Braz Dent J (2006) 17(3): 201-207 ISSN 0103-6440

Estrogen Deficiency and Periodontal Condition in Rats - A Radiographic and Macroscopic Study

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The purpose of this study was to evaluate the impact of ovariectomy-induced estrogen deficiency as a risk factor of periodontal disease in rats. Forty 90-day old female rats were either ovariectomized (OVX; n=20) or sham operated (SHAM; n=20). After 30 days, periodontitis was induced by placement of a cotton ligature around the upper second molars of 10 OVX and 10 SHAM animals. All animals were sacrificed 5 weeks later. Body weight was assessed before all surgical procedures. The left hemimaxillas were removed and the percentage of periodontal bone support was determined radiographically and buccal alveolar bone loss was determined macroscopically using an image-analysis software. Furcation involvement was also evaluated. Data were analyzed statistically by ANOVA at 5% significance level. Within the evaluated period, the ovariectomized rats gained more weight than the sham-operated animals ($p<0.001$). The animals in which periodontitis was induced had less bone support, greater alveolar bone loss and furcation involvement than those without ligature ($p<0.001$). However, there was no difference between ovariectomized and sham-operated animals ($p>0.05$). Based on the findings of this study, estrogen deficiency could not be considered as a risk factor for periodontal disease.

Key Words: periodontal disease, osteoporosis, ovariectomy.

INTRODUCTION

According to the latest Brazilian demographic census (2000) (1), the Brazilian population has nearly 12.5 millions women aged over 50 years and their estimated life expectancy is 68.82 years. Considering that menopause occurs around the age of 50, millions of Brazilian women are expected to live more than a quarter of their lives without the influence of ovarian hormones and dealing with the possibility of having osteoporosis.

Osteoporosis is worldwide defined as a systemic skeletal disease characterized by low bone density and microarchitectural deterioration of bone tissue, which leads to increased bone fragility and risk of fracture (2). This disease should be considered as a public health problem because of its social, physical and economical impact. Most cases of osteoporosis occur in postmenopausal women. After menopause, women experience estrogen deficiency and this condition is associated with a rapid increase in bone resorption.

Alveolar bone loss is the major cause of tooth loss in the geriatric population. Periodontitis has characteristic signs such as periodontal pocket, dental mobility, abscesses and tooth loss. However, tooth loss is not the only consequence of alveolar bone loss. The patient is also at higher risk for residual ridge resorption, which might compromise the placement of dentures. Like periodontitis, osteoporosis may be considered a "silent disease" because it reaches severe stages without showing obvious symptoms to the patients. In spite of having

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a multifactorial etiology, especially related to pathogenic bacterial plaque and susceptible individuals, periodontitis shares some risk factors with osteoporosis, such as a higher prevalence associated with tobaccoism and increased age in addition to the influence of some medications like steroids (3).

To date, some evidences, although not conclusive, give support to make an association between low systemic bone density and tooth and alveolar bone loss. If confirmed, the association between systemic bone loss and alveolar bone loss would explain the variability in periodontal disease progression and treatment outcomes (4).

Considering the limitations of cross-sectional studies because variables are difficult to be established and controlled, as well as the challenges involved in prospective studies in humans, the identification of an experimental animal model to define the relationship between osteoporosis/osteopenia and periodontitis is warranted. The purpose of this study was to investigate the influence of estrogen deficiency as a risk factor for periodontal disease in rats.

MATERIAL AND METHODS

The study protocol was performed in compliance with the bioethical principles for animal research and was approved by the Ethics in Research Committee of the School of Dentistry of São José dos Campos (UNESP, Brazil).

Forty 90-day-old adult female rats (*Rattus norvegicus, albinus,* Wistar) were randomly assigned to 2 groups: one group was ovariectomized bilaterally (OVX group) and the other was subjected to sham operation (SHAM group). The animals were weighted and anesthetized intramuscularly with a combination 2% xylazine (Rompum; Bayer, São Paulo, SP, Brazil; 13 mg/kg body weight) and ketamine (Francotar; Virbac, Roseira, SP, Brazil; 33 mg/kg body weight). After shaving the lateral abdominal area, the surgery started with incision of all tissue planes, close to the kidney and under the ribs. The ovaries were identified, exposed and hemostasis was achieved with silk suture #4.0 (Ethicon; Johnson & Johnson, São José dos Campos, SP, Brazil) and ligature of the superior portion the Fallopian tubes. The ovaries together with the surrounding adipose tissue, the oviduct and a small portion of the uterus were excised. The muscle was sutured with resorbable Catgut #4.0 (Cirumédica, Cotia, SP, Brazil) and the skin was sutured with silk suture #4.0 (Ethicon; Johnson & Johnson). In the SHAM group, the ovaries were exposed and repositioned in their original position to simulate surgical stress.

Thirty days postoperatively, 10 rats of each group were anesthetized again and a cotton ligature (Coats Corrente, São Paulo, SP, Brazil) was placed around the upper second molars to induce periodontitis. Each group was therefore subdivided into A (absence of ligature) and P (presence of ligature). The ligature was maintained for five weeks and, during this period, the animals were examined every 12 days under anesthesia to verify the presence of the ligature in the gingival sulcus. The animals were sacrificed 65 days after ovariectomy or sham surgery. The maxillas were removed and the left hemimaxillas were fixed in 10% formalin. All the animals were weighted at the day of surgery (day 0), 30 days post-surgery, when the cotton ligature was placed (day 30) and 65 days post-surgery (day 65) when they were sacrificed. The percentage of weight gain, since day 0, was calculated.

The left hemimaxillas were radiographed (Fig. 1a) by lingual side with a digital dental x-ray equipment (Gendex 765DC; Gendex Dentals, Des Plaines, IL, USA) at 7 mA and 65 kVp, with focus-object distance of 30 cm, exposure-time of 0.063 s. The hemimaxilla

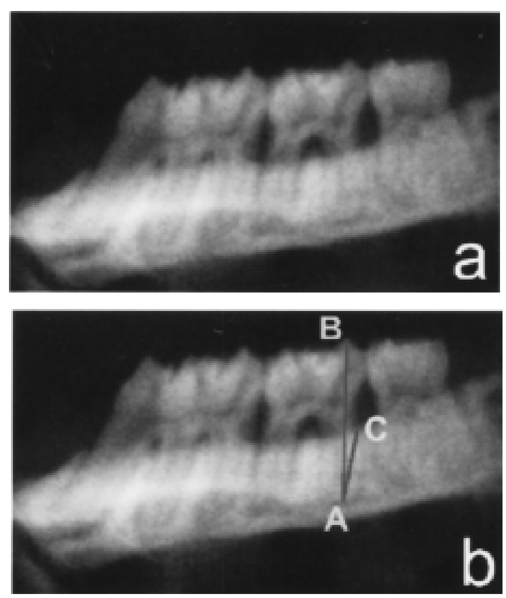

Figure 1. Analysis of distal bone support: a) radiograph of left hemimaxilla; b) illustration of the linear measurements performed.

was attached to the sensor apparatus with wax in order to obtain parallelism between lingual and buccal cusps, with superpositioning of the radiographic images of the cusps and roots.

The percentage of distal bone support (5) was calculated after the following linear measurements were made: distance between the distal root apex and the distal cusp top (AB segment; Figure 1b); distance between the distal root apex and the bottom of the deepest bone defect/top of bone crest distal to the tooth (AC segment - Figure 1b); and angle formed by these two segments (angle CÂB - Figure 1b). Image J 1.31p software (National Institutes of Health - USA; available from http://rsb.info.nih.gov/ij/Java1.31_03) was used according to the following formula: bone support (%) = [AC cos (CÂB) x 100]/AB. All measurements were confirmed three times in different moments. The average of the three values was determined as each animal value. Analysis of the coefficient of variation after repeated measurements was used for assessment of intraexaminer calibration.

After radiographic analysis, the same specimens were washed in tap water during 4 h to remove formalin residues, immersed in boiling water during 30 min and then treated with 1% papaine solution (Sigma Chemical Co., St. Louis, MO, USA) during 3 days. A new washing was done and the hemimaxillas were defleshed mechanically.

The level of furcation involvement of the upper second molars was also evaluated (two times in different moments) using a previously curved size 8 K-file, and was scored according to the following raking score scale: 0= no furcation involvement; 1= horizontal bone loss not involving all the extension of the furcation region; 2= horizontal bone loss involving all the extension of the furcation region.

For delimitation of the cementoenamel junction (CEJ) (5), after assessment of furcation involvement, the specimens were stained during 3 min in 1% methylene blue (Labsynth, Diadema, SP, Brazil) and had their buccal surfaces examined under stereomicroscopy. The image at X2.5 magnification was digitized and the area between CEJ and the top of buccal alveolar bone crest related with upper second molars (Figs. 2a and 2b) was evaluated using Image J 1.31p software. This area was denominated buccal alveolar bone loss. All measurements were taken in 3 distinct moments and the average was calculated as the specimen mean value. Intraexaminer calibration was also determined. All recordings were made by the same examiner, who was blinded to the groups to which the rats belonged.

Two independent variables were analyzed: *ovarian hormones* and *ligature to induce periodontitis*. The variable ovarian hormones had two levels: presence (SHAM group) or absence (OVX group). The variable ligature was analyzed in two levels as well: presence (P) and absence (A). For analysis of rat weight, the variable *time* was included (30 and 65 days). The dependent variables submitted to statistical analysis were percentage of *weight gain, distal alveolar bone support, furcation involvement* and *buccal alveolar bone loss*.

Data obtained from the measurements of body weight, distal alveolar bone support and buccal alveolar bone loss were submitted to descriptive statistics and analysis of variance. Tukey's test for multiple comparisons was used when needed. Significance level was set at 5%.

The following statistical softwares were used: Minitab *for Windows*, version 13.1 (2000, Minitab Inc., State College, PA, USA); Statistica *for Windows* 5.5 (2000, StatSoft Inc., Tulsa, OK, USA); and Statistix *for Windows* version 8.0 (2003, Analytical Software, Tallahassee, FL, USA).

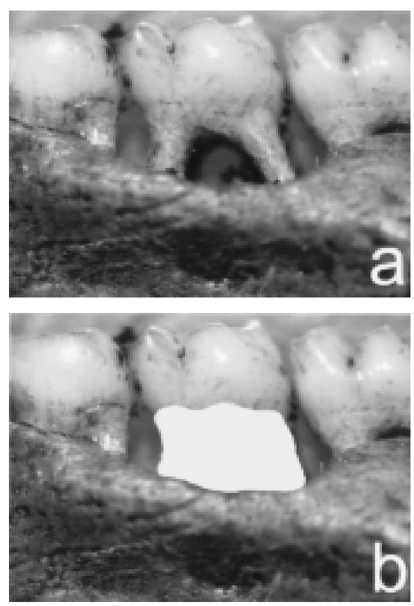

Figure 2. Analysis of buccal alveolar bone loss; a) macroscopic aspect of alveolar bone loss on left hemimaxilla; b) same figure with delimitation of the area under the cementoenamel junction and above the buccal alveolar bone crest.

RESULTS

Body Weight. The values of body weight of the animals at days 0, 30 and 65 were transformed in percentages of weight gain and were submitted to descriptive statistical analysis. After 30 days, the mean values varied between 8.57% (SHAM-A group) and 17.84% (OVX-A group) and standard deviation was between 4.45% (OVX-A group) and 5.93% (SHAM-P group). At day 65, the mean values varied between 16.50% (SHAM-A group) and 26.42% (OVX-A group) standard deviation ranged from 4.26% (SHAM-A group) to 6.52% (OVX-A group).

Weight gain results were analyzed by ANOVA for repeated measurements. Regarding ovarian hormones, it was observed that its absence influenced positively the weight gain (F= 26.91; p<0.001), i.e., the ovariectomized animals gained more weight (21.20 ± 6.86%) than the sham-operated rats (13.30 ± 6.23%). Regarding the variable time (F=93.21; p<0.001), comparing to day 0, the animals gained more weight at day 65 (21.07 ± 7.04%) than at day 30 (13.43 ± 6.19%). Other factors and interactions were not statistically significant (p>0.05). Figure 3 is a graphic representation of ovarian hormone X time interaction and illustrates data behavior, with more body weight gain by the OVX group and within the longest time interval.

Distal Bone Support. During radiographic analysis, it was observed that all animals with cotton ligatures showed the same type of bone resorption, predominantly vertical for the upper second molars, more accentuated in the mesial region, with furcation involvement and reaching the distal roots of the first upper molar and the upper third molar mesial roots.

The only significant effect was related to presence or absence of ligatures (F= 72.98; p<0.001); the animals in which periodontitis had been induced had less distal bone support in the upper second molar region (37.60 ± 7.73%) than those without ligatures (54.7 ± 3.41%). The results of descriptive statistics are on Figure 4.

For evaluation of intraexaminer calibration, the coefficient of variability of the three measurements performed in each digitized radiograph, in three different moments, was analyzed. The values of the coefficient of variability ranged from 0.608% to 4.933% (average= 2.611%).

Level of Furcation Involvement. During defleshing, two specimens from SHAM-P group were lost. The level of furcation involvement was evaluated two times by the same examiner, in distinct moments, and with 100% agreement between measurements. All animals without ligatures were scored 0 and those with ligatures were scored 2. No animal received intermediate scores. Thus, significant differences were related only to the presence/absence of ligature.

Buccal Alveolar Bone Loss. For the specimens with induced periodontitis, the findings of stereomicroscopic analysis showed predominantly

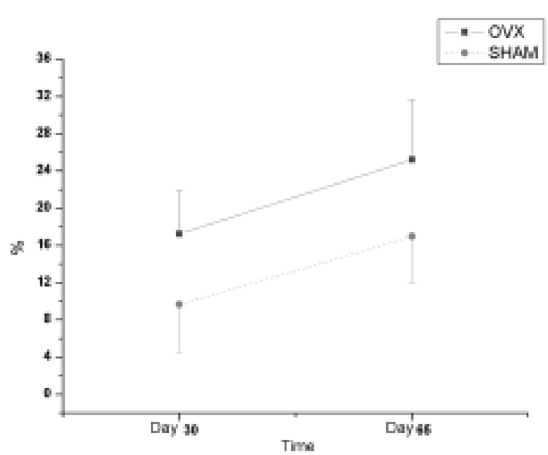

Figure 3. Means and standard deviation of the percentage of body weight gain referring to the interaction between time and ovarian hormones.

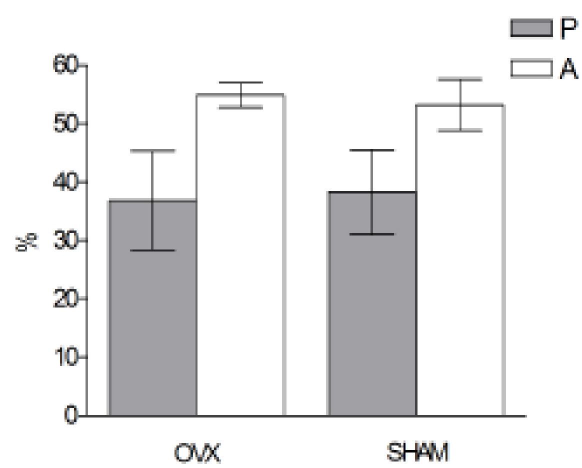

Figure 4. Means and standard deviation of the percentage of distal bone support referring to the interaction between ovarian hormones and ligature.

vertical bone resorption involving the area from the upper first molar distal root to the mesial side of the upper third molar. Bone resorption was much accentuated in the buccal surface, especially in the mesial root of the second upper molars. In the lingual side, the resorption was moderate and more horizontal; when vertical, it was less accentuated. In the animals without induced periodontitis, the bone level was located slightly apical to the CEJ.

Figure 5 is a graphic representation of the descriptive statistics of buccal alveolar bone loss. There was statistically significant difference ($F=332.70$; $p<0.001$) between animals with induced periodontitis (1.648 ± 0.300 mm^2) and those without ligature (0.361 ± 0.059 mm^2). The presence or absence of ovarian hormones did not influence the values significantly.

For evaluation of intraexaminer calibration, the coefficient of variability was calculated from three measurements on the digitized images in different moments. The coefficient of variability ranged from 0.294% to 3.665% (average = 1.650%).

DISCUSSION

Animal models have been used for investigation of pathogenic mechanisms of periodontal diseases as well as for treatment modalities. Rats have several similarities with humans regarding periodontal anatomy, dental plaque formation and composition, histopathology of periodontal lesions and basic immunology (6). It was therefore an acceptable model for the study performed.

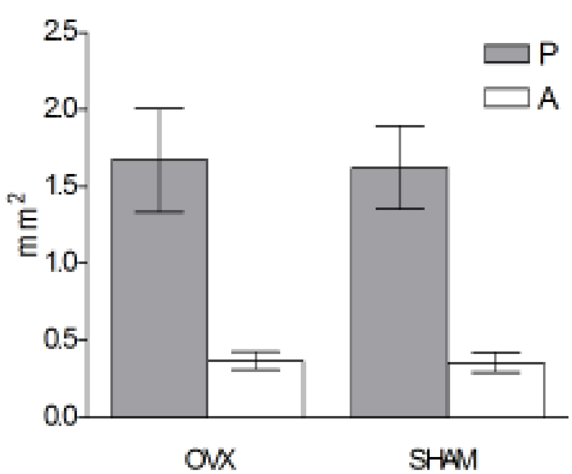

Figure 5. Means and standard deviation of buccal alveolar bone loss referring to the interaction between ovarian hormones and ligature placement (mm^2).

The experimental model for osteopenia induced by ovariectomy in female rats is also commonly used and is very useful for evaluation of problems related to bone loss in postmenopausal women. The literature shows that the effects of estrogen deficiency on bone characteristics as size, mass and density are site-dependent, the cancellous apendicular and axial bones being the most investigated for osteopenia due to a higher incidence of spontaneous fractures observed at these skeletal sites (7). Both animal and human studies show conflicting results about the influence of estrogen deficiency on alveolar bone loss. The effects of ovariectomy on bone tissue are not immediate. It usually takes more than three months for the effects of ovariectomy be evident in the mandible (8). Marques (9) evaluated the morphology of maxillary alveolar bone in ovariectomized rats and found evident characteristics of osteoporosis in the molar region 40 days postovariectomy, while Tanaka et al. (10) observed reduction of interradicular septae in the lower molars 60 days after ovariectomy. Another investigation (11) found alterations in the periodontal ligament of rats 90 days after ovariectomy. In the present study, the animals were evaluated 65 days after ovariectomy, but no alterations related to estrogen deficiency were observed. Perhaps, longer periods of time would provide different results.

However, like the present investigation, other studies did not find maxillary or periodontal alterations secondary to ovariectomy. Moriya et al. (12) evaluated the alveolar bone loss in ovariectomized rats with and without a calcium deficient diet and concluded that osteoporosis itself would not be capable of causing periodontal disease. The findings of another work (7) showed that estrogen deficiency was insufficient to cause maxillary osteoporosis in rats over an 11-week period. According to Zaffe et al. (13), due to their peculiar masticatory habits yielding huge loads on oral bones, rats would not be a suitable experimental animal model for studying oral bone loss related to osteoporosis. They believe that to worsen oral osteopenia, it would be necessary to combine gonadectomy with mechanical unloading of mandibular or maxilla bones, such as molar extraction.

In a previous study in which periodontal disease was induced in ovariectomized rats (14), it was observed histologically that estrogen deficiency may directly affect alveolar bone regardless of plaque accumulation and may also increase bone loss resulting from ligature-induced periodontitis. Our results disagree with those. In the present work, the ovarian hormones did not show a significant effect, which could be attributed to the methodology used in both works because Duarte et al. (14) waited 81 days to observe the results of ovariectomy and evaluated the lower first molar region instead of upper second molar.

Several studies referring to the association of mineral bone density and oral alterations indicated that the occurrence of tooth loss, periodontal disease and alveolar bone loss is more prevalent in individuals with decreased bone density. A positive relation between systemic bone loss and periodontal alterations was reported by different authors (15,16). Other researchers (17,18) did not find a direct relation between mineral bone density in postmenopausal women and oral alterations. Our results are consistent with those because we were not able to relate absence of ovarian hormones with periodontal alterations.

Ovariectomy induces an increase in food intake and a concomitant increase in body weight (19). In the present study, the success of ovariectomy in reducing the level of ovarian hormones could be evaluated by the significant body weight alterations in the animals. The factor time also indicated a significant body weight gain. In addition to being considered mature and independently of their hormone status, the animals grew during within the study period. Weight gain seems to yield a partial protection against of long bone osteopenia. After ovariectomy, animals' weight gain is primarily associated with an increase in the number of adipose cells, which increases the total activity of aromatase, a specific enzyme capable of converting adrenal-synthesized androgen precursor into estrogen. Postovariectomy body weight gain could somehow compensate estrogen blood levels (20). By stimulating animal food intake, ovariectomy could increase the mechanical load applied to the alveolar bone due to increased masticatory function, which could modify the direct effects of estrogen deficiency in the stomatognathic system (8). For these reasons, in some studies, feeding of ovariectomized animals is restricted to the same amount consumed by the sham animals. The lack of food control in this study might have affected the results.

A consensus has not yet been reached regarding the existence of a relationship between periodontal disease and ovariectomy. This subject needs to be further investigated to determine whether controlling certain variables can elucidate the questions still not answered. Under the tested conditions and based on the results obtained, it may be concluded that estrogen deficiency cannot be considered as a risk factor for periodontal disease.

RESUMO

O objetivo deste trabalho foi avaliar a influência da ausência de hormônios ovarianos induzida por ovariectomia como fator de risco para a doença periodontal em ratas. Quarenta ratas com idade de 90 dias foram submetidas a ovariectomia (OVX; n=20) ou cirurgia simulada (*sham*) (SHAM; n=20). Após 30 dias, ligaduras de algodão foram colocadas ao redor dos segundos molares superiores de 10 animais OVX e 10 animais SHAM. Cinco semanas após, todos os animais foram sacrificados. As ratas foram pesadas antes de todos os procedimentos cirúrgicos. As hemimaxilas esquerdas foram removidas e determinaram-se a porcentagem de suporte ósseo, através de radiografias, e a área de perda óssea alveolar, macroscopicamente, após maceração, com auxílio de programa computacional, além do grau de envolvimento de furca. Os dados foram submetidos à análise estatística por meio de ANOVA com nível de significância de 5%. No período avaliado, as ratas ovariectomizadas apresentaram maior ganho de peso que aquelas submetidas à cirurgia simulada (*sham*) ($p<0.001$). As ratas em que foi induzida periodontite apresentaram suporte ósseo estatisticamente menor e maior perda óssea alveolar e envolvimento de furca que as demais ($p<0.001$). Porém, não foram encontradas diferenças significantes entre os animais ovariectomizados e não-ovariectomizados ($p>0.05$). Com bases nos achados deste estudo, a deficiência de hormônios ovarianos não pôde ser considerada como fator de risco para a doença periodontal.

ACKNOWLEDGEMENTS

The authors would like to thank to the Brazilian National Council for Scientific and Technological Development (PIBIC/CNPQ) for granting financial support.

REFERENCES

1. Brazilian Institute of Geography and Statistics (IBGE). Rio de Janeiro. Demographic census 2000: universe results. Rio de Janeiro: IBGE, 2000. Available from: http://www.ibge.gov.br/home/estatistica/populacao/censo2000/tabelabrasil111.shtm. Latest access on September 1, 2006.
2. Genant HK, Cooper C, Poor R, Reid I, Ehilich G, Kanis J et al. Interim report and recommendations of the World Health Organization task-force for osteoporosis. Ostoporos Int 1999;10:259-264.
3. Jeffcoat MK, Chesnut CH. Systemic osteoporosis and oral bone loss: evidence shows increased risk factors. J Am Dent

Assoc 1993;124:49-56.

4. Krall EA. The oral effects of osteoporosis. Nutr Clin Care 2001;4:22-27.
5. Klausen B, Evans RT, Sfintescu C. Two complementary methods of assessing periodontal bone level. Scand J Dent Res 1989;97:494-499.
6. Klausen B. Microbiological and immunological aspects of experimental disease in rats: a review article. J Periodontol 1991;62:59-73.
7. Teófilo JM, Azevedo ACB, Petenusci SO, Mazaro R, Lamano-Carvalho TL. Comparison between two experimental protocols to promote osteoporosis in the maxilla and proximal tibia of female rats. Braz Oral Res 2003;17:302-306
8. Kuroda S, Mukohyama H, Hondo H, Aoki K, Ohyama T, Kasugai S. Bone mineral density of the mandible in ovariectomized rats: analyses using dual x-ray absorptiometry and peripheral quantitative computed tomography. Oral Dis 2003;9:24-28.
9. Marques KP, Taveira LAA. Microscopic investigation of periodontal and alveolar bone morphology in ovariectomized rats. J Appl Oral Sci (formerly Rev Fac Odontol Bauru) 1998;6:9-18.
10. Tanaka, M, Ejiri S, Toyooka E, Kohno S, Ozawa H. Effects of ovariectomy on trabecular structures of rat alveolar bone. J Periodont Res 2002;37:161-165.
11. Glickman I, Quintarelli J. Further observations regarding the effects of ovariectomy upon the tissues of the periodontium. J Periodontol 1960;31:31-37.
12. Moriya Y, Ito K, Murai S. Effects of experimental osteoporosis on alveolar bone loss in rats. J Oral Sci 1998;40:171-175.
13. Zaffe D, Paganelli C, Cocchi D. Induction and pharmacological treatment of oral osteopenia in rats. Minerva Stomatol 1999;48:45-62.
14. Duarte PM, Goncalves PF, Sallum AW, Sallum EA, Casati MZ, Humberto Nociti F Jr. Effect of an estrogen-deficient state and its therapy on bone loss resulting from an experimental periodontitis in rats. J Periodontol Res 2004; 39:107-110.
15. Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ. The relationship between bone mineral density and periodontitis in postmenopausal women. J Periodontol 2000; 71:1492-1498.
16. Inagaki K, Kurosu Y, Kamiya T, Kondo F, Yoshinari N, Noguchi T, Krall EA, Garcia RI. Low metacarpal bone density, tooth loss, and periodontal disease in Japanese women. J Dent Res 2001;80:1818-1822.
17. Elders PJ, Habets LL, Netelenbos JC, van der Linden LW, van der Stelt PF. The relation between periodontitis and systemic bone mass in women between 46 and 55 years of age. J Clin Periodontol 1992;19:492-496.
18. Hildebolt CF, Pilgram TK, Dotson M, Yokoyama-Crothers N, Muckerman J, Hauser J, Cohen S, Kardaris E, Vannier MW, Hanes P, Shrout MK, Civitelli R. Attachment loss with postmenopausal age and smoking. J Periodontal Res 1997;32:619-625.
19. Yoneda N, Saito S, Kimura M, Yamada M, Iida M, Murakami T, Irahara M, Shima K, Aono T. The influence of ovariectomy on ob gene expression in rats. Horm Metab Res 1998;30:263-265.
20. Davidge S, Zhang Y, Stewart KG. A comparison of ovariectomy models for estrogen studies. Am J Physiol Regul Integr Comp Physiol 2001;280:R904-907.

Accepted July 8, 2006