Research Results in Pharmacology

Research Results in Pharmacology 10(4): 29–41
UDC: 615.017
DOI 10.18413/rrpharmacology.10.517

Research Article

Epoetin-alfa-induced osteogenesis in the bone organoid model

Natalia A. Nadezhdina[1], Oleg S. Gudyrev[1], Sergey V. Nadezhdin[1], Lyudmila M. Danilenko[1], Aleksandr A. Dolzhikov[1], Kristina K. Kremleva[1], Tatyana G. Pokrovskaya[1]

1 Belgorod State National Research University, 85 Pobedy St., Belgorod 308015 Russia

Corresponding author: Natalia A. Nadezhdina (nadezhdina.nat@yandex.ru)

Academic editor: Mikhail Korokin ♦ **Received** 25 August 2024 ♦ **Accepted** 11 November 2024 ♦ **Published** 28 December 2024

Citation: Nadezhdina NA, Gudyrev OS, Nadezhdin SV, Danilenko LM, Dolzhikov AA, Kremleva KK, Pokrovskaya TG (2024) Epoetin-alfa-induced osteogenesis in the bone organoid model. Research Results in Pharmacology 10(4): 29–41. https://doi.org/10.18413/rrpharmacology.10.517

Abstract

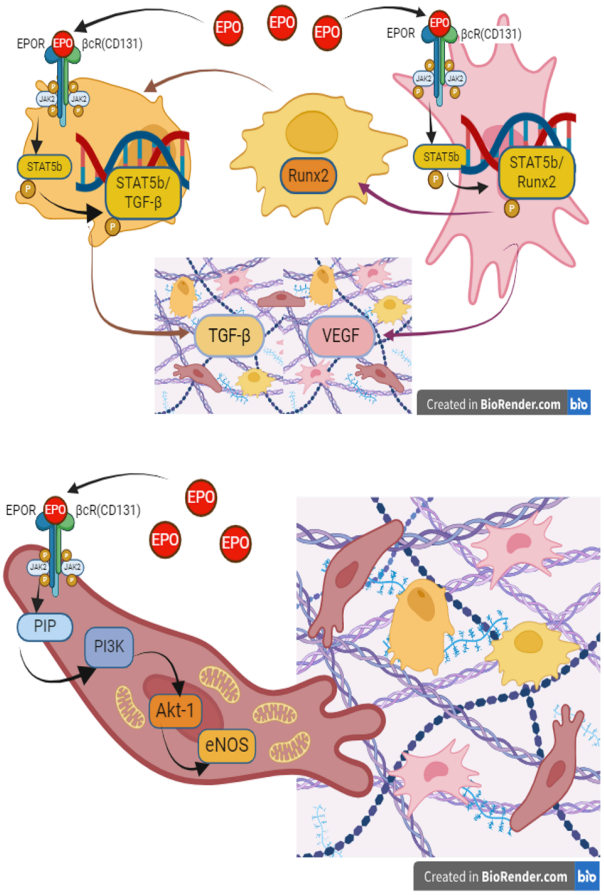
Introduction: Erythropoietin interacts ETwith EPOR/βcR(CD131) and activates the non-canonical pathway JAK/STAT and PI3K/AKT. Phosphorylated STAT subunits were found to get dimerized and translocated into the nucleus to activate the expression of the Runx-2 and TGF-β genes. **The purpose of the study** was to evaluate the effectiveness of osteogenesis when using epoetin alpha as an osteoinducer in an experimental model of a bone organoid.

Materials and Methods: The study was conducted on human mesenchymal stem cells (hMSC) and trabecular bone organoids (TBO). Photometry and microscopy were used to assess the effects of drugs on proliferation and differentiation of hMSC and the formation of osteogenic tissue in TBO. The volume of connective tissue structures and the activity of mitochondria was determined in cells that form fibrovascular osteogenic tissue in TBO.

Results: Epoetin alpha was found to have a dose-dependent osteoinductive effect in TBO. At a dose of 200 IU/mL, epoetin alpha has an inhibitory effect, whereas at a dose of 20 IU/mL, it activates the proliferation and differentiation of hMSC in the osteogenic direction by inducing osteogenesis in TBO.

Conclusion: Epoetin alpha has a protective effect on osteogenesis in TBO inducing the proliferation of osteoblasts and endotheliocytes, stimulating the growth of connective tissue structures of the bone organoid with the formation of osteogenic tissue along with increasing the activity of mitochondria and reducing intracellular generation of reactive oxygen species.

Graphical abstract



Keywords

epoetin alfa, trabecular bone organoid, osteogenesis

Introduction

Currently, bone tissue defects, which usually result from injury, bone infection, or removal of bone tumors, account for a significant part of limb fractures and cause a decrease in the quality of patients' life. Therefore, effective treatment and therapy of bone diseases is of great clinical value. It must be stressed that a 115%-increase in musculoskeletal diseases is forecast between 2020 and 2050, which is indicative of low efficiency of therapy with the existing drugs and rehabilitation methods (GBD 2021 Other Musculoskeletal Disorders Collaborators 2023).

However, before entering the market, most drugs for treating bone diseases require thorough *in vitro* and *in vivo* testing, for instance, drugs to impede bone resorption, to stimulate bone growth, angiogenic and anti-inflammatory drugs. The main disadvantages of traditional testing methods are organ toxicity, long-term cycle and high cost of research (Horvath et al. 2016). The use of bone organoids significantly reduces testing cycles and their cost. On the other hand, organoid methods can more accurately simulate physiological and pathological conditions at the cellular level compared to 2D culture. Bone organoids currently provide a sufficient resource for testing drugs claiming to be osteoinductive, thus making large-scale preclinical *in vivo* studies unnecessary (Zhao D et al. 2024). Therefore, bone organoids are an excellent model for predicting the result of bone regeneration, determining the effective therapeutic dose and identifying the mechanisms of the therapeutic effect of drugs.

Today, the treatment of diseases of various etiologies involves using drugs obtained through genetic engineering methods, among which Human recombinant erythropoietin (rHu-EPO) is of particular interest, as it has a pleiotropic effect (Nairz et al. 2012). In addition to the regulation of hematopoiesis, erythropoietin was shown to mediate the bone tissue formation, as it affects on hematopoietic and stromal stem cells, but the effects and mechanisms of osteogenesis in bone tissue regeneration with the use of erythropoietin have not been fully studied. In view of the above, the search for new therapeutic methods and more effective treatment

strategies remains an urgent task of contemporary regenerative pharmacology.

The rationale that rHu-EPO can affect osteogenic cells lies in the nature of the interaction between progenitor osteogenic cells and hematopoietic cells (Yin et al. 2006). The *in vivo* osteogenic activity was shown to increase after direct ablation or bone marrow injuries (Gazit et al. 1990). Studies in *in vivo* experiments when modeling bone injury in mice showed the effectiveness of high concentrations of rHu-EPO ranging from 500 IU/kg/day to 5000 IU/kg/day; however, the effect of treatment was offset in a long treatment with EPO (Holstein et al. 2007; Garcia et al. 2011). These results suggested that rHu-EPO has an osteoinductive effect only in the initial phases of osteogenesis.

In view of the above, the fibrovascular phase of osteogenesis comes to the forefront, with fibroblasts, osteo-progenitor cells and mesenchymal stem cells (MSCs) being the main cell component. Osteo-progenitor cells and MSCs are stimulated by cytokines and a hypoxic medium for proliferation, concentration and differentiation into osteoblasts by forming a matrix (Schindler et al. 2008).

Thus, there are potential grounds for studies in which the main emphasis is on determining the expression of the transcription factor Runx-2 along with the assessment of the growth of connective tissue structures and the formation of osteogenic tissue of the bone organoid in comparison with drugs with inhibitory effects both towards osteoblasts and endothelial cells (Hofmann et al. 2022; Berg et al. 2023).

The aim of the study was to evaluate the effectiveness of osteogenesis when using rHu-EPO epoetin alpha as an osteoinducer in an experimental model of a bone organoid.

Materials and Methods

Cell line

In the experiments, hMSCs were used, which were isolated from adipose tissue, passage 5 (BioloT Ltd, Russia).

In vitro experimental model

To evaluate the efficiency of osteogenesis when using rHu-EPO epoetin alpha as an osteoinducer, there was developed a model of a trabecular bone organoid using hMSCs. The method of forming a trabecular bone organoid involves a series of consistent operations performed with cultivation of hMSCs. To create a bone organoid, a method was developed for populating a matrix made of highly purified xenocollagen type I (a demineralized spongy sheet Bioplast-dent manufactured by VladMiVa JSC, Russia) and jointly cultivate hMSCs, osteoblasts and endotheliocytes.

The technical result is achieved through a two-stage process of cultivating hMSCs. The first part of thawed suspension of hMSCs in the amount of $1.0x10^6$ is added to a culture flask of 25 cm^2 in DMEM-F12 culture medium, 10% FBS, 1% antibiotics (PanEco Ltd., Russia), which contains osteoinductive agent: 10 mm of sodium β-glycerophosphate, 100 nm of dexamethasone, and 50 μg/mL of ascorbic acid (Pittenger et al. 2008). The second part of the hMSC suspension in the amount of $1.0x10^6$ is added to a culture flask of 25 cm^2 in DMEM-F12 culture medium, 10% FBS, 1% antibiotics (PanEco Ltd., Russia), with the medium containing endothelial inductive agent: FGF – 10 ng/mL and VEGF – 50 g/mL (Khan et al. 2017). All hMSCs were cultivated for 21 days, replacing the culture medium every third day of cultivation.

At the second stage of the cultivation process, hMSCs were removed from the 25 cm^2 flask using trypsin (0.05%) and EDTA (0.53 mmoL) and applied onto matrix on both sides in an amount of $0.04x10^6$. hMSCs cultivated in the culture medium with the osteoinductive agent were applied onto the matrix first, then hMSCs cultivated in the culture medium with the endothelial inductive agent were applied. hMSCs were cultivated for 27 days in DMEM-F12 culture medium, 10% FBS, and 1% antibiotics (PanEco Ltd., Russia), with the culture medium replaced every third day of cultivation. During 27 days of hMSC cultivation, native hMSCs were added to the matrices on the 9th day, followed by the replacement of the culture medium with a fresh DMEM-F12, 10% FBS, and 1% antibiotics (PanEco Ltd., Russia) with the osteoinductive agent. Further, native hMSCs were added on the 18th day, followed by the replacement of the culture medium with a fresh DMEM-F12, 10% FBS, and 1% antibiotics (LLC PANECO, Russia) with the endothelial inductive agent.

In the course of a two-stage cultivation process using culture media containing osteogenic and endotheliogenic agents, hMSCs multiply and differentiate into osteoblasts and endotheliocytes with the formation of connective tissue osteogenic islands characteristic of direct osteogenesis in the demineralized sponge bone matrix.

Drugs used in the experimental study

The studied drug is human recombinant erythropoietin epoetin alpha (Epocrin® in vials of 2000 IU/mL, Reg. No. PN003686/01, manufactured by the Federal State Unitary Enterprise "State Research Institute of Highly Pure Biological Products" of the Federal Medical and Biological Agency of Russia). Before use, the drug was stored at a temperature of +4°C. The comparison drugs are: zoledronic acid (concentrate for preparing infusion solution, vial of 4 mg/5mL, Reg. No. LP-006151, manufactured by Life Science OCPC, Russia), and bevacizumab (Avastin® concentrate for preparing infusion solution, vial of 400mg/16mL, Reg. No.LS-000533, manufactured by F. Hoffmann-La Roche Ltd, Switzerland).

Evaluation of influence of rEPO, zoledronic acid and bevacizumab on proliferation of human stem cells

After passage, the hMSC suspension in the amount of $2.1x10^6$ was put into a 75 cm^2 flask, followed by adding 20 mL of DMEM-F12 with L-glutamine, 10% of fetal bovine serum (FBS), a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 6 days in the incubator at +37°C and 5% CO_2. After 6 days, the cells were removed from the bottom of the flask by using a 15 mL of 0.05% tripsin solution with ethylene diamine tetraacetic acid and a 15 mL of spent culture medium DMEM-F12 with L-glutamine, 10% FBS, a 1% solution of penicilline-streptomycin (PanEco Ltd., Russia) was added to the flask. Then the cells were centrifugated at 200 g for 5 minutes and resuspended in the DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia).

hMSCs in the amount of $0.02x10^6$ (per well) were seeded into wells of 96-well plates with DMEM-F12

culture medium with L-glutamine, 10% FBS, 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. After 24 hours of incubation, rHu-EPO epoetin alpha at dilutions of 1:10, 1:100, 1:1000, and 1:10000 was added to hMSCs in wells. Into the wells of another 96-well plate, bevacizumab and zoledronic acid at dilutions of 1:100, 1:1000, and 1:10000 were added. One experimental group included 18 wells of a 96-well plate. Control – hMSCs cultivated in DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia).

After 72 hours of hMSC cultivation, a 20 µL of an MTT working solution (3-[4,5-dimethyltiazole-2-IL]-2,5-diphenyl tetrazolium bromide) was added to each well of a 96-well plate and incubated for another 2 hours in a CO_2-incubator. After 2 hours, the plates were removed from the CO_2 incubator, and the medium in each well was replaced with a 50 µL of the DMSO solution to dissolve formazan. Using a Multiskan FC reader (Thermo Scientific, USA), the optical density in each well was determined at 540 nm corrected by 620 nm background absorption (Xue-Gong et al. 2010).

The proliferation index (PI) of hMSCs was calculated by the formula: PI (%) = OD of treated cells 540-620 nm / OD of control sample 540-620 nm x100 %, where OD is the optical density of the formazan solution.

Evaluation of influence of recombinant erythropoietin on the differentiation of human mesenchymal stem cells in the osteogenic direction

After passage, the hMSC suspension in the amount of $2.1x10^6$ was put into a 75 cm^2 flask, followed by adding a 20 mL of DMEM-F12 with L-glutamine, 10% FBS, a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia).and cultivated for 6 days in the incubator at +37°C and 5% CO^2. After 6 days, the cells were removed from the bottom of the flask by using a 15 mL of a 0.05% tripsin solution with ethylene diamine tetraacetic acid and a 15 mL of spent complete culture medium DMEM-F12 with L-glutamine, 10% FBS, a 1% solution of penicilline-streptomycin (PanEco Ltd., Russia) was added to the flask. Then the cells were centrifugated at 200 g for 5 minutes and resuspended in the DMEM-F12 complete culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia).

hMSCs in the amount of $0.02x10^6$ (per well) were seeded into wells of 96-well plates with DMEM-F12 culture medium with L-glutamine, 10% FBS and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 18 wells of a 96-well plate. After 24 hours of hMSC incubation, rHu-EPO epoetin alpha at dilutions of 1:10, 1:100, 1:1000, and 1:10000 was added to hMSCs in wells. Into the wells of another 96-well plate, bevacizumab and zoledronic acid at dilutions of 1:100, 1:1000, and 1:10000 were added. Control – hMSCs cultivated in DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia).

After 7 days of hMSC cultivation, 50 µL of BCIP/NBT working solution (Sigma-Aldrich, USA) was added to each well of the plate and incubated for 2 hours in a CO_2-incubator (Jerome et al. 2019). Using a photometer with a wavelength of 450 nm, with the reference wavelength of 620 nm (Multiskan FC, Thermo Scientific, USA), the optical density of a solution of alkaline phosphatase was determined. The differentiation index (DI) of hMSCs was calculated by the formula: DI (%) = OD of treated cells 450-620 nm / OD of control 450-620 nm x100%, where OD is the optical density of alkaline phosphatase solution.

Evaluation of influence of drugs on the proliferation of osteoblasts and endothelial cells in the trabecular bone organoid

Trabecuelar bone organoids were placed into 96-well plates with DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 12 organoids. After 24 hours of incubation, to Group 1 (control), a new DMEM-F12 culture medium was added with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) without adding drugs. To Group 2, a fresh culture medium was added containing bevacizumab at a dilution of 1:100. To Group 3, a fresh culture medium containing zoledronic acid at a dilution of 1:100 was added. To Group 4, a fresh culture medium containing rHu-EPO epoetin alpha at a dilution of 1:100 was added. After 10 days of cultivation of organoids, immunofluorescence staining was performed with antibodies.

To evaluate the proliferation of osteoblasts in the trabecular bone organoid, an osteoblast marker – transcription factor Runx-2 – was used (Bruderer et al. 2014). Primary antibodies were directed against transcription factor Runx2 (Abcam, Great Britain), and then secondary antibodies (Goat Anti-Mouse IgG H&L (Alexa Fluor® 488), Abcam, Great Britain).

To evaluate the proliferation of endothelial cells in the trabecular bone organoid, an endotheliocyte marker was used – cell-cell contacts CD 31 (PECAM-1) (Pusztaszeri et al. 2006). Against CD 31, there were used primary antibodies (Anti-CD31 antibody [P2B1], Abcam, Great Britain) and secondary antibodies (Goat Anti-Mouse IgG H&L (Alexa Fluor® 488, Abcam, Great Britain)).

The study using antibodies was carried out on the basis of the manufacturer's manual (Abcam, Great Britain), bone organoids were removed from the holes of the 96-well plate and transferred to the bottom of 35-mm Petri dishes (SPL Lifesciences, Korea); then primary and secondary antibodies were introduced. Fluorescence was recorded using a C1 Plus confocal laser microscope (Nikon, Japan). Using EZ-C1 FreeViewer software (Nikon, Japan), the fluorescence intensity (FI) was determined in relative units (RU). The proliferation index (PI) of osteoblasts and endotheliocytes was calculated by the formula: PI (in %.) = FI of cells of an experimental group / FI of cells of control X100%, where FI is fluorescence intensity.

Evaluation of influence of drugs on the formation of osteogenic connective tissue in trabecular bone organoid

Trabecuelar bone organoids were put in 96-well plates with a DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 12 organoids. After 24 hours of incubation, to Group 1 (control), a new DMEM-F12 culture medium

was added with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) without adding drugs. To Group 2, a fresh culture medium was added containing bevacizumab at a dilution of 1:100. To Group 3, a fresh culture medium containing zoledronic acid at a dilution of 1:100 was added. To Group 4, a fresh culture medium was added containing rHu-EPO epoetin alpha at a dilution of 1:100. After 10 days of cultivation of organoids and removal of the culture medium, 100 µL of BCIP/NBT working solution (Sigma-Aldrich, USA) was added to each well of the 96-well plate and incubated for 2 hours in a CO_2-incubator (Jerome et al. 2019). After removal of organoids from wells, the optical density of a solution of alkaline phosphatase was determined using a photometer with a wavelength of 450 nm, with the reference wavelength of 620 nm (Multiskan FC, Thermo Scientific, USA). The osteogenic activity index (OAI) of a drug was calculated by the formula: OAI (%) = OP of treated cells 450-620 nm / OP of control 450-620 nm X100%, where OP is the optical density of a solution of alkaline phosphatase.

Evaluation of influence of drugs on the volume of the formed connective tissue structures in the trabecular bone organoid

Trabecuelar bone organoids were placed in 96-well plates with DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 12 organoids. After 24 hours of incubation, to Group 1 (control), a new DMEM-F12 culture medium was added with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) without adding drugs. To Group 2, a fresh culture medium was added containing bevacizumab at a dilution of 1:100. To Group 3, a fresh culture medium containing zoledronic acid at a dilution of 1:100 was added. To Group 4, a fresh culture medium was added containing rHu-EPO epoetin alpha at a dilution of 1:100. After 10 days of organoid cultivation, staining was performed with Calcein AM fluorescent dye (Invitrogen, USA) (Dai et al. 2017). At the first stage of the work, bone organoids were removed from plate wells and transferred to the bottom of 35-mm Petri dishes (SPL Lifesciences, Korea). Then, a culture medium containing 2 µm of Calcein AM fluorescent dye was added to Petri dishes and cultivated in an incubator for 30 minutes at +37°C and 5% CO_2. Then Calcein AM fluorescence was registered using a C1 Plus confocal laser microscope (Nikon, Japan). Using an XYZ-axis scanning (x=1.27 mm, y=1.27 mm, z=700 µm) of the bone organoid and the EZ-C1 Freeviewer software (Nikon, Japan), the volume (in mm^3) of connective tissue structures was determined in the organoids of the experimental groups.

Evaluation of influence of drugs on the functional activity of mitochondria in cells forming fibrovascular osteogenic tissue in trabecular bone organoid

The functional state of mitochondria was evaluated using the MitoTracker Red CMXRros fluorescent dye (Thermo Fisher Scientific, USA) depending on the membrane potential of mitochondria of living cells (Gambini et al. 2020). To do this, trabecular bone organoids were placed into 96-well plates with a DMEM-F12 culture medium with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 12 organoids. After 24 hours of incubation, to Group 1 (control), a new DMEM-F12 culture medium was added with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) without adding drugs. To Group 2, a fresh culture medium was added containing bevacizumab at a dilution of 1:100. To Group 3, a fresh culture medium containing zoledronic acid at a dilution of 1:100 was added. To Group 4, a fresh culture medium was added containing rHu-EPO epoetin alpha at a dilution of 1:100. After 10 days of organoid cultivation, staining was performed with MitoTracker Red CMXRos fluorescent dye. At the first stage of the work, bone organoids were removed from plate wells and stransferred to 35 mm Petri dishes (SPL Lifesciences, Korea). Then, a culture medium containing 200 µm of MitoTracker Red CMXRos fluorescent dye was added to Petri dishes and cultivated in an incubator for 30 minutes at +37°C and 5% CO_2. Then MitoTracker Red CMXRos fluorescence was registered using a C1 Plus confocal laser microscope (Nikon, Japan) and the EZ-C1 Freeviewer software (Nikon, Japan). The scanning area was 635.5 x 636.5 µm. The mitochondrial activity index (MAI) of cells forming fibrovascular tissue was calculated by the formula: MAI (%.) = FI of cells of experimental group / FI of cells of control X 100 %, where FI is fluorescence intensity.

As the intracellular redox state is directly dependent on the functioning of mitochondria, intracellular generation of reactive oxygen species was measured using Calcein AM fluorescent dye (Invitrogen, USA) in living cells (Uggeri et al. 2000; Kuksal et al. 2017). Trabecular bone organoids were placed in 96-well plates with a DMEM-F12 culture nutrient with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) and cultivated for 24 hours in an incubator at +37°C and 5% CO_2. One experimental group included 12 organoids. After 24 hours of incubation, to Group 1 (control), a new DMEM-F12 culture medium was added with L-glutamine, 10% FBS, and a 1% solution of penicillin-streptomycin (PanEco Ltd., Russia) without adding drugs. To Group 2, a fresh culture medium was added containing bevacizumab at a dilution of 1:100. To Group 3, a fresh culture medium containing zoledronic acid at a dilution of 1:100 was added. To Group 4, a fresh culture medium was added containing rHu-EPO epoetin alpha at a dilution of 1:100.

After 10 days of TBO cultivation, staining was performed with 2 µm of Calcein AM fluorescent dye; cells were cultivated for 30 minutes in an incubator at +37°C and 5% CO_2. Then fluorescence was registered using a C1 Plus confocal laser microscope (Nikon, Japan) and the EZ-C1 FreeViewer software (Nikon, Japan) with transforming fluorescence into pseudocolors. The scanning area was 635.5 x 636.5 µm. The oxygen oxidative activity index (OOAI) in fibrovascular tissue of bone organoids was calculated according to the formula: OOAI (%.) = FI of cells of an experimental group / FI of cells of control X100 %, where FI is pseudo-fluorescence intensity.

Statistical analysis

All data were processed using descriptive statistics. The data obtained in the research are presented as M (mean arithmetic) ± SD (standard deviation). The data were checked for normal

for normality, intergroup differences were analyzed by parametric (ANOVA) and non-parametric (ANOVA by Kruskal-Wallis test) methods. Statistical analysis was performed using Statistics 10 software, Microsoft Excel 2016. Significant differences were determined at $p<0.05$.

Results

Evaluation of influence of recombinant erythropoietin on proliferation of human mesenchymal stem cells

While analyzing optical density indicators, it was found that when rHu-EPO epoetin alpha at a dilution of 1:10 was added to the cultural medium, there was a decrease in the indicators, and the differences when compared to control were significant at $p \leq 0.01$. It is worth noting that optical density indicators increased at a dilution of 1:100; however, at an increased dilution, there was a decrease in the indicators; there were no significant differences with control. The optical density of formazan solution at 1:10 dilution was 1.38±0.05 cu, while in the control the indicator was 1.47±0.08 cu. At 1:100 dilution, the optical density of formazan solution was 1.52±0.09 cu, at 1:1000 dilution – 1.50±0.06 cu, and at 1:10000 dilution – 1.49±0.05 cu (Fig. 1).

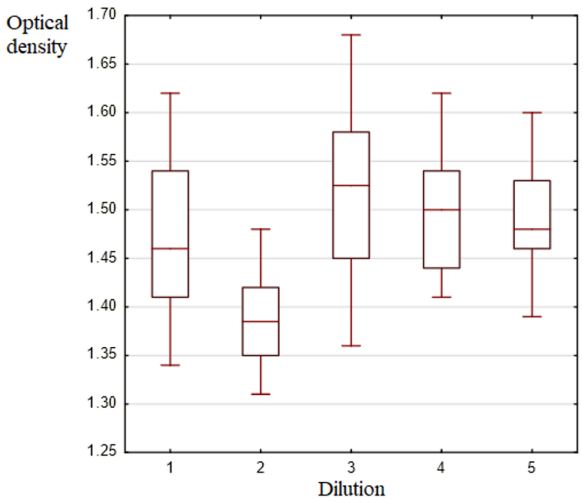


Figure 1. Optical density (cu) of formazan solution in experimental groups. 1 – control, 2 – 1:10 dilution, 3 – 1:100 dilution, 4 – 1:1000 dilution, 5 – 1:10000 dilution.

When adding rHu-EPO epoetin alpha at 1:10 dilution into a culture medium, the proliferation index (PI) was 93.88%, whereas at 1:100 dilution, it was higher and amounted to 103.40%; the tendency of reduced proliferation is observed at 1:1000 dilution when PI is 102.04% and at 1:10000 dilution when PI is 101.36%.

Evaluation of influence of zoledronic acid and bevacizumab on proliferation of human mesenchymal stem cells

The evaluation of optical density indicators made it possible to establish that adding the zoledronic acid at the dilutions of 1:100 and 1:1000 to culture medium resulted in decreased values of optical density compared to that in control, with differences being significant at $p \leq 0.05$. An increase in the values of optical density is observed at a dilution of 1:10000, which indicates a decreased inhibitory effect of zoledronic acid. The optical density of formazan solution at 1:100 dilution was 0.39±0.04 cu, at 1:1000 dilution – 1.52±0.09 cu, at 1:10000 dilution – 0.48±0.05 cu, whereas in the control it was 0.55±0.05 cu (Fig. 2).

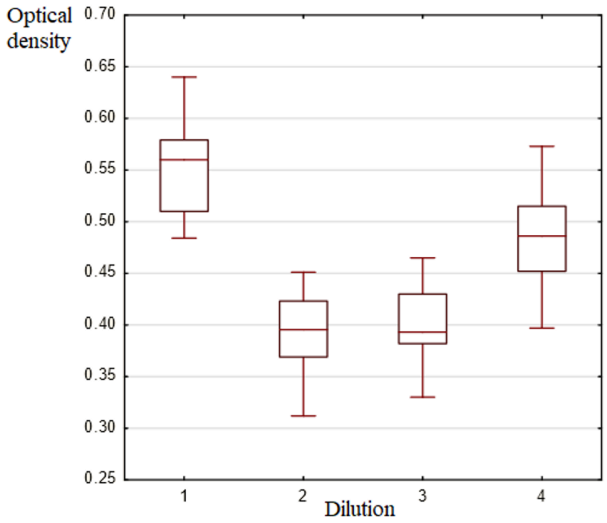


Figure 2. Optical density (cu) of formazan solution in experimental groups with zoledronic acid added to culture medium. 1 – control, 2 – 1:100 dilution, 3 – 1:1000 dilution, and 4 – 1:10000 dilution.

Calculation of the proliferation index showed that when adding zoledronic acid at a dilution of 1:100 to the culture medium, this indicator is the lowest compared to that at other dilutions and amounted to 70.91%, whereas at a dilution of 1:1000, it was 72.73%, and at a dilution of 1:10000, it was the highest, amounting to 87.27%.

Calculation of the optical density indicators of formazan solution showed that when adding bevacizumab at a dilution of 1:100 to the culture medium, the indicators decreased in comparison with those in the control group and other experimental groups (differences compared to control are significant at $p \leq 0.05$). When adding bevacizumab at higher dilutions, there was an increase in the optical density of formazan solution. The optical density of formazan solution when adding bevacizumab at a dilution of 1:100 was 0.45±0.06 cu, whereas in the control, the indicator was 0.68±0.04 cu, at 1:1000 dilution – 0.51±0.04 cu and at 1:10000 dilution – 0.52±0.06 cu (Fig. 3).

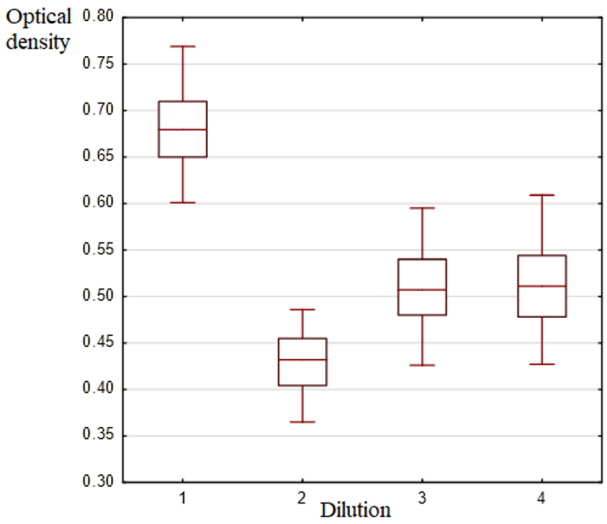


Figure 3. Optical density (cu) of formazan solution in experimental groups when adding bevacizumab to the culture medium. 1 – control, 2 – 1:100 dilution, 3 – 1:1000 dilution, and 4 – 1:10000 dilution.

The proliferation index, when bevacizumab at 1:100 dilution was added to the culture medium, was the lowest in comparison with that in other experimental groups and amounted to 66.18%, whereas at 1:1000 dilution it was 75.0% and at 1:10000 dilution it was 76.47%.

Evaluation of influence of recombinant erythropoietin on the differentiation of human stem cells in an osteogenic direction

The optical density analysis showed that when adding rHU-EPO epoetin alpha to the culture medium, there was an increase in the expression of alkaline phosphatase, both at a dilution of 1:100 and at a dilution of 1:1000; when compared with the control, differences were significant at $p<0.01$. The optical density of alkaline phosphatase solution diluted at 1:100 was 0.22±0.05 cu, whereas at a dilution of 1:1000 it amounted to 0.24±0.07 cu and in the control – 0.12±0.04 cu. Adding rHuEPO epoetin alpha diluted at 1:10 and 1:10000 to culture medium caused no increase in the expression of alkaline phosphatase in comparison with that in the control group, in which optical density amounted to 0.14±0.05 cu and 0.18±0.09 cu at 1:10 dilution and 1:10000 dilution, respectively (Fig. 4).

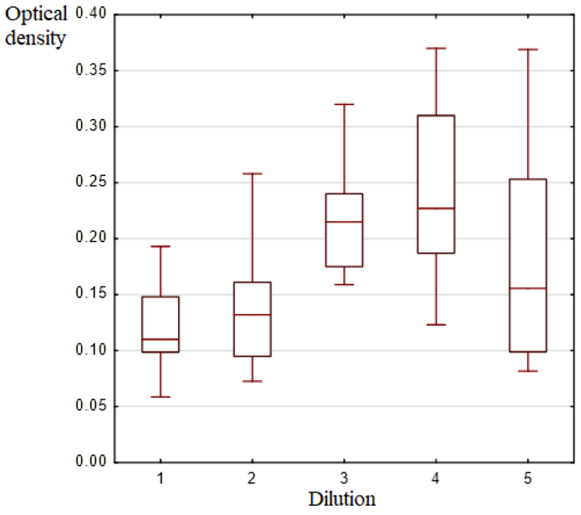


Figure 4. Optical density (cu) of alkaline phosphatase solution in experimental groups. 1 – control, 2 – 1:10 dilution, 3 – 1:100 dilution, 4 – 1:1000 dilution, and 5 – 1:10000 dilution.

The hMSC differentiation index when adding rHu-EPO epoetin alpha diluted at 1:10 and 1:10000 to the culture medium was the lowest and amounted to 116.67% and 150%, respectively. The greatest increase in the hMSC differentiation index was registered at a dilution of 1:1000 – 200% and at a dilution of 1:100 – 183.33%.

The study found that the hMSC differentiation activated in the osteogenic direction when the rHu-EPO epoetin alpha was added to the culture medium at dilutions of 1:100 and 1:1000 in comparison with control at $p\leq0.01$, whereas no hMSC differentiation was induced at dilutions of 1:10 and 1:10000.

Evaluation of influence of drugs on the proliferation of osteoblasts and endothelial cells in the trabecular bone organoid

The analysis of the results found that FI of ostoroblasts expressing transcription factor Runx2 in Group 4, when rHu-EPO epoetin alpha was added to the culture medium, was higher in comparison with that in groups of comparison and control, with differences being significant at $p<0.05$. In Group 4, FI was 7.71±0.38 cu, whereas in Group 2, FI was 4.47±0.28 cu, in Group 3, FI was 1.44±0.10 cu, and in Group 1, FI was 6.42±0.46 cu (Figs 5 and 6).

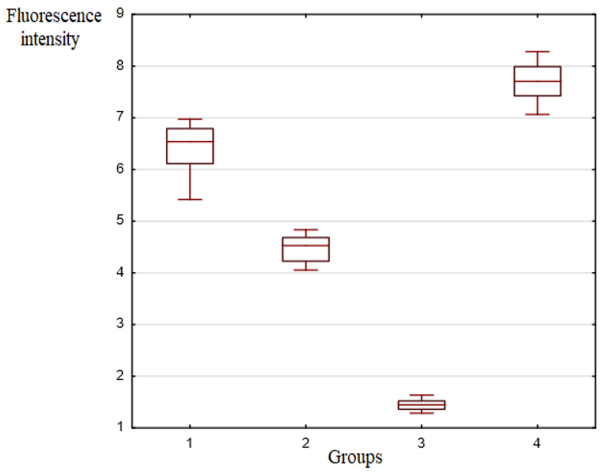


Figure 5. Fluorescence intensity (cu) of ostoblasts expressing transcription factor Runx2 in experimental groups of bone organoids. Group 1 – control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 – rHu-EPO epoetin alpha.

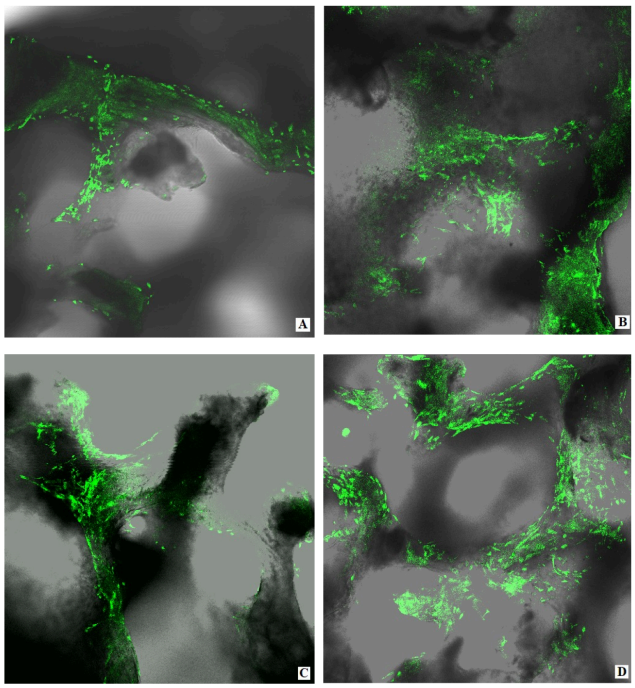


Figure 6. Optical sections, green fluorescence Runx2 in osteoblasts in bone organoid matrix: A – Group 1 (control), bone organoid No. 1; B – Group 2 with bevacizumab, bone organoid No. 2; C – Group 3 with zoledronic acid, bone organoid No. 3; D – Group 4 with rHu-EPO epoetin alpha, bone organoid No. 4; X200.

The ostoblast proliferation index was higher in Group 4 and amounted to 120.09%, while in the groups of comparison drugs it was 69.63% in Group 2 and 22.43% in Group 3. Evaluation of FI in endothelial cells expressing cell-cell contact protein CD31 made it possible to establish that FI was higher in endotheliocytes

of bone organoids in Group 4 in comparison with that in the groups of comparison drugs and control, with differences being significant at $p<0.05$. In Group 4, FI was 6.54±0.46 cu, whereas in Group 2 FI was 1.42±0.12 cu, in Group 3 FI was 2.35±0.16, and in Group 1 (control) FI was 3.23±0.38 cu (Figs 7 and 8).

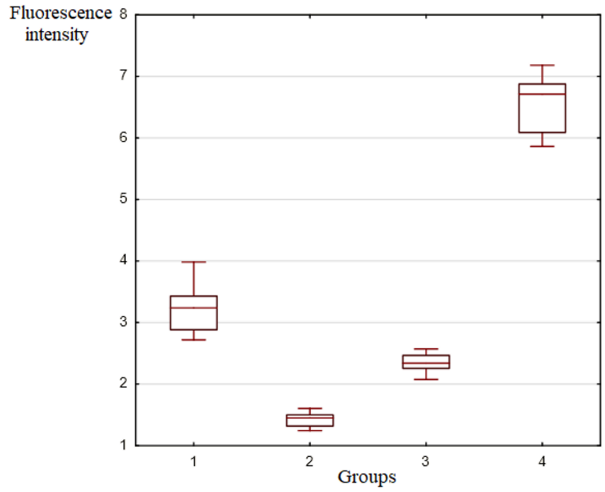


Figure 7. Fluorescence intensity (cu) of endotheliocytes expressing cell-cell contact protein CD 31 in the experimental groups of bone organoids. Group 1 – Control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 – rHu-EPO epoetin alpha.

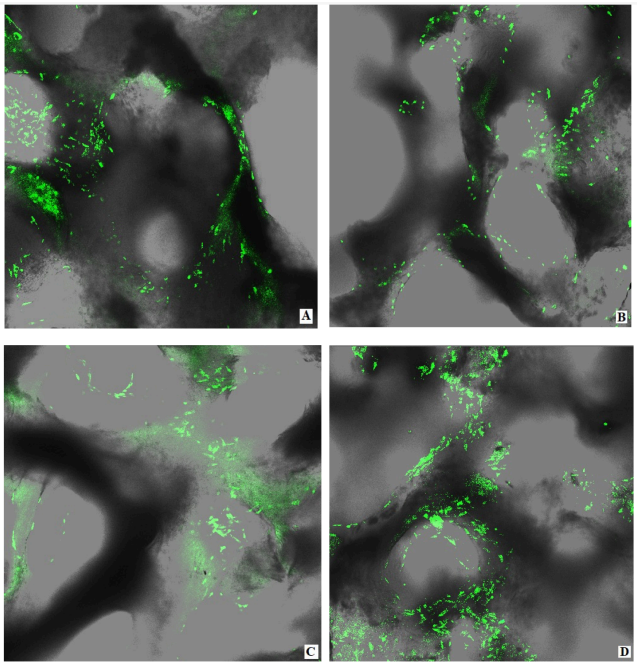


Figure 8. Optical sections, green fluorescence of CD31 in endotheliocytes in bone organoid matrix: A – Group 1 (control), bone organoid No. 1; C – Group 2 with bevacizumab, bone organoid No. 2; C – Group 3 with zoledronic acid, bone organoid No. 3; D – Group 4 with rHu-EPO epoetin alpha, bone organoid No. 4; X200.

The endotheliocyte proliferation index was higher in Group 4 compared to that in groups of comparison drugs and amounted to 202.48%, whereas in Group 2 this indicator was 43.96% and in Group 3 – 72.76%.

The results obtained indicate the proliferation activation of osteoblasts and endotheliocytes in bone organoids when rHu-EPO epoetin alpha is added to the culture medium. At the same time, comparison drugs – bevacizumab and zoledronic acid – reduce the proliferation of osteoblasts and endotheliocytes in bone organoids when compared to such in control.

Evaluation of influence of drugs on the formation of osteogenic connective tissue in trabecular bone organoid

An analysis of optical density revealed that when rHu-EPO epoetin alpha is added to the culture medium, the expression of alkaline phosphatase in the bone organoid increased in comparison with such in control and groups of comparison drugs, with differences being significant at $p<0.05$. The optical density of alkaline phosphatase solution in Group 4 was 0.40±0.02 cu, whereas in Group 2 it was 0.21±0.02 cu, in Group 3 it was 0.23±0.01 cu, and in Group 1 (control) it was 0.27±0.02 cu (Fig. 9).

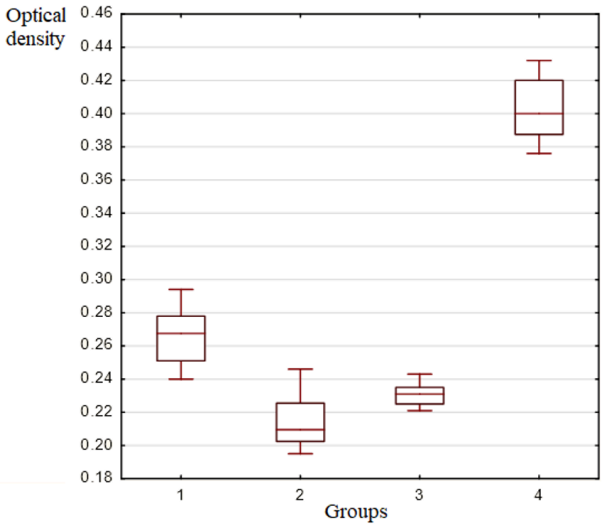


Figure 9. Optical density (cu) of alkaline phosphatase solution in the experimental groups. Group 1 – Control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 – rHu-EPO epoetin alpha.

The osteogenic activity index, when rHu-EPO epoetin alpa is added to the culture medium (Group 4), amounted to 148.15%, whereas in the groups of comparison drugs it was lower and amounted to 77.78% with bevacizumab added to the culture medium and to 85.19% with zoledronic acid added to the culture medium.

The expression of alkaline phosphatase was found to increase in the group of bone organoids when rHu-EPO epoetin alpha was added to the culture medium, which indicates more osteogenic connective tissue in comparison with such in control and groups of comparison.

Evaluation of influence of drugs on the volume of formed connective tissue structures in the trabecular bone organoid

After scanning a bone organoid section along the axes X, Y, and Z (X = 1.27 mm, Y = 1.27 mm) and processing the data by using specialized software, an increase in the thickness of formed connective tissue structures was found when rHu-EPO epoetin alpha was added to the culture medium in comparison with such in control and groups of comparison drugs, with differences being significant at $p<0.05$. The thickness of the connective tissue layer in Group 4 was 115.02±2.88 μm, whereas in

Group 2 it was 62.67±8.28 μm, in Group 3 it was 51.83±8.08 μm and in Group 1 (control) it was 102.33±7.83 μm.

The determination of the geometric indicators of the optical sections obtained while scanning the organoids showed that the volume of the formed connective tissue structures increased when rHu-EPO epoetin alpha was added to the culture medium and amounted to 0.19 mm^3 compared to such in control where it was 0.17 mm^3. At the same time, there is a decrease in the volume of the formed connective tissue structures in the groups of comparison, both when adding bevacizumab to the culture medium – to 0.10 mm^3 and in the group when adding zoledronic acid to the culture medium – to 0.08 mm^3 (Figs 10 and 11).

The addition of rHu-EPO epoetin alpha to the culture medium promoted growth of connective tissue structures of the bone organoid, whereas under the influence of comparison drugs – bevacizumab and zoledronic acid, there was a decrease in the formation of connective structures with further degradation, which is accompanied by a decrease in the volume in comparison with such in control.

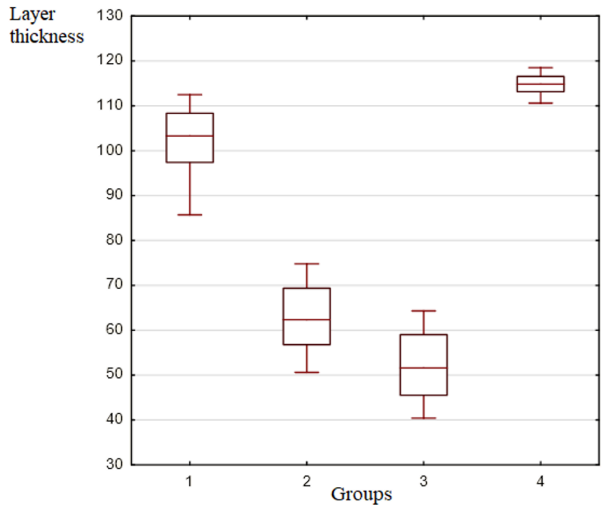


Figure 10. Thickness of the layer of formed connective tissue structures (μm) in experimental groups. Group 1 – Control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 –rHu-EPO epoetin alpha.

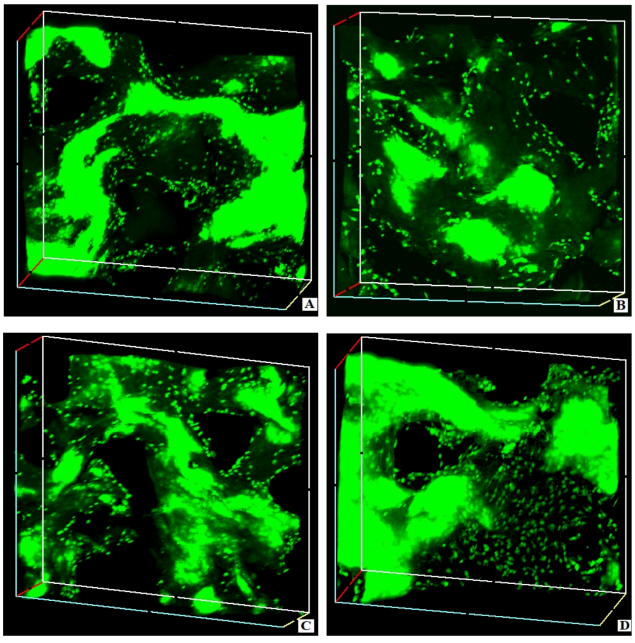


Figure 11. 3D reconstruction, volume of connective tissue structures in bone organoid, green fluorescence Calcein AM: A – Group 1 (control), bone organoid No. 3; B – Group 2 with bevacizumab, bone organoid No. 3; C – Group 3 with zoledronic acid, bone organoid No. 1; D – Group 4 with rHu-EPO epoetin alpha, bone organoid No. 3; X200.

Evaluation of influence of drugs on the functional activity of mitochondria in cells forming fibrovascular osteogenic tissue in trabecular bone organoid

The results of the study of mitochondrial activity showed that FI of MitoTracker Red CMXRos reagent in Group 4, when rHu-EPO epoetin alpha was added to the culture medium, was 2.95±0.11 cu, whereas in control it was 2.25±0.04 cu, and in groups of comparison drugs, it was 1.40±0.03 cu when zolandic acid was added to the culture medium and 1.67±0.05 cu when bevacizumab was added to the culture medium, with differences being significant at $p<0.05$ (Figs 12 and 13).

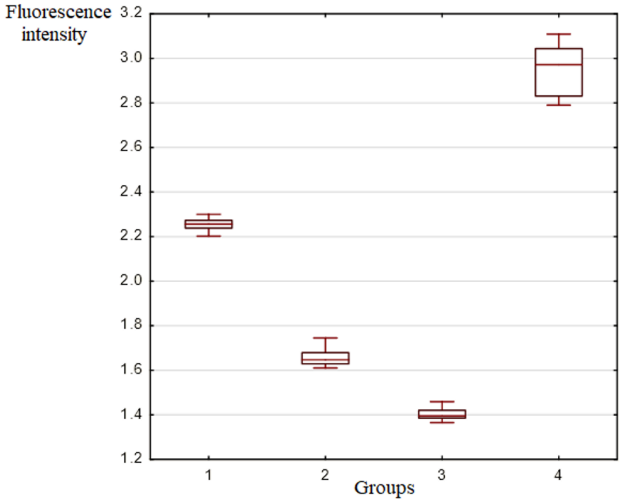


Figure 12. Fluorescence intensity (cu) of active mitochondria in fibrovascular tissue of bone organoids in experimental groups. Group 1 – control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 – rHu-EPO epoetin alpha.

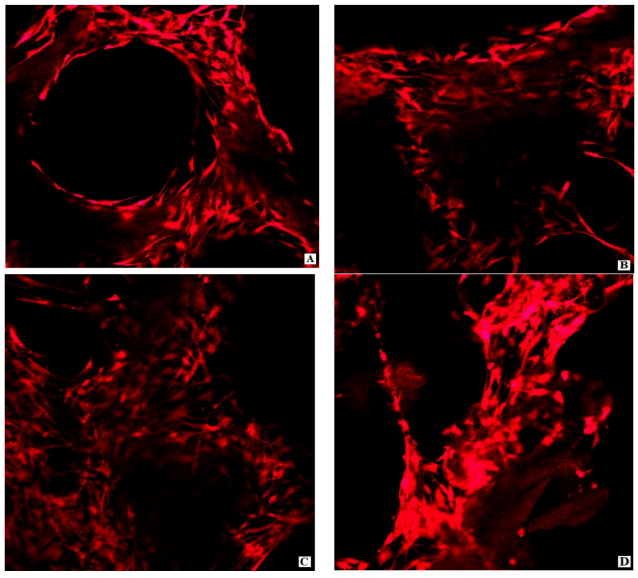


Figure 13. Optical sections, red fluorescence of MitoTracker Red CMXRos reagent of active mitochondria in fibrovascular tissue of the bone organoid: A – Group 1 (control), bone organoid No. 3; C – Group 2 with bevacizumab, bone organoid No. 2; C – Group 3 with zoledronic acid, bone organoid No. 3; and D – Group 4 with eHu-EPO epoetin alpha, bone organoid No. 5; X200.

The mitochondrial activity of cells forming fibrovascular tissue in Group 4 increased, when compared to such in groups of comparison drugs, and amounted to 131.11%, whereas in Group 2 this indicator was 74.22% and in Group 3 – 62.22%.

An analysis of the series of optical sections of bone organoids in order to determine FI characterizing intracellular oxidative activity showed significant differences in experimental Group 4 with rHu-EPO epoetin alpha in comparison with such in control and groups of comparison drugs, with differences being significant at $p<0.05$.

FI in cells that form fibrovascular tissue of the bone organoid was 4.69±0.61 cu when rHu-EPO epoetin alpha was to the culture medium, whereas in Group 1 it was 6.83±0.63 cu at $p\leq0.05$. Along with this, FI in groups of comparison drugs was higher than such in control and the experimental group. FI was found to be 9.69±0.36 cu when bevacizumab was added to the culture medium, but when zoledronic acid was added to the culture medium, FI increased to 12.43±1.07 cu, with $p\leq0.05$ (Figs 14 and 15).

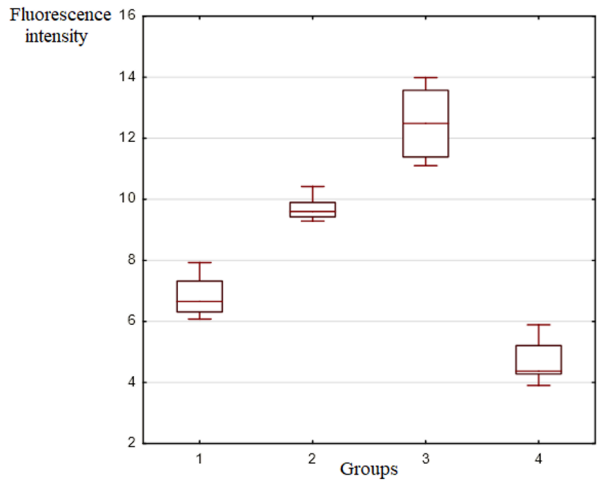


Figure 14. Fluorescence intensity (cu) of reactive oxygen species in fibrovascular tissue of bone organoids in experimental groups. Group 1 – control, Group 2 – bevacizumab, Group 3 – zoledronic acid, and Group 4 – rHu-EPO epoetin alpha.

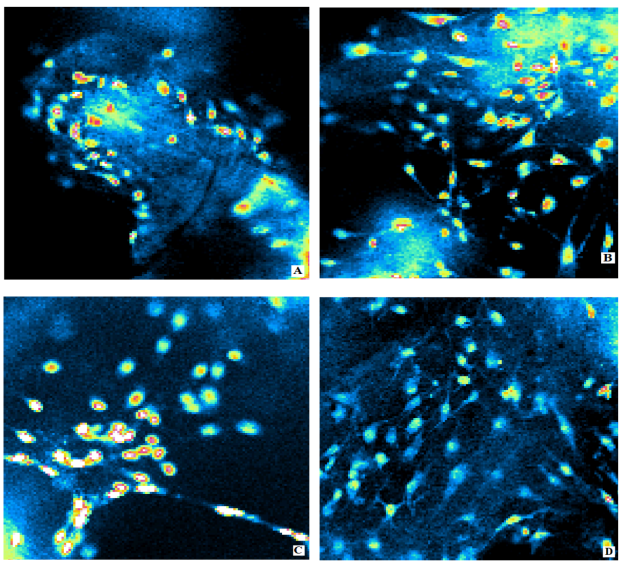


Figure 15. Optical sections (pseudocolor), oxidative activity of oxygen in cells that form fibrovascular tissue of the bone organoid: A – Group 1 (control), bone organoid No. 4; B – Group 2 with bevacizumab, bone organoid No. 3; C – Group 3 with zoledronic acid, bone organoid No. 1; D – Group 4 with rHu-EPO epoetin alpha, bone organoid No. 5; X200.

It is important to note that intracellular pseudo-fluorescence in all experimental groups was localized in the center of the cell, where the nucleus and mitochondria are located.

The oxidative activity of oxygen in cells forming fibrovascular tissue in Group 4 was the lowest and amounted to 68.67%, whereas in Group 2 this indicator was 141.87% and in Group 3 – 181.99%.

Discussion

Erythropoietin is a glycoprotein hormone, which is the main regulator of erythropoiesis (Jelkmann 2004). Though the name of this protein reflects its function as an erythropoietic hormone, this molecule also has pronounced pleiotropic effects as a cytokine and growth factor for non-hemopoetic cells (Zubareva 2019; Belyaeva 2020). The pleiotropic effects are exerted due to the presence of the heterodimeric EPOR/CD131 receptor (tissue-protective receptor, TPR) on the surface of non-hemopoetic cells. Heterodimer EPOR/CD131 does not support erythrogenesis, but transmits signals like homodimer signals through activating JAK2, MAPK and PI3K kinases and through phosphorylating STAT5 (Stepenko 2024). Other signaling pathways initiated by EPOR/CD131 included the regulation of the binding activity of NF-kB family members (Broxmeyer 2013).

The osteoinductive effect of rHu-EPO epoetin alpha when activating osteogenesis in the trabecular bone organoid is apparently due to the interaction of rHu-EPO epoetin alpha with a heterodimer receptor EPOR/βcR(CD131) on the surface of hMSCs with further activation of a non-canonical signal pathway signal JAK/STAT shown in Figure 16.

Activation of Janus Kinaze 2 (JAK2) occurs both in hMSCs and in osteoblasts. After activation, JAK2 induce STAT5 phosphorylation in the cytoplasm, and after dimerization, STAT5 are translocated into the nucleus. In the nucleus, STAT5 dimers bind to specific DNA-elements, which leads to the regulation of target genes responsible for the expression of transcription factors that regulate cell migration, proliferation, differentiation, and apoptosis. Once in the nucleus of a hMSC, STAT5 forms a functional complex with Runx2, which leads to an increase in the Runx2 transcriptional activity and the expression of genes involved in the differentiation of osteoblasts (Dieudonne et al. 2013). Besides STAT5 is assumed to form a functional complex with TGF-β in the nucleus of ostoblasts, which leads to an increased transcriptional activity of TGF-β (Tsiftsoglou 2021).

The addition of rHu-EPO epoetin alpha to a culture medium resulted in an increased activity of mitochondria and a decrease in intracellular generation of reactive oxygen species in cells that form fibrovascular tissue of the bone organoid. Increased mitochondrial biogenesis can be due in this case to the activation of the non-canonical signal pathway JAK/PI-3K/Akt-1 and the endothelial nitrogen oxide syntase (eENOS). eNOS is involved in the regulation of mitochondrial biogenesis as a redox regulator (Dimmler et al. 1999; Fisslthaler et al. 2003). In this case, mitochondrial breathing was not inhibited with an increased NO level, which did not lead to an increased generation of mitochondrial ROS and depolarization of mitochondria (Rakhit et al. 2001) as shown in Figure 17.

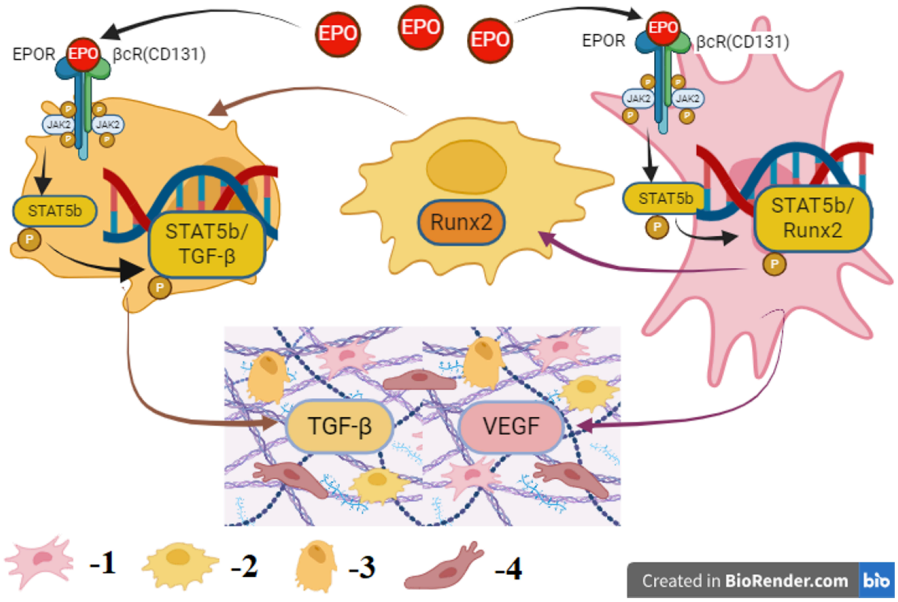


Figure 16. Hypothetical mechanisms of osteoinductive action of rHu-EPO epoetin alpha in the process of osteogenesis. 1 – MSC, 2 – pre-osteoblast, 3 – osteoblast, 4 – endotheliocyte.

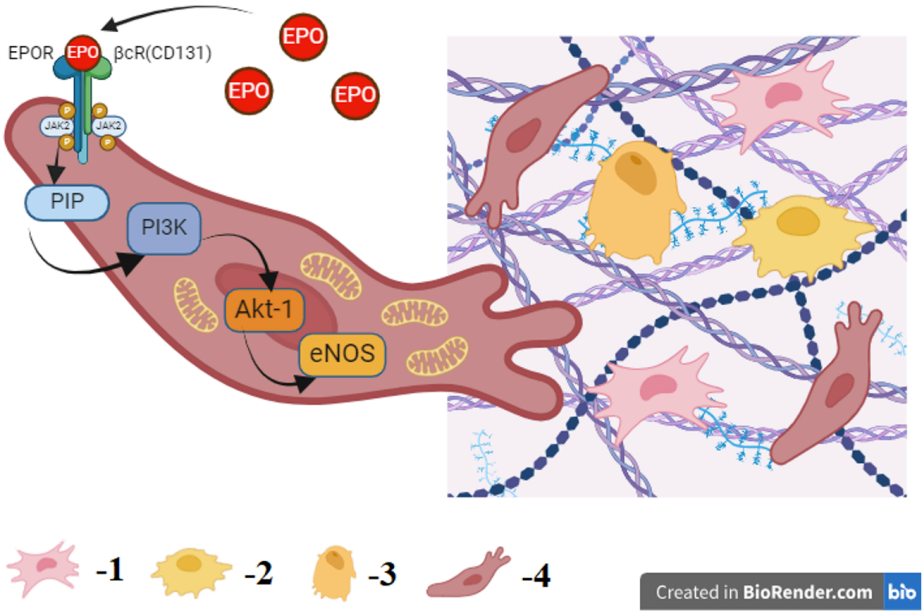


Figure 17. Hypothetical mechanisms of activating mitochondrial biogenesis under the influence of rHu-EPO epoetin alpha in the osteogenesis process. 1 – MSC, 2 – pre-osteoblast, 3 – osteoblast, 4 – endotheliocyte.

The addition of bevacizumab and zoledronic acid to the culture medium caused a decrease in the activity of mitochondria and an increase in the generation of reactive oxygen species.

The results of the study show the dose-dependent protective effect of rHu-EPO epoetin alpha that activate non-canonical signaling pathways JAK/STAT and JAK/PI-3K/Akt-1, which leads to induction of transcription factors and endothelial nitrogen oxide synthase in the experimental model of the bone organoid.

The use of rHu-EPO epoetin alpha leads to an increase in the hMSC differentiation in an osteogenic direction, increases the proliferation of osteoblasts and endotheliocytes, stimulates the growth of connective tissue structures of the bone organoid with the formation of osteogenic tissue, enhances the activity of the mitochondria and reduces intracellular generation of reactive oxygen species. The influence of the comparison drugs with the active substance of bevacizumab or zoledronic acid on osteogenesis in the organoid bone tissue has a completely opposite trend n comparison with rHu-EPO epoetin alpha.

The pharmacological activity of rHu-EPO epoetin alpha in the bone organoid tissue is ensured by a combined targeted effect on various molecular links of osteogenesis, which has high potential for the correction of bone regeneration and restoration of the functionality of the injured bone.

Conclusion

Human recombinant erythropoietin epoetin alpha has a dose-dependent osteoinductive effect in the model of trabecular bone organoid. The dose of 20 IU/mL is the best for preclinical studies of the therapeutic efficacy of human recombinant erythropoietin epoetin alpha on the model of the trabecular bone organoid, since it promotes the differentiation of human mesenchymal stem cells in an osteogenic direction. The study showed that human recombinant erythropoietin epoetin alpha added once to the culture medium has a protective effect on osteogenesis in the model of the trabecular bone organoid, inducing the proliferation of osteoblasts and endotheliocytes, stimulating the growth of the connective tissue structures of the bone organoid with the formation of the ostegenic tissue while increasing the activity of mitochondria and reducing intracellular generation of reactive oxygen species. At the same time, human recombinant erythropoietin epoetin alpha at a dose of 200 IU/mL has an inhibitory effect on the differentiation of human mesenchymal stem cells in an osteogenic direction. It is worth noting that bevacizumab at a dose of 0.25 mg/mL or zoledronic acid at a dose of 8 μg/mL have an inhibitory effect on osteogenesis in the experimental model of the trabecular bone organoid, inhibiting the proliferation of osteoblasts and endotheliocytes, inhibiting the growth of connective tissue structures of osteoogenic tissue, reducing the mitochondrial activity and increasing intracellular generation of reactive oxygen species.

Conflict of interest

The authors declare the absence of a conflict of interests.

Funding

The authors have no funding to report.

Acknowledgments

The authors have no support to report.

Data availability

All of the data that support the findings of this study are available in the main text.

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Author contributions

- **Natalia A. Nadezhdina**, otorhinolaryngologist, Children's Regional Clinical Hospital, postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: nadezhdina.nat@yandex.ru; **ORCID ID:** https://orcid.org/0000-0002-6425-3635. The author took part in the design of the study, in conducting experimental work and analyzing the material.

- **Oleg S. Gudyrev,** Candidate of Sciences (Medicine), Associate Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: gudyrev@mail.ru; **ORCID ID:** https://orcid.org/0000-0003-0097-000X. The author participated in the review and analysis of literary sources.

- **Sergey V. Nadezhdin**, Candidate of Sciences (Biology), Associate Professor, Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: nadezhdin@bsu.edu.ru; **ORCID ID:** https://orcid.org/0000-0001-8444-6330. The author took part in the experimental work.

- **Lyudmila M. Danilenko**, Doctor Habil. of Sciences (Pharmacy), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: Danilenko_L@mail.ru; **ORCID ID:** https://orcid.org/0000-0001-6101-8712. The author analyzed the results and edited the text of the article.

- **Aleksandr A. Dolzhikov,** Doct.or Habil. of Sciences (Medicine), Professor of the Department of Human Anatomy and Histology, Belgorod State National Research University, Belgorod, Russia; e-mail: dolzhikov@bsu.edu.ru; **ORCID ID:** https://orcid.org/0000-0001-7425-8416. The author analyzed the results and edited the text of the article.

- **Tatyana G. Pokrovskaya,** Doctor Habil. of Sciences (Medicine), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: pokrovskaia-tg@mail.ru; **ORCID ID:** https://orcid.org/0000-0001-6802-5368. The author analyzed the results and edited the text of the article.

- **Kristina K. Kremleva,** postgraduate student of Department of the Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: kremlevakristi@yandex.ru; **ORCID ID:** https://orcid.org/0009-0009-7595-6022. The author edited the text of the article.