Comparison of the Methods for Seeding Human Bone Marrow Mesenchymal Stem Cells to Macroporous Alginate Cryogel Carriers

Yu. A. Petrenko[1], R. V. Ivanov[2], V. I. Lozinsky[2], A. Yu. Petrenko[1]

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We performed a comparative study of the localization, distribution, metabolic activity, and surface properties of human bone marrow mesenchymal stromal cells after static and perfusion seeding to macroporous alginate cryogels. A simple perfusion system for mesenchymal stromal cell seeding to macroporous alginate cryogel sponges proposed in this study resulted in rapid and uniform distribution of cells within the whole volume of the scaffold preserving functional and morphological properties of the cells.

Key Words: *mesenchymal stromal cells; macroporous agarose cryogel sponges; perfusion and static seeding; tissue engineering*

Tissue engineering is a rapidly developing trend in biotechnology; it combines the principles of cell transplantology, science of materials, and engineering and is aimed at the development of functioning tissues for subsequent transplantation.

Mesenchymal stromal cells (MSC) characterized by high proliferative potential and capable of specific differentiation are a promising cell type for bioengineering [7]. Various synthetic and natural polymers and materials are now used as scaffolds in tissue engineering [9]. Alginate matrixes due to their high biocompatibility [10], resorption capacity, and other properties are widely used as porous sponge scaffolds in tissue engineering [8,13]. Cryogels, macroporous polymer gels formed in frozen media at sub-zero temperatures when the solvent crystals play a role of porogens, are a promising type of scaffolds [1,2].

An important stage in creation of bioengineering constructs is the choice of the method for cell seeding to the 3D scaffold, because the peculiarities of cell distribution within the scaffold determine successful formation of the full-value tissue [15]. Technically simple static seeding is often used for the majority of cell types and carriers, but it has some drawbacks: unequal cell distribution, low seeding efficiency, cell condensation, *etc*. [16]. Dynamic seeding with the use of perfusion, centrifugation, negative pressure, *etc*. is more effective [5,12,16]. It provides higher efficiency of cell seeding and distribution within the scaffold.

We performed a comparative study of the localization, distribution, metabolic activity, and surface properties of human bone marrow (BM) MSC after static and perfusion seeding to macroporous alginate cryogels. A simple method of perfusion seeding ensuring rapid and equal cell distribution within macroporous cryogels without impairing their adhesion properties and metabolic activity is described.

MATERIALS AND METHODS

We used adherent fraction of human MB cell suspension isolated from iliac crest needle biopsy specimens with strict adherence to the norms and rules of biomedical ethics. Stromal cells were cultured in α-MEM containing 15% FCS, 50 U/ml penicillin, 50 mg/ml

[1]Institute of Cryobiology and Cryomedicine Problems, National Academy of Sciences of Ukraine, Khar'kov; [2]A. N. Nesmeyanov Institute of Elementorganic Compounds, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** yu.a.petrenko@gmail.com. Yu. A. Petrenko.

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streptomycin, and 2 mmol/ml L-glutamine at 37°C, 5% CO_2, and 95% humidity. Passage 3-4 cells were used in the study.

Alginate cryogels in the form of disks (2 mm thickness, 10 mm diameter) were prepared according to a patented technology [3] with subsequent activation of the polysaccharide matrix with divinyl sulfone and covalent binding of type A gelatin (Sigma) to macropore walls as described elsewhere [5].

Two seeding techniques were used (Fig. 1). Static method (Fig. 1, *a*) consisted in application of a minimal volume (20 µl) of concentrated cell suspension (3×10^5 cells/ml) on the surface of a 3D scaffold using an automated pipette. The scaffold with cells was incubated for 3 h at 37°C and then transferred into wells of a 24-well plate containing 1 ml medium.

For seeding by the perfusion method (Fig. 1, *b*), two 1-ml syringes connected with an elastic plastic tube were used. Porous alginate scaffold was placed in one syringe (its diameter corresponded to the inner diameter of the syringe), 100 µl cell suspension (3×10^5 cells/ml) was placed in another syringe, and the scaffold was gradually saturated with cells by gently moving the syringe piston back and forth. The scaffold saturated with cells was incubated in a syringe for 3 h at 37°C and then transferred into wells of a 24-well plate containing 1 ml culture medium.

Metabolic activity of cells after their incorporation into macroporous scaffolds was evaluated using Alamar Blue indicator (AB; Serotec) reflecting integral activity of redox enzymes in cells [11]. To this end (10%), AB solution was added to the culture medium 24 h after cell seeding to the scaffold. After 3-h incubation at 37°C, the medium containing AB was taken and the degree of AB reduction was determined using a Tecan GENios plate reader (Austria) at excitation and emission wavelengths of 550 and 590 nm, respectively. The data were presented as the difference between the experimental and control sample (without cells) and expressed in arbitrary units of fluorescence.

Cell localization and distribution in the scaffold were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide). To this end, macroporous alginate scaffolds with embedded cells were incubated at 37°C for 3 h in a medium containing MTT (Sigma, 5 mg/ml), the medium was then removed and the scaffolds were transferred to colorless Hanks saline and examined under a stereomicroscope at ×40. For visualization of cell distribution, adhesion, and morphology within the opaque alginate scaffold, the MSC suspension was labeled with a DiOC18 (3,3'-dioctadecyloxacarbocyanine) fluorescent dye as described previously [14]: 1 µg/ml DiOC18 was added to the tube with cell suspension at constant agitation and incubated for 30 min at room temperature. Then the suspension was washed with Hanks saline, transferred to culture medium, and 24 h after seeding the scaffolds were examined under a confocal laser microscope (Carl Zeiss). Concentrated DiOC18 (1 µg/ml) in dimethylsulfoxide was obtained from Institute of Scintillation Materials, National Academy of Sciences of Ukraine.

The data were processed statistically by Student *t* test using Origin 4.0 software.

RESULTS

During culturing on adhesion plastic, the stromal MB cells formed a monolayer of typical fibroblast cells with typical "flows". During passages 3-4 the mean rate of attaining subconfluent monolayer was 4 days. According our previous data, stromal cells of the

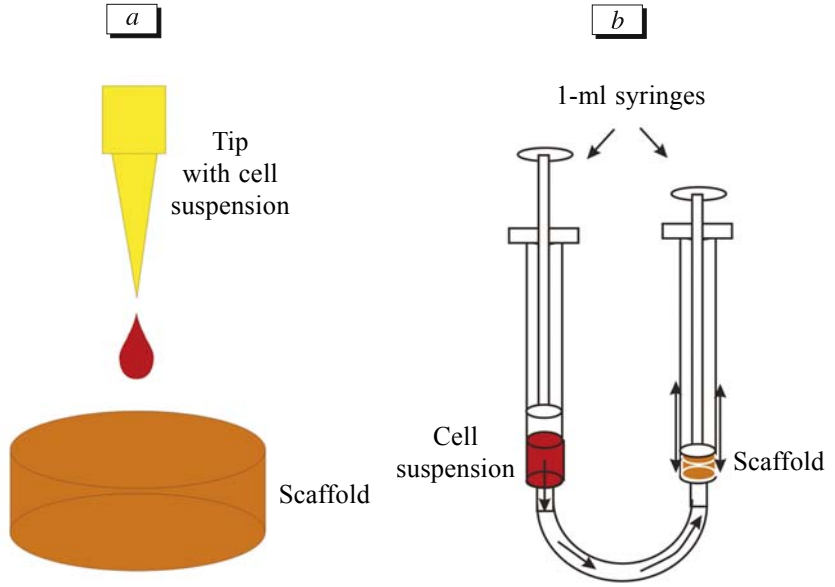

Fig. 1. Schematic presentation of the methods for seeding cells to macroporous alginate cryogel scaffolds. *a*) static method; *b*) perfusion (dynamic) method.

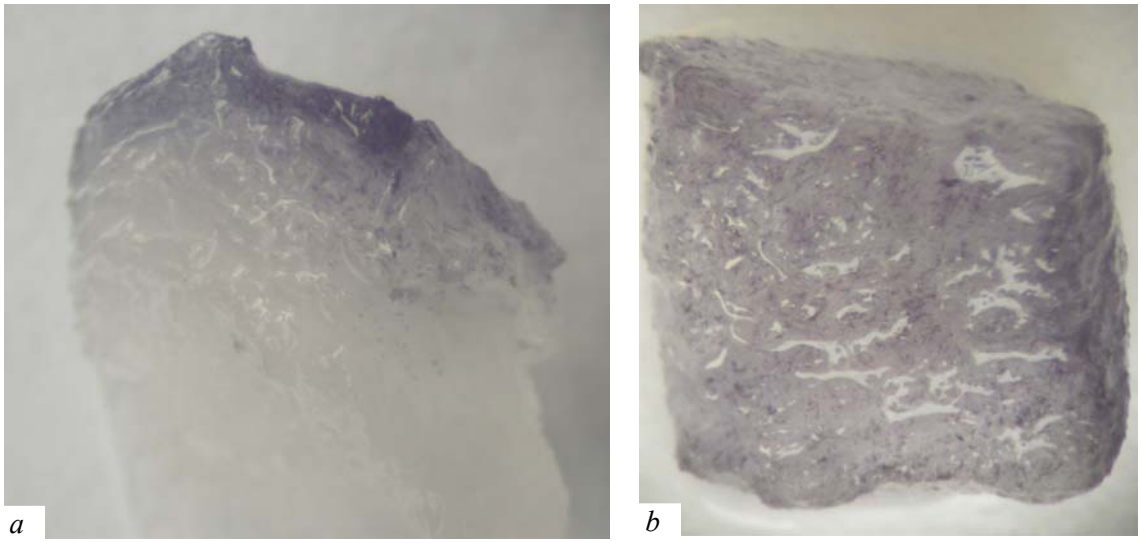

Fig. 2. Metabolic state and distribution of cells over the scaffold after seeding cells to macroporous alginate cryogels by static and perfusion methods. *a*) cell distribution over the scaffold after static seeding; *b*) cell distribution over the scaffold after perfusion seeding evaluated by MTT test.

specified passages had the typical phenotype $CD29^+$, $CD44^+$, $CD73^+$, $CD105^+$, $CD34^-$, $CD45^-$ and can differentiate towards adipogenic and osteogenic lineages [4,6], *i.e.* can referred to MSC.

The results of seeding the macroporous scaffolds by applying the cell suspension to the surface of the alginate sponge or by gentle perfusion were different. After static seeding most cells were located in the surface area of the scaffold and only few cells enter deep into it (Fig. 2, *a*). The use of perfusion method ensured more equal distribution of cells over the 3D structure of the alginate cryogel: MSC were located not only on the surface, but also over the entire volume of the scaffold (Fig. 2, *b*).

Metabolic activity of MSC was quantitatively evaluated by AB test. We previously showed that AB is reduced by mitochondrial respiratory chain enzymes of live cells [11]. The levels of AB reduction by human MSC seeded to macroporous alginate sponges by the two compared methods after 24-h culturing (Fig. 3) suggest that the cells embedded into the scaffolds can reduce AB, *i.e.* are viable. Fluorescence intensities in samples seeded by using static and perfusion methods were similar (25.159±2621 and 30.008±1332 arb. units, respectively). This can be explained by equal amount of seeded cells, which remained in metabolically active state irrespective of their location and distribution and similarly reduced AB (Fig. 3).

Metabolic activity of fibroblasts-like cells, MSC included, largely depends on their adhesion to the surface and their shape. Fluorescent label DiOC18 incorporated into membrane lipid bilayer allowed to evaluate these parameters. Human BM MSC adhered to the pore surface and flattened acquiring a fibroblast-like morphology and forming typical flows (Fig. 4). Moreover, microscopic examination revealed no cell concentration and agglutination, which were often observed after using static methods of seeding.

Thus, a simple perfusion system for MSC seeding to macroporous alginate cryogel sponges proposed by us ensured rapid and uniform distribution of cells within the entire volume of the scaffold preserving functional and morphological properties of the cells. Combined use of two indicators of cell metabolic activity (AB and MTT) allows specific evaluation of the degree of population, localization of cells in macroporous 3D scaffolds, and their metabolic state.

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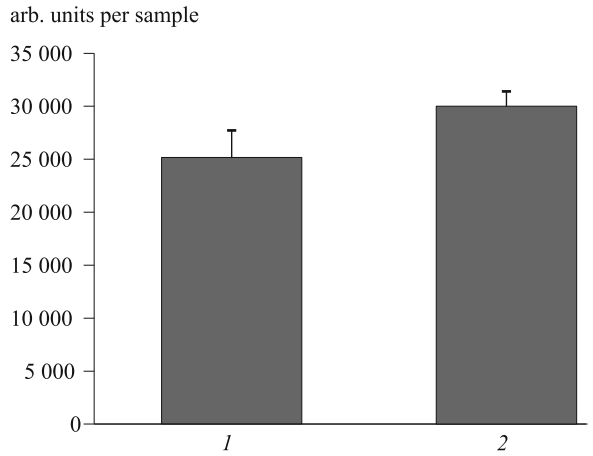

Fig. 3. Reduction of AB (arb. units) by human BM MSC 24 h after seeding macroporous alginate cryogels by static (*1*) and perfusion methods (*2*).

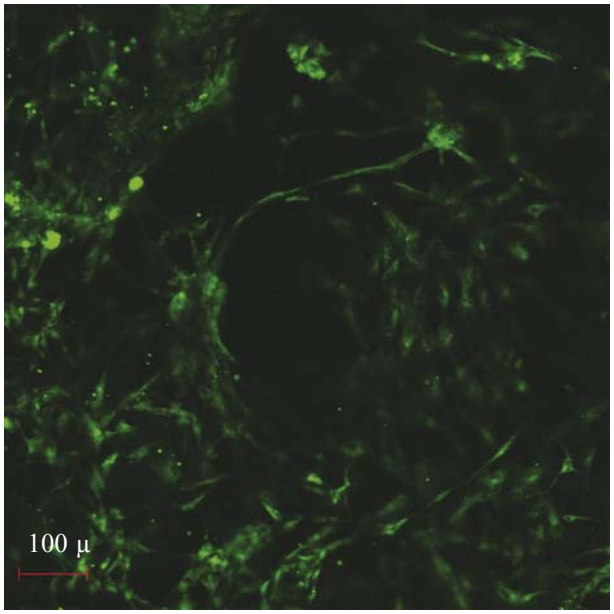

Fig. 4. BM MSC preliminary stained with green fluorescent dye DiOC18 24 h after seeding to macroporous alginate cryogels by perfusion method, ×100.

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