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Estrogen treatment and periodontal disease progression: an experimental study in ovariectomized rats

Tratamento com estrógeno e progressão da doença periodontal: Estudo experimental em ratas ovariectomizadas

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Abstract

The aim of this study was to investigate different periods of estrogen replacement therapy onset on the progression of experimental periodontitis in ovariectomized rats. Sixty five female Wistar rats were ovariectomized and divided into two groups, experimental and control that received 17ß estradiol or vehicle, respectively. Each group was subdivided into five subgroups that started the treatment immediately, one, two, three and four weeks after the ovariectomy. A month after ovariectomy, a cotton ligature was placed around the maxillary second molars. Thirty five days after ligature placement, the animals were sacrificed. The macroscopic, radiographic, microscopic and histometric aspects of the periodontal area were analyzed. The results indicated that estrogen-deficient state may not have a direct effect on the resorption of alveolar bone adjacent to the maxillary second molar roots, once no differences between test and control groups were detected. Under the conditions of this experiment, estrogen replacement therapy did not delay the progression of induced periodontitis.

Keywords

Estradiol; Osteoporosis; Ovariectomy; Periodontal diseases.

Resumo

O objetivo deste estudo foi investigar a ação da terapia de reposição de estrógeno iniciada em diferentes períodos na progressão da periodontite induzida em ratas ovariectomizadas. Sessenta e cinco ratas Wistar foram ovariectomizadas e divididas em dois grupos, experimental e controle, os quais receberam 17β estradiol ou veículo, respectivamente. Cada grupo foi subdividido em cinco subgrupos, os quais iniciaram o tratamento imediatamente, uma, duas, três e quatro semanas após a ovariectomia. Um mês após a cirurgia, um fio de algodão foi colocado ao redor dos segundos molares superiores. Trinta e cinco dias após a colocação do fio, os animais foram sacrificados. Foram analisados os aspectos macroscópicos, radiográficos, microscópicos e histomorfométricos da região periodontal. Os resultados mostraram que o estado de deficiência de estrógeno não possui efeito direto sobre a reabsorção do osso alveolar adjacente às raízes dos segundo molares, uma vez que não houve diferença entre os grupos experimental e controle. Dentro das limitações deste estudo concluiu-se que a terapia de reposição de estrógeno não retardou a progressão da periodontite induzida.

Palavras-chave

Estradiol; Osteoporose; Ovariectomia; Doença periodontal.

Introduction

A hypothesis of an increased risk for periodontal disease due to systemic disorders has long been proposed [1]. A reduction of estrogen levels observed in women after the menopause results in accelerated bone loss, and can lead to osteoporosis. Since osteoporotic changes have been observed in oral bone [2-7], and loss of alveolar bone is a prominent feature in periodontal diseases, osteoporosis may play a significant role in the progression of periodontal disease.

Hormone replacement therapy clearly decreases bone turnover, prevents postmenopausal bone loss and reduces fractures [8]. There is evidence that estrogen therapy (ET) reduces postmenopausal osteoporotic fractures, including hip fractures [9]. Many systemic ET-containing products have regulatory agency approval for prevention of postmenopausal osteoporosis through long-term treatment, although the use of hormone should be consistent with treatment goals, benefits and risks [9]. The optimal time to initiate ET and optimal duration of therapy is still controversial given the fact that the benefit-risk ratio should be considered. Emerging data reveal that the timing of HT initiation in relation to proximity to menopause is important. The sooner the treatment starts after menopause it seems to have a strong impact on long-term health outcomes [9].

In the late 90's Hildebold [10] reviewed 141 papers of the literature on the possible association between osteoporosis and oral bone loss, and concluded that such an association exists. Hara et al. [2] demonstrated that estrogen deficiency caused a reduction in the bone volume of the mandible and tibia. Estrogen deficiency also caused osteoporotic changes and thinned alveolar bone in the interradicular septum of the rat first molar [3]. The authors suggested that this phenomenon might accelerate destruction of alveolar bone and tooth loss, especially in elderly women affected by periodontal disease.

To determine the long-term changes in bone turnover of the alveolar bone in an estrogen-deficient condition, the same researches [11] investigated changes in OVX rats over a longer time after ovariectomy (1 year). The results of bone histomorphometry analysis revealed that bone loss and trabecular fragmentation had occurred in rats' mandibular alveolar bone.

Using the estrogen deficiency animal model, Yang et al. [12] observed that ovariectomy significantly decreased the ratio of bone volume/soft tissue volume and trabecular thickness, whilst significantly increasing trabecular separation and structure model index in both mandible and tibia. High-resolution micro-CT images demonstrated detailed microarchitecture of trabecular bone, and the authors found that there was a significant positive correlation between the body of the mandibles and the metaphysis of the tibia for the size of marrow spaces and the shape of trabeculae. However, there was no significant correlation for bone volume/bone tissue and trabecular thickness. In a cross-sectional study, Nicopoulou-Karayianni et al. [13] evaluated 665 women, aged 45–70 years and established a significant association between osteoporosis and tooth loss after adjusting the effect for age and smoking. Elsubeihi and Heersche [14] investigated the effect of ovariectomized rats at bone changes in edentulous and dentate mandibles and compared these to changes in tibiae and femorae. The authors concluded that OVX caused bone loss in the edentulous mandible as well as in the proximal tibiae and femorae of rats, however it had no effect on the dentate mandible. Ames et al. [5] using three dimensional micro-computed tomography images found that the total volume of mandibular bone was significantly smaller for the OVX group compared to the normal group. Their results suggest a decrease in the mechanical stability of alveolar bone that could play a significant role in development of periodontal disease.

In studies of elderly women, estrogen replacement therapy use is consistently associated with greater tooth retention and a reduced likelihood of edentulism [15]. It was demosntrated that estrogen inhibits osteoclast-like cells formation induced by human periodontal ligament fibroblast [16]. Estrogens attenuate osteoclastogenesis and stimulate osteoclast apoptosis, but the molecular mechanism and contribution of these effects to the overall antiosteoporotic efficacy of estrogens remain controversial [17].

Therefore, the purpose of this study was to discuss an animal model experiment and attempt to determine the effects of estrogen deficient state and its therapy, on different periods of onset, on alveolar bone loss resulting from an experimental periodontitis in rats. For this study the periodontal aspect was determined by macroscopic, microscopic and radiographic analysis and by measuring the percentage of bone at interradicular region.

Material And Methods

Animals/ experimental design/ligature placement/

Sixty-five female Wistar rats, 3 months of age, weighing approximately 250g, were used in this study. The animal experimental protocol was in agreement with the UNESP* Committee of Animal Care and was in accordance with the Brazilian College of Animal Experiments (COBEA). The animals underwent surgical intervention to remove both ovaries. After surgery, the rats were divided into experimental (E) (n=40) and control (C) (n=25) groups that were injected subcutaneously with 17 β estradiol (Sigma Chemical, St. Louis, MO, USA) dissolved in soil oil at a dosage of 5 μg / 100 g body weight (ERT), or soil oil only, respectively, at different beginning time intervals. The first groups E0 (n=8) and C0 (n=5), started the treatment or placebo immediately after the ovariectomy. The rats included in the other four groups (E1, E2, E3, E4) and (C1, C2, C3, C4) started the treatment or placebo at one, two, three, and four weeks after ovariectomy, respectively. Four weeks after ovariectomy a cotton ligature was placed around the maxillary second molars bilaterally of all animals (Figure1).

The ligatures were kept for five weeks and after that the animals were sacrificed as can be observed at the experimental design (Figure 2). The left hemimaxillae were used for macroscopic and radiographic analysis and the right ones were routinely processed for decalcified sections in a mesio-distal direction (5 μm) for microscopic analysis and histomorphometric measurement. Success of OVX was confirmed at necropsy for failure to detect ovarian tissue, by observation of marked atrophy of the uterine horns in rats not given estrogen therapy, and by the quantitative measurement of estradiol level in serum by 125I radioimmunoassay.

Macroscopic analysis

For the macroscopic analysis it was evaluated the furcation involvement degree of second maxillary molar and measured the total alveolar bone loss around this tooth. The left side of maxillae were boiled for 5 min, left for three days in a papain 1% solution (Sigma-Saint Louis, Missouri, EUA), and defleshed mechanically. The specimens were immersed into methylene blue (Labsynth-Diadema, SP Brazil) to delineate more clearly the cementoenamel (CEJ) junction [18]. Scores were established for the furca involvement: 0 – no furcation involvement; 1 - horizontal bone loss not involving all the furcation extension; 2 - horizontal bone loss from side to side of the furcation. This evaluation was performed using a stereomicroscope. For the measurement of the total alveolar bone loss, the left side of the maxilla was oriented so that buccal and lingual cusps were superimposed prior to measurement, and then it was photographed in a stereomicroscope (25x). The area of the CEJ and the alveolar bone top was calculated (mm2) using an Image J 1.31p (National Institute of Health – USA – http://rsb.inf.nih.gov/ij/Java1.31 03 (Figure 3).

Radiographic analysis

Radiographs of the left hemi maxilla permitted to evaluate the periodontal bone support. An intrabuccal digital radiographic system RVGui (Trex-Trophy Radiology Inc. Marne-la-Vallée, France) was used, with a focus-film distance of 30 cm, exposure time at 0,063 s, and incidence of the Rx from lingual surface. The pictures were taken using a Trophy 2000 (Optiview; Trex-Trophy). The linear distance from the apex of the distal root (A) to the distal cusp tip (B), the bottom of the deepest bony defect distal to the tooth (C), and the angle between the two segments (CÂB), were measured using Image J 1.31p computer software (Figure 4). Periodontal bone support (PBS) was calculated from the formula:

$$PBS(\%) = \frac{AC\cos(C\hat{A}B)x100}{AB}$$

Microscopic analysis and Histomorphometric measurement

The specimens obtained from the right hemi maxilla were stained with HE and Masson, and a descriptive analysis of the periodontal region of the second molar was performed using an optical microscope. Measurements from 10 sections for each rat from all the groups were averaged to allow inter group analysis. The region of interest was the interradicular septum of the second molar. This site was histologically observed at magnification of x100. The images were then captured and analyzed in an image analyzer (software Leica Qwin-Quantitative Imaging Solution, Leica Microsystems) and bone histomorphometry was performed. Bone percentage was taken from 0.37mm^2 area, in a rectangle

established from the cemento at the bifurcation of the maxillary second molar roots (Figure 5).

Statistical analysis

Data were expressed as mean values and standard deviation (SD) of each group. The comparison among groups was performed using ANOVA, t-Student, and non-parametric Kruskal Wallis test and Mann Whitney, with statistical software MINITAB for Windows, 13.1 (2000, Minitab Inc., State College, PA, EUA). The difference was considered significant when $p<0.05$.

Results

The absence of ovaries and the analysis of uterine horns confirmed the success of the ovariectomy surgery. A comparative analysis of serum estradiol between the experimental groups and control shows that E0, E1, and E2 were statistically different from C0, C1 and C2, respectively. However, E3 and E4 did not differ from C3 and C4. (Table 1)

Table 1 – Comparative serum estradiol levels between Experimental and Control groups

Time	Control	n	Experimental	n	t(Student); (gl =11)	
					T	p
G0 – immediately	15.06	5	31.6	8	2.65	0.022*
G1 – 1 week	6.68	5	38.5	8	2.49	0.030*
G2 – 2 weeks	0.45	5	12.49	8	3.63	0.004*
G3 – 3 weeks	1.41	5	8.97	8	1.72	0.114
G4 - 4 weeks	1.38	5	9.37	7	1.94	0.081

*p < 0.05

The macroscopic examination of the specimens revealed predominantly vertical bone resorption around all the roots of the second molar, also involving the distal roots of the first and third molar mesial. In some specimens bone resorption extended to the furcation region of the first and third molars. We observed more pronounced bone loss in the vestibular region, compared to the lingual aspect of displaying a large concavity extending from first to third molars. In some specimens the resorption appeared more horizontal. There was no statistically significant difference in the first two and last treatment period. The scores for the furca involvement degree, in the macroscopic analysis were between 1 and 2, and there were no significant difference between the groups regardless of the estrogen status (Table 2).

Table 2 - Mann Whitney test comparing the scores from the bifurcation of the maxillary second molar roots between groups E and C

Groups	E0 x C0	E1 x C1	**E2 x C2**	**E3 x C3**	E4 x C4
p	0.72	1.00	0.88	0.43	0.52

Radiographic analysis indicated an alveolar bone height loss around the second maxillary molar of all groups, but no statistically difference was observed as can be analyzed in table 3.

Table 3 – Comparative radiographic interproximal alveolar bone loss between Control and Experimental groups

Time	Control	Experimental	*t(Student); (gl =11)*	
			T	p
G0 – immediately	40.204	40.487	0.09	0.93
G1 – 1 week	43.565	43.277	0.09	0.93
G2 – 2 weeks	40.624	41.866	0.42	0.68
G3 – 3 weeks	42.157	40.932	0.35	0.73
G4 - 4 weeks	38.384	41.283	1.38	0.20

P < 0.05

Decalcified 5 µm sections of second molar alveolar bone of the right hemi maxilla demonstrated severe periodontal disease, regardless of the group. Microscopic analysis shows that junctional epithelium gradually underwent pathologic changes, including ulceration and apical migration of epithelial attachment. An inflammatory cell infiltrate containing lymphocytes, macrophages and polymorphonuclear leukocytes (PMN) appeared in the connective tissue. One of the most striking characteristics of the periodontium was the heavy impaction of hair, which seemed to contribute to loss of bone, attachment and the severity of inflammatory response, sometimes revealing a formation of a granuloma with giant cells around the hair (Figure 6).

On histological findings of the interradicular septum of the second maxillary molar, the bone was compact, with mineral apposition rate and osteoclasts (Figure 7).

Some specimens showed a portion of alveolar crestal bone exposed to the oral cavity, devoid of bone cells, and with colonies of microorganisms adhered to it.

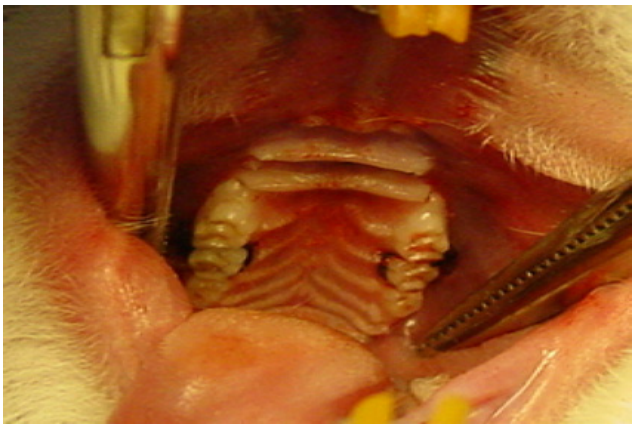

Figure 1 - Image illustrating cotton ligature placed around the maxillary second molars.

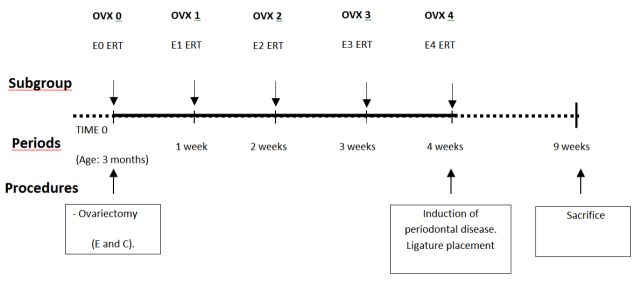

Figure 2 - Experimental design representing the experimental (E) and control (C) groups.

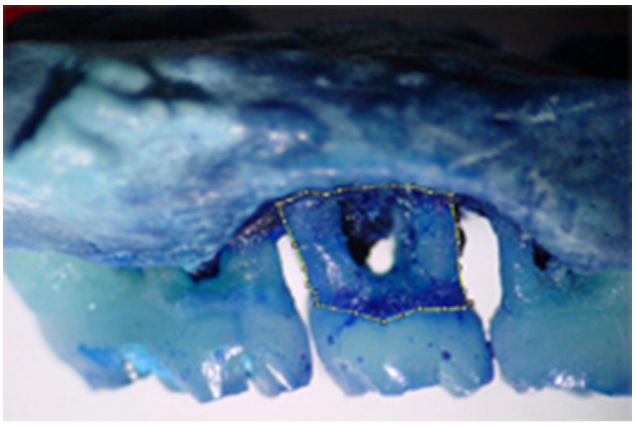

Figure 3 - Macroscopic aspect of the left hemi maxilla with area of the total alveolar bone loss.

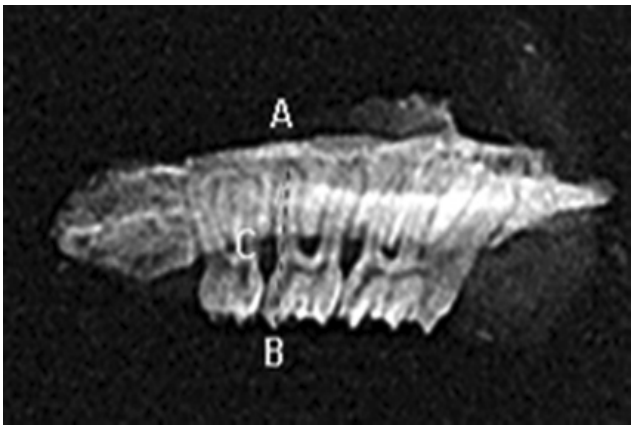

Figure 4 - Radiographic aspect of the left hemi maxilla with the linear distance used to measure the periodontal bone support.

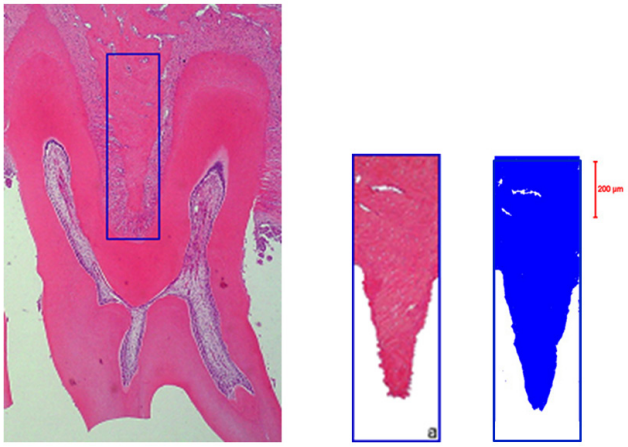

Figure 5 - Method to assess the percentage of bone at the bifurcation of the maxillary second molar roots area.

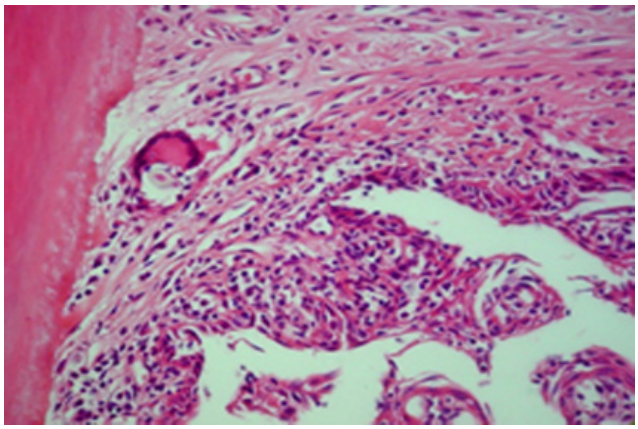

Figure 6 - Photomicrography illustrating an inflammatory response in the interproximal region with giant cell around the hair.

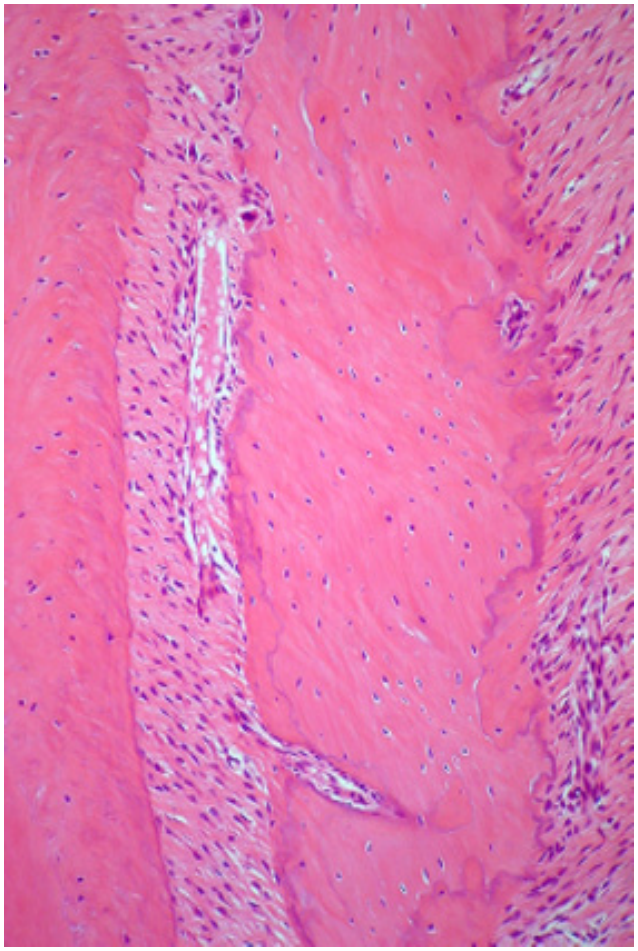

Figure 7 - Photomicrography illustrating bone reversal lines and osteoclasts indicating the high bone turnover.

Discussion

Both periodontal disease and osteoporosis are serious public-health problem, and their prevalence increases with advancing age. It has been long established that estrogen deficiency increases bone turnover and induces an imbalance between resorption and formation, and thereby accelerates skeletal losses and can lead to osteoporosis [19].

Since both periodontal diseases and osteopenia/osteoporosis are bone resorptive diseases, it has been hypothesized that this systemic condition could be a risk factor for the progression of periodontal disease. Ames et al. [5] observed an increased bone remodeling at the alveolar region compared to the external and internal borders of rats mandible and this results suggests a decrease in the mechanical stability of alveolar bone that could play a significant role in the development of periodontal disease. Progressive systemic bone loss could have significant dental implications. Papers addressing the relationships among osteoporosis, estrogen deficiency and periodontitis have been published [2,3,6,20-23]. It has been suggested that estrogen deficiency may be a risk factor for tooth loss [3,13,24,25], might affect dental implant success or compromise osseous support in totally edentulous subjects, leading to difficulty in wearing dentures. However the contribution of osteoporosis to tooth loss is difficult to demonstrate because teeth can be lost for several reasons other than the loss of bone support [26]. The wide divergence in the literature may be related to several factors such as the sample used, the method of analysis, the region, nutritional status, the time of analysis and the association with other drugs [2,14,18,26-30].

From our data it was combined a macroscopic, radiographic, microscopic and histomophometric analysis of the periodontal condition associated with the estrogen-deficient state and its therapy. In a previous study it was demonstrated that estrogen replacement therapy initiated immediately after OVX was an important factor in preventing trabecular bone loss in the tibia of OVX rats (data not shown). Recent data support the initiation of hormone therapy around the time of menopause to prevent osteoporosis [31]. It was reported [32] that estrogen treatment provides an increase in bone mass of the lombar spine (1.3%), femoral neck (0.9%), forearm bone (0.4%) and, when in conjunction with calcium, it increased to 3.3%, 2.4%, and 2.1%, respectively. It is also important to highlight that currently no hormone therapy has government approval for the treatment of osteoporosis, however many systemic HT products have government approval for the prevention of postmenopausal osteoporosis [31].

In the present study, it was demonstrated that estrogen depletion was not an important factor in the etiology of alveolar bone loss, as well as in

the progression of experimental periodontitis. In contrast to our study, Duarte et al. [21] found that estrogen administration prevents the direct effect of an estrogen-deficient state on alveolar bone. Although both studies analyzed the furcation region, we evaluated second maxillary molars, while Duarte evaluated mandibular first molars. This may have influenced the results, once the interradicular septum bone in the jaw seems to be narrower and therefore to present less trabecular bone.

Tivesten et al. [33] observed the effects of estrogen and androgen treatment on trabecular bone but not on cortical bone parameters in ovariectomized rats. It has been demonstrated that the estrogen receptor-α in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone [17]. The absence of significant changes in our results might also be attributed to the period of the experiment. Induction of periodontal disease in longer periods of time was sufficient to demonstrate that the duration of the estrogen deficiency caused great influence on alveolar bone loss. Amadei et al. [26] observed that increased bone loss was only observed when the ligature was applied after 90 days of estrogen deficiency.

Some studies in the literature report that a diet low in calcium may have a greater relationship with decreased bone mineral density of the alveolar bone of rats than estrogen deficiency [34]. In the present study the rats consumed water and diet ad libitum, and according to Jing et al. [35], pair-feeding is not essential to modulate bone mineral density or bone strength in rats. The results obtained in this study can also be justified by the presence of chewing forces, which probably often modulate bone loss observed after OVX at other parts of the skeleton [2,34]. In addition to the importance of estrogen deficiency in the general health status, studies of the possible influence with respect to the periodontium, as well as longitudinal and controlled clinical studies are required to provide information as to the best approach to deal with this condition.

Conclusion

Estrogen deficient state might not have a direct effect on the alveolar bone adjacent to the maxillary second molar roots. The local factors seem to be much more relevant in the progression of periodontal disease than the bone status resulting from ovariectomy. Under the conditions of this experiment, different time of estrogen replacement therapy did not delay the progression of induced periodontitis.

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