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MODULATION OF MESENCHYMAL STROMAL CELLS PROPERTIES BY THE MICROENVIRONMENT IN 3D CULTURE

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The aim of the research *was to compare the shape, viability, metabolic and proliferative activity of mesenchymal stromal cells (MSCs) during cultivation in hydrogels and macroporous scaffolds.*
Materials and methods. *Human adipose tissue MSCs were isolated from lipoaspirates of healthy adult donors after obtaining informed consent. Hydrogels were obtained from platelet-poor human blood plasma and alginate polymer, cross-linked with calcium ions in microspheres. Macroporous scaffolds were prepared from plasma by the cryotropic gelation method. Morphology and viability of cells within carriers were assessed using vital dyes. Metabolic and proliferative activity of MSCs was studied by the Alamar Blue test on the 1st, 3rd and 7th day of 3D culturing.*
Results. *Three-dimensional blood plasma scaffolds had a branched pore structure with a size sufficient for cell proliferation and migration. When plasma proteins were cross-linked with L-cysteine, almost all MSCs were viable, attached to the pore surface, spread and proliferated, filling carrier cavities. In plasma hydrogels, MSCs occupied spaces and acquired a fibroblast-like morphology, maintaining viability. In alginate microspheres, MSCs were uniform distributed throughout the gel volume, kept their spherical shape, had high viability. The highest metabolic activity of MSCs was observed in macroporous scaffolds, the lowest one in alginate microspheres. During cultivation, the activity of cells in macroporous scaffolds and plasma hydrogels increased significantly, which indirectly indicated the proliferation processes.*
Conclusions. *Properties of MSCs during 3D cultivation significantly depend on the microenvironment: in blood plasma carriers, cells acquire a fibroblast-like morphology and proliferate, while in alginate microspheres, they remain spherical and do not proliferate.*
Keywords: *mesenchymal stromal cells, three-dimensional cultivation, blood plasma hydrogel, alginate microspheres, macroporous scaffolds*

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1. Introduction

Mesenchymal stromal cells (MSCs) are finding increasingly broad applications in cell-based regenerative medicine due to a range of attractive and beneficial properties: multilineage differentiation potential, ease of isolation and widespread distribution in the body, high proliferative activity, and the ability to expand in culture, synthesis, and secretion of a wide array of factors capable of altering the metabolism and fate of neighboring cells. Among these properties, described in numerous monographs and reviews [1], two truly unique properties deserve special attention. Firstly, MSCs have the capability to differentiate into various types of connective tissue cells: osteoblasts, fibroblasts, adipocytes, and chondrocytes. This property enables them to serve as functional substitutes during transplantation and makes these cells an indispensable tool in tissue engineering – the discipline of creating bioengineered tissue equivalents for replacement or renewal of damaged tissues [2]. Secondly, the synthesis and secretion of numerous water-soluble trophic factors – proteins, hormones, and other biologically active molecules – into the surrounding environment, collectively referred to as the "secretome," is another noteworthy aspect. Additionally to secretome, release of nano- and micro-sized vesicles (exosomes and endosomes) containing a multitude of factors, including tetraspanins (CD63, CD81, CD9), heat shock proteins that modulate the physiology of target cells, as well as matrix RNA, microRNA, and pre-microRNA capable of reprogramming adjacent cells by interacting with their surface receptors and exhibiting paracrine effects, makes the media, conditioned by MSCs, a separate and promising tool in regenerative medicine [3]. These properties already have been applied in clinical trials for cell therapy of graft-versus-host-disease, myocardial infarction, stroke, diabetes mellitus, etc. (http://www.clinicaltrials.gov). For the therapeutic administration of MSCs, large amounts of cells are required. Conventional monolayer (2D) culturing does not allow maintaining of MSC properties during long term expansion, while 3D culturing establishes better conditions for MSCs growth [4].

There are multiple types of 3D systems that form a unique spatial microenvironment for cells and are able to support their growth and maintain viability. The microenvironment, along with physical factors (such as magnetic fields, pressure, vibration), chemical factors (partial pressure of oxygen, ion concentration, free oxygen forms), and other ones, can significantly

impact the behavior of MSCs including plasticity and proliferation [5].

Alginate hydrogel is biocompatible, semi-permeable material, widely used to form microspheres for 3D culturing, targeted drug and cell delivery [6]. It has been shown, that encapsulation into alginate microspheres could protect many cell types from acute immune response upon infusion. Earlier, we demonstrated that encapsulation of MSCs in alginate microspheres led to a cessation of division while retaining the cell ability to differentiation into osteogenic, adipogenic, and chondrogenic lineages [7, 8]. In another alginate-based 3D system, namely macroporous sponge-like carriers (scaffolds), MSCs retained their proliferation ability and similar to microspheres were capable to multilineage differentiation in the presence of inductors [9]. In the first system, cells are surrounded by a homogeneous hydrogel, while in the second system, they are situated at the interface between phases – the solid surface of the pores and the liquid culture medium. The observed difference in the behavior of MSCs in these two 3D systems with the distinct microenvironment remains not completely understood.

The aim of this study was to elucidate the role of the microenvironment in the manifestation of some MSCs properties in different 3D systems. For this purpose, a comparative assessment of the shape, viability, and proliferation of MSCs within hydrogels, made of alginate and human blood plasma, as well as in macroporous scaffolds, was conducted.

Human blood plasma was chosen due to its high relevance in today's context, especially when there is an urgent need for biocompatible and readily available materials, which exhibit high biological activity, particularly in combat conditions. A distinctive property of blood plasma is its ability to form hydrogels in the presence of the proteolytic enzyme thrombin and Ca^{2+} ions. This characteristic enables the use of blood plasma as a fibrin gel for military and disaster medicine. On the other hand, the fibrin hydrogel can be applied as a cell carrier in 3D culturing and for targeted delivery of cells to injury sites in regenerative medicine. To address the latter objective, it's possible to use macroporous scaffolds as well [10]. In this context, it's evident that cell carriers, made from blood plasma, satisfy modern requirements for medical materials.

2. Materials and Methods

Cell Isolation and Culture. After receiving the written consent of informed healthy volunteer donors, human adipose tissue MSCs were isolated from the lipoaspirate of adult patients in strict accordance with the recommendations of the World Medical Association Declaration of Helsinki. MSCs were obtained by collagenase digestion using the previously described method [11, 12]. Isolated MSCs were cultured in T25 adhesive polystyrene cell culture flasks (TPP, Switzerland) at 37 °C, 5 % CO_2, and 95 % humidity in Minimal Essential Medium-α modification (α-MEM, Sigma-Aldrich, USA) containing 10 % fetal bovine serum (Biowest, France), 50 μg/ml penicillin (Biowest, France), 50 μg/ml streptomycin (Biowest, France), and 0.2 mM L-glutamine (Sigma-Aldrich, USA). Complete medium changes were performed every 3–4 days. On reaching 80 % confluence, the cells were trypsinized, counted with a hemocytometer, and subcultured for 4 passages. *In vitro* expanded MSCs at passage 4 were harvested, characterized as previously described [13], and used in further experiments.

Blood Plasma Preparation. To avoid the impact of platelets and platelet-derived factors on the properties of MSCs during *in vitro* culture, we have chosen platelet-poor plasma as a less bioactive substance. The whole blood units of 5 different volunteer adult donors were collected at Kharkiv Regional Blood Service Center, accredited by the Ministry of Health of Ukraine. Before the collection, all donors were tested according to current Ukrainian legislative guidelines. One part of the blood (30 ml) was collected in the presence of an anticoagulant and processed by two-stage centrifugation to separate platelet-poor plasma ($<5 \cdot 10^4$ platelets/μl) from a platelet-rich plasma fraction [14]. Additionally, another 10 ml of blood was collected without an anticoagulant and stored at room temperature for 30 min for spontaneous coagulation. The obtained blood serum was aliquoted and stored at -20°C until further use.

Preparation of Plasma-Based Hydrogel. The platelet-poor plasma fraction was mixed with 10 % calcium chloride solution and blood serum in a ratio of 9 : 0.25 : 0.75. To obtain MSCs, embedded in a hydrogel, the platelet-poor plasma was preliminarily supplemented with a cell suspension in a concentration of $5 \cdot 10^6$ cells per 1 ml of the final mixture.

Preparation of Plasma-Based Scaffold. The blood plasma-based macroporous scaffolds were prepared by cryogelation of a mixture of blood plasma with 5.2 mM urea and 0.04 mM L-cysteine [15]. In some experiments, L-cysteine was changed for N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC). Samples were poured in the plastic Petri dishes (35 mm in diameter; the layer thickness was 2 mm) and frozen at -15 °C for 3 h. The obtained disks were stored in the 70 % aqueous ethanol prior to use. 5 mm diameter disks of blood plasma-based macroporous scaffolds were thoroughly washed 5 times with Hanks solution and then twice with a culture medium and seeded with cells as we described early [9].

Cell encapsulation in alginate microspheres (AMS). MSCs were washed with a medium of 0.15 M NaCl, 25 mM HEPES (pH 7.4) and encapsulated according to protocol [7]. Briefly, cells were resuspended in 1.2 % w/v solution of sterile filtered low viscous sodium alginate (Sigma-Aldrich, USA) at a cell concentration of 1.2–1.6×10^6 cells/ml. Cell suspensions were placed in a sterile syringe and sprayed in a solution containing 100 mM $CaCl_2$, causing gelation of the alginate as microspheres containing the cells. Alginate encapsulated MSCs were left in the solution of $CaCl_2$ for 10 min for complete polymerization, and thereafter twice washed with a solution of 0.15 M NaCl, containing 25 mM HEPES. The method resulted in AMS with sizes over the range of 500–700 μm as assessed by microscopy.

Morphology, Viability, and Metabolic Activity of MSCs within 3D carriers. Cell morphology was assessed using double fluorescent staining with fluores-

cein diacetate (FDA) and ethidium bromide (EB) [16]. FDA/EB staining was analyzed with Zeiss LSM 510 META (Carl Zeiss, Germany). Confocal images were obtained along the z-axis with 20 μm intervals at an excitation wavelength of 488 nm for FDA and 543 nm for EB.

To test cell metabolic and proliferative activity, MSCs were cultured either seeded into a plasma-based macroporous scaffold, encapsulated in alginate microspheres, or in a plasma-based hydrogel. The Alamar Blue test (AB, Serotec Ltd, USA) was performed as described previously [11, 17]. Briefly, on the 1st, 3rd and 7th day of sample culturing, MSCs were incubated for 3 h in the complete medium, containing 10 % AB. The fluorescence level of AB solution was measured by a TECAN GENios microplate reader (Tecan Genios, Austria) with an excitation wavelength of 550 nm and an emission wavelength of 590 nm. The ratio of the fluorescence intensity of experimental and blank sample (without cells) was used as AB value and expressed in relative fluorescence units (RFU). Each experiment was repeated in triplicate.

Statistical analysis. Data of the AB test were presented as mean ± SD. Each experiment was repeated in triplicate. The statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. $P<0.05$ was considered statistically significant.

3. Results

Three-dimensional plasma-based scaffolds exhibited a branched, wide-porous structure with pore sizes ranging from 100 to 250 μm, sufficient for cell proliferation and migration. When blood plasma proteins were cross-linked with L-cysteine, MSCs adhered to the surface of the pores, spread out, had a fibroblast-like shape, and proliferated, filling the scaffold cavities (Fig. 1A). Almost all cells remained viable, as indicated by FDA/EB staining. Cross-linking of blood plasma proteins with EDS resulted in intense staining with ethidium bromide, which normally does not penetrate the plasma membrane of viable cells [16]. These data indicate the toxicity of EDS and the inadvisability of its further use in experiments (Fig. 1B).

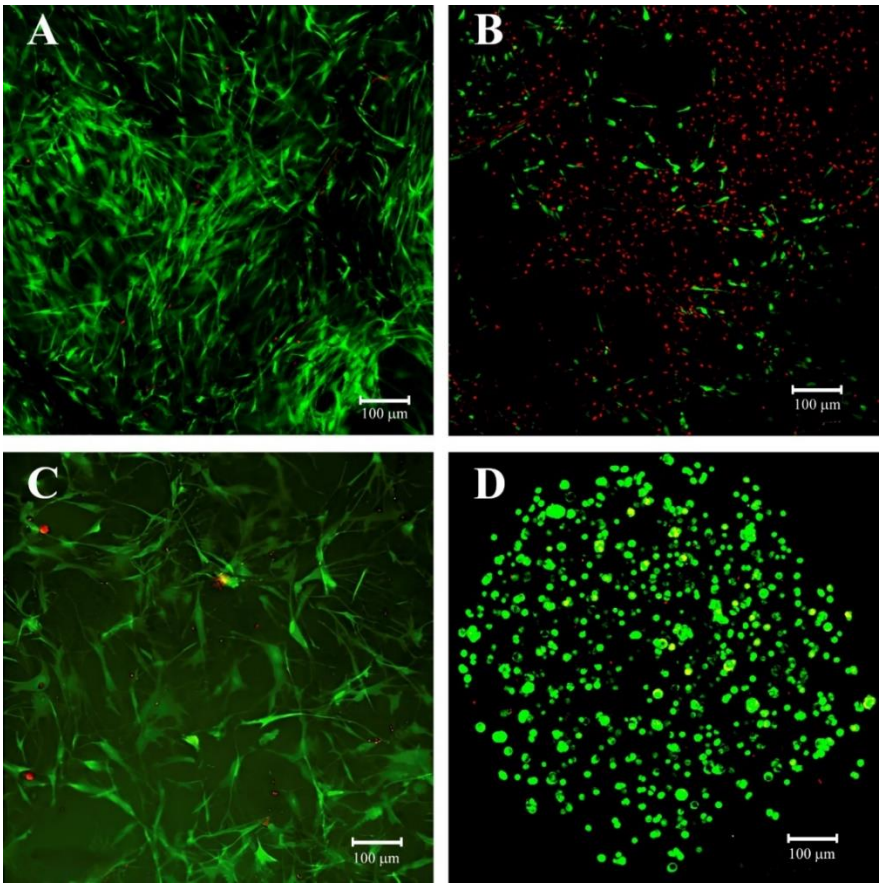

Fig. 1. Confocal microscopy images of viability (FDA/EB staining) and morphology of MSCs after culturing for 7 days within 3D carriers. A – plasma macroporous scaffold, treated with L-cysteine; B – plasma macroporous scaffold, treated with EDC; C – plasma hydrogel, D – alginate microspheres

When MSCs were placed in the plasma-based hydrogel, they evenly distributed within the volume, and similarly to macro-porous scaffold, they acquired a fibroblast-like shape (Fig. 1C). Live and dead analysis revealed only few cells, stained with EB, while the majority of cells remained viable.

Alginate microspheres, obtained by the spray method, were of a round shape with well-defined contours, fairly homogeneous, and their diameter ranged from 500 to 700 μm. During the 1-week of culturing, the microspheres remained stable: they did not collapse or aggregate. The encapsulated cells were evenly dis-tributed throughout the volume of alginate microspheres (Fig. 1D). Cells, encapsulated into the alginate hydrogel, had a spherical shape. For the purpose of accurate cell viability counting and demonstration, several images were overlaid and summed. Calculated MSCs viability values in microspheres were not less than 98 %.

Metabolic activity in different microenvironments was assessed using the AB-test after 1 day of cell seeding into 3D carriers, and then after 3 and 7 days. From Table 1, it can be seen, that even at 1st day, the levels of fluorescence of the restored AB form significantly differed in all examined samples. The highest level was observed in 3D macroporous scaffolds, intermediate values were found in the blood plasma-based hydrogel, and the lowest levels were seen in the alginate microspheres. Specifically, the values within the alginate microspheres were 2.5 and 3.4 times lower than those in the blood plasma-based hydrogels and macroporous scaffolds, respectively.

During the cultivation of MSCs, the level of AB fluorescence significantly increased in macroporous scaffolds and blood plasma-based hydrogels, while it remained unchanged for cells in alginate microspheres (Table 1).

Table 1

Alamar blue assay of MSCs, seeded into 3D carriers (RFU per well) (M±SD, n=7)

Carrier type	Time of culturing		
	1 day	3 days	7 days
Plasma-based macroporous scaffolds	3.12±0.25	4.55±0.42*	5.78±0.62*
Plasma-based Hydrogel	2.33±0.12	3.58±0.13*	4.15±0.36*
Alginate microspheres	0.91±0.14	1.18±0.25	0.97±0.26

*Note: * – values are significantly higher versus that measured on day 1 of culturing ($p<0.05$). Values in each type of carrier are significantly different versus other type at the same time of culturing ($p<0.05$).*

The accumulation of the restored form of AB indicates the activity of oxidation-reduction processes in cells, and its increase during cultivation is considered to be proportional to proliferative activity [17]. Consequently, MSCs within plasma-based carriers could actively proliferate during culturing, whereas within alginate microspheres – they did not. It is interesting that this arrest of MSC proliferation in alginate microspheres is reversible and can be restored by transferring of cells back to the monolayer culture [7].

4. Discussion

It is known that cell shape is directly coupled to cell division and gene expression patterns and hence cell fate can be altered by choice of adhesive substrate [18]. Our results indicate that the reversible stop of proliferation is caused by the low adhesive properties of alginate, which prevent cells from spreading and block division. In this context, the primary role is played not by the physical properties of the environment (hydrogel structure) but by the adhesive properties of the surface. Previously, we demonstrated that MSCs, seeded in macroporous alginate sponges with pore diameters allowing cell division and migration, also did not proliferate. However, chemically coupled gelatin to the surface of pores imparted adhesiveness, accompanied by cell proliferation [9].

In the current study, two types of carriers for MSCs were investigated: hydrogels and macroporous sponges. Hydrogels were composed of blood plasma or alginate. Within plasma-based macroporous scaffolds cells are located at the interface between the solid phase (pore walls) and the liquid phase (culture medium). In this 3D system, MSCs could adhere, spread, and proliferate, as evidenced by the accumulation of AB. In contrast, in hydrogels MSCs exist in a homogeneous environment an exhibit an altered behavior pattern. In the blood plasma-based hydrogel, MSCs proliferated at a rate roughly comparable to that observed within the macroporous scaffolds. Notably, the fact that AB fluorescence values after 1 day of culturing is lower in the hydrogel compared to the scaffold draws attention. It could be speculated, that on the solid adhesive surface of the scaffold pores, cells adhere more rapidly, spread, and manage to divide. In contrast, within the hydrogel composition, cells expend more time on cell adaptation and their division is postponed.

It should be noted, that alginate microspheres as a type of hydrogels have a number of advantages in terms of cell transplantation for regenerative medicine. Alginate microspheres reliably immobilize cells and can be of controlled sizes, making them suitable for precisely localized cell delivery to desired sites through injection. Additionally, a crucial benefit is that cells, encapsulated within alginate microspheres, exhibit prolonged viability in the recipient's body during transplantation, since the alginate gel serves as an immune isolator and protects cells from attack by the host's immune system [19]. From this it

follows that a great success will be the development of capsules that would support cell proliferation while maintaining a stable form and immune isolation properties. In an attempt to increase cell adhesion on alginate microspheres extracellular matrix proteins or protein fragments have been employed. The most commonly used molecules include collagen, gelatin (product of collagen hydrolysis), and arginylglycylaspartic acid (RGD) peptide, which is the functional adhesion sequence in several extracellular matrix proteins [20]. The results of this study suggest that a similar effect can be achieved by modifying alginate with components from blood plasma, which will be the subject of our further research. Solving this task will enable the rapid preparation of capsules in field conditions, possessing adhesive and immune-isolating properties, capable to support cell proliferation.

5. Conclusions

Properties of mesenchymal stromal cells, such as shape and proliferation ability, depend on the microenvironment in 3D culture.

When cultivated in 3D carriers based on blood plasma (hydrogel and macroporous scaffold), MSCs acquire a fibroblast-like morphology and can proliferate, while in alginate microcapsules cells remain spherical and stop division.

Modification of alginate microspheres forward to improve their adhesive properties is a promising approach for its application in regenerative medicine projects.

Conflict of interests

The authors declare that they have no conflict of interest regarding this research, including financial, personal, authorship or any other kind of conflict that could influence the research and its results, presented in this article.

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Data Availability

Data will be made available on reasonable request.

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