

Formation of Tissue-Engineered Construct of Human Cartilage Tissue in a Flow-Through Bioreactor

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We performed culturing of a cell-engineered construct of human cartilage tissue consisting of biopolymer microstructured collagen-containing hydrogel, human adipose tissue mesenchymal stromal cells, and induction chondrogenic culture medium in a specially designed flow-through bioreactor. On day 16 of the experiment, human adipose tissue mesenchymal stromal cells acquired flattened shape typical for chondroblasts, demonstrated high proliferative activity, and formed extracellular matrix. The observed histological changes in the cultured system attested to the beginning of the formation of a tissue-engineered construct of human cartilage tissue.

Key Words: *flow-through bioreactor; cell- and tissue-engineered constructs; biopolymer matrix; mesenchymal stromal cells; cartilage tissue*

The development of new approaches to the treatment of cartilage tissue defects based on the use of tissue-engineering technologies and regenerative medicine attracted much recent attention [10,11]. The existing technologies can be divided in two main groups [3]: 1) *in situ* implantation of cell-engineered construct (CEC) of human cartilage tissue for stimulation of regenerative processes in the damaged articular cartilage and 2) creation of a tissue-engineered construct (TEC) of the human cartilage tissue from CEC in a bioreactor.

In both cases, CEC of the human cartilage tissue consisting of resorbable matrix, autologous chondrocytes and/or stem cells, and induction chondrogenic culture medium containing necessary signal biomolecules is first cultured *in vitro*.

When using CEC of the human cartilage tissue for stimulation of the regeneration of the damaged cartilage, the matrix provides functional activity of cell at the site of implantation during a period sufficient for unfolding of the regenerative processes in the cartilage

tissue. In the bioreactor, the matrix plays a role of a temporary artificial extracellular matrix that provides conditions for cell proliferation/differentiation and production of the extracellular matrix.

Extracellular matrix components (collagen, hyaluronic acid and its derivatives), fibrin, alginate, gelatin, and chitosan are most often used as resorbable matrices (carriers, scaffolds) [5,8,9]. Methods of implantation of matrix-bound autologous chondrocytes are not free from some drawbacks, the main of these is traumatic procedure of obtaining biopsy specimens from healthy part of the cartilage and the possibility chondrocyte dedifferentiation *in vitro*, including fibroblast-like transformation. An alternative approach is replacement of autologous chondrocytes with mesenchymal stromal cells characterized by multilinear (adipogenic, osteogenic, and chondrogenic) differentiation potencies [1,5,7]. We have previously demonstrated the possibility of *in vitro* formation of cartilage TEC under static conditions from CEC consisting of a biopolymer microstructured collagen-containing hydrogel matrix (BMCH) and human adipose tissue mesenchymal stromal cells [12]. It was shown that structures formed during culturing and consisting of human adipose tissue MSC differentiated into chon-

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drogenic lineage cells started to produce extracellular matrix components. However, histological analysis of these structures showed that they were formed primarily on the surface of the biopolymer hydrogel matrix.

Our aim was creation of TEC of the cartilage tissue in dynamic environment of a flow-through bioreactor during culturing of human adipose tissue MSC on a BMCH matrix in a chondrogenic culture medium.

MATERIALS AND METHODS

Bioreactor. The formation of TEC from cartilage tissue CEC was performed in a bioreactor designed by us (Fig. 1). BMCH matrix with cells was placed onto a nylon membrane with pore size $10\ \mu$ (Merck Millipore Ltd.) located on a porous substrate of a bioreactor operational container.

Preliminary testing on NIH 3T3 mouse fibroblasts showed that nylon membranes were not toxic for cells and did not affect cell morphology. It has to be noted that only single cells attached to the nylon membrane surface without further proliferation.

The bioreactor has two independent circulation systems. In each system, reservoir with culture medium was connected simultaneously to two operational containers (height 5 mm, bottom diameter 22 mm). Constant medium circulation at a preset flow rate was provided by four peristaltic pumps (MasterFlex) located outside the CO_2 incubator. Medium circulation was regulated via a controller connected to a computer (Harvard Apparatus) using ORCA Controller Software. The operational containers and reservoirs with medium were placed in the incubator (37°C , 90-100% relative humidity, 5% CO_2 , and 20% O_2).

Matrix. BMCH from the product line of implantable heterogeneous gel composition (Sphero GEL, registration No. PhSR 2012/13033, July 15, 2015, Biomir Servis) used in the clinical practice for reparation of the defects of soft and cartilage tissues [2,6] was chosen as the matrix. Previous tests [4] have demonstrated that of the existing product line, the optimal matrix for CEC and TEC is BMCH with the following characteristics: mean microparticle size $145.79 \pm 0.09\ \mu$, elastic modulus $1170 \pm 12\ \text{Pa}$, viscous modulus $62.9 \pm 7.9\ \text{Pa}$, turgescence $\geq 86.6 \pm 3.0\ \text{wt}\%$, and time of biodegradation up to 9 months.

Preparation of mesenchymal stromal cells and composition of CEC of the human cartilage tissue. Human adipose tissue MSC were obtained from a healthy donor at the V. I. Shumakov Federal Research Center of Transplantology and Artificial Organs after signing the informed consent form. The cells were isolated and cultured by standard methods [12] in MesenPRO RS complete growth medium for mesenchymal human stem cells without glutamine (Gibco)

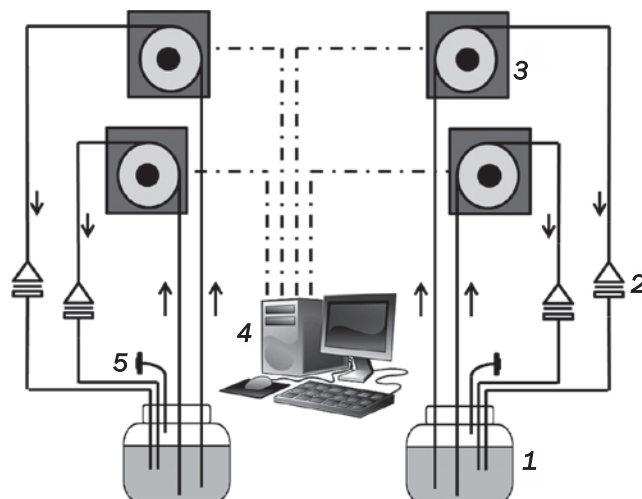


Fig. 1. Scheme of a bioreactor. 1) Reservoir with culture medium, 2) operational container, 3) peristaltic pump, 4) computer-operated controller, 5) sterile filter.

supplemented with MesenPRO RS Growth Supplement (Gibco) and 2 mM L-glutamine (PanEco). Passage 2 cells were used for creation of CEC.

All manipulations were performed under aseptic conditions. The cells were harvested from plastic with TrypLe reagent (Invitrogen) after rinsing the monolayer with Versene (PanEco). Then, the cell suspension in complete growth medium was centrifuged and brought to a concentration of 6×10^6 cells/ml. For creation of cartilage CEC, 500 μl suspension were mixed layer-by-layer with 1 ml BMCH biomatrix. The resultant sample was applied to a nylon substrate and placed in the bioreactor.

Vital staining of cells with LIVE/DEAD complex. After certain time intervals, the cartilage tissue CEC was taken out of a bioreactor container, placed in a Petri dish, and stained with LIVE/DEAD fluorescent dyes (Invitrogen). This complex consists of two components: calcein AM yields green fluorescence of live cells recorded at $\lambda = 515\ \text{nm}$, while ethidium homodimer-1 enters into cells through damaged cell membrane and binds with DNA, which results in red fluorescence recorded at $\lambda = 635\ \text{nm}$. The dyes are characterized by low background fluorescence and good diffusion into hydrogel, which allows using them for evaluation of cell condition in the sample. The dyes were mixed in sterile phosphate buffer DPBS and added to Petri dish with a TEC fragment. After 30-min incubation at room temperature, the sample was photographed under a Nikon Eclipse TS100 fluorescent microscope.

Histological examination of samples. The samples were fixed in 10% buffered formalin (for at least 4 h), rinsed in running water for 15 min, dehydrated in increasing alcohol concentrations (two portions of

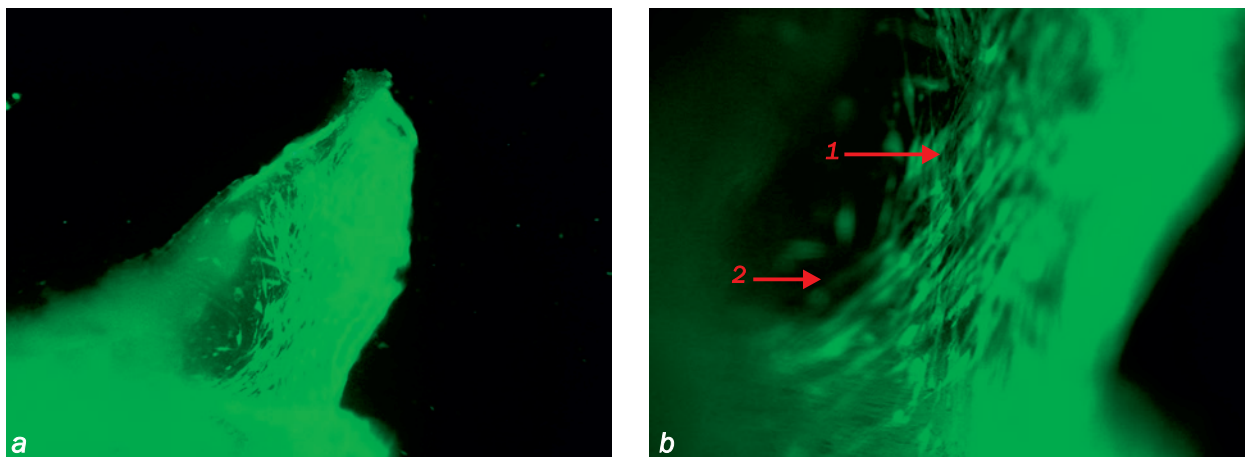


Fig. 2. Surface of sample No. 3: 14 days of culturing in STEMPRO differentiation medium under flow conditions. MSC on the surface of BMCH microparticle. Staining with LIVE/DEAD fluorescent dyes, $\times 50$ (a), $\times 100$ (b). 1) Cell cluster; 2) BMCH.

70, 80, and 96% ethanol; 5 min each), treated for 5-7 min in ethanol—chloroform mixture, then in chloroform, and embedded in paraffin. The sections (4-5 μ) obtained on a Leica RM3255 microtome were deparaffinized, rehydrated, and stained routinely with hematoxylin and eosin, alcian blue (for detection of mucopolysaccharides), and by Masson's method (for visualization of the connective tissue). The samples were analyzed and photographed with a digital camera under a Nikon Eclipse microscope.

Staining of paraffin sections of samples with hematoxylin and eosin. The sections were stained with Mayer's hematoxylin for 10 min, washed in running water for 3-5 min, rinsed with distilled water, stained with 1% eosin for 30-50 sec, rinsed in distilled water, dehydrated in ascending ethanol concentrations (70, 80, and 96%), clarified in carbolxylol, xylene, and embedded in balm.

Staining of paraffin sections with alcian blue. To prepare alcian blue solution (Sigma), 1 g dye was diluted in 100 ml 3% acetic acid. The solution was filtered and applied to deparaffinized and rehydrated sections for 30 min; then, the sections were rinsed in distilled water and poststained for 10 min with hematoxylin. The slides were placed in running water for 3-5 min, dehydrated in ascending alcohols and xylene, and embedded in balm.

Masson's staining of paraffin sections. Deparaffinized rehydrated sections were stained with Weigert's iron hematoxylin for 2 min, rinsed with running water for 15 min, and stained with acid fuchsine for 2 min. Then, the sections were shortly rinsed with distilled water and placed in 1% solution of phosphatungstic acid for 10 min. Then, the acid solution was removed, the slides without rinsing were placed in aniline blue for 1-2 min, shortly rinsed with tap water, and differentiated in 1% solution of acetic acid for 10 min. After

dehydration in ascending alcohols and clarification in xylene, the sections were embedded in balm. After these procedures, the connective tissue fibers were stained blue, cell nuclei were black or dark-blue, and cell cytoplasm was raspberry pink or red.

RESULTS

Experimental design. In long-term experiment, cartilage tissue CEC samples consisting of 1.5 ml MSC suspension in MesenPRO RS growth medium mixed layer-by-layer with 1 ml BMCH were applied onto nylon membranes (Millipore). The final concentration of cells in the mixture was 2×10^6 cells per 1 ml CEC. Four operational containers of the bioreactor with CEC were incubated for one day in MesenPRO RS growth medium under static conditions. Then, all four containers were placed in a bioreactor system filled

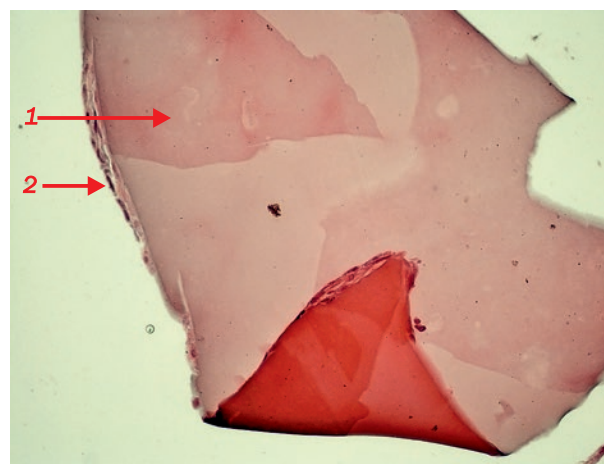


Fig. 3. Sample No. 1: Culturing of CEC in MesenPRO RS growth medium for 24 h under static conditions and 24 h under flow conditions. MSC growth on the carrier surface. Hematoxylin and eosin staining, $\times 200$. 1) BMCH; 2) cells.

with the growth medium and incubated under flow conditions at 0.5 ml/min flow rate (volume of circulating medium 110 ml). After 24 h incubation in circulating growth medium, one container (sample No. 1) was removed from the bioreactor and the growth medium was replaced with STEMPRO medium (Gibco) inducing chondrogenic differentiation. On the next day (48 h of culturing under flow conditions, including 24 h in differentiation medium), the second container (sample No. 2) was removed from the bioreactor. At this term, seal failure in one container the system was detected and that container was excluded from the study. On day 14 of culturing in differentiation medium, the last container (sample No. 3) was removed from the bioreactor. The differentiation medium was replaced weakly.

Analysis of sample using fluorescent vital LIVE/DEAD dye. Samples Nos. 1 and 3 were additionally stained with vital LIVE/DEAD fluorescent dye (Invitrogen) according to manufacturer's instructions for evaluation of cell viability. On the next day after the start of the experiment, numerous cells adhered and flattened on hydrogel particles. In 2 weeks after medium replacement for differentiation one, proliferation of adherent cells on BMCH microparticles and growth of the cell mass were seen (Fig. 2); somewhere, confluent layers with high cell density formed on the hydrogel surface.

Histological analysis of the samples. Histological analysis of sample No. 1 showed that the cell mass on the hydrogel surface after 2-day culturing in MesenPRO RS growth medium (24 h under static conditions and 24 h under flow conditions) was insignificant. The cells found on some BMCH particles had flattened elongated shape and oval nuclei. Attached cells were located on the surface of hydrogel particles and did not penetrate into the biomatrix (Fig. 3).

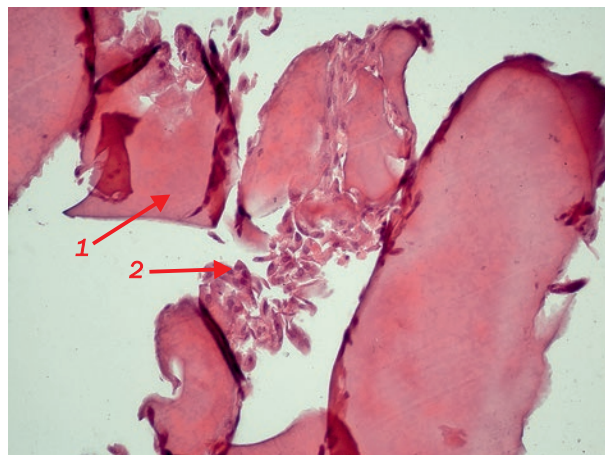
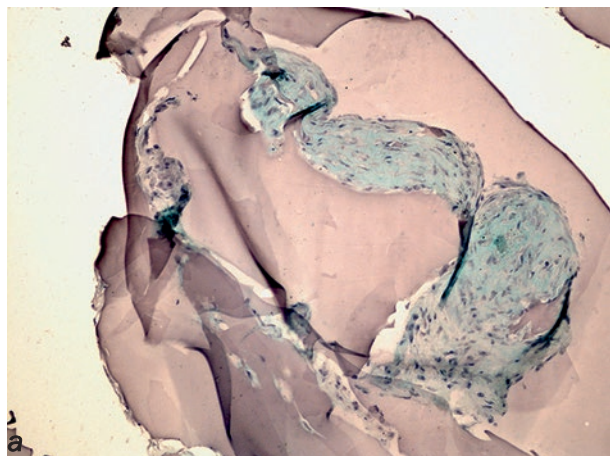


Fig. 4. Sample No. 2: culturing for 24 days in STEMPRO differentiation medium under flow conditions. MSC on the surface of BMCH microparticle. Start of the formation of 3D cell structure and MSC penetration into the biomatrix. Hematoxylin and eosin staining, $\times 200$. 1) BMCH; 2) cell cluster.

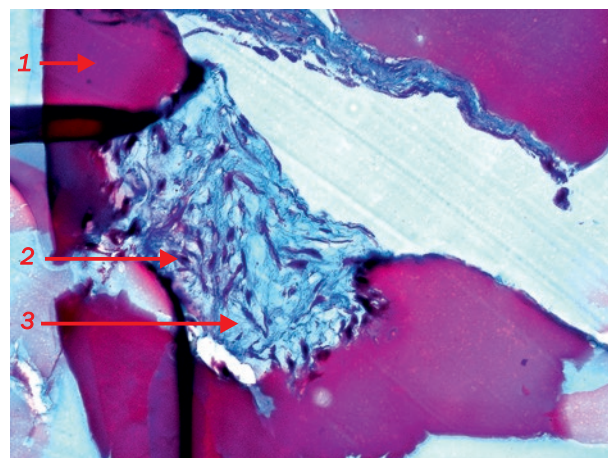


Fig. 5. Sample No. 3: culturing for 14 days in STEMPRO differentiation medium under flow conditions. Collagen fibers in the extracellular matrix. Masson's staining, $\times 200$. 1) BMCH; 2) cell; 3) extracellular matrix.

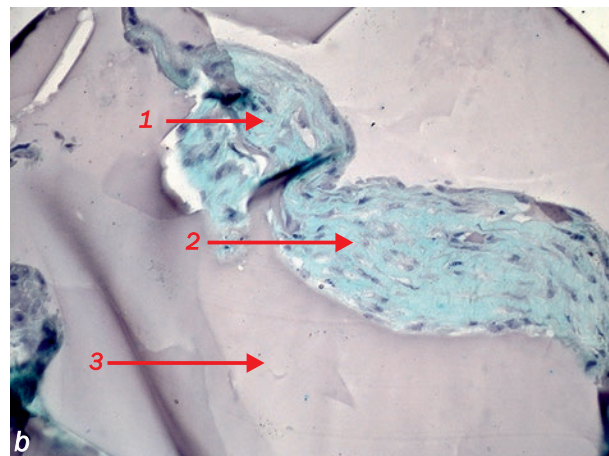


Fig. 6. Sample No. 3: culturing for 14 days in STEMPRO differentiation medium under flow conditions. Positive staining of the extracellular matrix with alcian blue, $\times 100$ (a), $\times 200$ (b). 1) Cells; 2) extracellular matrix; 3) BMCH.

In 24 h after replacement of the growth medium with STEMPRO differential medium (sample No. 2), appreciable increase in the cell mass and cell spreading on the surface of practically all hydrogel fragments were observed. The number of multilayered cell groups consisting of two morphologically different cell types (flattened cells and cigar-shaped cells with round nucleus) increased in comparison with sample No. 1 (Fig. 4). Penetration of cells into the biomatrix accompanied by resorption of the hydrogel was also noted. The cells acquired flattened shape and formed layers.

After 14-days flow incubation in STEMPRO differential medium (day 16 of the experiment), an increase in cell mass was observed in sample No. 3 with progressive increase in the proportion of extracellular matrix; these changes proved not only proliferative, but also high functional activity of cells. The cell/matrix proportion was shifted towards the latter. Most cells in these 3D structures had fibroblast-like shape.

Masson's staining for connective tissue revealed not only the presence of collagen in the extracellular matrix, but also matrix structure: thin blue fibers formed a net surrounding fibroblast-like cells. The highest density of collagen fibrils was observed in the immediate proximity to the producer cells (Fig. 5).

The use of alcian blue for visualization of mucopolysaccharides in the extracellular matrix yielded weak blue-green staining that was interpreted as a positive reaction to glycosaminoglycans appearing in cell-synthesized extracellular matrix (Fig. 6). These results attest to differentiation of MSC into chondrocytes and formation of TEC.

Significant difference in the histological pattern of CEC cultured under static and flow conditions were revealed. Culturing of MSC with BMCH in the chondrogenic medium under static conditions [12] for 14 days led to the formation of 3D structures (microspheres) with a diameter $\sim 30\text{--}300\ \mu\text{m}$; no microspheres were observed during CEC culturing in a flow-through bioreactor. On days 14 and 28 under static conditions and on day 14 under flow differentiation conditions, no type II collagen was found in the constructs, which can be explained by short differentiation time. During static culturing of CEC, positive staining with antibodies to human collagen II was detected in the cytoplasm only on day 42 of chondrogenic differentiation [12].

Thus, our experiments demonstrated active proliferation of human adipose tissue MSC with progressive accumulation of extracellular matrix produced by cells after 14-day culturing with BMCH in a bioreactor in chondrogenic medium at a flow rate of 0.5 ml/min.

Histochemical analysis of samples at these terms revealed the presence of collagen and glycosaminoglycans. The observed morphological changes in the cartilage tissue CEC cultured in the bioreactor attested to initial stages of cartilage tissue TEC formation on day 16 of the experiment.

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REFERENCES

1. Basok YB, Sevastianov VI. Tissue engineering and regenerative medicine technologies in the treatment of articular cartilage defects. *Vestn. Transplantol. Isskustv. Organov.* 2016;18(4):102-122. Russian.
2. Saykovskiy RS, Savenkova NA, Averyanov AV, Lisitsa AV. The effectiveness of Sphero@gel in the treatment of knee osteoarthritis. *Klin. Praktika.* 2013;(3):4-10. Russian.
3. Sevastianov VI. Technologies of tissue engineering and regenerative medicine. *Vestn. Transplantol. Isskustv. Organov.* 2014;16(3):93-108. Russian.
4. Sevastyanov VI, Perova NV. Bio-polymer heterogenic hydrogel Sphero@gel — an injection biodegradable implant for substitutive and regenerative medicine. *Prakt. Medistina.* 2014;(8):120-126. Russian.
5. Sevastianov VI, Perova NV, Nemets EA, Surguchenko VA, Ponomareva AS. Experimental and clinical applications of biocompatible materials in regenerative medicine. *Biocompatible Materials.* Sevastianov VI, Kirpichnikova MP, ed. Moscow, 2011. P. 237-252. Russian.
6. Solovyeva I, Shesternya N, Perova N, Sevastyanov V. Coadministration of heterogeneous biopolymer hydrogel and hyaluronic acid in osteoarthritis: the first experience. *Vrach.* 2016;(1):12-17. Russian.
7. Bajek A, Gurtowska N, Olkowska J, Kazmierski L, Maj M, Drewa T. Adipose-derived stem cells as a tool in cell-based therapies. *Arch. Immunol. Ther. Exp. (Warsz).* 2016;64(6):443-454.
8. Bernhard JC, Vunjak-Novakovic G. Should we use cells, biomaterials, or tissue engineering for cartilage regeneration? *Stem Cell Res. Ther.* 2016;7(1):56.
9. He B, Wu JP, Kirk TB, Carrino JA, Xiang C, Xu J. High-resolution measurements of the multilayer ultra-structure of articular cartilage and their translational potential. *Arthritis Res. Ther.* 2014;16(2):205.
10. Liu M, Yu X, Huang F, Cen S, Zhong G, Xiang Z. Tissue engineering stratified scaffolds for articular cartilage and subchondral bone defects repair. *Orthopedics.* 2013;36(11):868-873.
11. Stubbs AJ, Howse EA, Mannava S. Tissue engineering and the future of hip cartilage, labrum and ligamentum teres. *J. Hip Preserv. Surg.* 2015;3(1):23-29.
12. Surguchenko VA, Ponomareva AS, Kirsanova LA, Skaleckij NN, Sevastianov VI. The cell-engineered construct of cartilage on the basis of biopolymer hydrogel matrix and human adipose tissue-derived mesenchymal stromal cells (in vitro study). *J. Biomed. Mater. Res. A.* 2015;103(2):463-470.