Features of bone remodeling and osteoreparation processes in modeling femoral fracture in genetically modified mice with impaired enzymatic regulation of steroid hormone metabolism

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Abstract

Introduction: The aim of this study was to evaluate the processes of bone remodeling and osteoreparation in modeling femoral fracture in mice with zero expression of 11β-HSD2 (11β-HSD2−/−) or both 11β-HSD2 and apolipoprotein e (11β-HSD2−/−/ApoE−/−).

Materials and Methods: The experimental study was conducted on 60 male mice weighing 24-30 g. The study used male mice that lacked the expression of 11β-HSD2 (knockout mice with the Hsd2−/− genotype) and male mice that lacked the expression of 11β-HSD2 and apolipoprotein E (double knockout mice with the Hsd2−/−/ApoE−/− genotype). The control group includes wild type C57bl/6 animals. Modeling of a fracture of the proximal metaphysis of the femur was performed in animals at the age of 6 months using a closed technique. Fracture fusion and bone remodeling and osteoreparation processes were evaluated 6 weeks after fracture modeling.

Results and Discussion: It has been shown that a violation of the regulation of steroid hormone metabolism in groups of animals with the Hsd2−/− and Hsd2−/−/ApoE−/− genotypes leads to an increase in the number of ungrown fractures by 3 and 3.5 times, respectively, in comparison with wild-type animals. It was found that the microcirculation level of the proximal metaphysis of the femur in the area of the formed bone callus in the group of animals with the genotype 11β-HSD2−/− significantly decreased from 92.07±4.33 perfusion units (PE) in the group of wild-type animals to 82.67±3.54 PE (p=0.0002) in the group of animals with the genotype Hsd2−/−/ApoE−/− and up to 75.85±5.64 (p<0.0001) in the group of mice with the Hsd2−/−/ApoE−/− genotype. When calculating the coefficient of endothelial dysfunction, an increase in the coefficient of endothelial dysfunction was found from 1.297±0.19 in intact animals to 2.115±0.45 (p<0.00001) in the group of mice with the Hsd2−/−/ApoE−/− genotype and to 2.41±0.04 (p<0.00001) in the group of mice with the Hsd2−/−/ApoE−/− genotype.
In animals with impaired cortisol metabolism, there was a slowdown in the formation of bone tissue in the fracture area, bone trabeculae had a smaller width, large amounts of fibrous and connective tissue were observed in the lumen between bone fragments, and an increase in intertrabecular spaces was noted.

**Conclusion:** The close relationship between the metabolism of 11β-HSD2 and NO, confirmed in this study, can be considered as a promising pharmacotherapeutic target. It is obvious that approaches to changing the activity of 11β-HSD have significant therapeutic potential in the treatment of osteoporosis, bone remodeling disorders and osteoreparation in fractures against the background of formed osteoporotic changes in the violation of steroid hormone metabolism.

**Keywords**

osteoporosis, bone density, steroid hormone metabolism, transgenic animals, 11β-HSD2, Apoe, Hsd2<sup>-/-</sup>, Hsd2<sup>-/-/ApoE</sup>-

**Introduction**

To date, it is known that osteoporosis (OP) is a disease that is the most common cause of fractures among the elderly, characterized by a decrease in bone mass with increasing, depending on the age of the person, consequences for public health (Engström et al. 2012; Zhao et al. 2019). As a rule, a fracture is the first manifestation of osteoporosis, and previously a person may not notice the signs of this disease.

Changes in the concentrations and activity of steroid hormones are important factors in the development of osteoporosis. Elevated cortisol levels block the differentiation and formation of osteoblasts, which dramatically reduces the activity of osteosynthesis processes and leads to a decrease in bone density. As a result, the balance between the processes of bone formation and osteoresorption is disrupted. The key enzymes involved in the metabolism of cortisol are 11β-hydroxysteroid dehydrogenase - 11β-HSD type 1 (11β-HSD-1) and type 2 (11β-HSD-2). These enzymes catalyze the interconversion of active glucocorticoids (cortisol and corticosterone) and inert 11-ketoforms (cortisone and 11-dehydrocorticosterone), thereby regulating the function and activity level of steroid hormones in tissues (Zhang et al. 2017). 11β-HSD is widely distributed in the body in various organs such as kidneys, heart, and vascular endothelial cells. It is known that 11β-HSD converts inactive 11-dehydrocorticosterone into active corticosterone, whereas 11β-HSD-2 has the opposite
Materials and Methods

The experiment was performed at the Center for Preclinical and Clinical Studies of Belgorod State National Research University according to The Rules of Laboratory Practice, approved by Order No.708n of the Ministry of Health of the Russian Federation of August 23, 2010, in strict compliance with The European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Directive2010/63/EU). The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (Minutes №15/10 of October 29, 2021). Vivisection was performed in compliance with the ethical principles of treating laboratory animals outlined in The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS No.123).

Experimental animals

The experimental study was performed in 60 male mice, weighting 24-30 g. The animals were kept in accordance with the rules of laboratory practice for preclinical studies on the territory of the Russian Federation. The animals were kept under the standard conditions corresponding to the sanitary rules on the organization, equipment and maintenance of experimental biological clinics (vivariums) No. 1045-73, approved by the Ministry of Health of the USSR on April 6, 1973 and GOST R 53434-2009, in the individually ventilated cages (Tecniplast S.p.A., Italy) designed for keeping small laboratory animals. The bedding was sawdust, sterilized by ultraviolet irradiation. Special pellet feed for small laboratory rodents and pre-treated water disinfected with UV irradiation were used. In each cage, microclimate was created and supported by an individual ventilation system. All the animals had been acclimatized and quarantined for at least 10 days before the experiment.

Experimental groups

The experimental groups included:
1 – 20 male C57Bl/6J mice (WT+BF) with femoral fracture modeling;
2 – 20 male mice lacking 11β-HSD2 (BF+Hsd2−/−) with femoral fracture modeling;

Modeling of a femoral fracture

Modeling of a femoral fracture was performed using a closed technique (proposal for technical improvement No. 1975-11 dated November 15, 2011. “Closed technique for modeling a metaphyseal fracture in a small laboratory animal”). A cutting blade with silicone tubes on the cutting edges was used; external force was applied to the proximal metaphysis of the femur until the appearance of characteristic signs of fracture (specific pathological movements, crepitation of the fragment, change in the axis of the limb), the load was applied perpendicular to the axis of the limb (Fig. 1). The fracture was stabilized and fixed with a screw clamp and a spiral drill needle from the distal to the proximal femur, intramedularly through the bone canal and was firmly fixed in the cortical layer of the proximal femur. The control was carried out by the absence of pathological movements in the fracture area. The protruding part of the needle was cut short. Next, the wound was sutured with a single suture through all layers.

Figure 1. X-ray of the left femur of a wild-type animal with an experimental fracture of the proximal metaphysis of the femur.

Study design, study of microcirculation and calculation of the coefficient of endothelial dysfunction

The fracture of the femoral metaphysis was modeled in animals at the age of 6 months. Fracture fusion and bone remodeling and osteoreparation processes were evaluated 6 weeks after fracture modeling. Further, the level of microcirculation in bone tissue was measured in animals of the experimental groups. The measurement was performed in the proximal metaphysis of the left femur using a laser Doppler flurometer (LDF). For the animals with a fracture, a hole was made in the area of the callus formed after a fracture of the femur. A needle sensor was used to measure microcirculation. Endothelium-dependent vasodilation reactions were performed (in response to a single intravenous injection of acetylcholine solution at a dose of 40 mcg/kg) and endothelium-independent vasodilation (in response to a single intravenous injection of sodium nitroprusside solution at a dose of 30 mcg/kg). To confirm the role of endothelial dysfunction in the development of regional microcirculation...
disorders, the coefficient of endothelial dysfunction was calculated based on laser Doppler flowmetry data. The coefficient of endothelial dysfunction was calculated as the ratio of the area of the triangle above the microcirculation recovery curve in response to the administration of nitroprusside to the area of the triangle above the microcirculation recovery curve in response to the administration of acetylcholine. Microcirculation parameters were measured with a Biopac systems MP150 laser Doppler fluorometer and a TSD144 sensor. The data of the laser Doppler fluorometer were processed and recorded by the AcqKnowledge software version 3.9 - 4.2, microcirculation was measured in perfusion units (PU).

**Study of bone density**

The study of bone density and the assessment of the formation of callus using the In Vivo FXPRO system (Bruker, USA) was carried out 4 weeks after the fracture modeling, when the age of the animals was 7 months. In the In-Vivo FX PRO system, the principle of software evaluation of bone density is based on testing bone mineralization in an experimental animal by modeling cylindrical symmetry on an analytical X-ray image of a segment of a long tubular bone with appropriate calibration based on a Ca₃(PO₄)₂ sample immersed in water. Measurements of the density of long tubular bones suggest that the selected bone segment is a flat projection of a cylinder in cross section, where the outer shell of the cylinder is a homogeneous substance of higher density (bone tissue), and the inner substance of lower density (bone marrow). It is assumed that the medium in which the cylinder is immersed is water and may have zero depth (air). The software performs a "cylindrical fitting" procedure to extract important parameters of the selected bone segment from a high-resolution X-ray image. Based on the program evaluation, data characterizing various indicators of the selected bone segment is generated. The "Bone Column Density" and "Marrow Column Density" indicators characterize the degree of attenuation of X-ray radiation per unit depth of the material. This is an analogue of the "optical density", often used to characterize the attenuation of light passing through a colored substance. The indicators provide a fairly accurate assessment of the density of the bone column, which directly depends on the measured basic contrast of the digital radiograph. The "Bone Density" indicator is determined by the density of the bone column and is calculated from the "Bone Column Density" indicator, taking into account the depth of the tissue sample and the effect of this depth on the density of a similar Ca₃(PO₄)₂ column. The indicator is expressed in g/cm³. And finally, the "Bone Surface Density" indicator characterizes the average "coverage" of the material projected onto a flat surface. It is calculated based on the values of the density of the column of bone tissue and radii adjusted for the depth of the tissue and the effect of this depth on the density of a similar column Ca₃(PO₄)₂ (Korokin et al. 2022b). In this study, we based on the results of the “Bone Density” index (BD, g/cm³) for the proximal metaphysis and distal metaphysis of the right femur to confirm the development of osteoporosis.

**Histological examination**

To analyze the processes of bone remodeling and osteoreparation against the background of osteoporosis, a morphological study of the place of a callus formation in the metaphysis of the femur was carried out. For this purpose, during sample preparation, decalcification was previously carried out (Zamboni fixative and decalcification with a 20% solution of ethylenediaminetetraacetate and 5% sucrose-0.1 M tris at +4°C), preparation of sections using a rotary microtome and staining them with hemotoxylin and eosin. The resulting histological preparations were subjected to light microscopy. To perform histomorphometry of bone tissue, a pre-calibrated Image J software (versions 1.39-1.43) was used, in which the width of bone trabeculae was measured and expressed in micrometers.

**Quantitative PCR**

To isolate total RNA, femoral bones were removed and placed to liquid nitrogen for 30 seconds. Immediately afterwards, frozen samples were homogenized in TissuLyser (ThermoFisher, USA) using bead disruption of the tissue (Carter et al. 2012). The homogenized samples were incubated for 10 minutes at 37°C in the Extract RNA solution. After lysis of the sample in the reagent, it was subjected to chloroform cleaning; the sample was collected and washed with isopropyl alcohol and 70% ethyl alcohol. The concentration of the obtained RNA was measured on an IMPLEN NanoPhotometer® spectrophotometer and adjusted to a concentration of 30 ng/μL. Reverse transcription was performed using the MMLVRTSK021 kit in accordance with the manufacturer’s protocol (Evrogen). The mixture was gently mixed and heated at 70°C for 2 minutes to melt the secondary RNA structures and then anneal the OligoDT primer. After that, the samples were transferred to ice. The entire reaction mixture was incubated for 60 minutes at 40°C in a T100 ThermalCycler (Bio-Rad). To stop the reaction, the mixture was heated at 70°C for 10 minutes (Korokin et al. 2022a). The resulting cDNA was diluted to a concentration of 1 ng/μL. The level of gene expression was evaluated relative to the values of the Gapdh reference gene. The expression at a specific point was calculated using the formula: Gene expression = [(Ct(Gapdh)/Ct(Gene of Interest)) (Table 1).
As a result of measuring bone density at these points using the In Vivo FX PRO system (Bruker, USA), it was found that in wild-type animals (WT), bone density at the time of animal withdrawal from the experiment was 5.932±0.582 g/cm$^3$.

Results and Discussion

At the first stage, 6 weeks after modeling fractures of the proximal femoral metaphyses, we analyzed the number of non-fused fractures in the experimental groups. Figure 2 shows that out of 20 wild-type animals in the WT+BF group, fractures did not heal in 2 animals (10%) – in these animals, restriction of support on the limb concerned was observed during their lifetime, pathological mobility of fragments relative to each other was noted under anesthesia, and failure of the bone callus was macroscopically detected. In the group of animals with genotype 11β-HSD2$^{-/-}$, there were 6 animals out of 20 (30%) with non-fused fractures, in the group of animals with genotype 11β-HSD2$^{-/-}$ApoE$^{-/-}$, fractures did not heal in 7 animals out of 20 (35%).

![Figure 2](image.png)

Figure 2. Assessment of the viability of osteoreparation processes 6 weeks after modeling fractures of the proximal metaphyses of the femurs in experimental groups (n=20, % of non-healed fractures).

Also, to confirm osteoporotic changes in animals 6 weeks after fracture modeling, the values of the "Bone Density" index (BD, g/cm$^3$) in the area of the proximal and distal diaphysis of the right femur were measured and studied (Fig. 3).

![Figure 3](image.png)

Figure 3. Example of bone density measurement using the In-Vivo FX PRO system (Bruker, USA). Note: A – WT+BF – wild type mice with femoral fracture modeling; B – BF+Hsd2$^{-/-}$ – knockout male mice with null expression of the 11β-HSD2 with femoral fracture modeling; C – BF+Hsd2$^{-/-}$ApoE$^{-/-}$ – double knockout male mice with null expression of the 11β-HSD2 and Apolipoprotein E with femoral fracture modeling.

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Statistical data processing

The statistical data were processed using the Statistica 10.0 software. Shapiro-Wilk and Spiegelhalter (normtest package) normality tests were performed for the obtained data; the equality of variances was assessed using the Levene’s test (lawstat package). Depending on the type of distribution and the equality of variances, the significance of the results obtained was evaluated using parametric (ANOVA) or non-parametric (Kruskal-Wallis test) one-way analyses of variance, and as a post-hoc analysis to identify intergroup differences, the Student’s t-test or the Mann-Whitney test were used, respectively, with the Benjamini-Hochberg correction for multiple tests. The results were considered reliable at $p\leq0.05$. 

Results and Discussion

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1 – distal diaphysis of the right femur; 2 – proximal diaphysis of the right femur.
with zero expression of 11β-HSD2 showed a statistically significant decrease in bone density to 5.137±0.625 g/cm³ (p=0.0004 compared with wild-type animals). We found the most pronounced decrease in bone density in the group of animals with the Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> genotype of male double-knockout mice with zero expression of 11β-HSD2 and apolipoprotein E. The value of bone density in this group was 4.759±0.625 g/cm³ (p=0.000042) (Fig. 4).

It is noteworthy that the bone density in the group of animals BF+Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> was statistically significantly lower than in the group of animals BF+Hsd2<sup>-/-</sup> (p=0.031) (Fig. 4).

Further, during the study, it was found that the microcirculation level of the proximal metaphysis of the left femur in the area of the formed bone callus in the group of animals with the genotype 11β-HSD2<sup>-/-</sup> significantly decreased from 92.075±4.33 PE in the group of wild-type animals to 82.67±3.54 PE (p=0.0002) in the group of animals with the HSD2<sup>-/-</sup> genotype and up to 75.85±5.64 (p<0.0001) in the group of mice with the Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> genotype (Fig. 5, LDF). When conducting tests with endothelium-dependent (intravenous administration of acetylcholine) and endothelium-independent vasodilation (intravenous administration of sodium nitroprusside) with calculation of the coefficient of endothelial dysfunction, an increase in CED was found from 1.297±0.19 in the intact animals to 2.115±0.45 (p<0.00001) in the group of mice with the Hsd2<sup>-/-</sup> genotype and to 2.41±0.04 (p<0.00001) in the group of mice with the Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> genotype (Fig. 5, CED).

Bone biomaterial was taken for further morphological studies. Histological sections of the proximal sections of the left femur in the area of the formed bone callus were subjected to microscopy and histomorphometry. Osteoporotic changes in skeletal bones were histologically confirmed in mice with the Hsd2<sup>-/-</sup> and Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> genotypes. In wild-type animals, the formation of a bone callus was completed in the fracture area-bone tissue was formed, the width of the bone trabeculae was comparable to that of the intact bone section. In the animals with impaired cortisol metabolism, there was a slowdown in the formation of bone tissue in the fracture area; bone trabeculae had a smaller width; large amounts of fibrous and connective tissue were observed in the lumen between bone fragments, and an increase in intertrabecular spaces was noted. The described phenomena were more typical for the group of double knockouts with the Hsd2<sup>-/-</sup> and Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> genotypes. The average width of the bone trabeculae of the proximal metaphysis of the left femur (in the area of the formed bone callus) in wild-type animals was 54.94±5.12 microns. The width of bone trabeculae in

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**Figure 4.** Bone density in animals of experimental groups after 6 weeks of fracture modeling. Note: WT+BF – wild type mice with femoral fracture modeling; BF+Hsd2<sup>-/-</sup> – knockout male mice with null expression of the 11β-HSD2 with femoral fracture modeling; BF+Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> – double knockout male mice with null expression of the 11β-HSD2 and Apolipoprotein E with femoral fracture modeling.

**Figure 5.** Indicators of microcirculation (LDF) in bone tissue and coefficient of endothelial dysfunction (CED) and bone alkaline phosphatase (B-ALP) concentration in animals in experimental groups with experimental femur fracture. Note: WT+BF – wild-type mice with femoral fracture modeling; BF+Hsd2<sup>-/-</sup> – knockout male mice with null expression of the 11β-HSD2 with femoral fracture modeling; BF+Hsd2<sup>-/-</sup>/ApoE<sup>-/-</sup> – double knockout male mice with null expression of the 11β-HSD2 and Apolipoprotein E with femoral fracture modeling.
Figure 6. Width of bone trabeculae (A) and micrographs of histological preparations of bone tissue WT (B), Hsd2−/− (C) and Hsd2−/−ApoE−/− (D) male 8 month-old mice (n=20, Hematoxylin+eosin, х200). Note: WT+BF – wild type mice with femoral fracture modeling; BF+Hsd2−/− – knockout male mice with null expression of the 11β-HSD2 with femoral fracture modeling; BF+Hsd2−/−ApoE−/− – double knockout male mice with null expression of the 11β-HSD2 and Apolipoprotein E with femoral fracture modeling.

Gene expression analysis showed that genetically modified animals are characterized by increased mRNA expression of pro-apoptotic factors p53 and Bax along with reduced mRNA expression of anti-apoptotic factor Bcl2 (Fig. 7). We also analyzed the expression of mRNA factors associated with the development of endothelial dysfunction in animals against the background of metabolic disorders of steroid hormones. We found a statistically significant decrease in the expression of the eNOS and Vegf-a genes in the groups of animals with the Hsd2−/− and Hsd2−/−ApoE−/− genotypes (Fig. 8). It is noteworthy that in the group of animals with absent expression of 11β-HSD2 and ApoE (BF+Hsd2−/−ApoE−/−), the expression of mRNA genes responsible for endothelial NO synthase and apolipoprotein E was statistically significantly lower than in the animals of the WT+ Hsd2−/− group (Fig. 8).

Conclusion

The human skeleton is in a state of constant renewal, in which new bone structures are formed in response to the destruction of old bone structures. At a young age, the processes of osteosynthesis proceed faster than the processes of osteodestruction, which leads to an increase in bone mass. After the age of 20, this process slows down, and most people reach their peak bone mass by the age of 30 (Weaver et al. 2016; Rozenberg et al 2020).

Osteoporosis is a disease without any pathognomonic clinical signs, which usually remains undiagnosed until a pathological fracture or fracture occurs as a result of low-force exposure (de Souza 2010; Nogués et al. 2022). An osteoporotic fracture, usually affecting the hip and spine, affects a person’s performance and normal functioning, leading to chronic pain and long-term rehabilitation (Vidal et al. 2019). To date, it has been recognized that osteoporosis-related fractures are associated with increased mortality, with the exception of forearm fractures. Between 20% and 40% of people suffering from femoral fractures die within a year (Ballane et al. 2014).

Pharmacological and non-pharmacological methods are used to prevent fractures in patients with osteoporosis.
Non-pharmacological strategies include a healthy lifestyle such as a balanced diet, regular exercise, smoking cessation, limited alcohol consumption, etc. (Nogués et al. 2022).

Pharmacological correction strategies include antiresorptive and anabolic drugs and are indicated for patients with a high or very high risk of fractures. Both treatments have been shown to increase bone strength (Lou et al. 2019). The main drugs currently available for the treatment of osteoporosis are: bisphosphonates, anti-RANKL antibodies (denosumab), selective estrogen receptor modulators, estrogen replacement therapy, monoclonal anti-sclerostin antibodies (romozozumab), strontium ranelate and parathyroid hormone analogues (Tabatabaei-Malazy et al. 2017; Camacho et al. 2020; Nogués et al. 2022).

Despite the wide range of available pharmacological agents, the problem of prevention and treatment of fractures associated with osteoporosis is an urgent task of pharmacology. In order to study new pharmacological agents and form ways to search for new treatment strategies, new experimental models of the pathological process are primarily needed. Previously, we showed that
a violation of the metabolic regulation of steroid hormone metabolism in animals with zero expression of 11β-HSD2 (genotype Hsd2-/-) leads to the development of signs of osteoporosis – bone density decreases, which is accompanied by a decrease in the width of bone trabeculae and the level of microcirculation in bone tissue. The resulting endothelial dysfunction is accompanied by an increase in the coefficient of endothelial dysfunction. Additional zero expression of the AроE gene in double knockout mice with the Hsd2-/-ApoE-/- genotype leads to an increase in the severity of changes associated with impaired bone remodeling processes and, in addition to a more pronounced change in bone density, bone trabecular width, microcirculation and endothelial dysfunction coefficient. Also, an increase in the concentration of biochemical markers of bone resorption was found in such animals (Korokin et al. 2022a).

In this study, we studied the features of bone remodeling and osteoreparation processes in genetically modified mice with impaired enzymatic regulation of steroid hormone metabolism when modeling a femoral fracture in a closed manner.

It has been shown that a violation of the regulation of steroid hormone metabolism in groups of animals with the Hsd2-/- and Hsd2-/-ApoE-/- genotypes leads to an increase in the number of ungraven fractures by 3 and 3.5 times, respectively, in comparison with wild-type animals. These facts confirm that in animals with no expression of 11β-HSD2, bone resorption processes due to activation of osteoclast differentiation prevail over osteosynthesis processes. The previously obtained results were confirmed in this study – in animals of experimental groups, 6 weeks after modeling fractures of the proximal metaphysis of the femur (total age of animals was 7.5 months), a decrease in bone density was shown, which was more pronounced in the group of animals with absent expression of 11β-HSD2 and ApoE.

Thus, it has been shown that the vascular endothelium plays one of the key roles in the formation of the pathogenetic pathway leading to bone resorption processes against the background of vascular endothelial dysfunction (Deuchar et al. 2011). The role of the vascular endothelium in the realization of the osteotropic effects of 11β-HSD2 is shown when evaluating the coefficient of endothelial dysfunction in experimental groups and the expression of genes characterizing the functioning of the vascular endothelium. The close relationship between the metabolism of 11β-HSD and NO, confirmed in this study, can be considered a promising pharmacotherapeutic target. It is obvious that approaches to changing the activity of 11β-HSD have significant therapeutic potential in the treatment of osteoporosis, bone remodeling disorders and osteoreparation in fractures against the background of formed osteoporotic changes in the violation of steroid hormone metabolism.

Conflict of Interests

The authors declare no conflict of interests.

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