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Original article

Experimental osteoporosis in sheep – mechanical and histological approach

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Abstract

The implementation of new methods of osteoporotic therapy requires tests on animal model. The use of sheep as model has numerous advantages over other animals. The aim of this study was to describe the change in parameters in sheep with osteoporosis induced using steroids and ovariorectomy methods as opposed to the parameters in healthy sheep. The study was performed on female „merinos" breed sheep divided into the three groups: negative control (NC) – healthy animals, positive control (PC) – ovariorectomized animals and steroid control group (SC) – in which methylprednisolone was administered. This paper presents histological and ultrastructural examination with mechanical comparative tests for force/strength values as well as indentation tests of joint cartilage. The obtained results confirm the loss of bone mass associated with mineral composition content in bones, which has an influence on bone strength.

Key words: osteoporosis, animal model, indentation, mechanical tests, SEM x-ray

Introduction

Osteoporosis is characterised by reduced bone mass and microarchitectural deterioration of bone tissue. These changes result in reduced bone strength and increased risk of bone fractures. The most prone to fracture are: neck of femur, wrist, pelvis, and lumbar vertebrae. Fractures occur without direct cause or they follow minor injuries which would not pose any threat for healthy bones. The risk of disease increases with age (Cummings and Melton 2002, Poole and Compston 2006). Osteoporosis and related fractures are a major cause of pathogenicity and mortality in the aging population (Bartl et al. 2007). It is estimated that only 25% of patients recover from hip fracture, and more than 50% require care, including 20% who require constant care. Mortality during the year amounts to 40% (Spławiński et al. 1997). Osteoporosis has become one of the priorities of modern medicine, which is seeking new effective drugs. Treatment consists

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mainly of healing of fractures as well as increasing bone mass and bone strength (David et al. 1996). Secondary osteoporosis is associated with other diseases or their effects (steroid osteoporosis associated with hyperparathyroidism caused by malabsorption of vitamins C, and D, long immobilization, chronic treatment with heparin and the abuse of alcohol and nicotine). The causes of osteoporosis are different in specific cases, but the disease always involves jeopardizing the equilibrium between the activity of bone formation and the resorption of stimulating agents. Stimulators of osteogenesis include: estrogens, androgens, calcitonin, insulin-like growth factors (IGF-1, IGF-2), interferon-gamma (IFN-γ), prostanoids, mechanical forces and parathyroid hormone. Pacemakers of osteoresorption are 1.25 (OH) 2D3, thyroid hormones, glucocorticoids, tumor necrosis factors-alpha (TNF-α), transforming growth factor-α (TGF-α), free radicals, immobility. The emergence of new generation antiosteoporotic drugs still requires testing them on animal models. Therefore the aim of this study was to describe as accurately as possible the changes occurring in the bone under the influence of experimentally-induced osteoporosis in sheep in the case of methylprednisolone use.

Materials and Methods

The study material consists of „merinos" breed sheep, female, aged 5-6 years, weighing 50-60 kg (N=49). Every sheep was weighed before the start of testing. Animals were divided into three groups. The first group consisted of sheep which underwent ovariectomy and a steroidal therapy (SC) (n=35). The second and third groups of sheep were considered reference groups (PC n=7 and NC n=7). The NC group (negative control) underwent neither ovariectomy nor the steroidal therapy at a later stage (KS). The PC group (positive control) consisted of sheep which only underwent ovariectomy. All the animals during the test were kept in the same conditions. In order to reduce their physical activity sheep were kept in boxes of the area of 10 m^2, three animals per one box. Throughout the testing period, in order to maintain the diet with a low content of protein and mineral components, the sheep were fed twice a day with hay only (water without limits). After a two-week acclimation period the SC and PC group animals underwent ovariectomy.

Thirty days after the surgery, methylprednisolone at a dose of 150 mg/sheep started to be administered. The injections were repeated four times at 20-day intervals. 21 days after the last application of steroidal medication, the animals were subjected to euthanasia. At the final stage, through dissection, bone samples were collected for further studies.

Histological examinations

The study material for histological examinations included bone fragments from proximal and distal femoral epiphyses, distal humeral epiphyses and proximal and distal radioulnar epiphyses. The material was fixed in 4% formalin solution buffered with calcium carbonate for a period of 3 days.

After fixation the material was rinsed in running water and was subjected to decalcification in two phases: in 10% disodium edetate for a period of 10 days and in a mixture of formic acid plus sodium citrate for a period of 25 days. The material was then dehydrated in an alcohol series and embedded in paraffin. 4-6 μm thick sections were stained with haematoxylin and eosin according to the Goldner-Mason and Van Gieson method. The samples were analysed using a Nikon ECLIPSE 80i microscope in polarised and non-polarised light. With the application of NIS-elements AR software, the morphometric analysis of thickness of articular cartilage and its associated bone tissue was performed and bone porosity was measured. In addition, epifluorescence analysis of hydroxyapatite present in bone and cartilage tissues was performed. A level of epifluorescence and distribution of crystals in bone trabeculae were analysed with the use of the microscope specified above and with the application of Nikon UV-2A filters characterised by inducing waves with their length of EX 330-380.

For the examination of the bones in the scanning electron microscope (SEM), bone samples were fixed in a 3.5% solution of glutaraldehyde on 0.1M phosphate buffer at pH 7.2 – 7.4 for 3 hours and then washed in buffer and fixed in a 1.0% solution of osmium tetroxide, prepared on the basis of phosphate buffer. These samples were dehydrated in a series of alcoholic – acetone increasing solutions and sputtered with gold.

The material was analyzed in a Zeiss LEO-435 scanning electron microscope equipped with a Roentec X-ray microanalysis device.

Cartilage tissue indentation examinations

Specially designed brackets were used for the testing of indentation of cartilage tissue originating from sheep epiphyses and menisci. They allowed the samples and their arrangement to be controlled precisely so that an indenter was always directed perpendicularly to the examined surface. An indenter of 1 mm diameter was used. The linear velocity of the indenter during measurement was 1 mm/min. Initial loading of the samples was 1N. The maximal force (pressure) determined for the selected measuring

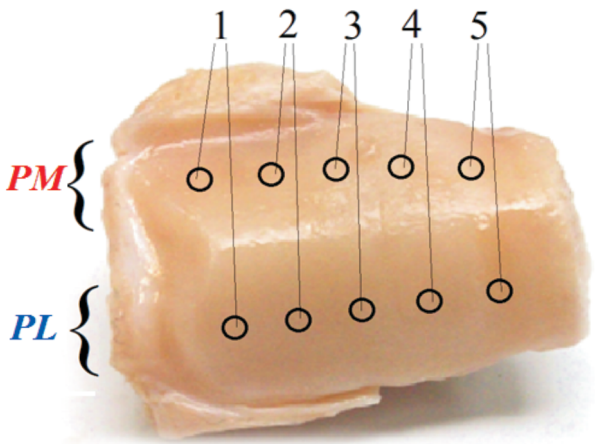


Fig. 1. The layout and description of the measurement points 1-5 (PM – medial part – PL Lateral part) for patella in indentation test.

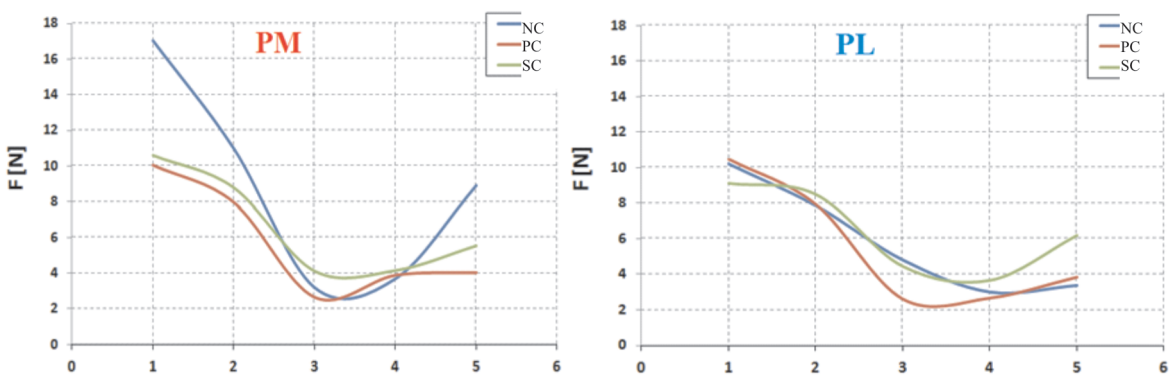


Fig. 2. Fmax values obtained in the indentation test of patellar cartilage measurements for 3 groups: the NC the PC and the average values for SC.

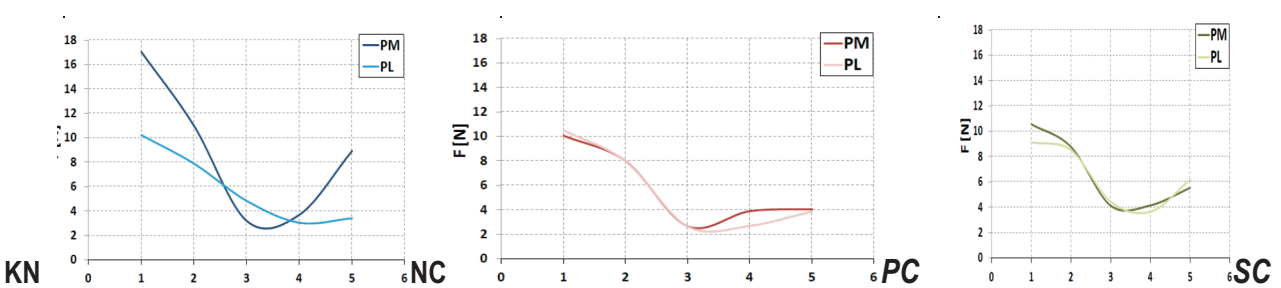


Fig. 3. Fmax distribution of values obtained in measuring the indentation for each point 1-5, (PM – part of the medial, PL – lateral part) of the patella for groups NC, PC and SC.

points was recorded for the indenter at a depth of 0.3 mm. The examinations were performed using an MTS Synergie 100 universal strength testing machine.

Mechanical tests

Cubic samples with dimensions of 10x10x10 mm were prepared in order to determine the mechanical parameters of bone tissue of proximal femoral bone epiphyses, proximal humeral bone epiphyses and patellae originating from sheep from each of the examined groups. The samples were cut out at a speed of 0.05 mm/s with the use of an Accutom-5® metallographic cutter STRUERS with a cutting precision of 5 µm, and additionally equipped with its own cooling system. The samples were prepared in such a manner that the direction of their load was compliant with the direction of force (pressure). One sample was cut from every bone. The samples – until they were meas-

ured – were stored in shallow multi-well plates at a temperature of -20°C. All tests were performed in a room with an air temperature of 22-23°C and air humidity of 55-57%. The tests were performed with the use of an MTS MiniBionix 858 strength testing machine. The measurement of every sample and the acquisition of measurement data were controlled by means of FlexTest software developed by MTS. The samples were loaded using an MTS 242.02 hydraulic cylinder within a range of motion of 100 mm. The level of force/strength reaction of the samples being stretched was measured using an MTS 661.19F-03 device. The evaluation of levels of indentation force/strength of cartilage tissues within proximal femoral bone epiphyses was performed at 9 measurement points. In order to run comparative analyses on the obtained results, a level of indentation force/strength was read out from the force/strength-displacement chart for displacement of 0.15 mm. The levels of indentation force/strength of cartilage tissue within proximal humeral bone epiphyses were determined for two circles: one inner circle for which the force values were recorded for 7 measurement points and one outer circle – also for 7 measurement points. The levels of indentation force/strength of cartilage tissue tests within patella and its epiphysis were performed at 5 measurement points within two areas (PM – medial section and PL – lateral section), as shown in Fig. 1. The measurements of the force values were read out from the force /strength-displacement chart, as in the previous stages, for displacement of 0.15 mm. The next step of the examinations was to measure the maximal force/strength of indentation of menisci for which measurements at 5 selected points were performed, as presented in detail in Figs. 2, 3. The properties of cartilage tissue heavily depend on its hydration, and therefore each of the examined samples was stored and constantly hydrated in a PBS solution. The examinations were conducted using an MTS Synergie 100 machine equipped with an indenter (ϕ=1mm). The samples were loaded (burdened) to a displacement of 0.3 mm. The maximal force/strength was recorded for each of the measurements.

Data are presented as mean standard deviation. A two-sided, nonpaired t -test was used to analyze the morphometric data obtained from bone morphometry, element content and biomechanical tests using Statistica 6.0 software. Results were regarded as statistically significant if $p<0.05$. A test of interaction was done on all subgroups to establish if the difference in effect size between subgroups was statistically significant.

Results

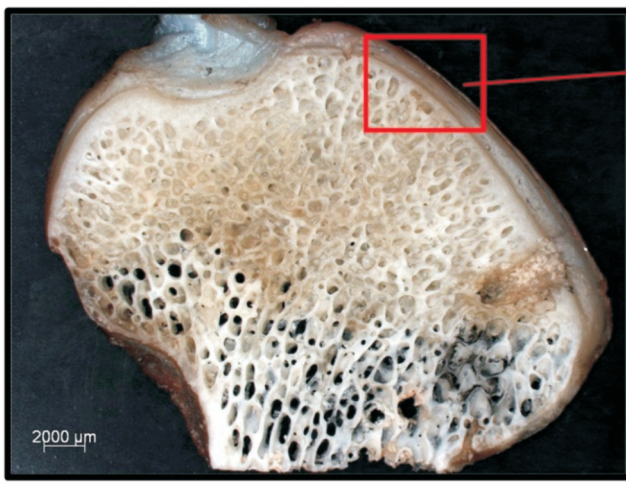
Morphometric analysis

In the analysed bone samples originating from the steroidal group (SC), repeated changes were noted primarily within the structure of articular cartilage tissue and the directly adjacent bone tissue. Changes in the structure of cartilage tissue were related primarily to thickness changes of individual areas having an impact on its final thickness. In some places residues of cartilage tissue inside bone trabeculae could be seen. This demonstrated a rapid rate of bone formation in the period preceding the experiment. The cartilage was smooth and without significant degenerative changes on all articular surfaces. The relation of collagen fibres to proteoglycans within surface layers was normal. Bone tissue lying directly under the articular cartilage demonstrated a varying range of organisation – from simple sets resembling gothic arches, falling directly in contact with articular cartilage, through thicker structures, up to bone tissue having its own osteons with numerous interstitial lamellae. The individual thickness values obtained in the morphometric examinations are presented in the charts.

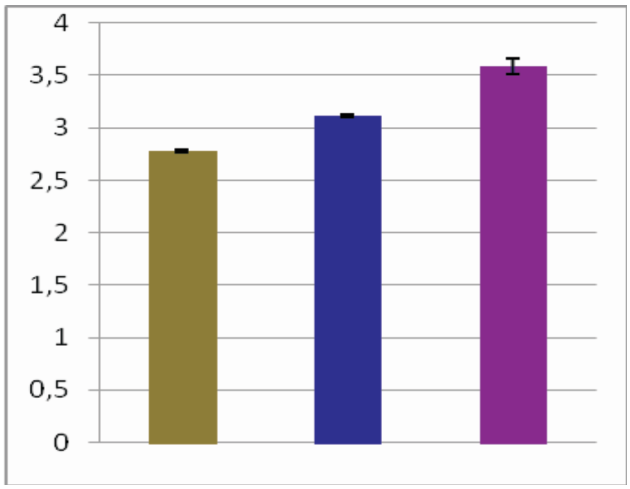
The maximal average thickness of bone tissue was observed at proximal femoral bone, proximal and distal humeral epiphyses. The minimal thickness of bone tissue was found in the distal femoral bone epiphyses.

The maximal average thickness of cartilage tissue was observed in the proximal femoral bone and proximal radioulnar epiphyses. The minimal thickness was found in the distal radioulnar and distal humeral epiphyses. In order to determine more precise changes taking place within tissues of the examined articulations, the ratio of the thickness of cartilage tissue to bone tissue was specified. The ratio will reduce in areas of mineralisation disturbance. Increased thickness levels of both articular cartilage and bone tissues were observed in the steroidal group. In practical terms this indicates that thicker bone tissue is needed to maintain the mechanical properties of joints. It also indicates that the mineralisation and synthesis of bone tissue (and not its resorption) are the major cause of changes occurring in osteoporosis (Fig. 4).

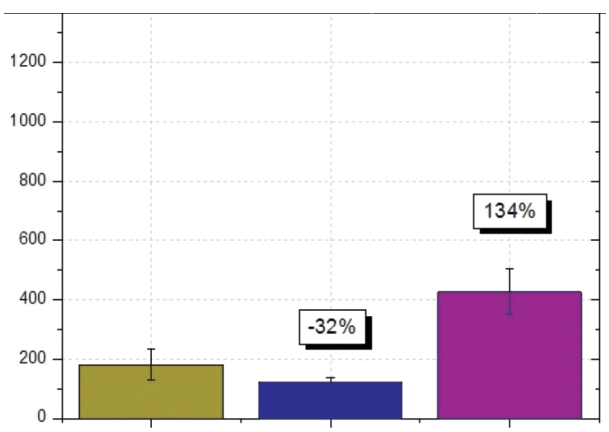
In morphometrical analysis of bone porosity level it was found that highest osteoporosity was noted in the SC group and lowest in the PC. This indicates that the use of both osteoporosis inducing factors – ovariorectomy and steroid therapy – results in the fastest bone loss. This is 3 times faster when compared to the NC group and 4 times to the PC group.



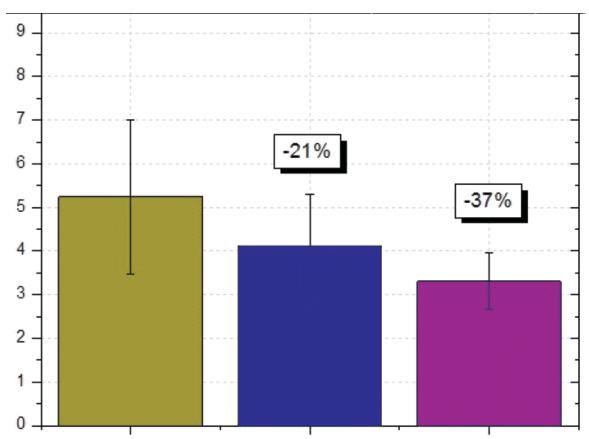
Localization of measuring area of the thickness of bone and cartilage in the proximal femoral epiphysis



Bone porosity Green – NC Navy blue – PC Violet – SC



Thickness of the bone tissue: Green – NC Navy blue – PC Violet – SC



Relative thickness of the cartilage and bone: Green – NC Navy blue – PC Violet – SC

Fig. 4. Results of bone morphometry.

Table 1. Elements distribution in bone samples before and after experiment.

	NC		PC		SC	
	before	after	before	after	before	after
C	44.865	69.06	43.01	20.31	21.34	72.46
Ca	6.6	1.53	4.38	11.56	11.25	1.24
O	43.225	28.04	47.51	58.55	59.83	25.21
Na	1.121	0.24	1.92	1.84	0.86	0.21
Mg	0.25	0.035	0.25	0.14	0.18	0.03
P	3.815	1.095	2.63	6.97	6.54	0.85

SEM X-ray microanalysis

The analysis of calcium and phosphorus content in the examined samples (which are connected with the process of mineralisation of bone tissue) and calcium/phosphorus ratio clearly demonstrated that the highest level of calcium and phosphorus occurred in the NC group. There was also a normal calcium/phosphorus (Ca/P) ratio in this group.

As for the PC group, the ratio decreased by 21%. It can be therefore concluded that there occur disorders in the process of mineralisation in animals treated with ovariectomy. The conclusion is additionally confirmed by the results obtained from the analysis of the element composition of cartilage and bone tissues. The content of calcium in the examined samples decreased by 20% and phosphorus by 7%. The Ca/P ratio also decreased by 15% (Table 1).

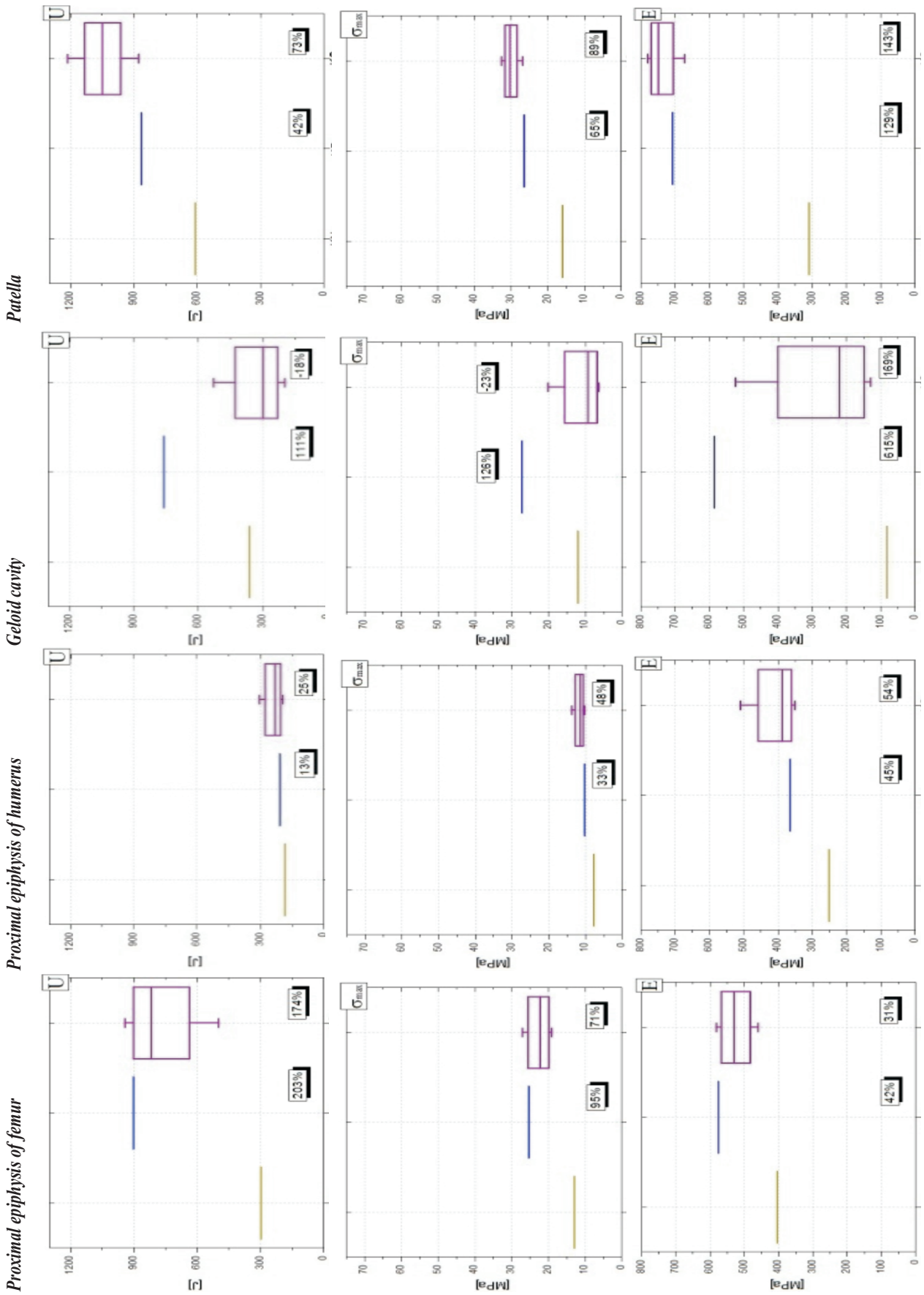


Fig. 5. Comparison chart of Young's modulus (E), compressive strength (smax) and strain energy (U) obtained for samples of bone for proximal epiphysis of the femur and the humerus, the glenoid cavity and the patella Green – NC Navy blue – PC Violet – SC

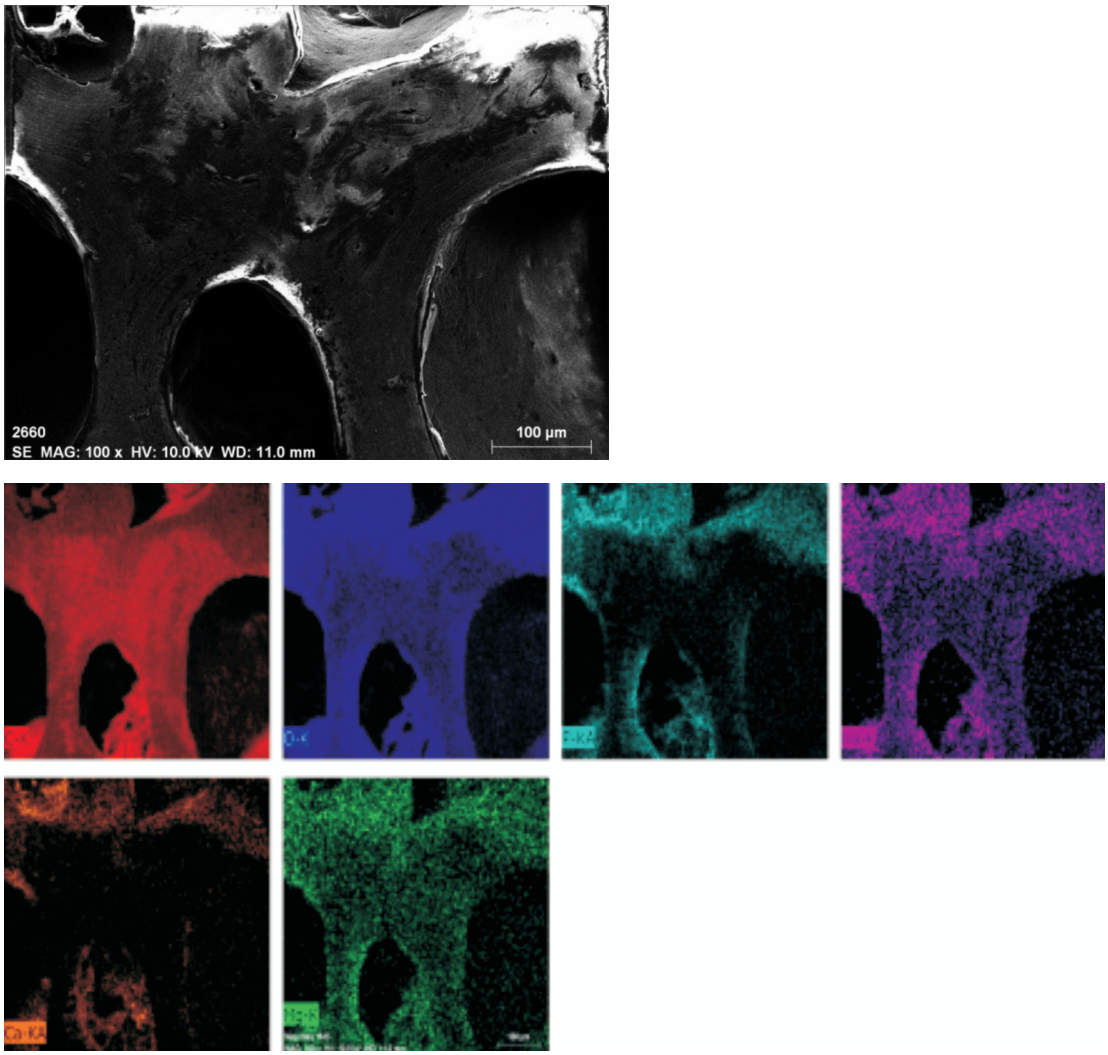


Fig. 6. Steroid group (SC). Sample of elements distribution. The head of the femoral proximal epiphysis. The elements from left to right, top to bottom: carbon, oxygen, phosphorus, sodium, calcium and magnesium. Decreased level of phosphorus, sodium and calcium in trabecules can be observed.

The detailed distribution analysis of elements showed that their distribution in the NC group was at a normal, steady level. In particular, this refers to carbon which occurs in all the observed samples. Calcium and phosphorus atoms are present in bone trabeculae, at some places within the surface, which is related to the presence of osteogenetic cells. A reduced amount of calcium within the area of bone trabeculae – while maintaining a normal distribution of phosphorous – was observed in the positive control group. This demonstrates that the process of mineralisation is disturbed. The disturbed process of mineralisation is also seen in the group of steroidal animals. Within femoral bones, in contrast to other groups, these disturbances also apply to phosphorus, sodium and oxygen. Magnesium is evenly distributed throughout the examined ample. There is almost no calcium inside the bone trabeculae – it is possibly replaced by magnesium salts (Fig. 6).

Mechanical tests

The results of biomechanical examination for the three study groups are shown in Fig. 5. The obtained data show that the highest force/strength values (7.5-9.4 [N]) in the measurement of indentation were obtained for the central points of the medial menisci (Points 7-9). For the lateral menisci, the highest strength values were obtained for Point 2 (8.4 N). The lowest strength values (2.3-3.8 [N]) were obtained at the end of the menisci at the attachments of cruciate ligaments. The highest strength values were obtained from menisci taken from sheep belonging to the negative control group.

Analysis of the results obtained from the strength examinations of the mechanical properties of bone tissues shows increased values of the Young's modulus (42%), a large increase in compressive strength (94%) and an even larger increase of deformation (strain)

energy (202%) in animals treated with ovariectomy. Following the introduction of steroidal medications into the experiment, in comparison to the positive control group, the following strength parameters are reported: the Young's modulus (8% down), compressive strength of bone tissue (12% down) and deformation (strain) energy (10% down).

Discussion

Although there have been numerous clinical observations and experimental studies, osteoporosis remains an area of interest to numerous scientific fields. Sheep were used in the experiment to examine changes occurring in the process of remodelling of bones after the treatment of ovariectomy and steroidal drug implementation, since as was observed by Chavassieux et al. (1993), sheep are a perfect model of large animals to be examined in terms of the growth of their bones and assessment of their repair.

The injection of high doses of methylprednisolone may induce numerous possible side-effects such as serious system infections or fleece loss (Lill et al. 2002, Ding et al. 2010). After analysing the literature thoroughly, the following pattern of application of methylprednisolone was adopted: 150 mg / sheep at long time intervals (every 20 days). It was applied intramuscularly, not subcutaneously as in the other studies (Schorlemmer et al. 2003). This method was selected since steroids applied intramuscularly are absorbed faster than those applied subcutaneously. Moreover, the intramuscular application is recommended when a long period of reaction is expected. Due to the 20-day intervals between applied subsequent doses, an extended observation of reaction to the medication was thus recommended. Oheim et al. (2012) in their studies claimed that ovariectomy in sheep leads to rapid and considerable losses of bone mass as early as 3 months after the treatment. In contrast, according to Turner et al. (1995) bone mass can be reduced after 6 months. However, some studies suggest that ovariectomy can cause significant losses of bone mass and changes in the micro-architecture of bones more than 12 months after treatment (Newton et al. 2004). According to Chavassieux et al. (1997) and Ding et al. (2010), the introduction of glucocorticosteroids in sheep leads to faster and more considerable losses of bone mass, changes in micro-architecture and abnormalities in bio-mechanics. The changes observed hitherto are comparable to those noted in humans.

In the case of the positive control group (PC), relevant information on the influence of ovariectomy on the examined supporting tissues is provided by analysis of the ratio between the thickness of cartilage and bone tissues obtained in the histological examinations, which was confirmed in the experiments conducted by An et Gruber (2003). Observed changes in the calcium distribution refer primarily to linking areas within bone trabeculae (rarely bone trabeculae themselves). This means that there are local processes connected with the remodelling of bone tissue (and to a lesser extent with osteocyte-based osteolysis). These processes differ from one another: the remodelling involves osteoclasts and osteoid resorption, while the osteocyte-based osteolysis is related to activated osteocytes and does not entail bone degradation (Teti et Zallone 2009). A major difference in this process refers to the fact that the resorption of bones involves their restoration connected with the proliferation and differentiation of osteoblasts, the quantity and maturation rate of which can be modified by steroids. This implies a decrease in bone mass and a degree of mineralisation. A reduced level of mineralisation and intensified processes of osteolysis through osteoclasts and their presence in bone trabeculae both within areas under articular cartilage and in deep layers were observed in the steroidal group (SC).

Articular cartilage showed no significant deviations from its standard characteristics. No sign of osteogenesis intensification was observed in the examined material. Once again this was confirmed by the analysis of its elements composition which showed that the lowest percentage of calcium occurred in the SC group (lower by nearly 50% compared to the NC group). In addition, the group was characterised by the most extensive reduction of phosphorus (by 32%). A low Ca/P ratio was also observed, which indicates that the provision of calcium to bones in the course of mineralisation or its removal from bones can be disturbed as a result of the process of osteocyte-based osteolysis (Teti et Zallone 2009).

At the final stage of the research the mechanical properties of bone tissue were tested in static uniaxial compression testing. A relevant issue related to the mechanics of bones is to determine their deformation (strain) and stress characteristics as the basis for defining material properties of the bone tissue. The values of the following mechanical parameters comprise the outcome of the measurements: Young's modulus (E) (relative elasticity modulus), compressive strength (smax) and deformation (strain) energy (U). The determination of these parameters allows a range of tissue degradation to be assessed by comparing its material properties with the characteristics set for healthy tissue. A bone is considered to be a biphasic material composed of a collagen matrix (of a low elasticity modulus) and hydroxyapatite crystals embedded therein (of a high elasticity modulus). The

analysis of the results showed that in case of the PC group in relation to the NC group there was an increase in all the parameters (being the most evident for proximal femoral bone epiphysis with an increase in deformation (strain) energy by more than 200% and compressive strength by more than 90%). In contrast, in the SC group all the mechanical parameters were characterised by reduction except only for the samples from the patella and humeral bone epiphysis, which showed little increase. Based on these results it can be concluded that steroidal medications cause a fall in the elasticity parameter of bone tissue which – in the case of the SC group – becomes less durable and more susceptible to deformation, leading to a fall in compressive strength in this group. A fall in deformation (strain) energy indicates a loss of capability of bones treated with steroidal medications to absorbe energy, and thus a lack of ductility. At the same time it provides evidence that such bones become less durable and more vulnerable to fractures.

Additional examinations included the testing of indentation of cartilage tissue within epiphyses and menisci. These are endurance tests performed on the basis of „mapping" methods. A matrix (array) of points was set on the articular surfaces of the examined samples and at these points the mechanical properties of the cartilage were analysed by exerting compressive force at a perpendicular angle to these surfaces. After analysing the results it can be observed that the highest force, i.e. the highest compressive strength, was represented by the samples coming from sheep in the NC group. Cartilage of articular surfaces from the samples taken from animals in the PC group in the case of patellae, femoral bones and glenoid fossae proved to be the least durable. Articular surfaces of humeral bones were the least durable in the SC group. During a thorough observation of the placement of indentation points at patellae, it was interesting to notice that the lowest strength (the least durability) was observed for points which were considered to be the most vulnerable, also under the reconnaissance arthroscopy. The examination of the indentation of articular cartilage is a unique procedure. Therefore it was decided to additionally examine the indentation of menisci in order to broaden the study material. The studies of the indentation of menisci were also performed by Sweigart and Athanasiou (2005), who examined the structure in pigs, by Proctor et al. (1989) who examined menisci derived from cattle or by Meena et al. (1995) who studied menisci in humans. No examinations on the indentation of menisci in sheep were found in the literature. Based on the obtained results, it can be concluded that the maximal strength values (7.5-9.4 [N]) when examining the indentation were obtained for the central points of medial menisci (Points 7-9). As for lateral menisci, the highest strength values were obtained for Point 2 (8.4 N). The lowest strength values (2.3-3.8 [N]) were obtained at the end of menisci at the attachments of cruciate ligaments. The highest strength values were obtained for menisci taken from sheep belonging to the negative control group.

The use of 150 mg/sheep of methylprednisolone induces changes in bone remodelling activity and alters bone mineralisation. These changes influence bone biomechanical properties in various ways. Changes observed in the „clinical approach" and this paper may be used in the control and monitoring of bone tissue metabolism in different experiments associated with alteration of bone tissue turnover.

Acknowledgements

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