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STUDY OF THE INFLUENCE OF L-NORVALINE, ROSUVASTATIN AND THEIR COMBINATION ON THE LEVEL OF MICROCIRCULATION IN BONE TISSUE IN EXPERIMENTAL OSTEOPOROSIS AND FRACTURES ON ITS BACKGROUND

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Abstract. The experiment was carried out in female white Wistar rats to investigate the effect of L-norvaline, rosuvastatin and their combination on parameters of blood supply to the bone tissue on models of experimental osteoporosis and osteoporotic fractures. It was found that the studied drugs prevent reduction of microcirculation in osteoporotic bone tissue as well as in callus tissue of experimental osteoporotic fractures and it positively influences the course of reparative regeneration of bone tissue.
Keyword: osteoporosis, osteoporotic fracture, microcirculation, endothelial dysfunction, L-norvaline, rosuvastatin, Bivalos.

Introduction

The World Health Organization defines osteoporosis as “a systemic disease of skeleton, characterized by low density of bone tissue and its microarchitecture defects”, that leads to high bone fragility and high fracture risks. The modifiable factors of osteoporotic disorders are low level of calcium and vitamin D, sedentary life-style, smoking, alcohol abuse, long-term glucocorticoid therapy and rheumatoid arthritis. The non modifiable factors includes advanced age, genetic predisposition, rapid weight loss and menopause [1, 2, 3].

According to the National Osteoporosis Foundation, ten million people in the USA have osteoporosis and 80% of them are women. Fractures are considered one of the main reasons of physical inactivity in elderly women, this significantly reduces their life quality.

Russian and foreigner orthopedic trauma specialists, state that fractures of neck of the femur is the most typical osteoporotic complication and the mortality rate is up to 30% during the first six months.

The number of surgical procedures on bone fractures using different implants is growing every year, including the use of endoprosthesis for large joints. According to recent investigations, each fifth operation of primary endoprosthesis replacement includes one re-replacement manipulation. This is generally connected with the development of aseptic instability due to osteoporotic disorders in bone tissue.

Blood circulation plays a significant role in bone tissue normal functioning. An important feature of the bone microvasculature is that the vessel wall does not have muscle and connective tissue layers, it consists only of the endothelial layer. Consequently, the humoral regulation between the osteocytes and blood is mediated by the endothelium. According to many authors, the imbalance between the processes of bone resorption and osteogenesis is based on endothelial dysfunction [4, 5, 6, 7, 8, 9, 10].

Norvaline (α-aminovaleric acid, propyl glycine) is a unconventional amino acid which is part of some proteins, in animals it is the antagonist of valine and leucine. L-norvaline is an inhibitor of arginase (a pharmacological agent with mechanism of action directed in blocking the arginase activity, thus increasing the concentration of L-arginine and, consequently increasing nitric oxide in serum) an enzyme that catalyses the cleavage of L-arginine, a substrate for nitric oxide, that is a powerful vascular endothelial mediator functioning on ornithine and urea [11, 12, 13, 14, 15].

L-norvaline is one of those substances, however its effect on the bone tissue microcirculation has not previously been investigated. This fact is the reason for the present research.

It is widely known that statins have an indirect corrective effect on endothelial dysfunction through the normalization of lipid profile and by direct effect on the endothelium (regardless of changes in lipid profile).

Possible mechanisms for improvement of endothelial functioning in the administration of statins is achieved through increased production of nitric oxide (NO), with beneficial effect on the expression of endothelial NO-synthase, decreased secretion of endothelin-1 and increased synthesis of prostacyclin, which positively affects the balance shift vasoconstrictors/vasodilators in favour of vasodilation, restoration of high content of plasma vascular endothelial growth factor (VEGF) and the induction of apoptosis in endothelial cells.

Rosuvastatin is a medication from statins group. In the available literature there is a wide variety of works about the role of statins in the correction of endothelial dysfunction, however, data about their effects on the bone tissue microcirculation is not sufficient.

Based on the powerful endothelial protective effect of arginase inhibitor, L-norvaline, and selective and competitive inhibitor of HMG-CoA reductase, rosuvastatin. A scientific interest on the study of their combined use has arisen, as implemented in this research.

Objectives. To study the effect of L-norvaline, rosuvastatin and their combination in the bone tissue microcirculation after experimental models of osteoporosis and fractures of proximal femur in Wistar rats.

Methods and materials. The experiment involved 240 female Wistar rats weighing 200-250g. Manipulations were performed with animals under general anesthesia (intraperitoneal injection of an aqueous solution of chloral hydrate at a dose of 300 mg/kg).

The animals were divided into eight groups – 20 rats each.

I – Intact – false bilateral oophorectomy surgery (performed abdominal incision without removal of the ovaries, followed by wound closure);

II – Control – true bilateral oophorectomy surgery (performed abdominal incision with removal of the ovaries, followed by wound closure) [16, 17, 18];

III – 8 weeks after oophorectomy and development of osteoporosis L-norvaline was daily administrated intragastrically, at a dose of 10mg/kg from the ninth to twelfth week [19];

IV – 8 weeks after oophorectomy, rosuvastatin was daily administered intragastrically, at a dose of 0.86mg/kg from the ninth to twelfth week;

V – 8 weeks after oophorectomy, a combination of L-norvaline at a dose of 10mg/kg, and rosuvastatin at a dose of 0.86mg/kg, was daily administered intragastrically from the ninth to twelfth week;

VI – 8 weeks after oophorectomy, a drug for comparison, Bivalos (strontium ranelate) was daily administered intragastrically at a dose of 171mg/kg from the ninth to twelfth week;

VII – 8 weeks after false oophorectomy, a model fracture of the proximal femoral metaphysis was performed;

VIII – 8 weeks after bilateral oophorectomy, a model fracture of the proximal femoral metaphysis was performed;

IX – 8 weeks after oophorectomy, a model fracture of the proximal femoral metaphysis was performed with further daily intragastrical administration of L-norvaline at a dose of 10mg/kg from the ninth to twelfth week;

X – 8 weeks after oophorectomy, a model fracture of the proximal femoral metaphysis was performed with further daily intragastrical administration of rosuvastatin at a dose of 0.86mg/kg from the ninth to twelfth week;

XI – 8 weeks after oophorectomy, a model fracture of the proximal femoral metaphysis was performed with further daily intragastrical administration of a combination of L-norvaline at a dose 10mg/kg and rosuvastatin at a dose 0.86mg/kg from the ninth to twelfth week;

XII – 8 weeks after oophorectomy, a model fracture of the proximal femoral metaphysis was performed with further daily intragastrical administration of a drug for comparison, Bivalos, at a dose 171mg/kg from the ninth to twelfth week.

Fractures were induced by close method using a cutting blade, the cutting edges were covered with rubber tubes and external force was applied perpendicular to the axis of the limbon the proximal

metaphysis of the femur, the pressure was applied until the characteristic signs of fracture (abnormal motility and crepitations on bone fragments) were present. After that the fractures were stabilized by K-wires (1 mm in diameter) inserted intramedullary from the distal to the proximal part of the femur.

For measurement of the level of microcirculation in the proximal femoral metaphysis, conducted after 12 weeks (on the eighty-fifth day), equipment manufactured by Biopac systems were used: namely the polygraph MP_{150} unit with laser Doppler flowmetry (LDF) module $LDF_{100}C$ and invasive needle probe TSD_{144}. A hole was drilled in the cortical layer of bone tissue with a depth of 2-3mm, into which the needle of sensor probe was placed. The data of laser Doppler flowmetry was processed and recorded by software AcqKnowledge version 4.1, the values were expressed in microcirculatory perfusion units (PU).

To confirm the endothelial vessels dysfunction and the degree of correction of the studied medication after oophorectomy in the I, II, III, IV, V and VI groups, tests were conducted based on the endothelium dependent vasodilation (EDVD) and endothelium non dependent vasodilation (ENVD) in response to intravenous bolus solutions of acetylcholine at a dose of 40μg/kg and sodium nitroprusside at a dose of 30μg/kg, respectively. After that the coefficient of endothelial dysfunction (CED) was calculated based on the LDF on the bone. It is defined as the ratio of the triangular area over recovery curve of microcirculation in response to the administration of sodium nitroprusside to the triangular ares over the recovery curve of microcirculation in response to the administration of acetylcholine.

Statistical analysis of primary data was carried out in the program Microsoft Excel. “Descriptive statistics” was used to calculate the average value (M) and error of the mean (m). Assessment of the statistical significance of differences between groups was based on “two-sample t-test with different variances”. The differences were considered statistically significant for values of $p < 0,05$.

Results. Experiment results found that the "intact" animals level of microcirculation in the proximal metaphysis of the femur, 12 weeks after the beginning of the experiment, was 99.91 ± 3.41 PU, which was significantly greater than in the control group of rats with osteoporosis (58.75 ± 3.76 PU). Based on the calculation of the coefficient of endothelial dysfunction, a significant increase of this index was found in rats with osteoporosis (CED = 2.57 ± 0.23) compared to "intact" animals (CED = 1.28 ± 0.18), indicating the formation of signs of endothelial dysfunction, including the microcirculation in the bone tissue (Table 1).

Table 1

Effect of L-norvaline, rosuvastatin and their combination on coefficient of endothelial dysfunction and microcirculation of proximal femoral metaphysis of rats in modeling of endothelial dysfunction and osteoporosis by bilateral oophorectomy (M ± m, n=20)

Groups of animals	CED	Microcirculation, PU
Intact	1.28 ± 0.18	99.91 ± 3.41
Control (osteoporosis)	2.57 ± 0.23 *	58.75 ± 3.76 *
L-norvaline 10mg/kg	1.64 ± 0.21 **	92.46 ± 2.29 **
Rosuvastatin 0.86mg/kg	1.72 ± 0.18 **	81.88 ± 3.39 **
L-norvaline 10mg/kg + Rosuvastatin 0.86mg/kg	1.68 ± 0.25 **	88.02 ± 3.03 **
Bivalos 171mg/kg	2.44 ± 0.19	67.48 ± 2.98

Note: CED – coefficient of endothelial dysfunction; * – $p<0.05$ as compared to intact animals; ** – $p<0.05$ as compared to control group.

Thus, after 12 weeks of bilateral oophorectomy female rats developed dysfunction of vascular endothelium, including the microcirculation of bone tissue. The consequence of the endothelial dysfunction is the decreased regional microcirculation, which leads to an imbalance of bone remodelling and consequently, the appearance and development of osteoporotic changes.

The treatment of osteoporosis using recombinant L-norvaline, rosuvastatin and their combination has shown an improvement in microcirculation of proximal femoral metaphysis of laboratory animals (92.46 ± 2.29 PU, 81.88 ± 3.39 PU and 88.02 ± 3.03 PU respectively), in contrast to the reference drug Bivalos, despite the trend towards improvement (67.48 ± 2.98 PU, $p=0.077$). Moreover L-norvaline 10mg/kg, rosuvastatin 0.86mg/kg, and their combination has also endothelial protective effect, effectively reducing the rate to values CED 1.64 ± 0.21 (for L-norvaline), 1.72 ± 0.18 (for rosuvastatin), and 1.68 ± 0.25 (for a combination) that can cause improvement of regional microcirculation. In BIVALOS endotheliotropic properties were found (CED = 2.44 ± 0.19) (Table 1).

The experimental models of proximal femoral metaphyseal fractures showed a similar pattern. The reparative regeneration processes in rats with osteoporosis had a statistically significant decrease in microcirculation in callus tissue (66.59 ± 3.61 PU) compared with animals with hip fractures without osteoporosis (89.30 ± 4.75 PU). The fracture consolidation in animals with osteoporotic femoral

fractures occurred in 55% of cases, while in animals without osteoporosis in 75% (Table 2).

Table 2

Effect of L-norvaline, rosuvastatin and their combination on microcirculation of callus tissue and consolidation in proximal femoral metaphysis of rats in modeling of osteoporotic fractures (M ± m, n=20)

Groups of animals	Microcirculation, PU	Consolidation, %
Fracture	89.30 ± 4.75	75
Osteoporotic fracture (OF)	66.59 ± 3.61 *	55 *
OF + L-norvaline 10mg/kg	107.14 ± 3.37 **	100 **
OF + Rosuvastatin 0.86mg/kg	94.34 ± 2.54 **	100 **
OF + L-norvaline 10mg/kg + Rosuvastatin 0.86mg/kg	104.1 ± 3.90 PU **	100 **
OF + Bivalos 171mg/kg	70.39 ± 2.39	100 **

Note: * – $p<0.05$ as compared to animals with fractures; ** – $p<0.05$ as compared to osteoporotic fractures group.

L-norvaline at a dose of 10mg/kg effectively increased microcirculation parameters in callus tissue in proximal femoral metaphysis up to 107.14 ± 3.37 PU, that creates favorable conditions for fracture consolidation on the background of osteoporosis. Rosuvastatin at a dose of 0.86mg/kg increased microcirculation parameters up to 94.34 ± 2.54 PU and its combination with L-norvaline up to 104.1 ± 3.90 PU (Table 2).

In comparison the drug Bivalos tended to increase the microcirculation in the callus of the proximal femoral metaphyseal fracture in osteoporotic rats without passing the threshold of statistical significance (70.39 ± 2.39 PU, $p = 0.386$). It is worth mention that consolidation of experimental osteoporotic fractures under the influence of L-norvaline, rosuvastatin and their combination occurred in 100% of cases (Table 2).

The drug used for comparison – Bivalos – had a tendency to increase microcirculation parameters in callus tissue in proximal femoral metaphysis in rats with osteoporosis, but it didn't overcome the statistically significant numbers (70.39 ± 2.39 PU, $p=0.386$). Experimental fracture consolidation using Bivalos also occurred in 100% cases (Table 2).

Conclusion:

1. Twelve weeks after bilateral oophorectomy in female rats, signs of endothelial dysfunction were present, including the microcirculation of bone tissue, with a consequent reduction of regional indicators of microcirculation, which may adversely affect the "quality" of bone, leading to osteoporosis.

2. The administration of L-norvaline at a dose 10mg/kg, rosuvastatin at a dose 0.86mg/kg, and the combination of L-norvaline and rosuvastatin at 12 weeks after bilateral oophorectomy, unlike the reference drug Bivalos, has endothelial protective action, manifested in the reduction of endothelial dysfunction, as well as significantly increase in the level of microcirculation in the metaphyseal bone of the proximal femur, favourably influencing the processes of bone remodelling.

3. Consolidation of osteoporotic fractures of the femur in rats at 12 weeks after oophorectomy was accompanied by decrease of microcirculation in callus tissue, consequently leading to poor consolidation parameters.

4. The administration of L-norvaline 10mg/kg, rosuvastatin 0.86mg/kg and their combination had increased microcirculation parameters in callus tissue in rats with proximal femoral metaphysis fracture and provided favorable conditions for reparative regeneration, which led to an increase in performance of experimental consolidation of osteoporotic fractures. Bivalos also had a positive influence on the consolidation of femoral fractures of osteoporotic rats.

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