



Effects of Low Molecular Weight Heparin on Alveolar Bone Loss in Wistar Rats

Harry Juan Rivera Oballe¹, Marcelo Lazzaron Lamers², Francisco Wilker Mustafa Gomes Muniz³, Tobias Rauber Spuldaro¹, Eduardo José Gaio¹, Cassiano Kuchenbecker Rösing¹

¹Department of Periodontology, Faculty of Dentistry, UFRGS – Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
²Neurosciences and Oral Pathology, Faculty of Dentistry UFRGS – Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
³Department of Periodontology, Faculty of Dentistry, UFPEL – Universidade Federal de Pelotas, Pelotas, Brazil.

Correspondence: Cassiano Kuchenbecker Rösing, Rua Ramiro Barcelos, 2492, 90035-003 Porto Alegre, RS, Brasil. Tel: +55-51-3308-5318. e-mail: ckrosing@hotmail.com

This study aimed to assess the effects of low molecular weight heparin (LMWH) on alveolar bone loss (ABL), blood count, and counting of megakaryocytes and adipocytes in male Wistar rats. 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). LMWH was applied for 60 days at doses of 1 ml/kg/day. Blood samples were collected at baseline, 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. Mean ABL in the side with ligature showed significantly different between C (0.35 ± 0.07 mm) and Hp+DP (0.49 ± 0.09 mm) groups ($p < 0.001$), between PD (0.55 ± 0.11 mm) and Hp (0.32 ± 0.06 mm) groups ($p < 0.001$) and between Hp and Hp+DP groups ($p < 0.001$). No significant differences were found among groups for ABL in the side without ligature. Animal weight, food intake, and water consumption showed no statistically significant difference among groups. Megakaryocytes and adipocytes were counted using optical microscopy and no statistically significant differences were found. Within-groups, there were an increase and a decrease, respectively, in the counting of lymphocytes ($p = 0.005$ for C and $p = 0.009$ for Hp+PD groups only) and leukocytes ($p = 0.003$ for C, $p = 0.001$ for PD, $p = 0.002$ for Hp, and $p < 0.001$ for Hp+PD groups). There was no decrease in the number of platelets in the three collection periods. LMWH was not able to affect ABL, but it may change the blood counting, especially increasing lymphocytes.

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, triggering a production of pro-inflammatory cytokines, which leads to a destruction of the tooth support structures (1). Periodontitis is characterized by clinical attachment loss, presence of periodontal pockets and bleeding upon probing.

Low molecular weight heparins (LMWH) are the most widely used drugs worldwide for the prophylactic treatment of thromboembolic disorders (2). These are complex drugs that play the role of inhibiting the blood clotting cascade, acting on the inhibition of antithrombin (3). LMWH are also used to prevent and treat thrombotic diseases, such as venous thrombosis and pulmonary thromboembolism (3). In recent years, the levels of thromboembolism in patients have been reduced due to LMWH (4). The literature also reports a decrease in the pro-inflammatory cytokines levels after LMWH is administered in rats with acute pancreatic (5). Moreover, LMWH treatment decreased the expression of T helper cell-1 (Th-1), Th2, and Th17 (6), cells that are directly associated with the pathogenesis of periodontal disease. In contrast, there is no study that associate the effects of LMWH and destructive periodontal disease.

Therefore, this study aimed to evaluate the effects of LMWH on alveolar bone loss, blood count, and counting of megakaryocytes and adipocytes in Male Wistar rats, using morphometric and histological analyses. 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.

Material 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 home cages were of white plastic boxes, covered by a metallic class, 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. Moreover, the present study followed the ARRIVE reporting guideline (7).

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 h light and dark cycles, humidity 55% and temperature $22\pm 2^\circ\text{C}$. The experimental procedures performed in the present study are expressed in Figure 1.

Sample Size Estimation and Randomization

The sample size estimation for this study was based on a previously published study that assessed alveolar bone loss on male Wistar (8). It was found that nine animals per group was sufficient to detect a mean difference between groups of 0.2 mm of alveolar bone loss, accepting a power of 90% and an alpha error of 5%. An attrition rate of approximately 10% was added to the sample size, totaling 40 animals. 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 a website (radomizer.org). The animals were identified with distinct color markers in their tails. One of the researchers (FWMGM), not involved in the morphometric analysis in the jaws, performed the randomization.

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 (Ethicon® Bridgewater, NJ, USA), around the second right upper molar. No further substance was administered in the animals.

Heparin (Hp) group (N=10): The animals received low molecular weight heparin (Clexane® Sanofi Aventis, France, 40 mg 0.4 mL) subcutaneously. No ligature-induced periodontitis was placed in the 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®, 40 mg 0.4 mL) 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

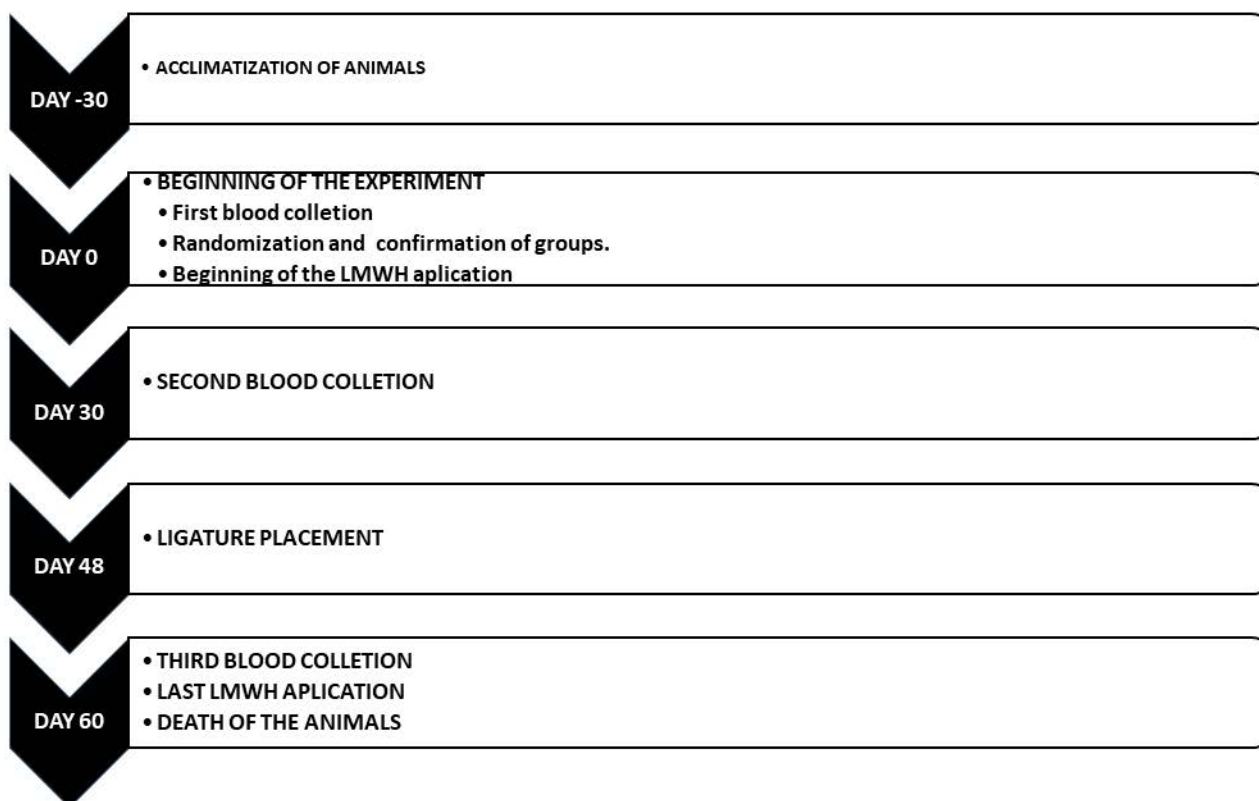


Figure 1. Flowchart of the experiment stages.

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 were performed at 08:00 am at days 0, 30 and 60. Isoflurane (Biochimico®, São Paulo, SP, Brazil) was used as an anesthetic. Both procedures were performed under a veterinarian supervision, not involved in the authorship of this study. The veterinarian was blind to the group allocation in all experimental procedures. 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 with EDTA, 1 mL of blood were collected. The tubes were stored in boxes and stored 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 day-30, 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®). 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 isoflurane (Biochimico®) under a veterinarian supervision. The black silk ligature was placed on the second maxillary right with the help of two Castro Viejo needle holders, light and scissors. 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. In the end of the study, it was observed that no ligature was lost.

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 of the researchers (HJRO, FWMGM or TRS) and the leftovers were discarded twice a week along with the grids. Regarding the consumption of water, 500 mL bottles were used in each box. The bottles were white opaque, and it was completed every other day, when the water consumption was measured by one of the 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

At day-59, the last dose heparin was applied, and the animals were fasted, remaining only the bottles with water. At day-60, they were previously anesthetized with 100 mL isoflurane (Biochimico®). The veterinarian also supervised the animals anesthetic procedure and performed the cardiac puncture for the third blood collection. Using 21G hypodermic needles and 10 mL syringes, the left ventricle was punctured and the blood was collected. The collected blood was placed in microtubes with 1 mL EDTA, stored in a styrofoam box with ice and then transferred to LACvet-UFRGS for analysis.

Subsequently, the maxillae were removed and placed in labeled and labeled flask submerged in a 10% buffered formalin solution. Thymus were also removed from each animal, and were submerged in a 10% buffered formalin solution. The right femurs were also extracted 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®, Gravataí, RS, Brazil) for a period of 4 h. After this time, the jaws were washed completely and submerged in clean water for 24 h. 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, for both the buccal and palatal sides of the right and left segments, in order to evidence 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, as described elsewhere (9). All the photos were analyzed by a software (Image J®, National Institutes of Health, USA), and, in each photo, the distance from the cemento-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 within 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, 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.

For calibration and training in counting megakaryocytes and adipocytes, a collaborator (MLL) with experience in the methodology trained the examiner for two weeks within one week-interval. It was demonstrated a ICC coefficient of 0.98.

Preparation of Femurs and Thymus

For the femur initial demineralization, Gracey and Padua Lima periodontal cures 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 24h, until it presents 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 (10). The distal and mesial parts of the femurs were cut and discarded. The central part of the femurs was cut in a sagittal form and embedded in paraffin. Afterwards, it was read for histological cuts.

For the thymus preparation, 10% buffered formalin was used and placed on a clean glass surface to be cut. The thymus was cut into three equal parts and submerged in 70° alcohol. To initiate the inclusion processing, the thymus was processed for 24 h and then placed in stainless steel for paraffin wax placement and the 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., Japan). The cuttings were done by a collaborator assisted by an experienced laboratory technician, using a microtome (Lupetec®) with a thickness of 0.50 µm. After the cuts, the operator took the blades to an oven at a continuous temperature of 55.6 °C for 24 h in order to eliminate any paraffin residue. After 24 h, the slides were removed and prepared to be stained by Hematoxylin and Eosin (HE) technique, according to a standard procedure.

Histological Analysis of Femurs and Thymus

For the femurs and thymus analyses, a binocular optical microscope (Olympus®, Tokyo, Japan) and a software were used to in order to obtain the microphotographs. The femurs and thymus were divided into five random parts, where the operator (HJRO) was able to take the microphotographs in 40X in each area. The researcher (HJRO) manually counted the megakaryocyte cells and the adipocytes on each microphotograph. The number of megakaryocyte and the adipocytes for each animal was determined by the sum of the five photographs.

Statistical Analysis

To evaluate the normal distribution 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 baseline and day-60, food and water consumption. One-way ANOVA assessed the differences among groups in mean body weight, food and water consumption, megakaryocytes counting, alveolar bone loss, and in platelet, leukocyte and lymphocytes counting. The comparison within groups for the platelet, leukocyte, and lymphocytes counting were analyzed by two-way ANOVA test. Whenever necessary, the Tukey post-hoc test was used the assessed the differences within 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 Statistics IBM®, College Station, TX, 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 shows that spontaneous mean alveolar bone loss in the side without ligature. No statistically significant differences were observed among groups ($p > 0.05$).

Table 1 shows the body weight at baseline and day-60. No statistically significant difference was observed among groups ($p = 0.925$). Significantly increase in body weight was observed for all groups ($p < 0.001$), with no statistically significant difference among groups ($p = 0.902$). Moreover, no statistically significant differences in water and food consumption were demonstrated among groups (Table 1). The number of megakaryocytes (Fig. 4) and adipocytes (Fig. 5) showed no statistically significant differences among groups ($p = 0.731$ and $p = 0.911$, respectively). Additionally, in Figures 4 and 5, it was detected that megakaryocytes and adipocytes are histologically normal.

Figures 6, 7, and 8 demonstrate cell counts in blood, and Table 1 shows the number of animals with normal blood counting at each experimental period. Mean platelet $\times 10^3/\mu\text{l}$ counts are demonstrated in Figure 6 in all three experimental periods. It was detected no statistically significant difference among groups at baseline ($p = 0.467$), day 30 ($p = 0.549$), and day 60 ($p = 0.266$) (Table 1). In the comparison within groups it was detected a statistically significant decrease in platelet count in PD group between baseline versus day 30 ($p = 0.007$) and day 60 ($p = 0.005$), while in the heparin group a statistically significant decrease was detected only between baseline and day 60 ($p = 0.035$).

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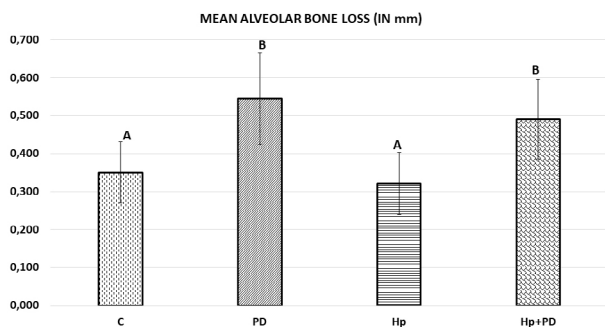


Figure 2. Mean alveolar bone loss in the sides with ligature (right sides). Different letters mean statistically significant differences by One-Way ANOVA test ($p < 0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

Figure 7 shows the mean leukocytes counting in all experimental periods. No statistically significant difference was observed among groups at baseline ($p = 0.636$), day 30 ($p = 0.100$), and day 60 ($p = 0.354$). Overall, a decrease in the leukocytes counting was detected in within all groups throughout the study.

Figure 8 shows the mean (\pm SD) percentage of lymphocytes. Again, no statistically significant differences were detected among groups in at baseline ($p = 0.677$) and day 60 ($p = 0.594$). In contrast, at day 30, a statistically significant difference was detected between the C and Hp groups ($p = 0.036$) and between the Hp and Hp+PD groups ($p = 0.005$).

Discussion

The present study aimed to evaluate the effect of the use of 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 an inflammatory disease, triggered by subgingival biofilm, that affects the supporting structures of the tooth with destruction of the connective tissue and alveolar bone loss (11). Microorganisms housed in the subgingival region produce inflammation and many immune reactions play an important role in the formation and progression of the disease (12). 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 (13).

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

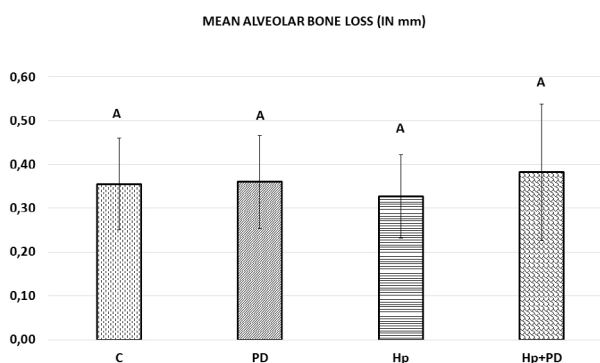


Figure 3. Mean alveolar bone loss in the sides without ligature (left sides). Different letters mean statistically significant differences by One-Way ANOVA test ($p < 0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

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 (14). Another study evaluated the effect of serum rabbit antiplatelet in the induction of thrombocytopenia on delayed periodontal healing (15). 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-induced. Therefore, it must be hypothesized that different antiplatelet/anticoagulant agents may produce different effects on the periodontal breakdown. The literature also shows that heparin effect on inflammatory stimulus is dependent on its dosage and the duration of the inflammation (16), which partially explain the results found in the present study. In this sense, therapeutic doses of LMWH may be expected to be safely used in patients with periodontitis. However, an increase in the number of lymphocytes may be expected in these individuals.

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

Table 1. Baseline and final weight, food, water consumption, and blood counting throughout the study in all experimental groups

	C	PD	Hp	Hp + PD	p value
Weight \pm SD at baseline (in g)	280.00 \pm 22.15	289.00 \pm 21.98	285.60 \pm 28.14	280.20 \pm 42.47	0.925*
Weight \pm SD at day 60 (in g)	400.57 \pm 56.11	396.69 \pm 64.15	409.47 \pm 69.00	398.47 \pm 59.95	0.902*
Mean daily food consumption (in g)	26.50 \pm 0.31	26.52 \pm 1.92	24.96 \pm 4.99	27.71 \pm 1.32	0.732*
Mean daily water consumption (in mL)	45.59 \pm 1.99	44.33 \pm 2.89	40.63 \pm 7.83	52.98 \pm 15.23	0.476*
Platelet					
First blood collection					
Normal count - n (%)	2 (28.6%)	3 (30.0%)	6 (60.0%)	5 (50.0%)	0.353#
High count - n (%)	5 (71.4%)	7 (70.0%)	4 (40.0%)	5 (50.0%)	
Second blood collection					
Normal count - n (%)	7 (100.0%)	8 (80.0%)	6 (60.0%)	7 (70.0%)	0.277#
High count - n (%)	0 (0.0%)	2 (20.0%)	4 (40.0%)	3 (30.0%)	
Third blood collection					
Normal count - n (%)	7 (100.0%)	8 (88.9%)	10 (100.0%)	7 (70.0%)	0.125#
High count - n (%)	0 (0.0%)	1 (11.1%)	0 (0.0%)	3 (30.0%)	
Leukocytes					
First blood collection					
Normal count	5 (71.4%)	8 (80.0%)	9 (90.0%)	7 (70.0%)	0.699#
High count	2 (28.6%)	2 (20.0%)	1 (10.0%)	3 (30.0%)	
Second blood collection					
Normal count	7 (100.0%)	8 (80.0%)	8 (80.0%)	10 (100.0%)	0.282#
High count	0 (0.0%)	2 (20.0%)	2 (20.0%)	0 (0.0%)	
Third blood collection					
Normal count	7 (100.0%)	8 (88.9%)	8 (80.0%)	10 (100.0%)	0.332#
High count	0 (0.0%)	1 (11.1%)	2 (20.0%)	0 (0.0%)	
Lymphocytes					
First blood collection					
Normal count	3 (42.9%)	4 (40.0%)	2 (20.0%)	3 (30.0%)	0.719#
Low count	4 (57.1%)	6 (60.0%)	8 (80.0%)	7 (70.0%)	
Second blood collection					
Normal count	3 (42.9%)	6 (60.0%)	2 (20.0%)	6 (60.0%)	0.228#
Low count	4 (57.1%)	4 (40.0%)	8 (80.0%)	4 (40.0%)	
Third blood collection					
Normal count	5 (71.4%)	5 (55.5%)	6 (60.0%)	3 (30.0%)	0.348#
Low count	2 (28.6%)	4 (44.4%)	4 (40.0%)	7 (70.0%)	

*One-way ANOVA test; #chi-square test.

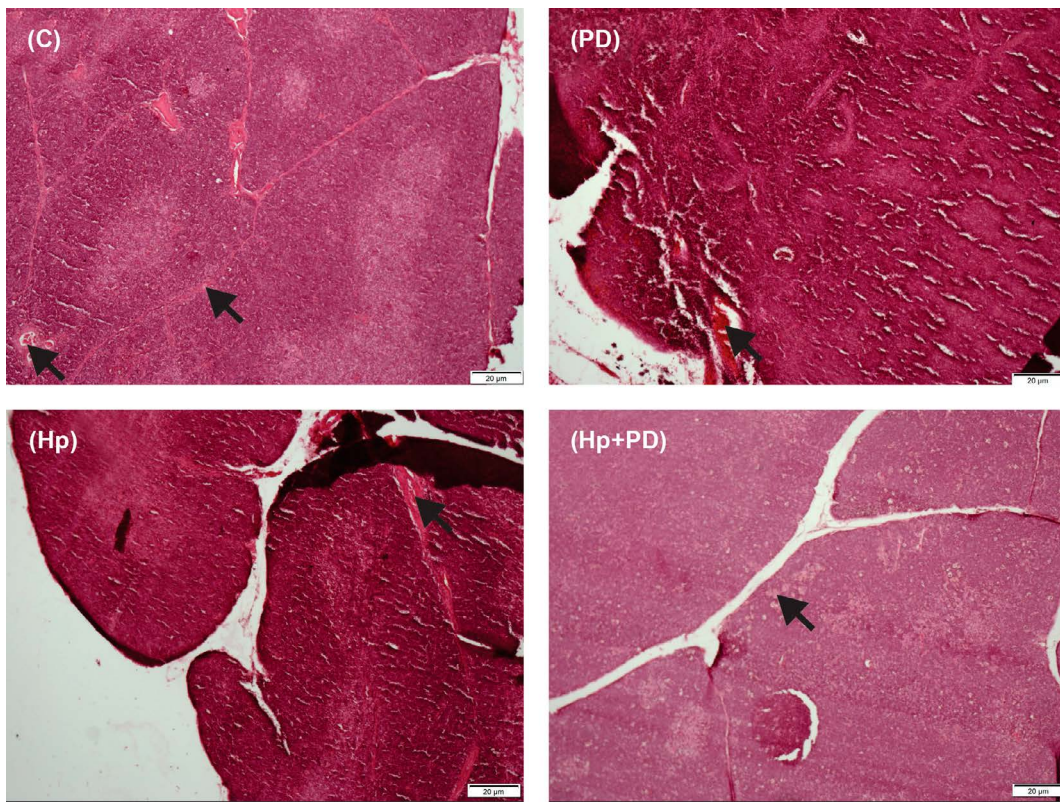


Figure 4. Photomicrographs of the femurs, showing the megakaryocytes cells detected in the experimental groups. Arrows shows the megakaryocytes cells observed. C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

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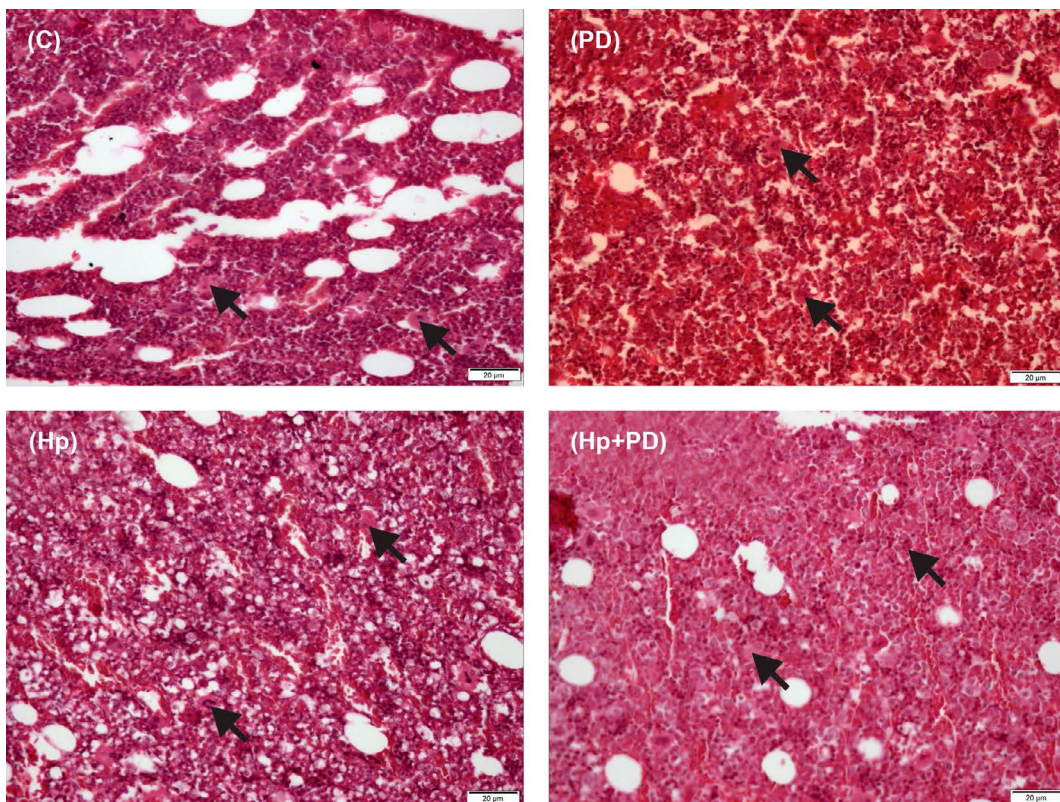


Figure 5. Photomicrographs of the thymus, showing the adipocytes cells detected in the experimental groups. Arrows shows the adipocytes cells observed. C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

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.

Megakaryocytes are cells located in the bone marrow that have the responsibility of platelet formation. Megakaryocytes are cells that are 10 to 17 times the size of a red blood cell, with a diameter of 50-100 μm (17). Megakaryocytes are precursor cells of hematopoietic cells in the marrow. Being produced in the liver, spleen, kidneys and bone marrow. 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.

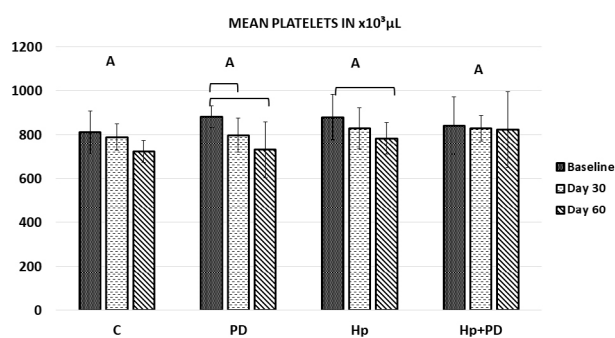


Figure 6. Mean platelets ($\times 10^3$) at baseline, day 30, and day 60. Analysis among groups was performed using One-way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between groups ($p < 0.05$). Brackets means statistical significance within groups ($p < 0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

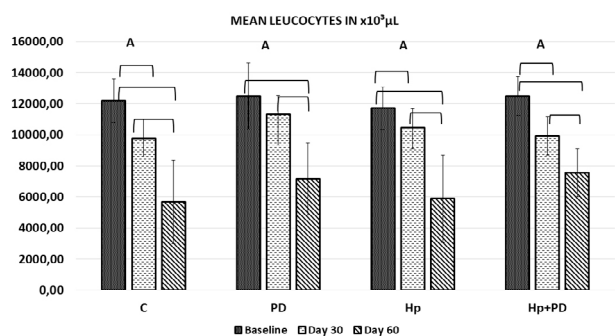


Figure 7. Mean leucocytes ($\times 10^3$) at baseline, day 30, and day 60. Analysis among groups was performed using One-way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between groups ($p < 0.05$). Brackets means statistical significance within groups ($p < 0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

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. This gland produces defense cells against infectious microorganisms and other harmful elements (18). Studies reveal that the thymus with its lymphoid structures is dependent on the precursor lymphopoietic 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 (19). 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 (20).

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 and in the inflammatory response (21). Low molecular weight heparin is fractions of unfractionated heparin obtained by enzymatic depolarization with molecular weight of 1000 to 10,000 daltons (2). Studies have demonstrated the great advantages of using LMWH in the treatment of thromboembolism (3) and against deep venous thrombosis (22). 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 (23). There have been studies evaluating the induction of severe thrombocytopenia in short time using antiplatelet agents such as rabbit antiplatelet serum in a 2 mL dose to evaluate pro-and anti-angiogenic VEGF

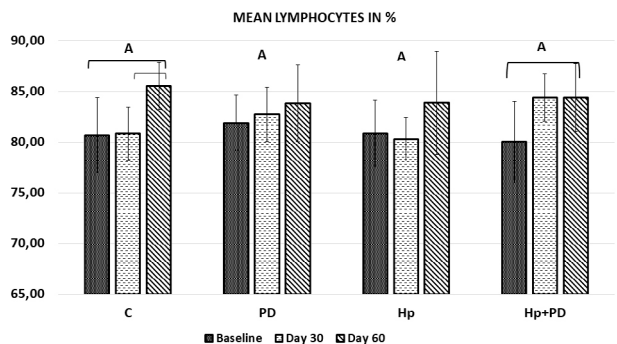


Figure 8. Mean lymphocytes ($\times 10^3$) at baseline, day 30, and day 60. Analysis among groups was performed using One-way ANOVA test followed by Tukey post-hoc, and within groups was two-way ANOVA. Different letters mean statistically significant differences between groups ($p < 0.05$). Brackets means statistical significance within groups ($p < 0.05$). C: control group; PD: periodontal disease group; Hp: Heparin group; Hp+PD: Heparin + Periodontal disease group.

growth factor and induced alveolar bone loss in wistar rats (15), showing that thrombocytopenia retarded periodontal healing in periodontitis. Other studies have been carried out evaluating antiplatelet agents, such as Clopidogrel and Aspirin, in the inflammatory response where they play an important role in the immune response on induced periodontitis (14). 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 (24). There are few studies that relate leukocyte levels to periodontal disease in patients with infectious diseases (25). 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. 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 (26). Studies have shown that some interleukins such as IL-10 reduce their levels during resolution of periodontitis (27). 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 (28).

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 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 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 doses of LMWH used in the present study was not able to induce thrombocytopenia, that is significant lower platelet counting. Therefore, it may be hypothesized that higher doses of LMWH would induce thrombocytopenia and, consequently, the animals would present a significant higher alveolar bone loss in the sites with ligature. In this sense, future studies should try higher doses of LMWH in order to induce thrombocytopenia and, afterward, evaluate the alveolar bone loss that occurred in these animals.

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.

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Resumo

O presente estudo objetivou verificar o efeito da heparina de baixo peso molecular (HBPM) sob a perda óssea alveolar (POA), contagem de células sanguíneas, megacariócitos e adipócitos em ratos Wistar machos. Quarenta ratos Wistar de 60 dias foram randomicamente divididos em quatro grupo: Controle (C), Doença Periodontal (DP), Heparina (Hp) e Heparina + Doença Periodontal (Hp+DP). HBPM foi aplicada durante 60 dias em doses de 1 mL/kg/dia. Coletas sanguíneas foram realizadas nos dias 0, 30 e 60. No dia 49, os grupos DP e Hp+DP receberam indução de doença periodontal por ligadura ao redor do segundo molar superior direito. No lado esquerdo, verificou-se perda óssea alveolar espontânea. A média de POA no lado com ligadura mostrou-se estatisticamente diferente entre os grupos C ($0,35 \pm 0,07$ mm) e Hp+PD ($0,49 \pm 0,09$ mm) ($p < 0,001$), entre os grupos DP ($0,55 \pm 0,11$ mm) e Hp ($0,32 \pm 0,06$ mm) ($p < 0,001$) e entre os grupos Hp e Hp+DP ($p < 0,001$). Nenhuma diferente significativa foi observada entre os grupos no lado sem ligadura. Peso dos animais, consumo de ração e ingestão de água não mostraram diferenças significativas entre os grupos. Megacariócitos e adipócitos foram contados por microscopia óptica e nenhuma diferença significativa foi encontrada. Dentro dos grupos, houve um aumento e uma diminuição, respectivamente, na contagem

de linfócitos ($p=0,005$ no grupo C e $p=0,009$ no grupo Hp+DP somente) e leucócitos ($p=0,003$ no grupo C, $p=0,001$ no grupo DP e $p=0,002$ no grupo Hp e Hp+DP). Não houve diminuição no número de plaquetas nos três períodos de coleta. HBPM não foi capaz de modificar a POA, porém modificou a contagem de células sanguíneas, especialmente aumentando o número de linfócitos.

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