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Evaluation of biocompatibility and osteoconductivity of a hybrid cell-tissue graft for bone regenerative medicine

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Abstract

Aim – to evaluate in vitro the biocompatibility and osteoconductivity of a hybrid graft based on a bioorganic matrix, human bone marrow mesenchymal stromal cells (BM-MSC) and osteogenic growth factors.

Material and methods. Bioorganic matrices were studied for biocompatibility with human BM-MSC culture used in traumatology and orthopedics. For promoted osteogenic differentiation of BM-MSCs, allogeneic plasma enriched with soluble platelet factors was used. The osteogenic potential of BM-MSCs by the synthesis of mRNAs of early (transcription factor 2 (Run X2), alkaline phosphatase (ALP)) and late genes (osteopontin (OSP)) of osteogenesis was analyzed. The properties of cell adhesion and proliferation of MSCs in the conditions of a three-dimensional hybrid graft by the MTT test and fluorescence microscopy were assessed.

Results. The biocompatibility of the studied bioorganic matrices with human BM-MSCs was established. The collagen matrix promoted rapid cell adhesion and proliferation between the scaffold fibrils. It has also been established that allogeneic platelet-rich plasma affects the osteogenic differentiation of human BM-MSCs in vitro, increasing the expression of marker genes RunX2, ALP, OSP. When modeling a hybrid graft in vitro, the formation of a tight contact between the alloimplant and collagen biopolymer using MSCs was shown.

Conclusion. The biological properties of the developed hybrid cell-tissue graft characterize its biocompatibility and osteoconductivity of its constituent components, which makes it promising for use in regenerative medicine, especially in reconstructive surgery of bone defects.

Keywords: human bone marrow mesenchymal stromal cells, scaffolds, biopolymers, bioorganic matrix, biocompatibility, platelet-rich plasma, hybrid cell-tissue graft.

Conflict of interest: nothing to disclose.

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Abbreviations
alPRP – allogenic plasma enriched with soluble platelet factors; DNA – deoxyribonucleic acid; U – action units; ELISA – enzyme-linked immunosorbent assay; BM – bone marrow; LBM – lyophilized bone matrix; MSC – mesenchymal stromal cell; MTT-assay – a colorimetric assay for assessing cell metabolic activity; OM – osteogenic medium; RT-PCR – reverse transcription of PCR; FGM – full growth medium; PCR – polymerase chain reaction; RNA – ribonucleic acid; sGAGs – sulfated glucosaminoglycans; PC – platelet concentrate; PBS – phosphate buffered saline; FBS – fetal bovine serum; EBS – embryo bovine serum.

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Оценка биосовместимости и остеокондуктивности гибридного клеточно-тканевого трансплантата для регенеративной медицины костной ткани

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Аннотация

Цель – оценить биосовместимость и остеокондуктивность in vitro гибридного клеточно-тканевого трансплантата для регенеративной медицины костной ткани на основе биоорганического матрикса, мезенхимальных стромальных клеток костного мозга (КМ-МСК) человека и остеогенных факторов роста.

Материал и методы. Исследованы на биосовместимость с культурой КМ-МСК человека биоорганические матриксы, используемые в травматологии и ортопедии. Для направленной остеогенной дифференцировки КМ-МСК использовали аллогенную плазму, обогащенную растворимыми факторами тромбоцитов. Остеогенный потенциал КМ-МСК анализировали по синтезу последними мРНК ранних (фактора транскрипции 2 / Run X2, щелочная фосфатаза / ALP) и поздних генов (остеопонтин / OSP) остеогенеза. Свойства клеточной адгезии и пролиферации КМ-МСК в условиях трехмерного гибридного трансплантата оценивали с помощью МТТ-теста и флуоресцентной микроскопии.

Результаты. Установлена биосовместимость исследуемых биоорганических матриксов с КМ-МСК человека. Отмечена быстрая адгезия и пролиферация клеток между волокнами используемых матриксов. Также установлено, что аллогенная плазма, обогащенная растворимыми факторами тромбоцитов, достоверно влияет на остеогенную дифференцировку КМ-МСК человека in vitro, усиливая экспрессию маркерных генов RunX2, ALP, OSP. При имитации трехмерного гибридного клеточно-тканевого трансплантата in vitro показано формирование плотного контакта между аллогенной спонгиозой (костной тканью) и биоорганическим матриксом с помощью остеогенно предифференцированных КМ-МСК.

Выводы. Биологические свойства разработанного гибридного клеточно-тканевого трансплантата характеризуются биосовместимостью и остеокондуктивностью, что делает его перспективным для применения в регенеративной медицине, особенно в реконструктивной хирургии костных дефектов.

Ключевые слова: мезенхимальные стромальные клетки, костный мозг, биополимеры, биоорганический матрикс, биосовместимость, обогащенная тромбоцитами аллогенная плазма, гибридный клеточно-тканевый трансплантат.

Конфликт интересов: не заявлен.

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Список сокращений

алПОРФТ – аллогенная плазма, обогащенная растворимыми факторами тромбоцитов; ДНК – дезоксирибонуклеиновая кислота; ЕД – единицы действия; ИФА – иммуноферментный анализ; КМ – костный мозг; ЛКМ – лиофилизированный костный матрикс; МСК – мезенхимальная стромальная клетка; МТТ-тест – колориметрический тест для оценки метаболической активности клеток; ОС – остеогенная среда; ОТ-ПЦР – обратная транскрипция ПЦР; ППС – полная питательная среда; ПЦР – полимеразная цепная реакция; РНК – рибонуклеиновая кислота; сГАГ – сульфатированные глюкозаминогликаны; ТК – тромбоцитарный концентрат; ФСБ – фосфатно-солевой буфер; ФТС – фетальная телячья сыворотка; ЭТС – эмбриональная телячья сыворотка.

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INTRODUCTION

Bone reconstruction surgery, such as auto- and alloplasty, has become the ‘gold standard’ in the restoration of critically sized bone defects that are not capable of self-healing and maintain chronic inflammation. Alloplasty is the more prevalent and acceptable choice for patients since the use of allogenous implants does not entail additional traumatization of the donor area and eliminates development of potential complications (bleeding and purulence). The evolution of the alloplasty method to repair non-healing defects would be regenerative medicine involving decellularized biomaterial, cell graft, osteogenetic growth factors, implantation of devitalized allogeneic bone equivalent and biomedical cellular products of living cells, biomaterial and growth factors. Several cell populations are associated with bone tissue and its formation, but the most important are osteoblasts, osteocytes, and osteoclasts, which are responsible for bone formation (osteogenesis), maintenance, and resorption, respectively. These cell populations come from the bone marrow mesenchymal stromal cells (BM-MSC). Compared to MSCs from the adipose tissue and the umbilical vein, the BM-MSC manifest a credibly higher efficiency of osteogenic differentiation, which accounts for their therapeutic perspective in cell transplantology and regenerative medicine of supporting tissue [1–6].

The osteogenic differentiation of BM-MSC *in vitro* is easily stimulated in the monolayer cell culture by the addition of the ß-glycerophosphate, hydrocortisone or ascorbic acid, which is confirmed by the increased expression of the early (Runx2 – Runt-associated transcription factors 2; ALP – alkaline phosphatase; BMP2 – bone morphogenetic protein 2 and osteonectin) and the late genes (OSP – osteopontin, osteocalcin) of the osteogenic differentiation [7]. Other growth factors also can perform as inductors of osteogenic differentiation towards BM-MSC, namely, the representatives of the TGF-ß superfamily (transforming growth factor ß); IGF-1 (insulin-like growth factor 1); FGF (fibroblast growth factor); PDGF (platelet-derived growth factor) и VEGF (vascular endothelial growth factors) [1, 8].

The proliferation of BM-MSC and their differentiation to osteoblasts are also regulated with their cellular micro-environment and signaling molecules in the processes of the bone tissue remodeling and repair of non-healing defects. The conjunctive use of BM-MSC with osteoconductive carriers (matrices, scaffolds) may be an effective alternative to replacement of bone

defects with autologous grafts and allogenic implants [9, 10]. Regardless of the treatment method, a necessary condition for bone regeneration is the use of biomaterials that have an adhesive surface for the attachment of osteogenic cells and are capable of transmitting signals for the differentiation of the latter into osteoblasts in response to osteoinduction [11]. The introduction of cells into the bone defect area by injection is ineffective due to the impossibility of their long-term local presence with the receipt of a stimulating effect to start the tissue regeneration process, therefore it is advisable to transplant them on carriers (matrices, scaffolds) made of biopolymers [12].

Depending on their nature, the cellular carriers may be divided into the following groups: inorganic materials such as titanium or tricalcium phosphate ceramics [13]; synthetic biopolymers, such as polyhydroxy acids (polyglycolic acid, polylactic acid, polydioxanone); natural biopolymers such as collagen, gelatin, chitosan or agarose [14]; combined (composite) materials [15].

In relation to stimulation of regeneration, such carriers should be bioresorbable and have an osteoinductive, osteoconductive or combined effect.

The matrices and scaffolds are to have a porous structure to allow cell adhesion, proliferation and differentiation and to be permeable for bioactive substances [16–19]. The response of cells may depend on the physical and biological properties of the biomaterial used as a carrier. Such properties as topography, roughness, chemical composition, surface energy and charge, mimicking the extracellular matrix of native tissue, regulate cell morphology and thus differentiation; vital is the presence of bioactive ligands that can provide anchoring sites for cell attachment [20, 21].

Composite carriers that combine porosity (microstructure), bioactivity (osteoinductivity, osteoconductivity), bioresorbability and properties close to the native extracellular matrix of bone tissue can be potentially considered as the most promising matrices or scaffolds for stem cells in bone tissue engineering. The stimulation of osteogenic differentiation of BM-MSC cells with such osteoinductive and osteoconductive carriers may be amplified by the use of osteogenic growth factors present in the blood cells, specifically, in the platelets [22–25].

Over 30 growth factors were identified in the platelet granules, including bone tissue regeneration factors: TGF-ß (transforming growth factor ß) and BMP (bone morphogenetic protein) that modulate cell proliferation, stimulate genesis of osteoblasts and extracellular bone matrix, retard its degradation, and show an immunosuppressive effect. In addition to platelet factors, plasma proteins such as fibrin, fibronectin and vitronectin are also active participants in osteogenesis [26]. R.E. Marx *et al*. (1998) [27] used in their patients the autologous grafts taken from the ilium and saturated with autologous plasma enriched with soluble platelet factors (PRP, Platelet Rich Plasma) to reconstruct the maxillofacial defects and found that this method increases the speed of bone tissue formation and fosters its higher density. Other researchers did not report such prominent advantages of the PRP in its combination with the demineralized allogenous bone matrix in hybrid cellular tissue graft to regenerate the bone in nude mice [28].

AIM

To evaluate in vitro the biocompatibility and osteoconductivity of a hybrid cellular tissue graft based on a bioorganic matrix, human bone marrow mesenchymal stromal cells (BM-MSC) and osteogenic growth factors, for the regenerative medicine of bone tissue.

MATERIAL AND METHODS

Specimens of bioorganic matrices

The study focuses on four commercially available bioorganic matrices:

• “Collapan”, the composition of which includes a biopolymer serving as the basis of the organic component of bone tissue, namely, type I collagen, and hydroxyapatite with added antibiotic lincomycin hydrochloride (“Intermedapatit”, Russia)[1];

• “Osteomatrix”, highly purified bone matrix with natural histoarchitecture, preserved organic collagen (25%) and mineral (75%) components, containing not lesss than 1.5 mg/cm^3 of affinity-bound bone biopolymers sulfated glucosaminoglycans (sGAG) (“Connectbiopharm”, Russia)[2];

• “Lyostypt”, resorbable biopolymer collagen sponge manufactured from natural bovine tendons (B. Braun, Spain)[3];

• “Lyophilized bone matrix” (LBM), immunologically safe and sterile bone allogenous bioimplant prepared from post-vital donor material (“Minsk Scientific and Practical Center for Surgery, Transplantology and Hematology”, Minsk, Belarus) [29, 30].

Production of plasma enriched with soluble platelet factors

All clinical procedures and collection of donor blood were carried out in accordance with the safety rules described in the World Health Organization guidelines [31]. For this purpose, allogeneic platelet rich plasma (alPRP) was used. It was obtained at the Republican Scientific and Practical Center for Transfusiology and Medical Biotechnology of the Ministry of Health of the Republic of Belarus. To produce the alPRP, platelet concentrate (PC) of the blood of donors was used; the donors were to be allowed to donate blood and not to have markers of viral infections (hepatitis B/HBV, hepatitis C/HCV, human immunodeficiency virus/HIV) after the enzyme-linked immunosorbent assay (ELISA)

[1] Available at: https://collapan.ru
[2] Available at: https://bioimplantat.ru/
[3] Available at: https://catalogs.bbraun.com/en-01/p/PRID00000356/lyostypt-local-haemostatic-agent

and molecular genetic assay (in a polymerase chain reaction or similar assay).

The platelet concentrate (50×10^{12} cells per dose) was produced from donor blood drawn from the ulnar vein (450 ml of blood) with the special kit YCELLBIO-KIT ("BIONIR", Russia). Following that, the blood was centrifuged in two stages to remove red and white blood cells (1550 rpm, 400 g, 20 min) with subsequent concentration of platelets on the laboratory centrifuge Liston C 2201 ("Liston", Russia) at 2450 rpm, 1000 g, 20 minutes. In the resulting alPRP, the amount of platelets was calculated using the Sysmex XN-300 hematological analyzer (Sysmex Corporation, Germany); the amount was to be at least 1.25 10^9/ml. The alPRP from 6 to 10 donors was stored in subsamples at the temperature of -30°C for 24 months. Before using the alPRP, the platelets were activated by a cycle of freeze-thawing with subsequent sedimentation of the cell debris by centrifuging (2900 rpm, 1400 g, 20 minutes). After the activation, the alPRP was used within the first hour [32].

Production of MSC culture from human bone marrow

To produce the BM-MSC culture, 10 ml of bone marrow (BM) was drawn from the iliac crest of healthy donors after obtaining the informed consent according to the resolution of the Ethics Committee of the Republican Scientific and Practical Center of Traumatology and Orthopedics of the Ministry of Health of the Republic of Belarus (Protocol No.3 dated 14.04.2021). The bone marrow punctate contained 20 U of high-molecular heparin per 1 ml of BM to deactivate the blood coagulation process. The BM punctate was then diluted 1:1 with Dulbecco phosphate buffer saline solution without calcium and magnesium (PBS) (Sigma-Aldrich, USA). Further, 20 ml of bone marrow was layered to 15 ml of medium (to separate the lymphocytes), the medium being a mixture of Ficoll and sodium diatrizoate with the density of 1.077 g/ml (Lonza, Switzerland) in 50 ml spin tubes (Corning, USA). The tubes were centrifuged at 450 g for 20 minutes. The mononuclear fractions were gathered in spin tubes with subsequent rinsing in α-modified Eagle's medium with α-MEM ribonucleosides (Gibco, USA) with 2% fetal bovine serum (FBS) (Sigma-Aldrich, USA) by centrifuging at 450 g and exposure for 10 minutes. The extraction of human BM-MSC from the mononuclear fraction was by the method of plastic adhesion in the concentration of 0.3–0.6×106 cells/cm2 in T75 culture tubes (Sarstedt, Germany).

The cells were then cultivated in the CO2-incubator at 5% CO_2 concentration (ESCO Cell Culture, Singapore) and at 37°C in the α-modified Eagle's medium (α-MEM) with ribonucleosides (Gibco, USA), augmented with 10% embryo bovine serum (EBS) (Sigma-Aldrich, CIIIA), 40 mM/ml glutamine (Gibco, USA) and 100 U/ml benzylpenicillin ("Pharm-Sintez", Russia) and 0.1 mg/ml streptomycin (Gibco, USA), i.e. in the full growth medium (FGM) [33]. The BM-MSC were reproduced in T75 culture tubes (Sarstedt, Germany) with initial concentration of 300.0×10^3 cells (passages 1 and 2). The culture medium was changed twice a week. When the cells reached 80-90% of confluence, they were disengaged using trypsin/EDTA (Sigma-Aldrich, USA), and plated again in the concentration of 1500 cells/cm^2 (passages 2-3). The BM-MSC were then immunophenotyped as CD90+, CD105+, CD45-, CD34- by flow cytometry. The results were checked on the FACS flow cytometer (Becton Dickinson, USA). The viability was assessed by the method of exclusion of the trypan blue (at least 90%) [34].

Assessment of biocompatibility of bioorganic matrices with BM-MSC

The carrier matrices were used in an experiment with 10 mg samples. The matrix samples were then cured in an the α-MEM medium with 10% FBS, glutamine and antibiotic medium for 24 to obtain the supernatants. The human BM-MSC was plated to the 24-well cell plate (Sarstedt, Germany) in the initial concentration of $100.0\times10^3/cm^2$ and cultivated for 24 hours. After the cell expansion, the FGM was removed from the plate cells and the prepared specimens of the carrier matrices (native specimens and their supernatants) were introduced directly to the wells with the human BM-MSC. The cultivation was at 37°C and 5% concentration of CO_2 in the CO_2-incubator (ESCO CelCulture, Singapore) in 200 μl 0f FGM. The biocompatibility and adhesion capacity of CM-MSCs on the surface of matrices and their supernatants were assessed in direct contact after 24-hour incubation after 1 and 7 days of co-cultivation by labeling with H33342 (Sigma-Aldrich, USA) at a concentration of 1 μg/ml. For this purpose, human BM-MSCs populated on carriers were incubated with a solution of H33342 of the appropriate concentration at 37°C for 20 minutes. Following the tinting, the cells were flushed twice with PBS, and the FGM was then added [35]. Labeled cells were analyzed using a Leica DM200 fluorescence microscope (Leica Microsystems, Germany) at ×100 magnification.

The cytotoxicity of the carrier matrices and their supernatants upon direct contact after 24-hour incubation after 1 and 7 days of co-cultivation was assessed using the MTT assay (Sigma-Aldrich, USA) [36]. The MTT reagent, at 5 mg/ml was introduced to the PBS to each well of the plate and incubated at 37°C for 4 hours. Then, dimethylsulfoxide (Serva, Germany) was added to each well to dissolve the formazan crystals that had formed as the result of the activity of living cells, and the tinted supernatant was detected at 570 μm on the BioTek® ELx 800 reader (BioTek, USA).

The experiments were performed in the following test groups where the human BM-MSC were cultivated together with the following bioorganic matrices: (1) "Collapan", (2) "Osteomatrix", (3) "Lyostypt", (4) LBM and (5) without a specimen in the FGM with similar cell concentrations as control. The viability of the BM-MSC, after joint cultivation with carrier matrices and their supernatants was calculated using the following **equation (1)**:

$$(OD_{exp} / OD_{control})\times100\%$$

Respectively, the cytotoxicity of the carriers and their supernatants was calculated using the following **equation (2)**:

$$100\% - (OD_{exp} / OD_{control}) \times 100\%$$

where OD_{exp} – optical density of eluates from wells with cells and carriers or supernatants; $OD_{control}$ – optical density of eluates from wells with cells cultivated only with FGM.

The quantitative criteria for assessing the cytotoxicity of the carrier matrices and their supernatants were as follows: low toxicity – death of up to 30% of cells (viability above 70%); medium toxicity – death of up to 30–50% of cells (viability 50–70%); high toxicity – death of over 50% of cells (viability below 50%).

As the basis for the creation and evaluation of a complex osteoconductive transplant at subsequent stages of the experiment, the bioorganic carrier that demonstrated the lowest cytotoxicity in relation to BM-MSC was selected.

Evaluation of expansion of human BM-MSC on a bioorganic carrier

In the wells of the 24-well cell plate, bioorganic carrier with the lowest identified cytotoxicity was placed. Human BM-MSC of the first passage was plated to the carrier with concentrations of the inoculate at 50.0×10^3, 100.0×10^3 and 300.0×10^3 and cultivated for 7 days in the FGM with and without added 5% alPRP. The cells were then removed with a trypsin/EDTA solution (Sigma-Aldrich, USA). The quantity of viable cells was calculated by the method of exclusion of the trypan blue in the Goryaev chamber [37].

Evaluation of the osteogenic differentiation of human BM-MSC

After two passages, the human BM-MSC were plated to T25 vials (Sarstedt, Germany), 8000 /cm^2 each, for subsequent quantitative polymerase chain reaction (PCR assay) in real time in 35 mm Petri dishes for visible light microscopy after staining. The experiments studies four variants of cell cultivation: BM-MSC cultivated in FGM (control specimen); BM-MSC cultivated in FGM with 5% alPRP; BM-MSC cultivated in osteogenic medium (OM); BM-MSC cultivated in osteogenic medium (OM) with 5% alPRP.

The osteogenic medium contained the α-MEM environment with addition of 10% FBS, 10 mM β-glycerophosphate (Sigma Aldrich, USA), 50 mcg of ascorbic acid (Sigma Aldrich, USA) and 0.1 μM dexamethasone (Sigma Aldrich, USA) [40]. The differentiation of BM-MSC was performed in the OM within four and seven days, the cultural medium being changed twice. The specimens of both osteogenic-induced and non-differentiated BM-MSC were cultivated in the presence of alPRP in the final concentration of 5%.

The *in vitro* evaluation of the osteogenic differentiation of the human BM-MSC was performed by real-time PCR with reverse transcription (RT-PCR) to identify expression of mRNA of the RUNX2, ALP and OSP genes on the fourth and seventh days of cell cultivation. Intravital imaging of osteogenic-induced human BM-MSC and *in vitro* assessment of mineralization (formation of calcium deposits) were also performed using von Kossa staining on day 21 of cell induction.

Genes	Forward primer 5' – 3'	Reverse primer 5' – 3'
RUNX2	CACTGGCGGTGCAACAAGA	TTTCATAACAGCGGAGGCATTTC
ALP	GGTGGAAGGAGGCAGAATTG	TCAGAGTGTCTTCCGAGGAG
OSP	CACAGCATCTGGGTATTTGTTG	CGACCAAGGAAAACTCACTACC
GapDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT

Table 1. *Nucleotide sequences for the RT-qPCR primers*
Таблица 1. *Нуклеотидные последовательности праймеров для ОТ-ПЦР*

The RT-PCR was performed in several stages [33, 38]. Firstly, primary extraction of the total RNA from the human BM-MSC cultivated in the T25 vials (Sarsedt, Germany) was performed. The extraction was done with the TRI Reagent (Sigma Aldrich, USA) according to the manufacturer's manual. Then the samples of total RNA were used to obtain complementary DNA (cDNA) by reverse transcription. The reaction was performed in 20 μl mixture of 2 mcg of RNA, 5 μM Oligo (dT18)-primer (Thermo Fisher Scientific, USA), 1 mM of deoxyribonucleotide triphosphate (dNTP) (Thermo Fisher Scientific, USA), 40 U of RNase inhibitor Ribolok (Thermo Fisher Scientific, USA), 1 μl (200 u/ml) of reverse transcriptase RevertAid Premium and 5X buffer (Thermo Fisher Scientific, USA). Amplification was carried out for 30 min at 50°C. Reverse transcriptase was inactivated by heating at 85°C for 5 min.

The obtained cDNA was amplified as follows: 10 minutes at 95°C (polymerase activation) with subsequent amplification of 40 cycles at 60°C for 1 minute. In order to detect the osteogenic differentiation markers (RUNX2, ALP and OSP), the following primer pairs were used with the following nucleotide sequences **(Table 1)** [39].

Data were normalized relative to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GapDH). The real-time RT-PCR was performed on the CFX96 Touch REAL thermocycler (Bio-Rad, USA). The results were processed in the Bio-Rad CFX Manager software suite.

Relative gene expression was calculated by Livak's method using the following **formula (3)** [40]:

$$Gene\ expression = 2^{-\Delta Ct}$$
$$\Delta Ct = [Ct\ (marker\ gene) - Ct\ (GapDH)]$$

Osteoblast formation and mineralization were assessed in Petri dishes (Sarstedt, Germany). The BM-MSC were stained with 1-2% solution of nitrate of silver for 45–60 minutes under UV light, then rinsed with distilled water and fixed with sodium thiosulfate for 5 minutes. The stained cells were then washed with deionized water, dried and evaluated using a light microscope Leica DM IL LED (Leica Microsystems, Germany) at ×100 magnification. Morphological transformation was characterized by a change in cell

shape to cuboid with intracellular calcium phosphate deposits stained black [41].

***In vitro* modeling of the hybrid cellular tissue graft**

While developing the hybrid cellular tissue graft to be used in regenerative medicine for the replacement of bone defects, we identified its major components: human BM-MSC pre-differentiated to an osteogenic phenotype; alPRP. To simulate the environment (bone tissue), we used the allogenetic spongiose (surgical specimen). The macroscopic picture was described, the absolute number of pre-differentiated BM-MSCs in the graft was determined at the end of the 1st , 2nd and 3rd week of cultivation, and cell adhesion and distribution in the graft were assessed. To that end, intra-vital cell staining with fluorescent dye Hoechst 33342 was used (Sigma Aldrich, USA).

Statistical processing of data

The data from the studies are presented as the mean ± standard error of the mean (M±SEM) in the GraphPad Prism 6.0 software suite (GraphPad Software, Inc., USA). The reliability of differences in the variation series was assessed using the paired Student's t-test. Differences were reliable at $p < 0.05$.

RESULTS AND DISCUSSION

Phenotypical characteristics of human BM-MSC

The immunophenotype of human BM-MSC of the second passage was characterized with expression of specific markers CD90+ and CD105+ and absence of markers CD34- and CD45-. The number of cells in the culture that were expressing the marker CD90+ was 99.9±0.14%, and those expressing the marker CD105+ was 99.14±1.23%. There was a minor quantity of cells expressing the markers CD34- (0.45±0.20%) and CD45- (0.30±0.07%). The culture was sterile (no bacteria or fungi), and the viability of the resulting cells was never below 90%.

Biocompatibility of bioorganic matrices with human BM-MSC

The main property of biopolymers and carrier matrices based on them when creating grafts for the restoration of large bone tissue defects should be the absence of a toxic effect on osteoprogenitor cells, as well as assistance in their attachment, proliferation and osteogenic differentiation. This stimulates the cells to synthesize new extracellular matrix on the surface of the carrier and its integration with the native bone tissue. Therefore, the carriers are to be compatible with BM-MSC supporting the cellular activity and mechanical integrity to foster a successful process of healing the defect of the bone tissue [11].To evaluate the cytotoxicity of matrix carriers, the MTT assay was used and intra-vital staining of cells with the fluorescent dye Hoechst 33342 (Sigma Aldrich, USA).

Specimens	Supernatant (% of living cells)		Matrix carrier (% of living cells)	
	1 day	7 days	1 day	7 days
Colapan	82,76 ± 22,35	89,03 ± 15,69	87,56 ± 32,56	93,35 ± 1,56
Osteomatrix	84,25 ± 10,14	91,31 ± 13,08	88,83 ± 9,89	92,32 ± 10,58
Lyostypt	101,72 ± 4,86	124,52 ± 7,30	88,63 ± 8,47	96,55 ± 10,91
LBM	101,44 ± 12,14	80,74 ± 3,35	87,90 ± 0,55	90,85 ± 9,14

Table 2. *Viability of BM-MSCs during cultivation in vitro with the carriers and their supernatants in direct contact. Data are expressed as M ± SEM*

Таблица 2. *Жизнеспособность КМ-МСК человека при культивировании in vitro с носителями и их супернатантами при прямом контакте. Данные представлены как M ± SEM*

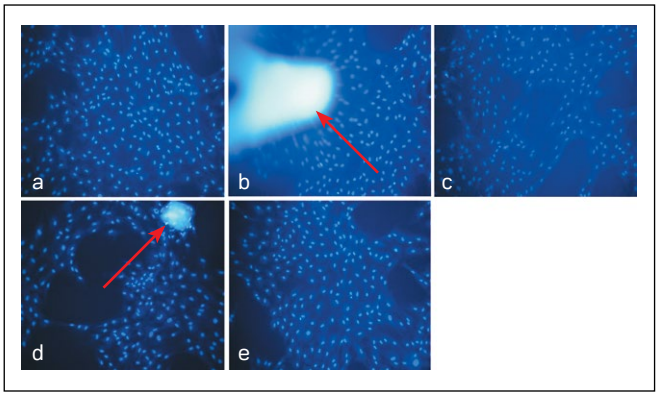

Figure 1. *Nuclei labeling of Hoechst 33342 BM-MSCs after one day of cultivation with different carriers: (a) CCM (control sample); (b) Osteomatrix; (c) LBM; (d) Kollapan; (e) Lyostypt. Image taken at 100x magnification.*

Рисунок 1. *Прижизненное окрашивание ядер КМ-МСК человека Hoechst 33342 после 24 часов культивирования с различными носителями: (a) ППС (контроль); (b) Остеоматрикс; (c) ЛКМ; (d) Коллапан; (e) Лиостипт. Изображение увеличено в x100.*

Staining with Hoechst 33342 identified nuclei of labeled cells which confirmed the cytocompatibility and capacity of human BM-MSCs for adhesion on carriers and their supernatants at direct contact **(Fig. 1)**.

Figure 1 shows the presence of flat cylindrical non-condensed nuclei of BM-MSCs and their formation of a homogeneous monolayer of living cells when cultured with carriers: red arrows show the adhesive capacity of the latter for cells.

The results of experiments on the cytotoxicity of carrier matrices and their supernatants in direct contact with BM-MSCs are presented **in Table 2**.

When assessing acute cytotoxicity after 24 hours of cultivation, the cell viability ranged from 82.76% to 100%. Cytotoxicity was found to be absent in the supernatants of “Lyostypt” and “LBM” carriers at all times of monitoring as compared to the control specimen. The matrix carriers demonstrated a similar effect on BM-MSCs. All four observed carriers had minor cytotoxicity that brought about the death of not more than 30% cells (low toxicity). Only the cultivation of BM-MSCs with “Lyostypt” showed the greatest viability of cells at direct contact after 24 hours and 7 days, and even a moderate proliferation of cells with its supernatant.

Proliferative activity of human BM-MSC on bioorganic carriers

BM-MSCs are prospective candidates for regenerative medicine in repairing bone defects. However, the introduction of BM-MSC to the osseous lesion does not always perform well due to cell migration with blood or tissue fluid or due to introduction of an insufficient amount of cells. Therefore, the use of bioorganic matrix carriers in the composition of a hybrid cell-tissue graft, capable of

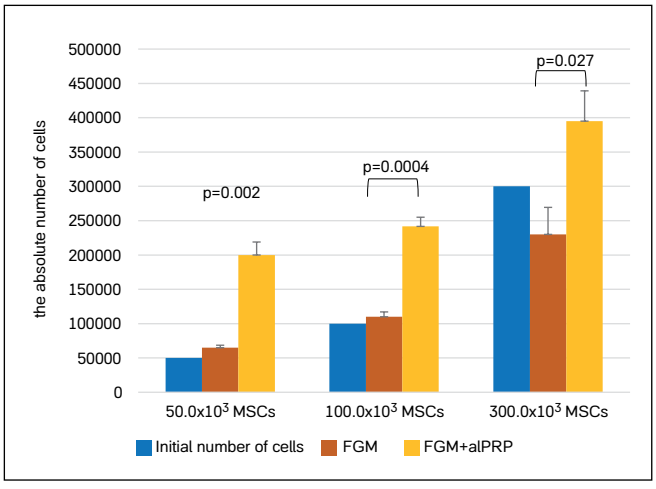

Figure 2. *Proliferative activity of different concentrations of BM-MSCs on the "Lyostypt" bioorganic carrier over a period of 7-d cultivation in vitro.*

Рисунок 2. *Пролиферативная активность различных концентраций КМ-МСК на биоорганическом носителе «Лиостипт» в течение 7 суток культивирования in vitro.*

being populated by cells and performing the function of a framework to facilitate the plastic closure of a defect, is an urgent task of tissue engineering [40]. The results of the assessment of the proliferative activity of human BM-MSC with the selection of their optimal concentration for populating the bioorganic matrix "Lyostypt" as the carrier with the lowest cytotoxicity are shown **in Figure 2**.

During the cultivation of the human BM-MSCs in the FGM (Fig. 2) without alPRP, by day 7, minor proliferation of cells was observed when 50.0x103 and 100.0x103 cells were introduced ($p \geq 0.05$), and a decrease in the initial amount of cells was seen in the higher introductory dose of $300.0x10^3$ cells ($p \leq 0.05$). With 5% alPRP added to the FGM, significant increase of proliferative activity of the cells was identified. The number of BM-MSC increased by 3.3 times ($p = 0.02$), by 2.6 times ($p = 0.0004$) and by 1.3 times ($p = 0.026$) versus initial seeding concentration of 50.0x103, 100.0x103 and 300.0x103 cells per cm2, respectively. The viability of BM-MSC was high in all samples and amounted to 99% in four series of the experiment. Thus, the increase in the proliferative activity of BM-MSC on the carrier was due to the presence of growth factors contained in alPRP.

Osteogenic differentiation of human BM-MSC

BM-MSCs have a significant potential for regenerative medicine, e.g. for patients with post-traumatic or post-surgery bone damage. BM-MSCs are capable of replacing the damaged cells, differentiate and synthesize the extracellular bone matrix in the lesion area. They may also indirectly contribute to tissue regeneration by secreting growth factors [40]. The studies of L. Meesuk *et al*. (2022) [42] showed that osteogenic differentiated BM-MSCs may produce more osteogenic factors than non-differentiated cells. This approach will be more efficient in repairing the bone defects.

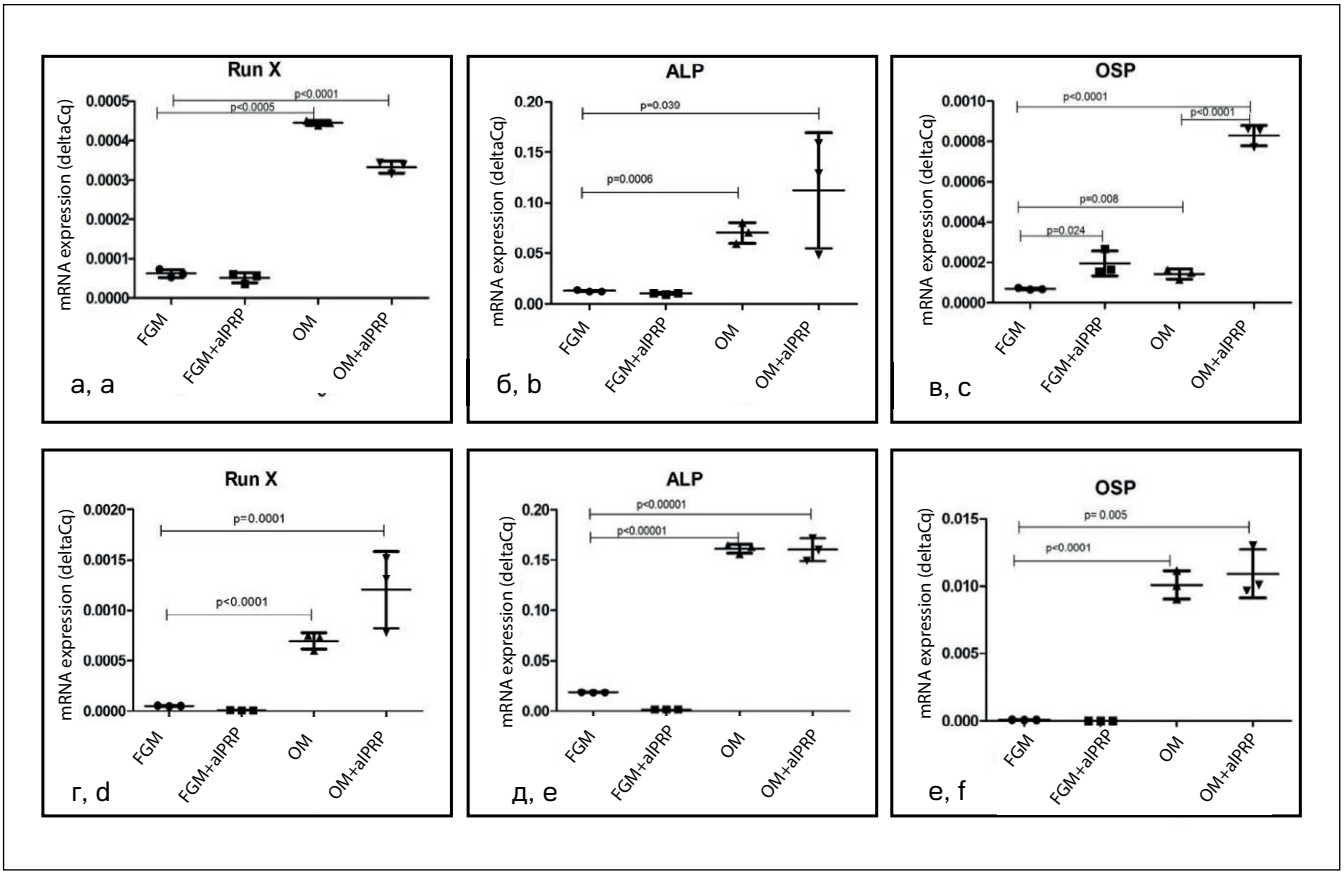

Figure 3. *mRNA expression of osteogenic genes: (a, d) RunX, (b, e) ALP, (c, f) OSP after 4 (top row) and 7 (bottom row) days of cultivation BM-MSCs. Data are expressed as M ± SEM.*

Рисунок 3. *Экспрессия мРНК остеогенных генов: (а, г) RunX, (б, д) ALP, (в, е) OSP после 4 (верхний ряд) и 7 (нижний ряд) суток культивирования КМ-МСК. Данные представлены как M ± SEM.*

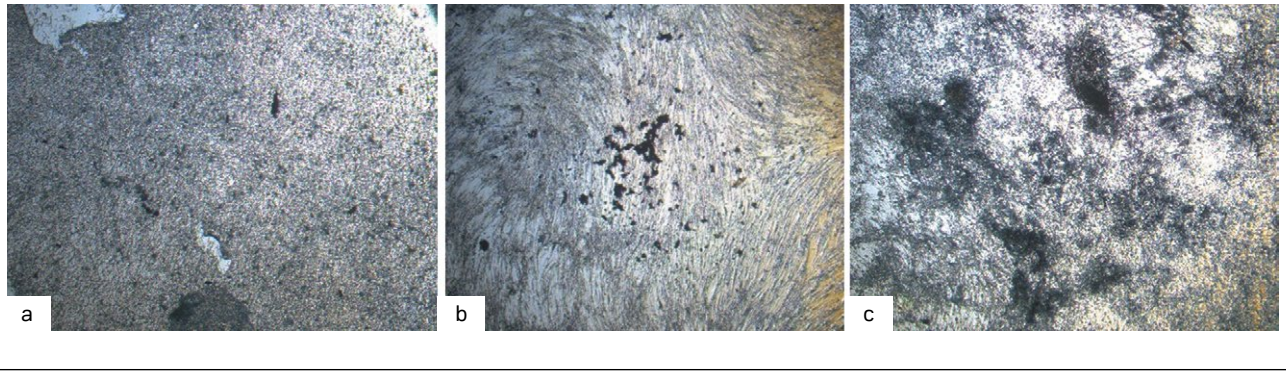

Figure 4. *Von Kossa staining of BM-MSCs: (a) cells cultivated in CCM; (b) cells cultivated in OM; (c) cells cultivated in OM, supplemented with 5% alPRP. Images are taken at 100x magnification.*

Рисунок 4. *Окрашивание фон Косса КМ-МСК человека: (а) клетки, культивированные в ППС; (b) – клетки, культивированные в ОС; (c) – клетки, культивированные в ОС с добавлением 5% алПОРФТ. Изображения сделаны при 100-кратном увеличении.*

In the in vitro experiments, the osteogenic potential of the cells was evaluated by the expression of molecular genetic markers (mRNA of osteogenic genes RUNX2, ALP, OSP) by RT-PCR in real time and Von Kossa staining of the cell monolayer with subsequent identification of calcium ossificates in the latter. The results of RT-PCR analysis of the expression of mRNA of osteogenic genes (RunX, ALP, OSP) are shown in **Fig. 3**.

After 4 days of BM-MSC cultivation in the OM, an increase in the expression of marker genes by osteogenic-induced cells was identified. The synthesis of mRNA of the RunX gene (**Fig. 3a**), ALP (**Fig. 3b**) and OSP (**Fig. 3c**) increased by 7.1 times ($p = 0.001$), 5.5 times ($p = 0.001$) and 2.1 times ($p = 0.008$) respectively versus non-differentiated BM-MSC cultivated in the FGM. The addition of 5% alPRP to the OM during the differentiation of BM-MSC increased the expression of the OSP gene by 12.0 times ($p = 0.0001$) versus non-differentiated cells cultivated in the FGM, and by 5.9 times ($p < 0.0001$) versus osteogenic-induced cells cultivated in the OM without alPRP. The presented differences are statistically significant.

After 7 of cultivation in the OM, the cells continued increasing the expression of the RunX (**Fig. 3d**), ALP (**Fig. 3e**) and OSP genes (**Fig. 3f**). Statistically valid results were obtained as to the growth of expression of the following genes: ALP – growth by 8.67 times ($p < 0.0001$) and OSP – by 22.9 times ($p < 0.0001$), respectively, versus non-differentiated BM-MSC cultivated in the FGM. The differences are statistically significant. At the same time, addition of 5% alPRP to the OM during differentiation of BM-MSC at this time did not result in a credible increase of gene expression in comparison with osteogenic-induced BM-MSC cultivated in the OM without alPRP. The synthesis of mRNA of the genes remained on a comparable level ($p \geq 0.05$).

Thus, cultivation in the OM credibly fosters an increased expression of RunX genes by the cells, and, respectively, osteogenic differentiation of BM-MSC. The addition of alPRP to the OM credibly strengthens expression of genes and osteogenic differentiation of human BM-MSC in the initial stage (up to day 7 of cultivation).

In the period from the 4th to the 7th day of cultivation in the OM (including addition of alPRP), there occurs re-differentiation of BM-MSC. In this period, the cells start expressing osteogenic markers, yet no calcium deposition starts in them. After 21 days of osteogenic differentiation, it was shown that the morphology of BM-MSC changed from spindle to cuboid. After Von Kossa's staining of the specimens of differentiated cells with nitrate of silver, additionally, intracellular deposits of calcium phosphate were found (stained black). The changes were most manifested in the cells cultivated in the OM with 5% alPRP added (**Fig. 4**).

In vitro modeling of the hybrid cell-tissue graft

The results of in-vitro co-cultivation of the components of the hybrid cell-tissue graft under the conditions of the required microenvironment (bone tissue) are shown in **Figure 5**.

After the incubation of all the graft components in vitro, at the end of the first week a close contact of the allogeneic spongiosa and "Lyostypt" matrix carrier were seen, as well as formation of a gel-like microenvironment (blue arrow), represented by the alPRP elements. At the end of the third week, all components of the graft were seen in the dense gel-like medium and close interaction of fragments of the allogeneic spongiosa with the matrix carrier by the formed monolayer of cells in the form of the BM-MSC band (red arrow).

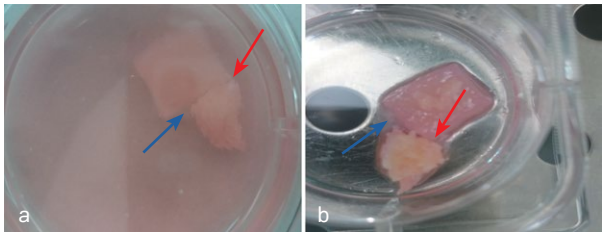

Figure 5. *Co-cultivation of graft components in vitro: (a) one week, (b) three weeks. Image taken at 50x magnification.*

Рисунок 5. *Совместное культивирование компонентов трансплантата in vitro: (а) 1 неделя, (b) 3 неделя. Изображения сделаны при 50-кратном увеличении.*

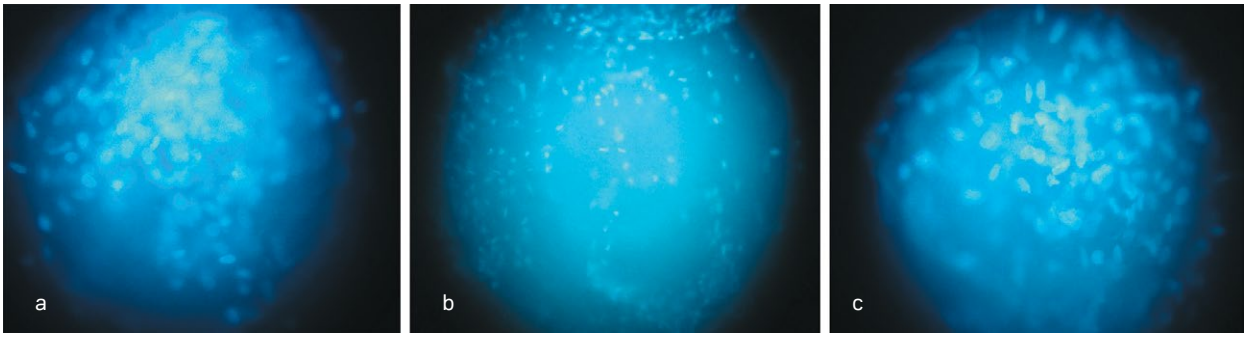

Figure 6. *Live labeling of Hoechst 33342 of osteogenically predifferentiated BM-MSCs in vitro graft modeling: (a) one week, (b) two weeks, (c) tree weeks. Images are taken at 100x magnification.*

Рисунок 6. *Прижизненное окрашивание красителем Hoechst 33342 остеогенно-предифференцированных КМ-МСК при моделировании трансплантата in vitro. Изображения сделаны при 100-кратном увеличении: а) одна неделя, б) две недели, в) три недели.*

Specimen	Week 1(x thousand cells)	Week 2 (x thousand cells)	Week 3 (x thousand cells)
Hybrid graft	557,5 ± 24,75	530,0 ± 49,50	380,0 ± 7,07

Table 3. *Proliferative activity of osteogenically predifferentiated BM-MSCs in graft in vitro*

Таблица 3. *Пролиферативная активность остеогенно предифференцированных КМ-МСК в составе трансплантата in vitro*

In the next stage, the absolute quantity of the osteogenic pre-differentiated BM-MSC were identified in the composition of the hybrid graft in the end of the first, second and third weeks of in vitro incubation (**Table 3**).

It is seen from the results presented in the table that during the first two weeks of incubation, no significant decrease in osteogenic pre-differentiated BM-MSC in the graft was observed. As the cultivation period increased to three weeks, the number of cells decreased in relation to the initially seeded number due to the dense distribution of the latter between the collagen fibers of the matrix carrier. Some of the cells migrated into the allogeneic spongiosa and formed a dense monolayer between the bone and the carrier.

Staining with the fluorescent dye Hoechst 33342 showed a uniform population of the biopolymer collagen sponge with osteogenic pre-differentiated BM-MSC and high viability of the cells between the collagen fibers of the matrix carrier in all stages of cultivation (**Fig. 6**).

Moving on to the discussion of the results, it is worth noting that allogeneic bone implants are a common and effective approach to replacing bone defects. New methods of tissue engineering and cell technologies can give this area a second wind and find application in replacing long-term non-healing bone defects, when cell-free osteoinductive implants or MSC without matrix carriers cannot be effective [19, 43]. In such situations, tissue engineering using bioorganic matrix carriers, or scaffolds, osteogenic cells and factors that promote regeneration and vascularization of transplants is of practical interest for the treatment of injuries or replacement of bone defects [19, 44]. BM-MSC are used for hybrid cell-tissue grafts more often that other cells since they are better characterized, readily available, and demonstrate a prolific osteogenic potential. At the same time, the clinical efficacy of such a graft depends significantly on the number of cells seeded onto the carrier, or scaffold, on biocompatibility of the latter, and other factors [43–45].

In bone tissue regenerative medicine, various bioorganic carriers are used, therefore the viability, adhesion and proliferation of BM-MSC on the matrix will depend on the chemical nature and structure of the latter [11, 46]. In this research, we studies the cytotoxicity of four commercially available bioorganic carriers based on collagen (“Lyostypt”), гидроксиапатита/collagen (“Collapan”), bone matrix with sGAGs (“Osteomatrix”), and lyophilized bone matrix (“LBM”), analyzing the viability of BM-MSC in their joint cultivation. All four studied matrix carriers had low cytotoxicity, causing the death of no more than 30% of cells. However, the “Lyostypt” collagen sponge showed the lowest cytotoxicity and the highest biocompatibility with human BM-MSC, which created optimal conditions for cell proliferation on the matrix. Therefore, we used “Lyostypt” as the basis (matrix) to produce the hybrid cell-tissue osteoconductive graft consisting of the carrier for the cells, osteogenic pre-differentiated BM-MSC and alPRP factors with subsequent evaluation of proliferative activity and osteogenic differentiation of the cells. In their experiment, J. Zheng et al. (2022) also demonstrated that cultivation of human MSCc on a collagen sponge with hydrogel fostered their proliferation and strengthened their chondrogenic differentiation by means of formation of the alternative extracellular microenvironment [47].

Scientific data indicate conflicting results of the influence of soluble individual factors (lysates and relysates of platelets) and platelet-rich plasma on human MSC differentiation. Thus, the use of plasma enriched with platelet factors showed a positive effect on the saturation of matrices (β-tricalcium phosphate, calcium-deficient hydroxyapatite) of MSC and on the proliferation of MSC in these matrices, but had little effect on the osteogenic potential of MSC [48].

Thus, a characteristic feature of modern tissue engineering and regenerative medicine is the combination of matrices with various biologically active components and their

saturation with cells, mainly MSC, with subsequent targeted differentiation of the latter. Earlier research [49, 50] and this study showed that platelet derivatives (lysates and relysates of platelets, alPRP) are effective components for stimulating the expansion and osteogenic differentiation of BM-MSCs in vitro. This approach allows obtaining a sufficient number of cells to create a hybrid cell-tissue graft with its subsequent use to replace bone defects. Moreover, the composition of the structure in the version of a bioorganic matrix carrier based on collagen, osteogenic predifferentiated BM-MSC and alPRP factors is optimal for potential clinical application.

CONCLUSIONS

1. The bioorganic matrices studied for biocompatibility with the human BM-MSC culture showed low in vitro toxicity towards cells (no more than 30%). With duration of BM-MSC cultivation with biomaterials increased to 7 days, the cells preserved high viability and increased their proliferative activity. The lowest cytotoxicity and the best biocompatibility was seen in the carrier from resorbable biopolymer collagen sponge.

2. The biological and structural characteristics of the collagen matrix carrier reliably promoted the adhesion of BM-MSC, their uniform distribution and proliferation.

3. The alPRP used as the source of soluble factors made an impact on the osteogenic differentiation of the human BM-MSC: addition of 5% alPRP to the osteogenic medium sped up the mineralization process (deposition of calcium salts in the cell matrix) and expression of genes of osteogenic differentiation (RUNX2, ALP and OSP).

4. The hybrid cell-tissue graft is characterized with safety, efficiency and biocompatibility with bone tissue making it suitable for clinical use in regenerative medicine when replacing the bone defects.

ADDITIONAL INFORMATION	ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ
Compliance with ethical standards. Informed consent was obtained from the study participants in accordance with the decision of the Ethics Committee of the Republican Scientific and Practical Center for Traumatology and Orthopedics of the Ministry of Health of the Republic of Belarus (protocol No. 3 dated 04/14/2021).	***Соблюдение этических норм.*** От участников исследования получено информированное согласие по решению этического комитета Республиканского научно-практического центра травматологии и ортопедии Министерства здравоохранения Республики Беларусь (протокол №3 от 14.04.2021 г.).
Study funding. This research received no external funding.	***Источник финансирования.*** Исследование проводилось без спонсорской поддержки
Conflict of interest. The authors declare that there are no obvious or potential conflicts of interest associated with the content of this article.	***Конфликт интересов.*** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с содержанием настоящей статьи.
Contribution of individual authors. N.N. Danilkovich – investigation, writing-original draft preparation. S.M. Kosmacheva – conceptualization, methodology and study design; A.G. Ionova – investigation, data curation; K.A. Krivorot – investigation, data curation. A.N. Mazurenko – data analysis, visualization. D.G. Alekseev – article review and editing. All authors gave their final approval of the manuscript for submission, and agreed to be accountable for all aspects of the work, implying proper study and resolution of issues related to the accuracy or integrity of any part of the work.	***Участие авторов.*** Н.Н. Данилкович – проведение исследования, подготовка рукописи. С.М. Космачева – концепция методология и дизайн исследования. А.Г. Ионова – проведение исследования, сбор данных. К.А. Криворот – проведение исследования, сбор данных. А.Н. Мазуренко – обработка данных, визуализация. Д.Г. Алексеев – редактирование рукописи. Все авторы одобрили финальную версию статьи перед публикацией, выразили согласие нести ответственность за все аспекты работы, подразумевающую надлежащее изучение и решение вопросов, связанных с точностью или добросовестностью любой части работы.

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