Methodological aspects in the study of periodontal breakdown in rats: influence of the presence and time of ligature

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ABSTRACT

The aim of the present work was to evaluate the effect of different times on alveolar bone loss (ABL) and whether the presence of ligature on one side affects ABL on the contralateral site.

This is a secondary analysis of databases from studies conducted at the Federal University of Rio Grande do Sul. Included studies used ligature-induced periodontal disease in rats. In order to be included, the studies were required to have a control group without any ligatures and an intra-group control. Three studies were included, which used different time periods: 2 weeks with ligature and 8 weeks without ligature; 5 weeks with ligature and 17 weeks without ligature; 22 weeks with and without ligature. Animals were raised similarly and sacrificed by decapitation. Maxillae were defleshed with 9% sodium hypochlorite. Pictures were taken and five measurements were obtained from each image.

The presence of ligature generated significantly greater ABL compared to sides without ligature. Comparing sides with ligature, ABL was lower at 2 weeks than at 5 and 22 weeks. Sides without ligature showed no significant difference between 8 and 17 weeks for spontaneous periodontitis. However, after 22 weeks, animals exhibited significantly greater ABL when compared to other periods. The presence of ligature on one side did not influence ABL on the contralateral side.

Two weeks of ligature-induced periodontal disease seems to be sufficient to demonstrate significant ABL. Teeth without ligature contralateral to teeth with ligature may be considered sound controls, thereby reducing the amount of animals needed in periodontal research.

Key words: *Periodontal diseases; periodontitis; rats.*

Aspectos metodológicos no estudo da destruição periodontal em ratos: influência da presença e tempo de ligadura

RESUMO

Objetivo: avaliar o efeito de diferentes períodos experimentais e se a presença de ligadura em um dos lados afeta a perda óssea alveolar (POA) no lado contralateral.

O presente estudo trata-se de uma análise secundária dos bancos de dados de estudos realizados na Universidade Federal do Rio Grande do Sul. Os estudos incluídos utilizaram o modelo de indução de doença periodontal por ligadura em ratos. Os estudos necessitavam possuir grupo controle sem ligadura, assim como controle intra-grupo. Foram incluídos 3 estudos, com diferentes períodos de análise: 2 semanas com ligadura e 8 semanas sem ligadura; 5 semanas com ligadura e 17 semanas sem ligadura; 22 semanas com e sem ligadura. Os ratos foram criados nas mesmas condições, sacrificados por decapitação, as maxilas retiradas e os tecidos moles removidos com hipoclorito de sódio 9%. Tomadas fotográficas foram realizadas e cinco mensurações foram obtidas de cada imagem. A presença de ligadura gerou uma perda óssea alveolar significativamente maior quando comparado ao lado sem ligadura. Nos lados com ligadura um período de 2 semanas mostra menor perda óssea alveolar que 5 e 22 semanas. Lados sem ligadura foram avaliados e 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. Duas 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: *Doenças periodontais; periodontite; ratos.*

INTRODUCTION

Periodontitis is highly prevalent worldwide 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 to the body of evidence, with increasing translational potential[5]. Experimental periodontitis models have been used to understand the etiopathogenic processes involved in periodontal disease, and to study new therapeutic agents and other factors associated with periodontitis[6-10]. Rats are often used in studies of experimental periodontitis because their anatomy in the molar region is very similar to that of humans[11-15].

Periodontitis and bone loss in rats may be spontaneous, as described in some recent studies,[16,17] or induced. There is clear evidence in 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[6,12]. The latter is based on the creation of a bacterial retention factor created by ligature placement, mimicking what happens in humans faster and more intensely. The use of ligatures as a periodontal disease induction model has been suggested by some authors as a representative model for studying the pathogenesis of periodontal disease[13]. However, issues such as the possible trauma generated by the presence of ligature and potential loss of ligatures during the trial period should be taken into account when the study is planned. The "split mouth" design uses ligatures, for example, on one side but not on the contralateral site, which serves as a control. If the "split mouth" design is used, the possibility of a crossover effect should be considered, since the presence of an irritant on one side may affect the contralateral site. Regarding time of periodontal disease induction, there is no consensus in the literature. Some authors report larger alveolar bone loss levels in the first 7-15 days after placement of the ligature[14], though periods of 4 or more weeks are widely used[15].

There are different methods proposed in the literature to measure alveolar bone loss in rats: histometric, morphometric, radiographic measurements and computed tomography. They are all widely used, accurate and capable of detecting alveolar bone loss in rats[18]. To date, no single method is considered the gold standard for the measurement of periodontal disease in rats, so the method should be chosen according to the purpose of the study.

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

MATERIALS AND 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 were retrieved from eligible studies of periodontal disease models induced by ligature in rats conducted by the periodontology research group at Federal University of Rio Grande do Sul. To be eligible for the analysis, a study was required to include 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 eligible studies, of which 3 met the inclusion criteria. These three studies used different 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 periodontal breakdown, and Study 3: 22 weeks for both spontaneous and induced periodontal breakdown.

Animals

All studies included utilized 45 to 60 day-old male Wistar rats (weighing 250-350g). Animals were housed in groups of 4-5 under a 12-hour light/dark cycle at room temperature (22°C ± 2°C) with free access to water and standardized rat chow (Nuvilab CR-1, NUVITAL®, Curitiba, PR, Brazil). The animals remained throughout the experimental periods at two different locations, according to the study, with the same routines. A total 88 rats were included in the analysis.

The studies included in this analysis followed important aspects of methodological care, as provided in the ARRIVE Guidelines[19]. For example, randomization, blinding, calibration of examiners, reproducibility and care in the handling of animals, especially in the reduction of pain and discomfort, were observed in all included.

Periodontal disease model

Periodontal disease was induced by placing a silk ligature (Ethicon, Johnson & Johnson, São Paulo, Brazil) on the right upper second molar with the knot tied on the buccal side[20-23]. The contralateral 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. All animals were sacrificed by decapitation.

Specimen preparation

Maxillae were removed, sectioned, and defleshed in 9% sodium hypochlorite for 2 hours and the remaining soft tissue was removed mechanically, after which the specimens were washed and dried. For better visualization of the cemento-enamel junction, maxillae were stained with 1% methylene blue, following Fernandes et al[24].

Morphometric analysis

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

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 the 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 converted into millimeters using as reference the markings of the endodontic ruler to which the hemimaxillae were attached. Fig. 1 shows buccal aspects of specimens without (Fig. 1A) and with (Fig. 1B) periodontal disease inductionused in one of the three studies.

For all included studies, specimen preparation, photographs and morphometric analysis were performed at the Laboratory of Periodontology of the Federal University of Rio Grande do Sul, following the methods proposed by Fernandes et al[24].

Statistical analysis

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

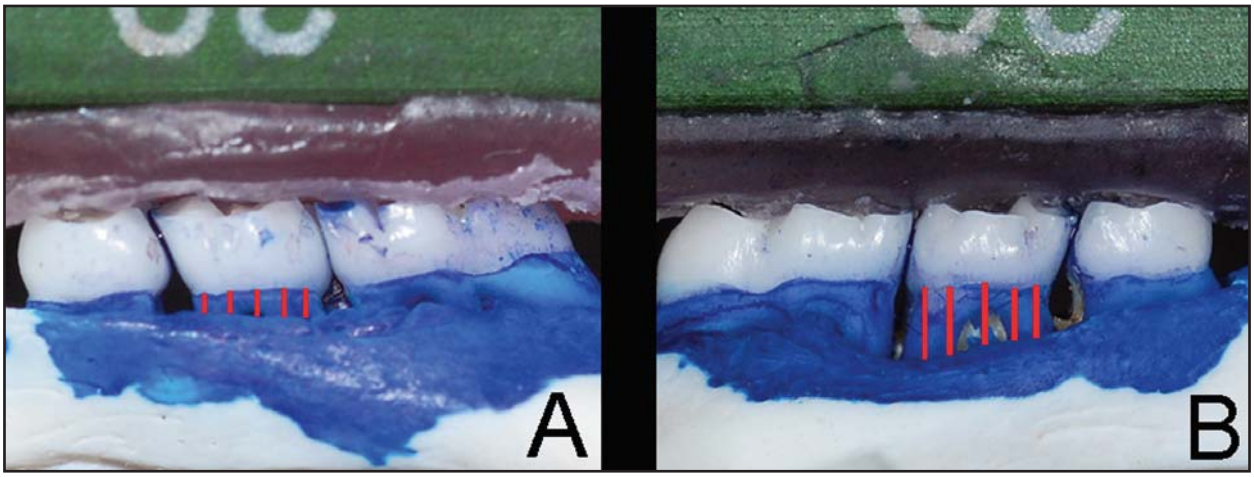

Fig. 1: Representative photograph of a specimen illustrating morphometric analysis in maxillae without (A) and with (B) ligature-induced periodontal breakdown.

RESULTS

Sites with ligature exhibited significantly higher ABL than sites in which periodontal disease was not induced (characterized as spontaneous ABL), showing that the model was effective in reproducing alveolar bone loss, which isone of the most important signs of periodontal disease (Figs. 2 and 3). The difference between ABL values was 40%, 54% and 57% for Studies 1, 2 and 3, respectively, depending on the experimental period (the longer the experimental period, the greater the periodontal destruction). Figs. 2 and 3 show mean ABL for sites with and without ligature, according to experimental period, respectively. For sites with ligature, ABL was significantly lower at 2 weeks of periodontal breakdown than at 5 and 22 weeks. However, no significant difference was observed between 5 and 22 weeks, showing that a 5-week period is sufficient to produce signs of periodontal disease (Fig. 2).

Sites without ligature showed no significant difference in ABL between 8 and 17 weeks. However, at 22 weeks, a statistically significant difference was detected, showing that longer periods are needed to produce spontaneous ABL in Wistar rats (Fig. 3).

Fig. 4 shows the comparison between control sites in animals submitted or not submitted to ligature-induced periodontal disease. No statistically significant difference was observed between 22 and 5 weeks, suggesting that the presence of ligature on one side does not affect mean alveolar bone loss at the contralateral site.

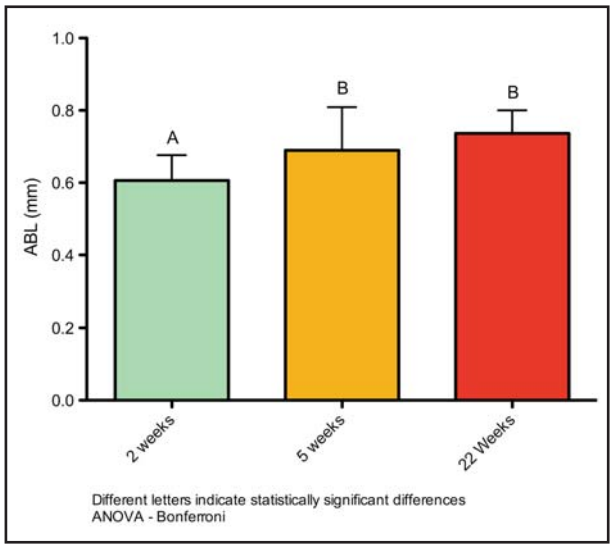

Fig. 2: Mean Alveolar Bone Loss (ABL) for sites with ligature according to experimental period.

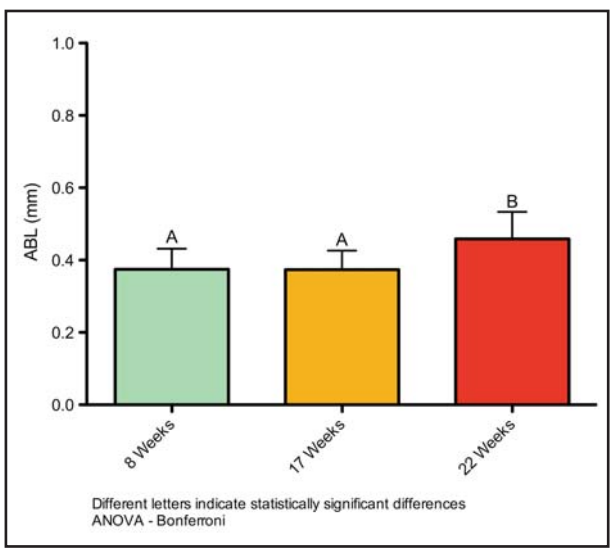

Fig. 3: Mean Alveolar Bone Loss (ABL) for sites without ligature according to experimental period.

DISCUSSION

This is a methodological study, the aim of which was to achieve better understanding of the effect of induction time and of a potential crossover effect of the presence of ligature on the contralateral side in studies using ligature-induced periodontal breakdown in rats.

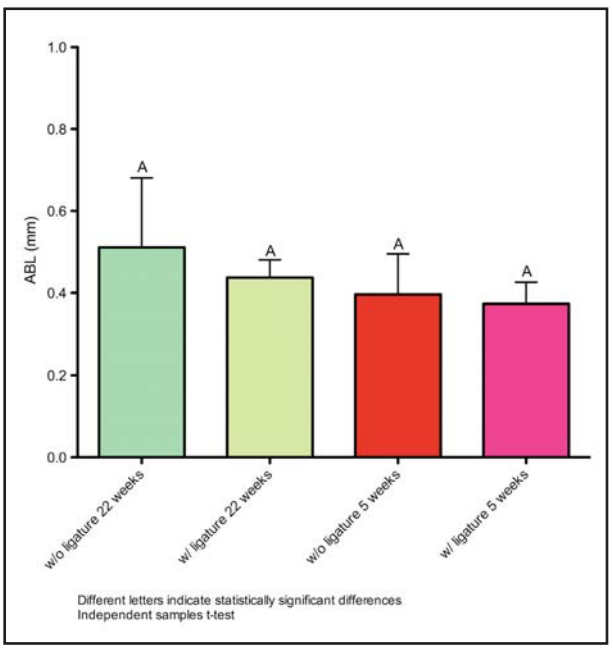

Fig. 4: Comparison of Alveolar Bone Loss (ABL) at control sites for animals with and without ligature.

Studies utilizing animal models are essential to understanding etiopathogenic aspects of periodontal diseases. Several studies have been performed using different animal strains[25,8]. Wistar rats are one of the most widely used species in studies on pathogenesis of periodontal diseases.They are non-isogenic rats that present variability in immune response that is similar to humans[26], as well as having similar anatomical characteristics to humans[11].

In periodontal research in animals, discussions have arisen regarding which the best models of induction are, which the best methods of analysis are, and whether it is really necessary to induce the disease[14]. Some studies have thus looked at naturally occurring periodontal disease, which could be an interesting way of demonstrating the effect of different exposures without the high-intensity challenge[16, 17].

The present study utilized morphometric analyses for periodontal breakdown. Different methods have been used, including histology, morphometry and tomography. They lead to different approaches, but are considered reproducible and capable of demonstrating periodontal breakdown[18]. In addition, the sites of analysis (area, proximal, furcation, buccal, etc.) have also been studied, and have all been shown to be reproducible and capable of detecting occurrence of alveolar bone loss[27,28].

In animal research there has been much discussion of the "3Rs" recommendation: reduce, refine and replace[29]. In this regard, a "split-mouth" design (in which ligatures, for example, are placed on one side and the contralateral tooth serves as a control) enables the number of animals to be reduced, since the use of a totally non-manipulated/exposed control is unnecessary. However, to the best of the authors' knowledge, the literature has not yet addressed this point. Thus, the novelty of the present study resides in further enabling evidence-based choices of using contralateral sides as controls, thereby reducing the number of animals used in periodontal research.

An interesting point in periodontal research using animals is the time of induction of periodontal disease. There is no consensus in the literature demonstrating that any one time is better than another in terms of occurrence of periodontal breakdown. Studies have used different time intervals ranging from 1 week to months[30,14]. If it is possible to effectively establish a minimum induction time for periodontal breakdown, the principle of refining the method can be contemplated.

Considering the points raised, and that we are a research group with experience in studies of periodontal pathogenesis in rats, we decided to analyze data from our database to address these issues. In order to be included in the present study, the experiment was required to have a total control group, with no exposure either to an external agent or to periodontal disease induction, and a control group not exposed to an external agent, with ligatures on one side but not on the other. 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 used similar housing, temperature, food and liquid intake, and manipulation strategies, enabling direct inter-study comparisons. In addition, the laboratory procedures and analyses, including randomization, blinding and reproducibility, were performed identically. All these research principles support a consistent level of internal validity[31]. None of the included studies used the same induction time, therefore the analysis does not merge groups.

With regard to induction time, the results of the present study indicate that the amount of alveolar bone loss in ligature-induced models is time-dependent, i.e. at 2 weeks there is less alveolar bone loss than at 5 and 22 weeks. However, the comparison between 5 and 22 weeks does not demonstrate any additional breakdown.

This should be considered from different perspectives. One important point is that the studies that demonstrate sufficient effect after shorter periods (2 weeks, for example) were performed on isogenic rats[14]. On the other hand, it should be emphasized that the study included in this analysis with 2-week induction found statistically significant differences as compared to controls, indicating that periodontal breakdown was actually achieved. In one study, no statistically significant difference was observed between 29, 43 and 57 days of ligatures[32]. The benefit of using less experimental time relates to the cost-effectiveness of research.

It should also be noted that groups with ligatures always present significantly higher degrees of periodontal breakdown than groups without ligatures. However, some studies have shown some effects only at sites without ligatures, suggesting a

potential increased challenge that may mask the effect of the presence of naturally occurring biofilm[33]. This is not supported 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[34]. Thus, a 2-week period seems to be sufficient for ligature-induced periodontal breakdown; however, depending on the exposure variable to be tested, additional time may be necessary, and after 5 weeks the breakdown seems to leveloff.

In animals with spontaneous alveolar bone loss, periodontal breakdown takes longer. Moreover, the studies use different times, since the whole experimental time frame is considered, not only the induction time.

The present analysis also studied the crossover effect with the aim of better understanding one of the supposed biases of split-mouth designs. This bias is considered "supposed" because no published paper has provided support to this hypothesis. The present study endeavors to shed some light on this discussion, restricted to animal studies, which could nevertheless be further investigated in human clinical studies. The main basis for the hypothesis is that no drug or event with a known systemic effect should be part of the experiment.

The analysis in the present study demonstrates that the presence of a silk ligature on one molar has no statistically significant effect on the contralateral side. This is supported by the fact that the values of periodontal breakdown encountered at 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. The present results thus suggest that total control groups are unnecessary in periodontal disease studies in rats.

To conclude, two weeks of ligature-induced periodontal disease seem to be sufficient to demonstrate significant bone loss, and teeth without ligature contralateral to teeth with ligature may be considered sound controls, thereby reducing the amount of animals needed in periodontal research.

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