3D culture of murine adipose-derived multipotent mesenchymal stromal cells in hydrogel based on carbomer 974P

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ABSTRACT

Actual issues during tissue regeneration are to ensure the survival of transplanted cells at the site of their application and further activity, especially in case of local pathological alterations such as inflammation and ischemia. For this purpose, the matrices that can not only fill the defects of tissues, but also be scaffolds for cells are developed.

THE AIM *of this study was to evaluate the effectiveness of 3D cultivation of murine adipose-derived multipotent mesenchymal stromal cells (MSMCs) in hydrogel based on carbomer 974P.*

MATERIALS AND METHODS. *MSMCs were obtained from the adipose tissue of FVB-Cg-Tg(GFPU)5Nagy/J mice transgenic for GFP gene. The cells were phenotyped by flow cytometry and directly differentiated into osteogenic and adipogenic direction to confirm multipotent phenotype. MMSCs were cultured and directly differentiated into osteogenic direction in three-dimensional hydrogel scaffolds. For hydrogel preparation we used carbomer 974P with composition of glycerol, propylene glycol, triethylamine and agarose in original proportion.*

RESULTS. *The three-dimensional hydrogel based on carbomer 974P for the further engraftment with MMSCs was obtained. Modified protocols for the preparation of hydrogels based on carbomer and agarose and their rehydration by culture media for the 3D cultivation of adipose-derived MMSCs have been developed. The optimal concentration of MSMCs and the injection method for engraftment of hydrogels of the required form and size are selected. It was shown that adipose-derived MMSCs in 3D carbomer hydrogel preserve the potential of directed osteogenic differentiation.*

CONCLUSION. *Three-dimensional hydrogel based on carbomer 974P is capable to support cells, provide the necessary cytoarchitectonics, maintain intercellular interactions, which can promote further long-term survival and specialization of the graft.*

KEY WORDS: *adipose-derived multipotent mesenchymal stromal cells; 3-dimentional cell culture; hydrogel; carbomer 974P*

Nowadays cell and tissue technologies are the most promising method for treatment of various diseases instead of existing surgery approaches and drug therapy if well-known techniques are being demonstrated as less effective. Stem cell transplantation cannot only confer tissue regeneration; it can also facilitate endogenous repair process due to the host progenitor cells activation. However, the most important problem in tissue and organ reparation is a long-term engraftment of transplanted cells at target sites for further effective activity especially in case of pathological alterations of the injury site as an ischemia and inflammation [1].

The method of cell culturing is widely used in the basic research, biotechnology industry and in the clinics towards regenerative medicine. The majority of cell culture studies have been performed on 2D culture systems due to the simplicity of method and high cell viability [2]. The growth of cells as 2D monolayers has notably improved the understanding of cellular biology. However, almost of all cells in organism are surrounded by extracellular 3D matrix that provides complex biochemical and physical signals between cells of different types [3]. Thus, recently it has become more and more relevant to develop 3D cell culture systems

over the variety of matrixes. At present, more than 380 cell lines have been tested for their potential to form and maintain 3D spheroids [4].

It is shown *in vitro* that 3D spheroids of MMSCs produced higher levels of extracellular matrix proteins (including collagen I, fibronectin and laminin), compared with 2D cultured cells. Additionally, 3D culture increased the paracrine secretion of cytokines, including angiogenic factors (VEGF and bFGF), anti-apoptotic factors (epidermal growth factor and hepatocyte growth factor), the anti-oxidative factors (IGF, TNF-a) [5]. It is also known that 3D spheroids allow MMSCs to keep the multipotency, which does not occur in 2D cell culture [6]. The adoption of spherical shape could induce a rearrangement of cytoskeleton, potentially involved in regaining in earlier stages of potency via E-cadherin-mediated mechanism as well as influence on physical properties of cell stemness [7].

To modify a proper cytoarchitectonics in 3D systems, the next common characteristics should be considered: mechanical support, chemical compounds, cell grow stimulation and signaling, transportation of nutrients, gases and cell metabolic products [8]. There were a dozen types of scaffolds suggested by different biotechnological industries for the purpose of regenerative medicine over last years. The origin of platform, method of obtaining, physical and chemical properties, biological inertness, and ability to resorption are between advantages and disadvantages of matrix application. There are known more than 100 different matrixes both organic (collagen, gelatin, elastin, chitosan, chitin, fibrin, fibrinogen etc.) and non-organic origin [9, 10, 11]. Natural and plastic based materials are used as a background for 3D scaffolds [12]. Thus, such materials provide the proper extracellular matrix due to their structure (porosity, stringiness, permeability) and mechanical support. These scaffolds are appropriate for the mechanical support or biodegradation and can be used as stable transplants. 3D platforms provide biologically active environment and subsequent better biophysical and biochemical cell-cell interaction, proliferation, differentiation and extracellular matrix secretion [13].

Different types of hydrogel are applied as a basement for the 3D scaffolds in biomedicine. The hydrogels made from naturally occurred biopolymers (such as alginate and gelatin) are reliable materials due to their biocompatibility and potency to the biodegradation [14, 15]. Challenges in applying hydrogels for 3D tissue constructs are the control of cell material interaction and realization of inner space to allow cell ingrowth and communication. Furthermore, a challenge in constructing 3D hydrogel tissue scaffolds is the high total porosity (more 60 %), which influences on the cell seeding effectiveness [16, 17].

One of the promising options for creating a 3-dimensional matrix is hydrogels based on agarose and carbomer. Carbomer or Carbopol is a polyacrylicacid polymer, which shows a sol to gel transition in aqueous solution as the pH is raised above its pKa of about 5.5 [18]. Due to their properties, carbomer hydrogels realize the potential of scaffolds of natural origin, providing a 3D structure similar to living tissue, while remaining non-toxic and non-immunogenic [19]. On the other hand, due to the ability to regulate the physical and chemical parameters of these hydrogels, changing their proportions and modifications of the components, the necessary kinetics of degradation of the scaffold is achieved, which correlates with the integration of the graft into the recipient's tissues [20].

The aim of this study was to evaluate the effectiveness of 3D cultivation of murine adipose-derived multipotent mesenchymal stromal cells in hydrogel based on carbomer 974P.

MATERIALS AND METHODS

In our study we used FVB-C-Tg (GFPU) 5Nagy/J mice transgenic for green fluorescent protein (GFP) kindly provided by European Molecular Biology Laboratory, Monterotondo, Italy. The animals were kept under standard conditions at the vivarium of State Institute of Genetic and Regenerative Medicine with free access to food and water ad libitum. All experiments were performed in compliance with international principles of the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes (European convention, Strasburg, 1986), Article 26 of the Law of Ukraine «On protection of animals from cruelty» (No. 3447-IV, 21.02.2006) and all norms of bioethics and biosafety.

ISOLATION OF ADIPOSE-DERIVED MMSCs.

The 4-month-old female mice (n = 3) were euthanized by cervical dislocation under ether anesthesia. Under sterile conditions inguinal adipose tissue was incised, minced with scissors into fragments 1x1 mm then treated with 0.1 % collagenase type I in DMEM medium (*Sigma*, USA) for 90 min at 37 °C on shaker. After washing and filtration trough 70 µm strainer, cell suspension was cultured in a 25 cm^2 flask in complete DMEM culture medium supplemented with 15 % fetal bovine serum – FBS (*HyClon*, USA), penicillin 100 U/ml, streptomycin 100 µg/ml, 1:100 nonessential amino acids (all – *Sigma-Aldrich*, USA) in a CO_2-incubator (37 °C, 5 % CO_2, humidified atmosphere). When culture reached 80 % confluency, cells were subcultured using 0.25 % trypsin. Cells of 2 passages were used for hydrogel engraftment.

The phenotyping of cell cultures for CD44, CD73, CD90, CD45, CD117, and CD34 markers was performed using FACSAria cell sorter (*Becton Dickinson*, USA). Samples of 2•10^5 cells in 50 µl medium were incubated with fluorochrome-conjugated monoclonal antibodies anti-CD44-PE, anti-CD73-PE, anti-CD90-PE, anti-CD45-PE, anti-CD117-PE-Cy7, and anti-CD34-APC (BD Bioscience, USA) in a working concentration of 0.5 µg/ml for 30 min then were washed in CellWash (*BD Bioscience*, USA) by centrifugation at 300xg for 5 min. At least 2•10^4 cells per sample were recorded for analysis using BD FACSDiva 6.2.1 software. To compensate fluorochrome overlap for multiparameter analysis the control samples of cells without antibodies (unstained control), samples with each antibody alone (single stained control) and samples with a combination of several antibodies without one (fluorescence minus one – FMO control) were used [21]. Cell viability was determined using 7-AAD staining (*BD Bioscience*, USA).

DIRECTED DIFFERENTIATION OF ADIPOSE-DERIVED MMSCs

Osteogenic cell differentiation was induced by modified methods Li et al. [22]. At the second passage, culture medium was changed to medium for osteogenic differentiation which consisted of DMEM-F12 medium (*Sigma-Aldrich*, USA) supplemented with 10 % FBS (*HyClon*, USA), and contained 0.05 mM L-ascorbic acid 2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate (all – *Sigma-Aldrich*, USA). Osteogenic medium was changed every 3 days. After 21 days under standard conditions in a CO_2 incubator cell monolayer was washed with phosphate buffered saline (PBS), fixed with cold 4 % paraformaldehyde for 25-30 min than stained with Alizarin Red S (*Sigma-Aldrich*, USA) to detect calcium phosphate salts deposit in extracellular matrix which is one of the signs of osteogenic differentiation. In addition, the production of alkaline phosphatase was detected by BCIP/NBT staining (*Sigma-Aldrich*, USA) for 20-30 min in the dark. After that cells were washed with distilled water and intensity of staining was analyzed [23].

The adipogenic differentiation was induced in DMEM-HG medium (*Sigma-Aldrich*, USA) supplemented with 10 % FBS (*HyClon*, USA) containing 1 µM dexamethasone, 200 µM indomethacin, 500 µM isobutylmethylxanthine and 5 µg/mL insulin (all – *Sigma-Aldrich*, USA) [24]. The adipogenic medium was changed every 3 days for 14 days in culture. Cells were fixed in cold 4 % paraformaldehyde and stained with Oil Red O (*Sigma-Aldrich*, USA) to detect lipid droplets in the cytoplasm.

HYDROGEL PREPARATION AND CELL CULTURE IN HYDROGELS

For hydrogel preparation we used carbomer 974P combined with glycerol, propylene glycol, triethylamine and agarose in original proportion. Gel was heated to 83-85 °C and placed in plastic forms of necessary sizes (10 mm diameter, 5 mm high) with subsequent cooling to room temperature. Prepared scaffolds were lyophilized and sterilized under UV in a biosafety cabinet.

The hydrogels were rehydrated in complete culture medium, than MMSCs were seeded using pipette tip (n = 6) or injected by insulin syringe (n = 10) all over the surface of the scaffold at $4 \cdot 10^4$, $2 \cdot 10^5$ and $1 \cdot 10^6$ cells per each hydrogel. Cultivations were performed under standard conditions in a DMEM medium supplemented with 10 % FBS. The culture medium was replaced every 3-4 days.

DIRECTED OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED MMSCs IN CARBOMER HYDROGEL

On the 14th day of cultivation of hydrogels engrafted with $1 \cdot 10^6$ cells (n = 4), the complete culture medium was changed with an appropriate medium for osteogenic induction. Cells were cultured under osteogenic conditions for 14 days, the medium was changed every 3-4 days.

To evaluate the effectiveness of osteogenic induction, control and experimental samples of hydrogels were fixed in a 4 % solution of paraformaldehyde in 0.1 M phosphate buffered saline for 48 hours at +4 °C and stained with Alizarin Red S to detect calcium salts. The production of alkaline phosphatase was detected by BCIP/NBT staining.

Monolayer cultures of cells and engrafted hydrogels were examined using an inverted fluorescence microscope IX-71 (*Olympus*, Japan) with a camera DP-20 (Olympus, Japan) using a QuickPHOTO software (*Promicra, s.r.o.*, Czech Republic).
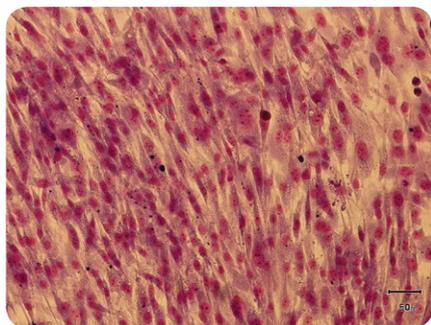

Fig. 1. Microphotograph of murine adipose-derived stromal cells culture, 2nd passage, light microscopy, azure-eosin staining, scale bar – 50 μm.

STATISTICAL ANALYSIS.

Statistical analysis was performed using Statistica v.5 software (StatSoft Inc., USA). The results are presented as mean ± standard error of mean (SEM).

RESULTS AND DISCUSSION

To confirm the proper characteristics of the adipose-derived MMSCs that were used for engraftment to the hydrogel, the obtained cultures were analyzed by morphology, immunophenotype and potential for directed differentiation into osteogenic and adipogenic direction [25].

In primary cultures, growth of spindle-shaped or polygonal cells with severe processes was observed. Further subcultures contributed to the formation of a more homogeneous population of fibroblast-like adhesive cells, which in a short time (2-3 days) doubled the population and formed a confluent monolayer. In some cases, growth was observed by increasing the cell colony size. Cell cytoplasm in this case was homogeneous, nuclei and nucleoli were clearly visualized (**Fig. 1**).

At the phenotyping of adipose-derived MMSCs from the initial to the second passages, there was an increase in the expression of the mesenchymal markers CD44 (up to 97.1 ± 2.2 %), CD90 (up to 95.9 ± 1.8 %) and CD73 (up to 64.3 ± 14,6 %) had been noted, which corresponds to the typical MMSC phenotype (**Fig. 2**). At the same time, low expression (<3 %) of hematopoietic markers CD45 (2.1 ± 0.8 %) and CD117 (2.2 ± 0.7 %) was observed. Expression of CD34 remained at 12 ± 4 % for the second passage. As it is generally known, the relatively high level of CD34 expression at the early passages is typical for the culture of adipose-derived MMSCs and may indicate a high potential of these cells for differentiation in the endothelial direction [26]. This feature of adipose-derived multipotent cells can contribute to activation of neoangiogenesis and better vascularization of grafts to ensure their survival. Cell viability after subculture from monolayer into a suspension for further 3D culture determined by 7-AAD was 92.4 %.
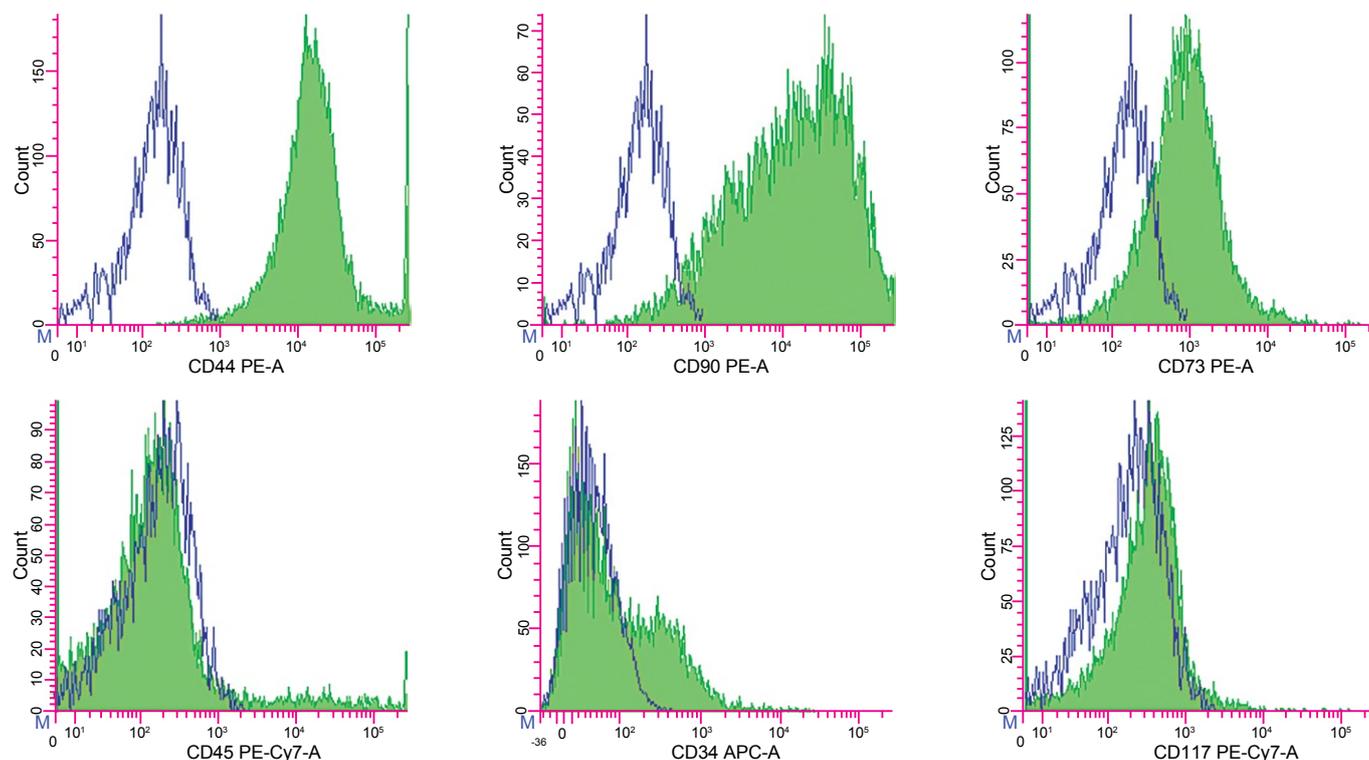

Fig. 2. Histograms of the expression of CD44, CD70, CD90, CD45, CD34 and CD117 markers in the culture of murine adipose-derived MSMC (green) compared to unstained controls (blue) according to flow cytometry, 2nd passage, BD FACSDiva software.
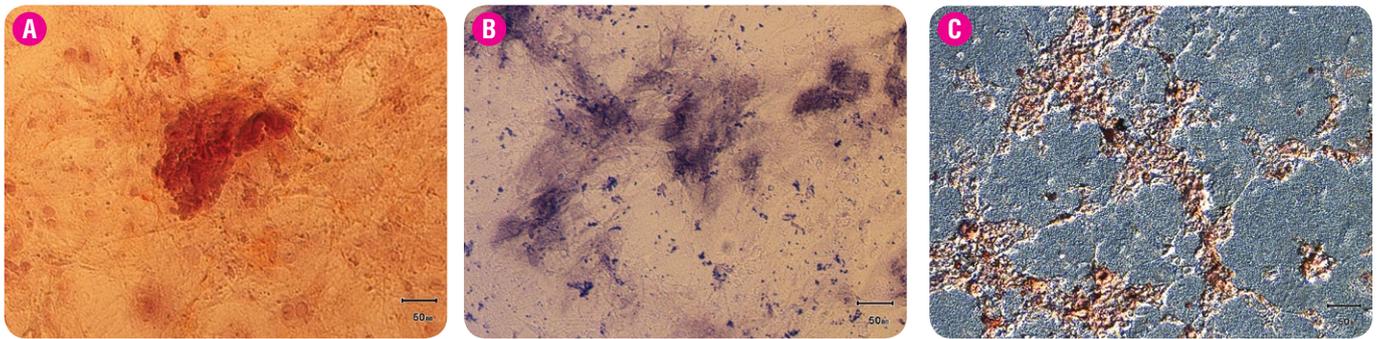
Fig. 3. Microphotographs of murine adipose-derived MMSCs, differentiated into the osteogenic direction (A-B), 21-day culture, staining with Alizarin Red S (**A**) and BCIP-NBT (**B**); and differentiated into the adipogenic direction (**C**), the 14th day of cultivation, staining with Oil Red O, scale bar – 50 μm.
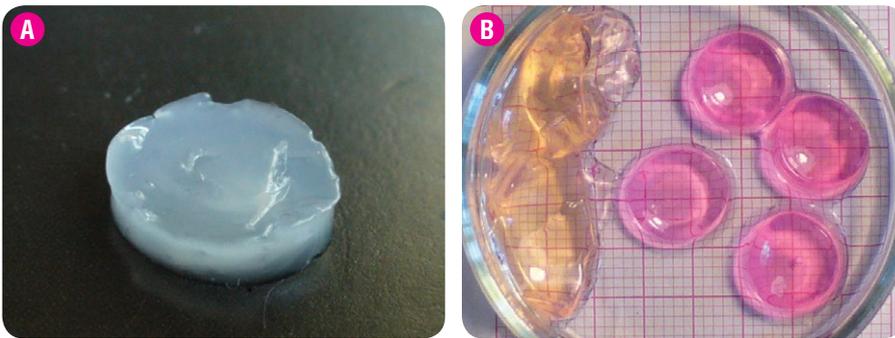

Fig. 4. Photo of hydrogels based on carbomer 974P and agarose: **A** – native hydrogel, **B** – hydrogels rehydrated with DMEM media.

The multipotent properties of the obtained murine adipose-derived MMSCs according to the ability to direct differentiation using histochemical staining for calcium salt deposits and alkaline phosphatase production at osteogenic induction or for lipid inclusions – at adipogenic differentiation were confirmed. On the 21st day of osteogenic induction, in the adipose-derived MMSCs culture an intensive mineralization of the extracellular matrix with calcium salts was observed which was detected by staining with Alizarin Red S (**Fig. 3A**), as well as high activity of alkaline phosphatase production, which was detected by BCIP-NBT staining (**Fig. 3B**). At the directed adipogenic differentiation after 14 days of cultivation in the appropriate medium, the presence of lipid droplets in the cytoplasm of the cells was detected (**Fig. 3C**).

Thus, according to morphological characteristics, immunophenotype and potential for directed differentiation, the obtained cultures of adipose-derived cells that were used in subsequent experiments, met the minimum criteria for MMSCs provided by International Society for Cellular Therapy [25].

For three-dimensional cell culture, hydrogels based on agarose and carbomer 974P were made. Using the original proportions of the initial components and modifying the time and temperature of their processing, it is possible to achieve a certain plasticity of the material to provide it with the necessary form (**Fig. 4A**) and elastic properties with further rehydration of the nutrient medium (**Fig. 4B**).

We obtained 3D cultures of murine adipose-derived MMSCs engrafted in hydrogel rehydrated with nutrient medium. It was shown that in the second week when 4•10⁴ cells were seeded to a cylindrical hydrogel with a diameter of 10 mm (volume ~400 mm³), only single cells penetrated to a depth of 1.5 mm from the surface of the hydrogel and acquired an elongated shape. Most cells retained the rounded form and were on the surface of the hydrogel. When seeding by pipette tip of 10⁶ cells to a hydrogel in the early stages of cultivation (up to 10 days), the cells were located on its surface and kept round. During the next 5-7 days, the cells penetrated the gel and acquired an elongated shape, contacting with each other. After 7-14 days after penetration, the number of intercellular contacts increased and they formed clusters and a network of adjacent cells, indicating their proliferation (**Fig. 5**).

An injection method for engraftment of MMSCs into the carbomer hydrogel using a syringe was proposed and the dependence of its efficiency on the initial number of cells was analyzed. When we injected 4•10⁴ or 2•10⁵ cells into a hydrogel at a depth of 1-2 mm, after 2 weeks in culture, the cells were located diffusively in the hydrogel, they acquired an elongated shape but did not contact with each other. When we injected 10⁶ cells into a hydrogel at a depth of 2-3 mm after 2 weeks of cultivation, the cells acquired polygonal form due to the numerous processes they contacted each other, forming a network of adjacent cells (**Fig. 6**).

Thus, a required concentration of cells and an optimal cell engraftment method for hydrogels of certain form and size for further *in vitro* studies were selected.

The method for directed osteogenic differentiation of adipose-derived MMSCs in carbomer hydrogel to assess their ability to multipotency under conditions of three-dimensional scaffold cultures have been developed. When hydrogels with cells in an osteogenic medium on the 14th day were stained with Alizarin Red S and BCIP-NBT dyes, a positive reaction on the calcium salt and alkaline phosphatase, respectively, on the periphery of the hydrogel and inside where cells localized was found (**Fig. 7**).

The obtained results indicate that adipose-derived multipotent mesenchymal stromal cells preserve the potential for directed differentiation under 3D culture in carbomer hydrogel, typical for two-dimensional monolayer cultures.

It is considered that regenerative potential of stem cells is realized with local cells environment (their niche), which provides direct cell-cell interaction and non-contact communications with the action of secreted signaling molecules of different type of cells [27]. The engineering of the stem cell microenvironment plays a crucial role in regenerative medicine and is considered as an approach to improve the regenerative potential of transplantation. [28]. For this purpose, culture matrixes are being developed. It is providing cells with the necessary tissue structure and reconstruction of the niche *in vitro*. [29]. The urgent task is to establish the influence of conditions of three-dimensional cultivation on the efficiency of survival, proliferation and potential for directed differentiation of multipotent stem cells of different origin.

Fig. 5. Microphotographs of adipose-derived MMSCs in a carbomer hydrogel after 10^6 cells seeding on hydrogel surface: **A** – 8th day of cultivation; **B** – 18th day of cultivation; **C** – 29th day of cultivation, scale bar – 50 µm.
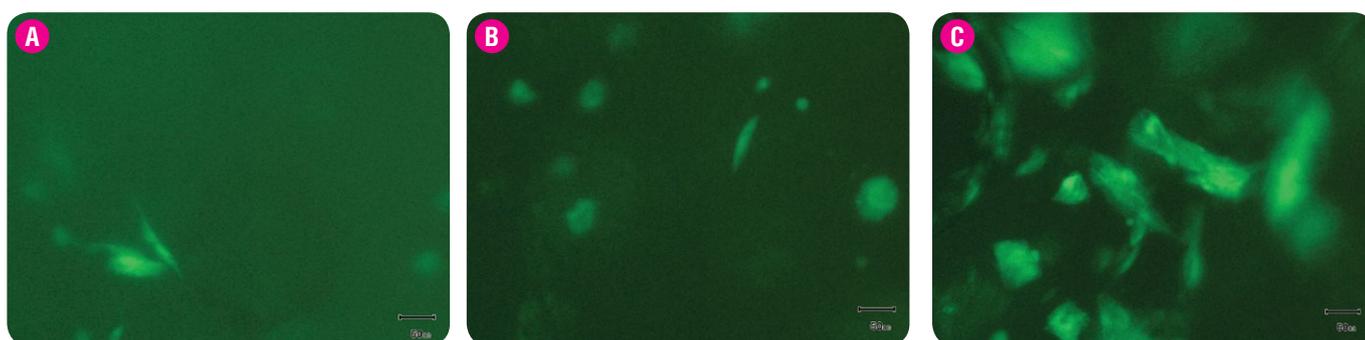

Fig. 6. Microphotographs of adipose-derived MMSCs in a carbomer hydrogel after cell engraftment into hydrogel by microinjection, 14th day of cultivation, fluorescence microscopy: **A** – initial dose of $4 \cdot 10^4$ cells; **B** – $2 \cdot 10^5$ cells; **C** – 10^6 cells, scale bar – 50 µm.
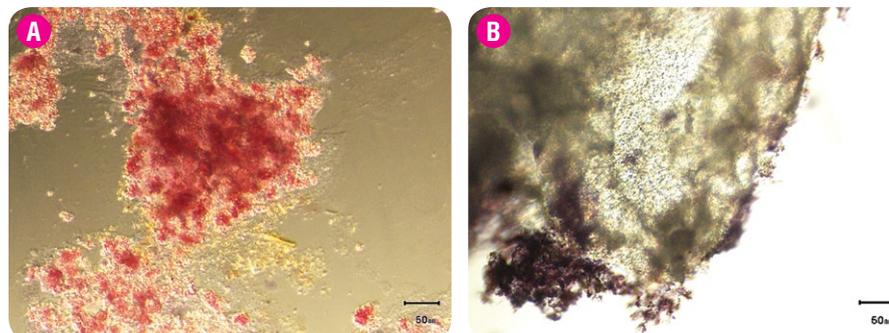

Fig. 7. Microphotographs of carbomer hydrogels fragments engrafted with murine adipose-derived MMSCs differentiated into osteogenic direction. Alizarin Red S staining for calcium phosphate salts (**A**, red) and staining with BCIP-NBT for alkaline phosphatase (**B**, brown), scale bar – 50 µm.

In addition to form-forming and supporting functions, matrixes can also regulate growth factors releasing during different time points providing not only cell proliferation but their direct differentiation *in vivo* as well [30, 31]. It is shown that cell-cell interaction with extracellular basement of biomaterial and growth factors provide stem cell differentiation and tissue growth [32, 33]. For example, bone marrow microenvironment factors (such as VEGF, TGF-β3, BMP-2) increase the survival of adipose-derived stem cells in the recipient organism [34, 35]. In our previous studies for multipotent stromal cells from subcutaneous adipose tissue, the possibility of a stage-by-stage osteogenic differentiation with prior chondrogenic induction was demonstrated, which partially reproduces *in vitro* conditions of niche for the laying and formation of bone in the body [36]. The combination of technologies for the creation of 3D hydrogel scaffolds and the directed differentiation of the engrafted cells opens wide prospects for regenerative medicine. Step-by-step, directed differentiation of multipotent cells *in vitro* in three-dimensional constructions will allow partially reconstruct the physiological mechanisms of tissue formation *in vivo* and obtain a spatially organized cell culture capable of surviving, proliferating and differentiating in a required direction.

Fast and reliable cells seeded over 3D scaffolds require development of new biotechnological approaches using three-dimensional culture model with subsequent evaluation of its safety and effectiveness, which is demonstrated in our study. The dynamic perfusion of transplants over bioreactor promotes 3D-matrix cell seeding and their directed induction. It is allowed to standardize the protocols of cell culture as well [37]. That is why further studies are needed to include in the composition of carbomer hydrogels different growth factors or cytokines responsible for chemotaxis, proliferation and tissue-specific differentiation of transplanted or endogenous stem cells.

Formed 3D scaffolds based on carbomer hydrogels can be used as transplants to study recovery processes at tissue injury in an *in vivo* experiments [19]. In general, the practical implications of this study will be the development of new biotechnological approaches for increasing effectiveness of adipose-derived MMSCs as an alternative source of adult stem cells in the injured ischemic tissue regeneration.

A dozen of the clinical application of matrixes within variety types of cells has been described in cases of bone, cartilage, intervertebral disk damages and skin injures. Furthermore, clinical trials were suggested to assess matrix efficacy and safety. These approaches are of the particular interest especially in reconstructive surgery in case of regeneration of soft tissues damages and wound healing [38, 39, 40].

CONCLUSION

Thus, three-dimensional hydrogels based on carbomer 974P are capable to mechanical support of cells growth providing the necessary cytoarchitectonics through the formation of intercellular contacts, and preserve the potential of multipotent cells for directed differentiation.

Improving the technology of cells engraftment in three-dimensional scaffolds based on the carbomer 974P will allow modeling their spatial organization and maintaining cell-cell interactions that can ultimately promote to the long-term survival and specialization of grafts for more effective repair of damaged tissues and organs.

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