OSTEOPOROSIS DELAYS THE BONE HEALING AFTER MANDIBULAR DISTRACTION OSTEOGENESIS IN POSTMENOPAUSAL OSTEOPOROSIS MODEL

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Abstract

This study aimed to evaluate the histological, histomorphometric and radiographic features of the bone repair in ovariectomized rats submitted to mandibular distraction osteogenesis. Thirty female Wistar rats were equally divided in ovariectomy and control group and submitted to mandibular distraction osteogenesis. After 7, 14 and 28 days the animals were sacrificed and mandibles were removed and radiographed. Then, the sections were submitted to histological and histomorphometric analysis. Histological analysis of distraction showed a fibrocellular connective tissue region involved by cartilage matrix that was gradually replaced by bone tissue. The complete ossification was observed only in the control group. Histomorphometry showed that the percentage of bone volume/tissue volume was statistically significant in the control group between the days 7 and 28. The radiographic evaluation did not show statistical differences between the groups and periods. In conclusion, osteogenesis induced by mandibular distraction osteogenesis in ovariectomized female rats was slower, suggesting the influence of estrogen in bone repair in the histological and histomorphometric analysis.

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Introduction

Developmental abnormalities may produce deformities in the jaws, causing functional and aesthetic problems to the patients. Some of these changes can be corrected by distraction osteogenesis. In distraction osteogenesis the bone is sectioned taking care to preserve the periosteum and its blood supply, and separated in a gradual and controlled by distraction devices, which enhance osteogenesis during bone lengthening.[1] However, a variety of systemic diseases can influence the course of bone repair in distraction osteogenesis.

Osteoporosis is the most common type of bone disease. It is characterized by systemic impairment of bone mass, strength, and microarchitecture, which increases the risk of bone fracture. With the increase in life expectancy, the medical and socioeconomic impact of osteoporosis has growth further.[2] In distraction osteogenesis, the surgeon should pay attention to the adverse effect of osteoporosis, on

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the outcome of the surgical procedure. An understanding of cellular events during distraction osteogenesis in osteoporotic bone may contribute to development of successful therapies to accelerate bone repair in this technique.

Estrogen hormone is considered a main factor for postmenopausal osteoporosis, because its deficiency creates conditions for the development of osteoporosis,[3] increasing the risk of bone fractures.[4-6] Postmenopausal osteoporosis has become more prevalent in the world population due to an ageing population,[7] thus requiring health professionals to better understand the bone changes that occur in this disease.

The ovariectomized animal model is commonly adopted to induce post-menopausal osteoporosis.[8] With the removal of the ovaries, estrogen deficiency occurs in the body of the animal and the consequent imbalance in bone remodeling process.[4,7,8] However, the influence of estrogen deficiency on distraction osteogenesis has been little studied and there are no studies investigating the effect of ovariectomy on bone repair in a rat model of mandibular distraction osteogenesis. Similarly to that observed in other areas of body, we hypothesized that ovariectomy might impair the bone repair during distraction.

The histological study is still the adequate method to evaluate osteogenesis. However, this analysis method is considered an invasive method. An alternative would be radiographic analyzing by means of digital radiography. Digital radiography has shown satisfactory results in the evaluation of small bone changes by examining the average pixel value and digital subtraction.[9,10] This technique may be able to detect changes in mandibular bone density in ovariectomized animals.

Therefore, considering that the acceleration of osteogenesis is one of the focuses of studies on distraction osteogenesis and that estrogen deficiency may impair bone repair in subjects with osteoporosis subjected to this surgical procedure, the present study aimed to evaluate the biological events occurred during mandibular distraction bone of ovariectomized rats by histological, histomorphometric and radiographic analysis. In addition, the present study examined the diagnostic accuracy of digital radiograph for detection of differences density between mandibles of the specimens from studied groups.

Methods

All animal procedures were in accordance with principles of laboratory animal care (NIH publication 85-23, 1985) and were authorized by the Ethical Committee for Animal Research of Dental School, Federal University of Bahia, Brazil (Protocol n^{o} 14/09). Thirty female *Wistar* rats weighing between 180 and 250g were randomly divided into two groups. The first group (OVX) comprised 15 animals in which bilateral ovariectomy were performed. The control group comprised 15 animals whose ovaries were not removed. After seven days, rats from both groups underwent right mandibular distraction. All of the animals received a standard pellet food and water ad libitum.

Surgical Procedures

After intramuscular anesthesia with a combination of ketamine hydrochloride (Francotar®, Parke Davis, Brazil), 50mg/kg, and xylazine (Anasedan®, Bayer SA, Brazil), 5mg/kg, OVX animals had the bilateral ovariectomy performed. Detailed description of the procedure has been published previously.[11] Seven days after ovariectomy, the animals of all the groups were anesthetized with same dose of xylazine and ketamine. Then, each animal had right mandible body partially osteotomized and two titanium screws (Neodent, São Paulo, SP, Brazil) placed 2 mm from the segmented borders. An orthodontic appliance (Dentaurum, Tokyo, Japan) was attached to the head screws with acrylic resin (Duralay, Brazil). Subsequently, the complete osteotomy was performed and this device was applied as a functional osteogenic distractor (Fig. 1). Detailed description of the procedure has been published previously.[12] After the surgery, the animals were treated with an antibiotic (Pentabiotic Veterinarian, Fort Dodge, Campinas, Brazil) and received special feeding conditions. After seven days, the devices coupled to the right mandible of the animal were activated at a 0.5 mm/day rate for 5 days, reaching a total length of 2.5 mm between the osteotomized bone edges.[12] Distraction osteogenesis was confirmed by measuring the distance between the screws at beginning and end of distraction phase.

Fig. 1. Distraction osteogenesis device implanted in right side mandible. Two titanium screws installed on the outer surface of the jaw positioned 2 mm from the edges of the osteotomy ends. Orthodontic expander anchored to titanium screws by acrylic resin.

Five animals from each group were anaesthetized as described previously and sacrificed at 7, 14 and 28 days after the first day of device activation and perfused through the heart with 4% paraformaldehyde in PBS buffer (pH 7.2). The estrogen deficiency by the ovariectomy was confirmed by clinical observation of the uterine atrophy after the euthanasia. The right and left mandibles were removed and further immersed in the same fixative for 24 hours.

Radiological Evaluation

The x-ray exposures were performed using Expert DC unit (Gendex Dental System, Italy), operating at 7mA, 65kVp and 0.160 seconds of exposure time. Each specimen was x-rayed with a digital system (DenOptix®-Dentsply International/Gendex Dental X-ray® Division, USA). The mandibles were fixed in a single wax to ensure reproducibility and similar positions for all specimens. The wax was fixed close to the support film in the acrylic positioner. In order to guarantee the standardization of the images, this support has an acrylic plate of 1.2 cm, simulating the soft tissue (Fig. 2A-B). An aluminum stepwedge was placed close to the specimen in all radiographs in order to calculate the radiopacity. Two markers of gutta-percha (radiopaque material) were also added to the margins of the bone defect, such as titanium screws, for facilitating the subsequent analyzes (Fig. 2C-D). A single storage phosphor plate of size 30mm x 40mm was used to obtain the images. After the X-ray exposure, the storage phosphor plate J 7J Jwas scanned. The files were exported from the software manufacturer and saved in TIFF format.

The storage phosphor plate was placed on a light box during two minutes to ensure that the image was completely erased. The tool of the ImageJ® 1.43u (National Institutes of Health, USA) program for obtaining the average of pixel values was used for each radiographic image.

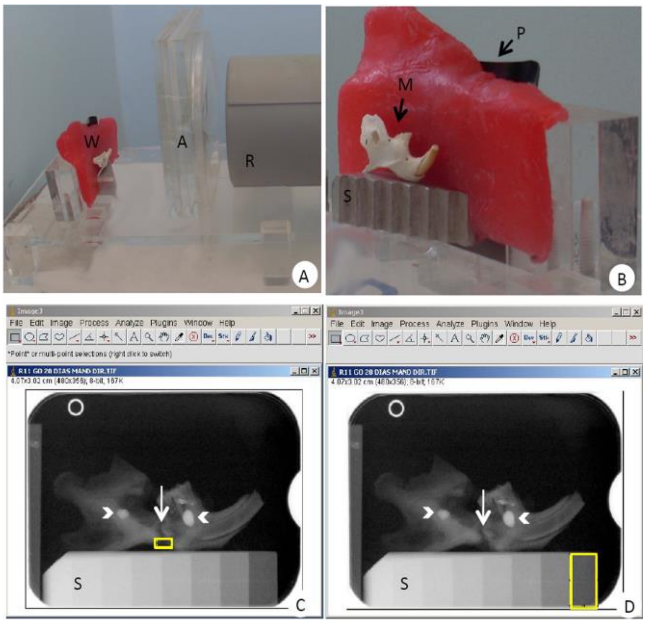

Fig. 2. (A) Image of acrylic positioner to perform the radiographs. A, Acrylic glass; W, Wax; R, x-ray apparatus. (B) Image of mandible (M) positioned in wax to perform the radiographs. S, aluminum scale; P, storage phosphor plate (DenOptix®). (C) and (D) digital radiographic images viewed at the program Image J for the right mandible of an animal subjected to distraction osteogenesis. Observe gap (arrow) created by osteotomy and distraction osteogenesis and markers (arrowheads) in the margins of the bone defect. S, aluminum scale. (C) Selection of the rectangular ROI (interest region) involving the center of the bone defect in the base region of mandible (yellow rectangle). (D) selection of the rectangular ROI highlighting the greatest area of one aluminum scale step (yellow rectangle).

A rectangular region of interest (ROI) was previously selected in the mandible base, involving bone defect by distraction osteogenesis, with about 0.023 mm^2 area. Then, values of the pixels from this ROI were obtained and each image was analyzed three times to ensure reproducibility of measurements (Fig. 2C-D).

Radiographs of the left mandibles were used as standard for this analysis which allows observing the changes in pixel values of the newly formed bone in each evaluated group, as well as between them and normal bone (left mandible). Each step of aluminum stepwedge was also measured in pixels. This procedure was repeated for each radiographic image. Thus, the average gray values obtained in the bone defect were converted into equivalent values in millimeters of aluminum (mmAl). Mathematical equations obtained from the radiopacity curves of the aluminum stepwedge were used to calculate the radiopacity of the bone defect in equivalent values of mmAl.

Histological Processing

After the x-rays, the specimens were decalcified in 4.13% ethylenediaminetetraacetic acid (EDTA) at pH 7.2 for 60 days. Then, the specimens were dehydrated and embedded in paraffin and the semi-serial sections (5μm thick) were sliced in the sagittal plane and stained with hematoxylin/eosin to histological and histomorphometric analysis. In the histological study, mandibular basal bone was examined for presence of bone and/or cartilage matrix interposed between distracted segments, cellular components, including osteoblasts, osteoclasts, fibroblast-like cells and inflammatory cells, and bone fusion according to periods of time and ovariectomy.

Histomorphometry

The amount of newly formed bone matrix in the distraction gap was quantified in the sections stained with hematoxilin/eosin using a histomorphometric parameter, the bone volume/tissue volume (BV/TV). The nomenclature and calculations were in accordance with the American Society of Bone and Mineral Research Histomorphometry Nomenclature Committee.[13] After obtaining the five images of two sections from tissue blocks of each group under the light microscope (BX-51, Olympus, Japan), the newly formed cancellous bone in the basal region of distracted gap was drawn, the proportion from the total area was measured using an image analyses system (Image-Pro Plus® 6.2 – Media Cybernetics, USA) in a ‘‘blinded’’ manner and its mean was recorded.

Statistical Analysis

In the present study, the variables time periods (7, 14 and 28 days) and groups (OVX and control) were compared. In the histomorphometry analysis, the BV/TV was analyzed using GraphPad Prism® version 5.00 for Windows (GraphPad Software, USA). The Kruskal-Wallis test with *post-hoc* Dunn's multiple comparison was used to determine the significance between the variables. The bone density values (equivalent in mmAl) was analyzed with SAS for Windows software (Version 9.1.3; SAS Institute Inc., Cary, NC). The Tukey test was applied to compare the means of the bone density between the variables. The significance level for all statistical analysis was set 5%.

Results

Clinical findings

After complete activation of the distractor, rats of all the groups showed deviation of the mandibular incisors from median line, causing feeding difficulties in some animals and therefore an ovariectomized animal from 14 days group was lost.

Histological Findings

In all groups, bone matrix formation was seen in gap and was oriented in the line of tension. In these areas, bone formation was through endochondral and intramembranous ossification. Histological examination showed a more decreased bone formation in OVX animals than in control group. In the control group, bone fusion occurred between bone extremities. At day 7, in both groups, the central zone of gap showed cellular connective tissue with condensation of fibroblast-like cells. Cartilaginous matrix formed on either side of the bone trabeculae surfaces in the gap (Fig. 3A-B). At day 14, in the control group, cartilaginous matrix was observed and it seemed to be replaced by bone through endochondral ossification and further remodeled into immature bone trabeculae with large marrow space. In the most of cases, there was union between cartilaginous matrix from the inner surfaces of the proximal and distal segments (Fig. 3C).

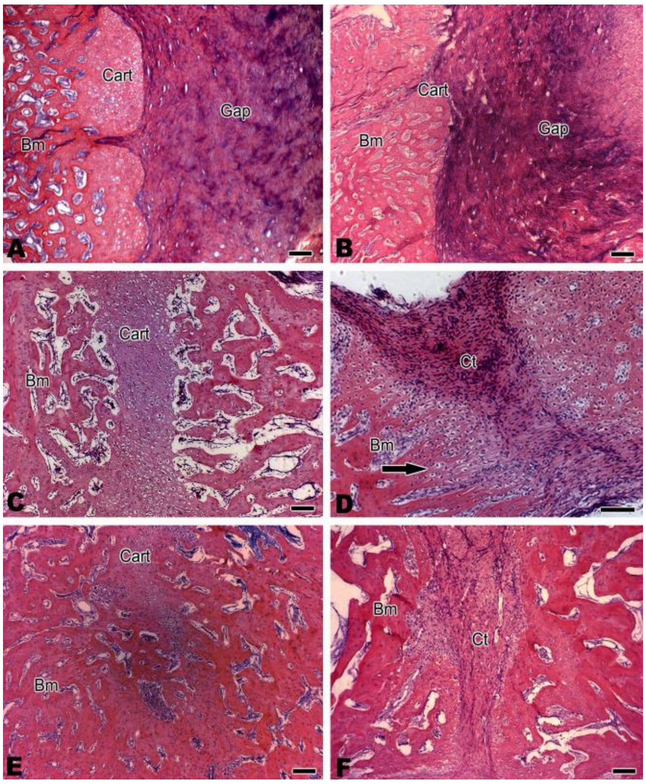

Fig. 3. Light micrographs showing several regions of the GAP between the bone ends in the mandibles after distraction osteogenesis of the control group in the day 7 (A), day 14 (C), day 28 (E) and the OVX group in the day 7 (B), day 14 (D) and day 28 (F). (A, B) Bone gap shows cellular connective tissue with condensation of fibroblast-like cells. Cartilaginous matrix is forming on either side of the bone trabeculae surfaces in the gap. (C) Cartilaginous union is observed between the bones ends. (D) Central area of bone gap remains filled by cellular connective tissue. Note that bone matrix formation is oriented in the line of tension (arrow). (E) Gap shows bone union with retention of small amount of cartilage. (F) Central area is occupied by thin cellular connective tissue. Note that there is no bone union between bone ends. Cart, cartilage; Bm, bone matrix; Gap, gap; Ct, cellular connective tissue. Scale=150μm. H&E.

In the OVX group, the central area was filled by cellular connective tissue with small amount cartilaginous matrix (Fig. 3D). At the day 28, the control group showed partial bone union in the peripheral zone of the gap, whereas central zone displayed retention of small amount of cartilage (Fig. 3E). In the OVX group, the central area was occupied by thin cellular connective tissue associated to small amount of cartilage matrix (Fig. 3F). In both groups, no sign of mature bone trabeculae was noted in the area.

Radiographic Findings

The radiographs of the control and OVX groups in 7, 14 and 28 days are seen in the Fig. 4.

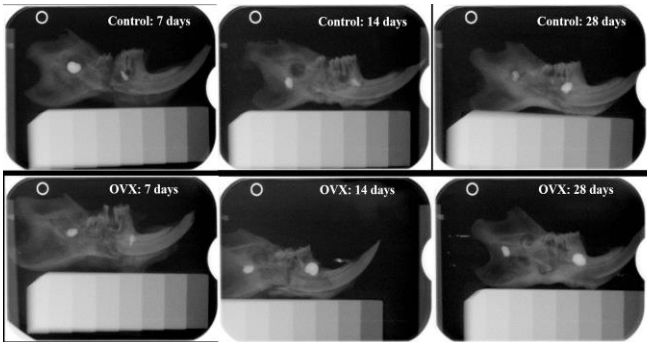

Fig. 4. Radiographs of the control and ovariectomized (OVX) groups in 7, 14 and 28 days.

It is possible to observe that the bone repair was similar in both groups. It was observed an increase of bone density values (equivalent in mmAl) of right mandibles subjected to distraction osteogenesis in control group animals along the 7 (0.51 mmAl), 14 (0.70 mmAl) and 28 days (0.78 mmAl). The same was not observed in OVX animals, the average of bone density decreased in day 14 (0.47 mmAl) compared to day 7 (mmAl 0.60), but no changes could be observed in day 28 (0.59 mmAl). In all groups, the values of the left mandibles of animals were bigger than those obtained from the right mandibles. Comparing the two groups along the periods, it was observed that the average of bone density (equivalent in mmAl) from right mandibles at the day 7 was higher in OVX (0.60 mmAl) compared to control group (0.51 mmAl) and values of the left mandibles were similar in OVX (1.03 mmAl) and control group (1.04 mmAl). At the day 14, the highest values were observed in the control group animals, even for the right mandibles (0.70 mmAl) and the left (0.92 mmAl) compared to the OVX (right = 0.47 mmAl, left = 0.85 mmAl). At the day 28 it was also observed higher values for the right mandibles in the control group (0.78 mmAl) compared to the OVX group (0.60 mmAl), however, the values obtained for the left mandibles were similar between the two groups (OVX = 0.93 mmAl and control = 0.92 mmAl). The amounts related to the right mandibles are illustrated in the table 1. The statistical analysis for these findings showed that the values of bone density images are not statistically significant ($p \leq 0.05$) between groups and among the periods.

Periods	Groups	
	Control	OVX
07 days	0.51 (±0.13)	0.60 (±0.16)
14 days	0.70 (±0.16)	0.47 (±0.06)
28 days	0.78 (±0.23)	0.59 (±0.20)

Table 1. Means (±Standard deviation) of the bone density equivalent in mmAl by radiographic images of right mandible related to the periods.

Histomorphometry

Histomorphometric analysis revealed differences in the percentage of BV/TV along the periods and groups. The percentage of BV/TV was statistically significant in the control group between the days 7 and 28 ($p \leq 0.001$). The percentage of BV/TV from OVX group samples increased when compared the day 7 (9.59 ± 7.007%) to the other periods: day 14 (42.3 ± 4.106%) and day 28 (35.35 ± 4.324%). However, no significant difference was noted among the periods. In all periods, BV/TV in the OVX group was lower than those on the control group. However, only in day 28, the percentage of BV/TV in the OVX group (35.35 ± 4.324%) was significantly decreased over the control group (63.85 ± 4.582%, $P < 0.05$). The values related to histomorphometric analysis are illustrated in the Fig. 5.

Discussion

The present study showed that the ossification of the animals from OVX group occurred more slowly with a lack of bone fusion in the last period and the percentage of bone volume was statistically lower in this group in day 28. Radiographic findings showed no difference statistically significant in bone density within the gap in the studied animals. The present results may contribute to management of women with postmenopausal osteoporosis who are submitted to distraction osteogenesis.

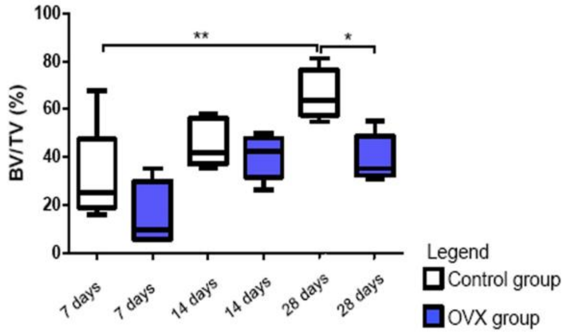

Fig. 5. Histomorphometric analysis of the amount of bone volume/tissue volume (%) of the right mandible of OVX animals and control groups. Values represent the median. Asterisk indicates significant difference calculated by the nonparametric Kruskal-Wallis test and post-test multiple comparison Dunn. * $p \leq 0.05$, ** $p \leq 0.01$.

The mandibular distraction osteogenesis has been used as an alternative treatment for facial bone reconstruction and their effectiveness in expanding the mandible was reported in the literature.[14,15] During distraction osteogenesis, the gap between the bone ends is amplified by the gradual growth of mandibular body and simultaneously filled by a newly formed bone matrix.[16] However, there is doubt if bone repair pattern is maintained after estrogen interruption. In order to mimic alterations in bone healing possibly observed in elderly postmenopausal women with ovarian failure, estrogen-deficient state by ovariectomy was constructed.[17,18] A study in the tibia bone of ovariectomized rats and underwent distraction osteogenesis demonstrated that estrogen deficiency affects the formation of callus distraction.[18] However, there are no studies about the influence of estrogen deficiency by ovariectomy in distraction osteogenesis in rat mandibular model. It could expand information about estrogen's action in bone tissue, contributing to understanding distraction osteogenesis technique and bone repair in osteoporotic bone.

Histological analysis was performed at basal bone of the mandibular distraction gap because this region is wider than other areas of the osteotomized bone in the rat mandible. Considering that new bone in the distraction gap is formed from cells originating from osteotomized bone edges, significant amount of bone matrix in repair area is an important factor for comparative analysis between sections from distraction osteogenesis groups, particularly if there is a histomorphometric analysis.[12,19] In addition, these areas are related to molar teeth and tissues that surround and support them, hindering histological evaluation and extrapolation of results.

The histological study of bone repair showed a slight difference between groups, and there was a lack of bone union in the OVX group. In both groups, there was bone mineralization occurring by endochondral and intramembranous ossification. In the mandibular distraction model, repair occurs predominantly by intramembranous ossification.[20] The presence of areas of endochondral ossification in our model can be associated a lack of stability in the distractor appliance,[21] because rat mandible is fragile and screws may not have been fixed properly. Some studies have displayed that a stable fixation is important for successful of bone repair, because it prevent trauma to initial tissues before new bone formation.[22,23]

In the fact, mineralization *in* mandibular bone distraction occurs predominantly via intramembranous ossification, although areas of endochondral ossification may also be observed.[24,25] In the long-bone distraction osteogenesis, repair occurs chiefly by endochondral bone formation in the early stage and subsequently by intramembranous (direct) bone formation. These differences may occur because the mandible differs from tubular bones in origin, structure and function.[26] Mandible is of ectomesenchymal origin and forms mainly through intramembranous ossification, with some portions of hyaline cartilage that will participate in secondary growth and which will later be replaced with bone formed by endochondral ossification.

Also small differences exist in bone pattern between the mandible and other bones in rats, if the study is done in experimental model of post-menopausal osteoporosis. Micro-CT images, for example, shown that both the mandible and the tibia of the OVX rats had a significantly decreased ratio of BV/TV compared with the normal rats. However, a greater percentile reduction of the ratio of BV/TV of the OVX rats occurred in the tibia (67%) compared with the mandible (18%),[27] confirming that the mandible shows specifics aspects that influence its repair after an ovariectomy. In addition, to the distinctive pattern of mineralization of the mandible, the mechanical stress due to masticatory function of the jaws may also explain the smaller percentile reduction of the BV/TV ratio of the OVX rats in the mandible compared with the tibia in response to oestrogen deficiency.[28] Therefore, extrapolation of long bone experimental findings to mandible must be made with caution, because they exhibit some difference in their pattern of repair.

When compared to control group bone repair in the OVX group occurred later and cases of incomplete bone union were observed in this group in the day 28. This finding was similar to results obtained by Arslan et al.[19], who reported that new bone matrix formed more slowly and is more osteoporotic in the tibial metaphysis after distraction osteogenesis in ovariectomized rabbit when compared to control subjects. In our study, the histological analysis was not able to detect a marked difference in the trabecular bone formation between the OVX and control groups. An important parameter for evaluating the estrogen deficiency is the bone histomorphometry.[13,29,30] Therefore, in order to confirm the small difference in bone matrix in the gap between the groups, it was performed a histomorphometric analysis to determine percentage of trabecular bone volume.[13] Through this analysis, a statistically significant difference was found between 7-day and 28-day control animals, indicating, according to some studies,[31,32] that mandibular distraction osteogenesis is a technique that increases the length bone with a concomitant deposition of bone matrix.

Our histomorphometric results showed that BV/TV increased from 7 to 28 days in the OVX group and was significantly reduced when compared to control group in the 28-day period. This suggests that new bone formed was affected by estrogen deficiency via ovariectomy, confirming its interference in the osteogenesis induced by distraction osteogenesis. Even finding no statistically significant differences between

OVX and control groups in the other periods it was noted that BV/TV was decreased in the OVX animals.

Histological and histomorphometric findings indicated new bone matrix was deposited slowly in OVX rats, leading to lack of union between the bone extremities in the last period. In addition, BV/TV in the OVX group had no significant difference between days 14 and 28, raising doubts about the final outcome of bone repair in mandibles of the OVX rats. Our findings indicated that, in the studied periods, estrogen deficiency in patients could be a contraindication for mandibular distraction osteogenesis, because increases risk of fracture after fixator removal, even if contention time is expanded in order to provide extensive amounts of matrix undergone mineralization and remodelling. It is possible that the fusion could occur if longer periods of study were employed, because the longest period in this study was 28 days. This period was insufficient to assess the complete fusion in both groups. Therefore, studies with longer periods are needed in order to evaluate the final outcome of repair and bone fusion in OVX animals.

Differences in the pattern of bone repair can also be detected by imaging analysis. In the present study, bone density in the radiographs was used to examine the mandibular bone density changes in the mandibular distraction osteogenesis. Comparing the two groups, bone density tended to be lower in the experimental group than control group, however it was not statistically significant. It is possible that bone mineral density in the repair area did not differ significantly between groups due to degree of mineralization and maturation of newly formed bone. Other possibility is that estrogen-deficient conditions by ovariectomy do not affect markedly the initial repair process, but largely modify new bone matrix deposited in the later period in the distraction osteogenesis. A study only observed radiological and histological osteoporotic changes in ovariectomized rats in the healing fracture model at 12 weeks after making the fractures[17]. Then, studies with longer periods are necessary to confirm these results.

In the present study, we used ovariectomy to induce estrogen deficiency. Slower bone healing in the OVX group was evident in the histological findings, demonstrating that an osteoporotic model had been established. In the present study, we chose to evaluate the bone healing in the right mandibles of the rats because it is an area subjected to several surgical procedures. Our results corroborates with other studies, which evaluated the effect of estrogen deficiency of bone healing in the maxilla,[33,34] mandible[28] and long bones, e.g. femur[35,36] and tibia.[37,38]

A remarkable finding was bone density values of left mandibles not subjected to distraction osteogenesis in both group were similar, indicating that, in the rat mandible, the ovariectomy only had influence in the mandible ramus submitted to surgical procedure. Mandibular distraction osteogenesis is a technique of applying controlled traction across the site of surgically produced bone disruption while it is healing. The mechanical forces are directed predominantly away from the site, and the technique takes advantage of the regenerative capacity of bone by creating and maintaining an active area of bone formation in the surgically created gap. The bone is lengthened along with its envelop. This technique may be used for: Deformity correction, lengthening, widening, bone transport, and alveolar ridge augmentation of the mandible, midface and upper face, in both congenital and acquired conditions.[39] The results of our research suggest that the surgeon should avoid surgical procedures performed in mandibles of individuals with osteoporosis, because changes in rates of estrogen may interfere in bone repair rather than in original bone.

Conclusions

Our findings showed that repair in the OVX rats was unfavourable, indicating the effect of estrogen deficiency caused by ovariectomy on the mandibular distraction osteogenesis. In addition, proposed method for radiographic analysis showed no statistically significant differences in density of bone matrix among groups and periods, indicating that this technique had poor accuracy to evaluation of bone repair in present model. The findings of this study need to be supported by experimental research of estrogen replacement in the ovariectomized rats to examine whether normal bone repair will be gained.

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Declaration of Interest

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