Scandinavian Journal of Laboratory Animal Science

2014, Volume 40, Number 5

Systemic Bone Mass Alterations and Ligature-Induced Periodontitis in Wistar Rats

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Summary

Chronic inflammatory conditions have been associated with pathological outcomes, resulting in events responsible for abbreviation of life or decreasing life quality in elders. Periodontal diseases have been associated with systemic disorders, but there is a lack of evidence concerning the relationship between periodontal inflammation and systemic bone conditions. The aim of the present study was to analyze the relationship between ligature-induced periodontitis and generalized bone mass alterations in Wistar rats. Periodontal disease was induced by the placement of silk ligatures around the maxillary left second molar of each animal of the test group (n=14). The control group did not receive ligatures. Systemic bone mass was determined by bone densitometry (double energy X-ray). After 30 and 60 days of ligature placement, bone densitometry analysis did not reveal statistically significant differences between test and control groups both for bone mineral density and bone mineral content (BMC), but at 60 days BMC was slightly lower in the test group. Total bone area was, at 30 days, higher in the test group than in controls. However at 60 days this relationship was inverted, with a higher bone area in the control group. The control group exhibited progressive bone growth, as shown by comparison of BMC between 30 and 60 days; this bone growth was significantly higher than in the test group.

Introduction

Understanding the natural ageing process is necessary for the comprehension of its impact on health conditions related to longevity and quality of life in humans. The presence of chronic inflammatory conditions has been associated with complex pathological outcomes, resulting in events responsible for shortening of life or decreasing life quality of older people.

According to Cimaz (*2002*), chronic inflammation can affect bone mass through a variety of mechanisms. It leads to alteration in the balance of the bone formation/resorption process, frequently resulting in decreased bone mass and density. Among contributing factors to osteopenia, the presence of inflammatory cytokines is considered a subject of special interest in scientific research (*Daci et al., 2002*).

In a study conducted by Jilka *et al.* (*1992*), increased bone resorption in ovariectomized rats could be corrected by the administration of neutralizing antibodies for interleukin-6. In addition, the action of interleukin-1 and 6 can have synergistic effects on bone resorption by osteoclasts (*Kurihara et al., 1990*). According to Pfeilschifter *et al.* (*1989*) and Thomson *et al.* (*1986*), interleukin-1 affects all lineage stages of osteoclast formation, and promotes additional effects over osteoclasis by stimulating prostaglandin

synthesis (*Boyce et al., 1989*). This influence occurs both locally and systemically. Interleukin-1 infusions induced a rise in bone resorption by osteoclast function at sites distant from the infusion point (*Boyce et al., 1989*).

Historically, the risk of developing generalised osteoporosis has been related to inflammatory levels. According to Ringe (*1996*), the presence of inflammatory chemical mediators originally from joints affected by chronic diseases can influence osseous metabolic characteristics, determining higher osteoclastic activities. Thus, in an initial rheumatoid arthritis phase, as an example, the best osteoporosis prevention may come from inflammation suppression.

According to Lerner (*1994*), periodontitis, via a similar mechanism to that associated with rheumatoid arthritis and osteomielitis, can induce host response processes that are able to provoke osteoclastic activity through inflammatory mediators, such as interleukins and tumor necrosis factor. Minne *et al.* (*1984*) developed an osteopenia model via inflammatory induction, and observed a generalized loss of trabecular bone after the injection of non-specific irritants. Associations between inflammatory periodontal disease and local or generalized bone metabolic alterations have also been reported in the literature. Tezal *et al.* (*2000*) conducted a study with 70 post-menopause women, in whom bone mineral density (BMD) and periodontal conditions were assessed. Multiple regression analysis adjusted by age, menopause age, estrogen supplementation, smoking and body mass index revealed significant correlations with BMD and alveolar bone loss. Periodontal attachment loss was consistently correlated to BMD, however without reaching statistical significance. The authors concluded that post-menopausal osteopenia is a risk factor for the occurrence of periodontitis, but without mentioning the inverse possibility. However, in the study, causality could not be determined.

In a study conducted by Takaishi *et al.* (*2005*), alveolar bone and lumbar spine BMD were significantly correlated, in 40 post-menopausal women. Additionally, alveolar bone BMD demonstrated correlations with periodontal parameters , like probing depth.

According to Taguchi (*2003*), the literature demonstrates diverse results when comparing mineral bone densities between the whole skeleton and the alveolar bone. The alveolar bone region measured and the sample sizes can account for these discrepancies. Additionally, results could be compromised by lack of control of confounding factors, and differences in periodontitis or osteoporosis cut-off points (*Wactawski-Wende, 2001*).

According to Pfeilschifter *et al.* (*1985*), studies showed generalized loss of trabecular bone in rats after the induction of inflammatory processes for a period of 3 weeks. These same authors stated that osteopenia induced by a single inflammatory lesion of sufficient expression was apparent even after 14 days of induction. This evidence supports the hypothesis that inflammatory processes of minor severity, but with chronic nature, can lead to considerable osseous effects.

The aim of the present study was to analyze the relationship between ligature-induced periodontitis and generalized bone mass alterations in Wistar rats.

Materials and Methods

Experimental Animals

Twenty-eight 180 days old male Wistar rats were included. The study protocol was approved by the São Lucas Hospital Ethical Committee.

Animals stayed under light/dark cycles of 12 hours and received standard feeding and water *ad libitum*. Animal body weight was measured at the moment of ligature placement *(baseline)* and after the experimental periods (30 or 60 days after ligature placement).

Experimental groups

The animals were randomly assigned to 2 groups: Test group (n=14) and Control group (n=14). Each group was subdivided into 2 experimental periods: 30 and 60 days, totaling 7 animals per group/period. Test animals received ligatures for induction of periodontitis and the controls were only manipulated in the same way at the same periods as the test animals.

Experimental Design

Periodontal disease was induced by the placement of silk ligatures (4-0 silk suture string – Johnson & Johnson -), at the left second molar, with the knot placed at the lingual site, in test animals (*Sallay et al., 1982*).

No ligature was lost or dislocated during the study. After 30 days of ligature placement, 7 randomly chosen animals of each group were sacrificed. Sixty days after the ligature placement the remaining animals were sacrificed (7 animals per group).

Maxillae of each animal were excised and fixed in a 10% formalin solution (pH 7,3) for 24 hours. Then, after being sectioned, they were decalcified in an Anna Morse solution for 14 days. Decalcified specimens were dehydrated in alcohol and embedded in paraffin.

Blocks were sectioned in a microtome, in 4 mm, sections were prepared and stained by haematoxylin/eosin. Each stained slice was examined using an AX 70 Olympus optical microscope.

Histological analysis and verification of effectiveness of the periodontitis-induction method

Histological preparations were made in a mesio-distal orientation, involving periodontal structures 30 days after the ligature placement in the test group, allowing descriptive analysis by optical microscopy. This observation served the purpose of confirming the effectiveness of the periodontitis-induction method.

Bone Densitometry

Systemic bone mass assessments were made at the end of each experimental period (30 and 60 days), before the histological analysis. Double energy x-ray for bone densitometry was applied, previously validated for animals in the study by analysis of reproducibility, tested by 10 sequential measurements with repositioning, with a standard position for further densitometry tests. Mean ± standard deviation and variation coefficient for the 10 BMC values were calculated.

For the densitometric analysis, the experimental animals were positioned on the densitometer in a standardized position, in order to avoid overlapping of corporeal parts, allowing the inclusion of the whole body (including the tail) on the evaluation area. The values generated by densitometry were analyzed and means per group were calculated.

Statistical analysis

A: Body Weight:
This parameter was analyzed by means and standard deviations at *baseline* and at the end of each observational period, for all animals. Means were compared between groups by Student's T-test.

B: Bone Densitometry:
Mean densitometric values for test and control groups [bone area, bone mineral density (BMD) and bone mineral content (BMC)] were compared by Student's T-test. Additionally, mean BMC values for 30 and 60 days were compared by Student's T-test.

The significance level adopted for the present study was 5%.

Results

The histologic descriptive analysis revealed periodontal bone loss in test animals, with higher degrees of inflammation and osteoclastic activity as compared to controls.

Table 1 shows the bone densitometry reproducibility. One rat was subjected to ten repeated measures of BMC. The low standard deviation (0.067) and variation coefficient (0.7%) demonstrates that reproducibility was achieved with the method.

Table 2 shows body weight, bone area, bone mineral density (BMD) and bone mineral content (BMC), analyzed by bone densitometry, 30 and 60 days after ligature placement. The mean values for each parameter were compared using Student's t-test.

Table 1. Reproducibility of BMC measurements

Measurements	1	2	3	4	5	6	7	8	9	10
BMC	9.494	9.559	9.567	9.592	9.561	9.706	9.491	9.551	9.650	9.513
Mean	9.568									
Standard deviation	0.067									
Variaton Coefficient	**0.7%**									

Table 2. Mean and standard deviation (S.D.) bone area, bone mineral content (BMC), bone mineral density (BMD) and body weight for 30 and 60 day animals in test and control groups.

	Test Group				Control Group			
	Area	BMC	BMD	Weight	Area	BMC	BMD	Weight
Mean 30 days	60.0	8.7368	0.1457	298.5	58.1590	8,4013	0.1444	273.35
S.D. 30 days	2.3141	0.6701	0.0065	21.27	2.2751	0,4436	0.0042	20.43
Mean 60 days	57.9968	8.8063	0.1517	354.0	61.4504	9.2869	0.1510	360.0
S.D. 60 days	4.1566	0.7162	0.0037	25.89	2.8559	0.6453	0.0048	29.17
p-value t-test 30 days:	Area: 0,093		BMC: 0,145		BMD: 0,327		Weight: 0,021	
p-value t-test 60 days:	Area: 0,047		BMC: 0,105		BMD: 0,376		Weight: 0,345	

In animals sacrificed at 30 days, the weight of the test animals at baseline was significantly higher than controls (252.28 vs. 224.14g, respectively). From baseline to 30 days, animals from both groups gained weight (p-values of 0.001 both for tests and controls). At 30 days, test animals continued to be heavier than controls. In animals sacrificed at 60 days, the controls, at baseline, were significantly heavier than the test animals (249.2 vs. 229.71g respectively). From baseline to 60 days, test and control animals gained weight (p-values of 0.000 for both groups), and at 60 days, no differences in body weight could be detected, suggesting that test animals gained more weight than controls.

A lack of significant differences between BMD and BMC values was observed between test and control groups for both experimental periods but at 60 days BMC was slightly lower in the test group.. In 30 day animals, bone area did not differ. However, in 60 day animals, the control animals had a significantly higher bone area when compared to the test group (61.45 vs. 58.00, respectively).

Additionally, the comparison of BMC values among 30 and 60 day periods for test and control animals showed that the control group had continuous bone growth, with BMC values significantly higher at 60 days than at 30 days (p-value 0.001). The test animals presented a compromised bone growth, with BMC without a significant difference between 30 and 60 days (p-value 0.856).

Discussion

Rats are used as an experimental model to investigate events associated with a wide diversity of diseases. The rat and the human body systems and physiology have similarities that allow the conduct of studies where humans cannot participate as an experimental model for ethical reasons (*Klausen, 1991*).

In the present study, periodontitis was induced by ligature placement around one molar in each experimental animal, in order to facilitate bacterial aggregation in the gingival crevice, leading to inflammation that causes local bone loss and loss of periodontal attachment around the teeth. Results of several studies show that this model leads to infection outcomes that are similar to what happens in humans (*Fiehn et al., 1992; Breivik et al., 2000; Leite et al., 2005*).

The experimental periods (30 and 60 days) were chosen to test the effects of more severe periodontal inflammation, but without allowing tooth loss. The biological cycle and life span of the rat are considerably shorter than for humans; periodontal disease evolution is also faster in the rat. This fact could compromise analysis of the continuity of the inflammatory impact for more than 30 days. An inflammatory process of a longer duration, with an increased cytokine synthesis in the periodontium (*Offenbacher et al., 1996*) and, as a consequence, a higher probability of systemic dissemination of these inflammatory mediators leading to outcomes distant from the gingival tissue (*Loffreda et al., 1998*), would provoke, hypothetically, a greater impact on the systemic bone mass. So, it was estimated that 60 days of inflammatory process would be of sufficient impact. However, due to the speed of periodontal breakdown in rats, at 60 days some of the animals could experience tooth loss, a fact that could compromise the proper evaluation of an inflammation of similar characteristics among all the experimental animals. In our study, no tooth loss was observed.

Both 30 and 60 days animals were included in the bone mass densitometric evaluation, in order to

observe if 30 days of periodontal infection would be sufficient to interfere with systemic bone mass.

The results of the present study show that the presence of an inflammatory disease located in a specific organ can provoke an impact on systemic bone conditions, similarly to other studies (*Heinrich et al., 2003; Ishihara & Hirano, 2002; Tracy, 2003*). Probably due to the low magnitude of an inflammatory process located only in one tooth and the continuous growth of the animals, the results did not reveal significant alterations in systemic bone mass, when the values for bone mineral density and bone mineral content were compared between the groups at 30 and 60 days. However, the bone area was higher in test animals (with periodontitis) at 30 days, but lower in test animals at 60 days.

The comparison of bone weight between baseline and 30 days and between baseline and 60 days emphasise the influence on bone development revealed by the bone area analysis. This influence, probably related to the periodontal inflammatory disease at 30 days was restricted, but after 60 days of periodontal inflammation as the only induced pathological process, was sufficient to compromise normal bone development. At 30 days, the test animals continued to be heavier than the controls (similarly to baseline) and with similar bone area. However, at 60 days, test animals presented a significant lower bone area, despite the fact that had gained more weight. These data support the hypothesis that periodontal inflammatory disease can influence the normal bone resorption/neoformation process systemically, but without affecting the general physical development of the experimental animals, probably not interfering with organic, behavioral or feeding patterns.
The BMC comparison between groups at 30 and 60 days revealed continuous bone growth in the control group. So, the group of animals without periodontitis had more bone area and also more acquisition of bone mineral content, showing higher bone dimensions. The test group, on the other hand, presented BMC values compatible to its lower bone area, emphasizing the compromised bone development.

Macroscopic examination of the skeleton by bone densitometry has been extensively used to diagnose situations involving osteopenia and osteoporosis in humans. The software used to examine small animals allowed the bone analysis with adequate levels of accuracy. However, significant results are only observed if the factors affecting the bone situation are of sufficient magnitude to induce considerable alterations to be detected on BMC and BMD values. In this interpretation, the cortical bone tissue presents great influence over the total calculation of bone area and bone quantity. On the other hand, the beginning of the bone loss process is primarily located on trabecular bone, leading to a lower volume of bone trabeculae and bigger intertrabecular spaces. Thus, it is expected that the effect of pathological processes of minor expression, but still capable of influencing bone mass conditions, can be more easily identified when the trabecular bone is analyzed independently. In these situations, bone densitometry may be of insufficient sensitivity to detect the systemic bone expression of an inflammatory process located around only one tooth. It would be of interest, according to the results of the present study, to conduct other studies in order to test the impact of periodontits induced in more that one tooth on the systemic bone mass in animals, observed by bone densitometry. It must be added, however, that overwhelming inflammatory conditions can affect not only the bone status, but also interfere in natural organic functions in experimental animals that can influence the systemic bone situation, acting as confounding factors for the specific inflammatory process under study.

In studies whose results show the relevance of a particular factor to provoke or modify a specific outcome, an adequate sample size is essential. If the effect is strong enough to generate an important impact over the outcome, eventually a small sample size could be sufficient to produce significant results. Otherwise, when the factor under study presents a minor impact over the outcome, the absence of statistically significant results can be related to a reduced number of experimental units. In the present study, an impact on systemic bone mass associated with the presence of periodontits (at 60 days BMC was slightly reduced), but the differences between the two experimental groups did not reach statistical significance. Probably the magnitude of the inflammatory process induced around one single molar was too modest to achieve statistical significance in our study. The number of experimental animals in the present study was similar to other studies where the rat model was used to verify the relationship of periodontal disease with systemic conditions or periodontal pathogens with antibiotic regimens. In the studies conducted by Fiehn *et al.* (*1992*) and Breivik *et al.* (*2000*), no more than 10 animals were included in each experimental group; in 2005, Orrico *et al.* (2005) included 9 animals per group; and in the

studies conducted by Chang *et al.* (*1994*) and Leite *et al.* (*2005*), 6 animals were present in each group. In each of these studies, periodontits was induced by the placement of ligatures, in a similar way to what was done in the present study.

Thus, sample size in the present study was arbitrarily selected based on prior studies with ligature-induced periodontal disease. Pioneer studies have the potential to reveal the strength and the impact of the factor tested over the outcome, thus making more favorable for future studies to choose a more appropriate sample size. The present study suggests the conduct of future investigations with a larger sample size to verify the influence of periodontits around more than one tooth over the systemic bone mass in rats.

According to the results of the present study, an effect provoked by periodontits on the systemic bone mass in rats was observed, however without reaching statistical significance. Additionally, it was also observed that the occurrence of periodontits was related to a compromised bone growth pattern in Wistar rats, verified by the comparison of bone mineral content between 30 and 60 days after periodontits induction, and by the bone area observed at the end of the 60-day experimental period.

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