Original paper

Experimental model of osteoporosis on 14 week old ovariectomised rats: a biochemical, histological and biomechanical study

*Tamara Popović[1], Ranko Šrbić[2], Milica Matavulj[3], Zoran Obradović[4], Sanja Sibinčić[5]

[1]High Medical School, Nikole Pašić 4a, 79000 Prijedor, Republic of Serpska, Bosna and Herzegovina
[2]Department of Farmacology, Faculty of Medicine, University of Banja Luka, Save Mrkalja 14, 78000 Banjaluka, Republic of Serpska, Bosna and Herzegovina
[3] Department of Biology and Ecology, Faculty od Science, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia
[4]Institute for Forensic Medicine, Zdrave Korde 1, 78000 Banja Luka, Republic of Serpska, Bosna and Herzegovina
[5]Health institution Medico-S, Jevrejska 58a, 78000 Banjaluka, Republic of Serpska, Bosna and Herzegovina

Summary. Ovariectomy is one of the most common ways of inducing experimental osteoporosis, since there are no meaningful differences in bone behavior between surgical and natural menopause. Because ovariectomized rat still represent the "gold standard" in studies of postmenopausal osteoporosis, the present study focuses on an animal model of osteoporosis which we developed for researching the effects of medicament and non-medicament treatment of this bone disease. Experiments were conducted on a total of 18 female Wistar rats aged fourteen weeks, randomly divided into three experimental groups: intact (INT) group and two groups of ovariectomized (OVX) animals (OVX I and OVX II), consisting of 6 animals each. Animals are ovariectomized using a ventral approach, which in our experience is more favorable than the midline dorsal skin incision or double dorsolateral approach, both of which are also commonly used for inducing experimental osteoporosis. The OVX I group consisted of bilateral ovariectomized females sacrificed six weeks after ovariectomy, while OVX II was sacrificed eleven weeks after ovariectomy. The group of intact, un-operated control animals (INT group) was sacrificed after eleven weeks together with animals of the OVX II group. Blood samples for measurement of serum alkaline phosphatase, osteocalcin, calcium and phosphorus level are collected from animals of the INT and OVXI group after six weeks and from animals of INT and OVX II groups after eleven weeks. Histological analyses were done on tibia while biomechanical measurements were done on the femurs of animals from INT, OVXI and OVX II groups. In animals of the OVX I group, levels of alkaline phosphatase, osteocalcin, phosphorus and calcium were statistically significantly increased (p < 0.01) compared to intact female (INT group) controls, indicating an increase in bone degradation. Histological analysis shows that the tibia of ovariectomized females are characterized by the appearance of fine thinned trabecular bone, with enlargement of Haversian channels in cortical bone and with gradual transition of lamellar (cortical) bone to cancellous (trabecular) bone. Biomechanical measurements also confirm significant changes in the quality of the bone after bilateral ovariectomy and show increasing fragility of the femur in the biomechanical bending test. In females that were sacrificed eleven weeks after ovariectomy (OVX II group), biochemical parameters also suggest accelerated bone degradation, but signs of stabilization of resorptive activity were present. Levels of alkaline phosphatase, osteocalcin, phosphorus and calcium were significantly increased in OVXII (p < 0.01) vs. INT, but were very close to those measured in the OVX I group. Histological findings for OVXII also indicate loss of longitudinal trabeculae, extension of the medullary canal and extension of the area with bone marrow 11 weeks after bilateral ovariectomy. This is consistent with the bone becoming more porous and increasingly fragile, resulting in lower and lower bone mass, with increasing holes and spaces. In agreement with this, as the bones become more porous, significantly reduced moment of force and deflection in bending was detected. With this experiment we demonstrated that ovariectomy using a ventral approach is a preferable method which provides 100 percent survival of animals as

*Corresponding author, e -mail: tamaralukac@yahoo.com

well as rapid wound healing without complications after the surgical procedure, while results obtained by histological and biomechanical analysis of the bones of ovariectomized animals reliably confirmed the existence of osteoporotic processes that progress over time. Thus, our animal osteoporotic model is suitable for testing the effects of drugs and physical procedures in postmenopausal osteoporosis.

Key words: bilateral ovariectomy with ventral approach, experimental osteoporosis, osteoporotic bone, rat.

INTRODUCTION

Demographic changes in the age structure of occidental populations is one of the main reasons that osteoporosis in recent decades has become a global problem of epidemic proportions. It is believed that 10% of the world's population suffers from osteoporosis. Periods of treatment of osteoporosis are very long and can last for years, or even decades. This is a major challenge for the safety of treatment and long-term effects of drugs used, as well as non-pharmacological therapies on osteoporotic bone. Therefore, the use of animal models of postmenopausal osteoporosis provides more uniform experimental material and allows for assessment of potential therapies (Bouillon et al. 1991). For the study of postmenopausal osteoporosis, the use of animal models can reduce problems associated with studying the disease in humans, such as time and variability in the behavior of test subjects. Moreover, clinical studies are expensive and require longer durations, which is one more reason why animal models play an important role in understanding osteoporosis research. Furthermore, since 1994 the U.S. Food and Drug Administration (FDA) requires data from animal models (FDA 1994) in their guidelines on giving food and medicine to animals for the preclinical evaluation of new drugs for treatment of osteoporosis. Until today there is no recognized animal model of spontaneous osteoporosis, because no mammals except for humans have spontaneous bone fractures in the course of their normal life cycle (Kimmel et al. 1999). Because of this, different experimental procedures to induce continuous bone loss in animals have been developed. Ovariectomy is one of the most common methods of induction of experimental osteoporosis since there are no meaningful differences in bone behavior between surgical and natural menopause (Wronski at al. 1989; Frost and Jee 1992). In addition to ovariectomy, immobilization with gypsum bandages and nerve or tendon cutting lead to severe bone loss in rats (Tarvainen et al. 1994). Rats and dogs are mainly used in experimental studies of osteoporosis, but experiments are also conducted on mouse, pig, sheep and all primates other than humans. Dietary regimes, such as lack of calcium in food and diet regimes with increased protein and phosphate also lead to osteoporotic phenomena and may cause excessive loss of bone mass (Jiang et al. 1997). In addition, inflammation can lead to generalized or localized bone loss (Kimmel et al. 1999).

Two types of ovariectomized rat models have been distinguished based on the ages of the rats, namely, the "aged rat model" and the "mature rat model". The aged rat model is based on animals aged 12 months and older, while the mature rat model is based on animals aged about 3 months. Although the "aged rat model" has many characteristics expected of an animal model, "mature rat models" are widely used to study ovariectomized bone loss because they are cheaper, easier to obtain, and the effects of ovariectomy occur within an acceptable time-frame. Mature or elderly couples are widely used in testing anti-osteoporotic drugs (Kalu 1991).

In light of the above, the aim of the present study was to present our experiences with an animal model of osteoporosis we developed for researching the effects of medicament and non-medicament treatment of this bone disease. The model was induced by bilateral ovariectomy with a ventral approach in rats aged 14 weeks; and validated by monitoring biochemical, histological and biomechanical characteristics of the osteoporotic process that occurs as a result of this surgical procedure.

MATERIAL AND METHODS

Experiments were conducted on a total of 18 female Wistar rats aged 14 weeks with an average of 200-220 g body weight. Animals were housed in plexiglass cages (six animals per cage) with a 12-hour light-dark cycle. Rats were allowed free access to water and commercially standard rodent food.

Animals were randomly divided into three experimental groups: a control intact group (INT), and two groups of bilateral ovariectomized animals (OVX I and OVX II), consisting of 6 animals each. The OVX I group was sacrificed six weeks after ovariectomy since a significant decrease in femur bone was observed. Control, unoperated INT animals were sacrificed after eleven weeks together with the OVX II group.

Experimental procedure

All experimental procedures on animals were performed with permission of the Ethical Committee on Animl Experiments of the Institute "Mlječanica", Republic of Srpska, Bosnia and Herzegovina.

Surgical procedure: bilateral ovariectomy

Bilateral ovariectomy was performed under general anesthesia. Female rats were anesthetized 15 minutes before surgery with ketamine (ketamine hydrochloride injection

USP 50 mg/ml Rotexmedica, Tritan, Germany) at a dose of 0.15 ml/100g body weight (bw) and with diazepam (Bensedin® amp. 10 mg/2 ml, Galenika Beograd) in a dose of 0.1 mg/100g bw. Anesthetics were administered intramuscularly in the gluteal region of each animal planned for operation.

Animals are ovariectomized with a ventral approach (Fig. 1), which we feel is more favorable than the often-applied midline dorsal skin incision or double dorsolateral approaches that are also commonly used for inducing experimental osteoporosis (Waynforth 1980).

Bilateral ovariectomy (longitudinal laparatomy) was performed taking into account asepsis and antisepsis. The operational field was shaved and washed with povidone foam. The incision is made longitudinal along the linea alba to the xyphoid process through the skin, fascia and muscle layer and after abdominal exploration the animal was gentle tilted 45° at kidney level so that the ovary, fallopian tube and uterine horn became visible. Ligature of the right ovary together with the surrounding fatty tissue and the upper third of the uterine horn (Fig. 1) was performed. Then these isolated structures were cut and the rest returned to the retroperitoneal space. The procedure was repeated for the left ovary through the same incision. The wound was closed in two layers (muscle and skin) using absorbable suture Vicryl 3 for muscles and fascia and Vicryl 2 for the skin. Antibiotic powder bacitracin (Bivacyn®, Lek, Ljubljana, Slovenia) was applied to the area to disinfect the skin after suturing. After surgery the animals were housed individually in cages provided with clean and dry bedding sets for a period of one week, in order to avoid hypothermia and prevent possible contamination. No animals were given antibiotic protection. After seven days the wound healed properly.
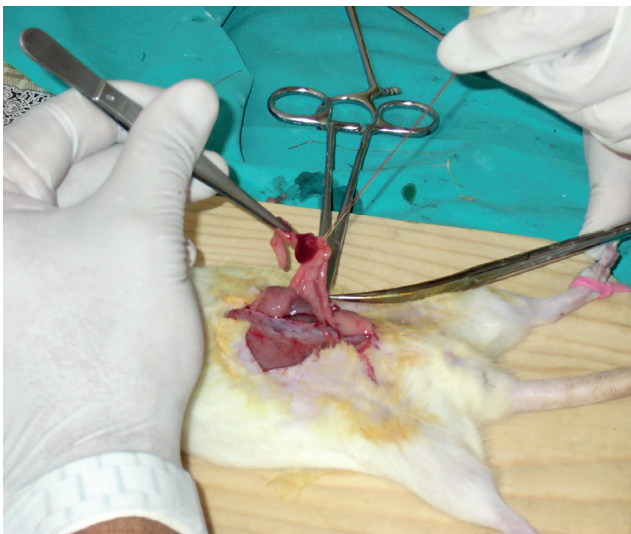

Fig. 1. Longitudinal laparotomy in a female rat.

Biochemical analyses of blood samples

For biochemical analyses, periorbital blood sampling with a heparinized slug was performed. Blood samples were taken from animals of the INT and OVX I group after six weeks and from animals of the INT and OVX II group after eleven weeks. Analyses were done from the serum, which was stored at -20 °C. Osteocalcin levels were determined by Elecsys 2010 (Roche Diagnostics GmbH, Mannheim, Germany) using reagents from the same company, via an immuno-diagnostic in vitro test-method (electrochemiluminiscence).

Alkaline phosphatase (AP) levels, serum calcium (Ca) and phosphorus (Ph) levels were analysed on a Hitachi 912 (Roche Diagnostics, GmbH, Mannheim, Germany). Colorimetric tests were done using reagents from Roche Diagnostics GmmH, Mannheim, Germany.

Histological analysis of bone

Histological analyses were done on tibia of animals from INT, OVXI and OVX II groups. Previously prepared left tibia were fixed in 10% formalin (10 ml bottle) and after 28 days of fixation, tibiae were dehydrated, decalcified by classical histological procedures, embedded in paraffin and cut into 5 μm thick longitudinal sections. These sections were stained with hematoxylin and eosin (HE), analyzed and photographed using a professional compound microscope Reichert-Jung, series 310.

Biomechanical analysis of bone

Biomechanical measurements were done on femurs of animals from INT, OVXI and OXII groups. After sacrifice, femurs were fixed in 10% formalin for four weeks, rinsed with plain water followed by cleaning soft tissue residues and drying at +37 °C for 72 hours. Dried bones were put in a separate holder for biomechanical testing of torsion and bending properties (Fig. 2). For these tests, a TOMI 2001 instrument specially designed for this purpose (Patent Office Belgrade, Official Gazette of the Intellectual Property No. 3/2003, p. 513) was used. The maximum bending moment of bone resistance (Mfmax) was calculated according to the formula

$$Mf_{max} = \frac{F \cdot li}{4}$$ (Nikolić et al. 1988), where F is the force in

N at which there was a fracture of the bone sample tested.

Statistical analysis

Numeric data from the field of descriptive statistics are shown as the mean +/- standard deviation. The significance of applied treatments on biochemical parameters and biomechanical measurements was tested by analysis of variance

Fig. 2. Rat femur prepared for biomechanical bending test.

while differences between means for two groups were compared with the Duncan multiple test of intervals. Statistical analyses were performed using StatSoft, Inc. STATISTICA (Data Analysis Software System), version 10, 2011. Probability values of $p < 0.05$ were considered significant.

RESULTS

There were no procedure-related deaths in the group of ovariectomized females. Also, no infection or wound dehiscence was detected on any operated animals. Together, this highlights the advantages of ovariectomy by a ventral approach, which allows fast and accurate access to the ovary, and causes much less trauma compared with the dorsal approach, where large muscle incisions or double dorsolateral

incisions are made, which may lead to excess bleeding, requiring the use of more sutures.

Results of biochemical analysis of serum

Biochemical analyses included determination of the levels of alkaline phosphatase, osteocalcin, calcium and phosphorus in the serum of females in all three experimental groups, in order to confirm osteoporosis induced by ovariectomy. The results of these measurements are summarized in Table 1.

Six weeks after ovariectomy, alkaline phosphatase (ALP) levels were significantly increased ($p < 0.01$) compared the control INT group, where levels were 78.5 U/L (Table 1). This trend was also present in the eleventh week after ovariectomy, and in the OVX II group levels were 152.7/L vs. 77.4 U/L in the INT group. Similarly, at same time, calcium levels in the OVX group six weeks after ovariectomy were significantly increased ($p < 0.01$), with levels of 2.88 mmol/L vs. 2.45 mmol/L in the control INT group (see Table 2). By the eleventh week after ovariectomy average serum calcium levels were 2.54 mmol/L in the OVXII group, compared to 2.39 mmol/L in the INT group - a statistically significant increased, but statistically significantly decreased compared to levels six weeks after ovariectomy.

Six weeks after ovariectomy phosphorous levels were also significantly increased ($p < 0.01$) (3.64 mmol/L) compared to INT controls, were levels were 2.25 mmol/L (Table 1). However, after eleven weeks, phosphorus levels in the OVX II were significantly reduced compared to OVX I (2.45 mmol/L, 3.64 mmol/L. respectively), but still significantly elevated compared to INT controls (Table 1).

Osteocalcin levels were significantly increased ($p < 0.01$) in OVX I six weeks after ovariectomy (26.8 ng/L) vs. INT, where levels were 12.8 ng/L.

The same upward trend was maintained in the eleventh week after ovariectomy in the OVX II group, where osteocal-

Table 1. Values of measured serum parameters in intact vs. ovariectomized female rats. Mean ± SD are given.

	Alkaline phosphatase (U/L)	Calcium Ca /s (mmol/L)	Osteocalcin (ng/L)	Phosphorous (mmol/L)
INT 6 weeks	78.5±6.70	2.45±0.020	12.8±1.38	2.25±0.017
OVX I 6 weeks	147.8±3.03 [a]	2.88±0.017 [a]	26.8±0.34 [a]	3.64±0.012 [a]
INT 11 weeks	77.4±6.54	2.39±0.020	12.6±1.46	2.15±0.012
OVX II 11 weeks	152.7±4.80 [a]	2.54±0.029 [a]	28.2±0.86 [a]	2.45±0.008 [a]

[a] $p < 0.05$.

Table 2. Femur deflection (mm) and bending force (N) in intact vs. ovariectomized groups of animals (INT, OVXI and OVX II).

Group of animals	Deflection (mm)	Force (N)
INT	0.817	119.7
OVX I	0.398[a]	119.8
OVX II	0.358[a]	118.3[a]

[a] $p < 0.05$.

cin levels were 28.2 ng/L, while in the INT group levels were 12.6 ng/L (Table 1).

Results of biomechanical testing of bone

The average amounts of force required for bone fracture in intact vs. ovariectomized animals (INT, OVXI and OVX II), were expressed in terms of the length of deflection (mm) (see Table 2).

The Duncan multiple range test confirms a statistically significant difference between the average value of femur deflection in intact (INT) vs. ovariectomized animals (OVX I and OVXII), indicating that bilateral ovariectomy led to an increase in bone fragility. The average bending force is decreased significantly ($p < 0.05$) in OVX II compared to the OVX I group, suggesting that ovariectomy decreases bone strength and increases bone fragility in a time-dependent manner.

The results of histological analysis of bone from intact and ovariectomized rats

Histological features of intact rat bones

During tibia preparation, no macroscopic changes in bones were observed of the tibia in any animal from this group. Also, a histological picture of its cross-section show normal histological bone structure. Specifically, at the periosteal side of the cortical bone, connective tissue and muscle are visible; while the endosteal side of the medullary canal is filled with bone marrow (Fig. 3a). The compact bone tissue contains Haverzian and Volkman channels in which blood vessels are embedded with some connective tissue. Bone lamellae in compact bone tissue are dense and oriented in different ways, but most are located concentrically around the Haversian canals (Figs 3b-c).

Histological characteristics of the bones of rats sacrificed six weeks after bilateral ovariectomy (OVX I group)

Macroscopic examination of tibia from females sacrificed six weeks after ovariectomy show no changes in shape and size. Microscopic examinations of cross sections of this tibia reveal that the medullary canal is expanded, with numerous small holes and resorbtion lacunae in the trabecular bone (Fig. 4). In cortical bone, enlargement of Haverzovih channels and gradual transition from lamellar to trabecular bone are visible (Fig. 4a). The trabecules of cancellous bone are short and parallel, oriented toward the stress line and connected by transverse short trabeculae; thus cancellous bone trabeculae contain structures in the form of the Latin letter H. Microscopically, these bones are characterized by

the appearance of fine thin trabecular bone, with enlargement of Haversian channels and a gradual transition from lamellar cortical bone to cancellous, trabecular bone (Fig. 4c).

Cortical bone changes are even more evident in the tibia of ovarioctomized rats (Fig. 5). Spreading of Haversian channels suggests that bone degradation predominates osteoblastic bone replacement, leading to the gradual transition of cortical to trabecular bone. The existence of so-called cement lines around Haversian channels within osteons - which is the interface between the 'fibers' (osteons) and extraosteonal bone matrix - provide evidence anabolic activity exists in osteoporosis, but is insufficient to maintain a balance with
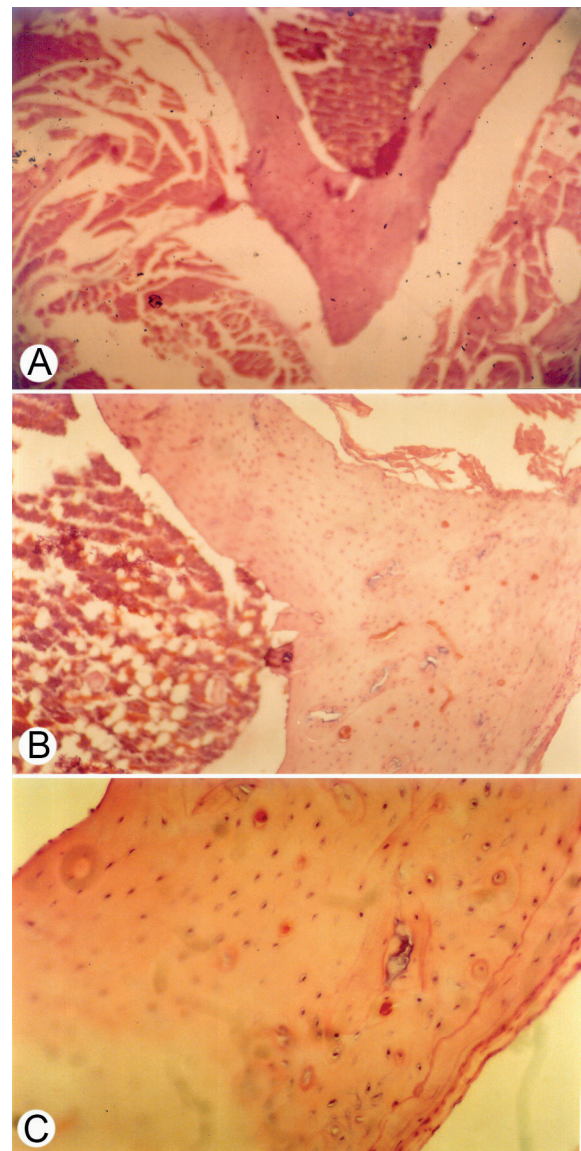

Fig. 3. Cross-section of the tibia of intact rat (INT group) stained with HE (A, oc. 10, obj. 4; B, oc. 10, obj. 10; C, oc. 10, obj. 25).
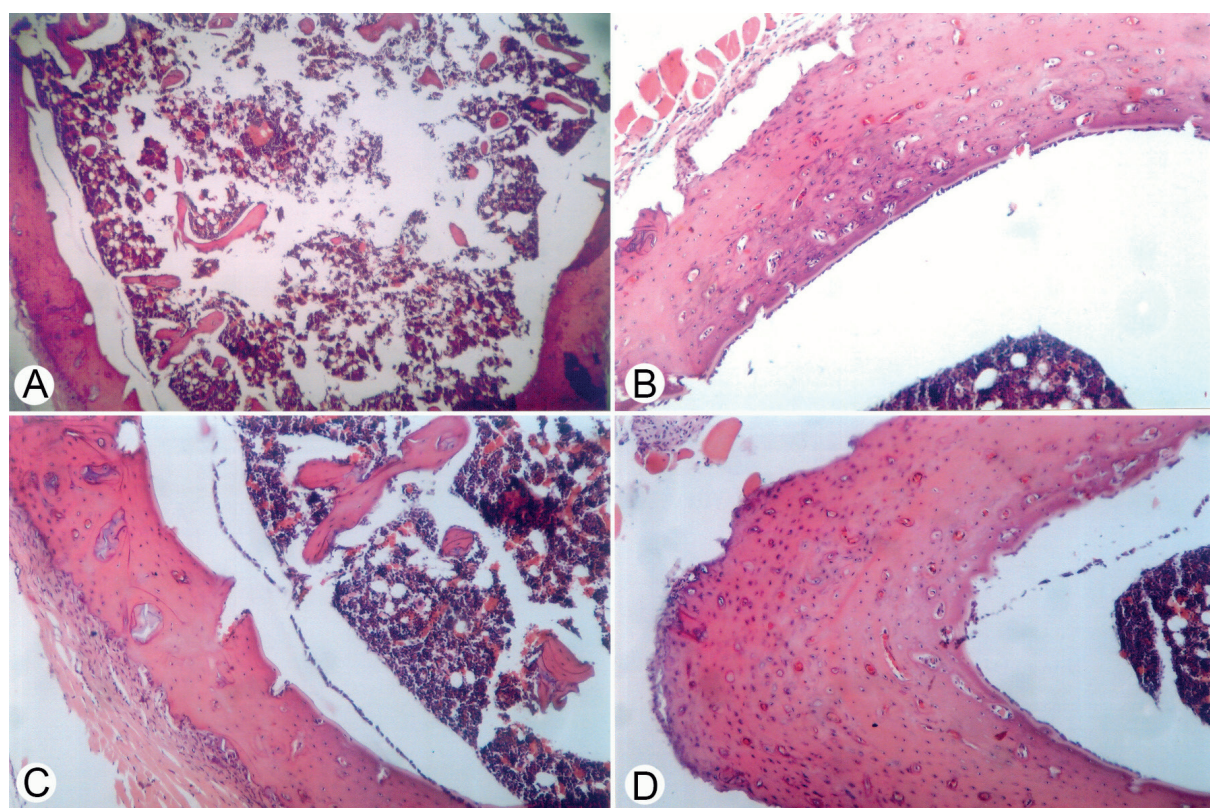
Fig. 4. Cross section of tibia from rats sacrificed six weeks after ovariectomy (OVX I group) stained with HE (A, oc. 10, obj. 4; B, C and D, oc. 10, obj. 10).

otherwise normal catabolic activity. The number of cement line indicates the number of attempts by the bone to compensate for the loss of tissue (Burr et al. 1988). The distance between these lines corresponds to the amount of bone tissue formed in each period of osteoblastic activity, and an increase in closeness (to complete the merger) suggests that bone compensation was not achieved (Figs 5a-b). Over time, the whole osteon becomes degraded, replaced by a cavity filled with bone marrow (Fig. 5c) and cortical bone assumes the histological characteristics of trabecular bone (Fig. 5d).

Histological characteristics of the bones of rats sacrificed eleven weeks after ovariectomy (OVX II group)

The outside tibia of females sacrificed eleven weeks after ovariectomy (OVXII group) does not deviate in shape and size from normal control animals. However, microscopic examination of cross sections of this tibia reveal further progress of osteoporosis. The medullary canal and area containing bone marrow are more extended. Also trabecular bone is increased compared to the OVXI group (Figs 6a-c). In some places in the cortical bone, the whole osteon is decomposed and replaced by a cavity filled with bone marrow. Thus, this cortical bone has histological features of trabecular bone

(Figs 6d, 7b) suggesting that the bone has become much more porous. Centers of enchondral ossification are visible here, while trabeculae are perforated and quite thinned. The trabeculae within cancellous bone are short, parallel and transversally connected, aligned towards the mechanical load distribution (Fig. 7b).

DISSCUSION

Osteoporosis is a global health problem associated with increased bone fractures, which in most cases result in severe disability. Thus, reliable osteoporotic animal models in which loss of bone mass is easy to achieve, are important for further examination of this bone disease, and development of new or improved therapies. In order to test new pharmacological and non-pharmacological treatments of osteoporosis, we attempted to establish a modified animal model of osteoporosis induced by bilateral ovariectomy. Because most rat models of osteoporosis use sexually mature females at least three months of age, in our experiment females were fourteen weeks old when they are ovariectomized. Although according to the literature a dorsal approach after Waynforthu (1980) is often used, in the present study we used an anterior approach, Ovariectomy by dorsal approach can be conducted
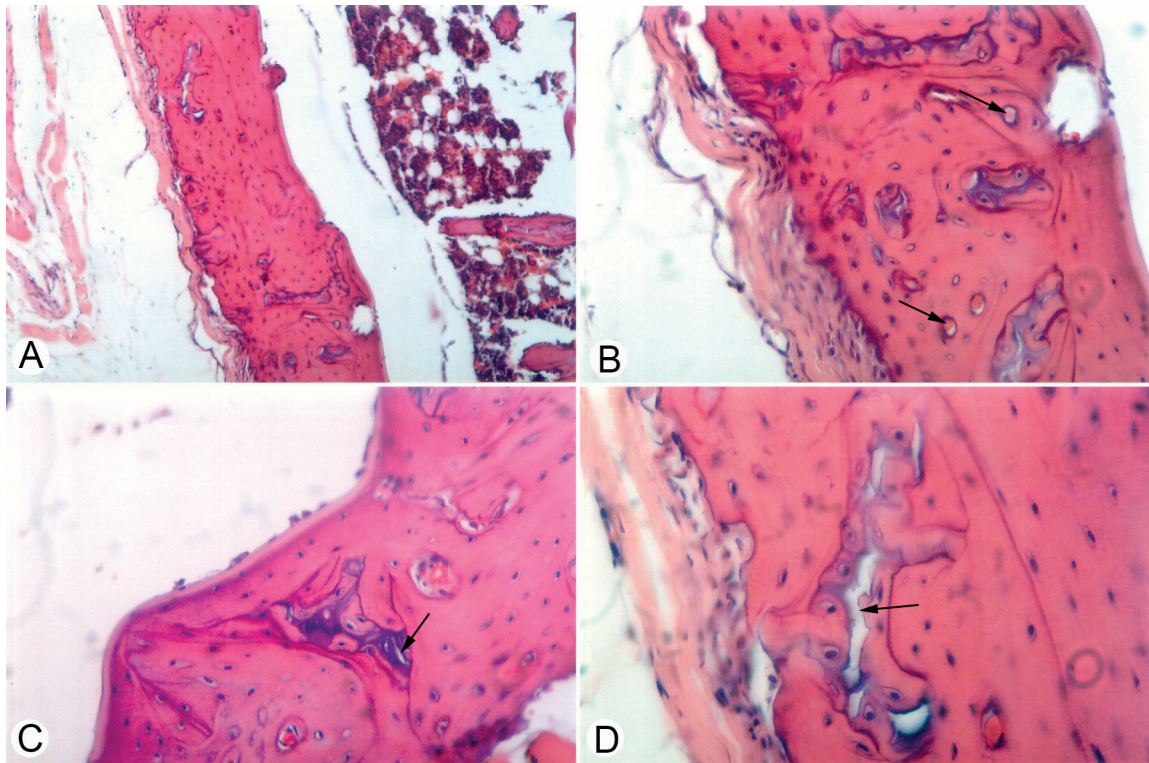
Fig. 5. Cross-section from the tibia of rats, six weeks after ovariectomy stained with HE. Note a number of small holes (B, arrows) and large resorption lacunae (C and D, arrows) (A, oc. 10, obj. 10; B and C, oc. 10, obj. 25; D, oc. 10 obj. 40).
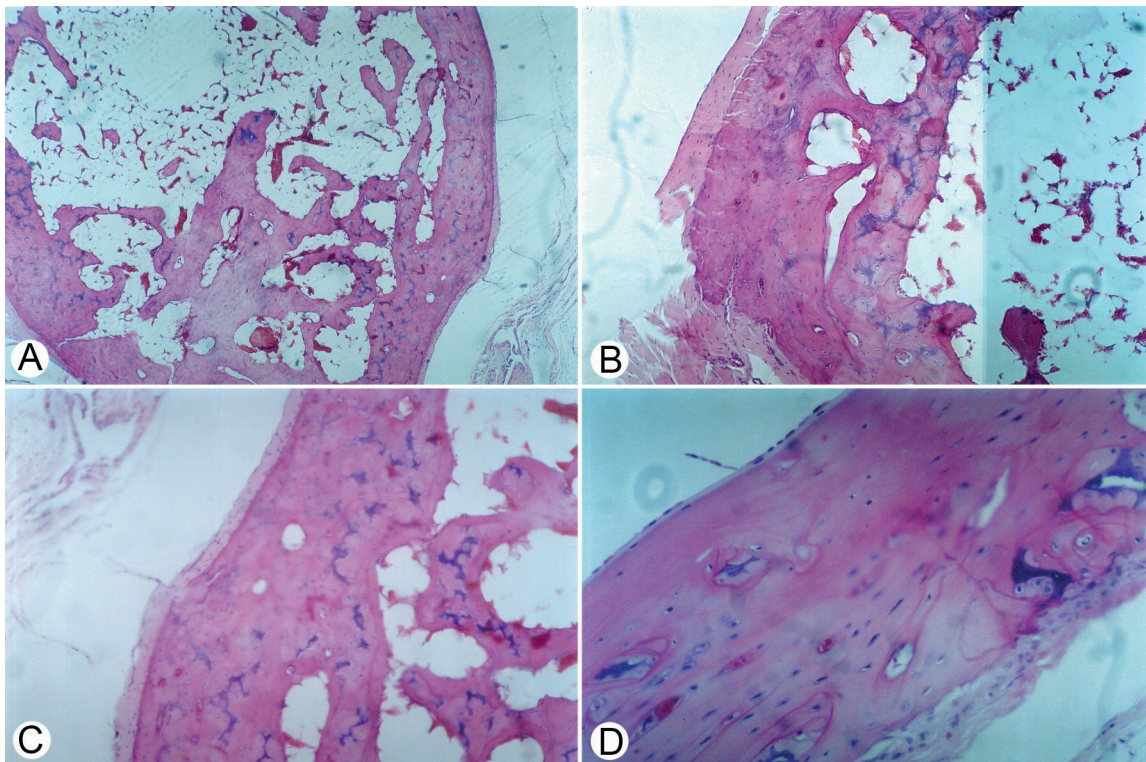

Fig. 6. Cross-section from the tibia of a rat, eleven weeks after ovariectomy (OVX II group), stained with HE (A, oc. 10, obj. 4; B and C, oc. 10, obj. 10; D, oc. 10 obj. 25).
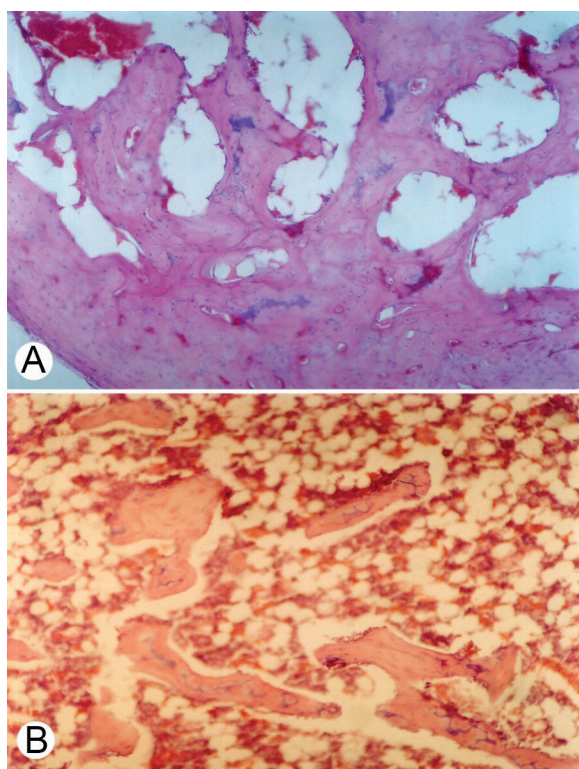
Fig. 7. Cross-section from the tibia of a rat eleven weeks after ovariectomy (OVX II) stained with HE (A and B, oc. 10, obj. 25).

via one of two methods (Lasota and Danowska-Klonowska 2004). In the first, ovariectomy is preceded by a mid-line dorsal skin incision, 3 cm long. After removing the ovary, the previous incision of the muscle requires suturing. In the second variant, ovariectomy is performed by two dorsolateral incisions, approximately 1 cm long above the ovaries. With the use of sharp dissecting scissors, the skin is cut almost together with the dorsal muscles and the peritoneal cavity is accessed. Because there is no need for muscle suturing, researchers chose this approach as more comfortable. Ventral access was recommended by Khajuria and coworkers (2010) as a new method of transversal ventral ovariectomy in female rats. This method is minimally invasive, technically easier, less time consuming and showed less wound healing duration. However, the ventral approach we chose (longitudinal laparotomy by the line alba to xyphoid process) provide the operator better visibility of the surgical field and diminish the risk of potential bleeding during surgery. This operation was performed under general anesthesia with ketamine and bensedin, which proved to be a very good anesthetic choice given that survival of animals post operation was 100 percent. Animal recovery after surgery passed normally with no wound infections even though animals did not receive post-operative antibiotic coverage. Only local antibiotic powder was used, while some laboratory animals in this period receive parenteral antibiotic coverage (Aydin et al. 2012). In

our experience, "matured osteoporotic model" develops in rats in the period of five to eleven weeks after bilateral ovariectomy. In the present study we chose six weeks after ovariectomy (OVX I group) for the first control period, in order to verify bone loss. Results of histological, biochemical and biomechanical analyses of bones from these animals confirmed the existence of an osteoporotic model. Histological images of the proximal part of the tibia here are characterized by the appearance of fine thin trabeculars, extending of Haversian and medullary canal and the gradual transition from lamellar (cortical bone) to spongiosa (trabecular bone). This is consistent with the findings of Chang and coworkers (2003), who showed by histomorphometric analyses that there is a significant loss of trabecular bone in the proximal part of the rat tibia one month after bilateral ovariectomy. Other studies, both on animal models and from clinical studies, confirm the loss of bone mass after the fall of estrogen levels (Recker et al. 2000; Compston 2001), in agreement with the finding that estrogens have multiple receptor-mediating effects on the activity of osteoblasts in order to ensure the right balance of bone resorption and formation (Riggs and Spelsberg 1996).

In animals from the OVX I group, levels of alkaline phosphatase, osteocalcin, and phosphorus were statistically significantly increased, while calcium levels were statistically non-significantly increased compared to intact conrol females (INT group). These results indicate an increase in bone degradation. Biomechanical measurements also confirm significant changes in the quality of the bone after bilateral ovariectomy and increased fragility in the bending test.

In females sacrificed eleven weeks after ovariectomy (OVX II group), biochemical parameters also indicate the existence of accelerated bone degradation. However, there is also a stabilization of resorptive activity. Although levels of alkaline phosphatase, osteocalcin and phosphorus were significantly increased compared to the intact group, these levels were very near those measured in the OVX I group. Histological findings also indicate that the extended period of 11 weeks after bilateral ovariectomy caused loss of longitudinal trabeculae, extension of the medullary canal and extension of the area with bone marrow. These results are consistent with the bone becoming more porous and increasingly fragile, in agreement with the significantly reduced moment of force and deflection in bending. Thus, results from the present study suggest that determination of biomechanical properties of bone substantially contribute to bone tissue analysis. In fact, significantly positive correlations were found between bone strength, elasticity and bone quality. Peng et al. (1994), using a "matured rat model", found significant decreases in maximal load of the femur neck (15.8%) and a reduction in the extreme load bending of the tibial body (8,7%) in ovariectomized rats. Ovariectomy was also found to reduce the ultimate load at pressure on the neck of the femur by 12% (Lepola et al. 1993) and decrease the stiffness and strength of

the proximal femur compared to controls (Bago et al. 1997); and caused 16.6% reductions in the maximum torque value (Peng et al. 1994). On the other hand, there are also finding that suggest no significant changes in the biomechanical properties of long bones induced by ovariectomy (Sano et al. 1999). Furthermore, some studies even show improvements in biomechanical characteristics after ovariectomy. Aronson and colleagues (1994) using a "matured rat model" found a significant increase in torsional resistance, strength changes and angular distortion of femur tested on torsion. The conclusion from all of these studies is that the "matured rat model" is adequate for experimental tests of different effects on bone tissue, which is also supported by our present research.

In addition to ovariectomy, osteoporosis in animal models can also be induced by immobilization with injection of BTX (Botulinum toxin Type A) in the femoral m. quadriceps of rats, mice or sheep (Atmaca et al. 2013; Lodberg et al. 2015). No statistically significant difference exists in the decline in bone mineral density between ovariectomized and BTX-treated rats (Atmaca et al. 2013). However, both methods of osteoporosis induction have their disadvantages. In ovariectomized animals, a decline in estrogen provides irreversible systemic effects not only on the bone, but also in other tissues and organs. On the other hand, the BTX model is easy to implement and provides reversible and regional changes in bones but the rate of bone loss is faster after ovariectomy. In our experiment we also showed that ovariectomy caused rapid (six weeks) loss of bone, induced weakening of bone mechanical properties and increased bone fragility. Treatment with BTX-A increases the power of healing in fixed fractures and leads to a reduction of callus, as it is made by hard fixation (Aydin et al. 2012). Hao and colleagues (2012) have investigated how local muscle atrophy and dysfunction affect fracture healing in BTX model fractures. By biomechanical testing they found that femur from animals treated with BTX have weaker mechanical properties with respect to callus quality compared to control femurs from animals treated with saline. Accordingly, these authors suggest that muscular atrophy and dysfunction here lead to mechanical instability of the callus. For these reasons, these researchers do not recommend the use of BTX-A models, and instead prefer OVX models for studies that estimate the treatment of fractures and osteoporosis at the same time. In contrast, ovariectomized rat models still represent the "gold standard" for studies related to postmenopausal osteoporosis (Tokuyama et al. 2015) and for testing the effects of physical modalities and antiresorptive therapy in estrogen deficient osteoporosis.

In the present study, we demonstrate that ovariectomy with a ventral approach is a comfortable surgical procedure which provides a 100 percent survival of animals as well as rapid wound healing without complications post-operation. Results obtained by histological and biomechanical analyses

of the bones of overioctomized animals reliably confirm the existence of osteoporotic processes that progress over time. Therefore, the animal osteoporotic model described in the present study is suitable for testing the effects of drugs and physical procedures in postmenopausal osteoporosis.

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