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Evaluation of *in vitro* biocompatibility of scaffolds for the repair of bone defects

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ABSTRACT

The use of bone scaffolds in traumatology and orthopedics is an extremely important issue. The growing number of cases of significant bone defects, in particular after revision arthroplasty, combat trauma and due to the introduction of new methods of reconstructive surgery of bones and joints, requires more detailed studies of the using different osteoplastic materials.

MATERIALS AND METHODS. *As scaffolds we used 4 types of materials that are most often used in the clinic for the correction of bone defects: ceramic hydroxylapatite, beta-tricalcium phosphate, allogeneic bone matrix treated with gamma irradiation, allogeneic bone matrix scaffold treated with gamma irradiation by local bone bank technology and a scaffold modified by delipidization. The effect of matrices on the viability of normal human fibroblasts (M19 cell line) in cell culture in vitro was studied. The viability of cells after their co-cultivation with scaffolds was determined by colorimetric method by staining with crystal violet. To obtain an osteoinductive effect, we used platelet-rich plasma (PRP), standardized by the method of Araki with some modifications. The proliferative activity of fibroblasts was assessed by the level of expression of the proliferation marker Ki-67 by immunocytochemical analysis.*

RESULTS. *It was found that the least pronounced antiproliferative effect is shown by allogeneic bone matrix treated with gamma irradiation. Data on the complex effect of co-cultivation of fibroblasts with scaffolds in the presence of PRP on cell viability and proliferative activity were obtained. It was found that PRP improves the survival of fibroblasts by 15-30 % and increases their proliferative activity by 35-75 %. Delipidization with 70 % ethyl alcohol for allogeneic bone matrix scaffolds, heat-treated by local bone bank technology, increased its biocompatibility with human fibroblasts.*

CONCLUSIONS. *According to the results of a comparative analysis of the impact of different scaffolds on the viability of normal human fibroblasts, it was found that scaffolds from allogeneic bone matrix have the least pronounced antiproliferative effect. Platelet-rich plasma has been shown to improve fibroblast survival and increase their proliferative activity during co-culture with scaffolds.*

KEY WORDS*: scaffold; allogeneic bone matrix; platelet-rich plasma; tissue engineering*

Tissue engineering is a relatively new multidisciplinary field that combines many fields (clinical medicine, biology, materials science, etc.), the main purpose of which is to restore and improve the tissues functions through the use of various techniques and approaches [1]. In this case, therapeutic agents in regenerative medicine often include only cells, while the most promising are combinations of cells with matrices (scaffolds) and soluble factors. The main requirements for scaffolds remain their safety and biocompatibility, which are ensured by the use of biomaterials with multifunctional properties, in particular to improve cell adhesion and proliferation [2].

Today, the active implementation of regenerative medicine technologies is taking place in orthopedics, as such promising strategies often avoid complications associated with traditional methods of treating bone diseases [3-4]. In addition, bone tissue is the second most common type of tissue for transplantation after blood transfusion. It is expected that due to the aging population, these new treatments will be even more popular in the future [5]. At the same time, there is always an acute shortage of donors, which is why a potential solution to such problems is the development and characterization of tissue-engineering technologies, which combine several components for a successful "regenerative complex".

Currently, different materials are used for the manufacture of scaffolds: inorganic and natural substances, synthetic polymers, composites with ceramics, etc. [2]. An extremely important characteristic of a scaffold is not only its compatibility with osteoinductive factors, including

cytokines and regenerative growth factors in platelets, including transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF); but also safety for the recipient and biocompatibility [6-8].

Therefore, in this study, we evaluated the effect of several variants of scaffolds on the viability and proliferative activity of cells *in vitro*. The human diploid fibroblast cell line was used in the study, because in the body it is a large group of connective tissue cells, which plays a significant role in tissue regeneration [9].

Scaffolds, which are most often used in traumatology and orthopedics as bone-plastic material, were selected for the study: ceramic hydroxyapatite, beta-tricalcium phosphate, allogeneic bone matrix treated with gamma irradiation and allogeneic bone matrix heat-treated by local bone bank technology. According to the literature, allogeneic scaffolds are characterized by high osteoconductivity, and hydroxylapatite is up to 70 % of the dry mass, which gives advantages to this type of ceramics among others [10-11]. Among allogeneic bone scaffolds, we chose two options – gamma-treated and heat-treated in the Scientific Center of Tissue and Cell Therapy of the State Institute of Traumatology and Orthopedics NAMS of Ukraine using local bone bank technology [12]. The method of sterilization of allogeneic bone scaffolds by gamma irradiation has been used for many years and has proven its positive qualities [13]. The advantage of the heat treatment technique is the possibility of validated production of allogeneic scaffolds in a hospital [14-15].

The analysis of the study results will assess the safety of the material of the selected matrices *in vitro* relatively to normal human fibroblasts in a comparative aspect. In addition, the complex effect of scaffold and platelet-rich plasma (PRP) as sources of platelet-derived growth factors TGF-β, IGF, VEGF, PDGF, bFGF, EGF and HGF [6-8, 16] on human viability and proliferative activity was investigated. This integral approach to the study of the effectiveness of applied technologies also allows to develop a certain algorithm for estimating the regenerative potential of various factors, to select the most successful experimental models for the further use in testing similar materials. The obtained data can be the basis for improving the biocompatibility of scaffolds with body tissues by physical or chemical factors modification for their further clinical use.

MATERIALS AND METHODS

Scaffolds. 4 types of materials were used as scaffolds: ceramic hydroxyapatite – HA (*Biomin*, Ukraine), beta-tricalcium phosphate – β-TCP (*Ceramed*, Portugal), allogeneic bone matrix treated with gamma irradiation – ABM-GI, (*Cenobioics*, UK) and allogeneic bone matrix processed by the technology of the local bone bank by thermal sterilization – ABM-TS (State Institute of Traumatology and Orthopedics of the NAMS of Ukraine, Ukraine) [12].

We also investigated the effect of ABM-TS modified by delipidization (ABM-TS + 70 % ethanol) on the viability and proliferation of human fibroblasts. The matrix was placed in 70 % ethanol (*Ukrspirt*, Ukraine) for 20 minutes at room temperature. After exposure, the matrix was washed three times with sterile saline (*Lekhim*, Ukraine) and used in experiments.

Cell line. Normal human fibroblasts of M19 cell line were obtained from the cell bank of human and animal tissue lines of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (*Kyiv*, Ukraine), cultured in 25 cm^2 cell culture flasks (*TPP*, Italy and *SPL*, Korea) in complete culture medium RPMI-1640 (*PAA*, Austria) supplemented with 10 % fetal calf serum (*PAA*, Austria), 40 μg/mL gentamicin (*Sigma*, USA) in a humidified atmosphere with 5 % CO_2 at 37 °C. The medium was changed every 2 days. After the cells formed a dense monolayer (3-5th day of growth), they were subcultured by dissociation using Versene solution with 1 mM EDTA (*BioTest Laboratory*, Ukraine). Cells of 8 passages were used in the study. Cell counting was performed in a Goryaev chamber with trypan blue vital dye (*Sigma*, USA) [17].

Platelet-rich plasma. PRP was obtained by the Araki method with certain modifications [18-19].The donors of blood to prepare PRP were three male volunteers aged 37-39, without acute and chronic diseases. 50 mL of venous blood was collected in vacuum tubes with dextrose citrate and centrifuged at 250 xg for 10 min using a benchtop centrifuge (*MICROmed*, China). Plasma was collected to new tubes and centrifuged at 2300 xg for 5 minutes. The platelet pellet was resuspended in 3 mL of platelet-poor plasma [20]. The platelet content in the product was more than 1 million cells per 1 μL.

Study design (Table 1).

Stage I: the study of the effects of different scaffold variants on the viability of normal human fibroblasts *in vitro* and select the scaffold that has the least significant effect.

Stage II: the assessment of the complex effect of scaffolds selected in stage I, in combination with PRP on the viability and proliferation of human fibroblasts.

The evaluation of cell viability. M19 fibroblasts were seeded on 96-well flat-bottom cell culture plates (*TPP*, Italy) in complete nutrient medium RPMI-1640 (*PAA*, Austria) supplemented with 10 % fetal calf serum (*PAA*, Austria), 40 μg/mL gentamicin at a concentration of $5 \cdot 10^4$ cells/mL (200 μL of cell suspension per well). At the same time, different variants of biomaterial were introduced into the corresponding wells: scaffolds, PRP. Different types of scaffolds were prepared in equal volume 0.02 g per well of a 96-well plate. The content of PRP was 10 or 20 % of the total nutrient medium in the well. The cells were incubated at 37 °C in the presence of 5 % CO_2 for 4 and 7 days.

The evaluation of cell viability was performed in a colorimetric assay using crystal violet dye (*Sigma*, USA). After incubation, the culture medium and scaffolds were removed from the wells, and 50 μL/well of dye was applied for 10 minutes, after which the wells were washed three times with water. Next, the plate was dried at room temperature for 3 hours and the dye was eluted with 96 % ethanol (100 μL/well) for 10 min using a mini-shaker PSU-2T (*Biosan*, Latvia) at 300 rpm. The data were recorded using a spectrophotometer Multiskan Plus (*Thermo Labsystems*, Finland) at a wavelength of 540 nm [20]. As a blank control, a well with a nutrient medium without cells was used, which underwent a standard staining procedure. The cell viability (X) in each well of the plate was calculated as a percentage by the formula:

$$X = \frac{A_1 \cdot 100\%}{A_0}$$

where A_0 is the average value of the optical density in the control wells of the cells; A_1 is the value of the optical density in each well of the experimental group or cell control group (to determine the SD of the cell control group).

The determination of cell proliferative activity. M19 fibroblasts were seeded in 24-well cell culture plates (*TPP*, Italy) in complete nutrient medium RPMI-1640 (*PAA*, Austria) supplemented with 10 % fetal calf serum (*PAA*, Austria), 40 μg/mL gentamicin at a concentration of $3 \cdot 10^4$ cells/mL (1 mL of cell suspension per well). At the same time, different versions of scaffolds were inserted in the appropriate wells in equal volume at 0.2 g per well. The content of PRP was 10 or 20 % of the total nutrient medium in the well. The cells were incubated at 37 °C in the presence of 5 % CO_2 for 4 and 7 days.

Immunocytochemical analysis. To prepare cytospin samples after cocultivation of human fibroblasts with scaffolds in the presence of PRP, a suspension of cells was resuspended in saline at a concentration of $1 \cdot 10^6$ cells/mL. 35 μL of cell suspension was added to cytocentrifuge cuvettes and centrifuged for 10 seconds at 180 xg, dried at room temperature and stored at -20 °C. Cytospin samples for immunocytochemical analysis were fixed in methanol + acetone (1:1) solution for 2 hours at -20 °C, then incubated for 20 minutes with 1 % bovine serum albumin. Then for 1 h monoclonal antibodies for the nuclear antigen of proliferating cells Ki-67

Table 1. Study design.

STAGE #	STUDIED SCAFFOLDS	ADDITIONAL FACTORS	CELL CULTURE TIME, DAY	CONTROL GROUP	STUDIED PARAMETERS
I	• Hydroxylapatite (HA) • Allogeneic bone matrix treated with gamma irradiation (ABMGI) • Allogeneic bone matrix heat-treated (ABMTS) • Beta-tricalcium phosphate (β-TCP)	-	4, 7	Normal human fibroblasts (M19 cells) without scaffolds	The evaluation of cell viability by colorimetric method by staining them with crystal violet (by the number of living cells relative to control).
II	• ABMGI • ABM-TS (without modification) • ABM-TS + 70 % ethanol(modified)	-	4, 7	M19 cells without scaffolds and PRP	The evaluation of cell viability by colorimetric method (crystal violet staining) compared to the control of M19 cells without scaffolds and PRP. The level of Ki-67 expression by immunocytochemistry.
	• ABM-GI • ABM-TS (without modification) • ABM-TS + 70 % ethanol (modified)	10 % PRP	4, 7	M19 cells + 10 % PRP	
	• ABM-GI • ABM-TS (without modification) • ABMTS + 70 % ethanol(modified)	20 % PRP	4, 7	M19 cells + 20 % PRP	

(*Thermo Scientific*, USA) were added. For visualization we used PolyVue peroxidase-conjugated detection system (*Thermo Scientific*, USA) with diaminobenzidine substrate. After the immunocytochemical staining, the slides were washed with water and stained with hematoxylin-eosin (*Thermo Scientific*, USA) for 15-30 seconds. The level of Ki-67 expression was assessed by H-score scale: S = 1xA + 2xB + 3xC, where S is the "H-Score", the value of which ranges from 0 (protein is not expressed) to 300 (strong expression in 100 % of cells); A – % of cells with low intensity staining; B – % of cells with moderate intensity staining; C – % of cells with strong intensity staining by microscopy using the AxioStarPlus microscope (*Carl Zeiss*, Germany) [21].

Statistical methods. Primary data processing was performed using the Excel 2016 software (*Microsoft*, USA). The experiments were performed three times (n = 3), results was presented as Mean ± SD. Statistical analysis of the obtained results was performed using the STATISTICA 6.0 software (*StatSoft Inc*., USA) according Student's t-test. The differences with a probability of at least 95 % ($p < 0.05$) were considered significant.

RESULTS AND DISCUSSION

At the first stage of the study, a comparative assessment of the impact of scaffolds of different origin and production on the viability of normal human fibroblasts of M19 line was performed (**Table 2**). Two time points were studied – the 4th and the 7th days in cell culture to obtain data on the growth characteristics of fibroblasts under conditions of exposure to scaffolds at different phases of the growth curve (exponential and stationary). The evaluation of the results was performed by staining the cells with crystal violet, which stains proteins and DNA in the cell.

As a result, it was found that the highest viability of M19 fibroblasts (relative to 100 % control of cells cultured without scaffold) was detected after co-cultivation with ABM-GI scaffold (**Table 2**). Ceramic scaffolds (HA and β-TCP) showed the worst results: fibroblast viability percentage were statistically significantly lower than control cells and other scaffold types by 65-80 % and 30-50 %, respectively, on both the 4th and 7th days of follow-up. The ABM-TS scaffold showed an average result, namely on the 4th day the viability percentage was 58 %, and on the 7th day – 51 % relative to 100 % control.

The detected antiproliferative effect of bioceramic scaffolds (HA, β-TCP) may be due to the high local concentration of calcium ions released from the materials [22]. A similar effect of bioceramic scaffolds was observed on normal hepatocytes of adult rats under conditions of their co-cultivation with matrix at non-physiological concentrations [23].

Table 2. The viability assay of human fibroblasts under the conditions of their co-cultivation with different scaffolds *in vitro*.

№	SCAFFOLD TYPE	THE TIME OF CO-CULTIVATION WITH SCAFFOLDS	
		4 DAYS	7 DAYS
		CELL VIABILITY, % (M ± SD)	
1	Hydroxyapatite (HA)	28.0 ± 6.0*◊	19.0 ± 4.0*◊
2	Allogeneic bone matrix treated with gamma irradiation (ABM-GI)	77.0 ± 2.0*#	64.0 ± 6.0*#
3	Allogeneic bone matrix heat-treated (ABM-TS)	58.0 ± 9.0*#	51.0 ± 3.0*#
4	Beta-tricalcium phosphate (β-TCP)	29.0 ± 4.0*◊	34.0 ± 2.0*#◊
5	Control sample (cells without scaffold)	100.0 ± 3.2	100.0 ± 2.8

*Note: * – $p < 0.05$ compared to control cells; # – $p < 0.05$ compared to the HA group; ◊ – $p < 0.05$ compared to the ABM-GI group.*

The least pronounced antiproliferative effect of ABM-GI can probably be explained by the fact that the doses of gamma irradiation used in the treatment of allogeneic scaffolds do not change the osteoconductive properties of bone tissue [24]. More pronounced antiproliferative effect of ABM-TS compared with ABM-GI is probably due to the fact that after heat treatment in the bone matrix there are lipid residues, which may worsen the osteoconductive properties of scaffold. For example, according to the literature, delipidization is mandatory for the protocol for the manufacture of scaffolds of xenogeneic origin [25].

Based on the previous results, the two most promising scaffolds ABM-GI and ABM-TS with the highest viability of normal human fibroblasts during co-cultivation were identified. These matrices were used for further studies of their effect on fibroblast proliferation *in vitro* and assessment of the complex effects of additional factors (PRP and modification of scaffold) to improve biocompatibility and minimize antiproliferative effects. It is know, that cells viability after the impact of matrix indicates its safety and biocompatibility.

In the second phase of the study, we used two approaches to improve the biocompatibility and osteoinductive characteristics of scaffolds when cultured with human fibroblasts, namely: (a) ABM-TS were modified by removing lipids and (b) addition of PRP, which is a source of cytokines

Table 3. The viability assay of human fibroblasts under conditions of their co-cultivation with different scaffolds and PRP *in vitro*.

№	SCAFFOLD TYPE	GROWTH MEDIA MODIFICATION					
		CELL CONTROL	+ PRP 10 %	+ PRP 20 %	CELL CONTROL	+ PRP 10 %	+ PRP 20 %
		CELL CULTURE DAY					
		4 DAYS			7 DAYS		
		CELL VIABILITY, % (N = 3, M ± SD)					
1	ABM-GI	73.0 ± 4.0*	99.0 ± 11.0▲	126.0 ± 3.0*	63.0 ± 6.0#	65.0 ± 5.0#▼	78.0 ± 4.0#
2	ABM-TS + 70 % ethanol (modified)	120.0 ± 2.0*—	101.0 ± 12.0—	140.0 ± 6.0*◊—	70.0 ± 3.0#—	88.0 ± 7.0—	91.0 ± 11.0—
3	ABM-TS (w/o modification)	47.0 ± 11.0*	39.0 ± 1.0*▲	50.0 ± 4.0*◊	39.0 ± 9.0#	43.0 ± 2.0#▼	44.0 ± 8.0#•
4	Control groups (M19 cells w/o scaffolds)	100.0 ± 3.8	125.0 ± 5.7*	114.0 ± 12.0	100.0 ± 4.1	101.0 ± 2.0	81.0 ± 13.0

Notes:

** – p < 0.05 compared to control cells w/o scaffold and PRP on the 4th day;*

 # – p < 0.05 compared to control cells w/o scaffold and PRP on the 7th day;

— – p < 0.05 compared to the group of ABM-TS w/o modification.

▲ – p < 0.05 compared to the group M19 + PRP 10 % on the 4th day;

▼ – p < 0.05 compared to the group M19 + PRP 10 % on the 7th day;

◊ – p < 0.05 compared to the group M19 + PRP 20 % on the 4th day;

• – p < 0.05 compared to the group M19 + PRP 20 % on the 7th day;

and growth factors (**Table 3**). The experiments used two concentrations of PRP – 10 and 20 % of the total nutrient medium volume, based on the results of studies of the impact of PRP on human cell proliferation *in vitro*, showed by other researchers [26].

The modification of the ABM-TS scaffold by delipidization significantly increased its biocompatibility with M19 human fibroblasts according to cell viability and proliferation data, in comparison with other variants of matrices. It is known, that the remains of lipids in the bone can be a barrier to cell engraftment and, as a consequence, adversely affect their biocompatibility and oseointegration. In addition, recipient's macrophages can recognize lipids as antigens and induce inflammatory responses, leading to increased bone resorption and fibrosis [27].

After the modification ABM-TS causes mitogenic effects on M19 cells – there was a significant increase in cell viability by 20 % on the 4th day of cultivation, compared with M19 cells without scaffold and PRP, and 73 % compared to group "M19 + ABM-TS" (**Table 3**). It should be noted that on the 7th day of incubation the mitogenic effect of "ABM-TS + 70 % ethanol" compared to M19 cells without scaffold and PRP was not observed, but the viability of this cells was significantly 30 % higher than in the group "M19 + ABM-TS".

The results presented in **Table 3** also show that 10 % of PRP in the culture medium significantly increases the viability of M19 cells on the 4th day of observation by 25 %, compared to cells without scaffold and PRP.

Thus, the increase in the viability of human fibroblasts M19 caused by the addition of 10 % or 20 % PRP of the growth medium was observed only on the 4th day of incubation, which may indicate its greater impact in the exponential phase of cell population growth. As the study did not provide the change of the medium and cell passaging after the addition of the PRP, on the 7th day fibroblasts under the impact of PRP formed a monolayer faster than control and, accordingly, due to contact inhibition, cell growth stopped.

The stimulating effects of PRP can be explained by its composition. It is known that PRP can contain a lot of growth factors and cytokines, in particular, PDGF, TGF-β, VEGF, EGF, FGF and IGF-1 [6-8]. According to the results of our previous studies [16], the presence of a significant concentration of TGF-β1 in the PRP was shown. TGF-β1 is a growth factor that plays an important role in the regulation of major cellular functions: provides metabolic activity of cells, including proliferation, differentiation and biosynthesis of macromolecules of extracellular matrix. Large amounts of this protein are found in platelets. The presence of receptors

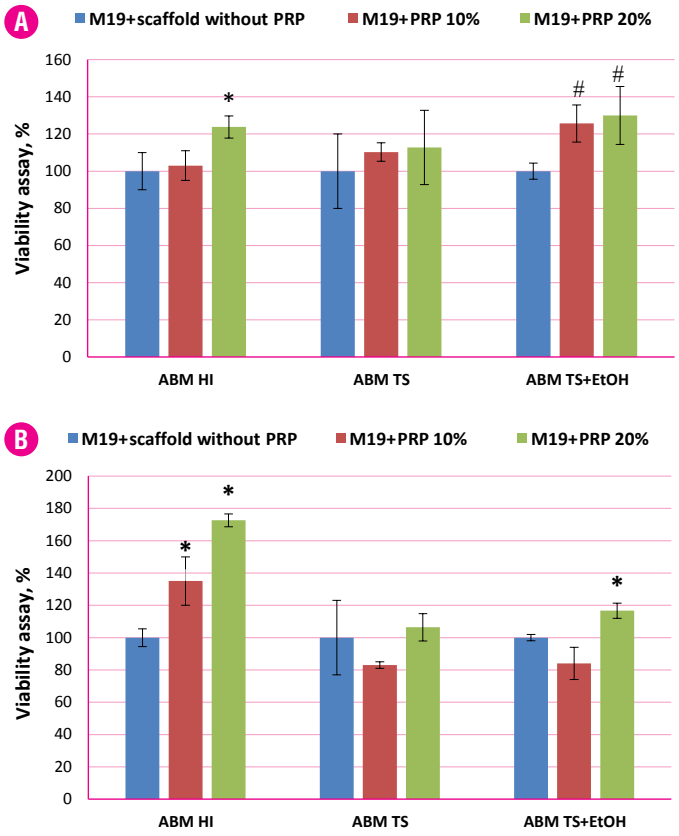

Fig. 1. The effects of PRP on cell viability under the conditions of their co-cultivation with different scaffolds: **A)** – the 4th day in cell culture; **B)** – the 7th day in cell culture.

Note:

** – p < 0.05 compared to the group "M19+ABM-GI" without PRP;*

 # – p < 0.05 compared to the group "M19+ABM-TS+70 % ethanol" without PRP.

for TGF-β1 on the surface of osteoblasts and chondrocytes suggests the participation of this factor in all stages of bone regeneration [28]. Also, TGF-β stimulates the proliferation of fibroblasts and changes in their phenotype, involving these cells in the bone regeneration [29].

It should be added that ABM-TS without delipidization on both the 4th and 7th day of incubation was characterized by a significant antiproliferative effect on M19 cells (**Table 3**): there was noted a statistically significant suppression of fibroblast viability by 40-50 %, compared with M19 cells without scaffold and PRP. Similar results were obtained at the first stage

Table 4. The expression level of the proliferation marker Ki-67 in human fibroblasts under conditions of co-cultivation with different scaffolds and PRP *in vitro*.

№	SCAFFOLD TYPE	CULTIVATION CONDITIONS					
		CELL CONTROL	+ PRP 10 %	+ PRP 20 %	CELL CONTROL	+ PRP 10 %	+ PRP 20 %
		CULTIVATION DAY					
		4 DAYS			7 DAYS		
		POINTS, ACCORDING TO THE H-SCORE					
1	ABM-GI	120.0 ± 10.0	140.0 ± 11.0	175.0 ± 15.0*†	80.0 ± 10.0	90.0 ± 5.0	90.0 ± 12.0
2	ABM-TS + 70 % ethanol (modified)	139.0 ± 10.0•	180.0 ± 16.0*•‡	210.0 ± 6.0*◊•‡	100.0 ± 10.0•	127.0 ± 7.0#▼•◘	130.0 ± 10.0#•◘
3	ABM-TS (w/o modification)	60.0 ± 10.0*	40.0 ± 5.0*▲	101.0 ± 4.0◊♦	70.0 ± 11.0#	89.0 ± 7.0	74.0 ± 6.0#
4	Control groups	119.0 ± 11.0 (M19 cells without scaffold and PRP)	144.0 ± 17.0 (M19 cells + PRP10 %)	180.0 ± 11.0* (M19 cells + PRP20 %)	94.0 ± 4.0 (M19 cells without scaffold and PRP)	98.0 ± 5.0 (M19 cells + PRP10 %)	110.0 ± 17.0 (M19 cells + PRP20 %)

Notes:

** - p < 0.05 compared to the control group (M19 cells w/o scaffold and w/o PRP) on the 4th day;*

 # - p < 0.05 compared to the control group (M19 cells w/o scaffold and w/o PRP) on the 7th day;

▲ - p < 0.05 compared to the group M19 + PRP 10 % on the 4th day;

▼ - p < 0.05 compared to the group M19 + PRP 10 % on the 7th day;

◊ - p < 0.05 compared to the group M19 + PRP 20 % on the 4th day;

● - p < 0.05 compared to the group of ABM-TS w/o modification;

† - p < 0.05 compared to the group of ABM-GI w/o PRP on the 4th day;

‡ - p < 0.05 compared to the group of ABM-TS + 70 % ethanol w/o PRP on the 4th day;

◘ - p < 0.05 compared to the group of ABM-TS + 70 % ethanol w/o PRP on the 7th day;

♦ - p < 0.05 compared to the group of ABM-TS w/o modification and w/o PRP on the 4th day.

of our research (**Table 2**). The presence of PRP in the culture medium did not significantly affect the biocompatibility of a similar matrix with fibroblasts. The obtained results suggest that in this case the addition of growth factors, which source is PRP, is not enough to improve the biocompatibility of such scaffold and only its modification, in particular by delipidization, can solve this issue.

For a comprehensive analysis of the impact of PRP on the viability of fibroblasts under conditions of their co-cultivation with scaffolds, we performed a comparative analysis of the results within each experimental group separately, using as a comparison group M19 cells + the corresponding scaffold without PRP (**Fig. 1**). The cell viability in each group for M19 + corresponding scaffold without PRP was presented as 100 %.

Interestingly, the addition of 10 % or 20 % of PRP to fibroblasts cultured in the presence of ABM-GI for 4 days led to a statistically significant increase in cell viability by 35 % or 72.6 %, respectively, compared to the group "M19 + ABM-GI" without PRP (**Fig. 1A**). A statistically significant increase in the viability of fibroblasts was also observed on the 4th day of the experiment under conditions of their co-cultivation with ABM-TS + 70 % ethanol + 20 % PRP by 17 % compared to the group "ABM-TS+70 % ethanol" without PRP (**Fig. 1A**).

On the 7th day of observation, there was a statistically significant increase in the viability of fibroblasts under conditions of their co-cultivation with ABM-GI in the presence of 20 % PRP by 23.8 %, compared to the group "M19 + ABM-GI" (**Fig. 1B**). In addition, co-cultivation of M19 cells with ABM TC + 70 % ethanol in the presence of 10 % or 20 % PRP also led to a statistically significant increase in fibroblast viability by 25.7 % and 30 %, respectively, compared to "M19 + ABM-TS + 70 % ethanol" (**Fig. 1B**). It should be noted that PRP did not significantly affect the biocompatibility of the scaffold ABM-TS without delipidization with normal human fibroblasts M19 (**Fig. 1**).

The obtained results show that 10 % or 20 % content of PRP in the nutrient medium of M19 fibroblasts under conditions of their co-cultivation with scaffolds based on allogeneic bone matrix significantly increases cell viability not only on the 4th but the 7th days of incubation. That is, in such conditions PRP promotes survival of normal fibroblasts for a long time. The obtained data suggest that the clinical application of such matrices in the combination with PRP will potentially increase the regenerative properties and the therapeutic effect will be observed longer.

In order to find possible mechanisms of the above-described changes in fibroblast viability under the conditions of their co-cultivation with different types of scaffolds in the presence of PRP, we studied the expression of the proliferation marker Ki-67 in M19 cells. Ki-67 is a nuclear protein associated with cell proliferation. The marker is expressed in phases S, G1, G2 and M of the cell cycle in the nucleus and indicates their proliferative activity.

According to the results of immunocytochemistry of human fibroblasts M19 after their exposure to scaffolds and PRP it was found that on the 4th day of the experiment, compared to M19 cells without scaffold and PRP, the following statistically significant changes in Ki-67 expression were detected: the decrease of the number of Ki-67+ cells by 50.4 % in the group "M19 + ABM-TS" and by 66.4 % in the group "M-19 + ABM-TS + 10 % PRP"; the increase in Ki-67 expression by 51.3 % in the group with 20 % PRP; the increase in expression by 47 % in the group "M19 + ABM-GI + 20 % PRP"; increase in the number of Ki-67+ cells and the increase of protein expression by 51.3 % in the group "M19 + ABM-TS + 70 % ethanol + 10 % PRP" and by 76.5 % in the group "M19 + ABM-TS + 70 % ethanol + 20 % of PRP "(**Table 4**).

On the 7th day of incubation of cells with scaffolds in the presence of PRP, the analysis of Ki-67 expression in fibroblasts showed that in comparison with M19 cells without scaffold and PRP, the following statistically significant changes in Ki-67 expression occur: the suppression of proliferation in the group "M19 + ABM-TS" by 25 %, and in the group "M19 + ABM-TS + 20 % PRP" by 21 %; the increase in Ki-67 expression in the group "M19 + ABM-TS + 70 % ethanol + 10 % PRP" by 35 % and in the group "M19 + ABM-TS + 70 % ethanol + 10 % PRP" by 38.3 % (**Table 4**).

The results presented in **Table 4** show that in human fibroblasts cultured with scaffold ABM-TS after delipidization on the 4th and 7th days of observation showed a statistically significant increase in Ki-67 expression in 2 and 1.5 times, respectively, compared to M19 + ABM-TS cells without modification. In the presence of PRP we observed a similar difference in

the expression of proliferation marker: in groups where cells were co-cultured with scaffold ABM-TS after delipidization in the presence of 10 % or 20 % PRP 2-4.5 times (the 4th day) and 1.7-1.9 times (the 7th day) higher expression index of Ki-67 was observed, compared to the group "M19 + ABM-TS" (**Table 4**).

Comparative analysis of the effect of PRP on the expression of Ki-67 in M19 cells under conditions of their co-cultivation with ABM-GI on the 4th day showed that only in the presence of 20 % PRP a statistically significant increase in marker expression by 45.8 % compared to M19 + ABM-GI cells without PRP was showed. On the 7th day of incubation of fibroblasts with the studied factors, no significant changes in the expression of Ki-67 were observed (**Table 4**).

The evaluation of the effect of PRP on the expression of Ki-67 in fibroblasts cultured in the presence of scaffold ABM-TS + 70 % ethanol, compared to the group "M19 + ABM-TS + 70 % ethanol" without PRP, shows that on the 4th and the 7th days incubation of cells with these factors the expression of Ki-67 was statistically significantly higher in the presence of PRP: 29.5 % (at 10 % PRP) and 51 % (at 20 % PRP) on the 4th day; 27 % (at 10 % PRP) and 30 % (at 20 % PRP) – the 7th day. Comparative analysis of Ki-67 expression in groups where M19 cells were incubated in the presence of ABM-TS and PRP did not show statistically significant changes in the studied parameter compared with the group "M19 + ABM-TS" without PRP (**Table 4**).

Therefore, the analysis of the proliferation marker Ki-67 in human fibroblasts under conditions of their co-cultivation with different types of scaffolds and in the presence of PRP indicate that the decrease in cell viability, which was shown above, after their interaction with scaffolds is due to a decrease in their proliferative activity (for group M19 + ABM-TS), and the increase of viability is due to active proliferation (for groups "M19 + ABM-GI + PRP", "M19 + ABM-TS + 70 % ethanol" and "M19 + ABM-TS + 70 % ethanol + PRP"). These facts confirm the results of the study of cell viability in co-cultivation with scaffolds and PRP.

CONCLUSION

According to the results of comparative evaluation of the impact of different scaffolds on the viability and proliferation of normal human fibroblasts, it was found that the least pronounced antiproliferative effect is caused by scaffolds based on allogeneic bone matrix.

It has been established that PRP improves the survival of fibroblasts and increases their proliferative activity. The additional modification of heat-treated allogeneic bone matrix scaffold by 70 % ethanol increased its biocompatibility with human fibroblasts. The delipidization of bone matrix scaffolds in combination with PRP provided the increase of their osteoinductive characteristic that can be followed by the increase of their regenerative properties and prolongation of the therapeutic effects.

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Оцінка в культурі клітин *in vitro* показників біосумісності скаффолдів для заміщення кісткових дефектів

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РЕЗЮМЕ

Використання кісткових скаффолдів в травматології та ортопедії є надзвичайно актуальним питанням. Зростання кількості випадків значних кісткових дефектів, зокрема після ревізійного ендопротезування суглобів, бойової травми та внаслідок впровадження нових методик реконструктивної хірургії кісток та суглобів, потребує більш детального з'ясування можливостей використання різних варіантів кістковопластичного матеріалу.

МАТЕРІАЛИ ТА МЕТОДИ. *Як скаффолди використовували 4 типи матеріалів, які найчастіше застосовуються в клініці для корекції кісткових дефектів – керамічний гідроксилапатит, бета-трикальцій-фосфат, алогенний кістковий матрикс, оброблений гамма-опроміненням, алогенний кістковий матрикс оброблений термічно за технологією локального кісткового банку та модифікований шляхом знежирення варіант скаффолду. Досліджували вплив матриксів на життєздатність нормальних фібробластів людини (клітинна лінія М19) в культурі клітин in vitro. Життєздатність клітин після їх співкультивування з скаффолдами визначали колориметричним методом шляхом фарбування кристалічним фіолетовим. Для отримання остеоіндуктивного ефекту використовували збагачену тромбоцитами плазму (ЗТП), стандартизовану за методикою Araki з деякими модифікаціями. Проліферативну активність фібробластів оцінювали за рівнем експресії маркера проліферації Ki-67 методом імуноцитохімічного аналізу.*

РЕЗУЛЬТАТИ. *Встановлено, що найменш виражену антипроліферативну дію проявляє алогенний кістковий матрикс, оброблений гамма-опроміненням. Отримано дані щодо комплексної дії співкультивування фібробластів із скаффолдами в присутності ЗТП на життєздатність клітин та їх проліферативну активність. Встановлено, що ЗТП покращує виживаність фібробластів на 15-30 % і підвищує їх проліферативну активність на 35-75 %. Деліпідізація 70 % етиловим спиртом скаффолду із алогенного кісткового матриксу, обробленого термічно за технологією локального кісткового банку, підвищувала його біосумісність щодо фібробластів людини.*

ВИСНОВКИ. *За результатами порівняльної оцінки впливу різних варіантів скаффолдів на життєздатність нормальних фібробластів людини встановлено, що найменш виражену антипроліферативну дію мають скаффолди створені на основі алогенного кісткового матриксу. Встановлено, що збагачена тромбоцитами плазма покращує виживаність фібробластів і підвищує їх проліферативну активність в присутності скаффолдів.*

КЛЮЧОВІ СЛОВА: *скаффолд; алогенний кістковий матрикс; збагачена тромбоцитами плазма; тканинна інженерія*