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Effect of Calcitonin Treatment on Bone Quality and Collagen Structure in Ovariectomized Rats

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ABSTRACT

The aim of this study was to investigate the effect of calcitonin on cortical bone quality and collagen structure at ovariectomized rat model. Forty five Sprague-Dawley female rats were assigned randomly to control group (CON, n=15) ovariectomized group receiving saline only (OVX, n=15), and ovariectomized group receiving salmon calcitonin (i.p.) (OVX-CT, n=15). Thirty rats were anaesthetized with ketamine and underwent bilateral ovariectomy via ventral incision. Ten weeks after ovariectomy, OVX-CT rats were treated with salmon calcitonin at the dose of 2 IU/kg body weight once per week for 12 weeks. BMD of the cortical femur was measured using dual energy X-ray absorptiometry, cross sectional area of the cortical femur was measured by computerized tomography and the length of the cortical femur was measured with a digital caliper. Tensile test was performed to measure stress, strain and toughness of cortical femur. In the OVX-CT group; stress, strain and toughness were not different than OVX group and significantly lower ($p<0.05$) than those of control group. Regular collagen fiber organization was observed in the CON group, whereas the parallel packing of fibrils was completely replaced by a random arrangement in OVX and OVX-CT rats. 12 weeks calcitonin treatment leads to significant increase of the BMD without any change in bone biomechanical parameters and collagen ultrastructure in ovariectomized rats.

Key Words: Calcitonin; bone biomechanics; collagen; stress

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INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mass, micro architectural deterioration of bone tissue and increase of fractures. Many bone-sparing drugs have been developed for the treatment and prevention of postmenopausal osteoporosis. Calcitonin is one of these drugs [1-3].

Salmon calcitonin (CT) is a 32-amino acid polypeptide drug and inhibits the osteoclastic activity. The inhibition of bone resorption by calcitonin is mediated in part by binding to osteoclast membrane receptors. It has been estimated that one osteoclast holds approximately one million calcitonin receptors (4). Flattening of the osteoclast ruffled borders and withdrawal of osteoclasts from sites of active bone resorption occurs upon exposure to calcitonin in vitro [5–9].

Parenterally injected calcitonin has beneficial effects on bone mass throughout the skeleton, including the proximal femur, at least in patients with established osteoporotic fractures [10]. In a multicenter, randomized, double-blind, placebo-controlled clinical trial, calcitonin has been shown to prevent incident vertebral fracture in osteoporotic postmenopausal women, although its effect on BMD was only modest [11].

Bone is a natural composite comprising mineral (mainly hydroxyapatite), organic (mostly type I collagen), and water phases [1]. Thus, the biomechanical properties of bone are dependent on the quality and spatial arrangement of these constituents [12,13]. Recent studies have shown that the mineral predominantly contributes to bone stiffness [14] whereas the quality of collagen matrix may predominantly determine the stress, strain and toughness of bone [15,16].

Although the effect of salmon calcitonin on BMD and fracture risk had been well documented, its effect on cortical bone biomechanical parameters and bone collagen structure were not known clearly. There are a few studies that investigated the effect of this drug on biomechanical parameters using different dose and different models [17-20]. Most of these studies investigated the effect of these drugs on prevention of osteoporosis. There are no enough data about the effect of calcitonin on osteoporotic cortical bone quality. Also there is no study to investigate the effect of this drug on bone collagen integrity.

The aim of this study was to investigate the effect of calcitonin treatment on cortical bone quality and collagen integrity.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Mersin University Medical Faculty approved the experiments described in this study. Forty five, twelve-week-old Sprague-Dawley female rats weighing 200-250 g each were used. The animals were acclimatized for 1 week to our laboratory conditions before experimental manipulation. They had free access to standard laboratory chow and water *ad libitum* was maintained on 12 h/12 h light dark cycle. The rats were divided into three groups: I, control (CON, n =15); II, ovariectomized (OVX, n =15) and III, ovariectomized+calcitonin (OVX-CT, n=15). The rats in the control group were neither operated nor treated with calcitonin. Thirty rats were anaesthetized with ketamine (Ketalar, Eczacibasi Pharmaceutical Co.) and underwent bilateral ovariectomy via ventral incision. Ten weeks after ovariectomy, ovariectomized rats were divided into two groups. OVX-CT rats were treated with calcitonin dissolved in saline by s.c. at a volume of 1 mL once per week for 12 weeks. OVX rats were given isotonic saline solution of equal volume. In previous studies [17], animals were treated with 2 IU/kg body weight dose of salmon calcitonin and the study was carried out using 2 IU/kg body weight dose.

BMD was measured before OVX as baseline, 10 weeks after OVX and a final at femoral region by dual-energy X-ray absorbsiometry (DEXA; Norland 45 XR) adapted to the measurement of BMD in small animals, with a scan speed of 1 mm/s and a resolution of 0.5x 0.5 mm. Before taking the measurement, the instrument was

calibrated by means of a Norland phantom. BMD measurements performed in-situ under ketamine anesthesia. The bone mineral density (mg/cm^2) was determined by the analysis of the cortical femoral region.

Cross sectional area of the cortical femur was scanned at 1mm resolution by computerized tomography (ARSTAR 40, Siemens, Erlangen, Germany) and the length of femoral shaft was measured with a digital caliper for the ultimate stress and strain calculations. Computerized tomography images were transferred to the personal computer and cross sectional area were calculated using the AUTOCAD software.

Biomechanical measurements were performed on the diaphysis of the left femur. Bones were resected, the soft tissue was cleaned, wrapped in gauze soaked in isotonic saline, and frozen at $-20^{o}C$ until testing.

Tensile test was performed to measure ultimate stress, ultimate strain and toughness of cortical femur. After thawing at the room temperature, the samples were tested using a biomaterials testing machine (BIOPAC MP 100, MAY 03, Santa Barbara, USA). The tensile loading speed in all tests was 2mm/min. The data were transferred to the computers translating to the numerical signals by 16 bit A/D converter for off line analysis. The sampling rate was chosen as 1000 sample/s. During mounting and testing of the specimens, Ringer's solution was regularly applied to prevent the bones from drying. Each specimen was subjected to a small initial preload (5 N) before actual testing. Load-displacement data were recorded using BIOPAC MP 100 Acquisition System Version 3.5.7 (Santa Barbara, USA). Load-displacement recordings were normalized by cross-sectional area and this curve was converted to a stress-strain curve. Stress/strain curves for each specimen were generated and the ultimate stress, ultimate strain and toughness were determined.

Histological Investigation

Cortical femurs were fixed with 2.5% gluteraldehyde, decalcified with 10%EDTA, postfixed with 1% osmium tetroxide, dehydrated in graded alcohol series, cleared with propylene oxide and embedded in epoxi resin. Thin sections (50-70 nm) of cortical femur were cut by Leica UCT-125 and contrasted with uranyl acetate and lead citrate Cortical sections were examined and photographed by transmission electron microscope (JEOL JEM-1011, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by using SPSS 10.0 software. After obtaining normal distribution (Kalmogorov-Smirnov), differences in BMD values within group were performed by using analysis of repeated-measures ANOVA. Differences between groups for BMD, stress, strain and toughness parameters were compared one-way ANOVA and Tukey's-Kramer Honestly Significant Difference test was performed for multiple comparisons. Data were expressed as means$\pm$standart deviations. Significance was set at $p<0.05$.

RESULTS

No significant differences were observed in baseline femoral region BMD between groups (Figure 1). After ten weeks, no significant differences were observed in the CON group compared with baseline ($p=0.677$). However, a remarkable decrease was observed in the femoral region BMD at OVX ($p=0.038$), and OVX-CT ($p=0.041$) groups. At the end of the experimental period, BMD was found significantly lower in OVX group ($p=0.027$) than that of the CON group. However, there were no significant differences between control and OVX-CT groups. The lines in Figure 2 represent load-displacement data from obtained cortical bone samples of control, OVX and OVX-CT rats. Load-displacement data was normalized by cross-sectional area and this curve was converted to a stress-strain curve (Figure 3).

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From the stress–strain curve, ultimate stress, ultimate strain and toughness were calculated. These values are shown in Table 1. In the OVX-CT group; ultimate stress, ultimate strain and toughness were not different than OVX group ($p>0.05$) and significantly lower than those of the control group ($p<0.05$).

Collagen integrity

Severe alterations in bone collagen fibrils were detected at the ultra-structural level. Regular collagen fiber organization was observed in the CON group (Figure 4), whereas the parallel packing of fibrils was completely replaced by a random arrangement in ovariectomized rats (Figure 5). Quite irregular organization of collagen fibers was observed in the the OVX-CT group similar to OVX group (Figure 6).

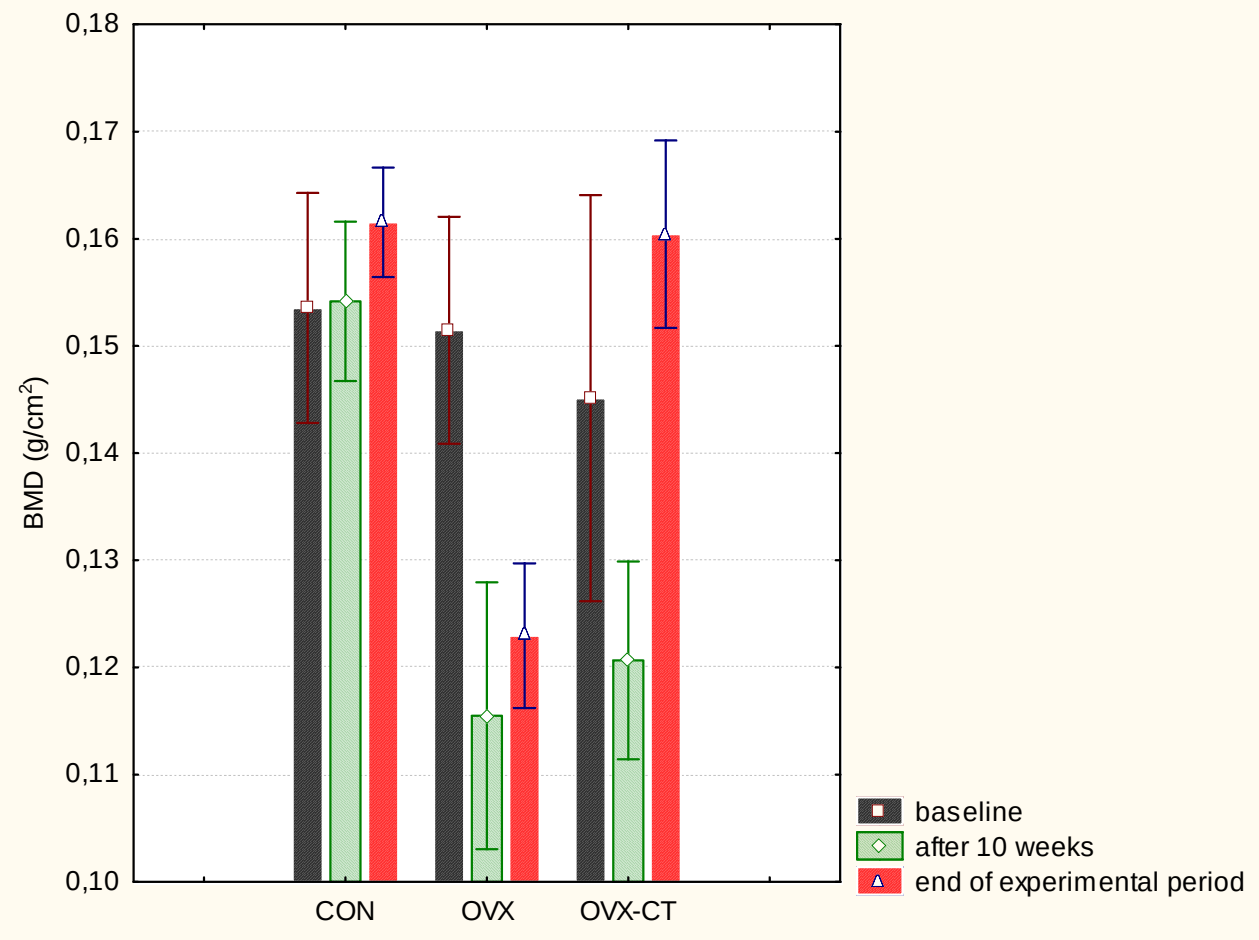

Figure 1. BMD values of the CON, OVX and OVX-CT groups.

Table 1. Values of stress, strain and toughness of cortical femur in control, OVX and OVX-CT groups.

Variables	Control	OVX	OVX-CT
Stress (MPa)	14.87±5.54	6.74±2.61*	5.86±1.39*
Strain	0.23±0.021	0.15±0.023*	0.11±0.03*
Toughness (MPa)	1.55±0.31	0.52±0.31*	0.43±0.26*

*Significant difference from control at $p<0.05$, Data are means±standart deviations

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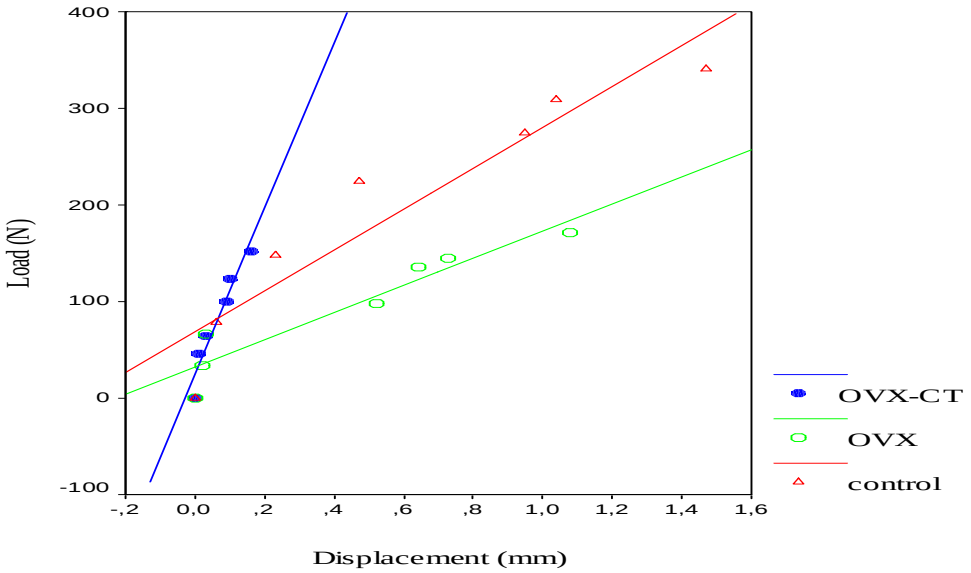

Figure 2. A typical force-displacement curves from obtained cortical bone samples of control, OVX and OVX-CT rats.

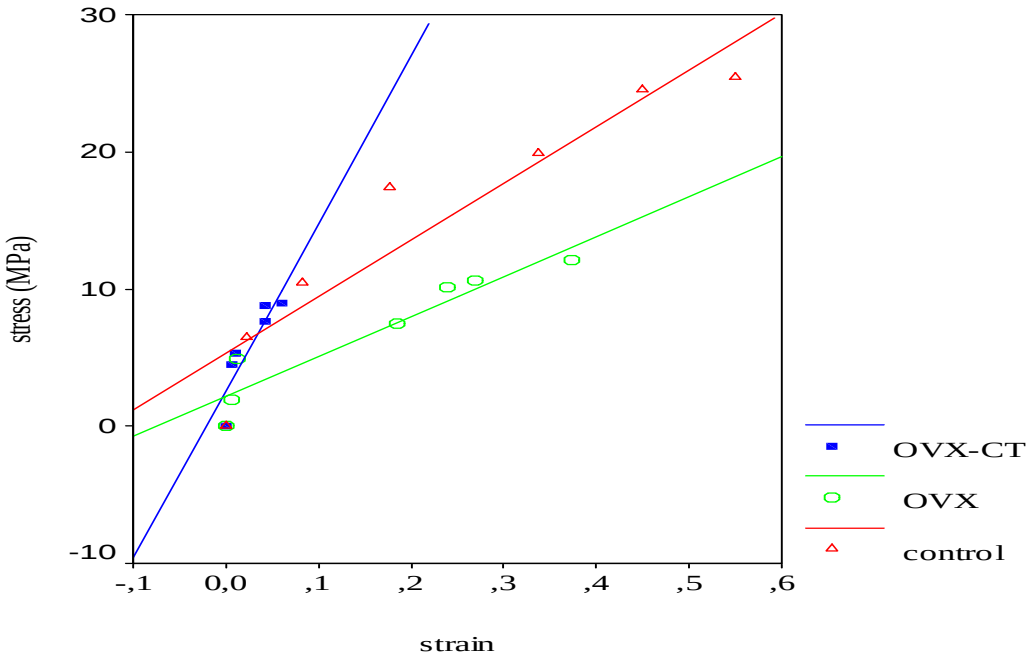

Figure 3. Samples of stress-strain curves in control, OVX and OVX-CT rats. When force-displacement curves are normalized by cross-sectional area, these curves can be converted to a stress-strain curves from which the properties of the tissue (intrinsic properties) itself can be estimated. The area under the stress-strain curve is the modulus of toughness, which is the energy required to cause failure of the bone matrix itself, independent of the bone's size or geometry.

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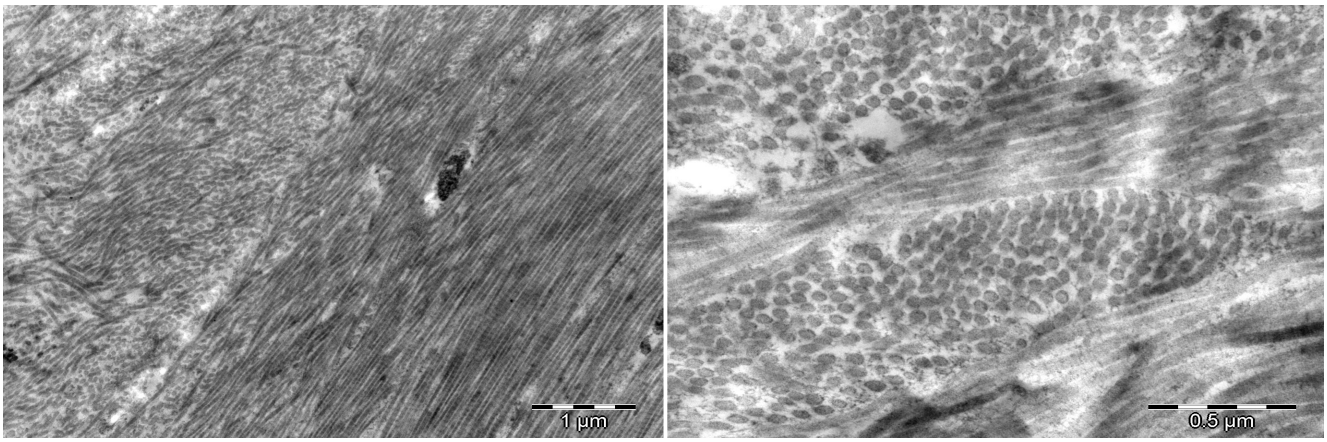

Figure 4. Collagen structure of the control cortical femurs

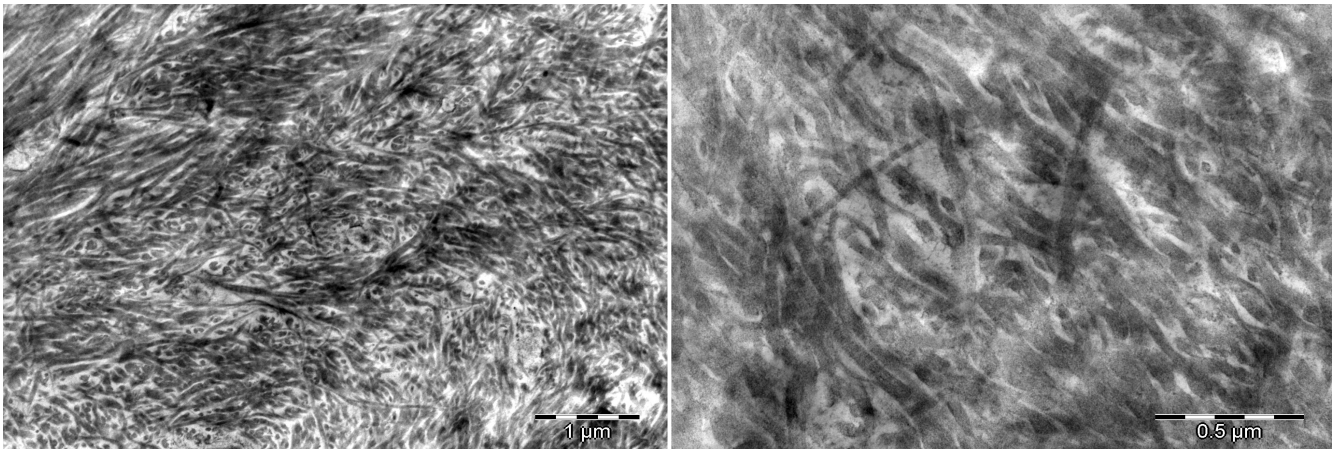

Figure 5. Collagen structure of the ovariectomy cortical femurs

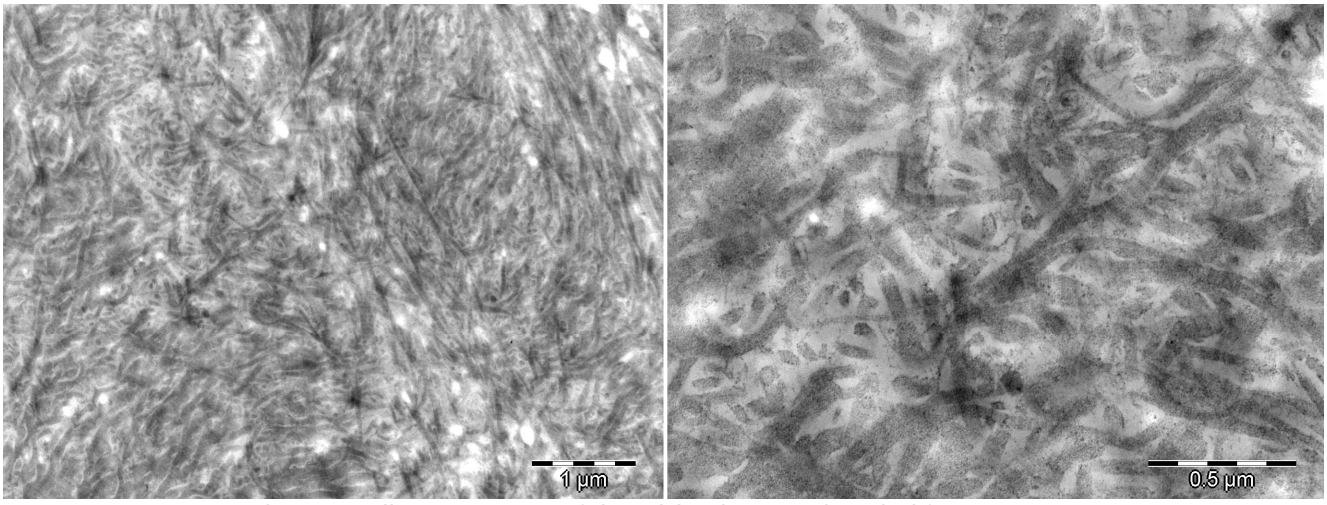

Figure 6. Collagen structure of the calcitonin treated cortical femurs

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DISCUSSION

The ovariectomized rat is a convenient and reliable model for studying the efficacy of pharmaceutical agents in post menopausal osteoporosis [21,22]. The pharmacological agents used to manage osteoporosis act by decreasing the rate of bone resorption, thereby slowing the rate of bone loss, or by promoting bone formation. Many synthetic agents such as calcium, calcitonin, hormones, bisphosphonates and selective estrogen receptor modulators (SERMs) such as Raloxifene and Droloxifene have been developed to treat osteoporosis [23-25].

In this study, the effect of calcitonin on biomechanical parameters and collagen integrity of osteoporotic cortical bone was investigated in a ovariectomized rat model. Although this drug leads to increase at the BMD levels, it was not affected positively bone biomechanical parameters and collagen integrity in osteoporotic bone. Studies in OVX rats using traditional 2D bone histomorphometry have shown that subcutaneous administration of calcitonin is associated with a partial inhibition of the OVX-induced increase in bone resorption, without detrimental effects on osteoid volume or mineralization lag time. Fukushima investigated the effect of calcitonin on histomorphometric and biomechanical parameters at ovariectomy and ovariectomy-steroid induced osteopenia in the rats and found that calcitonin treatment (10U/kg per day, SC 5 days a week, for 8 weeks) recovered maximum elastic load in femur [19]. Ogawa et al. were investigated the effect of salmon calcitonin on trabeculer microstructure and bone strength in ovariectomized rat and found that 15 IU/week calcitonin treatment prevent OVX induced changes at trabeculer microstructure and bone strength [20]. Also Wronski et al. were investigated the effect of calcitonin on bone histomorphometry and reported that calcitonin treatment has protective effect at early postmenopausal period in ovariectomized rats [26]. Contrary Giaveresi found that 2 IU/kg calcitonin dose did not significantly increase BMD and bending strength [17]. In our study, calcitonin treatment at the dose of 2 IU/kg for 12 weeks lead to significant increase of the BMD without any increase in bone strength and other bone biomechanical parameters. This result might be related with calcitonin dose and duration of treatment.

Bone quality varies with geometric properties (trabeculer network and macrostructure of the cortex and cortical shell) and material properties (matrix calcification and the composition and spatial arrangement of crystals, collagen fibers, and lamellae) [27]. Stress, strain and toughness were also important biomechanical parameters for bone strength. Stress, strain, and toughness contribute to bone collagen integrity [28]. We found that bone stress, strain, and toughness were reduced in the OVX-CT group. The decrease in stress, strain, and toughness may be related to deformation of collagen integrity. Collagen fibers organization were evaluated by transmission electron microscopy. We observed regular collagen fiber organization in the CON group but there was no preferential organization of the collagen fibers in OVX and OVX-CT groups. They were not as tightly packed and observed in somewhat randomly oriented bundles. Studies on rat femora suggest that the decline in bone's mechanical properties with age and osteoporosis may be dependent on the stability and cross-linking of the collagen [29]. Only a few studies on the osteoporosis-related changes in collagen and their correlation with the toughness of bone have been reported in the literature [19, 30], and there is no study regarding the effects of salmon calcitonin treatment on collagen networks and toughness of the cortical bone. Our biomechanical and histological results may suggest that any disturbances in collagen fibers results in the formation of low-quality bone tissue susceptible to deformation and fractures in OVX and OVX-CT groups. We observed that the treatment of salmon calcitonin were not affected positively bone quality in osteoporotic rats.

CONCLUSIONS

The results of current study showed that 12 weeks calcitonin treatment (2 IU/kg body weight) lead to significant increase of the BMD without any change in bone biomechanical parameters and collagen ultrastructure in ovariectomized rats.

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