AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1214-1224
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 619+616.5]:57.085.23

doi: 10.15389/agrobiology.2019.6.1214eng
doi: 10.15389/agrobiology.2019.6.1214rus

MAINTENANCE OF MULTIPOTENT MESENCHYMAL STEM CELLS OF FARM ANIMALS IN CRYOGELS BASED ON NATURALLY-DERIVED POLYMERS

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The authors declare no conflict of interests
Acknowledgements:
This work was performed as part of research work No. 0578-2018-0006 "Creation of new cell systems with desired properties based on mammalian stem cells, including farm animals for veterinary medicine, virology and biotechnology".
Received August 2, 2019

Abstract

Multipotent mesenchymal stem cells (MMSCs) of farm animals, whose growth in culture is determined by attachment to a solid substrate, are promising cellular material for veterinary medicine and biotechnology, as well as virology. One of the methods to overcome cell adhesion in suspension bioreactors in order to obtain a large number of cells with permanent properties of acceptable quality is the use of porous carriers formed from polymers of natural origin. Thus, for the first time we obtained data that allow us to make a scientific substantiation of the parameters for the cultivation of adhesive cultures of animal MMSCs using spatial protein-based cryogel carriers for subsequent suspension cultivation of the obtained constructs. The purpose of the work is to study the possibility to culture MMSC of farm animals in three-dimensional matrix sponges, i.e. the cryogels based on gelatin, blood plasma total protein and fetal bovine serum (FBS). MMSCs isolated from bovine bone marrow (BM) and adipose tissue (AT) and ovine BM, as well as mouse fibroblast STO cell line were used. We found that the optimal cell concentration for the settlement of the cell suspension by the method of natural absorption with swelling of squeezed sponges (0.24 cm^3 in volume) is 1.0×10^6 cells per 100 μl of medium for 2 hrs of saturation. The loading efficiency of MMSCs in sponge scaffolds is 98 %. The analysis of histological slices (at least 10 per sample) of three cryogels demonstrated the ability of all three-dimensional porous scaffolds to maintain cell culture for 14 days. Sponges were filled with cells that preserved morphology and proliferated in places of attachment to the polymer surface. The results of experiments on the effect of the matrix material on cell migration showed that all cells migrate from the monolayer in the volume of cryogel from the bottom and are not detected on the upper sides of the cryogels under study. On day 10 of culture, fibroblast STO cell line were detected in the volume of sponge scaffolds based on gelatin, blood plasma and FBS protein at a distance of 2990, 2871 and 1930 μm, respectively. MMSCs isolated from bovine AT migrated into the porous structure of matrix sponges to a depth of 607, 1364 and 657 μm, respectively. Expansion of MMSCs isolated from bovine and ovine BM in cryogels on the basis of different materials did not differ significantly from the migration of AT-MMSCs. The ability of farm animals' MMSCs on the early passages (2 to 3) and late passages (9 to 10) to attach to macroporous cryogels was not significantly different. Comparative analysis of the results of the experiments obtained in three replicates showed that the macroporous matrices based on gelatin, bovine blood plasma proteins and FBS support the viability of MMSCs during short-term culture, promote cellular adhesion, proliferation and migration. The obtained data allow us to predict the use of these cryogels as matrices for MMSCs of farm animals for research and practical use.

Keywords: multipotent mesenchymal stem cells, adipose tissue, bone marrow, farm animals, adhesion, migration, viability, cryogels, gelatin, bovine protein of blood plasma and serum-

derived scaffolds

The multipotent mesenchymal stem cells (MMSCs) capable of self-renewal and having the potential for adipogenic, osteogenic and chondrogenic differentiation in vitro are promising for veterinary medicine, cell and tissue engineering, virology and drug screening [1, 2]. The fundamental criterion for using the MMSCs' potential is reproducible and low-cost production of a sufficient amount of cells of consistently high quality. Common way of MMSCs production is adhesive culture in the presence of serum. However, current adhesive methods cannot ensure a suitable culture because of the dissimilar conditions that leads to changing the cells quality between batches. Besides, the methodology is laborious and cell yield is limited by substrate area for their growing that leads to limitations in scalability [3-5]. Microcarriers used in suspension bioreactors allow cell adhesion problem to be overcome [6-8]. Such systems need the improvement of the matrixes and adaptation of the cells culture parameters [9].

Porous gels are a promising substrate for matrixes in cell culture owing to their ability to imitate the main properties of most soft tissues. The cross-linked polymer chains detain a large amount of water, they facilitate the transportation of oxygen, nutrients, metabolites and soluble factors. Many of gels can be formed under conditions favorable for living cells, and can easily be modified for bestowing the desired physical and mechanical properties and the rate of degradation. Using natural biopolymers in bioengineered scaffolds makes it possible to most exactly imitate the structure, properties of tissues and organs, as well as to reproduce the microenvironment with the structure similar to that of natural cell niches. This provides optimal artificial niches for MMSCs population and contributes to their almost complete differentiation into the desired cell types [10].

In bioengineered structures, the pores inside the three-dimensional carrier must be interconnected, have the dimensions optimal for cells and sufficient area for their growth. The macroporous cryogels, formed in slightly frozen medium, meet these requirements [11]. They have some specific features compared to ordinary gels formed at the temperatures above the crystallization point of the solvent. Cryogels are characterized by macroporosity, and their macropores are interconnected [12, 13]. Depending on the initial concentration of components, their properties and regimes of cryogenic treatment, it is possible to obtain the macroporous matrixes with the pores with the cross section from tenths to 10 μm and supermacroporous (sponge) systems with the pores of tens and hundreds of micrometers. The cryogels based on materials of natural origin, which are the components of the extracellular matrix (ECM), for example, collagen, gelatin, etc., are of particular interest [14-17].

In a series of experiments, it was demonstrated that the protein-based cryogels (serum albumin, total blood serum protein) owing to their macroporous morphology can be used as porous substrates for cells cultivation [18, 19]. The main criteria of a biocompatible matrix must be the absence of cytotoxicity, maintenance of adhesion, migration, proliferation or the differentiation of the cells on its surface [20-22], as well as the mechanical strength and bioresorbability optimal for further use [23].

In this report, for the first time we present the data substantiating parameters of animal MMSCs adhesive cultures with bulky protein-based cryogel carriers for suspension culturing the obtained structures in a bioreactor.

Our objective was to assess suitability of cryogels based on gelatin, total blood plasma protein and blood serum of cattle fetuses culture as three-dimensional sponge matrixes for cell culture of mesenchymal stem cells of farm

animals.

Techniques. The experiments were carried out (2016-2019) with MMSCs from cattle bone marrow (BM) and adipose tissue (AT) isolated and characterized by us previously [24], from sheep BM [25], and with mouse fibroblasts of the STO line have been used.

For cells culture, the materials and reagents produced by the PanEco company (Russia) were used. The MMSCs were cultured in DMEM with the low glucose concentration (1 g/l), 10% fetal bovine serum (FBS) (GE Healthcare, USA) and 1× nonessential amino acids and antibiotics. The fibroblasts of the STO line were cultured in DMEM with 4 g/l glucose, 10% FBS, 2 mM L-glutamine; 50 μg/ml streptomycin, 50 U/ml penicillin (final concentrations). The cells were cultured at 37 °C in humidified atmosphere with 5% CO_2. For the long-time culture the MMSCs (5×10^3 cells/cm^2) were subcultured.

The porous matrixes for cells culturing were gelatin-based cryogels produced as previously published [26], and cryogels based on cattle total plasma protein or blood serum protein [27, 28].

Matrix sterilization procedure was as follows. The samples were placed in the wells of the sterile 24-well plate (Nunc, Denmark) and covered with ethyl alcohol for 1 hour. The alcohol was decanted, and the samples were washed three times with the Hanks' solution by adding 2 ml into each well. Then 1.5 ml of DMEM was added into wells and the plate was allowed overnight in an incubator for the complete removal of the ethyl alcohol. Before adding the cells, the cryogel was squeezed out with tweezers, and 100 μl of the cell suspension containing 1×10^6 cells was applied to the upper surface of the squeezed out matrix. Due to the rapid swelling of the sponge material, the cell suspension was pulled by capillary forces into the pore inner space and filled the carrier. The sponges were saturated with the cells for 2 hours in the Petri dish preventing the cells adhesion to the plastic at 37 °C in the CO_2 incubator, followed by transfer into 50 ml tubes with untreated surface and gas-tight covers, which simulated a minibioreactor.

Cattle and sheep MMSCs immobilization on the polymer matrixe pore surface was assessed by counting of the non-adsorbed cells. The efficiency of cell seeding was calculated as the difference from subtraction (cell number in the initial suspension minus cell number after immobilization) divided by the initial cell number.

The express analysis of the localization of the viable cells in the three-dimensional matrixes цфы performed using the fluorescein diacetate stain (FD) (Thermo Fisher Scientific, USA). FD was added to the culture medium at a final concentration of 25 μg/ml and incubated for 5 min at 37 °C. Then the sponges with cells were washed with the serum-free medium and examined (a fluorescence microscope, Carl Zeiss, Germany) at the fluorescence excitation wavelength of $\lambda = 450$ nm with closing filter G 247.

For studying cells migration ability, the MMSCs and STOs were seeded into the 24-well plates and grown up to a monolayer. The square 10×10 mm pieces of the cryogel cut out aseptically and washed with DMEM were placed on the surface of the cells monolayer with slightly pressing and cultured under the standard CO_2 conditions in the incubator for 10 days. Then the cryogels were washed with the Dulbecco's phosphate-buffered saline with the Ca^{2+} and Mg^{2+} ions (PBS-1) and fixed in the solution of 4% paraformaldehyde in PBS-1. At least 10 areas from the lower and upper sides of the cryogel, as well as the cross sections of the investigated matrixes were analyzed.

The cell analysis in the three-dimensional matrixes was performed using standard techniques for histological studies [29]. To prepare sections (a Mikrom

HM 525 cryotome, Thermo Scientific, Germany; fixation on the cryotome specimen stage with Neg-50 mounting medium, Thermo Fisher Scientific, USA), specimens were frozen at –15...–22 °C for 30-40 minutes, and 10-20 μm slices were made with one-off blades Microm Sec35e (Thermo Fisher Scientific, USA). The slices were mounted on Surgipath X-tra Adhesive slides with the adhesive coating (Leica Biosystems, Germany). Immediately after drying, they were fixed with methyl alcohol, washed and stained with hematoxylin and eosin or Giemsa stain (PanEco, Russia) according to the manufacturer's instructions. After the clarification in xylene, the stained preparations were embedded into the mounting medium. The morphological analysis was performed visually using the Axio Observer D.1 phase-contrast microscope (Carl Zeiss, Germany) with AxioVision Rel. 4.8 software (Carl Zeiss, Germany) for measurements.

The statistical processing was performed using the GraphPad Prism software (GraphPad Software, USA). The tables show the mean arithmetic values (*M*) and their standard errors (±SEM). The significance of the differences was assessed according to the Student's *t*-test at $p < 0.05$.

Results. The macroporous cryogels (sponges) based on the A type commercial gelatin were white-transparent 0.2-0.7 cm thick round disks 2.2-3.5 cm in a diameter (see Fig. 1, A) with the pore sizes from 50 to 100 microns. The cryogels from bovine blood plasma total protein and FBS had the similar cylindrical shape with the diameter of 1 cm and thickness of 0.6-1 cm (see Fig. 1, B, C).

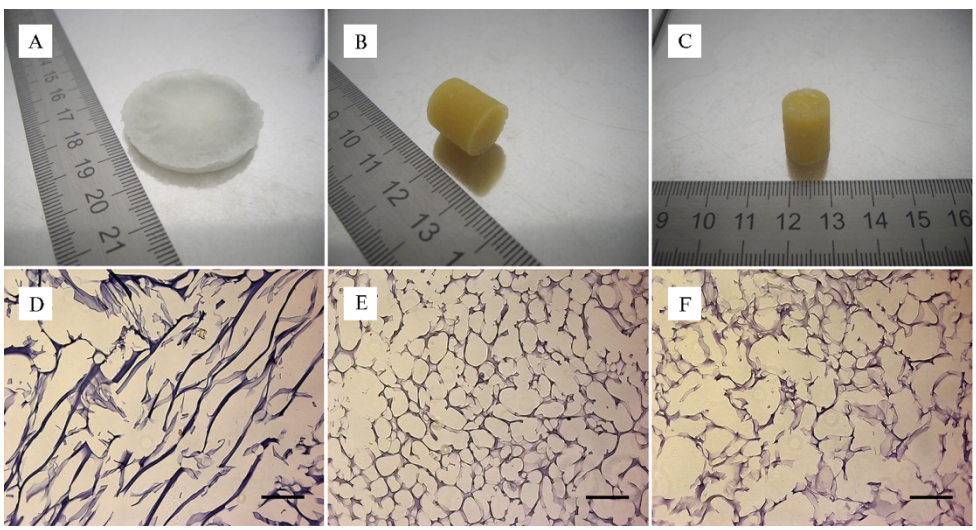

Fig. 1. Macro- and microstructure of the spongy cryogels based on gelatin (A, D)**, proteins of bovine blood plasma** (B, E) **and serum of the blood of cows' fetuses** (C, F). Giemsa staining, scale 500 μm (Axio Observer D.1, Carl Zeiss, Germany).

Cryogels have a system of branched interconnected pores ranging from 20 to 200 μm, which are resulted from formation of ice crystals in a frozen medium [11]. In our work, we found the 10 μm thick slices obtained at –20...–22 °C to be more suitable for histological analysis of cell distribution in cryogels. If a sponge has the greater porosity, the thickness of 20 microns is suitable for better visualization.

Histological analysis showed a macroporous structure of the matrixes, their strength and long-time stability during incubation in CO_2 (see Fig. 1, D-F). The gelatin-based sponges had interconnected large pores, 80-130 microns. The distinctive feature of such matrixes was the gradient changing of a pore size from the periphery toward the interior part as the result of the formation of ice

crystals while freezing of the initial solution of the biopolymer. The matrixes based on the total protein of bovine plasma and of FBS also had a macroporous structure with pores 70-170 microns in a diameter.

Within 24 hours after seeding MMSCs, the protein substrate had no cytotoxic effect on the cells. Our observations are consistent with the data obtained by other authors [13, 28, 30].

1. Efficiency of seeding multipotent mesenchymal stem cells into the gelatin cryogels (0.24 cm^3 matrix, $^1/_4$ part of the cryogel)

Cell counts	Growth medium, μl	Seeding efficiency, %
5×10^5	100	87
5×10^5	500	76
1×10^6	100	98
1×10^6	500	86

Experimental estimates of proper cells concentration and volume of the medium for seeding cells into cryogels have been made using the gelatin matrixes as an example (Table 1). The medium volume of 100 μl turned out to be optimal for saturation of gelatin matrixes. The optimum cells concentration for seeding by natural swelling of squeezed out 0.24 cm^3 sponges was 1.0×10^6 cells/100 μl of the medium during 2-hour saturation. The efficiency of MMSCs seeding into the matrix was 98%. The increase of the medium volume resulted in the loss of cellular material, probably due to an excess amount of the liquid phase. Previously, the dynamic (perfusion) seeding method was used for gelatin cryogels with high efficiency [30].

Cell seeding was performed with a special device which consists of two vessels connected by the flexible plastic tube. The porous carrier was placed into one of the vessels, wherein the porous matrix diameter matched the vessel's inner diameter, and about 100-200 μl of the cell suspension (1.3×10^6 cells/ml) was placed into the second vessel, after that the matrix was slowly saturated with the cells containing the growth medium by force of the gentle back-and-forth motion of the pistons in cylinders. The saturated matrix was allowed for 3 hours in the vessel placed into the CO_2 incubator, thereafter it was moved into the plate wells containing about 1 ml of the growth medium. To seed the cryogels with the cells, the technique of the sponge squeezing after the sterilization was applied. After the squeezing out for the removal of the medium residuals, the carriers quickly and easily restored their shape due to the elasticity of their material, while absorbing the cell suspension applied on their upper surface. Such method of loading the cells into the macroporous spongy matrixes by its efficiency was not inferior than the perfusion method and also was simple in performance.

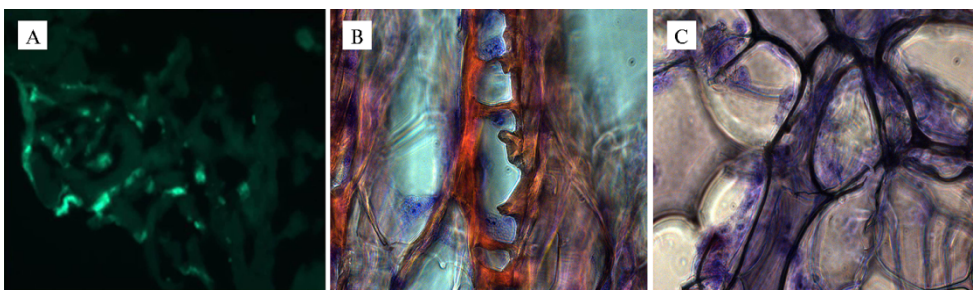

Fig. 2. Large-porous matrixes derived from gelatin (A, B) **and bovine plasma proteins** (C) **with multipotent mesenchymal stem cells from cattle bone marrow attached to the macropore walls.** MMSCs are stained with the fluorescein diacetate (A) and with the Giemsa stain (B, C) (Axio Observer D.1, Carl Zeiss, Germany, zoom ×200 (A) and ×400 (B, C).

The cell viability inside the spongy matrixed in dynamics, on day 7 and day 14 of culture has been studied. The results of the analysis of histological

staining of the slices (at least 10 for each sample) demonstrated the ability of three-dimensional porous matrix to maintain cells in culture for 14 days. These data were confirmed by staining cells with FDA (fluorescein diacetate), a hydrophobic non-fluorescent compound easily penetrating through the cell membrane into cells, where it is metabolized by cell esterases to fluorescein stain. The FDA having the green fluorescence appears only in the cytoplasm of the viable cells which have an intact cytoplasmic membrane, since FDA cannot penetrate through damaged cell membranes.

The histological comparison of slices of three cryogels showed the suitability of both gelatin matrixes and the matrixes based on total protein of bovine blood and of FBS for culturing MMSCs of farm animals. The sponges were filled with the cells which retained their morphology and proligerated at the places of their attachment to the macropore walls (Fig. 2).

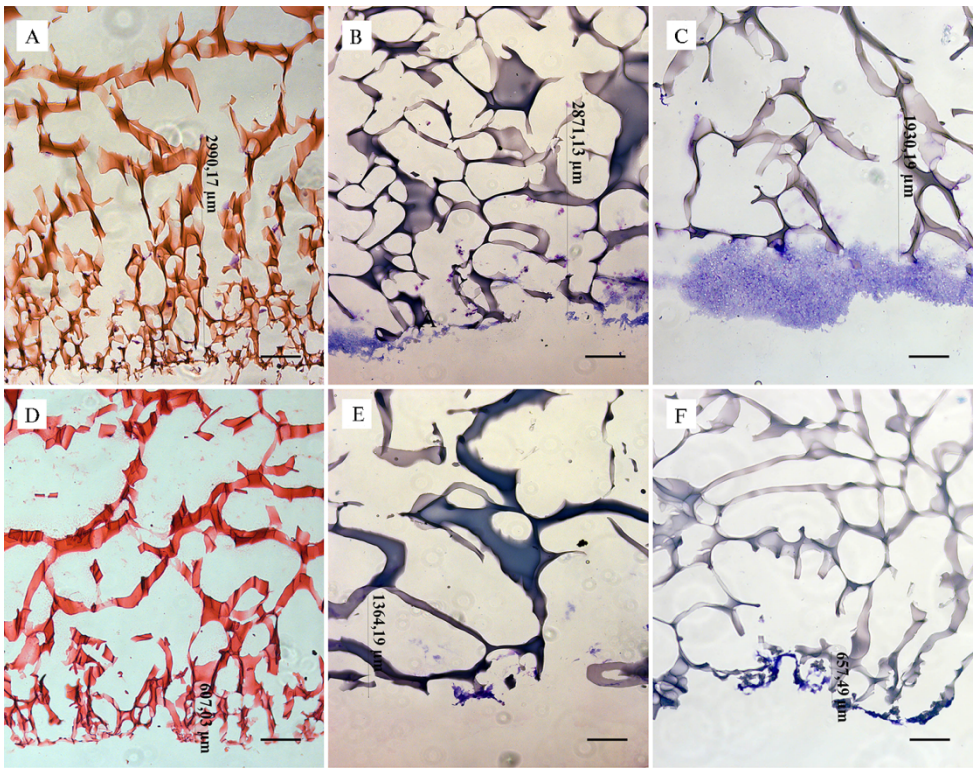

Fig. 3. The depth of cells spreading inside the matrixes on day 10 of culturing: the STO fibroblasts in the matrixes based on gelatin (A), protein of bovine blood plasma (B), FBS (C); the multipotent mesenchymal stem cells from adipose tissue in the matrixes based on gelatin (D), protein of bovine blood plasma (E), FBS (F). Staining with hematoxylin and eosin (A, D), and Giemsa staining (B, C, E, F), scale 500 μm (Axio Observer D.1, Carl Zeiss, Germany).

Histological examination of the effect of the matrix material on the cells migration (Fig. 3) revealed that all cells migrated from the monolayer's lower side into the cryogel depths and were absent on the upper sides of the sponges. The number of cells inside the cryogel was increasing when culturing. Thus, on the day 10, the STO fibroblasts penetrated into the matrixes based on gelatin, blood plasma protein and FDS to the distance of 2,990, 2,871 and 1,930 μm respectively (see Fig. 3, A, B, C). The MMSCs from cattle adipose tissue migrated to a depth of 607, 1,364, and 657 μm (see Fig. 3, D, E, F). MMSCs from bone marrow of cattle and sheep in the studied cryogels did not not differ significantly from migration of the AT MMSCs. The comparative analysis of the results obtained in three repeated experiments showed that the spongy cryogel ma-

tric based on gelatin, proteins of bovine plasma and FBS provided the MMSCs viability during the short-term culturing and promoted the cell adhesion, proliferation and migration. The structure of these cryogels turned out to be biocompatible for both mouse fibroblasts and MMSCs of farm animals. However, in case of this method, the immortalized mouse fibroblasts showed the significantly higher ability of adhesion and migration in all the cryogels.

Earlier, we showed the effect of the long-term culturing of the MMSCs from human AT on the efficiency of cells adhesion to the extracellular matrix proteins. The cells on the passages 2 and 17 of culturing differed in their ability to attach to the surface with previously applied ECM proteins, the fibronectin, collagen, and laminin. The cells attached to fibronectin and collagen on the passage 2 exceeded those on the passage 17 2 and 5 times respectively. At the same time, the number of cells adhering to laminin on the passage 17was 2 times more compared the passage 2 [31].

It was of interest to compare the bovine and sheep MMSCs on the early and late passages by their ability of adhesion to the investigated matrixes. For this, 1×10^6 cells/100 μl medium was layered on the cryogel surfaces. The cells adhesion on the early and late passages of culturing in the macroporous cryogels did not differ significantly (Table 2). This indicates that the bovine and sheep MMSCs retained their adhesive properties during culturing

2. Effect of early and late passages of the bovine and sheep multipotent mesenchymal stem cells on the adhesion to matrixes ($M\pm$SEM, $n = 3$)

Source of the MMSCs	Passage	Counts of cells attached to matrix, $\times10^5$	
		gelatin-based	based on bovine blood plasma protein
Bovine adipose tissue	2	8.0±0.10	7.8±0.13
	9	7.5±0.30	7.9±0.10
Bovine bone marrow	2	9.0±0.20	8.5±0.70
	10	8.7±0.01	8.5±0.23
Sheep bone marrow	3	7.2±0.17	7.0±0.50
	10	6.9±0.70	7.1±0.12

The analysis of the scientific literature showed that gelatin cryogels are used for culturing primary keratinocytes and human fibroblasts [31]. They formed the continuous layer of epithelium on the surface of the obtained in vitro spongy substrates. It was found out that fibroblasts are able to easily migrate deep into the porous structure of these carriers. The preclinical trials on pigs demonstrated the biocompatibility of gelatin cryogels and their nontoxicity to animals' organisms. There is the data on the addition to gelatin of other components which increase the strength of gelatin cryogels, for example, on the introduction into the initial composition of chitosan which contributes to maintaining the cells adhesion and proliferation [32-35].

MMSCs are considered a promising cellular material for the regeneration of animal joints and ligaments [1, 2]. The implantation of cellular preparations based on porous biodegradable matrixes is a method of introducing cells into the organism. Protein-based cryogels are used as porous substrates for tridimensional culturing of MMSCs and subsequent implantation of the obtained samples of certain tissue owing to their macroporous morphology and biodegradability [4, 9]. There is the data on using different cryogels based on gelatin [17], serum albumin [21] and total protein of blood serum [18-20] for this purpose. It was of interest to test cryogels in relation to the development in our laboratory of the method of creating "cultured meat in vitro" [10]. For this, we used a number of protein cryogels which served as the substrates for three-dimensional culturing the MMSCs from bovine and sheep bone marrow and adipose tissue. The re-

sults we got allow us to assume that the serum (plasma) of bovine blood includes the factors enhancing the adhesion and proliferation of MMSCs. As a result of cryotropic formation of gels based on blood plasma proteins, such components are embedded into the matrix of the formed cryogels, which create more favorable conditions for culturing stem cells compared to albumin-based carriers [19].

So, to date, the methods of cell biology including the three-dimensional culturing of mammalian cells is gaining research and practical priority importance in various fields of science and technology. We showed that using the cryogels based on gelatin and proteins of bovine blood plasma and fetal bovine serum makes it possible to adapt the adhesive culture of bovine and sheep multipotent mesenchymal stem cells (MMSCs) to the suspension culturing in a bioreactor. The data we got allow us to forecast the using of these cryogels as the matrixes for large-scale technologies of growing the MMSCs of farm animals.

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