

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

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ASPECTOS METODOLÓGICOS DE ESTUDOS DA DOENÇA PERIODONTAL EM
RATOS: INFLUÊNCIA DA PRESENÇA E DO TEMPO DE LIGADURA

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia pela Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

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RESUMO

SEIBEL, Luise. **Aspectos metodológicos de estudos da doença periodontal em ratos:** influência da presença e do tempo de ligadura. 2015. 24 p. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

Objetivo: avaliar a perda óssea alveolar induzida por ligadura em ratos Wistar em diferentes períodos de tempo e avaliar se a presença de ligadura em um lado afeta a perda óssea alveolar no lado contralateral nestes períodos de tempo.

Materiais e métodos: o presente estudo é resultado de uma análise secundária de bancos de dados, cujos estudos foram realizados pelo grupo de pesquisa da periodontia da Universidade Federal do Rio Grande do Sul. Os estudos selecionados incluíam o modelo de indução de doença periodontal por ligadura em ratos Wistar. Para serem incluídos na análise, os estudos precisavam possuir grupo controle que não tenha sido submetidos a indução de doença periodontal, assim como controle intra-grupos. Foram incluídos 3 estudos, que correspondiam a esses critérios, com diferentes períodos de análise. Estudo 1 com 2 e 8 semanas, Estudo 2 com 5 e 17 semanas e Estudo 3 com 22 semanas para os lados com e sem ligadura respectivamente. Os ratos Wistar dos três estudos, foram criados nas mesmas condições e, ao final de cada período experimental, os ratos foram sacrificados por decapitação, as maxilas retiradas e os tecidos moles química e mecanicamente removidos com hipoclorito de sódio 9%. Tomadas fotográficas foram realizadas e cinco mensurações foram obtidas de cada imagem.

Resultados: A presença de ligadura gerou uma perda óssea alveolar significativamente maior quando comparado ao lado sem ligadura. Considerando os lados com ligadura um período de 2 semanas com ligadura mostra menor perda óssea alveolar que 5 e 22 semanas. Quando os períodos de 5 e 22 semanas são comparados não se observa diferença estatisticamente significativa. Em relação aos lados sem ligadura avaliados, não observou-se diferença significativa entre 8 e 17 semanas para periodontite espontânea. No entanto a partir de 22 semanas os animais exibiram significativamente maior perda óssea alveolar quando comparado aos demais tempos experimentais. A presença de ligadura em um dos lados não influenciou a perda óssea do lado contralateral sem ligadura.

Conclusão: Pode-se concluir que 2 semanas de doença periodontal induzida por ligadura parece ser suficiente para demonstrar perda óssea significativa e a utilização de lados contralaterais de dentes com ligadura é possível de ser considerada como controles saudáveis, reduzindo o número de animais em pesquisa.

Palavras-chave: Ligadura. Periodontite espontânea. Periodontite induzida.

ABSTRACT

SEIBEL, Luise. **Methodological aspects in the study of periodontal breakdown in rats:** influence of presence and time of ligature. 2015. 24 p. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

Aim: The aim of the present study is to evaluate alveolar bone loss in different time points and to evaluate if the presence of ligature in one side affects alveolar bone loss in the contralateral site in these time periods

Material and methods: this study is the result of a secondary analysis of databases, which studies were performed by the research group of periodontics at the Federal University of Rio Grande do Sul. The selected studies included the model of periodontal disease induced by ligature in rats. To be included in the analysis, the studies needed to have control group that has not been subjected to periodontal disease induction, as well as intra-group control. It was included three studies that met these criteria, with different periods of analysis. Study 1 with 2 and 8 weeks, Study 2 with 5 and 17 weeks and Study 3 with 22 weeks, for the sides with and without ligature respectively. The Wistar rats of these studies were created under the same conditions and at the end of each trial period, the rats were sacrificed by decapitation and maxillae were defleshed with 9% sodium hypochlorite. Pictures were taken and five measurements were obtained from each image.

Results: The presence of ligature generated a significantly greater alveolar bone loss when compared to side without ligature. Comparing sides with ligature, a 2-week period shows lower alveolar bone loss than 5 and 22 weeks. When periods of 5 and 22 weeks are compared is not observed a statistically significant difference. The sides without ligature that were evaluated did not showed a significant difference between 8 and 17 weeks for spontaneous periodontitis. However, as from 22 weeks the animals exhibited significantly greater alveolar bone loss when compared to other experimental times. The presence of ligature in one side did not influence bone loss in the contralateral side without ligature.

Conclusion: It may be concluded that two weeks of ligature-induced periodontal disease seems to be enough to demonstrate significant bone loss and that utilizing the contralateral sides of teeth with ligature is possible to be considered sound controls, reducing the amount of animals in periodontal research.

Keywords: Ligature. Experimental periodontitis. Spontaneous periodontitis.

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1 APRESENTAÇÃO

A periodontite é uma doença com prevalência considerável em diferentes populações e constitui-se em importante causa de perda dos dentes especialmente em adultos. É influenciada por alguns fatores ambientais, comportamentais, biológicos e medicamentosos.

Diferentes modelos experimentais que mimetizam os processos etiopatogênicos envolvidos nas doenças periodontais têm sido propostos pela literatura especialmente com a utilização de ratos. Esta ampla aceitação e utilização justifica-se a medida que ratos apresentam importantes similaridades anatômicas, histopatológicas, microbiológicas e bioquímicas com os seres humanos. Considerando os diferentes modelos utilizados para reproduzir a etiopatogenia das doenças periodontais destacam-se os métodos que utilizam ligaduras, sendo esse considerado pela literatura um método bastante representativo. O mesmo baseia-se no pressuposto de que a presença da ligadura serviria como um fator de retenção de biofilme, favorecendo, portanto, a colonização da superfície e acelerando o processo de destruição periodontal.

No entanto, dúvidas ainda permanecem, especialmente sobre qual o período de tempo considerado ideal para a permanência da ligadura, bem como se a presença de ligadura de apenas um lado teria capacidade de aumentar a carga bacteriana na boca dos animais, gerando maiores destruições periodontais no lado contralateral.

Nesse sentido, o presente trabalho de conclusão de curso, além dos objetivos acadêmicos, busca elucidar alguns aspectos metodológicos envolvidos em estudos sobre um já consagrado modelo de indução das doenças periodontais que é a perda óssea induzida por ligadura. Trata-se de um experimento no qual utilizou-se bancos de dados de estudos em animais do grupo de periodontia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul. O presente estudo consiste de um artigo científico intitulado "Methodological Aspects In The Study Of Breakdown Of Periodontal Breakdown In Rats: Influence Of The Presence And Time Of Ligature" que será submetido para a revista Brazilian Oral Research.

2 ARTIGO CIENTÍFICO

Methodological Aspects In The Study Of Periodontal Breakdown In Rats: Influence Of The Presence And Time Of Ligature

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INTRODUCTION

Periodontitis is highly prevalent on worldwide population and is a major cause of tooth loss in adults.^{1, 2} It affects the underlying supporting structures of the teeth, resulting in loss of connective tissue and bone support.^{3, 4}

Animal models contribute as part of the body of evidence, with increasing translational potential.⁵ Experimental periodontitis models have been used to understand the etiopathogenic processes involved in periodontal disease, for the study of new therapeutic agents and other factors associated with periodontitis.⁶⁻¹⁰ Rats are often used in studies of experimental periodontitis since their anatomy in the molar region is very similar to that region in humans.^{11, 12}

There is clear evidence from the literature demonstrating bone loss in rats induced by the injection of lipopolysaccharides (LPS) from different bacterial strains including *P.gingivalis* or the use of ligatures in the gingival sulcus around molars.^{6, 13} The latter has its basis on the creation of a bacterial retention factor obtained by ligature placement that mimics faster and more intensely what happens in humans. The use of ligatures as periodontal disease induction model, has been suggested by some authors as an representative model to study the pathogenesis of periodontal disease.¹⁴ On the other hand, issues such as the possible trauma generated by the presence of ligature and the eventual loss of it during the trial period would be factors to be taken into consideration when the study is planned.

Considering the time of periodontal disease induction there is no consensus in the literature. Some authors state that larger alveolar bone loss levels are observed in the first 7-15 days after placement of the ligature.¹⁵ On the other hand periods of 4 or more weeks are widely used.¹⁶

Recently, some studies have observed the evaluation of spontaneous periodontitis models in rats, with interesting results.^{17, 18} However, there is possibility of a cross-over effect that should be looked, since the presence of an irritant in one side could affect the contralateral site. This model is referred to as a “split-mouth” design.

There are different methods proposed in the literature to measure alveolar bone loss in rats: histometric, morphometric, radiographic measurements and

computed tomography. All those methods are widely used, accurate and capable of detecting alveolar bone loss in rats.¹⁹ To date there is no method considered gold standard for the measurement of periodontal disease in rats. In this sense the method should be chosen according to the purpose of the study.

The aim of the present study is to evaluate alveolar bone loss in different time points and to evaluate if the presence of ligature in one side affects alveolar bone loss in the contralateral site in these time periods. The hypothesis to be tested is that the placement of a ligature in one side of the animal does not increase spontaneous alveolar bone loss in the contralateral side. In addition, a hypothesis that the time of ligature placement does not substantially increase bone loss after 2 weeks of ligature placement is raised.

METHODS

STUDY DESIGN

This is a secondary analysis of a database of studies in which periodontal breakdown was induced by ligature in Wistar rats. Data are retrieved from periodontal disease models induced by ligature in rats belonging to the periodontology research group of Federal University of Rio Grande do Sul were eligible for the analysis. To be included in the analysis, the study should present control groups that did not undergo periodontal disease induction and intra-group controls, i.e. sites contralateral to those in which periodontal disease was induced by ligature placement. The search in databases revealed 19 studies eligible for the analysis from which 3 met the inclusion criteria and were included. The studies included vary in experimental periods as follows: study 1 (2 weeks of ligature and 8 weeks of spontaneous periodontal breakdown) study 2 (5 weeks of ligature and 17 weeks of spontaneous periodontal breakdown) and study 3 (22 weeks for both, spontaneous and induced periodontal breakdown).

ANIMALS

All studies included used 45-60 days-old male Wistar rats (weighting 250-350g). Animals were housed in groups of 4-5 under a light/dark cycle of 12 hours and room temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) with free access to water and Standardized rat chow (Nuvilab CR-1, NUVITAL[®], Curitiba, PR, Brazil). The animals remained throughout the experimental periods in two different locations according to the study, with the same routines. A total of 88 rats were included in the analysis

QUALITY ASSESSMENT

The studies included in this analysis report important methodological care as stated in ARRIVE Guidelines.²⁰ For example, randomization, blinding, calibration of examiners, reproducibility and care in the handling of animals especially in the reduction of pain and discomfort were performed in all included studies.

PERIODONTAL DISEASE MODEL

Periodontal disease was induced by placement of a silk ligature (Ethicon, Johnson & Johnson, São Paulo, Brazil) on the right upper second molar with the knot made on the buccal side.^{21, 22} The contra lateral second molar was considered the intra-group control. Ligature placement was performed under general anesthesia with inhaled 5V% isoflurane (Isoforine[™] Cristália, SP, Brazil), vaporized in 100% oxygen by facemask or by intraperitoneal injection of 5% ketamine/2% xylazine (10 mg/kg—1:1). A veterinarian performed all anesthetic procedures.

SACRIFICE

All animals were sacrificed by decapitation.

SPECIMEN PREPARATION

Maxillae were removed, sectioned, and defleshed in 9% sodium hypochlorite for 2 hours and the remained soft tissue was removed mechanically. After this period, specimens were washed and dried. For a better visualization of the cemento-enamel junction, maxillae were stained with 1% methylene blue, according to Fernandes et al. (2007).²³

MORPHOMETRIC ANALYSIS

The morphometric analysis was performed by standard digital photographs. Pictures were taken using a 6.1 megapixel digital camera (Nikon™ Coolpix, Ayutthaya, Thailand) coupled to a tripod and equipped with 100mm macro-lenses with minimal focal distance. Specimens were fixed to an endodontic ruler, parallel to the ground. Photographs of the buccal and palatal aspects of right and left hemimaxillae were taken.

Measurements were made linearly from the cemento-enamel junction to the bone crest, using Adobe Photoshop™ CS4 software (Adobe Systems Inc., San Jose, CA, USA). Five measurements were performed on each surface of second molar both buccally and palatally (two on the distal root, two on the mesial root and one on the furcation). The measurements in pixels were then converted into millimeters using the markings of the endodontic ruler to which the hemimaxillae were attached as reference. Figure 1 shows an example of a specimen used in one of the three studies and the measurements made on the buccal surface.

For all included studies specimen preparation, photographs as well as morphometric analysis were performed at the Laboratory of Periodontology of the Federal University of Rio Grande do Sul and followed the methods proposed by Fernandes et al. (2007).²³

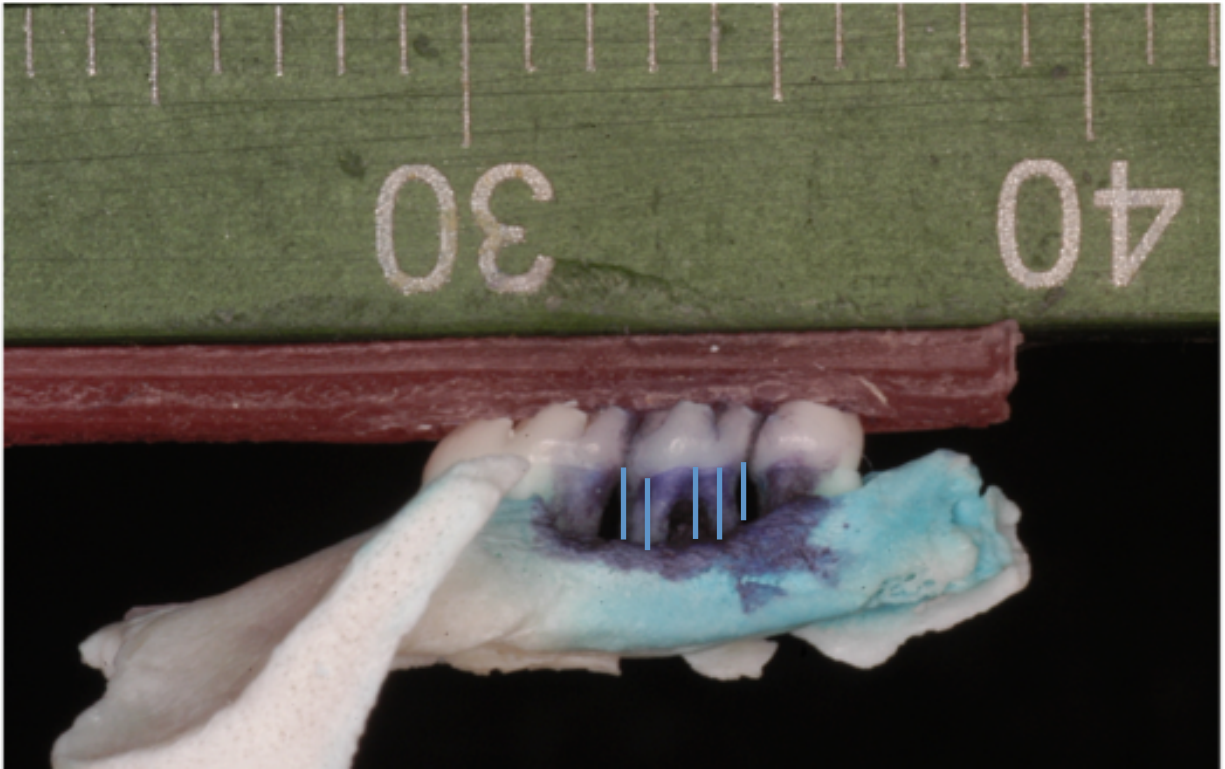


Figure 1: Representative photograph of a specimen illustrating the morphometric analysis in maxillae which alveolar bone loss was induced by ligature.

STATISTICAL ANALYSIS

The normality of data was checked by Shapiro-Wilk test and the data exhibited a normal distribution. Mean and standard deviations of Alveolar Bone Loss (ABL) in different time points were generated and compared by one-way ANOVA followed by Bonferroni multiple comparisons test. Comparisons between contralateral sites in animals with and without ligature were made by independent sample t-test. All analyses were performed in Stata 10.1 for Macintosh (Stata™, College Station, TX). The level of significance was set as .05.

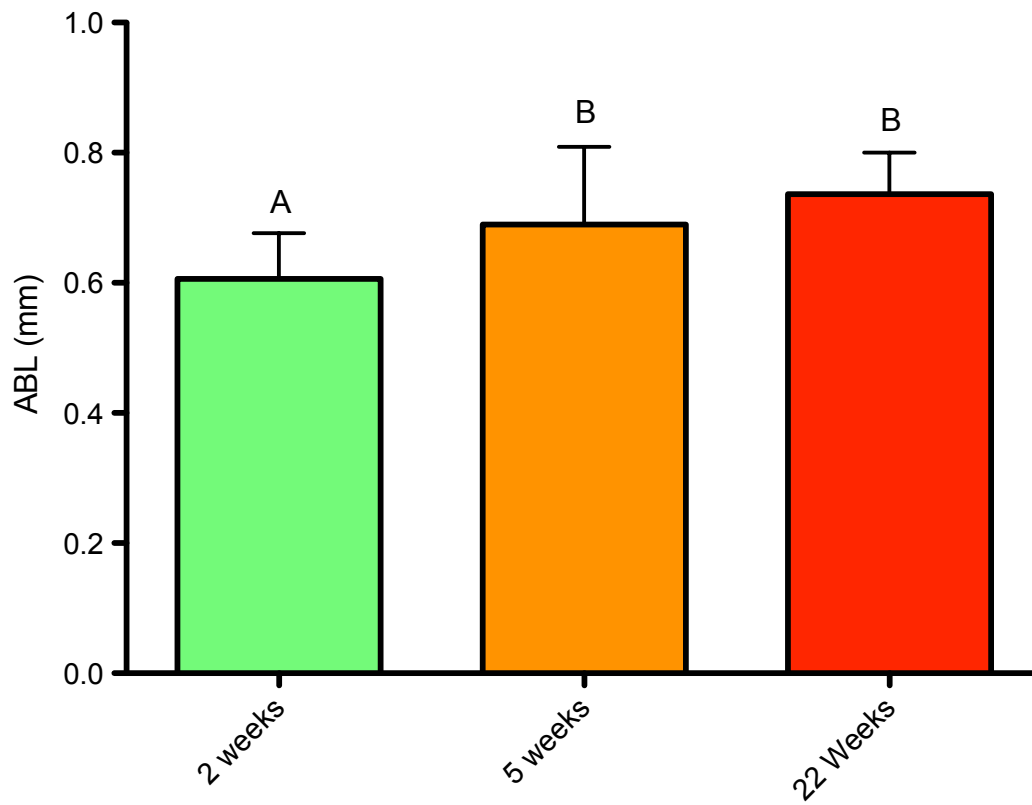
RESULTS

Sites with ligature exhibited significantly higher ABL as compared to sites in which periodontal disease was not induced (characterized as spontaneous ABL) showing that the model was effective in reproducing one of the most important signs of periodontal disease which is alveolar bone loss (Figure 2 and 3).

The % of difference between ABL was 40%, 54% and 57% for Studies 1, 2 and 3, respectively, dependent of the experimental period. This means that the higher the experimental period, the greater periodontal destruction experience rats exhibit. Figure 2 and 3 shows the mean ABL for sites with and without ligature, according to experimental period, respectively. For sites with ligature a period of 2 weeks of periodontal breakdown showed significant lower ABL when compared to 5 and 22 weeks. Additionally, when 5 and 22 weeks were compared, no significant differences were observed showing that a period of 5 weeks must be sufficient to reproduce the signs of periodontal disease (Figure 2).

When sites without ligature were evaluated, no significant differences in ABL were observed between 8 and 17 week. However when a period of 22 weeks was used significant differences must be detected, showing that higher periods must be necessary to reproduce spontaneous ABL in Wistar rats (Figure 3).

Figure 4 shows the comparison between control sites in animals submitted or not to ligature-induced periodontal disease. No statistically significant differences were observed between 22 and 5 weeks. This means that the presence of ligature in one side does not affect the mean alveolar bone loss on contralateral site.



Different letters indicates statistically significant differences
ANOVA - Bonferroni

Figure 2. Mean Alveolar Bone Loss (ABL) for sites with ligature according to experimental period

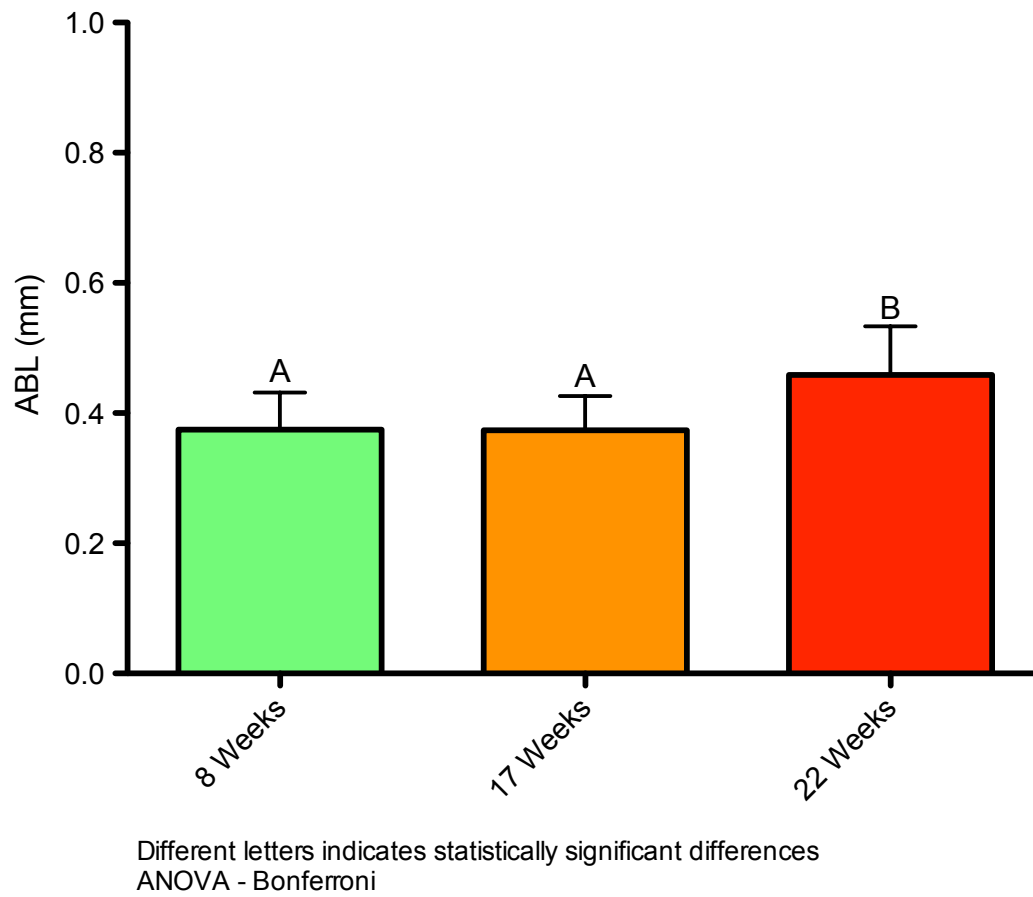
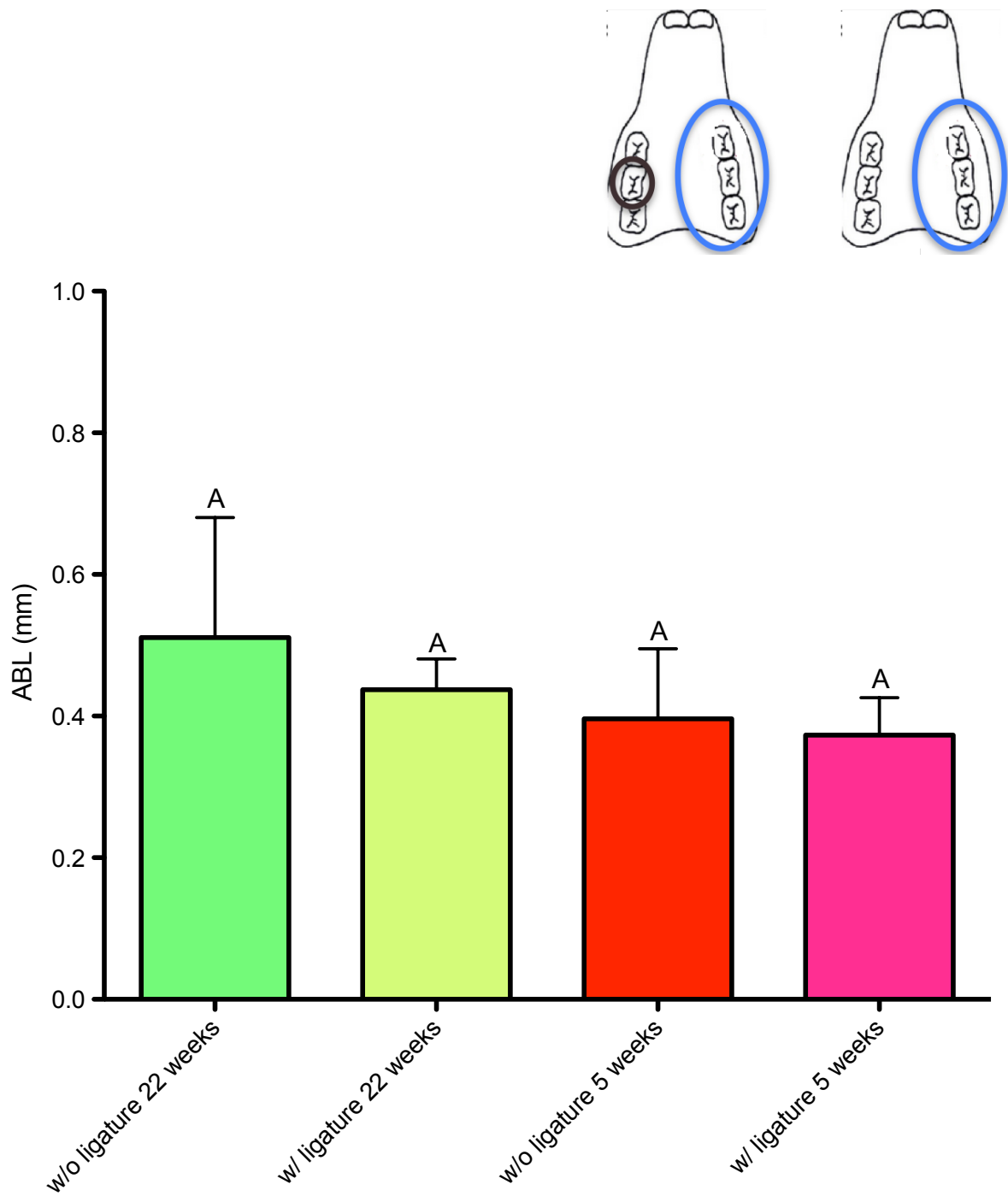


Figure 3. Mean Alveolar Bone Loss (ABL) for sites without ligature according to experimental period.



Different letters indicates statistically significant differences
Independent samples t test

Figure 4. Comparison of Alveolar Bone Loss (ABL) in control sites in animals with and without ligature.

DISCUSSION

The present study has a methodological nature and aimed at better understanding the effect of time of induction as well as a possible cross-over effect of the presence of ligature in the contralateral side in studies using ligature-induced periodontal breakdown in rats.

Studies utilizing animal models are fundamental in comprehending etiopathogenical aspects of periodontal diseases. Therefore, a number of studies has been performed using different animal strains.^{8, 24} Wistar rats are one of the most used species in studies of pathogenesis of periodontal diseases. They are non-isogenic rats that present variability in immune response, similar to humans.²⁵ In addition, the anatomical characteristics are also similar.¹²

In periodontal research in animals, discussions have been raised about what should be the best model of induction, of analysis as well as if there is really necessity of inducing the disease.¹⁵ Therefore, studies have looked at naturally occurring periodontal disease, that could be an interesting way of demonstrating the effect of different exposures, without the high intensity challenge.^{17, 18}

The present study utilized morphometric analyses for periodontal breakdown. Different methods have been used, including histology, morphometry and tomography. All of them lead to different approaches, however are considered reproducible and capable of demonstrating periodontal breakdown.¹⁹ In addition, the site of analysis (area, proximal, furcation, buccal, etc.) has also been studied and all of them have demonstrated capability of reproducibility and detecting the occurrence of alveolar bone loss.^{23, 26-28}

In animal research, a lot of discussion has been performed in terms of following the recommendation of the “3Rs”: reduce, refine and replace.²⁸ In this respect, if it is possible to use a “split-mouth” design (in which ligatures, for example, are placed in one side and the contralateral tooth functions as control), it is possible to reduce the number of animals, since the use of a totally non-manipulated/exposed control is not necessary. However, the literature, to the best of the authors’ knowledge, has not addressed this point yet. Thus, the novelty of the present study

resides in further allowing evidence-based choices of using contralateral sides as controls, reducing the number of animals in periodontal research.

One interesting point in periodontal research using animals is the time of induction of periodontal disease. There is no consensus in the literature demonstrating that one time is better the other in terms of occurrence of periodontal breakdown. Studies have used different time points, from 1 week to months.^{15, 29} If it is possible to effectively establish a minimum time of induction of periodontal breakdown, the principle of refining the method can be contemplated.

Taking into consideration the points previously raised, and considering a research group with experience in periodontal pathogenesis studies in rats, we decided to analyse data from our database to address these questions. Therefore, to be included in the present study, the experiment should have a total control group, with no exposure either to an external agent or to periodontal disease induction. Also, a control group not exposed to an external agent, however with one of the sides with and one of the sides without ligatures. From a database of 19 experiments, performed with similar protocols, three studies fulfilled these criteria and were included in the present analysis.

It should be emphasized that all experiments included present similar housing, temperature, food and liquid intake, manipulation strategies that allow inter-study direct comparisons. In addition, the laboratory procedures and analyses were performed identically, including randomization, blinding and reproducibility. All these research principles support a consistent level of internal validity.³⁰ None of the included studies had the same time of induction, therefore the analysis has no merging of groups.

In relation to the time of induction, the results of the present study indicate that there is a time-dependent relationship of amount of alveolar bone loss in ligature-induced models, i.e. after 2 weeks there is less alveolar bone loss as compared to 5 and 22 weeks. However, the comparison between 5 and 22 weeks does not demonstrate an additional breakdown.

This fact should be looked in different perspectives. One important fact is that the studies that demonstrate sufficient effect after shorter periods (2 weeks, for example) are performed in isogenic rats.¹⁵ On the other hand, in this respect, it

should be emphasized that the study included in this analysis with 2 weeks of induction, was able to detect statistically significant differences as compared to controls, which indicates that periodontal breakdown was actually achieved. In of the studies of our group, no statistically significant differences were observed between 29, 43 and 57 days of ligatures.³¹ The benefit of using less experimental time relates to cost-effectiveness of research.

It should also be remembered that groups with ligatures always present significantly higher degrees of periodontal breakdown that groups without ligatures. However, studies have achieved to demonstrate some effects only in sites without ligatures, suggesting a potential increased challenge that would mask the effect of the presence of a naturally occurring biofilm.³² This has no support by the mechanical effect of the presence of the ligature, since germ-free animals exposed to ligature-induced periodontal disease did not present significant periodontal breakdown.³³ In this respect, it is feasible to understand that a 2-week period seems to be sufficient for ligature-induced periodontal breakdown, however, depending on the exposure variable to be tested, additional time could be necessary and after 5 weeks the breakdown seems to level-off.

In animals with spontaneous alveolar bone loss, the time of occurrence of periodontal breakdown is bigger. Also, the studies use different times, since it is considered the whole experimental frame, not only the induction time.

The cross-over effect also studied in this analysis has as objective to better understand one of the supposed bias of split-mouth designs. This bias is considered “supposed”, since no published studies have given support to this hypothesis. The present study tries to shed some light into this discussion, restricted to animal studies, however that could be further investigated in human clinical studies. The main support of the hypothesis is that no drug or event that could have a known systemic effect should be part of the experiment.

The analysis of the present study demonstrates that the presence of a silk ligature in one molar has no statistically significant effect on the contralateral side. This is supported by the fact that the values of periodontal breakdown encountered in sites without ligature from animals exposed to ligature on the contralateral side does not differ from the mean values of spontaneous periodontal bone loss in animals not

exposed to any additional manipulation. In this respect, the present results raise the possibility of considering the lack of necessity of total control groups in periodontal disease studies in rats.

It may be concluded that two weeks of ligature-induced periodontal disease seems to be enough to demonstrate significant bone loss and that utilizing the contralateral sides of teeth with ligature is possible to be considered sound controls, reducing the amount of animals in periodontal research.

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3 CONSIDERAÇÕES FINAIS

A utilização de modelos animais para o estudo da etiopatogenia das doenças periodontais tem sido amplamente empregada na literatura periodontal. Modelos utilizando ratos Wistar são os mais comumente empregados. No entanto, dúvidas quanto ao período de tempo considerado ideal para indução de periodontite ainda permeiam a comunidade científica. Adicionalmente, a utilização de grupos controle intra-grupo também é questionada com base no argumento de que a colocação de ligadura de um dos lados teria capacidade de gerar um aumento na carga bacteriana da boca dos animais e, portanto, interferir na perda óssea no lado contralateral.

O presente estudo objetivou acrescentar ao corpo de evidências já existentes informações sobre o tempo ideal de indução de doença periodontal, bem como identificar se a presença de ligadura de um dos lados teria capacidade de gerar perda óssea adicional do lado controle intra-grupo. Os resultados demonstraram que um período de 2 semanas de indução de doença periodontal por ligadura parece ser suficiente para demonstrar uma perda óssea alveolar estatisticamente significativa e que a utilização dos lados contralaterais de dentes com ligadura é possível de ser utilizada como controle saudável.

O presente estudo tem sua importância destacada a medida que reduções no custo de um experimento que envolve modelos animais em periodontia pode ser verificada a medida que menores tempos experimentais podem ser empregados. Adicionalmente, a possibilidade de utilização de lados controle intra-grupo reduz, à semelhança da condição anterior, os custos, bem como o número de animais a serem utilizados em um experimento. Tais abordagens são de grande valia e estão de acordo com os princípios mais contemporâneos que envolvem a utilização de animais de laboratório previstas em importantes orientações divulgadas pela comunidade científica nacional e internacional.