Effects of Two Experimental Models of Osteoporosis on Alveolar Bone: Histopathologic and Densitometric Study

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Summary

We investigated the morphometric and histopathological changes associated with two different models of experimental osteoporosis and the effects of osteoporosis on mandibular and femoral bone density and found that bran bread induced osteoporosis may cause a decrease in BMD of mandible and femur compared to retinoic acid induced osteoporosis and osteoporosis may increase osteoclastic activity.

Abstract

Aim: The aim of this study was to compare two different models of osteoporosis radiographically and histopathologically and evaluate the effect of osteoporosis on alveolar bone.

Methods: Twenty two rats were involved in this study. Study groups are; Control (C, n=6) group; retinoic acid-induced osteoporosis group (EG-1, n=8) and bran bread-induced osteoporosis group (EG2, n=8). Retinoic acid induced osteoporosis was created by intraperitoneal injection of 70 mg/kg retinoic acid for 15 days. Bran bread induced osteoporosis was created with the replacement of normal rat diet to the bran bread for 45 days. The study period began with the osteoporosis induction. Rats were kept alive for 15 days and then sacrificed. Femurs and mandibles of the rats were extracted and digitalized radiographs were taken. Density measurements were performed on radiographs. Mandibles of the rats were examined morphologically and histopathologically. Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) and TRAP staining were performed on histological slides. **Results:** There was no significant difference in RANKL expression among groups ($p>0.05$). Femoral density was lower in the EG2 group than those of the others ($p<0.05$). Mandibular density was lower in the EG2 group than EG1 ($p<0.05$). TRAP positive osteoclast numbers were higher in the EG2 group than other groups ($p<0.05$). **Conclusion:** Within the limitations of this study, we concluded that bran bread induced osteoporosis may decrease mandibular bone density and may increase osteoclastic activity but both model of osteoporosis did not affect alveolar bone morphology.

Key Words: Experimental osteoporosis, Bone mineral density, Alveolar bone, Retinoic acid, Trap

Introduction

Osteoporosis is defined as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration with consequent increase in bone fragility and susceptibility to fracture"[1]. And it is considered to be present when Bone Mineral Density (BMD) is 2.5 Standard Deviations (SD) below the young normal. Osteoporosis and the bone loss as a characteristic of this disease may increase the onset and progression of periodontal disease and tooth loss. Both diseases share common risk factors and inflammatory cytokines in the mechanism of bone loss. Recent studies have examined the possible association between periodontal disease and osteoporosis, but the evidence is contradictory. Some studies have found an association between low skeletal BMD and periodontal bone loss and tooth loss [2-4], while others have not [5-7]. Jawbones especially mandible can be affected by osteoporosis. Horner et al. found that thinning of the mandibular cortex below 3 mm at the mental foramen was associated with low skeletal bone mass (or osteopenia) at the spine, femoral neck, or forearm [8]. Furthermore it is reported that osteoporosis affects the speed of alveolar bone resorption, bone remodeling and bone density after tooth extraction in jaws [9].

Bone remodeling in the jaws, like all other bones, results from the osteoblastic and osteoclastic activity. The activities of these cells are mainly associated with Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), a very important cytokine for differentiation and activation of osteoclasts [10,11]. Alteration in RANKL and its competitive inhibitor Osteo Prote Gerin (OPG) productions were found to affect bone resorption [12]. It was reported that the administration of serum RANKL to mice promoted osteoclast growth and activation, leading to osteoporosis [13]. Expression of RANKL is a good indicator of osteoclastic activity and bone loss.

To evaluate histopathological changes associated with osteoporosis animal studies would be beneficial. Some advantages of animal models of osteoporosis are it allows examination of bone-related alterations, it gives opportunity to try new treatment modalities and it is easier to induce experimental osteoporosis in rats. It is reasonable to use an Ovariectomized (OVX) female rat to induce post-menopausal osteoporosis in an animal study. However, the OVX model should be curtailed due to its longer and more complicated experimental process. In order to avoid these limitations of OVX model, utilizing its side-effect of osteoporosis, some investigations have successfully established an osteoporosis

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rat model by using retinoic acid [14-16]. In addition to retinoic acid, bran bread is also reported to be effective in inducing osteoporosis in wistar rats [17].

In the light of literature, the aim of this study was to compare two different models of osteoporosis radiographically and histopathologically and evaluate the effect of osteoporosis on alveolar bone.

Materials and Methods

Animals and experimental model

Twenty two male wistar rats, with an average weight of 300–320 g, were used in this study. They were housed in specially designed wire cages and maintained on a 12 h–12 h light–dark cycle with a constant room temperature of 23°C. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Cumhuriyet University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. The animals were randomly divided into three groups as follows:

1. Control group (n=6)

2. Retinoic acid-induced osteoporosis group (Experimental group 1, EG1, n=8)

3. Bran bread-induced osteoporosis (Experimental group 2, EG2, n=8)

Induction of Osteoporosis

Retinoic acid-induced osteoporosis: Eight rats in EG1 were administered retinoic acid [Sigma-Aldrich, St Louis, Missouri, USA] (70 mg/kg) [15]. Retinoic acid was mixed with olive oil and each rat administered 0.5 cc of this mixture intraperitoneally once a day for 15 days.

Bran bread-induced osteoporosis: In the group EG2, osteoporosis induction was achieved according to the procedure reported by Allam et al. [17]. Briefly, diets of the rats were changed to bran bread [17,18]. Rats were fed with only bran bread for 45 days. This diet lacks the major constituents of a basic diet, thus resulting in an elevated rate of bone resorption and osteoporotic changes as a result of the dietary restriction and calcium and magnesium deficiency.

Control rats did not received any injection. After osteoporosis was achieved in the groups, rats were kept alive for 15 days. At the day 15 osteoporotic rats and control rats were sacrificed by overdose of pentotal sodium. Right and left mandibles and right femurs were carefully removed and placed to 10% formalin solution for fixation.

Morphometrical evaluation

Left mandibles were stained with aqueous methylene blue (1%) to identify the Cemento-Enamel Junction (CEJ). The alveolar bone height was measured under a stereomicroscope (x25 magnification) [Stemi DV4, Carl Zeiss, Jena, Germany] by recording the distance from the CEJ to the alveolar bone crest. Measurements were taken at three points on both the buccal and lingual sides in mandibular first molar tooth to quantify the alveolar bone level. A mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (H.T) who was unaware of the identity of samples.

Bone density measurement

After the removal of the soft tissue around femur and left mandible, digitalized X-ray investigations and BMD measurements were performed. All radiographs were taken by same researcher (H.B.Y.) with a digitalized intraoral imaging system (Soredex Digora, Tuusula, Finland) in same conditions (Figure 1). Briefly, radiation tube was placed to a certain distance (10 cm) from a stable table. A certain distance was settled between bone and tube. A 5 mm metallic bar was placed onto a pink wax plate which has the same dimensions as the radiographic film. Radiographic films placed on the wax and standard radiographs were taken with the same setting of the system and then all images were transferred to an internationally acknowledged image analysis programme (ImageJ, National Institutes of Health, NIH). BMD measurements were performed in a standard-sized toothless area below the apices of the first molar in all images. BMD of femurs were measured from 3 points (head, body and condyle portions of the femur) and the mean average was calculated and recorded as the BMD of the femur.

Histopathological evaluations

Right mandibles were fixed in 10% neutral buffered formalin. The tissues were then decalcified in 10% ethylenediamine-tetra-acetic acid (EDTA), pH 7, with a change twice a week for 8 weeks until decalcification was completed and then the decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. The periodontal tissues in the mesial and distal part of the mandibular first molar tooth were observed. Histological analysis was performed by a single examiner (F.G.) who was also blinded to the identity of samples. Each sample was sliced into 5 mm continuous sections and prepared for Haematoxylin and Eosin (H&E) and histochemical staining for Tartrate-Resistant Acid Phosphatase (TRAP) and immunohistochemistry staining for RANKL (Figure 2). Osteoblast cells, i.e., forming surfaces, by the visibility of active bone formation surfaces that were bordered by the osteoid and cuboidal osteoblasts in the examined area were count.

Sections were subjected to Tartrate-Resistant Acid Phosphatase (TRAP) staining, to identify osteoclasts, using the TRAP staining kit (Sigma-Aldrich, St Louis, Missouri, USA) according to the manufacturer's protocol. Sections were counterstained with hematoxylin and analyzed using light microscopy (Nikon Eclipse, E 600, Tokyo, Japan).

RANKL Immunohistochemistry

After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for 2 h at 70°C. Then the sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 min, samples were incubated with primary antibodies overnight. The antibodies and conditions used were as follows: goat

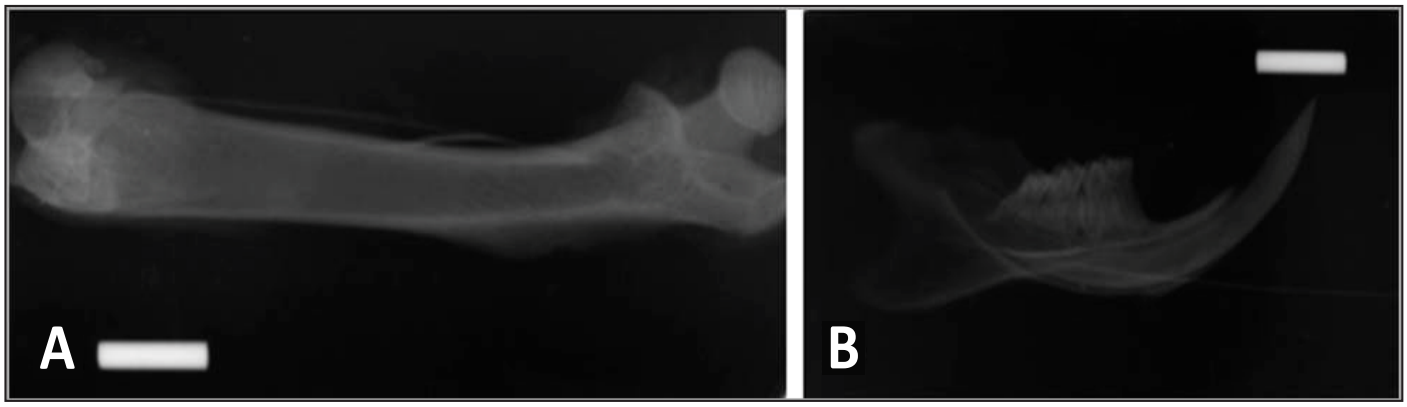

***Figure 1**. Representative images of the radiographs of femur and the mandible.**A**: An image of the control group **B**: An image of the EG1 group. incisor.*

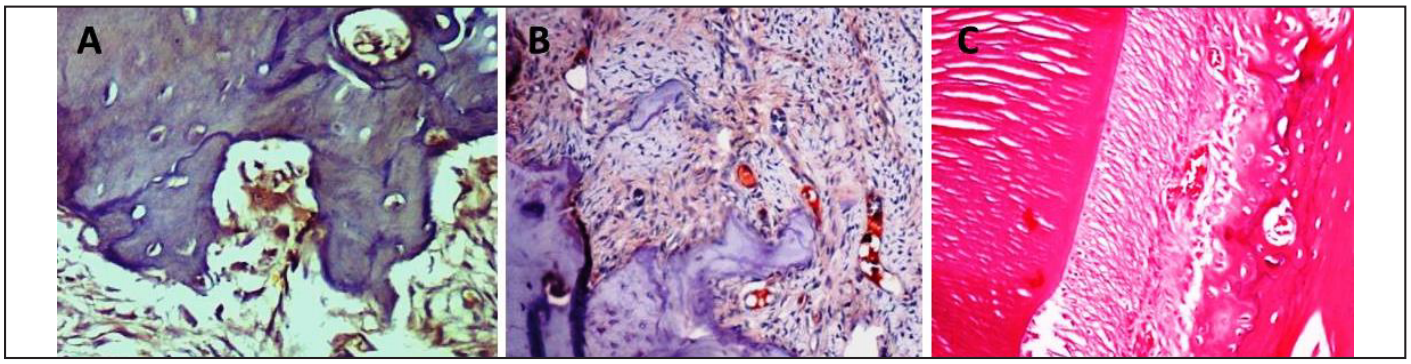

***Figure 2**. A histopathological view of the groups. **A:** TRAP staining of the EG2 group (x100) **B:** RANKL staining of the EG1 group (x100) **C:** Hematoxylin-eosin staining of the control group (x100).*

polyclonal anti-RANKL (N-19, 1:100) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing several times with phosphatebuffered saline, the sections were incubated with biotinylated immunoglobulin G for 30 min, washed several times with phosphate-buffered saline and reacted with streptavidin–horseradish peroxidase conjugated reagent for 30 min. Following three times, 5-min washes with phosphate-buffered saline, samples were incubated with AEC chromogen to visualize the immunoreactivity. Sections were counterstained with hematoxylin and analyzed using light microscopy.

Alveolar bone areas surrounding roots of the first molars were examined and RANKL evaluation was made by measuring the RANKL+ areas of the bone surrounding teeth. The percentage of RANKL+ area to the examined area was calculated. RANKL presence less than 25% of the areas surrounding teeth were scored as '1', %25-50 were scored as '2', %50-75 were scored as '3' and more than %75 were scored as 4.

Statistical analysis

Data were presented as mean ± SD or percentage as appropriate. Osteoclast numbers, alveolar bone loss, RANKL, mandibular and femur bone density analyzed with ANOVA followed by Tukey test for pair-wise comparisons. P values less than 0.05 were considered statistically significant. The alveolar bone loss and mandibular bone density were determined as the expected primary and secondary outcomes of the study, respectively.

Results

Experimental procedure was performed successfully, no complications arise.

Alveolar bone height measurements were shown in Table 1. There was no significant difference in alveolar bone height among the groups (p=0,187). Bone mineral density of mandibles in EG2 was significantly lower than the control group (p=0,021) while there was no significant difference between the EG1 and the control or the EG1 and the EG2 groups (p=0,140 and p=0,580 respectively) (Figure 3). BMD measurements of femur in the EG2 group was lower than those of the control and EG1 groups (p=0.007 and p=0,049 respectively) and the difference between the EG1 and the control group was not significant (p=0,589) (Figure 4).

Histological sections from the control group showed normal architecture in both the periodontal ligament and the alveolar bone tissues (Figure 2). Sections from the osteoporosis groups revealed thinning of the bony trabeculae with multiple resorption foci along the bone surface. The TRAP-positive osteoclast cell numbers and osteoblast cell numbers of the study groups were shown in the Table 1. The osteoclast number in the EG2 group was significantly higher than the other groups (p=0,002 vs the control group and p=0,045 vs the EG1 group). Although osteoblast number in experimental groups were decreased after osteoporosis induction, the difference in osteoblast numbers did not reach significance among the groups (p=0,242). There was no significant difference RANKL evaluation among the groups either (p=0.090).

Table 1. *Osteoblast numbers, osteoclast numbers and alveolar bone height measurements in the study groups. Data are given as mean ± standart deviation.**p<0.05 versus EG2

	Alveolar bone height (mm)	Osteoblastnumber	Osteoclastnumber
Control	0.58 ± 0.03	39.2 ± 20.5	9.5 ± 1.5*
EG1	0.59 ± 0.13	20.6 ± 6.6	14.2 ± 2.1*
EG2	0.56 ± 0.12	25.8 ± 3.7	18.0 ± 4.6

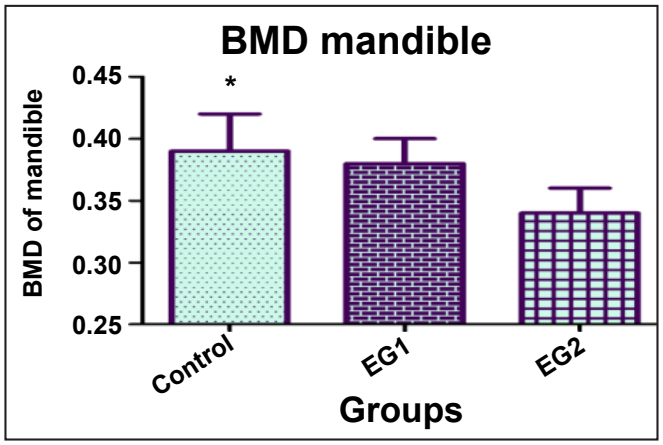

Figure 3*. Mean bone mineral density of mandibles in the study groups.* **p<0.05 versus EG2.*

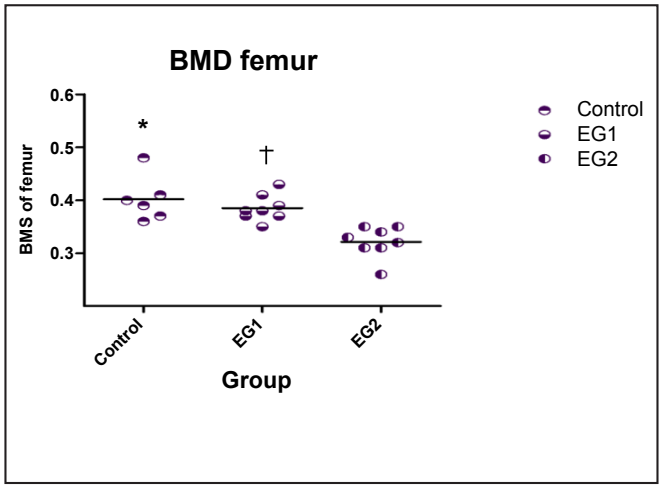

Figure 4*. Mean bone mineral density of femurs in the study groups.* **p<0.05 versus EG2;* †*p<0.05 versus EG2.*

Discussion

Osteoporosis is a chronic skeletal disease affecting bone biology and physiology. Along with long bones of the body, osteoporosis may affect jawbones and resulting modifications of alveolar bone may compromise the treatment and/or prognosis of periodontal diseases [19,20]. Animal models of osteoporosis are frequently used in the field of periodontology. The most used animal model for experimental osteoporosis is ovariectomy [21-23]. It is reasonable to use OVX female rats to achieve osteoporosis in an animal study. But it has some disadvantages. It is applicable only in female rats, requires a second surgery and simulates postmenopausal osteoporosis. Also it requires a sham operated control group and increases the number of the animals [24]. To avoid these limitations retinoic acid-induced osteoporosis and bran bread-induced osteoporosis might be considered.

In order to evaluate the implication of animal models of osteoporosis for clinical use and the relationship between alveolar bone height and osteoporosis we used two distinct rat model of osteoporosis. We aimed to compare the possible effects of both model of osteoporosis on alveolar bone and we examined the BMD of femur and mandible, alveolar bone height, histopathological changes in mandibles and RANKL expression in alveolar bone in osteoporosis. Both models of osteoporosis used in this study are easy procedures and both involved minimal trauma [15,17].

Alveolar bone loss, alveolar bone height, resorption of the residual ridge, the distance between cemento-enamel junction and alveolar crest, clinical attachment loss and erosion of the cortical mandible were also used as periodontal markers [6,8,9,14,17,25,26] and BMD of spine [3,4,9,26], BMD of femur [15,26,27], metacarpal cortical thickness [28], metacarpal index [29] as systemic markers of osteoporosis in order to investigate these two diseases.

Many studies investigated alveolar bone loss, systemic and local bone mineral density and risk of fracture in osteoporosis and periodontal diseases, some of them suggested a strong relationship while some results showed no association [6,8,15,25,27,28,30-32]. Wactawski-Wende et al. showed that bone mineral density of femur and spine may affect alveolar crestal height [25]. Payne et al. also showed a positive relationship between alveolar crestal height and spine and alveolar bone density [31]. In contrast Brennan-Calanan et al. found a negative correlation between alveolar crestal height and BMD of femur and spine [30]. Hattatoglu-Sonmez et al. reported that osteoporosis and a decrease in BMD spine and femur may result in an increase in clinical attachment loss and periodontal pocket depth in postmenopausal women [6]. More recently Marjanovic et al. stated that severe periodontal disease was not associated with osteoporosis in post-menopausal women [33]. This study showed that bran bread-induced osteoporosis resulted in lower BMD in both femur and mandible, when compared to controls and retinoic acid-induced osteoporosis. Similarly with previous studies [6,31], we found that osteoporosis did not affect alveolar bone height. However our results showed that osteoporosis caused a decrease in BMD of mandible. This result is similar to some other investigators [25,34,35]. We have also found that osteoporosis altered bone metabolism by increasing the number of osteoclast.

The body of the mandible and the posterior alveolar processes, consisting predominantly of cortical bone, are very similar to the diaphysis of long bones, while in the anterior alveolar processes of the mandible and in the alveolar processes of the jaw, bone architecture is mostly trabecular. According to some authors the rate of bone turnover at the level of alveolar processes would be greater than in long bones, so the loss of bone mass could manifest earlier at the alveolus than at other skeletal segments, thus, representing an early indicator of osteoporosis [2,35-37]. It has also been found that porosity of the jaws increases in case of osteopenia or osteoporosis [30]. Radiographic monitoring of the jaws could be beneficial in early diagnosis and maintenance of osteoporotic patients [38,39].

Allam et al. reported an increase in RANKL immunoreactivity, one of the strong indicators of osteoclastic activity in bone tissue [17] but we couldn't find any difference in the expression of RANKL in the study groups. However there was significant increase in TRAP+ osteoclasts in bran bread-induced osteoporosis. This can be considered as an increase in the osteolytic activity in alveolar bone. Also, retinoic acid caused an increase in osteoclast numbers but the difference did not reach significance.

Retinoic acid was found to be effective in the practicality of induction of the acute osteoporotic rat model. It was shown that serum calcium level, total body BMD, isolated left femora BMD had all significantly decreased in retinoic acid-induced osteoporotic rats [40]. However, some side effects including loss of appetite, weight loss, hair loss, decreased activity might arise. Therefore, the induction of osteoporosis by retinoic acid may be related to weight loss. However, we didn't observe loss of appetite, weight loss or hair loss in retinoic acid induced osteoporotic rats. It was also stated that bran bread diet causes some physiological side effects. These are weight loss, restriction of calcium and magnesium intake and increased bone resorption and all are due to the lack of essential nutrients [17]. Bran bread caused some weight loss but there was no other side effect.

Conclusion

Within limitations of animal study, our results revealed that bran bread induced osteoporosis may cause a decrease in BMD of mandible and femur compared to retinoic acid induced osteoporosis and osteoporosis may increase osteoclastic activity. Although we are unable to make definitive conclusions regarding the mechanisms of the effects of osteoporosis on alveolar bone, the present data showed that both experimental

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