Oral Diseases (2006) 12, 181–186. doi: 10.1111/j.1601-0825.2005.01184.x

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ORIGINAL ARTICLE

Mandible analysis in sex steroid-deficient rats

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The aim of this study was to evaluate, by morphologic techniques, the effects of sex steroid deficiency on mandible bone remodeling of female rats, in groups of different experimental periods and to compare the results with 90-day orquiectomized males. Female and male Wistar rats, 3 months old, were divided into experimental groups and at the end of each experimental period were killed, and mandibles were extracted. The left mandibles were prepared with rote technique bone and examined by a light microscope. Morphological analyses of the mandibles demonstrated resorption signals in the alveolar bone, after 30 days in ovariectomized females, but it was more intense 90 days after castration. The orquiectomized group exhibited some signals of resorption similar to the ovariectomized group of 60 days. Morphometric analysis of alveolar bone thickness in females after 60 days was in agreement with morphological results. However, the analysis of periodontal ligament thickness did not show any significant difference. There were variations in sexual hormone deficiency in the mandibles of males and females and they seemed to be more precocious in ovariectomized than in orquiectomized rats. It is important for a health professional to have knowledge about bone metabolism to improve the quality of life of postmenopaused and old people.
Oral Diseases (2006) **12**, 181–186

Keywords: mandibles; morphometry; resorption; castration; sex steroids

Introduction

There is a well-defined relationship between sex steroids and bone, and deficiency in these hormones results in augmented bone resorption. The success of a dental treatment depends not only on the correct diagnosis and ability of the dentist, but also on the knowledge of the biological mechanisms of bone remodeling. Osteoporosis is the most common bone metabolic disease. It is characterized by a decreased rate formation and a continuous bone resorption process, leading to fractures (Francischetto, 1993; Wowern, 2001), which are the cause of morbidity in old persons (Raisz, 1997; Netelenbos, 1998; Krahe, 2003; Sanfilippo and Bianchi, 2003; Tayeb, 2003). Human beings have a decreased bone mass after the age of 35. In males, the loss is slow, during all life. But in females, the bone loss is bigger and quicker, about 50% in peri- and post-menopausal period, caused by the lack of oestrogen protection (Gillespsy and Gillespsy, 1991; Jeffcoat and Chesnut, 1993; Assaf, 1999; Bandeira *et al*, 2000; Castro, 2001; Russo, 2001; Mattson *et al*, 2002; Oliveira, 2002). The loss in bone mass observed after ovariectomy or orquiectomy, shows the importance of sex steroid hormones in the genesis of osteoporosis (Cao, 2004). Among other factors, oestrogens and androgens are necessary to ensure normal skeleton growth, maturation and turnover. Oestrogen receptors (ERa and/or ERb) are present in osteoblasts, and oestrogen and androgen withdrawal increase bone resorption, *in vivo*, through an increase in the synthesis and sensibility of local cytokines, like interleukins 1 and 6, TNF and prostaglandins (Schwartz and McCormack, 1991; Raisz, 1998; Ramalho, 2000). Although androgen receptors have been identified in osteoblast culture, it is accepted that oestrogen is essential for the development of the skeleton in males.

Some studies (Elsubeihi and Heersch, 2002; Johnson, 2002; Teófilo, 2003) have demonstrated the early effect of oestrogen deficiency in the trabecular bone than in the cortical bone, being more marked in the femur than in the mandibles (Hiatt and Gartner, 1997; Grynpas, 2002).

The reduction in bone mass is related to a greater risk for oral bone loss, which directly influences dental stability (Jiang *et al*, 2003). After 50 years, the mandible cortical bone porosity augments. The alveolar region is more affected than body mandible and it is most evident in women (Wactawski-Wende, 1996; Cao, 2004). Age and oestrogen deficiency affect alveolar crestal leading

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Received 14 April 2005; revised 4 May 2005; accepted 29 June 2005

to tooth loss (Wowern, 2001; Sanfilippo and Bianchi, 2003).

In the present study we evaluated continuous changes in the mandible caused by sex steroid deficiency, through morphological analysis, and compared long-term alteration found in male and female rats.

Materials and methods

Wistar rats, 3 months old, were maintained in a temperature-controlled room (22–25°C) with light/dark cycle of 12/12 h, receiving commercial pellet chow (Nuvital) and water *ad libitum*. Sex steroid deficiency was induced by the removal of bilateral gonads under thiopental anesthesia. Animals were weighed at the beginning and at the end of the experiment.

Females were previously evaluated by vaginal cytology and divided into three groups: ovariectomized (OVX), ovariectomized receiving estradiol benzoate (OVX + EB, 0.7 μg/100 g BW/day), and control (C). The rats were evaluated at 10, 30, 60 and 90 days after castration.

Male rats formed two experimental groups: orquiectomized (ORQ) and control (C), being evaluated 90 days after castration.

At the end of each experimental period the animals were killed under general anesthesia and the mandibles were extracted and divided into two parts. The left mandibles were fixed in Bouin solution, decalcified in FAS solution (10% acetic acid, 0.85% NaCl, 10% formalin) for nearly 30 days, and embedded in paraffin. Eight-micrometer sections were cut and stained with hematoxylin–eosin (HE). A light microscope (model DMRBE; Leica Microsystems, Wetzlar, Germany) was used to examine sections of the mandible.

A morphometric analysis of the periodontal ligament and alveolar bone thickness was realized with captured images of mandible sections stained with HE, using a microscopic-video system (model DMRBE, sony video camera; Leica). The periodontal ligament thickness was determined by the distance between apical region of second molar and adjacent alveolar bone. Alveolar bone thickness was determined between roots of the first and the second molars, using ImageLab program – Sistem of Image Process (Softium Informática Ltda, ME, Brazil).

The results are reported as mean ± s.e.m., and ANOVA was used for the evaluation of statistical analysis, with the level of significance set at $P < 0.05$.

Results

Numeric values of females and males body weight are shown in Table 1. In females, the ovariectomized group expressed the largest gain in body weight during the experimental period (10, 30, 60 and 90 days), whereas the ovariectomized group receiving estradiol benzoate showed the smallest gain in body weight. The control male group had a significantly higher body weight than the orquiectomized group.

Light microscope mandible analysis, made on the second molar round apical crest 10 days after ovariectomy (Figure 1a) showed that the alveolar bone, periodontal ligament, dentin and cementum were well organized and integral, as observed for C (Figure 1a) and OVX + EB groups. Similar morphological characteristics were also described for C females after 30 and 60 experimental days.

Thirty days after ovariectomy, females showed some alterations – two distinct areas were observed in the periodontal ligament. The one near the cementum was the most cellular, having fibroblasts with round nuclei. The other, close to the alveolar bone, the collagen periodontal fibers without orientation, seemed to be more in number than fibroblasts, with elongated nuclei. On the bone surface some irregularities, associated with the presence of osteoclasts, were observed. Innumerable reversal lines were present, disoriented when compared with C group.

In the 60-day ovariectomized group, aspects of the periodontal ligament and alveolar bone were similar to

Table 1 Effect of castration on females and males rat body weight

Experimental time	*Groups*	*n*	*Initial body weight (g)*	*Final body weight (g)*	*Δ* Body weight (g)
10 days	C	7	230.6 ± 7.8	239.7 ± 8.64	9.1
	OVX	7	223.3 ± 9.16	246.9 ± 9.99	23.6
	OVX + EB	7	224.4 ± 7.18	229.1 ± 8.92	4.7
30 days	C	7	220.3 ± 7.11	256.7 ± 9.16	36.4
	OVX	7	216.1 ± 6.69	261.4 ± 11.9*	45.3
	OVX + EB	6	205.6 ± 4.56	223.9 ± 4.4*	18.3
60 days	C	9	205.9 ± 6.61	231.5 ± 4.52	25.6
	OVX	7	217.1 ± 5.13	275.1 ± 5.74*	58
	OVX + EB	6	221.3 ± 5.4	236.3 ± 7.11**	15
90 days	C	8	211.5 ± 5.59	257.1 ± 5,86	45.6
	OVX	8	217.9 ± 4.13	293.1 ± 8.98*	75.2
	OVX + EB	9	205.9 ± 2.62	242.6 ± 5.98**	36.7
90 days	C	5	327.6 ± 9.41	439.8 ± 11.22	112.2
	ORQ	6	341.9 ± 12.37	412.4 ± 11.73	70.5

Results are reported as mean ± s.e.m. *Δ* Body weight is the difference between weight at the end of the experimental period and initial body weight.
*$P < 0.05$ C *vs* OVX, **$P < 0,05$ C *vs* OVX + BE. Kruskal–Wallis/Dunn's multiple comparison for females and *t*-test for males.

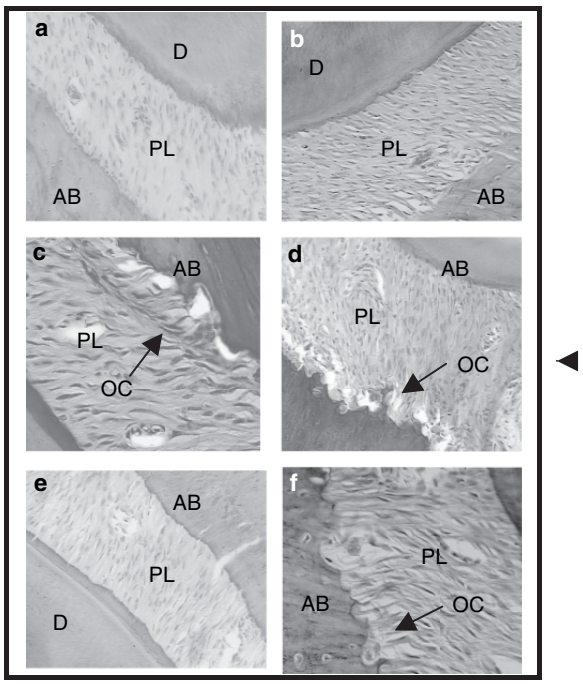

Figure 1 Photomicrographs of sex steroid deficient rat mandibles. Histologic section stained with HE at different stages of sex steroids deficiency in mandibles second molar apical area of female and male rats. Female group of 10 days: (**a**) control and (**b**) OVX; Female group of 90 days: (**c**) control and (**d**) OVX; Male group, 90 days: (**e**) control and (**f**) Orq. At 10 days there is no difference between mandible structures of C and OVX. At 90 days, the resorption process is evident by the presence of osteoclasts and disorganization of periodontal ligament in OVX. Some structural alterations can be observed at Orq group. AB: Alveolar Bone, PL: Periodontal Ligament, D: Dentin, OC: Osteoclast are evident (arrows). Original magnification ×200

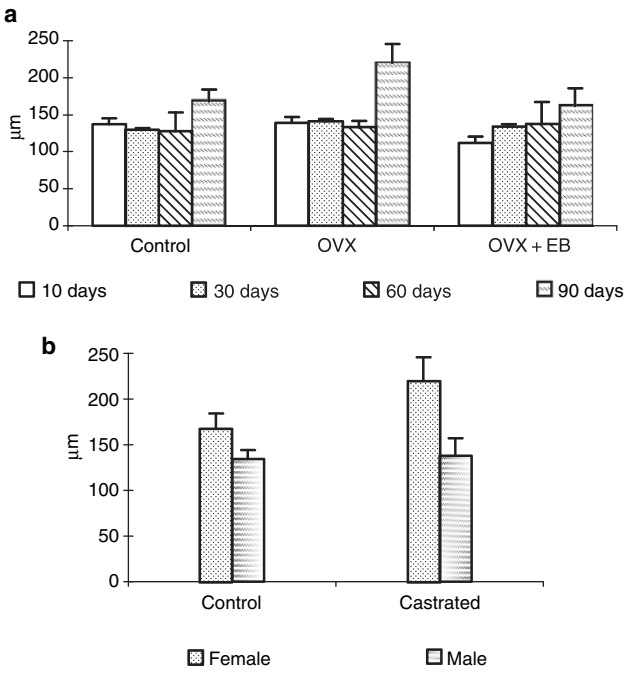

Figure 2 Morphometric periodontal ligament thickness analysis. (**a**) Periodontal ligament analysis among females with different experimental Groups (10, 30, 60 and 90 days after castration). (**b**) Periodontal ligament analysis between females and males with 90 days after castration

the 30-day group, but more intense. The intensity of alterations in the bone was more pronounced 90 days after ovariectomy (Figure 1d).

The 90-day control female group (Figure 1c) exhibited variations in morphological characteristics. The periodontal ligament was full of collagen fibers, although it was disoriented. The surface of the alveolar bone was irregular surface near the periodontal ligament. The periodontal ligament space seemed increased when compared with the other groups, C and OVX + EB. A large number of osteoclasts were observed at irregular areas of the bone surface and disoriented reversal lines were also demarked.

After 30, 60 and 90 days no significant differences between OVX + EB and C groups could be observed except the periodontal ligament that demonstrated a tenuous fibers disorganization at OVX + EB group.

In control males (Figure 1e) periodontal ligament fibers were more markedly inserted into the alveolar bone. The apical crest was complete and the alveolar bone had an homogeneous appearance, in spite of the presence of rare osteoclasts. The orquiectomized group (Figure 1f) exhibited some signals of resorption, similar to the OVX group, at 60 days. The periodontal ligament fibers were disorganized and without any pattern of insertion, suggesting an augmentation of periodontal ligament space. The alveolar bone showed disorganization, irregular surface and an apparent increase of osteoclasts in the whole root.

Morphometrical comparative analysis of periodontal ligament thickness from all female groups did not show any significant difference ($P > 0.05$) among C, OVX and OVX + EB, at 10, 30 and 60 days after ovariectomy. At 90 days, C and OVX + EB were visually different from the OVX group, but their mean values did not show any significant difference ($P > 0.05$). Control and treated groups were similar at all experimental periods. Male groups did not show any significant difference. However, when the measures were compared with females, 90 days after castration, the results suggested that the periodontal ligament was lower than in females, although there was no statistical significance (Figure 2 and Table 2). Morphometric analysis of alveolar bone thickness did not show any alteration in females after 10 and 30 days, neither in males at 90 days ($P > 0.05$). However, a significant difference between C and OVX rats at the experimental period of 60 days was observed, the thickness being lower in the OVX group ($P = 0.0052$). At 90 days the thickness was significantly lower in OVX when compared with OVX + EB ($P = 0.0028$), but not between C and OVX (Table 2).

Discussion

The increase in the life expectancy of Brazilians induced a greater awareness among doctors and dentists about common health problems of elderly people. Osteoporosis

Table 2 Periodontal ligament and alveolar bone thickness analysis

Experimental time	*Groups*	*n*	*Periodontal ligament thickness (μm)*	*Alveolar bone thickness (μm)*
10 days	C	7	136.9 ± 9.12	324.5 ± 5.57
	OVX	7	138.8 ± 9.14	362.5 ± 14.19
	OVX + EB	7	112.1 ± 357	320.4 ± 15.86
30 days	C	7	128.7 ± 5.71	311.8 ± 40.22
	OVX	7	140.6 ± 18.07	279.3 ± 28.29
	OVX + EB	6	133.8 ± 9.40	342.5 ± 28.27
60 days	C	9	128.2 ± 24.89	308.6 ± 16.31
	OVX	7	132.6 ± 8.51	238.0 ± 12.86*
	OVX + EB	6	136.9 ± 30.56	306.5 ± 10.40**
90 days	C	8	167.7 ± 16.21	199.8 ± 16.58
	OVX	8	220.4 ± 25.98	158.7 ± 8.22**
	OVX + EB	9	161.8 ± 24.09	240.0 ± 9.99
90 days	C	5	134.5 ± 10.17	330.7 ± 55.30
	ORQ	6	138.3 ± 19.4	267.7 ± 17.43***

The effects sex steroid deficiency on female (OVX) and male (ORQ) rat mandibles, compared to the respective controls, at various experimental periods. The number of experimental animals (*n*) are different per group. Data are reported as mean ± s.e.m. $^{*}P < 0.05$ (*C vs* OVX), $^{**}P < 0.05$ (*OVX vs* OVX + BE), $^{***}P < 0.05$ (*C vs* ORQ). Kruskal–Wallis/Dunn's multiple comparison for females and *t*-test for males.

is one of the most prevalent bone diseases among senior citizens and affects one in four postmenopausal women and this morbid clinical condition is represented by fractures.

Oral bone loss originating from osteoporosis is a great problem to dentists. The quantity and quality of the mandibular bone is mainly important to professionals planning prosthetic treatment and implants. So, knowledge of the relationship between sex steroids and bone metabolism helps the dentist to advise the patients about the risks of systemic and oral bone loss.

Bone alterations caused by sex steroid deficiency and/or age have been previously observed in women when compared with men (Jeffcoat, 1998; Cao, 2004). Based on this affirmation we studied a group of males after 90 days of orquiectomy.

In this study we observed that ovariectomy increases body weight and that estradiol benzoate treatment can revert this effect. Our results are in agreement with Lisbôa (1997), Marques and Taveira (1998) and Teófilo (2003), but to date, there is no plausible explanation. Contrary to results in females, castration in males did not induce body weight alterations, as reported by Lisbôa (1997).

Early signals of bone loss were observed by light microscopic analysis of the mandible. As expected, the effects of ovariectomy were more marked in control and treated rats. OVX rats had an evident oral bone loss and it was present in several experimental periods, mainly at 90 days, when the animals were almost 180 days old. Control rats of the same age also showed alterations, however less markedly.

Our results are in agreement with Marques and Taveira (1998) showing that age caused bone and dental morphological differences besides the influence of the endocrine system and body weight. In a review, Devlin *et al* (1990) correlated hormonal changes, like menopause, with mandible and generalized osteoporosis, determined by a decrease in the density of alveolar bone surface. These observations were confirmed by morphological results acquired in the experimental group of 30 days of castration. But our experimental groups are not compatible with Zaffe *et al* (1999), Jiang *et al* (2003), and Teófilo (2003), who did not find expressive alterations after 60 days or 11 weeks. Hsieh *et al* (1995) have shown that during aging, the effects of menopause on the bone are directly associated with the animal's age. This study justifies different reports found in the literature by utilizing animals with different ages and time of castration.

Morphological alterations of males are less expressive than of females. The orquiectomized group demonstrated compatible alterations with that observed in OVX females 60 days after castration; and the control group showed characteristics similar to those OVX + EB 30 days after castration. These reports are in agreement with Taguchi (1995) that desynchronization in bone remodeling is delayed and inexpressive in males compared with females. But, with the increase in the life expectancy of the population, the incidence of osteoporosis is on the increase in elderly males. Bone formation was constantly observed in OVX females, due to the presence of reversal lines (Yamashiro and Yamamoto, 2001). Nevertheless, as there is bone loss, the resorption mechanism is greater (Wronski *et al*, 1988). The characteristics became more evident when the time of castration increased, reinforcing the disorganization of new bone formation. Cellular evaluation suggests a progressive increase in the number of osteoclasts at 30, 60 and 90 days after ovariectomy. Histomorphometric analyses (Wronski *et al*, 1988, 1989; Wronski, 1988) confirm this suggestion. In terms of changes observed in the osteocytes, our results were very similar to Marques and Taveira (1998) and Shklar and Glickman (1956) who found an irregular distribution of these cells near the incremental lines. The morphological characteristics of incremental lines and

osteoclasts near the surface of the bone in OVX groups are attributed to increased bone remodeling (Piroshaw and Glickman, 1957; Marques and Taveira, 1998). There is a closer relationship between sex steroid deficiency and bone mineral density. Wronski *et al* (1989) and Devlin *et al* (1990) related a fast decrease in bone volume, increase in cancellous bone loss and osteoid surface extension, when they used several bone types and a control protocol.

In a 10-year (1989–1998) review, Birkenfeld (1999) verified that mandibles and maxillary osteoporosis alterations affect dental stability and retention, and a reduction in bone modification and an increase in bone retention after sex steroid treatment was observed. In our study, using estradiol benzoate treatment, the presence of incremental lines was more tenuous and homogeneous. These results are similar to those of Shklar and Glickman (1956), Li and Nishimura (1994); Sanfilippo and Bianchi (2003) and Cao (2004). They found that therapies which influence systemic mineral bone density may be associated with a reduction in teeth and alveolar bone loss.

As histological results of castrated males and females were very different compared with the control groups, we decided to determine periodontal ligament and alveolar bone thickness. morphometric analysis of the periodontal ligament showed some alterations that were more accentuated in the OVX group at 90 days, when compared with all female and male groups. Shklar and Glickman (1956), Marques and Taveira (1998), Teófilo (2003) have suggested an augmentation in periodontal ligament space in castrated females. Alveolar bone thickness showed a significant decrease after 60 days of ovariectomy, that was more accentuated after 90 days. But, at the same time control females also demonstrated a decrease in alveolar bone thickness, probably due to aging. Treatment with estradiol benzoate prevented bone loss, particularly after long-term castration (90 days), when the thickness was significantly higher in the OVX + EB group than in C and OVX groups. The two measures of thickness are complementary in that they show the evident bone resorption process at work in ovariectomized females. Despite the lack of statistical difference among groups periodontal ligament thickness is visually different in castrated females, in view of periodontal ligament increases as function of bone loss. So, the significant decrease of bone alveolar measures confirms the increased presence of osteoclast observed at morphological analysis of castrated groups. A similar result was also observed in older control females, who had an imbalance in the remodeling process. We have proved that a longer castration time (90 days) lead to severe morphological and morphometric alterations which can be prevented by estradiol benzoate treatment.

Castrated males were not different from 180-day-old control males, confirming that males are more protected than females. Furthermore, the results did not differ significantly from females (90 days) probably due to few males observed.

Conclusion

The effects of sex steroid deficiency caused by castration are distinct between male and female rats, in the long-term, with bone alterations more accentuated in females. In comparison with males, ovariectomized females had an increase in body weight that is influenced by age and experimental period. Oral bone remodeling resulting from lack of sex steroids, occurred 30 days after ovariectomy, being more evident after 60 and 90 days of castration. The same occurs with the periodontal ligament and alveolar bone thickness, which increased with age and the time of ovariectomy. We conclude that lack of sex steroid hormones and the age of females, but not of males, are deleterious to bone health.

Acknowledgement

We would like to thank PhDs Lais Carvalho, Denise Pires de Carvalho and Eliete Bouskela for assistance.

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