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Effect of Risedronate and Alendronate Treatment on Femoral Bone Quality and Collagen Integrity

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The aim of this study was to investigate the effect of risedronate and alendronate treatment on bone biomechanical parameters and bone collagen integrity during postmenopausal osteoporosis. Sixty Sprague-Dawley female rats were divided into four groups: **I**, control (CON, n = 15); **II**, ovariectomized (OVX, n = 15); **III**, ovariectomized + alendronate (OVX-ALN, n = 15) and **IV**, ovariectomized + risedronate (OVX-RIS, n = 15). Ten weeks after ovariectomy, OVX-ALN rats were treated with alendronate sodium at the dose of 1.75 mg/kg body weight and OVX-RIS rats received risedronate sodium at the dose of 0.5 mg/kg body weight using gavage twice per week for 12 weeks. Biomechanical measurements were performed at the femoral shaft and femoral neck of the left femur. In the OVX-ALN and OVX-RIS groups, strength, displacement, energy, strain and toughness were significantly increased when compared to OVX group. There were no significant differences between control and OVX-ALN and OVX-RIS groups for all biomechanical parameters at the femoral diaphysis and femoral neck. There were no statistically significant differences between the control, OVX, OVX-ALN and OVX-RIS groups with respect to biochemical parameters. Normal collagen fiber organization was observed in control group, whereas the parallel packing of fibrils were completely replaced by a random arrangement in ovariectomized rats. However, OVX-ALN and OVX-RIS groups showed more regular arrangement than OVX group. In conclusion, the positive effect on bone biomechanical parameters and collagen integrity in ovariectomized rat femur was observed following the treatment of risedronate and alendronate.

Key Words: Alendronate, Risedronate, Bone strength, Collagen, Bone biomechanic.

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INTRODUCTION

Osteoporosis is a major public health problem throughout the world and defined as a skeletal disorder characterized by compromised bone strength and increased risk of fracture. Oral bisphosphonates are used for more than ten years for the treatment and prevention of osteoporosis. They are analogs of inorganic pyrophosphate and inhibit bone resorption[1].

Risedronate, a potent bisphosphonate, is effective for the prevention and treatment of postmenopausal osteoporosis[2-4]. In clinical studies, risedronate reduced the risk of vertebral fracture by up to 65 % within the first year of treatment[2,3]. Alendronate sodium (4-amino-1-hydroxybutylidine-1,1-*bis*phosphonate) is a bisphosphonate which is poorly absorbed following oral administration and the assimilated fraction is rapidly bound to the bone remodeling units or is excreted in urine as the unchanged molecule[5,6]. The effect of alendronate on bone was shown by both *in vitro* and *in vivo* studies[7-11].

Bone is a natural composite comprising mineral (mainly hydroxyapatite), organic (mostly type I collagen) and water phases. Thus, the biomechanical properties of bone dependens 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]. In addition, it was found that osteoporosis is not just a simple loss of bone mass, but involves significant changes in the biochemical and physical properties of the collagen network[17].

Although the effect of these drugs on bone mineral density (BMD) and fracture risk had been well documented, their effect on bone biomechanical parameters and bone collagen structure were not known clearly. There are a few studies that investigated the effect of these drugs on biomechanical parameters using different dose and different models[18-21]. Most of these studies were investigated the effect of these drugs on the prevention of osteoporosis. There are no enough data about the effect of osteoporotic bone parameters. Also there is no study to investigate the effect of these drugs on bone collagen integrity.

The aim of this study was to investigate the effect of risedronate and alendronate treatment on osteoporotic bone, bone biomechanical parameters and bone collagen integrity during postmenopausal osteoporosis. Bone collagen structure was also evaluated. Ovariectomized rat model was used for this purpose and trabecular and cortical bone effect was evaluated separately.

EXPERIMENTAL

The Institutional Animal Care and Use Committee at Mersin University Medical Faculty approved the experiments described in this study. Sixty, 12-week-old Sprague-Dawley female rats weighing 200-250 g each were used. The animals were acclimatized for 1 week in experimental laboratory conditions before experimental manipulation. They had free access to standard laboratory chow and water *ad libitum* were maintained on 12 h/12 h light dark cycle throughout the experiment.

Rats were assigned randomly to 4 groups (n = 15) as: (**1**) control groups (CON, n = 15), (**2**) ovariectomized group: (OVX, n = 15) The rats receiving only saline, (**3**) ovariectomized group treated with alendronate (OVX-ALN, n = 15) and (**4**) ovariectomized group treated with risedronate (OVX-RIS, n = 15).

Forty five rats were anaesthetized with ketamine (Ketalar, Eczacibasi Pharmaceutical Co.) and underwent bilateral ovariectomy *via* ventral incision. Ten weeks after ovariectomy OVX-ALN rats were treated with 1.75 mg/kg body weight alendronate sodium dissolved in saline and OVX-RIS rats received risedronate sodium at the dose of 0.5 mg/kg body weight by gavage twice per week for 12 weeks. Cross sectional area of the femoral shaft and femoral neck were measured by computerized tomography (ARSTAR 40, Erlangen, Germany) and the length of femoral shaft and femoral neck were measured with a digital caliper.

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

Tensile test was performed to measure strength, stiffness, energy absorption capacity, stress, strain and toughness of femoral shaft and neck. After thawing at room temperature, the samples were tested using a biomaterials testing machine (May 03, USA). The tensile loading speed in all tests was set as 2 mm/min. The data transferred to the computers were translated into 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). Strength represents the maximum tensile force applied until a fracture is occurred. The slope of the linear portion of the load-displacement curve defined stiffness and the area under the load-displacement curve defined the energy absorption capacity. 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 stress, strain and toughness were determined. Stress values were calculated utilizing the following equation[22]:

$$\sigma = F/A$$

where σ = ultimate stress (MPa), F = failure load (N), A cortical area of the specimen (mm^2).

Strain was calculated from the following equation:

$$\varepsilon = \Delta L/L_0$$

where ε = strain, ΔL = change in the length (mm) and L_0 = original length.

Elastic modulus was calculated from the following equation:

$$E = \sigma/\varepsilon$$

where E = elastic modulus (GPa), σ = ultimate stress and ε = ultimate strain.

Biochemical analysis: While rats were under anesthesia, blood samples were collected from the heart and transferred into the tubes in the absence of anticoagulant. The samples were centrifuged within 0.5 h at 1500 x g for 5 min. Serum was separated and immediately frozen to -20 ºC until analysis. Serum alkaline phosphatase, calcium and phosphorous levels, free T_3 (FT_3), free T_4 (FT_4), parathormone, estrogen, progesterone and magnesium levels were measured using an auto analyzer in all groups.

Histological investigation: 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 epoxy resin. Thin sections (50-70 nm) were cut by Leica UCT-125 and contrasted with uranyl acetate and lead citrate. Sections were examined and photographed by Jeol JEM-1011 electron microscope.

Statistical analysis: Statistical analysis was performed by using SPSS 10.0 software. After obtaining normal distribution (Kalmogorov-Smirnov), the data were expressed as mean ± standard deviation (SD) and Tukey-Kramer Honestly significant difference test was used to compare different groups. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Femoral shaft results: The results of biomechanical tests for femoral shaft are shown in Table-1. In the OVX-ALN group; strength, displacement, absorbed energy, stress, strain and toughness ($p < 0.05$) were significantly higher than the ovariectomy group. Strength and stiffness were significantly lower than the control group. Displacement and strain were significantly higher than the control group ($p < 0.05$). Stress and toughness were not different than that of the control ($p > 0.05$).

TABLE-1
BIOMECHANICAL FINDINGS OF FEMORAL SHAFT IN CONTROL, OVX, OVX-ALN, OVX-RIS GROUPS

Variables	Control	OVX	OVX-ALN	OVX-RIS
Strength (N)	350.840±86.73	143.700±13.90†	184.730±26.96‡†	291.19±109.24‡
Displacement (mm)	1.640±0.45	0.900±0.28 †	2.550±0.86†‡	2.15 ± 0.73‡
Energy (mJ)	577.220±105.95	129.310±39.31†	447.780±162.65‡	604.35±99.98‡
Stiffness (N/mm)	223.770±74.32	176.510±67.71	82.830±44.32†	149.30±75.90
Stress (MPa)	30.460±5.44	14.100±3.86†	24.290±6.08‡	18.81±7.82†
Strain	0.058±0.017	0.030±0.0013†	0.083±0.027‡†	0.07±0.024‡
Toughness (MPa)	1.770±0.67	0.420±0.18†	1.360±0.45‡	1.26±0.44‡

†Significant difference from control at $p < 0.05$.
‡Significant differences from OVX at $p < 0.05$.

In the OVX-RIS group; strength, displacement, absorbed energy, strain and toughness were significantly higher than the OVX group ($p < 0.05$) however, there were no significant differences between control and OVX-RIS groups for strength, displacement, absorbed energy, stiffness and toughness ($p > 0.05$) but stress was significantly lower than the control group ($p < 0.05$).

Femoral neck results: The results of biomechanical tests for femoral neck are shown in Table-2. In the OVX-ALN group; strength, absorbed energy and toughness were significantly higher than the OVX group ($p < 0.05$). Displacement, stiffness, strain and stress were not changed when compared to the OVX group ($p > 0.05$). Stress and stiffness were significantly lower than the control group.

TABLE-2
BIOMECHANICAL FINDINGS OF FEMORAL NECK IN CONTROL, OVX, OVX-ALN, OVX-RIS

Variables	Control	OVX	OVX-ALN	OVX-RIS
Strength (N)	135.37±19.17	39.80±5.56†	92.71±23.78‡	100.83±31.71‡
Displacement (mm)	1.42±0.22	1.15±0.06	1.17±0.054	0.24±0.051†‡
Energy (mJ)	96.10±18.39	22.90±3.14†	81.45±13.12‡	79.13±10.95‡
Stiffness (N/mm)	97.19±21.68	34.67±5.12†	41.09±21.64†	96.07±21.4‡
Stress (MPa)	49.69±12.08	19.14±5.72†	25.12±10.21†	35.95±10.90‡
Strain	0.52± 0.07	0.40±0.027	0.48±0.014	0.97±0.016†‡
Toughness (MPa)	13.09±3.91	4.90±0.42†	11.39±4.13‡	11.89±3.85‡

†Significant difference from control at $p < 0.05$.
‡Significant differences from OVX at $p < 0.05$.

In the OVX-RIS group; strength, stress, absorbed energy, stiffness, strain and toughness were significantly higher than the ovariectomy group ($p < 0.05$). However, displacement was lower and strain was higher than the control group ($p > 0.05$).

Collagen integrity: Severe alterations in bone collagen fibrils were detected at the ultra-structural level. Normal collagen fiber organization was observed in control group (Fig. 1), whereas the parallel packing of fibrils was completely replaced by a random arrangement in ovariectomized rats (Fig. 2). However, OVX-ALN and OVX-RIS groups were shown more regular arrangement than OVX group (Figs. 3 and 4).

Biochemical results: There were no statistically significant differences between the control, OVX, OVX-ALN and OVX-RIS groups in serum calcium, phosphate, FT_3, FT_4, T_3 and T_4 ($p > 0.05$) (Table-3). Level of estrogen and progesterone were significantly lower in the OVX, OVX-RIS and OVX-ALN groups than those of the control group ($p < 0.05$).

In this study, the effect of risedronate and alendronate on osteoporotic bone has been investigated and found that these drugs were increased the cortical and trabecular bone quality and collagen integrity in ovariectomized rats.

Bone strength is related with bone macrostructure, microstructure and bone collagen structure. While the effects of risedronate and alendronate therapies on fracture risk have been relatively well established, the effect of bone biomechanics is relatively unknown[23]. Borah *et al.*[24] reported that risedronate preserved trabecular architecture and bone strength is tightly coupled both bone mass and architecture in ovariectomized minipigs. Ito *et al.*[25] found that risedronate improve bone mineral density and biomechanical parameters and maintining a plate-like structure as well

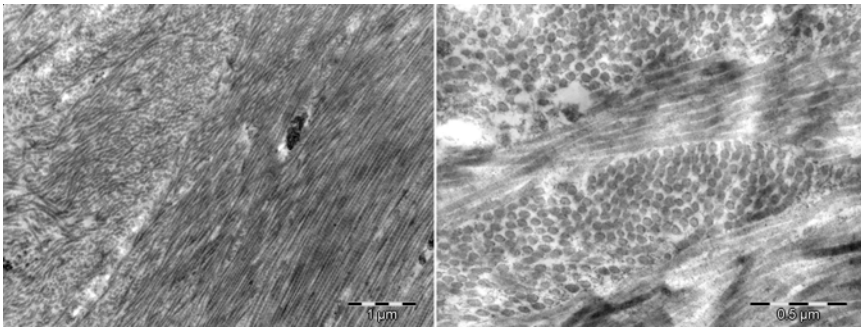

Figure 1. Collagen fibril organization in control group

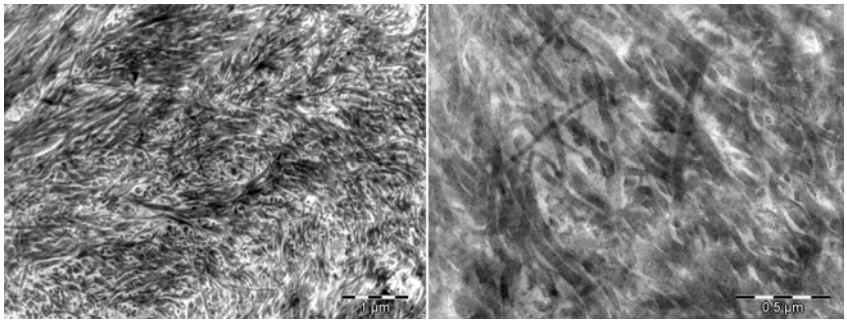

Figure 2. Collagen fibril organization in OVX group

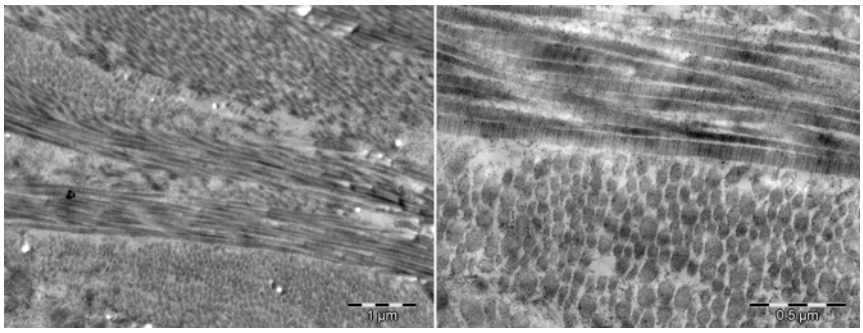

Figure 3. Collagen fibril organization in OVX-ALN group

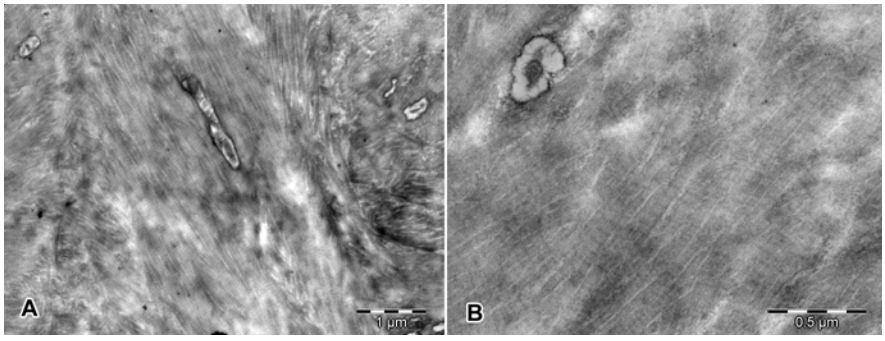

Figure 4. Collagen fibril organization in OVX-RIS group

TABLE-3
BIOCHEMICAL FINDINGS IN CONTROL, OVX, OVX-ALN AND OVX-RIS RATS

Variables	Control	OVX	OVX-ALN	OVX-RIS
Calcium (mg/dl)	9.65±0.26	7.31±2.32	8.67±1.95	8.95±0.92
Phosphate (mg/dl)	5.55±0.37	3.88±0.80	5.32±0.51	4.67±0.43
Magnesium (mg/dl)	2.34±0.086	2.11±0.31	2.19±0.37	2.31±0.25
Alkaline phosphatase (U/L)	275.00±36.90	235.85±73.5	273.85±69.8	266.85±73.5
FT_3 (pmol/L)	4.16±0.90	3.91±0.48	3.98±0.47	4.07±0.55
FT_4 (pmol/L)	24.48±4.77	24.76±3.95	28.37±4.32	31.23±6.55
T_3 (ng/mL)	1.16±0.66	0.73±0.043	1.05±0.06	0.93±0.09
T_4 (µg/dL)	4.73±1.83	3.88±0.61	4.36±0.38	4.24±0.26
Progesterone (ng/mL)	35.87±8.48	3.87±1.64†	9.64±1.35†	7.52±2.31†
Estrogen (pg/mL)	30.74±5.72	8.08±1.44†	12.32±4.36†	15.31±3.03†

†Significant difference from control at $p<0.05$

as connectivity of trabecula at ovariectomized rat tibia. Similarly Otomo *et al.*[26] reported that risedronate leads to increase of bone mass, bone strength and mineral-matrix ratio at dose of 0.1-0.5 mg/kg/d. There are several recent studies that support the role of alendronate on bone biomechanics. Azuma *et al.*[27] also show that continuous treatment for 2 months with alendronate did not reduce the biomechanical properties of the rat femur. They also showed that there was a correlation between the femoral neck BMD and ultimate load and stiffness. Guy *et al.*[28] reported that continuous alendronate treatment for 2 years leads to increase of vertebral compression and femoral bending. Hu *et al.*[29] demonstrated that alendronate increased the mechanical properties of healthy canine trabecular bone after short term treatment. They also found that alendronate increased the BMD and no improvement was observed in the diaphyseal mechanical properties. Recently, Iwamoto *et al.*[30] reported that alendronate treatment is effective on both cancellous and cortical bone histomorphometry and bone strength. The results of present study are consistent with these studies.

It is known fact that the collagen network is important for bone quality and stress, strain and toughness parameters of bone biomechanics are related to collagen matrix[31]. But a few studies was investigated the osteoporosis-related changes in collagen and their correlation with the toughness of the bone[16,32]. There is limited study regarding the effects of risedronate and alendronate treatment on collagen networks and toughness of the cortical bone. Durchschlag *et al.*[33] were evaluated long-term effects of *bis*phosphonates on bone mineral maturity/crystallinity and collagen cross-link ratio in triple iliac crest biopsies of osteoporotic women and found that long-term treatment with risedronate affects bone material properties (mineral maturity/crystallinity and collagen cross-link ratio). In our study, collagen integrity was also evaluated and risedronate and alendronate influenced the collagen integrity nearby the control.

As a conclusion, a positive effect was observed on bone biomechanical parameters and collagen integrity in ovariectomized rat femur following the treatment with risedronate and alendronate.

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