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Protective role of 3-oxypyridine derivatives in rats' steroid-induced osteoporosis associated with reduced oxidative stress and recovery of nitric oxide formation

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From the point of view of the mechanisms for the implementation of pathogenetic links in the development of steroid-induced osteoporosis considered in the paper, the increased risk of the oxidative stress in osteoblasts, as well as the development of the vessels endothelial dysfunction of the microcirculatory bloodstream in the bone tissue, are of particular interest. They lead to the impaired bone tissue trophism and progression of osteoporosis.

The aim of the study was research of the osteoprotective effects of a 3-hydroxypyridine derivatives composition on the model of steroid-induced osteoporosis.

Materials and methods. To model osteoporosis pathology, the animals (male Wistar rats) were injected with methylprednisolone (MP) at the dose of 5 mg/kg (intraperitoneally) every 5th day for 5 weeks. As a non-selective blocker of NO synthase, L-NAME was used at the dose of 25 mg/kg (intraperitoneally). Derivatives of 3-hydroxypyridine (hereinafter referred to as composition No. 1) were administrated at the dose of 50 mg/kg (*per os*) In all experimental groups, the level of microcirculation and the bone mineral density, as well as the analysis of histomorphological and biochemical samples, were assessed.

Results. The study results showed that composition No. 1 (50 mg/kg) has an osteoprotective activity, effectively prevents a decrease in the level of the regional bone tissue microcirculation and in the development of an endothelial dysfunction. That makes it possible to increase the bone mineral density and to slow down the thinning of bone trabeculae. In addition, composition No. 1 (50 mg/kg) reduces the production of reactive oxygen species and increases the NO bioavailability.

Conclusion. The data obtained indicate that the studied composition of 3-hydroxypyridine derivatives is considered a promising compound for the prevention and treatment of steroid-induced osteoporosis.

Keywords: 3-hydroxypyridine derivatives; osteoporosis; reactive oxygen species; oxidative stress; nitric oxide; endothelium

Abbreviations: ROS – reactive oxygen species; MP – methylprednisolone; L-NAME – L-Nitro-arginine methyl ester; NO – nitric oxide; GC – glucocorticoid; NFκB – nuclear factor-κB; RANK – receptor activator for nuclear factor kappa B; RANKL – ligand of receptor activator for nuclear factor kappa B; OPG – osteoprotegerin; NOS – NO-synthase; SOD – superoxide dismutase; MDA – malondialdehyde; EDC – endothelial dysfunction coefficient; LPO – lipid peroxidation; GP – glutathione peroxidase; CSF – colony-stimulating factor; eNOS – endothelial NO-synthase; NOX – nicotinamide adenine dinucleotide phosphate oxidase.

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Протективная роль производных 3-оксипиридина при стероид-индуцированном остеопорозе у крыс, связанная со снижением оксидативного стресса и восстановлением образования оксида азота

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С точки зрения рассматриваемых механизмов реализации патогенетических звеньев развития стероид-индуцированного остеопороза особый интерес представляет повышенный риск окислительного стресса в остеобластах, а также развитие эндотелиальной дисфункции сосудов микроциркуляторного русла костной ткани, приводящее к нарушению трофики костной ткани и прогрессированию остеопороза.

Цель. Изучить остеопротекторные эффекты композиции производных 3-оксипиридина на модели стероид-индуцированного остеопороза.

Материалы и методы. Для моделирования патологии остеопороза животным (самцы крыс линии Wistar) внутрибрюшинно в течение 5 недель вводили метилпреднизолон (МП) в дозе 5 мг/кг каждые 5 дней. В качестве неселективного блокатора NO-синтазы в работе использовали L-NAME в дозе 25 мг/кг, внутрибрюшинно. Производные 3-оксипиридина (в дальнейшем по тексту как композиция № 1), вводились в дозе 50 мг/кг перорально. Во всех экспериментальных группах проводилась оценка уровня микроциркуляции и минеральной плотности костной ткани, анализ гистоморфологических и биохимических проб.

Результаты. Результаты показали, что композиция № 1 (50 мг/кг) оказывала остеопротекторное действие, эффективно предотвращала снижение уровня регионарной микроциркуляции в костной ткани и развитие эндотелиальной дисфункции, что позволило увеличить минеральную плотность костей и замедлить истончение костных трабекул. Кроме того, композиция № 1 (50 мг/кг) снижала выработку активных форм кислорода и увеличивала биодоступность NO.

Заключение. Полученные данные свидетельствуют о том, что изучаемая композиция производных 3-оксипиридина, считается перспективным соединениям для профилактики и лечения стероид-индуцированного остеопороза.

Ключевые слова: производные 3-оксипиридина; остеопороз; активные формы кислорода; оксидативный стресс; оксид азота; эндотелий

Список сокращений: АФК – активные формы кислорода; МП – метилпреднизолон; L-NAME – L-нитро-L-аргининметиловый эфир; NO – оксид азота; ГК – глюкокортикоид; NF-кВ – ядерный фактор-кВ; RANK – активатор рецептора NF-кВ; RANKL – активатор рецептора лиганда NF-кВ; OPG – остеопротегерин; NOS – NO-синтаза; СОД – супероксиддисмутаза; МДА – малоновый диальдегид; КЭД – коэффициент эндотелиальной дисфункции; ПОЛ – перекисное окисление липидов; ГП – глутатионпероксидаза; CSF – колониестимулирующий фактор; eNOS – эндотелиальная NO-синтаза; NOX – никотинамидадениндинуклеотидфосфатоксидаза.

INTRODUCTION

Being widely used in various fields of medicine (rheumatology, pulmonology, hematology, gastroenterology, dermatology, and transplantology), glucocorticoids (GCs) remain one of the effective methods for the treatment of many inflammatory and autoimmune diseases, [1]. However, long-term GC therapy has a number of side effects, one of the most significant among which is steroid-induced osteoporosis. This is the most common form of iatrogenic and secondary osteoporosis [2], which causes a decrease in the bone mineralization and, as a result, fractures

in 30–50% of patients. In terms of prevalence, steroid-induced osteoporosis ranks second among all forms of osteoporosis, second only to postmenopausal and senile ones [3]. The pathophysiology of glucocorticoid-induced osteoporosis is determined by various factors [4–8], including the receptor activator for nuclear factor kappa B (RANK), its ligand (RANKL), and osteoprotegerin (OPG). As a member of the tumor necrosis factor (TNF) superfamily, RANKL regulates osteoclast differentiation, activation, and survival by binding to its cognate RANK receptor, which can interact with several TNF (TRAF) receptor-associated factors to activate signaling molecules [9]. Reactive oxygen species (ROS) are considered the main factors in the RANKL-induced effect on the bone tissue, including in steroid-induced osteoporosis [10–12], which makes it possible to determine one of the most important therapeutic strategies for correcting this pathology.

Another potential target for the treatment of osteoporosis may be nitric oxide (NO). Endogenous NO is formed from L-arginine as a result of a reaction catalyzed by an enzyme of the calmodulin-dependent NO synthases (the NOS family). Endothelial NOS (eNOS), of the three isoforms of NOS, contributes the most to the development of osteoporosis. Strong evidence for a role of NO in the osteoblast function comes from the eNOS knockout animal studies, which report severe defects in the bone formation and an osteoblast activity in both *in vivo* and *in vitro* studies. [13]. In addition, a preventive administration of NO donators (nitroglycyrin and L-arginine) delimits the bone loss, increases bone strength by reducing the development of osteoporosis [14, 15].

Recommendations for the treatment of glucocorticoid-induced osteoporosis include routine calcium and vitamin D supplementations, bisphosphonate therapy, selective estrogen receptor modulators, human monoclonal antibodies to RANKL and its intracellular factor, and a recombinant parathyroid hormone [16-18]. All pharmacological approaches are still controversial and show inconsistent and variable results, which may depend on age, sex, dose and duration of treatment. In addition, a long-term use of certain drugs can lead to serious complications, including kidney damage, venous thrombosis, and an increased risk of developing tumors.

In this regard, the search for new effective approaches for the correction of steroid-induced osteoporosis seems to be a very promising direction in pharmacology.

Derivatives of 3-hydroxypyridine belong to the simplest heterocyclic analogues of aromatic phenols and have a wide spectrum of activity, like antioxidant, antihypoxic, anti-inflammatory, anti-ischemic [19], cardio- and endothelioprotective activities [20]. A huge list of pharmacological effects suggests that a new complex of 3-hydroxypyridine derivatives, consisting of one molecule of 2-ethyl-6-methyl-3-hydroxypyridinium 3-pyridinocarbonoate and three molecules of 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate (hereinafter referred to as composition No. 1), obtained by topochemical synthesis (All-Union Scientific Center for the Safety of Biologically Active Substances, Staraya Kupavna, Russia) can become a promising compound for the prevention and treatment of steroid -induced osteoporosis.

THE AIM of the study was research of the osteoprotective effects of a 3-hydroxypyridine derivatives composition on the model of steroid-induced osteoporosis.

MATERIALS AND METHODS

Methods of obtaining and analysis

Chemical reagents necessary to prepare the compound were purchased from commercial suppliers who have a certificate for chemical products (Sigma-Aldrich, USA). The way of composition No. 1 synthesis consisted of the following stages: 26.0 g (0.1 g/mol) of 2-ethyl-6-methyl-3-hydroxypyridinium 3-pyridinocarbonoate was loaded into the homogenizer; while stirring, 93.2 g (0.3 g/mol) 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate was gradually added. The mass was homogenized for 10–15 min at a stirring speed of 300-400 rpm. Next, the particle size of the resulting powder which should be no more than 10 μm, was checked and, if necessary, additionally homogenized. The output was 119.0 g of white fine crystalline powder with a melting point of 139–143°C. The resulting compound is soluble in water with slight opalescence. The following was found out, %: C 62.48; H 7.98; N 9.39 C62 H94 N8O15; m.m. 1 191.46. The result of the calculation, %, was the following: C 62.50; H 7.95; N 9.41; O 20.14. The chemical formula of the compound (composition No. 1) is shown in Fig. 1.

Study design

All experimental studies were carried out in accordance with the Rules of Laboratory Practice approved by the Order of the Ministry of Health of Russia No. 708n, dated Aug 23, 2010, with strict observance of the European Convention for the Protection of Vertebrate Animals used for experiments or other scientific purposes (Directive 2010/63/EU). The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University of the Ministry of Health of Russia (Protocol No. 11/9 dated Feb 12, 2022). The vivisection was carried out in accordance with the ethical principles for the treatment of laboratory animals as set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS No. 123).

The experiments were performed on 60 healthy non-morbid male Wistar rats weighing 220–300 grams.

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The animals were obtained from the nursery "Stolbovaya" of the Institution of Science and Technology of the Federal Medical and Biomedical Institute of the Federal Medical and Biological Agency (Moscow Region), were kept under standard conditions that corresponded to the sanitary rules for the arrangement, equipment and maintenance of experimental and biological clinics (vivariums) No. 1045-73.

At the first stage, the laboratory animals were randomly divided into 6 experimental groups: group I – intact animals, intraperitoneally (ip) injected with saline; group II – the animals ip injected with methylprednisolone (MP) at the dose of 5 mg/kg for 5 weeks every 5th day; group III – the animals ip injected with L-NAME at the dose of 25 mg/kg for 35 days; group IV – the animals ip injected with methylprednisolone (MP) at the dose of 5 mg/kg+L-NAME 25 mg/kg ip for 35 days; group V – the animals intragastrically administrated with MP+composition No. 1 at the dose of 50 mg/kg ip with MP+L-NAME+composition No. 1 at the dose of 50 mg/kg BID for 35 days.

On day 36, the animals were withdrawn from the experiment with a further evaluation of densitometry, functional, biochemical and histomorphometric tests. The design of the experiment is shown in Fig. 2.

Bone density test

Densitometry in the animals was performed after a preliminary putting the animals into narcotism with a solution of tiletamine and zolazepam (60 mg/kg) and chloral hydrate (300 mg/kg). The indicator, expressed in g/cm^3, was determined for the proximal metaphysis, diaphysis, and distal metaphysis of the femur. The assessment of the bone density (BD) was carried out using the IN-VIVO MS FX PRO multifunctional laboratory X-ray unit manufactured by Bruker (USA) with a molecular imaging system using licensed Bone Density Software.

Vascular tests

The impact of the bone tissue on the microcirculatory bed is one of the promising approaches in the correction of osteoporosis, and therefore, in all the experimental groups, the microcirculation in the cancellous bone tissue of the proximal metaphysis of the right femur was assessed. To obtain the data on the bone microcirculation, BIOPAC Systems Equipment (USA) was used: an MP100-150 polygraph with an LDF100C laser Doppler flowmetry module and a TSD144 sensor. The results of the laser Doppler flowmetry (LDF) were recorded using Acq Knowledge software (versions 3.8–4.2). Microcirculation parameters were expressed in perfusion units (p.u.). After measuring the intraosseous microcirculation level, a test was performed for an endothelium-dependent vasodilation (acetylcholine 40 μg/kg intravenously (IV)) and an endothelium-independent vasodilation (sodium nitroprusside 30 μg/kg IV), with a further determination of the endothelial dysfunction coefficient (EDC) [21].

Biochemical blood assay

To assess the biochemical parameters in the animals of the experimental groups after conducting vascular tests, the blood was taken using a syringe from the tail vein, followed by the determination of the total calcium content (mmol/l) in the blood plasma by colorimetry with o-cresolphthalein and alkaline phosphatase (U/l) in the blood serum (colorimetric, kinetic methods), on the spectrophotometer SF-46 (LOMO, Russia). The serum levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) (Nanjing Jiancheng Biological Engineering Research Institute, China) were determined according to the manufacturers' instructions [22].

Morphofunctional assessment of bone tissue state

The object of the study for the histological analysis was tibias, which were initially fixed in 10% formalin. The proximal segment was dissected from the fixed bones for 1 cm from the articular surface of the condyles. According to the recommended protocol[1], the material was decalcified in the Surgipath Decalcifier II liquid (Leica, Germany). The decalcified fragments were automatically embedded in paraffin, followed, according to Mallory, by staining the 7 μm thick sections with hematoxylin and eosin. Micropreparations were studied by a scanning method under the microscope "Mikmed" with a video camera "DV1000". Using the McrAView 7.3.1.7 program (LOMO, Russia), the thickness of the bone trabeculae and the cortical bone of the diaphysis was measured.

Statistical analysis

The data were tested for the normal distribution using the Shapiro-Wilk test. Normally distributed data were compared using a conventional one-way analysis of variance (ANOVA) with Tukey's post-hoc test. The non-normally distributed data were compared using the Kruskal-Wallace test and Dunn's nonparametric post-hoc test. The differences were determined at a significance level of 0.05. The statistical analysis was carried out using GraphPad Prism 9.2.0 software (GraphPad Software, USA).

RESULTS AND DISCUSSION

The structure of the supramolecular complex (composition No. 1) was confirmed on the basis of the spectroscopic data: IR spectrum (ν, cm^{-1}): 3412 (OH) 3290 (NH), 2941 (CH), 2673 (N$^+$), 1781 (C=N–), 1634 (C=C), 1561 (COO–). The mass spectrum of the protonated supramolecular complex in the positive ion scanning mode [M+H] is M/z 1195.46, which corresponds to m.m. 1191.46. The ^{1}H NMR spectra are shown in Fig. 3.

[1] Decalcification. Cardiovascular Pathology (5th Edition), 2022. Available from: https/www.sciencedirect.com/topics/medicine-and-dentistry/decalcification

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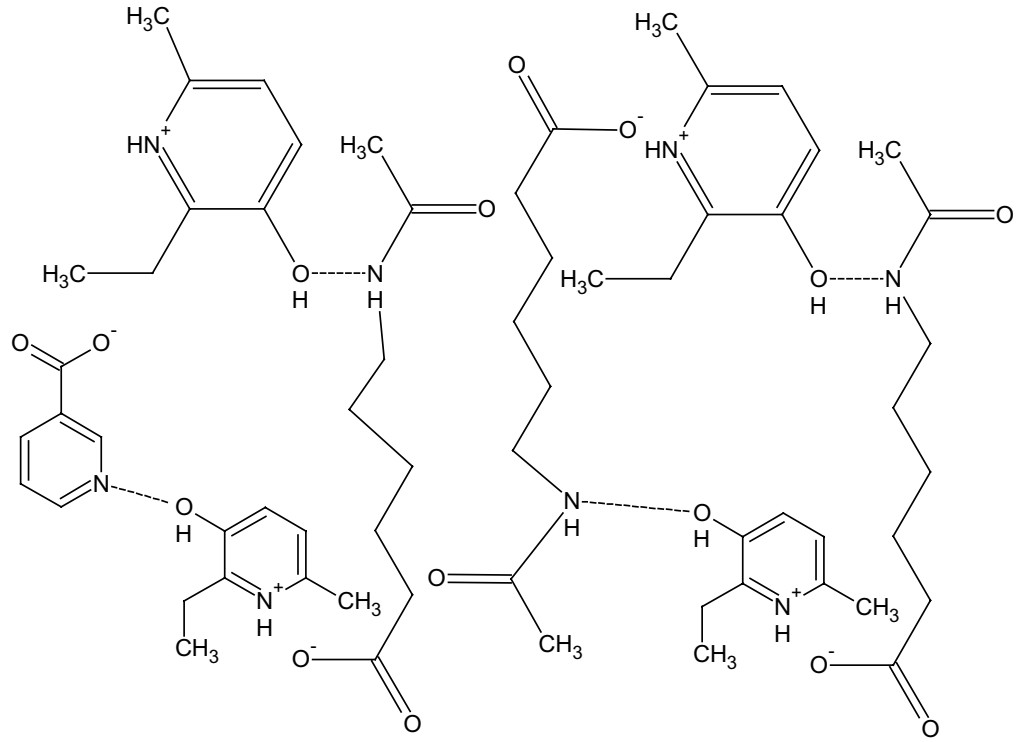

Figure 1 – Structural formula of the compound (composition No. 1)

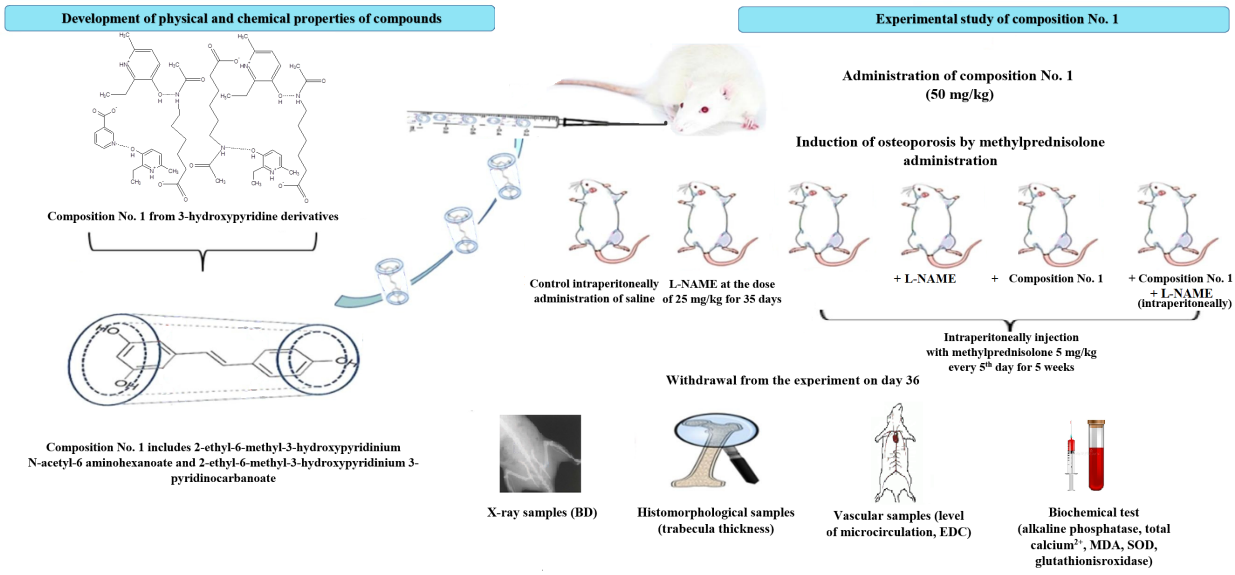

Figure 2 – Experiment design

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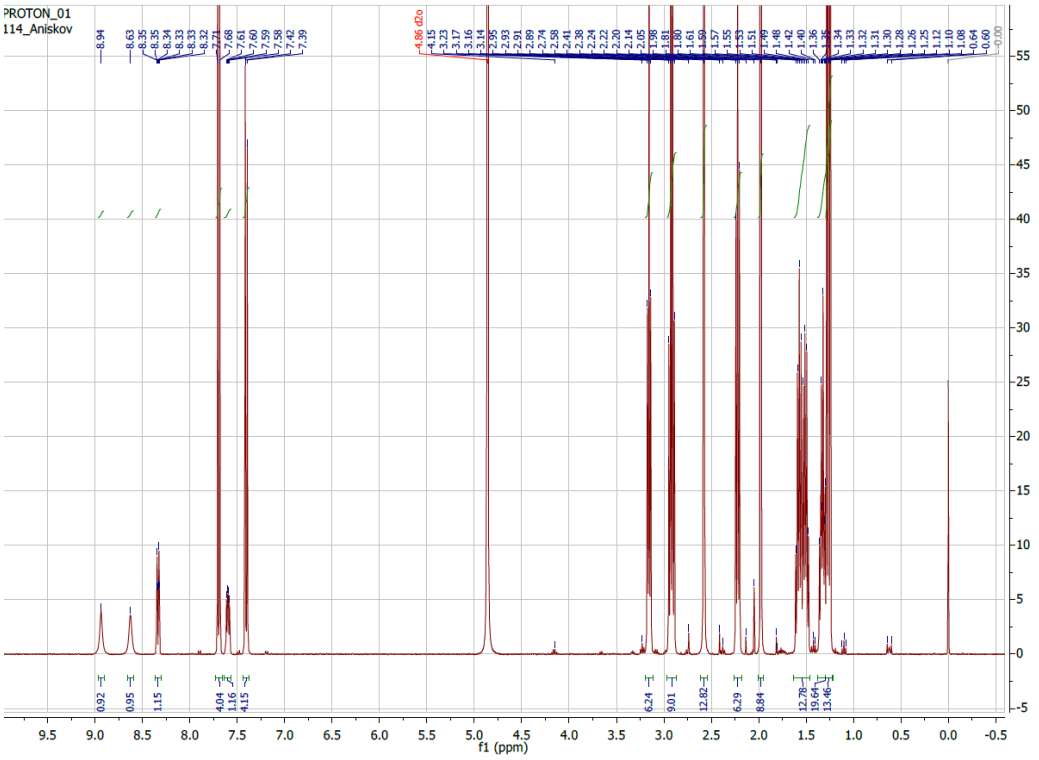
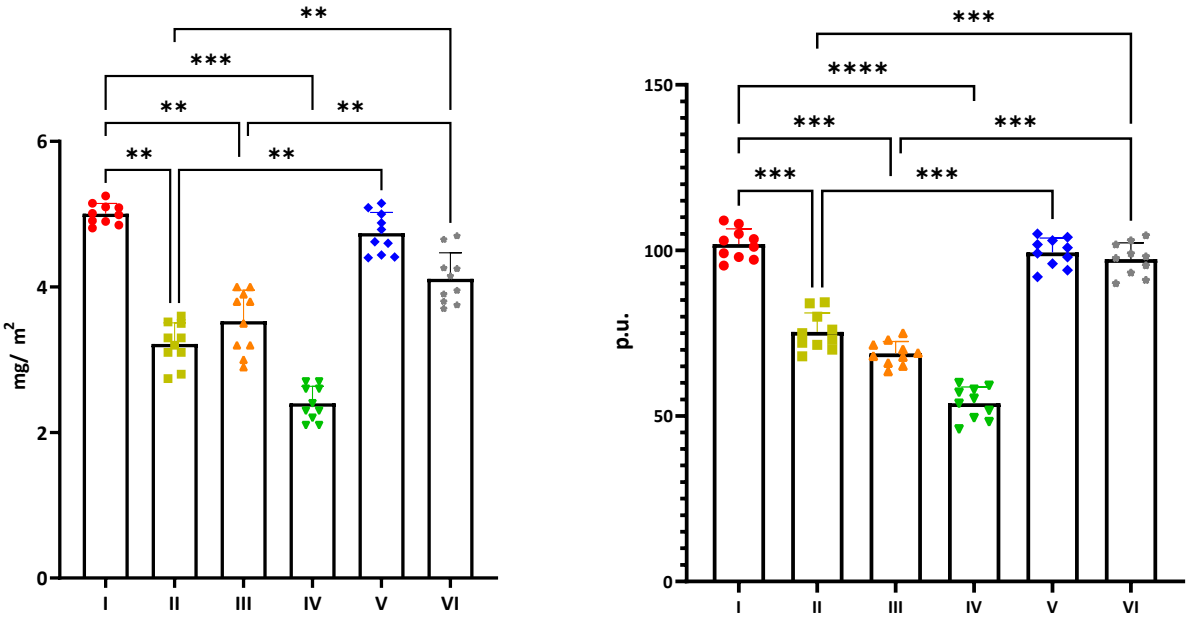


Figure 3 – ^{1}H NMR spectrum of composition No. 1



A **B**

Figure 4 – Effect of composition No. 1 on bone density (A) and the level of microcirculation (B) in steroid-induced osteoporosis

Note: (*here and in Fig. 3-5*): I – intact; II – MP; III – L-NAME; IV – MP+L-NAME; V – MP+composition No. 1; VI – MP+L-NAME+composition No. 1

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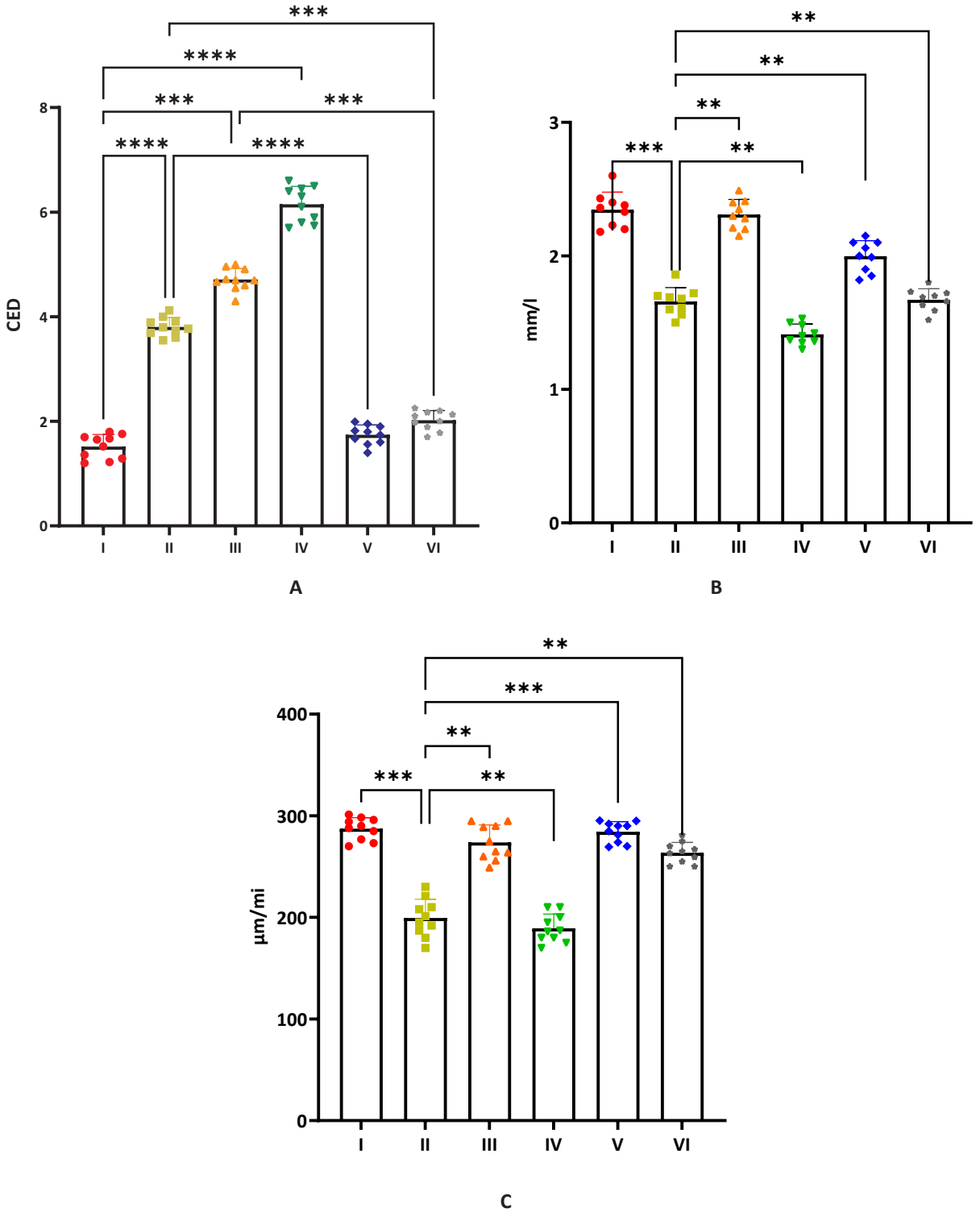

Figure 5 – Effect of composition No. 1 on coefficient of endothelial dysfunction (A), the level of alkaline phosphatase (B) and the calcium content in blood serum (C)

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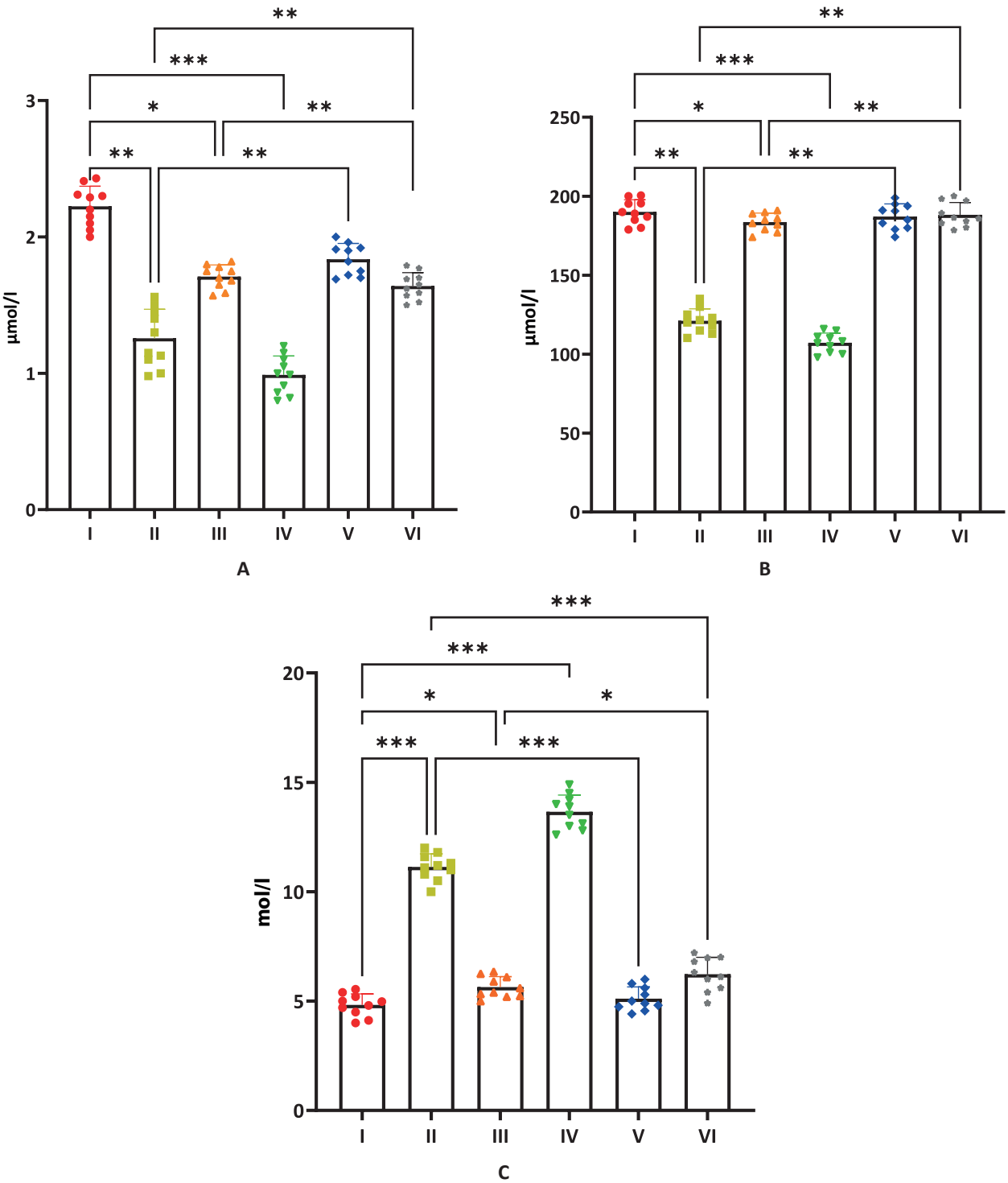
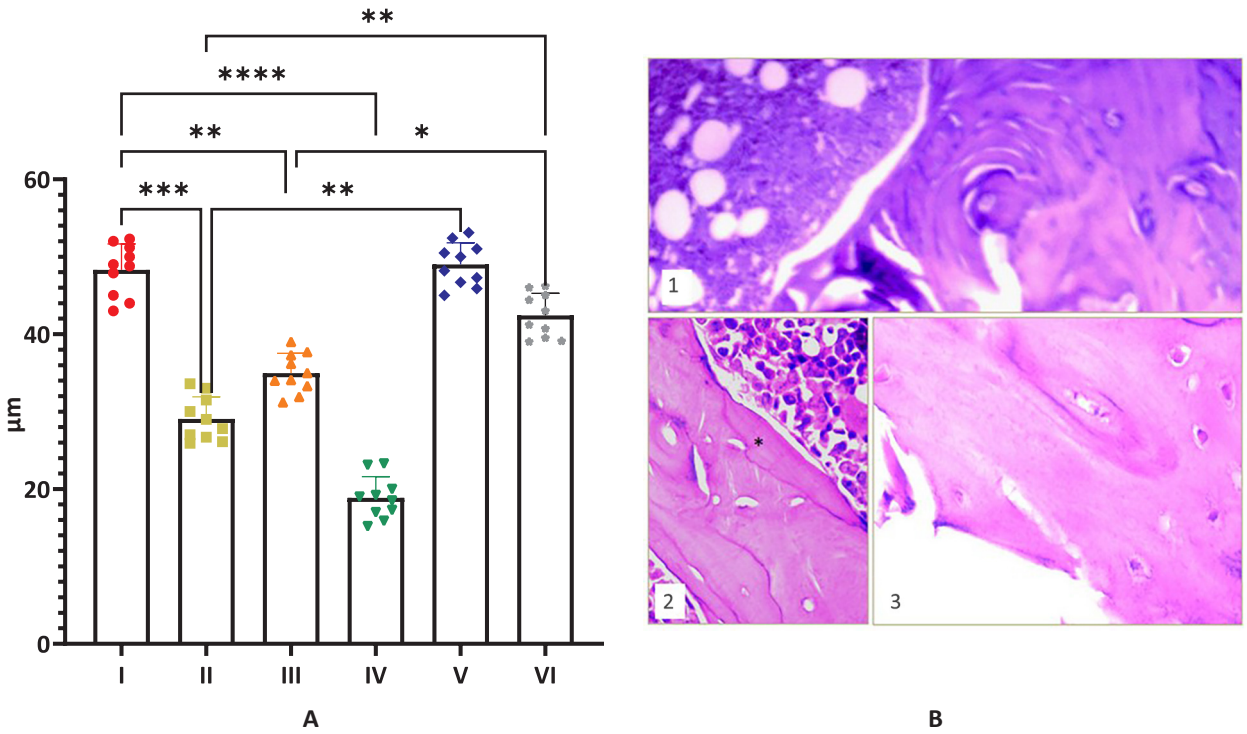


Figure 6 – Effect of composition No. 1 on the content of superoxide dismutase (A), the level of glutathione peroxidase (B) and malondialdehyde (C)



A B

Figure 7 – Effect of composition No. 1 on bone trabeculae thickness (A) and tibia microstructure (B)

Note: tibia microstructure in group VI: MP+composition No. 1+L-NAME; 1 – cortical bone and adjacent area of spongy substance without specific changes; 2 – bone trabecula *of spongy substance with a newly formed layer of lamellar bone tissue; 3 – intact internal structure of bone trabecula. Stained with hematoxylin and eosin, magnification ×100 (A), ×400 (B).

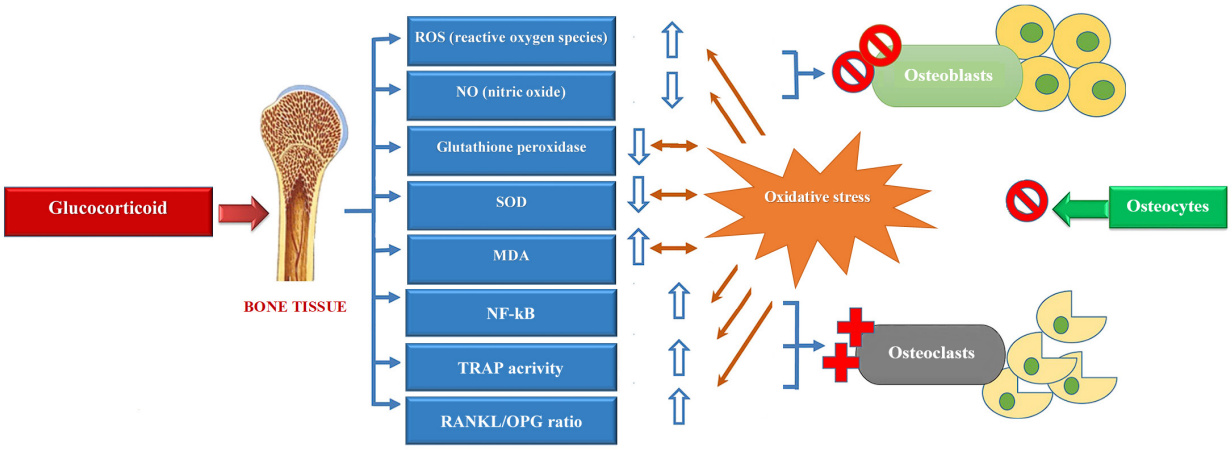


Figure 8 – Possible mechanisms of bone tissue damage in steroid-induced osteoporosis

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The ^{1}H NMR spectrum of the sample composition No. 1 showed the key signals associated with nicotinate anion: 8.94 (br s, 0.25H), 8.63 (br s, 0.25H), 8.34 (dt, J=8.0, 1.8 Hz, 0.25H) 7.60 (dd, J=7.9, 5.1 Hz, 0.25H) and 6-acetihexanoate anion 3.16 (t, J=6.9 Hz, 2H), 2.58 (s, 3H), 2.22 (t, J=7.4 Hz, 2H), 1.98 (s, 9H), 1.63–1.46 (m, 4H), 1.38–1.29 (m, 4H). The ratio of anionic residues is 1:3 (nicotinate/hexanoate). The key signals of 2-ethyl-6-methyl-3-hydroxypyridine were notified: 7.69 (d, J=8.7 Hz, 1H), 7.40 (d, J=8.7 Hz, 1H), 2.92 (q, J=7.6 Hz, 2H, (2-ethyl (CH2)), 1.26 (t, J=7.6 Hz, 4H, 2-ethyl (CH3)).

Composition of 3-hydroxypyridine derivatives prevents the development of steroid-induced osteoporosis

At the beginning of the experiment, significant differences in the baseline bone density were not observed in any of the 6 experimental animals' groups. In all the experimental groups, the mean values of the femur density were 4.91±1.03 mg/cm^2, which confirms the absence of clinical osteoporosis signs in all the experimental animals at the beginning of the experiment (Fig. 2A). However, after 5 weeks of the experiment, a significant and trustworthy decrease in the bone density by 37% (p <0.05) was noted in group II; in group III, there was a decrease by 29% (p <0.05) and in group IV – by 52% (p <0.05). A combined use of MP+L-NAME (group IV) contributed to the maximum reduction in the bone density (Fig. 4A). The administration of composition No. 1 led to the prevention of a decrease in the bone density, bringing the values closer to the group of intact animals (Fig. 4A).

The microcirculation analysis of the proximal metaphysis parameters of the femur in the intact animals showed the indicator of 101.1±4.15 p.u. (Fig. 4B), while in group II, it significantly decreased to the values of 72.09±3.26 p.u. (p <0.05); in group III – up to 67.8±4.12 p.u. (p <0.05); in group IV, there was a maximum decrease in the microcirculation index to the values of 55.3±4.12 p.u. (p <0.05). Against the background of the MP and L-NAME administration, the test compound, composition No. 1, effectively prevented a decrease in the level of the regional blood flow in the femoral bone tissue. The indicator of the microcirculation level in the MP+composition No. 1 group (group V) was 100.8±3.23 p.u.; in the MP+L-NAME+composition No. 1 (group VI) it was 98.1±4.79 p.u., respectively (p <0.05) (Fig. 4B).

A test of vasodilation with an endothelium-dependent (the intravenous acetylcholine administration) and endothelium-independent (the intravenous sodium nitroprusside administration) was conducted. In the calculation of the EDC, the following values were established: 1.22±0.01 (p <0.05) in the group of intact animals; 3.6±0.07 (p <0.05) in the MP group; 4.66±0.09 (p <0.05) in the L-NAME group; 6.31±0.04 (p <0.05) in the MP+L-NAME group (Fig. 5A).

The administration of composition No. 1 contributed to the correction of the endothelial damage. Thus, in the MP+composition No. 1 group, CED decreased to 1.4±0.02, in the MP+L-NAME+composition No. 1 group – to 2.1±0.03, respectively, which confirms an increase in the NO bioavailability with the administration of composition No. 1.

For a biochemical analysis of bone metabolism processes, the concentrations of Ca^{2+} and bone alkaline phosphatase (an osteosynthesis marker) were determined. In the MP and MP+L-NAME groups, there was a statistically significant decrease in serum calcium levels by 33% and 41% (p <0.05), respectively. In the rest of the experimental groups, no statistically significant difference was observed. The administration of composition No. 1 helped to prevent the loss of Ca^{2+} caused by the administration of MP and L-NAME (Fig. 5B).

After two weeks of the experiment, the MP and MP+L-NAME groups showed a significant decrease (p <0.05) in serum alkaline phosphatase levels. After 5 weeks of treatment the animals with composition No. 1, serum alkaline phosphatase levels remained significantly lower than in the MP and MP+L-NAME groups, which confirms the effectiveness of composition No. 1 in the treatment of bone metabolism disorders (Fig. 5C).

When studying the effect of composition No. 1 on the oxidative stress markers in the blood serum, it was found out that initially, the contents of SOD and glutathione peroxidase in the MP and MP+L-NAME groups were significantly lower in comparison with the group of intact animals (p <0.05) by 32 and 41.3%, respectively (Fig. 6A). The administration of composition No. 1 to the animals made it possible to statistically significantly increase the concentration of SOD and glucation peroxidase relative to the groups of animals with MP and MP+L-NAME (Fig. 6A and 6B). There was a change in the level of malondialdehyde (MDA) in the group of animals with MP and MP + L-NAME, in the form of a significant secretion increase to the level of 4.9±0.1 and 5.2±0.2 mol/l (p <0.05), respectively (Fig. 6C). The use of composition No. 1 as a pharmacological support made it possible to statistically significantly decrease the concentration of the lipid peroxidation product – MDA-relative to the group of the animals with pathology modeling (groups II and IV) (Fig. 6C).

To confirm the morphofunctional and biochemical samples, histomorphological studies of the animals' proximal femurs were carried out. When studying the intact animals' materials, no features that distinguish the structure of the studied tibia areas from the typical structure, were found out (Fig. 7A).

The bone trabeculae thickness averaged 47.9±1.8 µm. With the administration of MP and MP+L-NAME, the reproduction of the bone changes characteristic of osteoporosis was achieved. The bone trabeculae

thickness decreased and amounted to 31.5±2.2 and 23.1±1.3 μm, respectively (Fig. 7A), which characterizes thinning of the bone trabecula of the spongy substance.

A corrective effect of the studied composition No. 1 is evidenced by both qualitative and morphometric parameters of spongy substance trabeculae. The general architectonics of the cortical bone and spongy substance in the MP+composition No. 1 group approximated the intact animals. The group had both cellular manifestations of the osteoplastic activity and the result of imperfect osteogenesis in the form of lamellar bone structures on the surface of the bone trabeculas (Fig. 7B).

The variety of biological effects of steroid hormones and the complexity of their metabolic pathways make it difficult to fully understand the pathogenetic aspects of the steroid-induced osteoporosis development and its progression.

The oxidative stress can play a central role among a lot of factors contributing to the development of steroid-induced osteoporosis. This has been confirmed in a number of experimental studies [23]. For the pharmacological correction of the oxidative stress in steroid-induced osteoporosis, the influence of antioxidants of various chemical nature, i.e. natural pharmaceutical, is being studied. However, their osteoprotective activity is insufficient and requires a further experimental confirmation [24, 25].

For the creation of new drugs, the choice of compounds of well-studied chemical structures, in particular, pyridine derivatives, remains relevant as a precursor [19]. The validity of this direction lies in the fact that pyridines, while having a low toxicity, exhibit a wide range of pharmacological activity. The supramolecular complex studied in the work (composition No. 1) is represented as one molecule of 2-ethyl-6-methyl-3-hydroxypyridinium 3-pyridinocarbonoate and three molecules of 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate. 2-ethyl-6-methyl-3-hydroxypyridinium 3-pyridinocarbonoate has been proven to have antihypoxic, antioxidant, and endothelioprotective activities. The second component – the compound 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate is known as a tool able of accelerating the wound surface cleansing from the necrotic masses, reducing exudative processes, activating the growth of granulation tissue, vascularization and epithelialization of wounds; it also stimulates the formation of bone marrow, accelerating the healing of bone fractures [26].

Taking into account the relationship between the oxidative stress and the development of endothelial dysfunction in the violation of bone remodeling processes, in the framework of this study, the authors tried to assess the influence of the both factors on the bone tissue damage. As it is known, the structure of the bone tissue microvasculature differs significantly from the morphology of the vascular bed of other body tissues. Bone microvessels have only endothelium and do not have muscle or connective tissue layers. Thus, it is the endothelium that mediates the entire humoral regulation of the exchange between osteoblasts, osteoclasts, and blood. To confirm the contribution of NO to the development of osteoporosis, the animals were injected with a selective inhibitor of eNOS, the compound L-NAME. An intraperitoneal administration of L-NAME at the dose of 25 mg/kg daily for 35 days led to a decrease in the bone density, which was accompanied by a statistically significant decrease in the level of microcirculation in the bone, and an increase in the EDC, which indicates the involvement of the endothelial dysfunction in the development of osteoporosis. A combined administration of glucocorticosteroid MP at the dose of 5 mg/kg every 5th day for 5 weeks (intraperitoneally) and L-NAME at the dose of 25 mg/kg for 35 days (intraperitoneally) increased the bone tissue damage, herewith, significantly reducing microcirculation and increasing EDC.

Due to the high reactivity of free radicals, their action in the body is controlled by endogenous and exogenous antioxidants, as well as enzymes of the antioxidant system. The endogenous antioxidant system does not always cope with this process, leading to the development of various pathological conditions. In the course of the study, it was found out that under the conditions of the MP and L-NAME administration, composition No. 1 contributed to the activity preservation of endogenous antioxidant defense enzymes, a decrease in the intensity of lipid peroxidation, which was expressed in an increase in the activity of superoxide dismutase, glutathione peroxidase, and a decrease in the formation of MDA relative to the group of the animals with modeling steroid- induced osteoporosis. However, in the MP group, there was a more pronounced decrease in the amount of antioxidant enzymes SOD and GP, as well as an increase in the level of MDA. The L-NAME administration had no statistically significant effect on the level of antioxidant enzymes.

A colony stimulating factor (M-CSF) and a receptor activator ligand NF-κB (RANKL) are known to influence the osteoclast differentiation and lead to the abnormal bone resorption. The importance of RANKL induction of ROS production in modulating osteoclast differentiation is well known [27]. The stimulation of RANKL causes a significant increase in intracellular ROS due to the activation of the tumor necrosis factor receptor-associated (TNF-α) and nicotinamide adenine dinucleotide phosphate oxidase (NOX) [28]. This is confirmed by a number of works verifying the osteoprotective activity of antioxidants in osteoporosis, in particular, with the use of N-acetyl-L-cysteine and ascorbic acid [29].

Therefore, targeting intracellular ROS can represent a potential therapeutic approach to prevent a bone resorption and treat disorders of the bone metabolism.

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Another important link in the pathogenesis of osteoporosis is a decrease in the blood supply to the bones, accompanied by an endothelial dysfunction and leading to the inhibition of the osteoblast activity, as well to the increased activity of osteoclasts [30]. It is now known that NO has a direct stimulatory effect on osteoblasts, positively influencing the bone tissue. At the same time, many works show a relationship between the oxidative stress and the presence of the endothelial dysfunction [31, 32].

The oxidative stress leads to a decrease in the formation of endothelial NO, which, in turn, disrupts the microcirculation in the damaged bone tissue, *inter alia* steroid-induced osteoporosis. Thus, the oxidative stress generated by the MP administration in combination with the blockade of eNOS by the L-NAME administration, confirms the hypothesis of a detrimental effect of glucocorticosteroids on the bone tissue through an increase in ROS and a decrease in the NO production (Fig. 8).

CONCLUSION

Thus, summing up the study on the osteoprotective effect of a new compound based on 3-hydroxypyridine derivatives, composition No. 1 (50 mg/kg), it can be concluded that the studied compound prevents a decrease in the microcirculation level in the thigh bone tissue, exhibits a pronounced endothelioprotective effect, increasing the bioavailability of NO, and also improves the performance of biochemical and morphometric tests against the background of steroid-induced osteoporosis. The observed improvements can be associated with the compound's effect on reducing the ROS production and inhibiting the RANKL-induced NF-κB activation. However, further in-depth studies are required to elucidate the exact mechanism of action of the compound. The data obtained characterize the prospects of studying composition No. 1 for the correction and prevention of steroid-induced osteoporosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Anton P. Danilenko – article writing, research design developing; Konstantin S. Trunov – results evaluation and interpretation; Mikhail V. Pokrovsky – study and all stages of the experiment planning; Lyudmila M. Danilenko – article writing, results interpreting; Mikhail V. Korokin – statistical data processing, text editing; Oleg S. Gudyrev – methodology development, research conducting; Aleksey A. Khentov – results interpretation; Natalya P. Masalytina – sampling for histomorphological and biochemical studies, literature sources analysis; Irina A. Tatarenkova – literary sources analysis, text editing; Albina V. Cherednichenko – pathology modeling, experimental work carrying out; Elizaveta V. Boeva – pathology modeling, experimental work; Ivan S. Koklin – literature analysis, graphic materials preparation; Eduard I. Taran – literature analysis, graphic materials preparation.

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