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ORIGINAL ARTICLE

Influence of ovariectomy and masticatory hypofunction on mandibular bone remodeling

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INTRODUCTION: This study was designed to examine the effect of masticatory hypofunction and estrogen deficiency on mandible bone mass and compare this site with spine and femoral bone.

METHODS: Twenty-four rats were ovariectomized (OVX) or Sham-operated (Sham) and analyzed after feeding with hard diet (Hard) or soft diet (Soft). They were divided into four groups (GI)Sham-Hard; (GII)OVX-Hard; (GIII)Sham-Soft and (GIV)OVX-Soft. Bone mineral density (BMD) was measured in the spine and femur in the baseline and at the end of the study, and ΔBMD (final BMD − baseline BMD) was calculated. In mandible bone, BMD and histomorphometry were analyzed at the end of the experiment.

RESULTS: Sham rats showed higher spine (GI 13.5% *vs* GII: 0.74%, $P < 0.01$; GIII: 10.67% *vs* GIV: −4.36%, $P < 0.001$) and femur ΔBMD (GI: 14.43% *vs* GII: 4.42%, $P < 0.01$; GIII: 10.58% *vs* GIV: 0.49%, $P < 0.001$) than OVX, but no difference was observed in mandible BMD among these groups ($P > 0.05$). Soft-diet groups showed decreased mandible BMD compared with hard-diet groups (GIV *vs* GII, $P < 0.01$; GIII *vs* GI, $P < 0.01$). Similarly, mandibular condyle histomorphometry showed that soft-diet groups presented a significant decrease in trabecular thickness and volume (GIV *vs* GII, $P < 0.05$; GIII *vs* GI, $P < 0.01$) compared with hard diet.

CONCLUSION: Our results suggest that mandibular bone loss resulted from decreased of mechanical loading during mastication, and was not affect by estrogen depletion.

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Keywords ovariectomy; masticatory hypofunction; mandible; dual-energy X-ray absorptiometry ; histomorphometry

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Introduction

There is controversy if mandible bone and axial or peripheral skeleton, react similarly to estrogen deficiency bone loss. The estrogen is important for the growth and maintenance of the female skeleton. This sexual hormone has been shown to induce apoptosis in bone-resorbing osteoclasts and has an anti-apoptotic effect in osteoblasts, leading to an overall building of bone (Manolagas *et al*, 2002). Menopause leads to estrogen deficiency and changes in bone mass and it is major cause of osteoporosis in women. (Genant *et al*,1998).

The etiology of skeletal and oral bone loss in osteoporotic patients is presumed to differ. Age and estrogen deprivation are major etiologic factor in systemic skeletal bone loss (Seeman, 2004), while oral bone atrophy may be considered a consequence of local factors, such as absence of teeth, impaired adapted prostheses which lead to decrease of mastication (von Wowern, 2001; Seeman, 2004).

Studies in rats with skeletal osteoporosis have revealed that ovariectomy (OVX), by itself, does not promote alveolar bone loss or changes in the mandibular cortical thickness, requiring association with other osteopenic factors to induce significant mandibular bone loss (Moriya *et al*, 1998; von Wowern, 2001; Jiang *et al*, 2003). By the other way, it has been found a positive correlation between oral bone mass reduction and loss of teeth from patient with systemic osteoporosis (Hirai *et al*, 1993; Taguchi *et al*, 1995).

However, the majority of works reporting alterations caused by estrogen depletion and mandibular bone resorption did not take into consideration the loads generated during mastication, and their action on mandible bone mass. The growth of the condyle is known to be highly adaptable to functional factors, and the comparison of experimental studies is difficult by the lack of detailed analyses of the load distribution within the condyle. Meanwhile, it is unclear whether estrogen deficiency and/or masticatory hypofunction could affect the internal bone structure in the whole mandible in the different manner (Bresin *et al*, 1999; Huiskes *et al*, 2000).

Experimental studies in growing rodents have shown that reduced masticatory function causes morphological

changes in the mandible (Ito *et al*, 1988). A method used to alter the masticatory function is feeding the animals with a sotf diet (Kiliaridis *et al*, 1999; Tanaka *et al*, 2007). Altering the consistency of the diet in this way has been shown to cause overall size differences in the ramus region, angular and condylar processes (Ito *et al*, 1988). The softer diet consumed in modern societies is likely to generate reduced masticatory forces (Corruccini and Lee, 1984). Studies on growing rats have shown that masticatory hypofunction induced by a soft diet lead to reduced masticatory muscle strength that results in changes of the mandibular morphology and the alveolar bone microarchitecture (Kiliaridis and Shyu, 1988; Mavropoulos *et al*, 2004a,b).

Most elderly people perceive their oral health status as important to their quality of life, for a variety of physical, social and psychological reasons. The ability to eat is considered to be particularly important. It is therefore essential for dentists to understand the pathophysiological mechanism related to oral bone loss to preserve the good quality of life of these patients. Edentulous adults who received implant retained bridges had an improvement of both masticatory muscle strength and chewing ability (Jemt *et al*, 1993).

Therefore, the aim of this study was to assess the role of estrogen depletion and masticatory hypofunction in mandible bone, and compares this site with spine and femur bone.

Materials and methods

Protocol

Twenty-four 4-month-old female Wistar rats presenting an initial body weight of 240 g, provided by the Animal Center of the Medicine School of the University of São Paulo (FMUSP), Brazil, were maintained under constant conditions of temperature (20 ± 1°C) on a 12/12h light–dark cycle with *ad libitum* access to food and water.

As an estrogen-depletion bone loss osteoporosis model, 12 animals were bilaterally ovariectomized (OVX) and the control group ($n = 12$) was subjected to Sham surgery (Sham), after being anesthetized via intraperitoneal injection of 50 mg kg^{-1} ketamine (Ketalar; Parke-Davis Ache, São Paulo, Brazil) and 10 mg kg^{-1} Xylazine (Rompum; Bayer do Brasil S/A, São Paulo, Brazil). To induce masticatory hypofunction, the consistency of the diet was altered from a standard hard diet (Hard) to soft diet (Soft). Rats in the hard-diet group were fed a standard solid diet (Nuvital Nutrients S/A, Curitiba, PR, Brazil) The soft-diet animals were given the same diet in a powdered form, with water added in standardized proportions (2/5 = food/water) (Kiliaridis *et al*, 1999; Tanaka *et al*, 2007) and the maxillary incisors of these rats were trimmed bilaterally to the level of gingiva every other day (Hinton and Carlson, 1986). The bedding material of the cages of the soft-diet group was sifted to exclude large particles which could stimulate gnawing activity (Kiliaridis *et al*, 1999; Tanaka *et al*, 2007). The animals were randomized by weight into four groups ($n = 6$): GI: sham-operated rats fed on a hard diet (Sham-Hard); GII: ovariectomized rats fed on a hard diet (OVX-Hard); GIII: sham-operated rats fed on a soft diet (Sham-Soft); and GIV: ovariectomized rats fed on a soft diet (OVX-Soft).

Bone mineral density (BMD) was measured in the lumbar spine and total femur at the baseline and after 9 weeks (da Paz *et al*, 2001), and ΔBMD (final BMD − baseline BMD) was calculated. The animals were weighed each week during the experimental period. Because of the anatomic shape of the mandible that limits the measurement, the mandibles were dissected, and the right side was used to measure final BMD and the left side was submitted to histomorphometry.

The design was approved by the Animal Ethics Committee of COBEA (Brazilian College of Experimental Animals) in accordance with procedures set by UFAW (the Universities Federation for Animals Welfare).

Bone mineral density measurement

The bone mineral density was measured by dual-energy X-ray absorptiometry using a clinical densitometer (Hologic QDR-2000, Bedford, MA, USA) and software for small animals. *In vivo* reproducibility was checked by measuring the coefficient of variation (CV = 100 × s.d./mean) of five BMD measurements in three rats with mean weight of 227 g, resulting in 1.1% in the lumbar spine (L1–L4), 1.9% in total femur and 0.88% the hemimandible.

Bone mineral density of the entire right side of the mandibles were measured after dissection. The region of interest (ROI) was defined as a rectangular area that included: condylar, coronoid and alveolar process. The ROI measurement was set by hand for elimination of molar and incisors (crown). The samples were placed in a saline-filled container, lingual side up. The mandible was positioned so that the scanning was moved in a direction perpendicular to the occlusal plane. (Elovic *et al*, 1995).

Mandibular bone histomorphometry

The left mandibular bone were dissected and fixed in 70% ethanol, dehydrated through serially increased concentrations of ethanol, and embedded in methyl methacrylate without decalcification. After polymerization, the blocks were unilaterally ground up to the mid-sagittal surface of the mandibular bone. The samples obtained were sliced longitudinally at 5–10 μm thickness, using a Policut S microtome (Reichert-Jung, Heidelberg, Germany). The 5-μm sections were stained with 0.1% toluidine blue, pH 6.4, and at least two non-consecutive sections of each sample were examined. The histological measurement of the mandible was taken in the whole condyle, at the greatest convexity of the condylar process in its larger superior portion of the contour, in the subchondral bone directly below the anterior, central and posterior regions which is in articulation with the cranial part of the joint.

All histomorphometric indices were reported according to the standardized nomenclature recommended by the *American Society of Mineral Research* (Parfitt *et al*, 1987). The following histomorphometric parameters, measured at a standardized site below the growth plate,

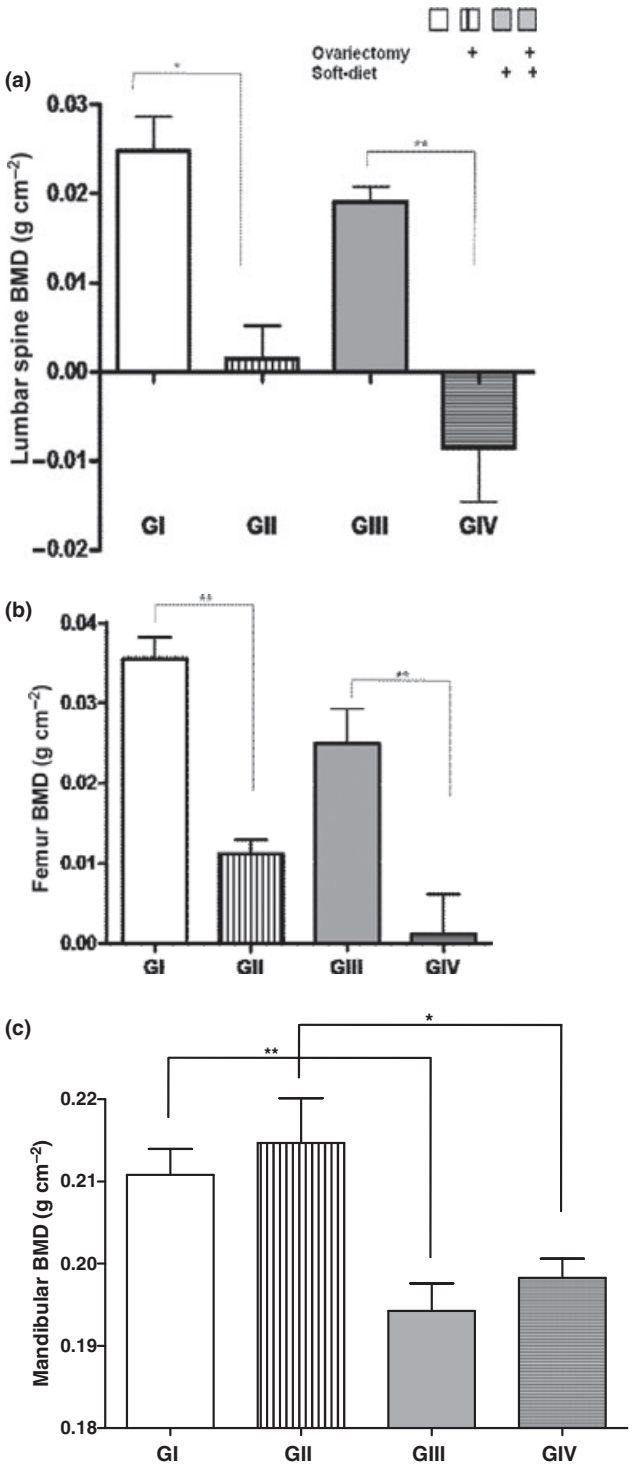

Figure 1 Bone mineral density change (ΔBMD) in lumbar spine (**a**); in femur (**b**); and the final BMD in the right mandible (**c**), compared Sham and OVX groups fed on Hard diet or Soft diet. #$P < 0.05$. *$P < 0.01$. **$P < 0.001$

were obtained: (i) bone volume (BV/TV, %); (ii) trabecular thickness (Tb.Th, μm); (iii) trabecular separation (Tb.Sp, μm); and (iv) trabecular number (Tb.N/mm). All animal data were obtained using blinded measurements.

Statistical analysis

All data are presented as the mean and standard deviation (s.d.). Analysis of variance (ANOVA) was used to determine significant differences between groups. Multiple analyses of variance were also used to study the effects of the two experimental factors, OVX (OVX *vs* SHAM) and soft diet (Soft *vs* Hard) on the variables under study. The Bonferroni test was used to perform *post hoc* comparisons between groups using Prism 3.0 (GraphPad Software, San Diego, CA, USA). Significance level of $P < 0.05$ was used for all comparisons.

Results

At the baseline of the experiment, no difference was observed between the four groups (GI *vs* GII *vs* GIII *vs* GIV) related to body weight (228.5 ± 7.03 *vs* 234.5 ± 3.83 *vs* 227.3 ± 8.11 *vs* 240 ± 3.98 g, $P = 0.27$), lumbar spine BMD (0.184 ± 0.009 *vs* 0.190 ± 0.007 *vs* 0.178 ± 0.007 *vs* 0.195 ± 0.12 g cm^{-2}, $P = 0.54$) and femur BMD (0.245 ± 0.008 *vs* 0.253 ± 0.008 *vs* 0.235 ± 0.007 *vs* 0.244 ± 0.010 g cm^{-2}, $P = 0.93$). The mean body weight at the end of the experiment showed no statistically significant difference between the four groups of rats (GI: 48.33 ± 7.34 g; GII: 57.33 ± 5.11 g; GIII: 42.33 ± 7.80 g; GIV: 43.5 ± 8.66 g, $P > 0.05$). Weight gain in the soft-diet group was quite good. Nutritional inadequacy was hardly a problem.

The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns in OVX group.

Bone mineral density

Sham groups vs *OVX groups*. A significant higher ΔBMD (g cm^{-2} and %) in lumbar spine was observed in the Sham groups compared with the OVX groups with the same consistency of diet (Hard: GI: 0.0248 ± 0.009 g cm^{-2} *vs* GII: 0.0014 ± 0.009 g cm^{-2}, $P < 0.01$ and Soft: GIII: 0.019 ± 0.004 g cm^{-2} *vs* GIV: −0.0085 ± 0.014 g cm^{-2}, $P < 0.001$), (Hard: GI: 13.51% *vs* GII: 0.74%, $P < 0.01$ and Soft: GIII: 10.67% *vs* −4.36%, $P < 0.001$) (Figure 1a).

Similarly, a higher ΔBMD (g cm^{-2} or %) in femur was demonstrated in the Sham groups compared with the OVX groups (Hard: GI: 0.0354 ± 0.006 g cm^{-2} *vs* GII: 0.0112 ± 0.004 g cm^{-2}, $P < 0.001$ and Soft: GIII: 0.0249 ± 0.010 g cm^{-2} *vs* GIV: 0.0012 ± 0.011 g cm^{-2}, $P < 0.001$); (Hard: GI: 14.43% *vs* GII: 4.42%, $P < 0.001$ and Soft: GIII: 10.58 *vs* GIV: 0.49%, $P < 0.001$) (Figure 1b).

By contrast, no significant difference in mandible BMD was observed comparing the Sham groups and the OVX groups using equivalent consistency of diet (Hard: GI: 0.2108 ± 0.007 g cm^{-2} *vs* GII: 0.2146 ± 0.005 g cm^{-2}, $P > 0.05$ and Soft: GIII: 0.1942 ± 0.008 g cm^{-2} *vs* GIV: 0.1982 ± 0.005 g cm^{-2}, $P > 0.05$) (Figure 1c).

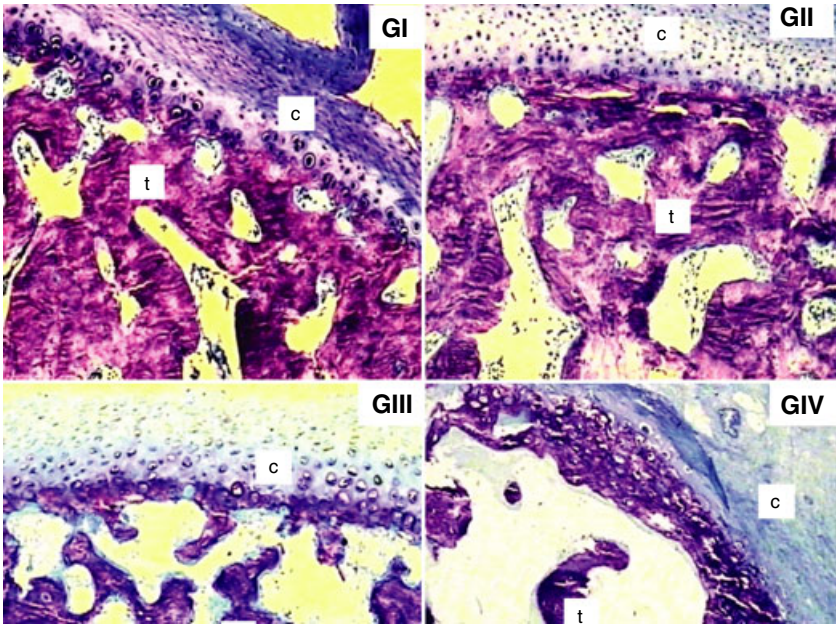

Figure 2 Histological changes in mandibular condyle: the trabecular thickness and number were lower in rats fed on soft diet (GIII, GIV) compare with those fed on hard diet (GI, GII). (Toluidine blue stain; ×100). (t) trabecular bone, (c) condylar cartilage, GI (Sham-Hard) – sham operated rats fed on a hard diet, GII (OVX-Hard) – ovariectomized rats fed on a hard diet, GIII (Sham-Soft) – sham operated rats fed on a soft diet, GIV (OVX-Soft) – ovariectomized rats fed on a soft diet

Hard- vs *soft-diet groups*. Lumbar and femur ΔBMD in rats fed on hard- and soft diets were comparable – values in the Sham groups were as follows lumbar ΔBMD: GI: 0.0248 ± 0.009 g cm^{-2} *vs* GIII 0.019 ± 0.004 g cm^{-2}, $P > 0.05$ and femur ΔBMD: GI: 0.0354 ± 0.006 g cm^{-2} *vs* GIII 0.0249 ± 0.010 g cm^{-2}, $P > 0.05$; and values in the OVX groups as follows lumbar ΔBMD: GII: 0.0014 ± 0.009 g cm^{-2} *vs* GIV: −0.0085 ± 0.014 g cm^{-2}, $P > 0.05$ and femur ΔBMD: GII: 0.0112 ± 0.004 g cm^{-2} *vs* GIV 0.0012 ± 0.011 g cm^{-2}, $P > 0.05$ (Figure 1a,b).

Differently, significantly lower mandible BMD was observed in the soft-diet groups compared with the hard-diet groups (Sham: GIII: 0.1942 ± 0.008 g cm^{-2} *vs* GI: 0.2108 ± 0.007 g cm^{-2}, $P < 0.01$ and OVX: GIV: 0.1982 ± 0.005 g cm^{-2} *vs* GII: 0.2146 ± 0.005 g cm^{-2}, $P < 0.01$) (Figure 1c).

Mandibular bone histomorphometry

The area of the whole condyle measurements were similar in the four groups: (GI: 2.07 ± 0.28 mm^2 *vs* GII: 1.90 ± 0.73 mm^2 *vs* GIII: 1.80 ± 0.46 mm^2 *vs* GIV: 1.53 ± 0.98 mm^2, $P = 0.598$).

Sham groups vs *OVX groups*. No significant difference in mandibular bone histomorphometry parameters (BV/TV, Tb.Th, Tb.Sp, Tb.N) were observed in the Sham groups compared with the OVX groups with the same consistency of diet (Hard: GI *vs* GII, $P > 0.05$ and Soft: GIII *vs* GIV, $P > 0.05$) (Figures 2 and 3a,b).

Hard- vs *soft-diet groups*. The mandibular bone histomorphometry parameters: BV/BT and Tb.Th were significantly lower in rats fed on soft diet compare with those fed on hard diet in the Sham groups (BV/TV: GIII: 18.56 ± 2.78% *vs* GI: 30.65 ± 3.49%, $P < 0.001$; Tb.Th: GIII: 41.41 ± 6.06 μm *vs* GI: 61.33 ± 6.00 μm, $P < 0.01$) and OVX groups (BV/TV: GIV: 19.85 ± 1.77% *vs* GII: 27.84 ± 3.34%, $P < 0.01$; Tb.Th: GIV: 42.91 ± 9.13 μm *vs* GII: 57.49 ± 9.66 μm, $P < 0.05$) (Figures 2 and 3a,b).

Discussion

The present study demonstrated that masticatory hypofunction is the major factor to induced mandible bone loss and cause deleterious effect on bone quality of in female Wistar rats.

Ovariectomy in rats is known to cause bone loss in vertebra and in femur (Wronski *et al*, 1988). However, the skeletal alteration associated with ovariectomy varies with the region observed, and may cause differential effects on the mandible, spine and femoral bone (Li *et al*, 1996; Yamashiro and Yamamoto, 1997).

Some reports showed a positive correlation between systemic osteoporosis caused by estrogen deficiency and low mineral density in the mandible (Tanaka *et al*, 2002; Yang *et al*, 2005). Yang *et al* (2005), demonstrated that a long-term estrogen deficiency in OVX rats is necessary to decrease the mandibular cortical thickness and suggested that in humans a considerable duration of estrogen deficiency may be required before a reduction of mandible cortical bone loss.

On the other hand, many studies have failed to detect mandible bone loss following OVX in animals (Moriya *et al*, 1998; Jiang *et al*, 2003). Some authors found that the mineral content and mechanical properties of the mandibles of OVX rats are similar to those of Sham operated rats, and that rat mandible bone did not show bone loss after OVX (Elovic *et al*, 1995; Yamashiro and Yamamoto, 1997). Jiang *et al* (2003), concluded that bone loss in the mandible caused by ovariectomy alone were lower than those caused by dietary calcium deficiency alone; in particular, OVX rats showed no

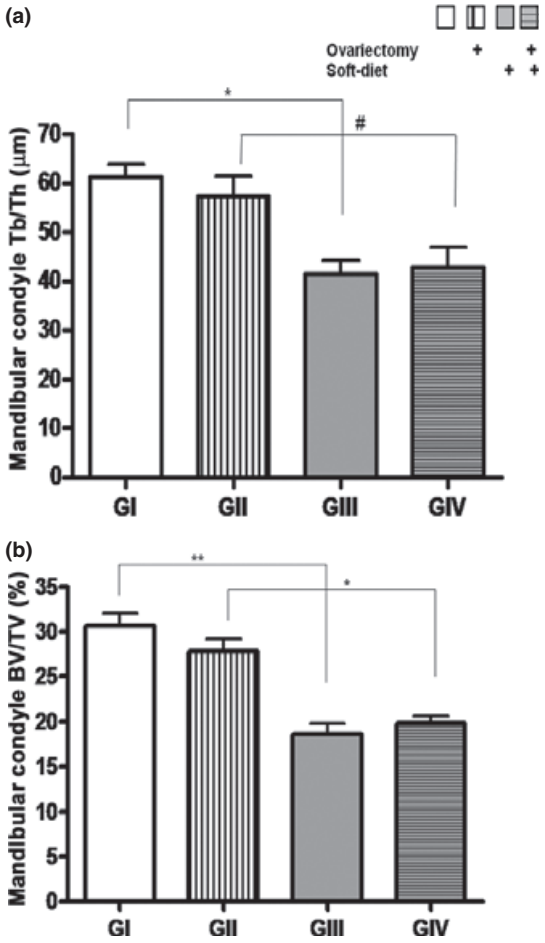

Figure 3 Histomorphometric parameters on mandibular condyle compared Sham and OVX groups fed on Hard diet or Soft diet: (**a**) trabecular thickness (Tb/Th) and (**b**) bone volume (BV/TV). $\#P < 0.05$. $*P < 0.01$. $**P < 0.001$

significant decrease in cortical BMC and BMD compared with Sham.

These controversies could be related to that fact that the majority of these studies did not analyze simultaneously the role of estrogen depletion and masticatory function in oral bone loss. Recently, an experimental study in adults rats showed that mandibular bone was less affected by ovariectomy and/or isocaloric protein undernutrition comparing this site with proximal tibia, suggesting that mechanical loading of alveolar process during mastication may protect the alveolar bone from the detrimental effects observed in other skeletal sites (Mavropoulos *et al*, 2007).

The mandible is loaded by forces applied to the teeth during mastication or biting and this factor seems to be more important than estrogen deficiency. It has been reported that mechanical loading accelerates formation and suppresses bone resorption (Sakata *et al*, 1999; Tanaka *et al*,2007). A switch to a soft diet linked with reduced forces applied to the mandible during mastication is assumed to result in a reduction of bone formation and increased bone resorption. The mandibular bone that was under conditions in intermittent loading by occlusion, frequently stimulates bone formation in opposition of bone resorption induced by estrogen deficiency (Tanaka *et al*, 1999, 2007). Our findings are in agreement with these literature data, and were related to the whole condyle hystomorphometric analysis and entire BMD mandible analysis. Some researchers demonstrated that a soft diet is also responsible for increase malocclusion from dental crowding, which implies narrower, shorter jaws (Yamamoto, 1996; Kiliaridis *et al*, 1999). It was also demonstrated that when rats' incisors were cut off and fed on soft diet to reduce mechanical loading on the condyle; the trabecular density of the condyles decreased significantly (Hinton and Carlson, 1986).

Mavropoulos *et al* (2004b) have also demonstrated that alteration of food consistency in young growing rats induced a lower mandibular alveolar bone, mineral density and decreased trabecular bone volume and thickness caused by a reduction of masticatory functional and mechanical demands. Other authors investigated the effect of OVX on the edentulous and dentate mandibles, comparing these to changes in tibia and femur in rats. The results showed that loss of bone mass in the edentulous mandible of OVX animals was similar to that occurring in tibia and femur, while lack of a significant effect of OVX on bone mass in the dentate mandible suggests that functional loading related to biting force prevented bone loss in the dentate mandible. (Elsubeihi and Heersche, 2009)

Bone is constantly adapting to its functional environment, by both modeling and remodeling. The altered masticatory function by feeding animals a soft diet, caused change in mandibular shape and induced alterations in bone mass, bone thickness and bone density (Kiliaridis *et al*, 1996; Bresin *et al*, 1999). Ödman *et al*, found that the animals fed a soft diet with a reduced mechanical loading had a smaller mandible with some significant shape adaptations. Compared with animals with normal masticatory function, the hypofunctional group showed morphological differences compared with the normal group (Ödman *et al*, 2008).

This is in agreement with observations in long bones, where functional alterations causes change in transversal and longitudinal shapes. Woo *et al* (1981) showed that the thickness of the total femur increased by 17% when young pigs were exercised. By contrast, the diameter of the immobilized femur of growing rats was reduced (Osako *et al*, 1991). The absence of physical activity may result in a negative effect on long bone mass. Likewise, jaw immobilization may cause the onset of osteoporosis-like alterations, such as expansion of the marrow cavity and reduction in trabecular bone width (Shimahara *et al*, 1991).

Equally, individuals with partial or total edentulism, who also displayed problems in masticatory function, showed a reduced amount of bone in the residual alveolar process and mandibular body (Klemmetti and Vainio, 1994; Taguchi *et al*, 1995).

The physical activity effect on bone mass is local (concentrated) and depends on type, duration and

intensity of the exercise. Mastication promotes better quality maxillary bones by generating occlusal forces when triturating food, with pressure varying according to the number of existing teeth, diet consistency and oral health. Functional occlusion is crucial in maintaining the volume and structure of the mandible. The mandibular bone reacts in response to functional loading of occlusion with an improvement of bone mass and their structural mechanical properties.

In conclusion, the findings of the experiments conducted in this work suggest that the groups submitted to masticatory hypofunction, which annulled the intensity of the occlusion forces, decrease bone mass and cause a deleterious effect on the bone quality in mandible. By contrast, the groups with normal masticatory function presented no mandible bone loss despite estrogen depletion. These results support the concept that the presence of normal occlusion forces may promote protection against the development of mandible osteopenia.

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Author contributions

IMF Patullo: study design, animal experimental management, statistical analysis and manuscript preperation; L Takayama and RF Patullo: animal experimental management and densitometric analysis; V Jorgetti: histomorphometric analysis; RMR Pereira: study design, statistical analysis and manuscript preperation.

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