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THE STUDY OF OSTEOPROTECTIVE PROPERTIES OF NANOPARTICULATED FORMS OF RESVERATROL AND LOSARTAN

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Abstract. The study demonstrated that endothelial dysfunction of bone microvasculature and deterioration of regional blood flow in bone developed eight weeks after ovariectomy in female white Wistar rats thus raising the risk of generalized osteoporosis. Nanoparticulated forms of losartan and resveratrol possessing endothelioprotective action effectively prevented reduction of regional microcirculation in bone tissue through keeping it at the same level as intact rats had. It allowed maintaining an adequate level of bone remodeling processes which was manifested in slowing of thinning of bone trabeculas and in prevention of possible microfractures in them.
Key words: osteoporosis, endothelial dysfunction, bivalos, losartan, resveratrol.

Osteoporosis (OP) is a systemic skeletal disease characterized by reduction of a bone mass in a unit volume and deterioration of the bone tissue microarchitectonics which results in increased bone brittleness and high risk of bone fracture. The main reason for OP development involves unbalance between two basic processes of bone remodeling, i.e. osteogenesis and osteoresorption [1].

Deterioration of bone vascular supply [10] causing osteoblasts activity inhibition as well as osteoclasts activation is one of significant stages in pathogenic mechanism of OP. Thus during the earlier investigations we've managed to establish close interrelation between sufficient bone tissue vascular supply and bone tissue quality which is evaluated though the thickness of bone trabeculas and bone tissue resistance to external influence [8].

As is known the structure of bone tissue microvasculature has essential differences as compared to the morphology of vasculature of the other body tissues. The bone microcirculatory vessel has only endothelium which mediates the whole humoral regulation of exchange between bone cells and blood. In the course of the earlier investigations carried out by us it was demonstrated that generalized hypoestrogenism-induced OP in female Wistar rats was accompanied by the signs of endothelial dysfunction (ED) development which might cause deterioration of regional vascular supply and might result in derangement of osteogenesis and osteoreparation processes and induce development of OP.

The current nosotropic therapy does not pay sufficient attention to the medicines with endothelioprotective properties and therefore having positive effect on bone tissue vascular supply. This is indicative of importance of studying osteoprotective action of medicines with proved positive endotheliotropic effects such as for example losartan and resveratrol. At the same time increase of efficiency and reduction of the number of adverse effects caused by a medicine is an urgent problem of the current experimental and clinical pharmacology, such purpose may be reached by a medicine nanoparticulation along with its dose reduction. Due to the above the present investigation is aimed at study of the anti-osteoporosis properties of nanoparticulated resveratrol and losartan.

The research techniques

The experiments involved 267 white Wistar female rats with the weight of 250±25 g. Systemic OP simulation was accomplished through intraperitoneal anesthesia with chloral hydrate solution in the dose of 300 mg/kg after which bilateral ovariectomy was performed [5]. Progression of generalized OP was evaluated eight weeks (on the 57th day) after the surgery.

The level of microcirculation was assessed in the tissues of proximal metaphysis of the femoral bone. For this purpose an animal was fixed on a surgical table [7] and a monocortical orifice was bored in the femoris after which a conductor shaft [6] was introduced in the

orifice in order to hold stably a sensor used for bone microcirculation measuring. The following equipment of Biopac Systems Company was used for obtaining microcirculation data: polygraphs MP100 and MP150 with a laser Doppler flowmetry (LDF) module LDF100C and a sensor TSD144. The LDF result recording was made via the program AcqKnowledge of 3.8.–4.2. versions, microcirculation value was expressed in perfusion units (PU).

Progression of hypoestrogenism-induced ED was assessed after measuring an intraosteal microcirculation level which was made trough a test for endothelium-dependent vasodilation (EDVD) as a reaction to bolus intravenous administration of acetyl choline solution in the dose of 40 μg/kg [16] and for endothelium-independent vasodilation (EIVD) as a reaction to bolus administration of sodium nitroprusside solution in the dose of 30 μg/kg [2]. In order to provide objective estimation of endothelial dysfunction in case of generalized OP an endothelial dysfunction coefficient (EDC) was calculated based on LDF data in the bone [4, 8].

Morphological examination of the femoral bone proximal metaphysis was made within an integral assessment of efficiency of both the studied medicines alone and their combinations in order to confirm OP progression. Slide plates with tissue specimens were analyzed by optical microscopy and photos of the bone trabeculas were taken by means of matching a camera lens and a microscope eyepiece. For the purposes of bone tissue histomorphometry the precalibrated program ImageJ of 1.39–1.43 version was used, with the aid of the mentioned program the bone trabeculas width was measured and expressed in millimeters.

We've chosen nanoparticulated forms of the medicine having endothelioprotective and osteoprotective effect as proved by the previous investigations, namely losartan potassium and resveratrol for investigation of anti-osteoporosis action [9]. In the course of experiments losartan (in the dose of 6 mg/kg) as well as its nanoparticulated form (hereafter «n-losartan», in the dose of 0.6 mg/kg) were administered intragastrically every day once a day over a period of eight weeks after osteoporosis simulation. Resveratrol (in the dose of 2 mg/kg) as well as its nanoparticulated form (hereafter «n-resveratrol», in the dose of 0.2 mg/kg) was introduced intraperitoneally according to the same regimen. Bivalos in the dose of 171 mg/kg was used as a reference substance.

A group of controls was represented by the group of animals with experimental OP but not receiving pharmacological correction. A group of intact rats included animals which were subjected to false surgery (false ovariectomy not involving ectomy of ovaries).

Statistical analysis of the obtained data was made in the Microsoft Excel program. «Descriptive statistics» was used for calculation of the mean value (M) and the standard error (m). «Two-sample t-test with different variances» was used for comparison of indices in the different groups of animals and determination of significance of differences between them. The differences having $p<0.05$ value were considered as statistically significant.

The research results

The LDF results allowed to ascertain significantly lower level of microcirculation in the bone tissue of the rats eight weeks after ovariectomy (61.52±3.74 PU; n=42) as compared to the intact animals (100.51±4.41 PU; n=30).

Microcirculation intensity reduction with the following blood flow indexes recovery was observed in response to systemic administration of acetyl choline and nitroprusside. For example microcirculation level decrease during EDVD tests in the group of intact animals amounted to 46.7±3.8% of the initial indices on the average, in the group of rats with experimental OP this index amounted to 38.9±3.8%. When EIVD reaction was checked the level of microcirculation in the group with intact rats reduced by 29.0±3.5% on the average as compared to the initial value and in the group of controls by 27.3±5.3%.

EDC in the group of intact animals constituted 1.3±0.2 and in the group with experimental OP EDC was significantly higher and made 2.4±0.2.

Osteoporosis-related changes in the skeletal bones were histologically proven for all of the rats eight weeks after ovariectomy, i.e. there was observed thinning of the bone trabeculas and extension of intertrabecular spaces. Besides some histological specimens demonstrated microfractures of the bone trabeculas. Statistically significant decrease of the average bone trabeculas width within the studied location was an objective criterion of OP progression eight weeks after the bilateral ovariectomy. Thus the average bone trabeculas width in the proximal femoral metaphysic in the rats with experimental OP (61.68±1.24 μm) was lower than that of the intact animals (97.69±1.02 μm) by 36.8%.

In the course of analysis of the studied medicines it was found out that losartan (n=35), resveratrol (n=20) and their nanoparticulated forms (n=20) as well as Bivalos reference substance (n=20) effectively prevented reduction of the level of regional blood flow in the femoral bony tissue (Fig. 1).

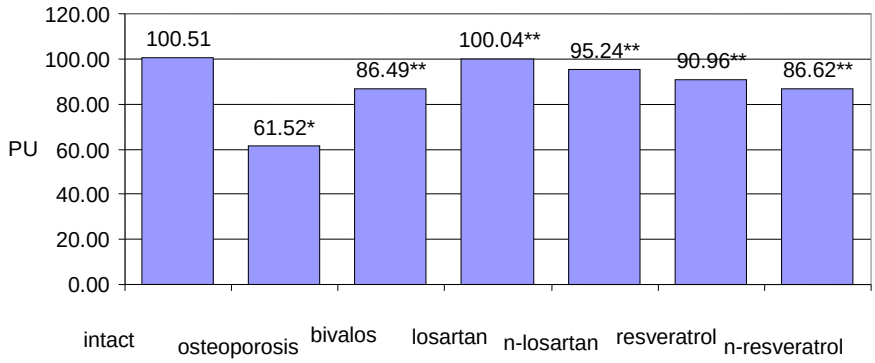

Figure 1. Results of the effect of the studied medicines on bone tissue blood supply 8 weeks after bilateral ovariectomy. Note. Hereinafter: * – p<0.05 as compared to the group of intact animals; ** – p<0.05 as compared to the group of rats with osteoporosis

The results of LDF in the groups of rats receiving bivalos, losartan, n-losartan, resveratrol and n-resveratrol approximated the indexes of the intact animals and had no statistical differences between each other as well as had statistically higher values than that in the group of controls.

It was found out that all of the studied medicines except for bivalos made the ratios between the areas of triangles above the curves characterizing recovery of the level of bone microcirculation due to administration of nitroprusside and acetyl choline equal to the same ratios of the intact animals. Hence the studied medicines induced statistically significant reduction of EDC to the following levels: losartan – 1.5±0.2, n-losartan – 1.5±0.2, resveratrol – 1.3±0.2, n-resveratrol – 1.3±0.1, thus demonstrating endothelioprotective action. The value of EDC in the group of animals receiving bivalos made 2.1±0.2.

Optical microscopy of the femoral bone cuts of the rats receiving medicines demonstrated preservation of the bone tissue structure and bigger width of bone trabeculas as compared to the rats having OP and not receiving drugs. It has been found that bivalos, losartan, n-losartan, resveratrol and n-resveratrol prevented reduction of the average bone trabeculas width to the level which the animals with experimental OP had however the average trabeculas width didn't reach the values characteristic for intact rats (Fig. 2).

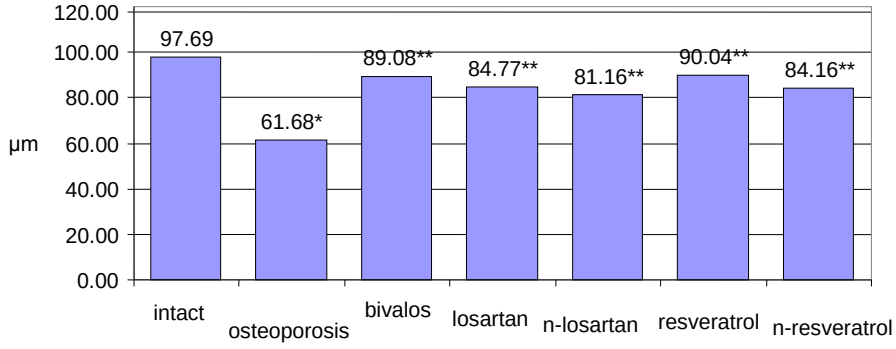

Figure 2. The results of the impact of medicines on the width of bone trabeculas

In the course of study of activity of combinations of the investigated medicines it has been found that the combinations of losartan in the dose of 6 mg/kg with resveratrol in the dose of 2 mg/kg (n=20), n-losartan in the dose of 0.6 mg/kg with resveratrol in the dose of 2 mg/kg (n=20), losartan in the dose of 6 mg/kg with n-resveratrol in the dose of 0.2 mg/kg (n=20) and n-losartan in the dose of 0.6 mg/kg with n-resveratrol in the dose of 0.2 mg/kg (n=20) more effectively prevented lowering of the level of regional blood circulation in the femoral bone tissue as against monotherapy with the stated medicines (Fig. 3).

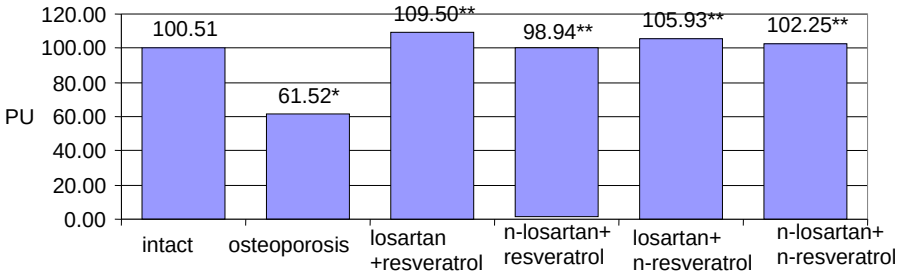

Figure 3. The results of the effect of combinations of medicines on the blood supply of the bone tissue

The results of LDF in the groups of rats receiving combined therapy had no statistical differences as compared to the indices of the intact animals and often even exceeded the same, had no statistical differences between each other and significantly exceeded both the values characteristic for the group of controls and the group of animals receiving bivalos.

It has been found that all of the studied drug combinations made the ratios between the areas of triangles above the curves characterizing recovery of the level of bone microcirculation due to administration of nitroprusside and acetyl choline equal to the same ratios of the intact animals. Hence the studied drug combinations induced statistically significant reduction of EDC to the following levels: losartan + resveratrol – 1.1±0.1, n-losartan + resveratrol – 1.2±0.1, losartan + n-resveratrol – 1.4±0.2, n-losartan + n-resveratrol – 1.2±0.1 thus demonstrating pronounced endothelioprotective action.

Optical microscopy of the femoral bone cuts of the rats receiving treatment by the studied drug combinations demonstrated preservation of the bone tissue structure and bigger width of bone trabeculas as compared to the rats having OP and not receiving medicines. It has been found that the combinations of the investigated medicines efficiently prevented reduction of average bone trabeculas width to the level which the animals with experimental OP had (Fig. 4).

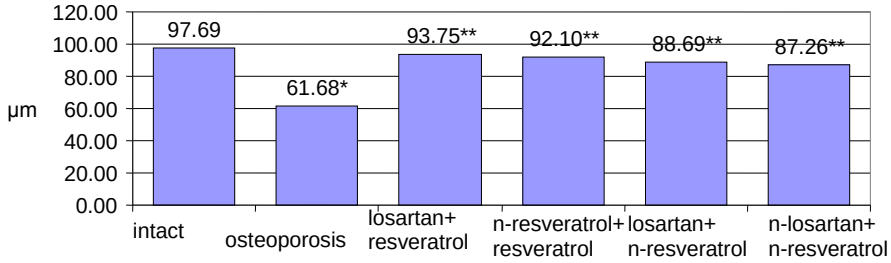

Figure 4. The results of the effect of combinations of drugs on the width of bone trabeculas

Discussion

Development of fully functional microcirculatory bloodstream and systematic condition of regional microcirculation in bone tissue have utmost value for maintenance of bone homeostasis. Impairment of bone tissue blood supply may induce occurrence of such pathologies of musculoskeletal system as osteonecrosis [12], osteomyelitis [17] or osteoporosis [11, 18]. Endothelium in mature vasculature plays the major regulatory role ensuring connection between other layers in a vascular wall and giving adequate response to their needs by means of secretion of mediators [3]. Therefore in our opinion endothelium of bone tissue vasculature being an essential part of a bone determines the state of regional microcirculation to a large extent hence it is responsible for maintaining homeostasis in a bone.

The above statement is approved by the works of a number of authors who declare for example that VEGF (a key regulator of a cascade of events resulting in vascular system formation and development) plays a significant role in the processes of remodeling [13] and repair of bone tissue injuries [14]. Thus it was demonstrated that inhibition of VEGF induced increase of the width of the femoral and tibial growth plates, reduction of angiogenesis intensity in the growth plates, disappearance of blood vessels in the metaphysial growth plate as well as decrease in intensity of formation of a bone

trabecular structure [13] and intensification of spongy bone tissue resorption [15].

At the present time the medicines having endothelioprotective properties are being actively studied as well as possible pathoegenetic mechanisms of their effect on vascular endothelium are being formulated.

Endothelioprotective influence of angiotensin receptor antagonists is obviously realized through AT1-receptors block which results in decrease of superoxide radicals production, reduction of NO bonding degree and its accumulation. Since AT1-receptors stimulation favors formation of superoxides which inactivate NO and stimulation of AT2-receptors results in vasodilation and intensification of natriuresis due to activation of the system of bradykinin, NO and cyclic guanosine monophosphate the effect of AT-2 (intensification of synthesis or inactivation of NO) depends on the type of receptors which have prevalent interaction with AT-2. Therefore it's obvious that with AT1-receptors block there exist favorable conditions for intensified functioning of free AT2-receptors which results in accumulation of NO.

Resveratrol as a representative of the group of phytoalexins induces endothelium-dependent relaxation of vasculature through intensification of nitric oxide production and the following growth of the level of cyclic guanosine monophosphate. At the same time the mentioned effects are diminished in case of administration of competitive inhibitors of NO-synthase, i.e. N^G- monomethyl-L-arginine and N^G-nitro-L- arginine. It is known that short-time effect of resveratrol in low concentrations (1-10 μM) on endotheliocytes results in increase of the quantity of the produced nitric oxide which can be explained by short-term intensification of eNOS and reduction of superoxide production in endothelium. Resveratrol promotes eNOS expression and VEGF in chronological order. On the other hand inhibition of NO production by eNOS inhibitors results in significant reduction of mitogenic and angiogenic effects stimulated by VEGF.

Use of nanoparticulated forms of losartan and resveratrol in the present study allowed achieving two-fold reduction of the doses of the tested medicines with maintenance of comparable therapeutic outcome. In addition the nanoparticulated forms of losartan and resveratrol demonstrated efficient osteoprotective action both for monotherapy and for combination of drugs.

Therefore the nanoparticulated forms of losartan (AT1-receptors blocker) and resveratrol (a representative of the group of phytoalexins) through their endothelioprtective effect on endothelium of microcirculatory bloodstream of bone tissue efficiently prevent decrease of intensity of regional blood supply in bone tissue in case of experimental osteoporosis and have osteoprotective action consisting in positive influence of such medicines on the processes of bone remodeling and restoration.

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