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Biomechanical Properties of Bones from Rats Treated with Sevelamer

Natasa Drača[1], Ana Tikvica[1], Domagoj Eljuga[2], Damir Semenski[3], Mladen Brnčić[3] and Slobodan Vukičević[1]

[1] University of Zagreb, School of Medicine, Laboratory for Mineralized Tissues, Zagreb, Croatia
[2] University of Zagreb, Zagreb University Hospital Center, Department of Surgery, Zagreb, Croatia
[3] University of Zagreb, Faculty of Mechanical Engineering and Naval Architecture, Department of Engineering Mechanics, Zagreb, Croatia
[4] University of Zagreb, Faculty of Food Technology and Biotechnology, Section for Thermodynamics, Zagreb, Croatia

A B S T R A C T

Sevelamer hydrochloride is used for ten years in patients on dialysis as a phosphate binder. We have previously shown that oral application of sevelamer prevents the bone loss and increases the bone volume in ovariectomized rats[1]. In this study we further analysed the biomechanical properties of bones from rats treated with sevelamer utilizing a threepoint bending test to determine the mechanical properties of the cortical bone of the mid-shaft femur, while the indentation test was used to determine the mechanical properties of cancellous bone in the marrow cavity of the distal femoral metaphysis. Parameters analyzed included: maximum load (F_u), stiffness (S), energy absorbed (W), toughness (T) and ultimate strength (σ). The intrinsic properties, stress, elastic modulus and toughness were determined from measured maximum load, strains, stiffness, energy absorbed, outer and inner diameters, and calculated bone cross-sectional moment of inertia. Sevelamer was given to rats for 25 weeks with a content of 3% of sevelamer in a standard diet, starting immediately following ovariectomy (OVX). Animals were divided to the following groups: (1) Sham; (2) Sham + sevelamer 3%; (3) OVX; (4) OVX + sevelamer 3%. Our results showed that sevelamer particularly influenced the rat trabecular bone by increasing the maximum load for 26.2%, energy absorbed for 24.2% and the ultimate strength for 26.2% in sham animals treated with sevelamer 3%, as compared to sham rats. Sevelamer 3% in OVX rats also increased the maximum load for 71.4%, stiffness for 70.7%, energy absorbed for 55.9% and the ultimate strength for 71.3% as compared to OVX controls. In the three bending test sevelamer had a very little effect on preventing loss of bone strenght in the cortical bone. These results collectively suggest that sevelamer improves bone biomechanical properties, mainly affecting trabecular bone quality in both normal and ovariectomized rats.

Key words: *biomechanical testing, threepoint bending test, indentation test, bone*

Introduction

Sevelamer hydrochloride (sevelamer, Renagel®), a non-calcium phosphate binder, has been shown to reduce coronary artery and aortic calcification and increase trabecular bone mineral density in patients with chronic kidney disease as compared to calcium containing phosphate binders[2,3]. It has been successfully used in clinics for more than 10 years[4,5]. We have recently found that sevelamer prevents bone loss and increases bone volume in normal and ovariectomized rats with an intact kidney function[1]. In order to further explore the role of sevelamer treatment on the bone biomechanical quality, we evaluated its effect in normal rats and in ovariectomized (OVX) rat model of osteoporosis with high bone turnover due to estrogen deficiency. Osteoporosis is characterized by low bone mineral density (BMD) and diminshed bone quality. Bone tissue quality depends, both on bone biomechanics and overall bone structure. Bone in humans and other mammals is generally classified into two types: cortical bone, also known as compact bone and trabecular bone, also known as cancellous or spongy bone (Figure 1). These two types are classified on the basis of porosity and the unit microstructure. Cortical bone is

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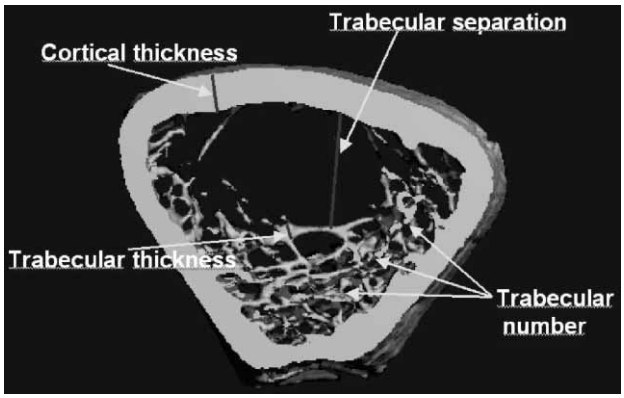


Fig. 1. Cortical and trabecular bone on the horizontal microCT cross section of rat femur.

much denser with a porosity ranging between 5% and 10%. It is primary found in the shaft of long bones and forms the outer shell around cancellous bone at the end of joints and the vertebrae. Trabecular bone is much more porous with porosity ranging anywhere from 50% to 90%. It fills the end of long bones and also makes up the majority of vertebral bodies. The major mechanical property differences between trabecular and cortical bone is the effective stiffness. Trabecular bone is more compliant than cortical bone and it is believed to distribute and dissipate the energy from articular contact loads. Trabecular bone contributes to about 20% of the total skeletal mass within the body while cortical bone contributes to the remaining 80%. However, trabecular bone has a much greater surface area than cortical bone. Within the skeleton, trabecular bone has a total surface area of 7.0x106 mm^2 while cortical bone has a total surface area of 3.5x106 mm^2. Trabecular bone may have bone volume fraction ranging from just over 5% to a maximum of 60%. Bone volume fraction is defined as the volume of bone tissue per total volume. The trabecular bone volume fraction varies between different bones, with age, and between species. The basic structural entity at the first level of trabecular bone is the trabeculae.Trabeculae are most often characterized as rod or plate like structures. In this study we have used two types of biomechanical tests: threepoint bending test and indentation test that are commonly used for investigating characteristics of bone. Bending tests are useful for evaluating the mechanical strength of bones from small animals, such as rodents. The three-point test is the most widely used. It is typically used on long bones of rodents to discriminate among species[6] and to test treatment effects on mechanical properties[7]. Threepoint bending test was used to determine the mechanical properties of the cortical bone of the mid-shaft femur. The holders for bone are perpendicular to the horizontal axis, and the force is applied downward, perpendicularly to the horizontal axis and at the midpoint of the specimen. The load is increased until the bone breaks. Force and displacement data recorded during the tests are used in combination with bone geometry parameters to compute mechanical properties. Cross-sectional area and shape are assumed constant along the longitudinal axis of the bone. Although this assumption is not met, it provides a means of comparing results across studies[8]. Indentation test imitate load that are sustained in vivo, for instance on the vertebrae. They can also be performed on small specimens. Force-displacement curves recorded during indentation tests usually show a gradual increase with no true plastic deformation. Microstructural bone damage with pore collapse eventually occurs. Failure is usually defined as the point beyond which stress drops. Stress and deformation are typically computed from force-displacement curves. Thus, stiffness, Young's modulus, deformation and stress at failure, deformation and stress at the elastic limit can be obtained[8]. Indentation test of the distal femoral metaphysis was used to determine the mechanical properties of cancellous bone in the marrow cavity of the distal femoral metaphysis.

Materials and Methods

Animal model

Six months old Sprague-Dawley rats were subjected to OVX. Animals were anesthetized with an intraperitoneal injection of thiopental at doses of 4 mg/kg body weight. Twelve animals were subjected to sham surgery during which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed in the remaining rats from the abdominal approach and the sevelamer therapy was initiated immediately following OVX to prevent the development of osteopenia. Sevelamer to the final concentration of 3% was mixed with the standard rodent diet »Mucedola» 4RF21 (Mucedola, Italy) and pelleted before use. Control animals received standard rodent diet »Mucedola» 4RF21 only. Food was given ad libitum to the animals divided into the following groups: (1) Sham (n=12); (2) Sham + sevelamer 3% (n=12); (3) OVX (n=12); (4) OVX + sevelamer 3% (n=12) for 25 weeks. All experiments and protocols were approved by the University of Zagreb Animal Care Committee at School of Medicine.

Specimen preparation

At the end of the experiment, animals were anesthetized, weighed and sacrificed by cervical dislocation. The femurs were removed and fixed in 70% ethanol. Before testing, the bone specimens were carefully cleaned from any remaining adherent soft tissue.

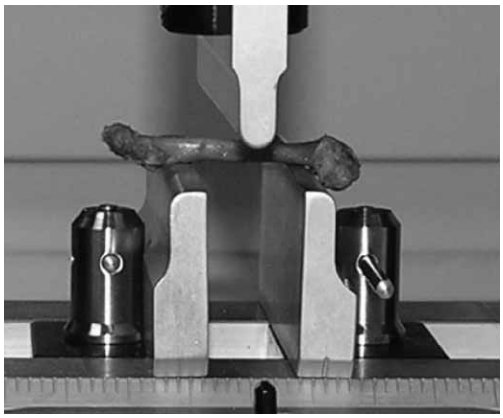
Biomechanical testing

Using a materials testing system (TA.HDplus Texture Analyser, The Stable Micro Systems, Godalming, UK), two types of mechanical testing were performed on the rat femur.

Threepoint bending test of the femoral shaft

De-fleshed whole femurs were used in the three point bending test. The anterior to posterior diameter at the

a)



b)

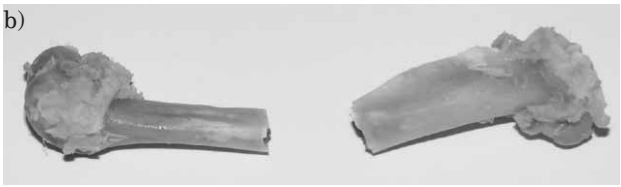


Fig. 2. a) Three point bending testing on the rat femur, b) The mid-shaft of the femur was subjected to three-point bending to failure.

midpoint of the femoral shaft was taken with an electronic caliper and subsequently recorded. Femurs were then placed on the lower supports of a three point bending fixture with anterior side facing downward. The span between the two lower supports was set at 15 mm. The upper loading device was aligned to the center of the femoral shaft. The mid-shaft of the femur was subjected to three-point bending to failure at a displacement rate of 0.1 mm/sec, as described by Turner and Burr[9], using a 30 kg load cell of the testing machine (TA.HDplus The Stable Micro Systems) (Figure 2). The locations of maximal load, stiffness and energy absorbed were selected manually and values were calculated by machine's software (Texture Exponent). The intrinsic properties, stress, elastic modulus and toughness were calculated from maximum load, stiffness, energy absorbed, outer and inner diameters and moment of inertia with the following equations:

From machine measurements

Maximum load (F_u)(N)

Stiffness (S) (N/mm)

Energy absorbed (W) (mJ)

From electronic caliper and microCT measurements

Outer (a, b) and inner diameters (a_1, b_1)(mm)

h, »cortical thickness» from microCT database at the midshaft of femur (mm) (Figure 1)

$$a_1 = a - 2h, \text{ (mm)} \quad (1)$$

$$b_1 = b - 2h, \text{ (mm)} \quad (2)$$

$$A = \frac{\pi(b^3a - b_1^3a_1)}{64}, \text{ (mm)} \quad 3)$$

Axial area moment of inertia

$$I = \frac{\pi(b^3a - b_1^3a_1)}{64}, \text{ (mm}^4\text{)} \quad (4)$$

Constant

Length between two supports L = 15mm

Derived parameters

Ultimate Strength (σ)

$$\sigma = \frac{F_uLb}{48I}, \text{ (N/mm}^2\text{)} \quad (5)$$

Young's modulus of elasticity (E) (extrinsic stiffness or flexural rigidity)

$$E = \frac{SL^3}{48I}, \text{ (N/mm}^2\text{)} \quad (6)$$

Toughness (T)

$$T = \frac{3Wb^2}{4LI}, \text{ (MJ/m}^3\text{)} \quad (7)$$

Indentation test of the distal femoral metaphysis

An indentation test was used to determine the mechanical properties of the cancellous bone in the marrow cavity of the distal femoral metaphysis as described previously[10,11]. A 3 mm segment of the distal femoral metaphysis was cut directly proximal to the femoral condyle with a low-speed diamond saw. The load was applied with a cylindrical indenter (of 2 mm diameter) to the center of marrow cavity on the distal face of the segment. The indenter was allowed to penetrate the cavity at a constant displacement rate of 0.1 mm/sec to a depth of 2 mm before load reversal (Figure 3). Maximum load, stiffness and energy absorbed were selected from load-displacement curve and processed by the machine's software.

From machine measurements

Maximum load (F_u)(N)

Stiffness (S) (N/mm)

Energy absorbed (W) (mJ)

Derived parameters

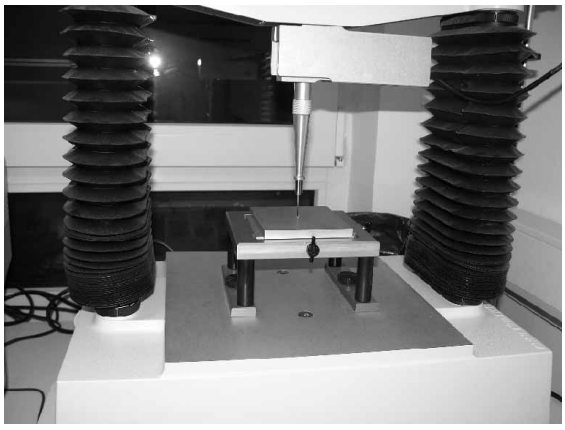


Fig. 3. Indentation testing of the rat femur.

Ultimate Strength (σ)

Stresses were calculated by using the following equation by using the radius r of indenter:

$$s = \frac{F_u}{A} = \frac{F_u}{r^2\pi}, \text{ (MPa)} \quad (8)$$

We used ANOVA Dunnett test for statistical analysis with $p<0.05$ as a significant value.

Results

Sevelamer improves biomechanical properties of trabecular bone on indentation test

Our results showed that sevelamer had a particular influence on trabecular bone. Sham animals treated with sevelamer had increased the maximum load for 26.2%, the energy absorbed for 24.2% and the ultimate strength for 26.2% as compared to sham rats, without reaching statistical significance. Sevelamer had no influence on the stiffness of the trabecular bone. On the contrary ovariectomized animals treated with sevelamer 3% significantly increased the maximum load for 71.4%, the stiffness for 70.7%, the energy absorbed for 55.9% and the ultimate strength for 71.3% as compared to OVX control animals (Figure 4, Table 1). Significantly higher values of Fu for animals receiving sevelamer were caused by the increased trabecular bone volume and number comparing to the OVX control group (Figure 5).

Sevelamer does not affect cortical femoral properties on three point bending test

In the three point bending test sevelamer had no effect on preventing loss of the cortical bone as shown in Table 1. There were no differences in either of measured parameters between sham and sham rats treated with sevelamer. Ovariectomized rats that were treated with sevelamer also showed no difference as compared to OVX control operated animals.

We conclude that sevelamer therapy improved the trabecular bone parameters as compared to OVX animals, but did not restore the mechanical properties of sham rats.

The influence of sevelamer on the trabecular bone was stronger than on the cortical bone according to the microCT measurements

OVX animals receiving sevelamer had increased trabecular bone volume BV (51%), trabecular number (43%), trabecular thickness (9%), cortical thickness (16%), mineral apposition rate (103%), bone formation rate (25%), and enhanced cortical and trabecular bone mechanical strength as compared with OVX rats (Figure 6).

Discussion

Bending tests constitute the preferred method for investigating rodent long bones[8]. Trabecular thickness measured by microCT correlates to stiffness and bending moment of femurs[12] and cortical thickness correlates to breaking force[13]. Three point bending test is a great indi-

TABLE 1
BIOMECHANICAL PARAMETERS OF THE FEMUR FROM ANIMALS TREATED WITH SEVELAMER 3% IN THE PREVENTION MODE OF THERAPY

Three Point Bending Test				
Parameters	Sham	Sham + sevelamer 3%	OVX	OVX+ sevelamer 3%
Fu (N)	232.80±39.19 O	221.31±15.77 O	176.07±22.79	165.68±31.68
S (N/mm)	277.54±40.93	283.81±73.30	259.85±30.54	258.33±52.65
W (mJ)	116.56±54.43 O	93.83±31.19	73.62±24.12	74.88±26.06
T (MJ/m^3)	10.29±4.62	8.10±3.06	7.18±2.44	6.90±2.63
Indentation Test				
Parameters	Sham	Sham + sevelamer 3%	OVX	OVX+sevelamer 3%
Fu (N)	61.59±20.43 O	83.43±37.29 O	3.86±3.52	13.48±4.67 O, S
S (N/mm)	120.78±56.3 O	116.6±21.94 O	16.78±19.18	57.29±37.69 O, S
W (mJ)	59.98±20.36 O	79.07±37.57 O	3.29±3.82	7.46±3.29 O, S
Σ (N/mm^2)	19.62±6.51 O	26.57±11.88 O	1.23±1.12	4.29±1.49 O, S

Animals were treated for 25 weeks immediately following OVX; n=12 in all groups; data are X±SEM.Femur diaphysis was subjected to three point bending to failure. Parameters analyzed include: maximum load (Fu), stiffness (S), energy absorbed (W) and toughness (T). Significant differences are indicated with respect to OVX control (O) and Sham (S) control rats ($p<0.05$ by ANOVA Dunnett test). Indentation test provided data on mechanical properties of trabecular bone. Parameters analyzed include: maximum load (Fu), stiffness (S), energy absorbed (W) and ultimate strength (σ). Significant differences are indicated with respect to OVX control (O) and Sham (S) control rats ($p<0.05$ by ANOVA Dunnett test)

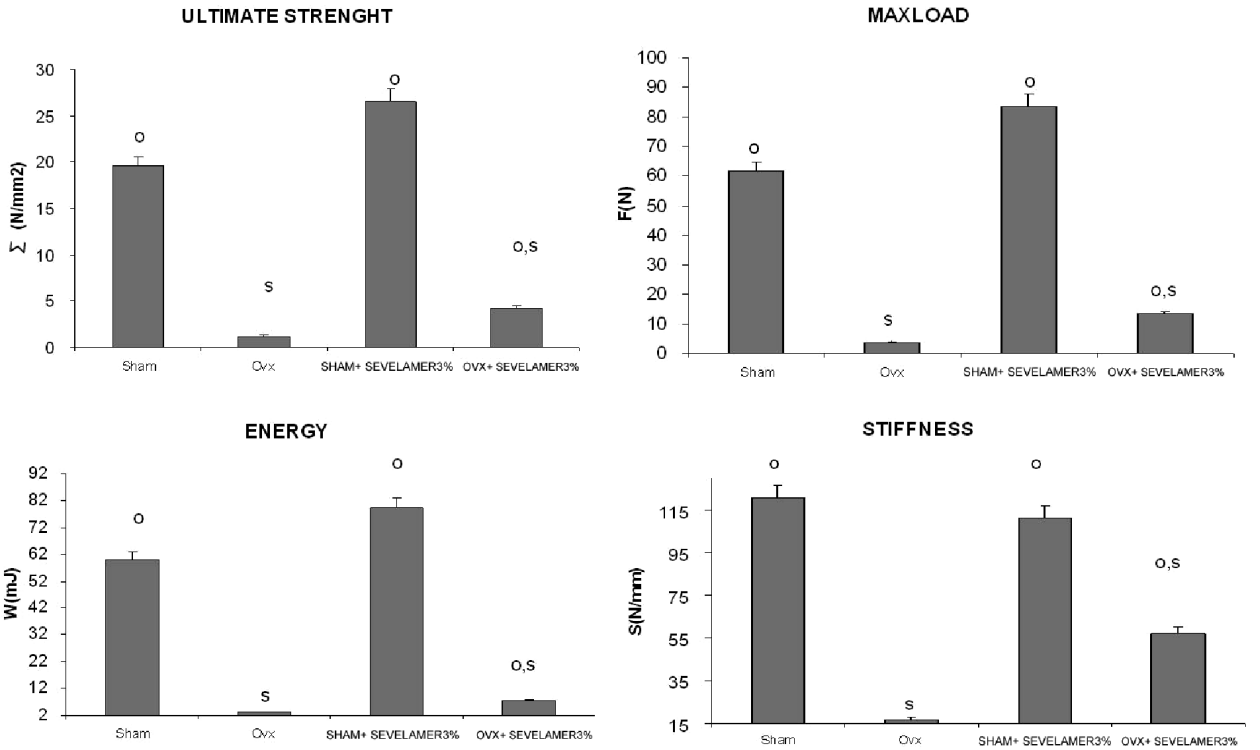


Fig. 4. Biomechanical parameters of the femur from animals treated with sevelamer 3% in the prevention mode of therapy; ultimate strenght(σ)(N/mm^2), maximum load(Fu)(N), energy absorbed(W) (mJ)and stiffness (S) (N/mm).Significant differences are indicated with respect to OVX control (O) and Sham (S) control rats ($p<0.05$ by ANOVA Dunnett test).

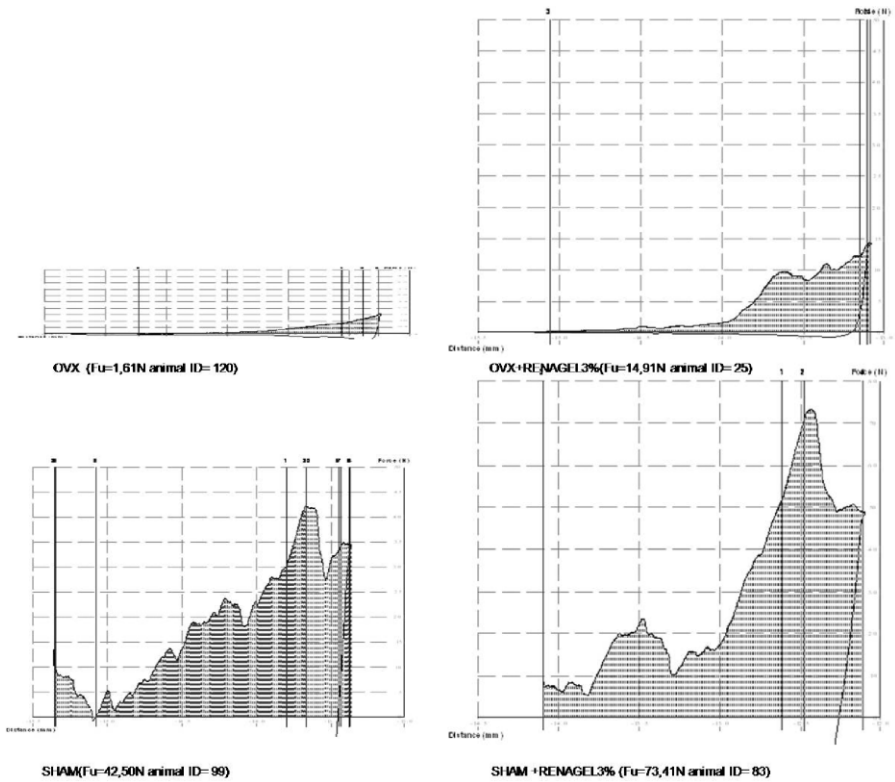


Fig. 5. Indentation test graph-comparison among the animals in different experimental groups; each animal identification number(ID) representable for each treated group.

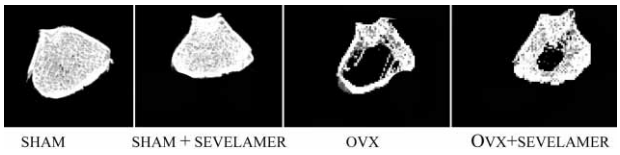


Fig. 6. MicroCT pictures; horizontal cross sections of rat femurs frome our data base correlated with our biomechanical testing. Each microCT picture is representative for each group.

cator for the biomechanical quality of cortical bone. In our previous studies, we explored sevelamer's potential to restore bone and it improved strenght of cortical bone as well as the trabecular bone[1,3]. Results were confirmed by additional imaging methods which examined the bone structure (DEXA, microCT). In this study, we evaluated the preventive influence of sevelamer showed by microCT and DEXA measurements of BMD. Sevelamer did not have an effect on the cortical bone of treated animals, but it had a significant effect on the trabecular bone. Correlations between indentation tests and apparent trabecular bone density have been investigated. It is known that BMD is a good predictor of fractures caused by com -pressive loads, most notably at the spine[13,14]. There are studies which have evaluated correlations between biomechanical bone properties and architectural properties as assessed using MRI and microCT. Pothuaud et al.[15] showed that the proportion of bone (bone volume/tissue volume, BV/TV) computed from MRI scans correctly estimated the mechanical properties of trabecular bone from lumbar vertebras. In animal studies, vertebral trabecular bone specimens were used to assess correlations between microarchitectural parameters and maximum compressive strengths[16]. Microarchitectural parameters (BV/TV, trabecular pattern formation-TBPF, structure model index-SMI, and trabecular number and separation) correlate significantly with failure stress, but the correlations varied according to the time between OVX and termination of the study. These indentation biomechanical results correlated with our microCT results. MicroCT data did not show such a difference as the biomechanical testing. Measurement errors can occur during biomechanical testing. Experimental errors during indentation tests on trabecular bone include artifacts caused by bone damage and friction and during three point bending tests cutting the specimens causes bone damage.

We used standardized test procedures to minimize the measurement error.

In a randomized clinical trial in which 111 hemodialysis patients were treated for 1 year with a calcium-based phosphate or sevelamer, patients who received a calcium based binder showed a decrease in thoracic vertebral trabecular bone density compared with sevelamer therapy and indicated a trend toward a decreased vertebralcortical bone density despite increases in serum calcium levels[1]. Also in patients who received sevelamer only, a decrease in cancellous bone trabecular separation during therapy was demonstrated, suggesting that sevelamer may improve trabecular bone structure and density[17].

Conclusion

Sevelamer has been shown to improve bone volume in preclinical models and clinical trials. Treatment with sevelamer increased the total body bone mineral density in the femur, tibia and lumbar spine and also increased the bone formation rate, cortical thickness, and bone volume, resulting in improved bone biomechanical properties[4].

We used the indentation test to determine the mechanical characteristics of trabeculae of the distal femoral metaphyses. Direct parameters such as maximal load, stiffness and energy absorbed were significantly increased in sevelamer treated rats as compared to OVX control animals; the ultimate strength showed the same trend. We showed that sevelamer treatment improved the trabecular bone parameters as compared to OVX animals, but did not restore the mechanical properties of sham rats. Sevelamer did not prevent the loss of cortical bone strength in treated animals as assessed by three point bending test.

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D. Eljuga

University of Zagreb, Zagreb University Hospital Center, Kišpatićeva 12, 10000 Zagreb, Croatia
e-mail: deljuga@vip.hr

BIOMEHANIČKA SVOJSTVA KOSTIJU ŠTAKORA TRETIRANIH SEVELAMEROM

S A Ž E T A K

Sevelamer hidroklorid koristi se već 10 godina u pacijenata na dijalizi kao fosfatni vezač. Naša prethodna istraživanja pokazala su da oralna primjena sevelamera sprečava gubitak koštane mase i povećava koštani volumen u ovarijektomiranih štakora[1]. U ovom ispitivanju analizirali smo biomehaničke karakteristike kostiju štakora tretiranih sevelamerom pomoću »threepoint bending» testa kako bi se odredila biomehanička svojstva kortikalne kosti središnjeg dijela femura, dok se za mehaničke karakteristike trabekularne kosti distalne femoralne metafize koristio test indentacije. Analizirani parametru su bili: maksimalno opterećenje (F_u), rigidnost (S), apsorbirana energija (W), čvrstoća (T) i maksimalna jakost (σ). Endogene karakteristike, stres, elastični modul i čvrstoća kostiju dobivene su pomoću izmjerenog maksimalnog opterećenja, rigidnosti, apsorbirane energije, vanjskih i unutarnjih dijametara kosti te izračunatog inercijskog momenta koštanog presjeka. Štakori su dobivali sevelamer 25 tjedana sa standardnom prehranom, počevši odmah nakon ovarijektomije (OVX). Životinje su podijeljene u slijedeće grupe : (1) Sham (lažno operirane životinje); (2) Sham + sevelamer 3%; (3) OVX; (4) OVX + sevelamer 3%. Naši rezultati su pokazali da je sevelamer osobito utjecao na trabekularnu kost povečavajući maksimalno opterećenje za 26,2%, apsorbiranu energiju za 24,2% i maksimalnu jakost za 26,2% u životinja koje su dobivale 3% sevelamera u usporedbi sa životinjama koje su dobivale standardnu prehranu. Sevelamer 3% u ovarijektomiranim štakorima je također povećao maksimalno opterećenje za 71,4%, rigidnost za 70,7%, apsorbiranu energiju za 55,9% i maksimalnu jakost za 71,3% u usporedbi sa OVX kontrolama. U »threepoint bending« testu sevelamer je imao mali utjecaj na sprečavanje gubitka jakosti kortikalne kosti. Ovi rezultati pokazuju da sevelamer poboljšava biomehanička svojstva kosti, prvenstveno djelujući na trabekularnu kost u normalnih i ovarijektomiranih štakora.