Electronic Journal of Biotechnology 69 (2024) 1–10

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

Research article

Formation of osteoconductive biograft with bioorganic scaffold, human mesenchymal stromal cells, and platelet-rich plasma with its evaluation *in vitro* ☆

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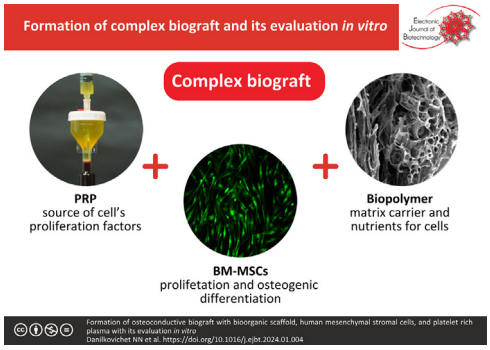
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 August 2023
Accepted 24 January 2024
Available online 25 March 2024

Keywords:
Allogeneic platelet rich plasma

ABSTRACT

Background: Complex graft bioengineering is an actual topic in bone defects' repair. For those, different scaffolds may be seeded with mesenchymal stromal cells and growth / differentiation factors. The natural role of platelet factors in reparative processes justifies the possibility of its usage for mesenchymal stromal cell proliferation and differentiation into osteoblasts *in vitro* in terms of the scaffold-based bioengineering. To develop and evaluate *in vitro* biocompatibility and osteoconductivity of a complex biograft based on a bioorganic scaffold seeded with human bone marrow mesenchymal stromal cells and saturated with growth and differentiation factors of allogeneic platelet-rich plasma.

List of abbreviations: ALP, alkaline phosphatase; alPRP, allogeneic platelet-rich plasma; AT-MSCs, mesenchymal stromal cells from adipose tissue; BM, bone marrow; BMP2, bone morphogenetic protein 2; CCM, complete culture medium; DNA, deoxyribonucleic acid; dNTP, deoxyribonucleotide triphosphate; DPBS, dulbecco phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FGF, fibroblast growth factor; GapDH, glyceraldehyde-3-phosphate dehydrogenase; HBC, hepatitis C; HBV, hepatitis B; IGF-1, insulin-like growth factor 1; LBM, lyophilized bone matrix; MSCs, mesenchymal stromal cells; MTT, methyltetrazolium test; OM, osteogenic medium; OSP, osteopontin; PDGF, platelet-derived growth factors; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; RUNX2, runt-associated transcription factors 2; sGAGs, sulfated glycosaminoglycans; SVF-AT, stromal-vascular fraction of adipose tissue; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factors; α-MEM, α-modified Eagle's medium.

☆ Audio abstract available in Supplementary material.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

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https://doi.org/10.1016/j.ejbt.2024.01.004

Biocompatibility
Biograft
Bioorganic scaffold
Bone reconstructive surgery
Cytotoxicity
Human bone marrow mesenchymal stromal cells
Osteoconductive biograft
Osteogenic differentiation
Proliferation
Regenerative medicine
Scaffolds

Results: The properties of viability and adhesion of human bone marrow mesenchymal stromal cells in four types of bioorganic scaffolds were evaluated with biochemical and immunomorphological methods. Scaffold with the least cytotoxicity was used as a basis for complex biograft formation, so as a carrier for cells and platelet-derived factors. Then, cell proliferation activity and osteogenic differentiation were estimated with biochemical, morphological, histochemical and molecular-biological methods. The study showed high viability of cells in all bioorganic scaffolds but the least cytotoxicity was the one based on xenogeneic collagen sponge. We also found that allogeneic platelet-rich plasma positively affects the proliferation and osteogenic differentiation of bone marrow mesenchymal stromal cells in a complex biograft *in vitro*.
Conclusions: The properties of the developed complex biograft characterize its biocompatibility and osteoconductivity and make it potentially suitable for regenerative medicine, particularly for reconstructive surgery of bone defects.
How to cite: Danilkovich NN, Kosmacheva SM, Ionova AG, et al. Formation of osteoconductive biograft with bioorganic scaffold, human mesenchymal stromal cells, and platelet rich plasma with its evaluation*in vitro*. Electron J Biotechnol 2024;69. https://doi.org/10.1016/j.ejbt.2024.01.004.

1. Introduction

Bone grafting is the gold standard for the regeneration of critical-sized non-healing defects in bone reconstructive surgery. Such defects are not capable of self-healing and maintain chronic inflammation. Alternatives include cell grafts, the usage of osteogenic growth factors and cell-free scaffolds, or the implantation of bone equivalents with a combination of cells, scaffolds, and osteogenic growth factors [1,2,3,4,5,6]. Several cell populations are associated with bone tissue, but the most important are osteoblasts, osteocytes, and osteoclasts, which are responsible for bone formation (osteogenesis), maintenance, and resorption, respectively. These cell populations originate from bone marrow-derived mesenchymal stromal cells (BM-MSCs).

In this regard, the therapeutic benefit of BM-MSCs is determined by the fact that they act as a source of progenitors of osteoblast and therefore demonstrate a higher potency for osteogenic differentiation than MSCs derived from adipose tissue and umbilical vein. Osteogenic differentiation of BM-MSCs *in vitro* is easily stimulated in monolayer cell culture by the addition of β-glycerol phosphate, hydrocortisone or dexamethasone and ascorbic acid, as confirmed by stimulation of the expression of early genes (Runx2 – Runt-associated transcription factors 2; ALP - alkaline phosphatase; BMP2 - bone morphogenetic protein 2, and osteonectin) and late genes (osteopontin, osteocalcin) of osteogenic differentiation [7]. Other growth factors are also able to act as osteodifferentiation inducers in BM-MSCs, namely the representatives of the TGF-β (Transforming Growth Factor beta) superfamily, IGF-1 (Insulin-like Growth Factor 1), FGF (Fibroblast Growth Factor), PDGF (Platelet-Derived Growth Factors) and VEGF (Vascular Endothelial Growth Factors) [1,8].

Thus, the proliferation of osteogenic cells and their differentiation into the osteoblasts are regulated by the cellular microenvironment and signaling molecules during the processes of bone tissue remodeling and traumatic defects' restoration. BM-MSCs are an important source for the regenerative medicine in case of non-healing bone defect restoration due to the ability of expanding and osteogenic differentiation *in vitro* [5,9]. The combined use of BM-MSCs with osteoconductive carriers (matrixes) is an alternative to the successful substitution of bone defects [10,11]. Regardless of the treatment method, the necessary conditions for bone regeneration are the usage of matrixes that have an adhesive surface for the osteogenic cells to attach and are able to conduct signals to differentiate the last one into osteoblasts in response to osteoconductive signals. The only injection of free cells into the bone's defect area is not effective because it does not provide long-term local presence so the stimulating effect on tissue regeneration will not be achieved. Therefore, the cells should be transplanted in defects with carriers (matrixes), made of biomaterials [12].

All biomaterials can be divided into several groups according to their origin: 1) inorganic, such as titanium or tricalcium phosphate bioceramics [13]; 2) natural biopolymers, such as collagen, gelatin, chitosan, agarose [14]; 3) synthetic biopolymers, for example, polyhydroxy acids (poly (glycolic acid), poly (lactic acid), polydioxanone); 4) combined (composite) biomaterials [15]. Applying to reconstructive bone surgery, such carrier biomaterials must have sufficient stiffness to retain their shape, so their second and more relevant name is scaffolds. In relation to the stimulation of bone regeneration, such scaffolds ought to be bioresorbable and have osteoinductive, osteoconductive or combined effect. To ensure cell attachment, proliferation, and differentiation, scaffolds should have a porous structure that is permeable to biologically active substances [16,17,18,19,20]. The response of cells may depend on the physical and biological characteristics of the scaffolds' biomaterial, such as topography, roughness, chemistry, surface energy, and charge. The above-mentioned features mimic the extracellular matrix of the native bone tissue, regulate cellular morphology, and hence differentiation, as well as the presence of bioactive ligands that can provide anchoring sites for cell attachment [21].

In the bioengineering of bone tissue, biomaterials that are combining the properties of porosity (microstructure), bioactivity (osteoinductivity, osteoconductivity), bioresorption, and are similar to the native bone extracellular matrix, can be considered as the most promising scaffolds for BM-MSCs. Stimulation of osteogenic differentiation of the last ones by such bioactive scaffolds may be intensified by the application of growth and differentiation factors available in blood cells, namely in platelets [22,23,24].

Over 30 growth factors have been identified in platelet granules, including factors for bone tissue regeneration: TGF-β and BMPs (Bone Morphogenetic Proteins), which modulate cell proliferation, stimulate the formation of osteoblasts and extracellular bone matrix, inhibit its degradation, and exhibit an immunosuppressive effect. In addition to platelet factors, blood plasma proteins, such as fibrin, fibronectin and vitronectin, are also active participants of osteogenesis [25]. Marx et al. [26] used in patients autogenous bone transplants soaked with plasma and platelet growth factors in the form of autologous PRP (Platelet Rich Plasma) for maxillofacial defect reconstruction and found that it resulted in quicker transplant maturation and higher bone density]. Con-

versely, other researchers did not report about such detectable benefits from the combination of PRP and demineralized bone matrix in complex biografts on a nude mouse model for bone regeneration [27].

We aimed to develop and evaluate *in vitro* biocompatibility and osteoconductivity of a complex biograft based on a bioorganic scaffold seeded with BM-MSCs and saturated with alPRP, containing growth and differentiation factors. It was hypothesized that the osteogenic properties of the bioorganic scaffold would be improved by BM-MSCs and alPRP usage, and their synergistic effect on cells' proliferation and differentiation in such complex biograft.

2. Materials and methods

2.1. Samples of bioorganic scaffolds

We sampled four commercially produced bioorganic scaffolds:

1) "Kollapan" includes nanoparticles of synthetic hydroxyapatite and collagen I type with added antibiotic lincomycin hydrochloride («Intermedapatit», Russian Federation) [28].
2) "Osteomatrix" is a highly purified bone matrix with preserved collagen (25%) and mineral components (75%) and natural architectonics, containing at least 1.5 mg/cm^3 of affinity-bound bone sulfated glycosaminoglycans (sGAGs) («Konektbiopharm», Russian Federation) [29].
3) "Lyostypt" is an absorbable biopolymer sponge made of natural bovine I type collagen fibers («B. Braun», Spain) [30].
4) Lyophilized bone matrix ("LBM") – immunologically safe, sterile demineralized human spongiosa prepared from post-vital donor biological material (State Institution "Minsk Scientific and Practical Center for Surgery, Transplantology and Hematology", Minsk, Republic of Belarus) [31].
5) All scaffolds are produced sterile in the form of granules of 0. 3 × 30 × 50 mm in size.

2.2. Obtaining allogeneic platelet-rich plasma

Blood was obtained from donors in accordance with safety rules marked in the World Health Organization handbook "The Clinical use of blood" [32]. Blood donors were admitted to donation and had no markers of viral infections (hepatitis B/HBV, hepatitis C/HCV, human immunodeficiency virus/HIV). It was confirmed by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) of molecular genetic testing. Donor's blood was collected from the elbow vein using a special YCELLBIO-KIT («BIONEER», Russian Federation). After that, the blood was centrifuged in two stages to remove erythrocytes and leukocytes (1550 rpm, 400 g, for 20 min) and concentrate platelets (2450 rpm, 1000 g, for 20 min) on a laboratory centrifuge Liston C 2201 («Liston», Russian Federation). In the obtained alPRP, the number of platelets was counted on the hematological analyzer Sysmex XN-300 («Sysmex Corporation», Germany) - their content had to be at least 1.25 × 10^9/ml. The alPRP from 6-10 donors were stored in aliquots at −30°C for up to 24 months. Prior to alPRP use, platelet activation was performed via a freeze–thaw cycle, followed by precipitation of cellular detritus by centrifugation (2900 rpm, 1400 g, for 20 min). After activation, alPRP was used during the first hour [33].

2.3. Obtaining and evaluation of bone marrow-derived mesenchymal stromal cells

The culture of BM-MSCs was obtained from 10 ml of human BM by iliac crest trepan biopsy in healthy donors. The decision of the Ethics Committee of the Republican Scientific and Practical Center for Traumatology and Orthopedics (Protocol No.3 from April 14, 2021) was issued, and all the participants have given informed consent. Biopsy material contained 20 units of high molecular weight heparin per 1 ml of bone marrow to inactivate the blood coagulation process. Then, biopsy material was diluted 1:1 in Dulbecco phosphate-buffered saline with calcium-magnesium free (DPBS) (Sigma-Aldrich, USA) followed by careful layering of 20 ml of the diluent onto 15 ml lymphocyte separation medium which is a mixture of Ficoll and sodium diatrizoate (Hypaque) with density adjusted to 1.077 g/ml (Lonza, Switzerland) in 50-ml centrifuge tubes (Corning, USA). The tubes were centrifuged at 450 g for 20 min. Mononuclear fractions were collected in centrifuge tubes, followed by washing in α-modified Eagle's medium with ribonucleosides (α-MEM) (Gibco, USA) with 2% fetal calf serum (FCS) (Sigma-Aldrich, USA) using centrifugation at 450 g for 10 min. Isolation of human BM-MSCs from the mononuclear fraction was selected by plastic adhesion at a concentration of 0.3–0. 6 × 10^6 cells per cm^2 in T75 culture flasks (Sarstedt, Germany).

Then BM-MSCs were cultured in a CO_2 incubator (5% CO_2) (ESCO CelCulture CO_2-incubator, Singapore) at 37°C in a complete culture medium (CCM), consisting of α-MEM (Gibco, USA), supplemented with 10% FCS (Sigma-Aldrich, USA), 40 mM/ml glutamine (Gibco, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (BioloT, Russian Federation) [34]. BM-MSCs were expanded in T75 culture flasks (Sarstedt, Germany) at the initial concentration of 300.0 × 10^3 cells (first and second passages). The CCM was changed twice a week. When BM-MSCs reached 80–90% confluence, they were detached using trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, USA) and then replated at 1500 cells/cm^2 (second and third passages). Next, BM-MSCs of the first passage were immunophenotyped on the expression of CD90, CD105, CD45, and CD34 antigens on a «FACScan» flow cytometer (Becton Dickinson, USA). Also, cell culture was checked for sterility (no viable bacteria and fungi) by the membrane filtration method. The viability of obtained BM-MSCs was assessed by the trypan blue exclusion method in counting chamber [35].

2.4. Evaluation of bioorganic scaffolds' biocompatibility

At this stage, we used 10 mg fragment samples of scaffolds. Also, the scaffolds' samples were kept in the CCM with 24-h exposition to obtain the scaffolds' supernatants. BM-MSCs were expanded in a 24-well culture plate (Sarstedt, Germany) with CCM at the initial concentration of 100.0 × 10^3/cm^2 for 24 h. After cultivation, the CCM was removed from the wells of the plate and the prepared biomaterial samples (scaffolds and its supernatants) were added to BM-MSCs. Next, 200 µl of fresh CCM were added to the wells of the plate and biomaterial samples with BM-MSCs co-cultured at 37°C and 5% CO_2 (ESCO CelCulture CO_2-incubator, Singapore).

The cytocompatibility and ability for adhesion of scaffolds and their supernatants to BM-MSCs in direct contact were evaluated after 24-h incubation within 1 and 7 d of co-cultivation by labeling with H33342 (Sigma-Aldrich, USA) at concentrations of 1 µg/mL. To accomplish this, the attached BM-MSCs were incubated with an H33342 solution of the respective concentration at 37°C for 20 min. After staining, the BM-MSCs were rinsed twice with DPBS and then, CCM was added [36]. The c BM-MSCs were analyzed using the «Leica DM2000» fluorescence microscope (Leica Microsystems, Germany) under × 100 magnification.

The cytotoxicity of the scaffolds and their supernatants in direct contact with BM-MSCs was evaluated and assessed after 24-h incubation within 1 and 7 d of co-cultivation by the MTT assay (Sigma-Aldrich, USA) [37]. MTT 5 mg/ml in DPBS was added to each well followed by incubation at 37°C for 4 h. Then, dimethyl-

sulphoxide (Serva, Germany) was added to each well to dissolve the formazan crystals produced by the activity of live BM-MSCs, and the colored scaffolds' supernatant was read at 570 wavelength on a «BioTek® ELx 800» reader (BioTek, USA). The experiments were carried out in the following test groups where BM-MSCs were co-cultured with next bioorganic scaffolds: (1) «Kollapan», (2) «Osteomatrix», (3) «Lyostypt», (4) LBM and (5) without scaffold in CCM with similar seeding concentrations as a control.

The viability of BM-MSCs after co-culturing with scaffolds and their supernatants was calculated according to the following [**Equation 1**]:

$$\frac{OD_{ES}}{OD_{CS}} \times 100\% \quad (1)$$

Accordingly, the cytotoxicity of scaffolds and their supernatants was calculated according to the following [**Equation 2**]:

$$100\% - \frac{OD_{ES}}{OD_{CS}} \times 100\% \quad (2)$$

where OD_{ES} is the eluate optical density from the well plate with BM-MSCs and scaffolds' supernatants; OD_{CS} is the eluate optical density from cells cultivated only in a CCM.

Qualitative criteria for assessing the cytotoxicity of scaffolds and their supernatants were as follows:

- low toxicity - death of up to 30% of cells (viability higher than 70%);
- medium toxicity - death of 30% to 50% of cells (viability 50–70%);
- high toxicity - death of more than 50% of cells (viability lower than 50%).

Scaffold with the least detected cytotoxicity was chosen as the basis to fabricate a complex biograft, namely a carrier for alPRP factors and MB-MSCs with followed evaluation of proliferation activity and osteogenic differentiation of the last ones.

2.5. Evaluation of BM-MSCs' proliferative activity on a bioorganic scaffold

Bioorganic scaffold with the least detected cytotoxicity was placed in the wells of a 24-well plate. MB-MSCs of the first passage were seeded on a scaffold at inoculum concentrations of 50.0×10^3, 100.0×10^3, and 300.0×10^3 and cultured for 7 d in the CCM with and without the addition of 5% alPRP. Then, cells were removed with the trypsin-EDTA solution (Sigma-Aldrich, USA). The number of viable cells was counted by trypan blue exclusion in the counting chamber [38].

2.6. Evaluation of BM-MSCs' osteogenic differentiation

BM-MSCs of second passage were adjusted in 8000/cm^2 concentration to a T25 plastic flask (Sarstedt, Germany) for subsequent reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis and in Petri dishes 35 mm in diameter (Sarstedt, Germany) for subsequent light microscopy. The three variants of cell cultivation were studied in the experiments of BM-MSCs osteogenic differentiation:

- BM-MSCs cultivated in the CCM (control sample);
- BM-MSCs cultivated in the OM;
- BM-MSCs cultivated in the OM supplemented with 5% alPRP.

The osteogenic medium (OM) was α-MEM supplemented with 10% FCS, 10 mM β-glycerol phosphate (Sigma Aldrich, USA), 50 μg ascorbic acid (Sigma Aldrich, USA), and 0.1 μM dexamethasone (Sigma Aldrich, USA) [38]. Next, osteogenic differentiation was estimated on the 4 and 7 d (here with single medium change) of cultivation by RT-qPCR analysis of mRNA expression of osteogenic genes RUNX2, ALP and OSP and on the 21 d of cultivation with medium change every 3 d by performing microscopy with assessing of BM-MSCs morphology and mineralization (intracellular calcium phosphate deposition) by von Kossa histological staining.

RT-qPCR was carried out in several stages [34,39]. Initially, we accomplished primary isolation of total RNA from BM-MSCs cultured in T25 flasks (Sarstedt, Germany). Extraction was performed using «TriReagent» (Sigma Aldrich, USA) following the manufacturer's instructions. RNA samples were stored at −80°C until the reverse transcription.

Thereafter total RNA samples were used to obtain complementary DNA (cDNA) using the reverse transcription method. The reaction was performed in a mixture with 20 μl of volume consisting of 2 μg RNA, 5 μM «Oligo(dT18) primer» (Thermo Fisher Scientific, USA), 1 mM deoxyribonucleotide triphosphate (dNTP) (Thermo Fisher Scientific, USA), 40 units of RNase inhibitor «Ribolock» (Thermo Fisher Scientific, USA), 1 μl (200 units/ μl) of reverse transcriptase «RevertAid Premium», and 5X buffer (Thermo Fisher Scientific, USA). Amplification was performed for 30 min at 50°C. The reverse transcriptase was inactivated by heating at 85°C for 5 min.

The obtained cDNA was amplified according to the following program: 95°C for 10 min (polymerase activation) with further amplification for 40 cycles at 60°C for 1 min. For the amplification of the markers of osteogenic differentiation (RUNX2, ALP, and OSP), RT-qPCR was performed with the usage of the following primers' pairs with corresponding nucleotide sequences (Table 1) [40]. Glyceraldehyde-3-phosphate dehydrogenase (GapDH) gene was used as a reference. For RT-qPCR, we used a «CFX96 Touch REAL TIME» detection amplifier (Bio-Rad, USA). The results were processed using Bio-Rad CFX Manager software.

Relative gene expressions were calculated with Livak's method according to the following [**Equation 3**] [41]:

$$Relativegeneexpresssion = 2^{-\Delta Ct}$$
$$\Delta Ct = [Ct(targetgenes) - Ct(GapDH)] \quad (3)$$

The onset of osteoblasts and their mineralization was assessed in Petri dishes (Sarstedt, Germany). BM-MSCs were stained with 1–2% silver nitrate solution for 45–60 min under ultraviolet light, then washed with distilled water and fixed with sodium thiosulfate for 5 min. Stained cells were afterward washed with deionized water, dried, and optically evaluated using the «Leica DM IL LED» light microscope (Leica Microsystems, Germany) under x100 magnification. Morphological transformation was characterized by a change in cell shape to cuboidal and intracellular calcium phosphate deposits stained black [42].

2.7. Statistics

Data are presented as mean ± standard error of the mean (M ± SEM) in GraphPad «Prism» 6.0 of least than three experiments. Each scaffold groups were tested in duplicate in one experiment. The significance of differences in variation series was performed

Table 1
Nucleotide sequences of primers.

Gene	Forward	Reverse
RUNX2	CACTGGCGGTGCAACAAGA	TTTCATAACAGCGGAGGCATTTC
ALP	GGTGGAAGGAGGCAGAATTG	TCAGAGTGTCTTCCGAGGAG
OSP	CACAGCATCTGGGTATTTGTTG	CGACCAAGGAAAACTCACTACC
GapDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT

using paired Student's t-test. Differences were significant at $p \leq 0.05$.

3. Results

3.1. Evaluation of obtained bone marrow-derived mesenchymal stromal cells

Immunophenotypic characterization of obtained human BM-MSCs showed the expression of specific markers CD90 and CD105 and the absence of CD34 and CD45 markers. The number of cells in the culture expressing marker CD90 was 99.9 ± 0.14%, marker CD105 – 99.14 ± 1.23%. An insignificant number of cells expressed marker CD34 (0.45% ± 0.20) and CD45 (0.30% ± 0.07) was revealed. The culture was sterile (no bacteria or fungi were present), and the viability of obtained cells never has been lower than 90%.

3.2. Biocompatibility of bioorganic scaffolds

The absence of toxic effects on BM-MSCs, opportunity for their adhesion, proliferation, and osteogenic differentiation are the main requirements for the scaffolds, which may be used in tissue engineering for the restoration of large bone defects. Such a combination of effects promotes the formation of a new natural extracellular matrix on the surface of the scaffolds, and its integration with native bone tissue. Therefore, scaffolds' biomaterials should have compatibility with BM-MSCs and ensure maintaining of cellular activity and mechanical integrity for the bone defect healing process [9]. To assess the cytocompatibility and adhesion ability of scaffolds, we used *in vitro* Hoechst 33,342 fluorescent staining (Sigma Aldrich, USA) and for the evaluation of cytotoxicity (cell viability) of scaffolds MTT assay was performed.

Hoechst 33,342 staining revealed labeled cells' nuclei which confirmed the citocompatibility and adhesion ability of scaffolds and their supernatants to BM-MSCs in direct contact (Fig. 1).

Fig. 1 shows the presence of flat cylindrical non-condensed nuclei of BM-MSCs and the formation of a uniform monolayer of living cells by them during cultivation with scaffolds: The red arrows show the adhesion ability of scaffolds for BM-MSCs.

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

When assessing acute cytotoxicity after 24 h of cultivation, cell viability ranged from 82.76 to 100%. The absence of cytotoxicity was established for the scaffolds' supernatant from the "Lyostypt" and the "LBM" at all periods of observation. Scaffolds in direct contact with BM-MSCs showed a similar effect on cells. All four studied scaffolds had insignificant cytotoxicity which caused the death of no more than 30% of cells (low toxic). Only cultivation of BM-MSCs with "Lyostypt" showed the highest cell viability in direct contact with the scaffold after 24 h and 7 d and even cell proliferation with its supernatant.

3.3. Proliferative activity of BM-MSCs on a bioorganic scaffold

Even though BM-MSCs are promising candidates for application in bone reparative regeneration, their pure delivery to the defect area is not always effective due to cell flushing by blood and tissue fluid, as well as cell migration. Therefore, the use of cytocompatibility scaffolds seeded with cells as part of a complex biograft with subsequent placement of the latter in the defect area for bone grafting is an actual task of bone tissue engineering [41]. The results of evaluation of BM-MSCs' proliferative activity with the selection of its optimal concentration on a bioorganic scaffold "Lyostypt", as the carrier with the least cytotoxicity, are shown in Fig. 2.

When cultivating BM-MSCs in CCM (Fig. 2**)** without alPRP, a slight increase in cells at a dose of 50.0×10^3 (initial number of cells / init.numb.of cells) and 100.0×10^3 ($p \geq 0.05$), but a decrease in the number of cells at a higher dose 300.0×10^3 cells ($p \leq 0.05$), was observed by 7 d. When adding 5% alPRP to CCM, a significant increase in proliferative activity of cells was found. The number of MSCs increased by 3.3-fold (p = 0.02), 2.6-fold (p = 0.0004) and 1.3-fold (p = 0.026) at the initial inoculum concentration of 50.0×10^3, 100.0×10^3 and 300.0×10^3 cells per cm^2 respectively. The viability of BM-MSCs was high in all samples and amounted 99% in four sets of experiments. Thus, for increasing the proliferative activity of BM-MSCs on a bioorganic scaffold, the presence of alPRP is more significantly important than the initial number of cells seeded per cm^2.

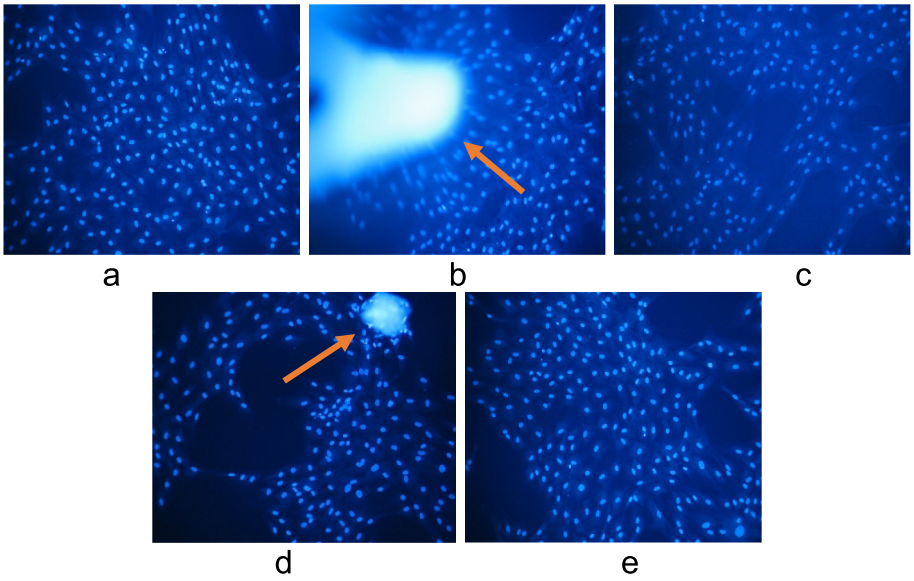

Fig. 1. Nuclei labeling of Hoechst 33,342 BM-MSCs after one day of cultivation with different scaffolds: (**a**) CCM (control sample); (**b**) Osteomatrix; (**c**) LBM; **(d)** Kollapan; **(e)** Lyostypt. Image taken at 100x magnification.

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

Samples	Supernatants (% of living cells)		Scaffolds (% of living cells)	
	1 d	7 d	1 d	7 d
Kollapan	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

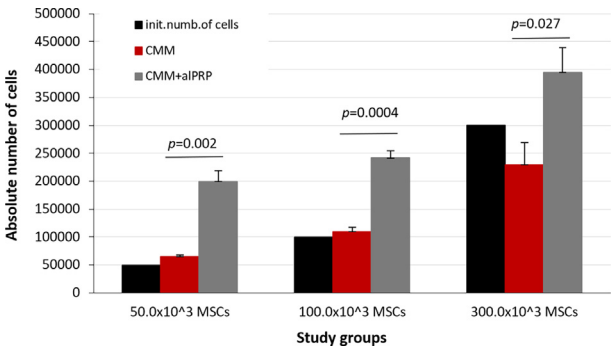

Fig. 2. Proliferative activity of different concentrations of BM-MSCs on the "Lyostypt" bioorganic scaffold over a period of 7-d cultivation.

3.4. Osteogenic differentiation of BM-MSCs

BM-MSCs have a potential for usage in regenerative medicine, for example, in patients with posttraumatic, including postoperative bone tissue defects, because these cells, differentiating, begin to synthesize extracellular bone matrix in the area of damage. Besides, BM-MSCs can indirectly promote bone tissue formation in the areas surrounding the defect due to the secretion of corresponding growth factors [41]. Studies by Meesuk et al. [43] showed that osteogenically differentiated BM-MSCs can produce more osteogenic factors than undifferentiated cells and, therefore, are more suitable for use in regenerative medicine for healing bone defects.

In experiments *in vitro*, the osteogenic potential of BM-MSCs was determined by RT-qPCR analysis of mRNA expression of osteogenic genes RUNX2, ALP and OSP followed by microscopy with assessing of cell's morphology transformation and mineralization (intracellular calcium phosphate deposition) by von Kossa histological staining. The results of RT-qPCR analysis of mRNA expression of osteogenic genes (RunX, ALP, OSP) are presented in Fig. 3.

After 4 d, an increase in mRNA expression of the target genes in BM-MSCs cultivated with OM was shown. The synthesis of mRNA of the RunX gene (Fig. 3a), ALP (Fig. 3b) and OSP (Fig. 3c) by differentiated cells increased by 7.1-fold ($p \leq 0.001$), 5.5-fold ($p \leq 0.001$) and 2.1-fold ($p \leq 0.008$) respectively, in relation to control (BM-MSCs cultivated in CCM). Adding 5% alPRP to the OM increased the expression of mRNA of the OSP gene (Fig. 3c) by 12.0 fold ($p \leq 0.0001$) in relation to control and by 5.9-fold ($p \leq 0.0001$) to BM-MSCs cultivated in OM without alPRP. The difference is statistically significant in all comparisons presented above.

After 7 d, the expression of mRNA of the RunX (Fig. 3d), ALP (Fig. 3e), and OSP (Fig. 3f) genes increased. Statistically significant difference was obtained for ALP (Fig. 3e) – 8.67-fold ($p \leq 0.0001$) and OSP (Fig. 3f) – 22.9-fold ($p \leq 0.0001$) in relation to control. The addition of 5% alPRP to the OM did not lead to a significant increase in mRNA expression of osteogenic genes in relation to BM-MSCs cultivated in OM without alPRP.

Thus, the addition of alPRP to OM significantly enhanced the osteogenic differentiation of BM-MSCs. On day 7, it was noticeably more pronounced than on day 4. The ratio of mRNA synthesis of RunX, ALP and OSP on 7/4 d of cultivation was 3.6-fold, 1.4-fold, and 13.2-fold, respectively. BM-MSCs cultured in OM and alPRP within 4 to 7 d are preliminarily osteogenically differentiated. In these terms, the cells already express osteogenic markers, but at the same time, calcium deposits are not formed yet.

After 21 d of cultivation, we checked that the morphology of BM-MSCs changed from fusiform to cuboidal. Staining with silver nitrate by von Kossa revealed intracellular deposits of calcium phosphate (stained black). Changes were maximally expressed in cells cultivated in the OM supplemented with 5% alPRP (Fig. 4).

4. Discussion

MSC-based tissue engineering approaches have attracted attention for the repairing of non-healing bone defects when large lesions exist and MSCs without matrices (carriers) or cell-free osteoconductive scaffolds are ineffective for grafting [18,44]. In such a situation, bone tissue engineering using a combination of biomaterials, osteogenic cells, and factors, contributing to bone regeneration and vascularization is of practical interest in bone regenerative medicine [18,45]. Therewith, BM-MSCs are more frequently used for complex graft construction because they are well characterized, easy for obtaining and exhibiting tremendous osteogenic potential. Nevertheless, the clinical efficacy of the graft's composition strongly depends on the number of seeded cells, the biocompatibility of scaffolds, and other factors [44,46,47].

Various biomaterials are used in bone tissue engineering, thus, the viability, adhesive capacity, and proliferation of MSCs will differ from the type of scaffolds [9,48]. So synthetic nanofiber scaffolds (Polycaprolactone – PCL; Poly-lactic acid – PLLA; Polyvinylidene fluoride - PVDF) have recently become widespread in tissue engineering due to its uniformity and high porosity provided a good resemblance to the physiological environment of the body. However, the most significant difference in these scaffolds is in the nature of the material and its degradation rate. Abazari et al. [49] and Mirzaei et al. [50] investigated PCL, PLLA and PVDF scaffolds and among them, PLLA showed the highest degradation rate. The viability of the cells on the nanofiber scaffolds was not significantly different, adipose-derived MSCs (AT-MSCs) revealed proliferation and expansion on such scaffolds, but osteogenic differentiation of AT-MSCs was detected on PVDF scaffold only [49,50].

In our study, we explored the cytotoxicity of four commercially produced bioorganic scaffolds based on collagen ("Lyostypt"), hydroxyapatite/collagen ("Kollapan"), bone matrix with sGAGs ("Osteomatrix"), and lyophilized bone matrix ("LBM") by analyzing the viability of BM-MSCs in its porous structure. All four studied scaffolds had low cytotoxicity, which caused the death of no more than 30% of cells (low toxicity). However, the collagen sponge «Lyostypt» showed the least cytotoxicity and the highest biocompatibility with human BM-MSCs, which promotes cell proliferation

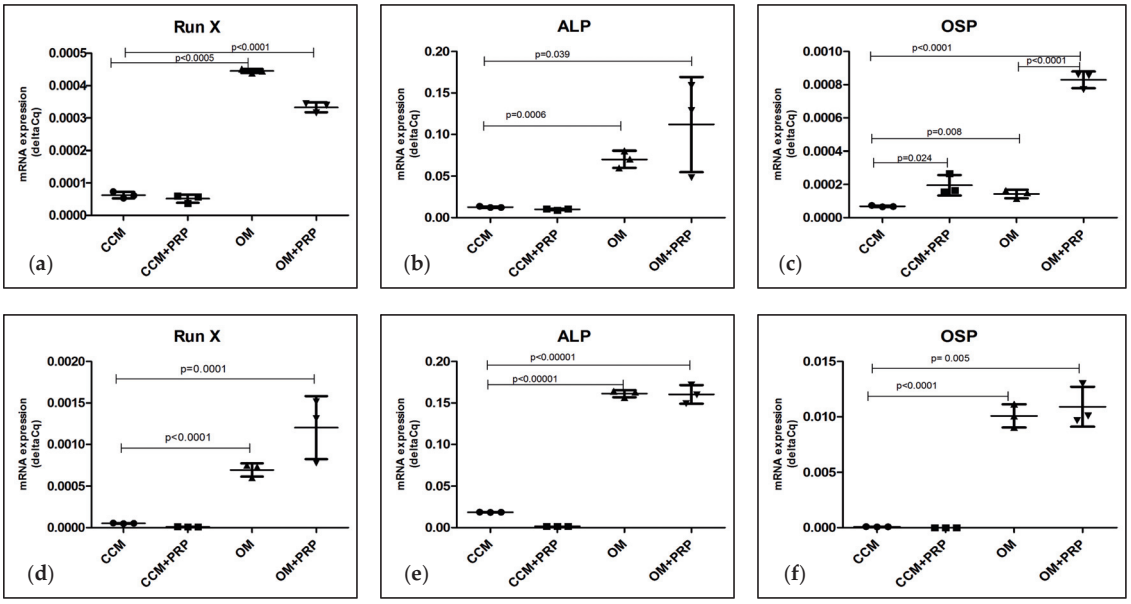

Fig. 3. mRNA expression of osteogenic genes: (**a, d**) RunX, (**b, e**) ALP, (**c, f**) OSP after 4 and 7 d of cultivation. Data are expressed as M ± SEM.

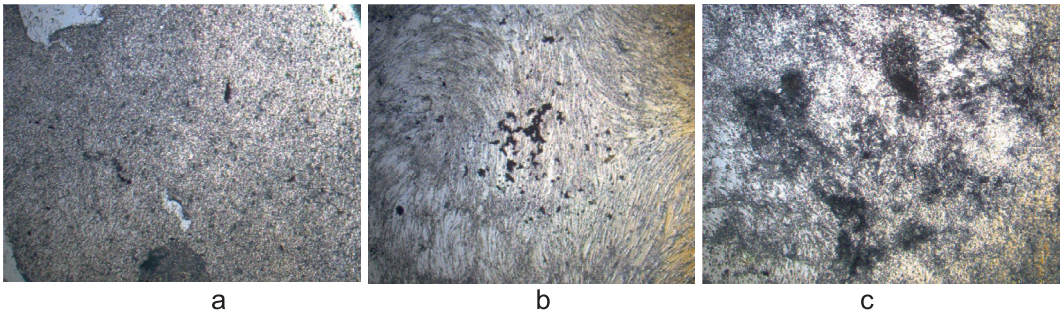

Fig. 4. Von Kossa staining of BM-MSCs: (**a**) cells cultivated in CCM; (**b**) cells cultivated in OM; (**c**) cells cultivated in OM, supplemented with 5% alPRP. Images are taken at 100x magnification.

in the pores of the matrix. Therefore, we used the «Lyostypt» as a basis (matrix) for creating a complex osteoconductive biograft, namely a carrier for MB-MSCs and alPRP factors with followed evaluation of proliferation activity and osteogenic differentiation.

Zheng et al. [51] showed that culturing human MSCs on a collagen sponge and hydrogel promoted proliferation and enhanced chondrogenic cell differentiation by creating a prototype extracellular microenvironment. However, scientific evidence indicates inconsistent results of the alPRP influence on human BM-MSC differentiation. Thus, the usage of alPRP with platelet growth factors has shown a positive effect on the BM-MSCs saturation and their proliferation in scaffolds (β-tricalcium phosphate, calcium-deficient hydroxyapatite), but had minor exposure on the properties of BM-MSCs osteogenic differentiation [52].

A characteristic feature of modern biotechnology in the field of scaffolding is the combining of matrices with various biologically active components. Platelet derivates (platelet lysate and relysate, alPRP) were shown earlier [53,54] and in the present study to be an efficient substance to drive BM-MSCs' expansion and osteogenic differentiation *in vitro*. Such an approach allows provide a sufficient number of cells for transplantation in bone regenerative medicine. Our study found the ability of alPRP to enhance the osteogenic differentiation of human BM-MSCs *in vitro* which gives a reason to consider this platelet derivatives as an effective component of the complex biograft to trigger BM-MSCs' transformation into osteoblast-like cells.

The main types of cultured cells used to create tissue engineering bone grafts include both multiple BM-mononuclear cells and a heterogeneous collection of cells contained within adipose tissue or so-called SVF-AT (Stromal-Vascular Fraction of Adipose Tissue). Meanwhile, they have a high complexity of the technological process for obtaining a large number of cells, the necessity to meet compliance standards of production (GMP – Good Manufacturing Practice) and laboratory quality control (GLP - Good Laboratory Practice), special storage conditions that are not always available for some medical institutions.

The latest and frontier approach in tissue engineering allows the usage of minimally manipulated cells to create *in situ* effective tissue-engineered constructs for bone regeneration. The Ficoll density gradient centrifugation and its modified versions are among the widespread and simple techniques for the manual BM-mononuclear cell concentration [55]. One of the most used devices for automatic BM-mononuclear cells and an SVF-AT isolation is Sepax [55,56]. Cells can be isolated and seeded into various scaffolds using selective retention technologies. Method of selective retention/filtration combines cell isolation and seeding and allows scaffold enrichment in 10–15 min. The actual method for intraoperative cell seeding is a static incubation of scaffold in a cell suspension. Also, prepared bone marrow aspirate may be placed on

the scaffold for 15–20 min for cell adhesion with followed implantation of the cell-enriched construct [57]. However, the use of specific pre- and intraoperative methods of cell isolation and seeding may be limited by the lack of quality control of cell culture at the last stages of the technological process, and uncontrolled quantitative isolation and seeding of cells on scaffolds. Limitations may also be due to strict legal regulation in some countries for the registration of cellular products and medical products containing living cells [48,55].

An alternative option for creating high concentrations of growth factors at the site of scaffold implantation is gene therapy using viral/non-viral vectors and stem cells. The effectiveness of gene therapy has been demonstrated with various vectors in some animal models, which makes it promising for the delivery of growth factors *in vivo* [58]. Walsh et al. [59] have demonstrated that the dual delivery of pBMP-2 (Bone Morphogenetic Protein-2) and pVEGF-A (plasma Vascular Endothelial Growth Factor-A) plasmid DNA using the 64-star-PLL (Phase Lock Loop) vector can be used to induce MSC-mediated osteogenesis on a range of collagen-based scaffolds with different macromolecular compositions. The 64-star-PLL-pDual gene-activated scaffold of just 4 weeks within a critical-sized rodent calvarial defect caused a 6-fold increase in a new bone formation compared to a pure collagen-based scaffold and a 4-fold increase compared to a 32-star-PLL-pDual gene-activated scaffold [59]. In a study by Presnyakov et al. [60], gene-activated hyaluronic acid-based hydrogels containing pVEGF-A were developed. Scaffold made of such hydrogel was implanted in the articular cartilage with subchondral bone defects in rabbits. Study has shown that healing of the articular cartilage and subchondral bone was effective at the observation periods of 30 and 60 d due to the formation of cartilage regeneration. However, after 90 d, remodeling of the newly formed cartilage regenerated into fibroelastic and fibrous connective tissues was observed [60]. Using gene-activated scaffolds has also some difficulties typical for this approach, such as sterilization, standardized control of the biological activity preservation of the gene constructs after the production cycle completion, the necessity to provide their controlled release from the scaffold structure after implantation, and adherence to standardized uniform requirements for their subsequent use in clinical practice.

In terms of further development of tissue engineering and scaffolding, creation of complex grafts containing living cells, combined usage of platelet-derived factors and vector-based gene therapy for directed differentiation of MSCs into osteoblast-like cells is prospective for application in regenerative medicine including reconstructive bone surgery.

5. Conclusions

Autologous and allogeneic bone implants are the classic approach to bone defect replacement. However, their obtaining is a complex surgical procedure, which is not always characterized by satisfactory results of bone defect regeneration in the implantation area. The approaches of regenerative medicine allow for reducing the relevance of the implant's disadvantages by replacing them with tissue-engineered biografts.

Modern biograft represents a complex tissue-engineered construct consisting of a cellular component, cell carrier matrix, saturated with proliferation and differentiation factors. We developed and studied *in vitro* a complex cell-tissue biograft based on BM-MSCs and bioorganic scaffold from a collagen sponge impregnated with alPRP as a source of factors for cell proliferation and osteogenic differentiation.

BM-MSCs have a significant potential for proliferation and osteogenic differentiation in conditions of a three-dimensional collagen scaffold impregnated with alPRP. Bioorganic scaffold of collagen sponge showed the lowest cytotoxicity, which characterizes its good biocompatibility with cells. Due to the structural characteristics of collagen sponge, BM-MSCs were easily adsorbed and distributed throughout the scaffold. The presence of alPRP in the composition of complex biograft creates conditions for adhesion, proliferation, and differentiation of BM-MSCs, which were confirmed by the increased expression of RUNX2, ALP and OSP genes and accumulation of calcium phosphate in cells, as well as changes in their shape.

6. Future prospects

Many researchers have been studying the synergetic effect of MSCs and growth factors with different scaffolds in stimulating bone regeneration. Although the results are very encouraging, some aspects remain underexplored. First, most experiments have explored *in vitro* and therefore, it is necessary to conduct additional studies and aggregate data about the *in vivo* mechanisms of MSCs in bone healing. Second, although MSCs on carrier matrices have been applied in clinical practice with patients, their efficacy is still inferior to that of bone autografts. Third, the efficacy of MSCs of various origins also needs further research and assessment. Surely, with the development of biotechnologies i.e. synthesis of new biomaterials, cultural methods and molecular techniques, MSCs will become more actual in the progress of bone restoration surgery [61].

Ethical approval

The study protocol was approved by the Ethics Committee of Republican Scientific and Practical Center for Traumatology and Orthopedics, with the approval number 3 from April 14, 2021.

Author contributions

- Study conception and design: N.N.D.; A.V.M.
- Data collection: K.A.K., A.N.M.
- Analysis and interpretation of results: S.M.K.; A.G.I.; E.I.P.
- Draft manuscript preparation: N.N.D.; N.O.; N.A.M.
- Revision of the results and approval of the final version of the manuscript: N.N.D.; D.G.A.

Financial support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare no conflict of interest.

Supplementary material

https://doi.org/10.1016/j.ejbt.2024.01.004.

Data availability

Data will be made available on request.

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