ISSN: 0975-766X
CODEN: IJPTFI
Available Online through *Research Article*
www.ijptonline.com

OSTEOPROTECTIVE EFFECTS OF NANOENCAPSULATED FORMS OF RESVERATROL AND LOSARTAN AND THEIR COMBINATION

Oleg S. Gudyrev[1], Natalya Y. Koklina[2], Alexander V. Faitelson[2], Mikhail V. Pokrovskiy[1], Galina A. Lazareva[2], Alexander A. Dolzhikov[1], Vladimir D. Lutsenko[1], Vladimir I. Shutov[1].
[1]Belgorod State University, 85, Pobedy St., Belgorod, 308015, Russia.
[2]Kursk State Medical University, 3, K. Marx St., Kursk, 305041, Russia.
Email: gudyrev@mail.ru

Received on 15-10-2016 *Accepted on 18-11-2016*

Abstract

The use of nanoencapsulated forms of losartan and resveratrol in this study led to a tenfold decrease in doses of studied drugs while maintaining comparable pharmacological activity. At the same time nanoencapsulated forms of losartan and resveratrol have demonstrated an effective endothelio- and osteoprotective action both as monotherapy and in combination.

Keywords: Osteoporosis, bivalos, losartan, resveratrol, nanocapsules.

Introduction

Osteoporosis – multifactorial systemic disease characterized by progressive decrease in bone mass and impaired bone structure, leading to an increased risk of skeletal fractures [1, 2]. The prevalence of osteoporotic bone fractures making it one of the prior problems of modern medicine. According to WHO data, the problem of osteoporosis as a cause of disability and mortality in patients from fractures, ranked fourth in the world among non-communicable diseases, behind diseases of the cardiovascular system, cancer pathology and diabetes. Osteoporosis is diagnosed more than 75 million people in the US, Europe and Asia. With the increase in life expectancy the world's population is expected to increase the frequency of osteoporotic fractures. However, the application of modern techniques of diagnosis and early begun prevention and treatment of osteoporosis reduce the risk, and in some cases, prevent the occurrence of fractures and their complications.

Modern drugs used in the treatment of osteoporosis can be divided into three groups: slowing bone resorption (estrogens, calcitonin); stimulators of bone formation (anabolic steroids, fluoride, strontium ranelate); multiaction (vitamin D). This division is relative, since these drugs in varying degrees, affect all processes of osteoregeneration.

However, the increase in the frequency of fractures on the background of osteoporotic changes in the skeleton proves that there is no reliable ways of medical therapy of this disease. This fact points to the urgency of the search for new ways of pharmacological correction of bone remodeling disorders in osteoporosis, proving thus the direction of this study. Known the significant role of microcirculatory blood supply to the bone tissue recovery processes [3, 4, 5]. Capillary blood vessels in the bones are significantly different from the structure of other body tissues microvessels. They lack muscle and connective tissue layers, there is only the endothelium, through which directly goes regulatory metabolic processes between osteoclasts, osteoblasts and blood [6, 7, 8]. Endothelial dysfunction may be one of the causes of blood supply to bone disorders, through the microcirculation deterioration able to lead to osteogenesis pathology, thereby causing an osteoporotic changes [9, 10, 11, 12].

Earlier experimental studies have demonstrated the positive osteoprotective effects of drugs, correcting endothelial dysfunction, such as angiotensin-converting enzyme inhibitor enalapril, angiotensin receptor blocker losartan and resveratrol – a natural substance found in large quantities in red wine grape [13, 14].

At the same time, there is no information in the public literature about anyone to use nanoencapsulated forms of resveratrol, losartan or other medicinal products with a positive effect on vascular endothelial function in the treatment of osteoporotic disorders.

Materials and methods

Experiments were carried out on 267 white female Wistar rats weighing 250±25 g. To simulate osteoporosis rats were anesthetized by intraperitoneal injection of chloral hydrate aqueous solution in a dose of 300 mg/kg, and the bilaterally ovariectomy operation was performed. Beginning from the next day after ovariectomy, as in the prevention of hypoestrogenism osteoporosis, studied drugs and combinations of drugs were administered for eight weeks. Osteoporosis as osteoprotective action of studied preparations and their combinations, were evaluated after eight weeks (on day 57) after ovariectomy, control animals were underwent false ovariectomy.

The level of microcirculation was evaluated in tissue of proximal femur. To get data on the state of the microcirculation in the bone we used equipment manufactured by Biopac systems: MP100 and MP150 polygraphs with laser doppler flowmetry (LDF) module LDF100C and invasive needle sensor TSD144. Registration and processing of the results of LDF was performed using the program AcqKnowledge version 3.8.-4.2., values of microcirculation were expressed in perfusion units (PU).

After measuring the level of microcirculation, conducted tests for the endothelium-dependent vasodilation (EDV) in response to intravenous administration of acetylcholine solution in a dose of 40 mg/kg and endothelium-

nondependent vasodilation (ENV) in response to intravenous administration of sodium nitroprusside solution in a dose of 30 mg/kg [15, 16]. With intravenous administration of acetylcholine and sodium nitroprusside we observed a decrease in the level of the microcirculation with subsequent normalization of parameter. For objectification of evaluation of development of endothelial dysfunction in developing osteoporosis, as well as to monitor the efficacy of the studied drugs and their combinations, on the basis of the results calculated coefficient of endothelial dysfunction (CED) as the ratio of the area of the triangle above the curve of microcirculation restoration in bone tissue in response to sodium nitroprusside to the area of the triangle above the curve of microcirculation restoration in bone tissue in response to the introduction of acetylcholine.

To evaluate the osteoprotective activity of studied drugs and their combinations carried out histomorphometry of proximal femur bone tissue. Biomaterial fixed in 10% formalin solution with subsequent filling in paraffin and stained with hematoxylin-eosin. The slides with histological preparations were subjected to light microscopy and photographed bone trabeculae. For histomorphometry of bone tissue used a pre-set program ImageJ version 1.39-1.43.

In order to study osteoprotective action we chose the traditional and nanoencapsulated forms of drugs losartan and resveratrol. Angiotensin receptor blocker losartan (Bloktran, Pharmstandard-Leksredstva, Kursk) was used in a dose of 6 mg/kg, representative of phytoalexins group resveratrol (Greensyn (Guangzhou) Co., Ltd.) – in a dose of 2 mg/kg, which is consistent with the available literature.

Nanoencapsulated forms of losartan and resveratrol were obtained with physico-chemical method. To determine the size of the nanocapsules was used method of analysis of the trajectories of the nanoparticles developed by Nanosight (UK). The measurements were carried out on Multiparameter Analyzer of nanoparticles Nanosight LM0 production of Nanosight Ltd. (UK) in the configuration of HS-BF (high-sensitivity video camera Andor Luca, a semiconductor laser with a wavelength of 405 nm and a power of 45 mW). The measurement results showed that the average size of the obtained nanocapsules of resveratrol in xanthan gum was 356 nm for losartan in sodium alginate – 168 nm. The highest concentration of the particles had sizes ranging from 50 to 200 nm. Thus, the nanocapsules have a necessary size for the study of their endothelioprotective properties within single compartment pharmacokinetic model.

The study of nanoencapsulated forms of losartan (further – n-losartan) and resveratrol (further – n-resveratrol) performed in doses of 0.6 mg/kg and 0.2 mg/kg, respectively. Also studied endothelio- and osteoprotective effects of combinations: nanoencapsulated form of losartan with resveratrol, nanoencapsulated form of resveratrol with losartan and the combination of nanoencapsulated forms of resveratrol and losartan in the previously mentioned doses.

As a comparison drug was chosen fairly common on the Russian market and effective drug for the prevention and correction of osteoporotic disorders – Bivalos (strontium ranelate) in a dose of 171 mg/kg. Studied drugs as monotherapy and in combination were administered daily once a day intragastrically as a suspension in 1% starch paste (losartan, n-losartan, Bivalos) or intraperitoneally (resveratrol, n-resveratrol) at the rate of 1 ml per 100 g of animal body weight.

All the animals were divided into groups by stratified randomization stratified by body weight, conditions of detention and food, as well as for operations and manipulations. During the experiment, the animals were kept in conditions of standard experimental biological clean room, the air temperature was 22-24 °C, lights – 12 h light/dark cycle, all rats received pellets and filtered water.

All the experimental data obtained in this study were analyzed using descriptive statistics (Microsoft Excel analysis package). In the group indicators were defined the average values (M) and the average error (m), the data in the text and tables presented in the form M±m. The analysis of statistically significant differences in intergroup comparisons was made based on heteroscedastic t-test (two-sample Student t-test with different variances). In the analysis of a large number of comparisons used t-test adjusted Newman-Keuls.

Results of the study

Study of Bivalos osteoprotective activity. According to the study protocol, osteoporosis was modeled by performing of bilateral ovariectomy operation in mature female Wistar rats. On 57 day after the surgery before necropsy of animals evaluated the level of the microcirculation in the proximal right femur. LDF results in bone tissue of rats revealed a significantly lower level of microcirculation in femoral bone tissue in rats with osteoporosis (61.52±3.74 PU) compared to control animals (100.51±4.41 PU). It was found that Bivalos in a dose of 171 mg/kg warned microcirculatory level reduction in femoral bone tissue in osteoporosis (86.49±4.99 PU). LDF results in a group of rats receiving Bivalos were significantly different from those in the control group of animals, and in the group of rats with osteoporosis.

After measurement the microcirculation in the femoral bone tissue carried out functional vascular tests. Calculate values of the coefficient of endothelial dysfunction for the microcirculatory level of the proximal femur in rats. Thus, in the control group of animals received CED=1.3±0.2, and in the group of rats with experimental osteoporosis CED=2.4±0.2. Bivalos in a dose of 171 mg/kg not normalized the proportion between the areas of triangles over curves of restoration of microcirculation level in bones during vascular tests and significant effect on the coefficient of endothelial dysfunction not provided (Table 1).

For further morphological studies produced the fence of bone biomaterial. Histological sections of the proximal femurs of animals subjected to microscopy and histomorphometry. Osteoporotic bone skeletal changes were confirmed histologically in all rats eight weeks after ovariectomy. During the microscopy we revealed pathological changes in the trabecular bone tissue of the femur in rats with experimentally-induced gipoestrogen osteoporosis. The spongy material found that the lattice network of trabecular bone was thinned, and sometimes partially interrupted as a result of the disappearance of the horizontal trabecular thinning and perforation of the bony plates themselves. In some histological preparations were defined trabecular bone microfractures, about lifetime occurrence be judged on the germination of the connective tissue at the site of the fracture that is the hallmark from the possible damage during sampling of biological material or production of histological preparation.

Table 1. The dynamics of microcirculation during functional vascular sampling in modeling of experimental osteoporosis and its correction with Bivalos in a dose of 171 mg/kg.

Group of animals	Reaction	The amount of reduction of microcirculation, PU	Time of normalization of microcirculation, sec.	The area of the triangle over the curve of microcirculation restoration, c.u.	CED
Control (n=42)	EDV	55.8±11.4	22.2±3.8	571.3±153.8	1.3±0.2
	ENV	36.1±8.7	40.3±4.6	697.2±171.2	
Osteoporosis (n=30)	EDV	28.9±3.9	17.2±3.3	246.9±69.2	2.4±0.2*
	ENV	21.2±4.6	47.7±4.0	520.3±138.7	
OP+Bivalos (n=20)	EDV	31.2±4.2	19.5±2.6	296.7±65.3	2.1±0.2
	ENV	26.4±5.1	45.1±3.9	589.6±156.4	

Annotation: here and everywhere below: c.u. – conventional units; CED – coefficient of endothelial dysfunction; OP – osteoporosis; * – $p<0.05$ compared with the control.

Objective criterion for osteoporosis in eight weeks after bilateral ovariectomy has been a significant decrease in the average width of bone trabeculae in the spongy tissue of the proximal femur. Thus, the average width of the bone trabeculae in this localization in rats with osteoporosis was 61.68±1.24 microns, which is significantly lower (36.9%) than the index in the control animals – 97.69±1.02 microns. In rats treated with Bivalos in a dose of 171 mg/kg, we

found no trabeculae microfractures and conservation of bone tissue structure. In morphometric study it was found that Bivalos prevented decrease in the average width of bone trabeculae. The specified parameter was significantly higher than in animals with experimental osteoporosis without treatment in studied localization (89.08±1.09 microns). Thus, the average width of the bone trabeculae in the proximal femur under the influence of Bivalos in a dose of 171 mg/kg was 44.4% higher than in animals with untreated osteoporosis.

The study of osteoprotective activity of nanoencapsulated forms of losartan and resveratrol

According to experimental design, n-losartan in a dose of 0.6 mg/kg and n-resveratrol in a dose of 0.2 mg/kg were administered daily once a day for 56 days after bilateral ovariectomy. It was found that the tested drugs prevent decrease in regional blood flow level in femoral bone tissue in rats with osteoporosis.

LDF results in the group of rats treated with n-losartan (95.24±1.96 PU) were significantly higher than those in the group of rats with untreated osteoporotic. No significant differences from similar values in groups of rats with osteoporosis who underwent Bivalos and losartan (100.04±2.29 PU) therapy, has not been revealed (p=0,111 and p=0.161 respectively). LDF results in animals treated with n-resveratrol (86.62±1.59 PU) were also significantly higher than the value of LDF in the group of rats with osteoporosis without treatment. No significant differences between the studied parameters in groups of rats with osteoporosis who underwent Bivalos and resveratrol (90.96±4.27 PU) treatment, also was not found (p=0,979 and p=0.347 respectively).

The results of the vascular tests presented in Table 2. CED indicators in both experimental groups were significantly different from the group of animals with osteoporosis. CED values in the groups of animals treated with nanoencapsulated forms of preparations did not differ significantly from the corresponding values of CED in the groups of animals treated with traditional forms of preparations (p>0.05).

Table 2. The results of the effect of nanoencapsulated forms of losartan and resveratrol on the coefficient of endothelial dysfunction, calculated on the results of laser doppler flowmetry in the bone

Group of animals	Reaction	The amount of reduction of microcirculation, PU	Time of normalization of microcirculation, sec.	The area of the triangle over the curve of microcirculation restoration, c.u.	CED
OP+losartan (n=35)	EDV	32.9±4.0	38.4±6.3	645.2±146.6	1.5 ±0.2**
	ENV	36.6±6.1	54.1±10.2	1016.2±284.3	

OP+resve-ratrol (n=20)	EDV	43.4±1.2	21.9±2.0	472.6±31.5	1.3 ±0.2**
	ENV	36.2±3.4	33.5±3.3	610.9±100.2	
OP+n-losar-tan (n=20)	EDV	35.6±3.4	24.7±2.1	421.7±82.6	1.5 ±0.2**
	ENV	33.9±5.2	36.4±3.5	605.5±125.1	
OP+n-resve-ratrol (n=20)	EDV	37.4±5.3	31.9±6.2	578.4±143.3	1.3 ±0.1**
	ENV	34.7±6.2	43.6±5.7	746.8±174.9	

Annotation: here and everywhere below: ** – $p<0.05$ compared with the osteoporosis group

At femoral bones slices microscopy of rats treated with n-losartan and n-resveratrol, found bone structure preservation of the proximal femur. In carrying out of morphometric studies indicated the prevention of reduce of the average width of the trabecular bone in the proximal femur in laboratory animals under the influence of nanoencapsulated forms of investigated drugs.

The average width of the trabeculae in rats treated with n-losartan (81.16±0.59 microns) significantly exceed those values in the group of rats with osteoporosis without treatment and was less than similar values in groups of rats with osteoporosis who underwent therapy with Bivalos and traditional form of losartan (84.77±0.60 microns). In animals treated with n-resveratrol (84.16±0.75 microns), the average width of trabeculae was also significantly higher than the values in the group of rats with osteoporosis, and also less than the values in the group of animals with osteoporosis who underwent Bivalos therapy. No significant differences from that in rats with osteoporosis treated with the traditional form of resveratrol (90.04±3.42 microns) was observed (p=0,101).

The study of osteoprotective activity of nanoencapsulated and traditional forms of losartan and resveratrol combinations

In accordance with the experimental design, as well as the fact that nanoencapsulated forms of losartan and resveratrol in 10 times smaller doses provide comparable in severity with traditional forms of drugs antiosteoporotic action, it was decided to study various combinations of these drugs on osteoporosis model.

Found that combination therapy with n-losartan and resveratrol prevents deterioration of the microcirculation in the bone of the proximal femur in rats, keeping indexes almost at the level of the control animals (98.94±2.55 PU), significant differences from the control group was not observed (p=0.815). There was excess over the indexes in rats treated with the comparison drug Bivalos (p=0.032). At the same time, there was no significant differences from groups of animals receiving n-losartan and resveratrol as monotherapy (p=0.258 and p=0.116, respectively).

It was found that the combination of losartan and n-resveratrol significantly improves microcirculation of the proximal femur (105.93±2.67 PU). The level of the microcirculation when using the designated combination

exceeded this index in rats with osteoporosis without treatment, as well as in rats with osteoporosis receiving Bivalos (p=0.001). No significant differences from the control groups was observed (p=0.420) as well as from the group of animals treated with losartan as monotherapy (p=0.112). However, it was observed an excess over the microcirculation in rats receiving n-resveratrol monotherapy (p<0.001).

It was found that the combination of n-losartan and n-resveratrol increases microcirculation in the bone of the proximal femur at developing osteoporosis (102.25±2.19 PU). The level of the microcirculation when using the designated combination in absolute terms, exceed the value of the control rats, but not significantly different from them (p=0.792), and also was significantly higher than in groups of rats, receiving on the background of osteoporosis therapy with Bivalos (p=0.006), n-losartan and n-resveratrol (p=0.022 and p<0.001, respectively).

The results of the vascular tests when using combinations of investigated preparations are presented in Table 3.

Table 3. The results of the effect of combination therapy with studied preparations on endothelial dysfunction coefficient value calculated based on the results of laser doppler flowmetry in the bone (n=20).

Group of animals	Reaction	The amount of reduction of microcirculation, PU	Time of normalization of microcirculation, sec.	The area of the triangle over the curve of microcirculation restoration, c.u.	CED
OP+losartan +resveratrol	EDV	54.8±3.5	26.0±2.1	709.8±60.4	1.1 ±0.1**
	ENV	44.7±5.8	36.7±2.4	810.0±83.6	
OP+n-losartan +resveratrol	EDV	41.7±5.3	24.8±2.7	493.8±117.7	1.2 ±0.1**
	ENV	32.6±6.2	35.1±6.4	567.4±135.2	
OP+losartan +n-resveratrol	EDV	49.3±7.2	26.4±5.2	624.2±124.4	1.4 ±0.2**
	ENV	38.6±5.3	43.4±7.8	826.4±182.7	
ОП+n-losartan +n-resveratrol	EDV	51.3±8.2	20.7±2.4	516.6±122.1	1.2 ±0.1**
	ENV	31.3±7.4	37.7±3.5	573.6±157.3	

The values of CED in rats treated with combination of n-losartan with resveratrol, losartan with n-resveratrol and nanoencapsulated forms of losartan and resveratrol, were not significantly different from those in the control group of animals, as well as significantly different in the smaller side from indicators in rats with osteoporosis without treatment and animals with osteoporosis receiving Bivalos. At the same time, there was no significant differences in CED values from groups of animals receiving monotherapy of studied drugs (p>0.05).

Microscopic examination of the proximal femur in rats who received the combination therapy with studied preparations, showed preservation of the structure of the bone as compared to rats with osteoporotic changes are not receiving treatment, no microfractures.

The width of bone trabeculae in the proximal femur in rats treated with a combination of n-losartan and resveratrol (92.10±0.49 microns), was significantly lower than the values of control rats and more than in rats with untreated osteoporosis and osteoporotic animals treated with the comparison preparation Bivalos (p=0.016). The average width of bone trabeculae in these rats exceeded that of animals treated with n-losartan monotherapy but did not differ significantly from index in the group of animals treated with traditional form of resveratrol monotherapy (p=0.554).

Revealed that the average width of trabeculae in the proximal femur under the influence of combination of losartan and n-resveratrol, despite the positive trend did not reach the values of the control animals (88.69±0.57 microns). Studied index in these rats was significantly higher than the value of animals with osteoporosis without treatment and did not differ from values in rats with osteoporosis received Bivalos (p=0.754), and also was significantly higher than in groups of animals treated with losartan and n-resveratrol as monotherapy (p<0.001).

Average trabeculae width in the investigated localization in animals treated with the combination of nanoencapsulated forms of preparations significantly exceeded the value in osteoporotic rats without treatment and was less than that of control rats (87.26±0.65 microns). This parameter was not significantly different from the values of animals with osteoporosis receiving Bivalos (p=0.161), and was significantly higher than those in groups of rats treated with nanoencapsulated forms of losartan and resveratrol as monotherapy (p<0.001 and p=0.004 respectively).

Conclusions

1. Bivalos in a dose of 171 mg/kg on the model of osteoporosis induced by bilateral ovariectomy has no endothelioprotective effect, but prevents deterioration of blood supply to the bone tissue by keeping it at 86.49±4.99 PU (compared to 61.52±3.74 PU in animals with osteoporosis), as well as increasing the average width of the trabeculae in the proximal femur by 44.4%, have expressed osteoprotective activity.
2. Nanoencapsulated forms of losartan in a dose of 0.6 mg/kg and resveratrol in a dose of 0.2 mg/kg on model of osteoporosis induced by bilateral ovariectomy, have a pronounced endothelioprotective action, manifested in the reduction of coefficient of endothelial dysfunction to a value of 1.5±0.2 and 1.3±0.1, respectively (compared to 2.4±0.2 in animals with osteoporosis). Studied preparations effectively prevents deterioration of blood supply to the bone tissue by keeping it at 95.24±1.96 and 86.62±1.59 PU, respectively, as well as increasing the average

width of the trabeculae in the proximal femur by 31.6% and 36.4%, respectively, have expressed osteoprotective activity.

3. Combination of nanoencapsulated form of losartan in a dose of 0.6 mg/kg and resveratrol in a dose of 2 mg/kg on model of osteoporosis induced by bilateral ovariectomy, has a strong endothelioprotective action, manifested in the reduction of coefficient of endothelial dysfunction to a value of 1.2±0.1. Studied combination of preparations effectively prevents deterioration of blood supply to the bone, keeping it at the level of the control animals – 98.94±2.55 PU, as well as increasing the average width of the trabeculae in the proximal femur by 49.3%, have expressed osteoprotective activity.
4. Combination of losartan in a dose of 6 mg/kg and nanoencapsulated form of resveratrol in a dose of 0.2 mg/kg on model of osteoporosis induced by bilateral ovariectomy, has a strong endothelioprotective action, manifested in the reduction of coefficient of endothelial dysfunction to a value of 1.4±0.2. Studied combination of preparations effectively prevents deterioration of blood supply to the bone, keeping it at the level higher than in control animals – 105.93±2.67 PU, as well as increasing the average width of the trabeculae in the proximal femur by 43.8%, have expressed osteoprotective activity.
5. Combination of nanoencapsulated forms of losartan in a dose of 0.6 mg/kg and resveratrol in a dose of 0.2 mg/kg on model of osteoporosis induced by bilateral ovariectomy, has a strong endothelioprotective action, manifested in the reduction of coefficient of endothelial dysfunction to a value of 1.2±0.1. Studied combination of preparations effectively prevents deterioration of blood supply to the bone, keeping it at the level of the control animals – 102.25±2.19 PU, as well as increasing the average width of the trabeculae in the proximal femur by 41.5%, have expressed osteoprotective activity.

Practical recommendations

The model of experimental osteoporosis induced by bilateral ovariectomy in female Wistar rats, can be recommended as a representative and reproducible model for studying the dynamics of osteoporotic changes in bones in laboratory animals, as well as assessment of endothelio- and osteoprotective effects of drugs. The results obtained in the course of the experimental study allow to recommend targeted clinical trials of osteoprotective action of nanoencapsulated forms of losartan and resveratrol, including combinations in osteoporotic changes in bones.

Prospects for further development of the theme

It looks promising study of osteoprotective activity of combinations of drugs with proven endothelioprotective activity and conventional treatment of osteoporotic disorders preparations, such as strontium ranelate, salmon

calcitonin, calcium supplements and others. It is also a promising idea to conduct clinical studies on the efficacy of nanoencapsulated forms of losartan and resveratrol in women with hypoestrogenic condition arising as a result of undergoing ovariectomy or postmenopausal and elderly men. Further, it is logical to assume that drugs with endothelioprotective properties may have osteoprotective pleiotropic effects, which may also be the subject of further studies.

References

1. Benevolenskaya, L.I., 2003. Guidelines on Osteoporosis. M.: BINOM. Knowledge Laboratory, 524 p.
2. Lazzarini, L., De Lalla, F., Mader, J.T., 2002. Long Bone Osteomyelitis Curr. Infect. Dis. Rep., 4: 439-445.
3. Alagiakrishnan, K., Juby, A., Hanley, D. et al., 2003. Role of vascular factors in osteoporosis. J. Gerontol. A Biol. Sci. Med. Sci., 58: 362-366.
4. Burkhardt, R., Kettner, G., Bohm, W. et al., 1987. Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone, 8: 157-164.
5. Szulca, P., 2012. Association between cardiovascular diseases and osteoporosis – reappraisal. Bonekey Rep., 1: 144-149.
6. Haigh, J.J., Gerber, H.P., Ferrara, N., Wagner, E.F., 2000. Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. Development, 127: 1445-1453.
7. Galagan, M.E., Shirokolova, A.V., Vanin, A.F., 1991. The antihypertensive effect of nitric oxide produced from exogenous and endogenous sources. Questions of Med. Chem., 37(1): 67-70.
8. Laursen, J.B., Rajagopalan, S., Galis, Z., 1997. Role of superoxide in angiotensin II–induced but not catecholamine-induced hypertension. Circulation, 95: 588-593.
9. Childs, S.G., 2005. Osteonecrosis: death of bone cells. Orthop. Nurs., 24: 295-301.
10. Chu, T.W., Wang, Z.G, Zhu P.F. et al., 2002. Effect of vascular endothelial growth factor in fracture healing. Zhongguo Xiu Fu Chong Jian Wai KeZaZhi, 16: 75-78.
11. Yao, Z., Lafage-Proust, M.H., Plouet, J. et al., 2004. Increase of both angiogenesis and bone mass in response to exercise depends on VEGF. J. Bone. Miner. Res., 19: 1471-1480.
12. Markov, X.M., 2005. Oxidative stress and endothelial dysfunction. Patol. Phys. and Exp. Ther., 4: 5-9.

13. Kochkarov, V.I., Pokrovsky, M.V., Korneev, M.M. et al., 2006. Endothelioprotective effects of resveratrol and its combination with enalapril and losartan in experimental modeling of deficiency of nitric oxide. Kuban Research Medical Gazette, 9 (90): 150-152.
14. Faitelson, A.V., Gudyrev, O.S., Pokrovsky M.V. et al., 2009. Vascular endothelium of bone as a target of pharmacological effects in experimental osteoporosis. Kuban Research Medical Gazette, 5 (110): 116-121.
15. Korokin, M.V., Pokrovsky, M.V., Artyushkova E.B. et al., 2008. Methods of experimental modeling of endothelial dysfunction. Allergology and immunology, 9 (3): 327.
16. Pokrovsky, M.V., Pokrovskaya, T.G., Kochkarov, V.I. et al., 2007. Pat. 2301015 Russian Federation, MPK[7] A61B 5/02. A method of evaluating endothelial dysfunction. Applicants and patent holders Pokorvsky, M.V., Pokrovskaya, T.G., Kochkarov, V.I. – 2005113243/14; petition 04.05.2005; publ. 20.06.07, Bull. 17. – 7 p.: ill.