needles and 10ml syringes, the left ventricle was punctured and the blood was collected. The collected blood was placed in microtubes for blood collection of 1 ml with EDTA previously identified and stored in a styrofoam box of with ice and then transferred to LACvet-UFRGS for analysis. The remained blood was filled into 4.0 ml EDTA blood collection tubes to be centrifuged at 2500 RPM for 15 minutes to separate the blood serum. Subsequently, the maxillae were removed and placed in labeled and labeled flask submerged in a 10% buffered formalin solution. Additionally, the palatine gingival tissue was removed and deposited in 1.5 ml Eppendorf tubes previously labeled and identified. The tubes were kept in liquid nitrogen. Thymus were also removed from each animal, and it was deposited in pots previously labeled and identified and submerged in a 10%

buffered formalin solution. The right femur was also extracted and deposited in pots previously labeled and identified and submerged in a 10% buffered formalin solution. The carcasses of the animals were placed in white plastic bags and then taken to specific freezers for disposal.

Preparation of the jaws for the morphometric analysis

The jaws were submerged in a 9% sodium hypochlorite solution MAZZAROLO® GRAVATAI, RS, BRAZIL for a period of 4 hours. After this time, the jaws were washed completely and submerged in clean water for 24 hours. Afterwards, they were re-washed and dried to remove and clean with an extra soft dental brush the remaining organic material that may have remained. The jaws were dyed with 1% methylene blue and with the aid of a medium-head Microbrush for both the buccal and palatal sides of the right and left segments, in order to differentiate the cemento-enamel. The jaws were placed on an endodontic ruler, using a wax, which was supported on two dense silicone bases. Photos were taken using a professional camera (Nikon® D3500, Nikon Co, Japan) with a 105 mm zoom and flash built into the camera lens. The photos were taken in a standardized manner in both upper second molar either by vestibular and lingual surfaces (Fernandes, Gaio, Oppermann, Rados, & Rosing, 2007). All the photos were analyzed by a software (IMAGE J®, NATIONAL INSTITUTES OF HEALTH, USA), and, in each photo, the distance from the cement-enamel junction to the alveolar bone crest was measured in five specific points: two in the mesial root, one in the furcation, and two in the distal root. The endodontic rule served as a parameter for converting the pixel measurements into millimeters.

Evaluator training and calibration

All photos were analyzed by a trained and calibrated examiner (HJRO). A test for intraexaminer reproducibility was performed with 10 randomly chosen photos measured twice by the same examiner with one-week interval. The intra-class correlation (ICC) showed a coefficient of 0.99 for alveolar bone loss.

Data Blindness and Reliability

After the standardized photos were taken, a research collaborator (FWMGN) performed a re-randomization of the photographs, in order to be able to measure them. The measurement of the pieces was done with the help of IMAGE J® WAYNE RASBAND PHOTO RETOUCHING SOFTWARE. NATIONAL INSTITUTE OF HEALTH, USA that allowed the transformation of pixels in millimeters. To this end, each half of the maxilla to be measured was as follows: a measurement that was performed was distance from the piece to the ruler for its transformation of pixels into millimeters and five measurements that went from the cemento-enamel junction to the alveolar bone crest, These measurements were performed both bucally and lingually in sides with or without ligature.

After the standardized photos were taken, one of the researchers (FWMGM) performed a randomization of the photographs. The calibrated examiner was not aware to the group allocation of each photograph until the end of the study.

Preparation of Femurs and Thymus

For the femur initial demineralization Gracey and Padua Lima periodontal curettes were used to remove any remaining soft tissue and leave the pieces completely clean. Afterwards, the femurs were submerged in a solution of 5% nitric acid for periods of 24 until it present a rubbery consistency. The total demineralization period was 11 days. In order to test the degree of demineralization an insulin needle was used, if the needle penetrated the bone, it meant that the demineralization of the femur was concluded (Paolillo et al., 2016). The distal and mesial parts of the femurs were cut and discarded. The central part of the femurs were cut in a sagittal form, to be included for 24 hours and immediately embedded in paraffin, being ready to perform the histological cuts.

For the thymus preparation, 10% buffered formalin was used and placed on a clean glass surface to be cut such that the thymus would be cut into three equal third following the same anatomical position in the rats, then deposited in previously identified cassettes and submerged in alcohol 70 degrees. To initiate the inclusion processing, the thymus were processed for 24 hours and then placed in stainless steel for paraffin wax placement, being ready to perform the future histological cuts.

Histological sections of the femurs and thymus

The embedded thymus and femurs were taken to a micrometer (LUPETEC® SÃO PAULO, SP, BRAZIL using high-shear FEATHER® SAFETY RAZOR CO., LTD, JAPAN). The cuttings were done by a collaborator assisted

by an experienced laboratory technician, using a microtome (LUPETEC® SÃO PAULO, SP, BRAZIL) with a thickness of 0.50 µm. Used microscopy slides LAMELA® SÃO PAULO, BRAZIL 25.4 x 76.2 mm and 1 mm thick, previously cleaned with 70% alcohol. After the cuts, the operator took the blades to an oven at a continuous temperature of 55.6 ° C for 24 hours to eliminate any paraffin residue. After 24 hours the slides were removed and prepared to be stained with Hematocillin and Eosin for histological analysis. The stains were made in Hematoxylin and Eosin (H&E), following the standard procedure for this staining.

Histological analysis of femurs

The histological slides were analyzed in the Laboratory of Pathology of UFRGS Dental School. for their analysis an binocular optical microscope (OLYMPUS®, TOKIO, JAPAN), and a software were used to realize the microphotographs. For the micrographical images the histological samples of the femur were divided into five (5) random fields, where the operator (HJRO) was able to take the microphotographs in 40X in each area. the researchers (HJRO) manually counted the megakaryocyte cells on each microphotograph. The number of megakaryocyte for each animal was determined by the sum of the five photographs.

Statistical analysis

To evaluate the normality of all continuous variables, the Shapiro-Wilk test was used. Paired T test was used to assess the difference within groups in

the following parameters: mean body weight at the beginning and end of the study, and in food and water consumption before and after ligature placement. In the platelet, leukocyte, and lymphocytes counting longitudinal analyses, the two-way ANOVA test was used. One-way ANOVA assessed the differences between groups in mean body weight, food and water consumption, megakaryocytes counting, alveolar bone loss, and in platelet, leukocyte and lymphocytes counting. Whenever necessary, the Tukey post-hoc test was used the assessed the differences between groups. All analyses were performed using the animal as the unit of analysis, except for the occurrence of periodontal destruction, in which the surface was the unit of analysis. Statistical evaluation was performed in SPSS (version 20.0 Statictics IBM®, College Station, Texas, USA), and the level of significance established was $p < 0.05$.

RESULTS

Figures 2 and 3 demonstrate the mean outcome of the present study: alveolar bone loss. In Figure 2, mean alveolar bone loss (in millimeters) in sides with ligature is demonstrated. A statistically significant difference between groups ($p<0.001$) was detected. In the post hoc analysis, statistically significant differences were found between C and Hp+PD groups ($p<0.001$), between PD and Hp groups ($p<0.001$), and between Hp and Hp+PD groups ($p<0.001$). Figure 3, reveals spontaneous mean alveolar bone loss in sides without ligature. No statistically significant differences were observed among groups.

After thirty days of acclimation, the experimental period started. Body weight at baseline and at day 60 are demonstrated in Figure 4. At baseline, mean (±standard deviation in grams) body weight were of 288.00(25.15),

284.60(21.98), 285.60(28.14) and 280.20(24.47), for C, PD, Hp and Hp+PD groups, respectively. No statistically significant difference was observed among groups (p=0.925). After 60 days, an increase in body weight was observed for all groups (p<0.001). Mean (± standard deviation in grams) were 442.29(30.67) for C group, 445.78(42.88) for PD group, 454.80(51.82) for Hp group and 442.80(31.48) for Hp+PD group, with no statistically significant difference among groups (p=0.902).

Figures 5 and 6 demonstrate, respectively, food and liquid intake during the experiment. Mean values of food intake were of approximately 25g, with no statistically significant difference among groups (p=0.732, Figure 4). Also, no statistically significant difference was found between the groups in liquid consumption (p=0.476, Figure 6).

Counts of megakaryocytes in femurs of the animals and adipocytes in the thymus were analyzed with no statistically significant differences among groups (p=0.731 and p=0.911, respectively).

Figures 7, 8, and 9 demonstrate cell counts in blood. Mean platelet x 10^3µl counts are demonstrated in Figure 7 in all three experimental periods. The results showed that in the first blood collect, in C group, mean (±standard deviation) was 810.57(± 97.77), with 5 animals with normal platelet counts and 2 with high platelet counts. In the PD group, these values were 880.50(48.94), with 3 animals presenting normal platelet values and 7 animals with high platelet counts. In the Hp group, 879.40(103.00) of mean platelet count, with 6 animals with normal platelets and 4 animals with high platelets. In the Hp+PD group, 849.44(134.42) was observed with 5 animals with normal amount of

platelets and 5 animals with high amount of platelets. No statistically significant difference among groups was detected at baseline (p=0.467).

In the second blood collect, the results were as follows: C group 788.14(44.19), with all animals displaying normal values of number of platelets; PD group, 797.80(77.25), with 8 animals with normal platelets and 2 with high platelets; Hp group, 828.11(93.81), with 6 animals with normal amount of platelets and 4 animals with a high amount of platelets; In the Hp+PD group, 828.80(58.02), with 7 animals classified as normal in the platelet counts and 3 with high number of platelets. In this second collect, no statistically significant difference was found among the experimental groups (p=0.549).

Regarding the third blood collection, the C group demonstrated 723.00(51.26), with 7 animals with normal platelet count; PD group, 730.78(127.67), with 8 and 2 animals with normal and high platelet counts, respectively; Hp group, 783.70(71.88), and all animals showed normal amount of platelets; Hp+PD group, 823.20(172.23), with 7 animals presenting normal amounts of platelets, and 3 of them with high platelet counts. In the evaluation between the experimental groups in the third collect, there is not statistically significant difference between experimental groups. (0.266), In the evaluation overtime, there was a statistically significant difference in the periodontal disease group of collect 1 versus collect 2 (p=0.007) and collect 1 versus collect 3 (p=0.005), in the heparin group we found a statistically significant difference in collect 1 versus collect 3 (p=0.035).

Figure 8 shows the mean leukocytes x 10^3µl in all experimental periods. At baseline, no statistically significant difference was observed among groups (p=0.636). It should be emphasized that the number of animals with

normal leucocyte counts was of 5, 8, 9 and 7 for C, PD, Hp, and Hp+PD groups, respectively. In the second blood collect, also no statistically significant difference was observed among groups, with 7, 8, 8 and 10 animals with normal counts in C, PD, Hp, and Hp+PD groups, respectively. At day 60 no statistically significant difference between experimental groups was observed (p=0.354). In the analysis within the groups, it was found a statistically significant difference in the control group in collect 1 versus collect 2 (p=0.002) and in collect 1 versus collect 3 (p=0.007) and in collect 2 versus collect 3 (p=0.015). In the periodontal disease group, it was found a statistically significant difference in collect 1 versus collect 3 (p=0.001) and collect 2 versus collect 3 (p=0.002). In the heparin group, it was found a significant difference in collection 1 versus collect 2 (p=0.015) and in collect 1 versus collect 3 (p=0.002) and in collect 2 versus collect 3 (p=0.002) and in the heparin + disease group (p=0.001) and in collect 1 versus collect 3 (p<0.001) and in collect 2 versus collect 3 (p=0.004).

Figure 9 shows the mean(±SD) percentage of lymphocytes. At baseline, the C group demonstrated 80.66(3.69); PD group presented 81.88(2.71); Hp group 80.29(2.12) and Hp+PD group 80.00(4.00), with no significant difference among groups (p=0.677). In the second blood collect, a statistically significant difference was between the C and Hp groups (p=0.036) and between the Hp and Hp+PD groups (p=0.005). In the third blood collect, the C group presented 85.51(2.31), PD group, 82.93(3.79), Hp group, 83.85(5.09), and Hp+PD group, 84.39(3.39). No statistically significant difference (p=0.594) was demonstrated among groups. In the analysis within groups, significant differences were encountered in the C group in collect 1 versus in the collect 3 (p=0.005) and in the collect 2 versus the collect 3 (p=0.001). In the Hp+PD

group, a significant difference was observed in collect 1 versus collect 3 ($p=0.009$).

DISCUSSION

The present study aimed to evaluate the effects of the use of Low Molecular Weight Heparin (LMWH), on alveolar bone loss, with morphometric analysis in male Wistar rats. LMWH was not able to alter alveolar bone loss both in spontaneous and ligature-induced periodontitis.

Periodontitis is a disease that affects the supporting structures of the tooth with destruction of the connective tissue and alveolar bone loss (Calisir, Akpinar, Poyraz, Goze, & Cinar, 2016). This is caused by subgingival biofilm (Kornman, 2008). Microorganisms housed in the subgingival region (Greenstein & Caton, 1990) produce inflammation and many immune reactions play an important role in the formation and progression of the disease (Chandy et al., 2017). Multiple studies have adopted the animal model of alveolar bone loss to be able to associate the effects of different drugs/diseases/conditions on pathogenesis of periodontal diseases. (Terrizzi et al., 2013) Studies showed that the prevalence of periodontal disease is increasing along with life expectancy. Therefore, the incidence of tooth loss due to periodontitis might assume the same trend (Graetz et al., 2017) .

In the literature, to the best of the authors' knowledge, there is no study evaluating the application of LMWH on spontaneous and ligature-induced periodontitis. In the present study, the presence of ligature (that allows biofilm accumulation) led to greater alveolar bone loss than in sides without ligatures, meaning that the model was effective in accumulating plaque. However, in spontaneous alveolar bone loss, the presence of heparin was not able to modulate the response.

The literature shows the influence of antiplatelet agents in the pathogenesis of periodontal diseases, and it was concluded that the systemic administration of aspirin and clopidogrel attenuate the systemic inflammation and the alveolar bone loss in Wistar rats submitted to ligature-induced periodontitis (Coimbra et al., 2011). Another study evaluated the effect of serum rabbit antiplatelet in the induction of thrombocytopenia on delayed periodontal healing (Spolidorio et al., 2010). It was concluded that the use of serum rabbit antiplatelet impaired periodontal healing. In the present study, LMWH did not alter the alveolar bone loss, either spontaneous or ligature-induce. Therefore, it may be hypothesized may not produce any effect on periodontal breakdown or that the dose of LMWH was too low to detect any significant alteration.

In animal studies, body weight is used to suggest general health. The evaluation was performed from the start of the experiment at the end of the experiment. No statistically significant difference was observed between the groups from baseline to the end of the study, with all animals in the study presenting weight gain. Additionally, in the total food and water consumption among the experimental groups there was no significant difference among groups. These facts suggest that neither procedure affected general health of the animals (Muluke et al., 2016).

Megakaryocytes are cells located in the bone marrow that have the responsibility of platelet formation (Branehog, Ridell, Swolin, & Weinfeld, 1975). Megakaryocytes are cells that are 10 to 17 times the size of a red blood cell, with a diameter of 50-100 μm (Bunting et al., 1997). Megakaryocytes are precursor cells of hematopoietic cells in the marrow. Being produced in the liver,

spleen, kidneys and bone marrow (Deutsch & Tomer, 2006). In the literature, there are no studies evaluating the production of megakaryocytes in relation to alveolar bone loss. In the results the mean and standard deviation of the number of megakaryocytes were evaluated. Noting that the difference between the groups was not statistically significant, it was attributed that there was no plaquetosis or thrombocytopenia.

The thymus is a primary lymphoid gland, responsible for the development of T cell production (Safieddine & Keshavjee, 2011). This gland produces defense cells against infectious microorganisms and other harmful elements (Laufer, Glimcher, & Lo, 1999). Studies reveal that the thymus with its lymphoid structures is dependent on the precursor lymphopoetic character as some natural or induced immune responses being different ways in relating relations to the lymphatic system. The immune response plays an important role in the genesis of periodontal disease (Yoshie, Taubman, Ebersole, Smith, & Olson, 1985). Several studies have shown that the immune response is a controversial issue, as it may interfere with gingival protection against inflammation, but on the other hand it has been shown to interfere with the immune response that contributes to periodontal destruction (Yoshie, Taubman, Olson, Ebersole, & Smith, 1987).

Platelets are anucleated coroplasmic fragments found in the blood and formed in the bone marrow. The main function is blood clot formation, participation in the immune response. (Zhan et al., 2017), and in the inflammatory response Low molecular weight heparin is fractions of unfractionated heparin obtained by enzymatic depolarization with molecular weight of 1000 to 10,000 daltons (Junqueira et al., 2017). Studies have

demonstrated the great advantages of using LMWH in the treatment of thromboembolism (Robertson & Jones, 2017) and against deep venous thrombosis (Khoursheed et al., 2013). In the literature, there is no study evaluating the effects of low molecular weight heparin on alveolar bone loss, but in counterpart studies have demonstrated the application of heparin may cause adverse effects (Larcan, Laprevote-Heully, & Toulemonde, 1986). There have been studies evaluating the induction of severe thrombocytopenia in short time using antiplatelet agents such as rabbit antiplatelet serum in a 2ml dose to evaluate pro-and anti-angiogenic VEGF growth factor and induced alveolar bone loss in wistar rats (Spolidorio et al., 2010), showing that thrombocytopenia retarded periodontal healing in periodontitis. Other studies have been carried out evaluating antiplatelet agents, such as Clopidrogel and Aspirin, in the inflammatory response where they play an important role in the immune response on induced periodontitis (Coimbra et al., 2011). The results indicated that systemic application of these two periodontitis inflammatory attenuating drugs do not affect the periodontal repair process when the stimulus is removed. The results of the present study demonstrated that there was no significant difference between the study groups and each of the blood collections. But that an analysis within the groups found significant differences in the periodontal disease group and within the heparin group as previously mentioned, this may have occurred the relation of the immune and inflammatory response in which the platelet is responsible.

Leukocytes are white blood cells, formed in the bone marrow has the function of defending the body against infectious diseases and allergies. They are present in the blood, lymph, lymphatic organs and various connective

tissues of the body. Leukocytes in conjunction with red blood cells and platelets form the configured blood elements. An increase in leukocytes will generate a leukocytosis otherwise a leukopenia will be more susceptible to infections. (Riedel-Caspari & Schmidt, 1990) There are few studies that relate leukocyte levels to periodontal disease in patients with infectious diseases (Goultschin, Attal, Goldstein, Boyan, & Schwartz, 2000). These studies concluded that periodontal treatment reduces bacterial load by reducing the number of leukocytes in patients diagnosed with neutropenia. Our results in each of the blood collections performed did not differ significantly between the experimental groups, but in the evaluation within the groups we found a significant difference in all groups.

Lymphocytes is a type of leukocytes present in the blood, they are produced in the bone marrow by means of change cells. These cells can differentiate into two types of specialized cells, the pre-bursicas and the pre-thymocytes which in turn will give rise to B and T cells respectively (Sasson et al., 1989). Periodontitis is usually generated by a bacterial process which triggers a set of immune reactions such as the proliferation of cytokines and pro-inflammatory cells that will lead to the recruitment of lymphocytes (Dogan et al., 2015). Studies have shown that some interleukins such as IL-10 reduce their levels during resolution of periodontitis (Ebersole et al., 2014). On the other hand, studies evaluating the immune response in the etiopathogenesis of periodontal diseases have evaluated that the development of media adaptive immunity by T lymphocytes that is highly dependent on antigen presenting cells associated to the innate immunity, where they produce different patterns of

cytokines that will contribute to the polarization and activation of specific T lymphocytes (Campbell, Millhouse, Malcolm, & Culshaw, 2016).

In the results of our study we observed a significant difference between the experimental groups in the second blood collection where the Hp+PD group compared to the C group reflected inflammatory and immune reaction. Our study had great advantages as the calibration, training and experience the researchers in the methodology applied. Another advantage of this study is the use of randomization, examiner blinding, sample size calculation and the stress control, allowing that there are not future biases of information. On the other hand, the limitations for the present study were that the first blood collection did not take place during the first seven days of the experiment, which was the time in which the clinical signs of coagulopathies started in the animals. Another limitation may be the single dose of heparin tested, as it is hypothesized that increased doses may induce thrombocytopenia.

The present study showed that low weight molecular heparin was not able to produce alveolar bone loss in the Wistar rats, but it was able to increase the leucocyte and lymphocytes quantity.

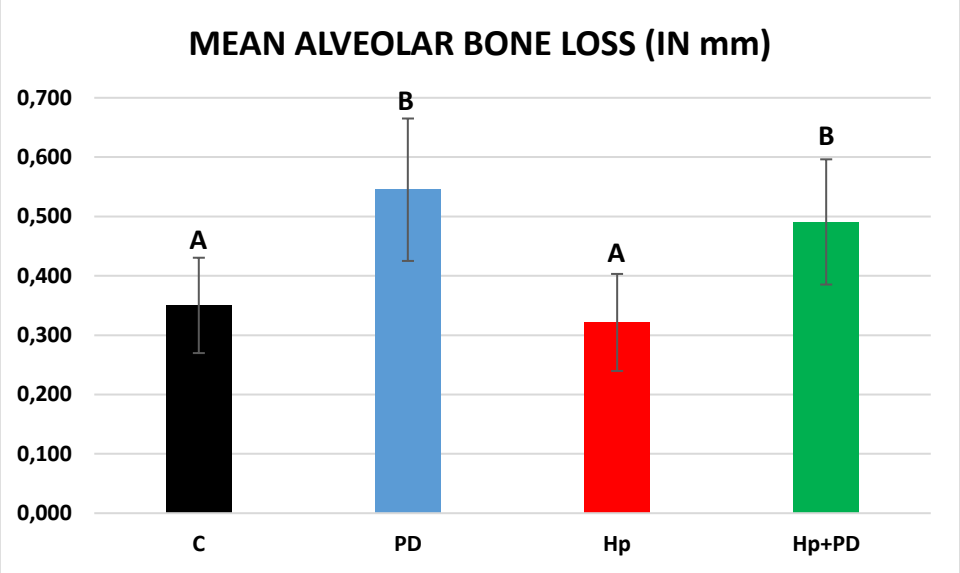

Figure 2: Mean alveolar bone loss in the sides with ligature (right sides). Different letters mean statistically significant differences by One-Way ANOVA test ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

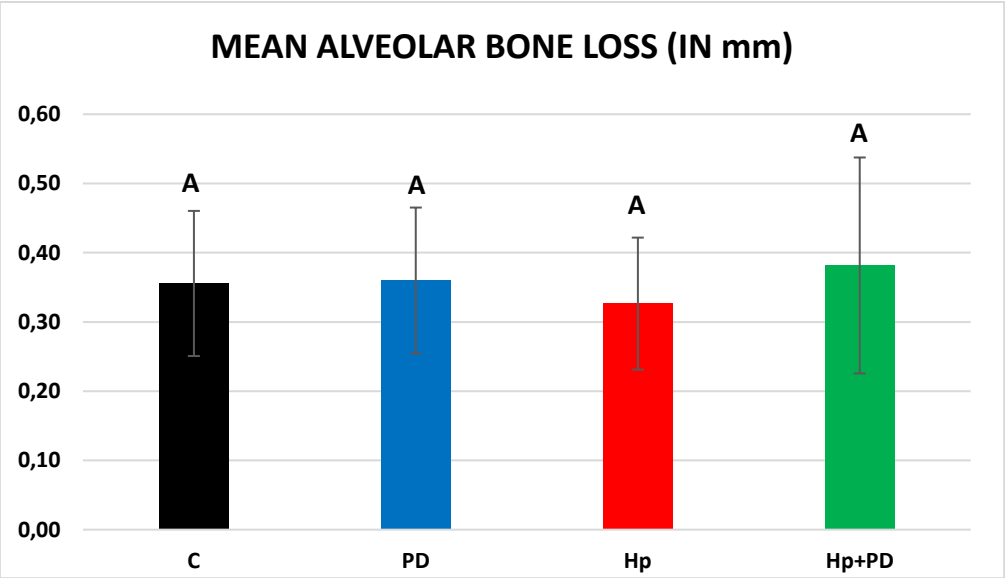

Figure 3: Mean alveolar bone loss in the sides without ligature (left sides). Different letters mean statistically significant differences by One-Way ANOVA test ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

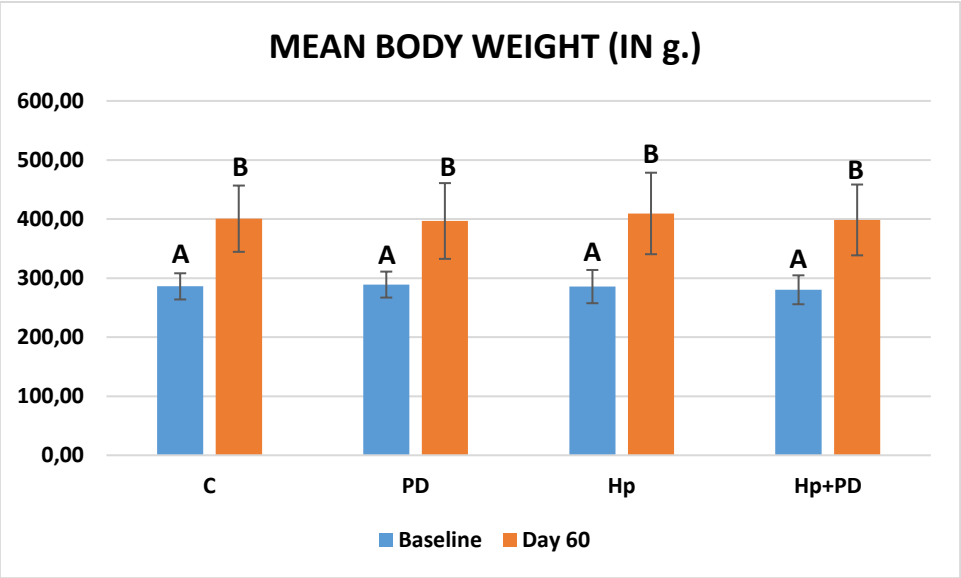

FIGURE 4: Body weight at baseline and at day 60 (end of the experiment). Analysis among groups was performed using One-Way ANOVA test, and within groups was t test for dependent samples ($p<0.05$). Different letters mean statistically significant differences between/within groups. C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

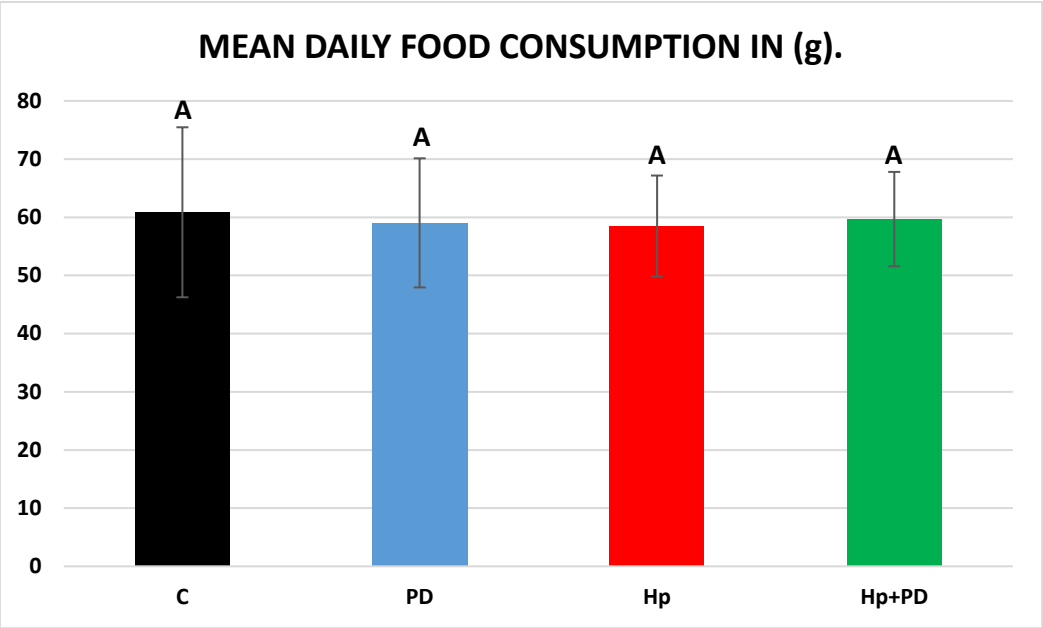

FIGURE 5: Mean daily food consumption during the experimental period. Different letters mean statistically significant differences by One-Way ANOVA test ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

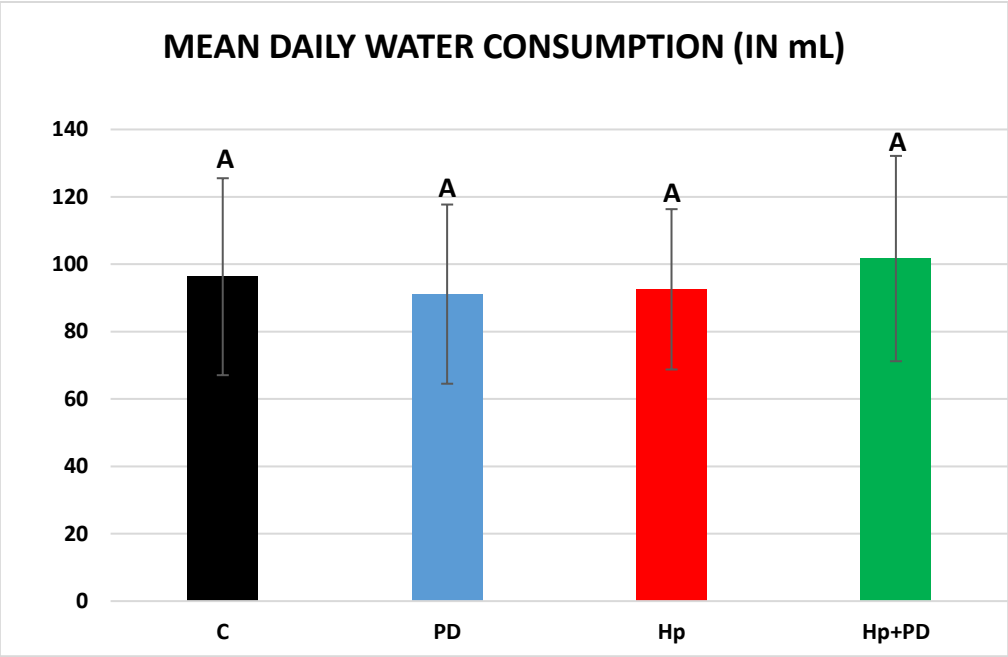

FIGURE 6: Mean water consumption during the experimental period. Different letters mean statistically significant differences by One-Way ANOVA test ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

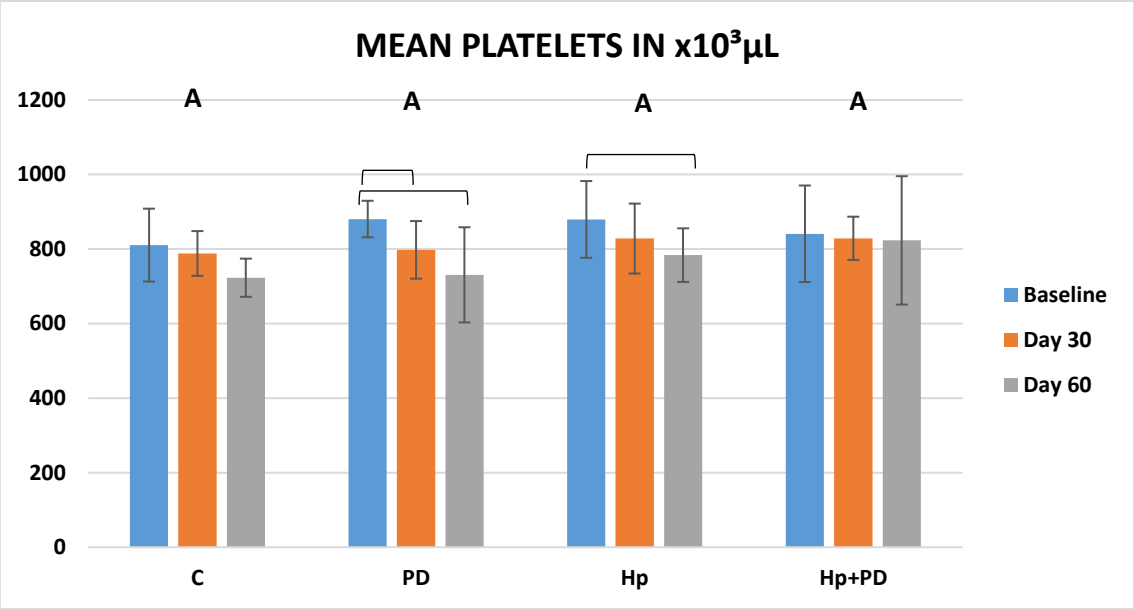

FIGURE 7: Mean platelets (x10³) at baseline, day 30, and day 60. Analysis among groups was performed using One-Way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between groups ($p<0.05$). Brackets means statistical significance within groups ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

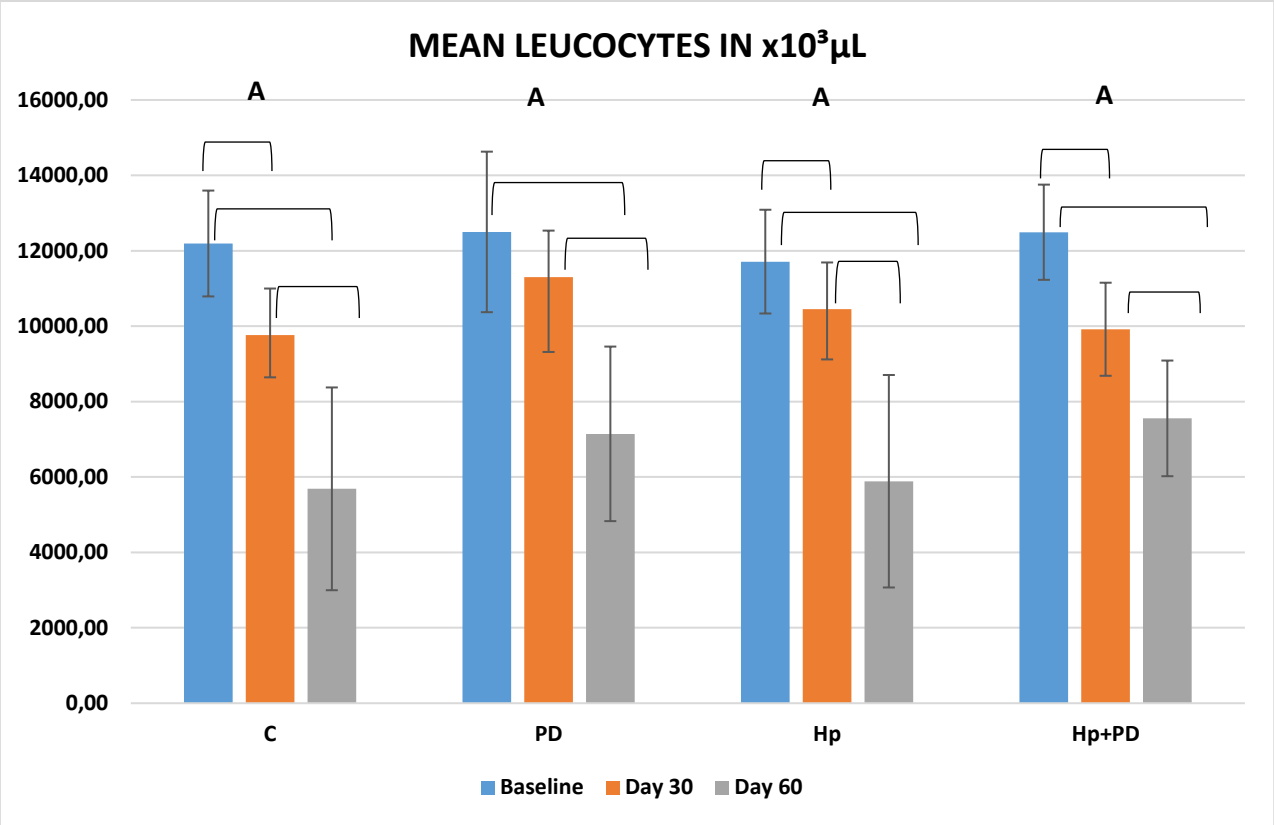

FIGURE 8: Mean leucocytes (x10³) at baseline, day 30, and day 60. Analysis among groups was performed using One-Way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between/within groups ($p<0.05$). Brackets means statistical significance within groups ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

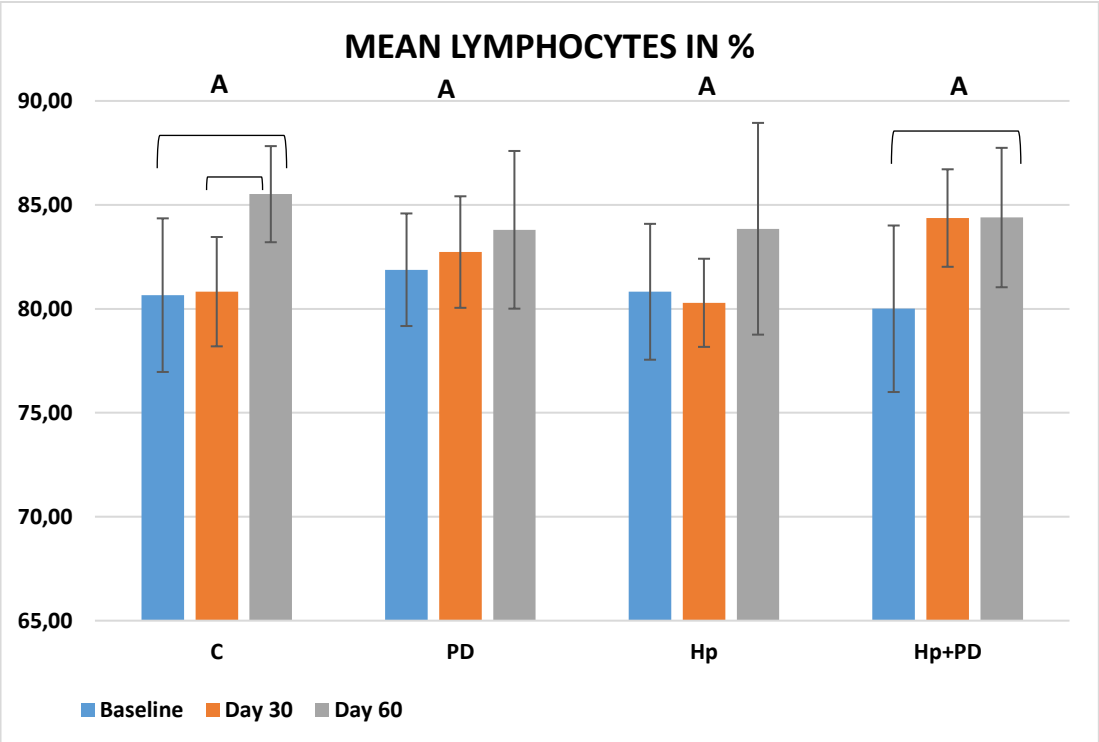

FIGURE 9: Mean lymphocytes (x10³) at baseline, day 30, and day 60. Analysis among groups was performed using One-Way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between/within groups ($p<0.05$). Brackets means statistical significance within groups ($p<0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

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IV. CONSIDERAÇÕES FINAIS

O objetivo do presente estudo foi avaliar os efeitos da heparina de baixo peso molecular na perda óssea alveolar em ratos Wistar. Estudos que relacionam os antiplaquetários com as doenças periodontais (Spolidorio et al., 2010) apresentam metodologias diversas como avaliação de cicatrização e processo inflamatório periodontal (Coimbra et al., 2011). Na literatura atual, as pesquisas que associam os efeitos dos anticoagulantes como a heparina de baixo peso molecular na perda óssea alveolar não existem. É neste sentido que por meio desta tese procurou-se estabelecer a relação entre os anticoagulantes e as doenças periodontais.

Em busca de determinar uma metodologia e criar uma base de dados disponíveis para continuar em pesquisar a associação entre as heparinas de baixo peso molecular e as etiopatogenias das doenças periodontais, é que esta tese foi desenvolvida.

Estudos em modelo animal são considerados importantes tendo em vista a plausibilidade biológica, permitindo uma melhor compreensão e explicação do processo biológico que acontece entre a etiopatogenia das doenças periodontais e os efeitos das heparinas.

A utilização das heparinas de baixo peso molecular atualmente tem um importante papel na prevenção e tratamento nas alterações tromboembolíticas.

Os resultados de presente estudo evidenciam que as heparinas de baixo peso molecular não tiveram a capacidade de produzir perda óssea alveolar adicional, mas sim a capacidade de produzir um processo inflamatório sistêmico. Assim, pode-se restringir a relação com processo inflamatório sistêmico, um reconhecido fator de risco a diferentes morbidades.

Como consequência dos resultados do presente estudo, novas reflexões são suscitadas, tentando compreender esta relação, permitindo que, do ponto de

vista das doenças periodontais, as heparinas de baixo peso molecular não apresentariam efeitos adversos significativos.

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