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COMPARATIVE STUDY OF CHONDROGENESIS OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS WHEN CULTURED IN COLLAGEN-CONTAINING MEDIA UNDER *IN VITRO* CONDITIONS

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In terms of method of production, collagen carriers are subdivided into materials obtained on the basis of extracellular matrix (ECM) components, particularly collagen-containing hydrogels and decellularized tissue. **Objective:** to compare in vitro the ability of biopolymer microheterogeneous collagen-containing hydrogel (BMCH) and tissue-specific matrix from decellularized porcine articular cartilage (DPAC) to support adhesion, proliferation and chondrogenic differentiation of human adipose-derived mesenchymal stem cells (hAMSCs). **Materials and methods.** For cartilage decellularization, we carried out treatment with surfactants (sodium dodecyl sulfate, Triton X-100) followed by exposure in DNAase. The metabolic activity of hAMSCs was assessed by PrestoBlue™ (Invitrogen, USA) staining. The morphological study of cell-engineered constructs (CECs) formed by culturing hAMSCs in the presence of matrices was performed using histological staining and scanning electron microscopy (SEM) with lanthanide contrasting. **Results.** The number of cells on the surface of both BMCH and DPAC increased within 14 days. Mitochondrial activity of the cells was 1.7, 1.7, and 1.3 times higher on days 3, 10, and 14 when cultured on DPAC compared to BMCH, respectively. On day 14 of cultivation in the chondrogenic culture medium, hAMSCs formed cell layers on the DPAC surface and on the BMCH surface. Cytoplasm of the cells included numerous granules, which, when stained, resembled the matrix itself. On the DPAC matrix surface, cells were more evenly distributed, whereas in the case of BMCH, cell adhesion and proliferation were observed only in certain areas. The ECM produced by the cells contained collagen and glycosaminoglycans (GAGs). **Conclusion.** The ability of DPAC obtained according to the developed protocol to form CECs with hAMSCs with uniform distribution of cells and their production of specific collagen- and GAG-containing ECM suggests that DPAC is effective in regeneration of damaged cartilage. Chondrogenic differentiation of hAMSCs was observed both when cultured with BMCH and with DPAC. When creating a tissue equivalent of cartilage in vitro, the advantage of using tissue-specific matrix over BMCH should be considered.

Keywords: cartilage tissue, chondrogenic differentiation, mesenchymal stem cells, decellularized matrix, tissue engineering.

INTRODUCTION

Articular cartilage is an avascular dense tissue with limited self-regeneration capacity, thus indicating the feasibility of developing therapeutic approaches to its repair using cell therapy and tissue engineering [1].

Today, autologous chondrocyte transplantation is the gold standard cell therapy for joint diseases. However, this method has a number of disadvantages, which primarily include traumatic biopsy and high probability of chondrocyte dedifferentiation during expansion [2]. Mesenchymal stem cells (MSCs) are considered as an alternative to autologous chondrocytes. By their nature, these cells are capable of directed differentiation into various mesenchymal tissues, including the cartilage, and have immunomodulatory properties. Note that due to its high chondrogenic potential, ease of isolation and minimal traumaticity, human adipose-derived tissue is seen as a promising source of MSCs for cartilage tissue engineering [3, 4].

To increase the efficiency of cell therapy of affected joints, MSCs are injected into the intra-articular capsule on biodegradable carriers – extracellular matrix (ECM) mimetics, which help maintain the viability and chondrogenic differentiation of MSCs [5].

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Collagen, the main structural protein of natural ECM cartilage, is used in most biomedical cell products for the treatment of cartilage defects [6]. Among Russian innovations, we can single out a biopolymer microheterogeneous collagen-containing hydrogel (BMCH) from the linear series of implantable heterogeneous gel composition *Sfero*®GEL (Biomir Service, Russia). The efficacy of using BMKG as a matrix in biomedical cellular products for regeneration of damaged articular cartilage, liver and pancreas, has been proven [7, 8]. From our point of view, of great interest for cartilage tissue engineering are decellularized carriers – biological ECM mimetics, obtained by removing cells and their fragments from tissue with maximum preservation of the structure and composition of natural ECM [9, 10]. It was shown that decellularized cartilage (DC) not only supports cell adhesion and proliferation, but also stimulates MSCs differentiation into chondrocytes [11–13]. A comparative assessment of the abilities of the obtained BMCH and DC to support adhesion, proliferation and chondrogenic differentiation of MSCs in vitro will make it possible to select a matrix that has the greatest potential for use in regenerative medicine to restore damaged cartilaginous tissue, which was the goal of this work.

MATERIALS AND METHODS

Heterogeneous gel *Sfero*®GEL

To create a cell-engineered construct (CEC), a composition of heterogeneous implantable gel from the *Sfero*®GEL linear series (Biomir Service, Russia) was chosen with the following characteristics:

– average microparticle size: 145.79 ± 0.09 microns;
– modulus of elasticity: 1170 ± 12 Pa;
– viscosity modulus: 62.9 ± 7.9 Pa;
– swellability: not less than 86.6 ± 3.0 wt.%.

Obtaining microparticles of articular cartilage

Pork femur and knee joints were obtained from a slaughterhouse (Promagro, Stary Oskol) after the slaughter of healthy animals (weight about 120 kg) in accordance with the European Directive 64/433/EEC. After refrigerated transportation (+4 °C), the cartilage was removed from articular surfaces with a scalpel and cut into 0.5 × 0.5 × 0.1 cm fragments. The resulting fragments were micronized using CryoMill (Retch GmBH, Germany). The fraction of microdispersed particles in the range of 100–250 μm was isolated by sieving the milling through a set of sieves with appropriate pore sizes.

Decellularization mode

Decellularization was performed by treating the microparticles in three changes of phosphate buffer (138 mM NaCl, 2.67 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) containing 0.1% sodium dodecyl sulfate and an increasing Triton X100 concentration (1%, 2% and 3%, respectively) at room temperature and occasional stirring. The samples were then placed in a buffer solution (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mol $CaCl_2$; pH = 7.6) containing 50 U/ml type I DNAase (New England Biolabs Inc., USA) for 48 hours at 37 °C. The matrix was washed in bidistilled water. It was sterilized by gamma irradiation at 1.5 Mrad dose.

Cell isolation

The source of human adipose-derived mesenchymal stem cells (hAMSCs) was the subcutaneous adipose tissue of a healthy donor taken from him with informed voluntary consent. A 2–3 g sample of the subcutaneous adipose tissue was shredded with a scalpel, subjected to double washing with cold (+4…+6 °C) Hanks' solution, and then incubated in 0.1% collagenase type I solution (Gibco, USA) at 37 °C for 20 minutes.

All cells were precipitated by centrifugation, resuspended in complete DMEM/F12 (1:1) growth medium supplemented with 10% bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Gibco, USA) and cultured until monolayer formation, changing the medium twice a week. The cells were transferred into suspension by treating with Versene solution at 37 °C for 1 minute, followed by addition of TrypLe™ dissociating agent (Invitrogen, USA). Third passage cells were taken for the experiments.

Cell differentiation

To confirm the presence of multipotent cells in the culture, experiments on its multidirectional differentiation were performed. Chondrogenic cell differentiation was performed in microspheres obtained by deposition of 2×10^5 cells in 96-well plates with a conical bottom. After 2 weeks of cultivation in chondrogenic culture medium (DMEM HG supplemented with GlutaMAX™ (Gibco, USA), 10% ITS+ (Corning, USA), 1% sodium pyruvate (Gibco, USA), 0.25% ascorbate-2-phosphate (Sigma-Aldrich, USA), 0.0001% dexamethasone (Sigma-Aldrich, USA), 0.002% TGF-β1 (PeproTech, USA) and 1% culture antibiotic-antimycotic (Gibco, USA)), the preparations were fixed in a 10% buffered formalin and embedded in paraffin. Sections were dewaxed, rehydrated, and stained with alcian blue. Adipogenic differentiation of the studied cultures was performed in DMEM/F12 medium containing 10% horse serum, 0.5 mM isobutyl methylxanthine and 60 mM indomethacin for 7 days. The preparations were then fixed in 4% paraformaldehyde and stained with oil red O. Differentiation of the obtained cultures into bone tissue was performed in serum-free DMEM/F12 medium supplemented with 0.2 mM ascorbic acid, 10 mM b-glycerophosphate calcium, 10-7 M dexamethasone (Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine (Gibco). The cells were cultured for 3 weeks,

changing the medium twice a week. Upon completion, the preparations were fixed in 4% formaldehyde solution and stained with alizarin red.

Previous studies [14] have shown that MSC cultures from all sources had a similar phenotype CD29, CD34, CD44, CD49b, CD45, CD73, CD90, HLA-DR, indicating a high content of multipotent mesenchymal cells. Most of these macromolecules are included in the list of markers recommended by the International Society for Cellular Therapy for characterization of MSC cultures [15].

Method for determining metabolic activity

The metabolic activity of cells was determined using a test with PrestoBlue™ reagent (Invitrogen, USA) according to the manufacturer's instructions. The method was based on the dehydrogenase activity of cells converting resazurin to resorufin, which can also be determined spectrophotometrically. Spectrophotometric analysis was performed on a Tecan Spark10 plate reader (Tecan, Austria). When studying metabolic activity, 2×10^4 hAMSCs were added per 5 mg of decellularized cartilage (DC). In the metabolic activity study, 2×10^4 hAMSCs were added per 100 μl of BMCH. Absorbance measurements were used to calculate the metabolic activity coefficient (K) using the formula:

$$K = \frac{117{,}216 \times Abs_{570} - 80{,}586 \times Abs_{600}}{155{,}677 \times Abs_{600} - 14{,}652 \times Abs_{570}} \times 100\%,$$

where Abs_{570} – absorbance at 570 nm wavelength, Abs_{600} – absorbance at 600 nm wavelength.

Creation of cell-engineered constructs

The CEC consisted of 1×10^6 cells and 5 mg of decellularized cartilage (DC) or 0.25 ml BMCH. Matrices were populated with cells by rotating them in tubes with culture medium on a Multi Bio 3D (Biosan, Latvia) ballerina type shaker platform. CECs were cultured in a growth culture medium for the first 5 days. Then the culture medium was replaced with a chondrogenic differentiation medium. The medium was replaced every third day. Samples were analyzed on days 14 and 42 in differentiation culture medium using histological staining methods.

Scanning electron microscopy

The morphology of the surface and the nearest subsurface layer of samples was studied together with employees of the Laboratory of Basic Research in Oph-

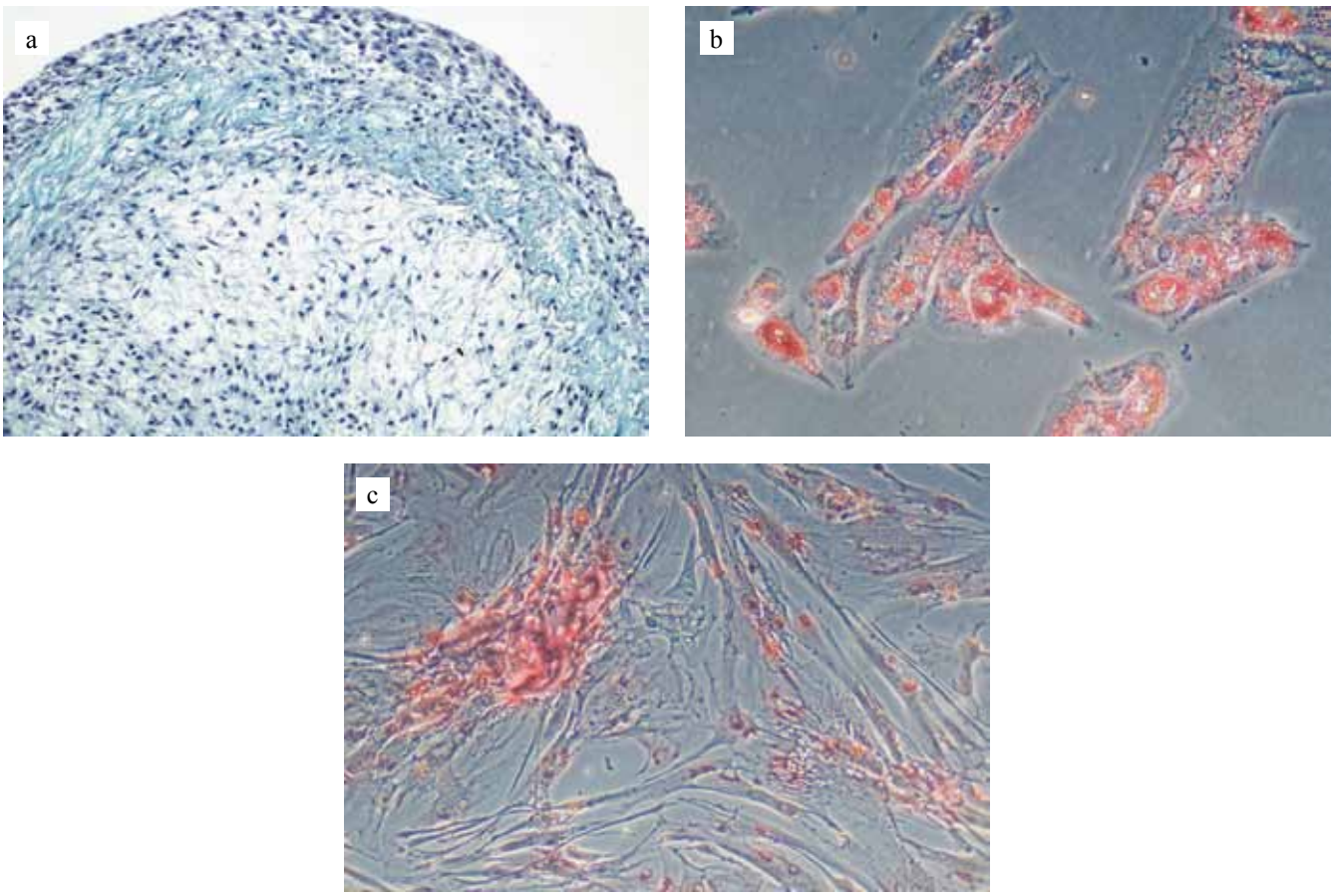

Fig. 1. Differentiation of hAMSCs in chondrogenic (a, alcian blue stain), adipogenic (b, Oil Red O stain) and osteogenic (c, Alizarin Red stain). 200× magnification

thalmology at the Research Institute of Eye Diseases in Moscow by scanning electron microscopy (SEM) using lanthanide contrasting [16].

Sample preparation of heavily watered samples for SEM is difficult because it requires their dehydration and sputtering of the conductive layer, and this leads not only to strong structural changes in such objects, but also complicates differentiation of cellular elements from the substrate. The lanthanide contrasting method allows observation of non-dehydrated biological samples in a low vacuum after keeping them in a saturated solution of a rare-earth metal. In this case, the native state of the studied object is preserved as much as possible, and the image obtained in backscattered electron detection mode carries extended information about intracellular structures [16].

The processing protocol included an initial wash, holding for 45 minutes in a BioREE contrast solution (Glaucon, Russia), and a final wash with distilled water. After contrasting, excess moisture was removed from the specimen surface using an airbrush and placed on the slide of an EVO LS10 microscope (Zeiss, Germany). Observations were performed in low vacuum (EP, 70 Pa), at an accelerating voltage of 20 kV.

Methods of histological analysis of samples

The specimens were fixed in a buffered 10% formalin solution, washed in running water and dehydrated in

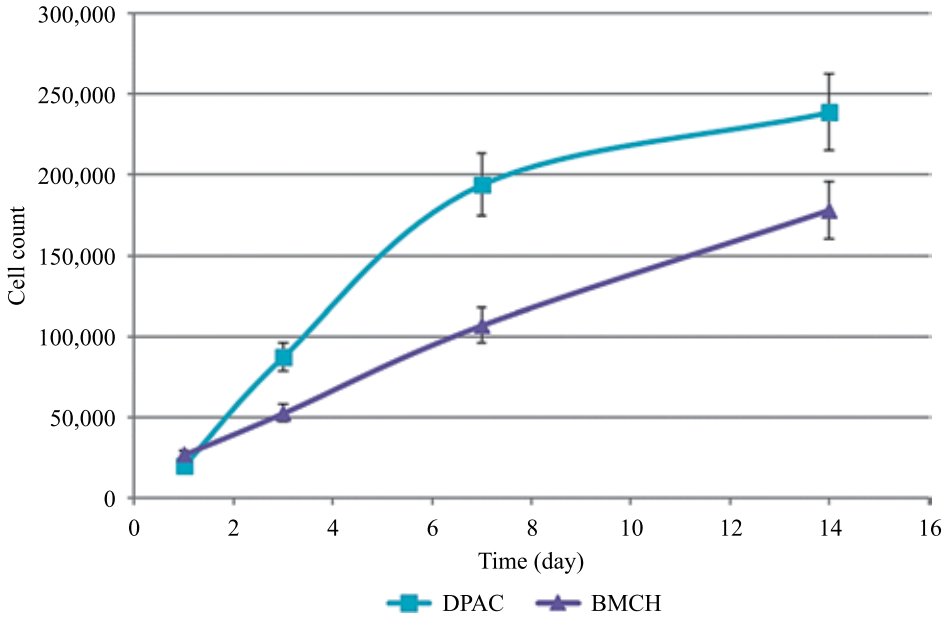

Fig. 2. Growth curve of hAMSCs when cultured in DPAC and BMCH

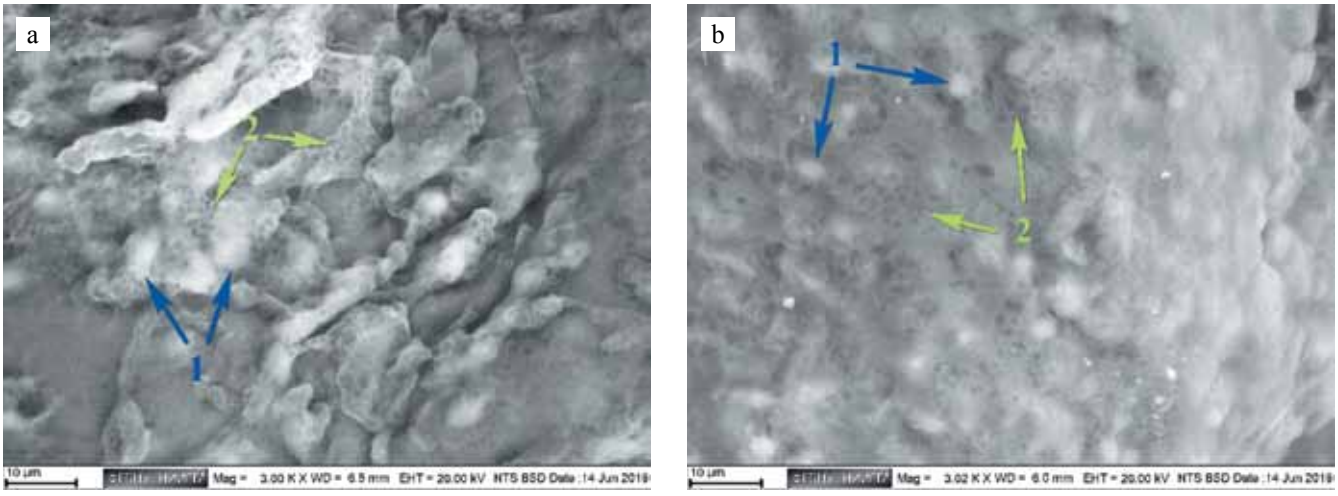

Fig. 3. Microphotographs of the CEC surface structure, including hAMSCs, cultured in DPAC (A) and in BMCH (B) in a chondrogenic differentiation medium for 14 days. SEM using lanthanide contrasting BioREE. The scale bar size is 10 μm. 1 – nuclei, 2 – intracellular vesicles

alcohol of ascending concentrations (in two portions of 70%, 80%, 96% ethanol), incubated in a mixture of ethanol and chloroform, in chloroform and embedded in paraffin. Sections 4–5 μm thick, obtained with a Leica RM3255 microtome, were dewaxed, rehydrated and stained by standard techniques, with hematoxylin and eosin, alcian blue to detect glycosaminoglycans (GAGs) and using Masson's method for connective tissue according to standard techniques.

Analysis and photography of the obtained preparations was performed using a Nikon Eclipse microscope equipped with a digital camera.

RESULTS AND DISCUSSION

Ability of hAMSCs to differentiate in chondrogenic, adipogenic and osteogenic directions is shown in Fig. 1.

The growth curves show that the number of cells on the surface of both BMCH and DC increased over 14 days (Fig. 2). On the first day measurements, optical density of the samples did not differ, indicating a similar cell population on the BMCH and DC surfaces at the start of the experiment and probably the same adhesive capacity of the carriers for MSCs in the amount initially deposited in CEC. However, a significant difference in the volume of the cell population from day three onwards revealed the effect of the carriers on the proliferative capacity of hAMSC culture. The mitochondrial activity of cells ensuring the conversion of blue resazurin into pink resorufin was 1.7, 1.7 and 1.3 times higher on days 3, 10 and 14, respectively, when MSCs were cultured on DC compared to BMCH. The shape of the growth curves was similar; however, in the case of BMCH, we observed an approach towards the stationary phase by day 14, whereas in the case of BMCH, no signs of slowing down in hAMSCs growth were visualized by day 14. Apparently, the slowdown in cell growth was associated with the limited area of the carrier for colonization, as the optimal amounts of growth factors and nutrients were maintained by regularly replacing the culture medium. Note that the results obtained allow us to estimate the count of hAMSCs with metabolic activity, whose presence in the samples is important for cell function in

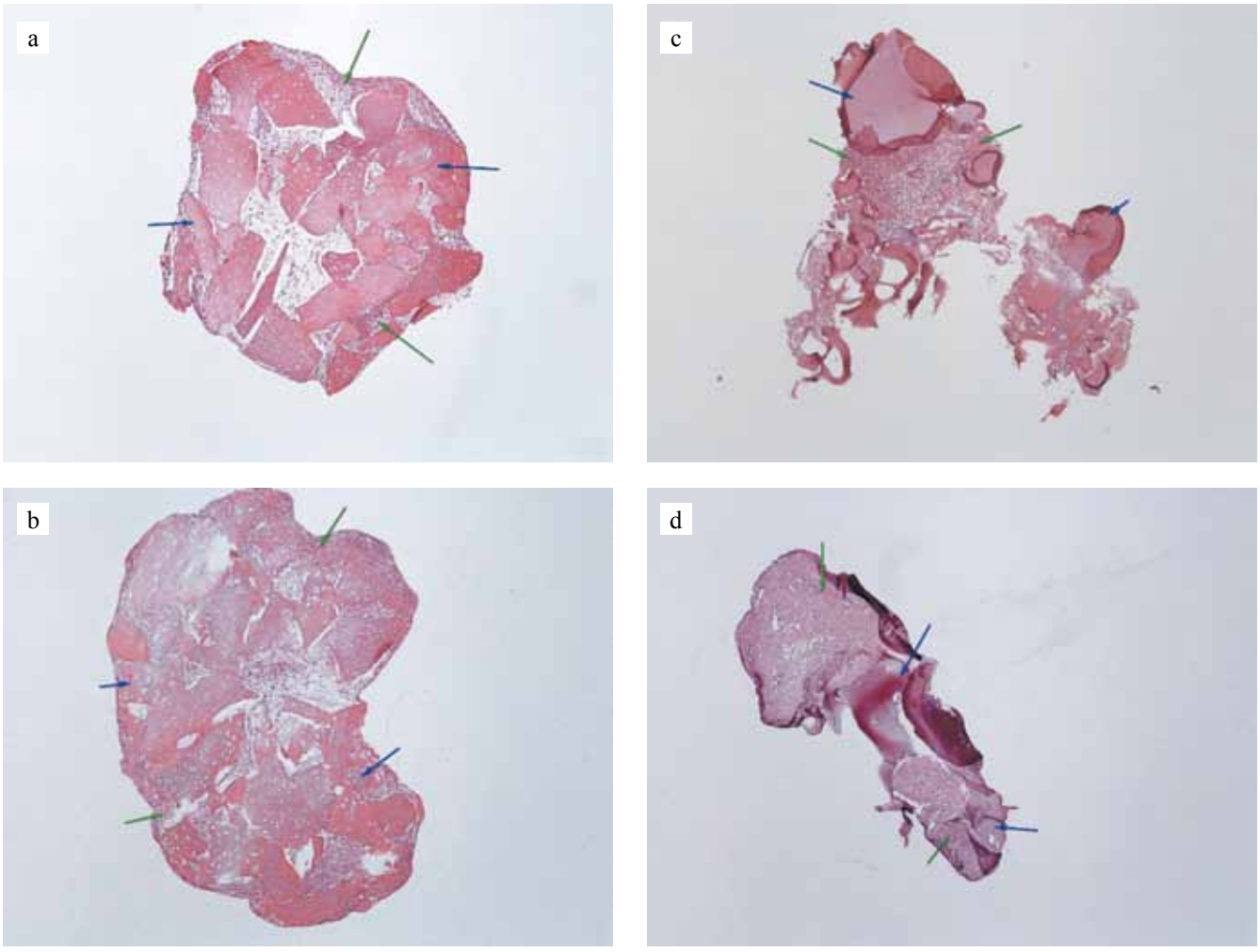

Fig. 4. Histological picture of CEC formation dynamics. H&E staining. 40× magnification. a, b – DPAC-based CECs, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

CECs, as mitochondrial dehydrogenases, cytochromes and dehydrogenases located in the cytoplasm take part in reduction of resazurin to resorufin [17]. The difference in the cell count when observed for 14 days (Fig. 2), indicates a greater ability of DC to stimulate the growth of hAMSCs compared to BMCH. Note that for a full in vitro comparative assessment of the prospects of using cell carriers in cartilage tissue engineering, it is necessary to investigate their ability to maintain chondrogenic differentiation of hAMSCs.

Fig. 3. shows the results of SEM morphology of CECs consisting of hAMSCs and DCs or BMCHs after 14 days of culture.

At 14 days of cultivation in chondrogenic culture medium, hAMSCs formed cell layers on both DC and BMCH surfaces (Fig. 3, a, b). In some cells, the use of lanthanide contrasting allowed visualization of intracellular structures due to accumulation of lanthanides in calcium- and phosphorus-rich areas (including Ca^{2+}-channels of various membranes, cell contact proteins). We have been able to identify nuclei and plasma membrane edges in some cells. Interestingly, the cytoplasm of the cells included numerous granules whose contents were similar in brightness to the matrix used. These intracellular vesicles may be related to both secretion of components of the extracellular cartilage matrix and resorption of the carrier. Both described processes were observed in histological studies of cartilage CEC specimens. Note that, summarizing this with the observed spread-eagled shape of cells, it can be concluded that physiological metabolic processes were actively taking place in the cells cultured on the surface of the tissue-specific carrier and the collagen-containing hydrogel.

As can be seen in Fig. 4, there was active fibroblast-like cell growth on the DC surface with formation of multilayer layers. The cells and their derived ECM bound numerous DC microparticles, forming large conglomerates.

In CEC samples including BMCH, a heterogeneous cell population forming stratified patches was observed.

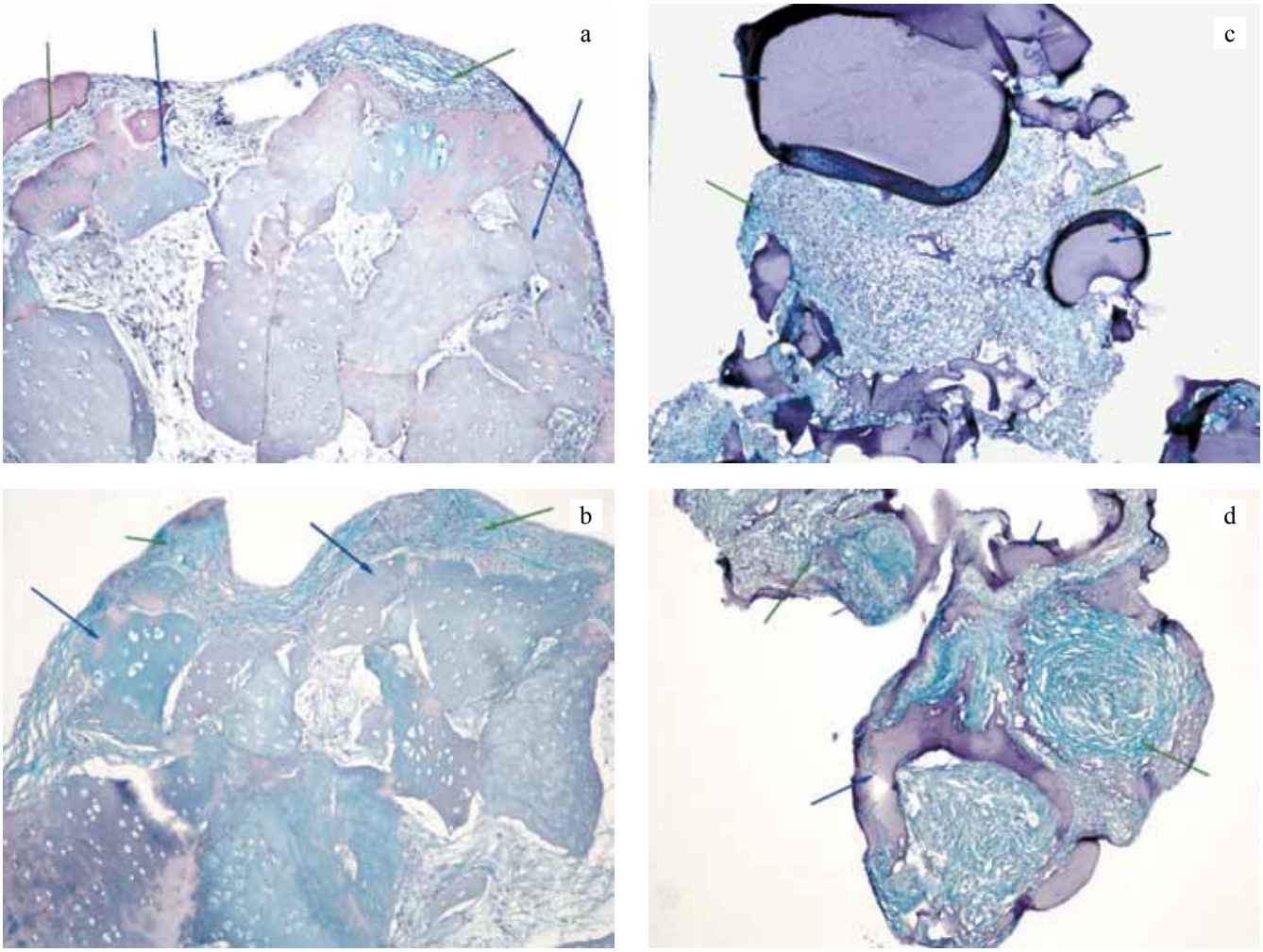

Fig. 5. Histological picture of cartilage CEC formation dynamics. Alcian blue stain for GAGs. 100× magnification. a, b – DPAC-based CEC, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

The cells sprouted into the BMCH thickness, which was accompanied by its resorption. Areas of cell layer destruction were also visualized. The sample contained areas not stained with hematoxylin – cell detritus.

The amount of cell detritus in both DC- and BMCH-based CECs increased over time. Note that cells were more evenly distributed on the surface of the DC matrix, while for BMCH, cell adhesion and proliferation were observed only in isolated areas. It should be noted that for both matrices, the cell mass volume did not visually differ. However, on the DC surface, cells with the ECM that were synthesized by them form thin strands, which leads to formation of aggregates of micro-dispersed particles. On the surface of the hydrogel matrix, hAMSCs are unevenly distributed across the surface in the form of large clusters, which can lead to nutrient deficiencies in the cell layer volume.

In cell-derived ECM in CECs including both types of matrices, local positive staining for GAGs and collagen in multilayer areas was observed at 14 days of cultivation in differentiation medium (Fig. 5, a, b and Fig. 6, a, b). Uniform ECM staining for GAGs was observed in CECs containing BMCH and in CECs with DC at 42 days (Fig. 5, b, d). By day 42, there was a significant increase in collagen content in the samples (Fig. 6, b, e).

Results obtained show that hAMSCs are capable of forming cartilage CECs when cultured in chondrogenic differentiation medium on collagen-containing matrices. It can be assumed that chondrogenesis of hAMSCs in CECs with ECM-derived collagen-containing mimetics was influenced by macromolecular components of ECM. Macromolecules such as hyaluronic acid, chondroitin sulphate and type II collagen have been extensively studied as cartilage repair agents. It is thought that the reason for their stimulating effect on chondrogenesis lies in their interaction with cells. For example, introduction of hyaluronic acid into a chondrocyte culture enhanced ECM synthesis. As a possible mechanism, it is suggested that the chondrocyte surface receptor CD44 first binds to the hyaluronic acid molecule that stimulates it and then interacts with the cytoplasmic domain of the TGF β1 receptor, ultimately regulating the genes responsible

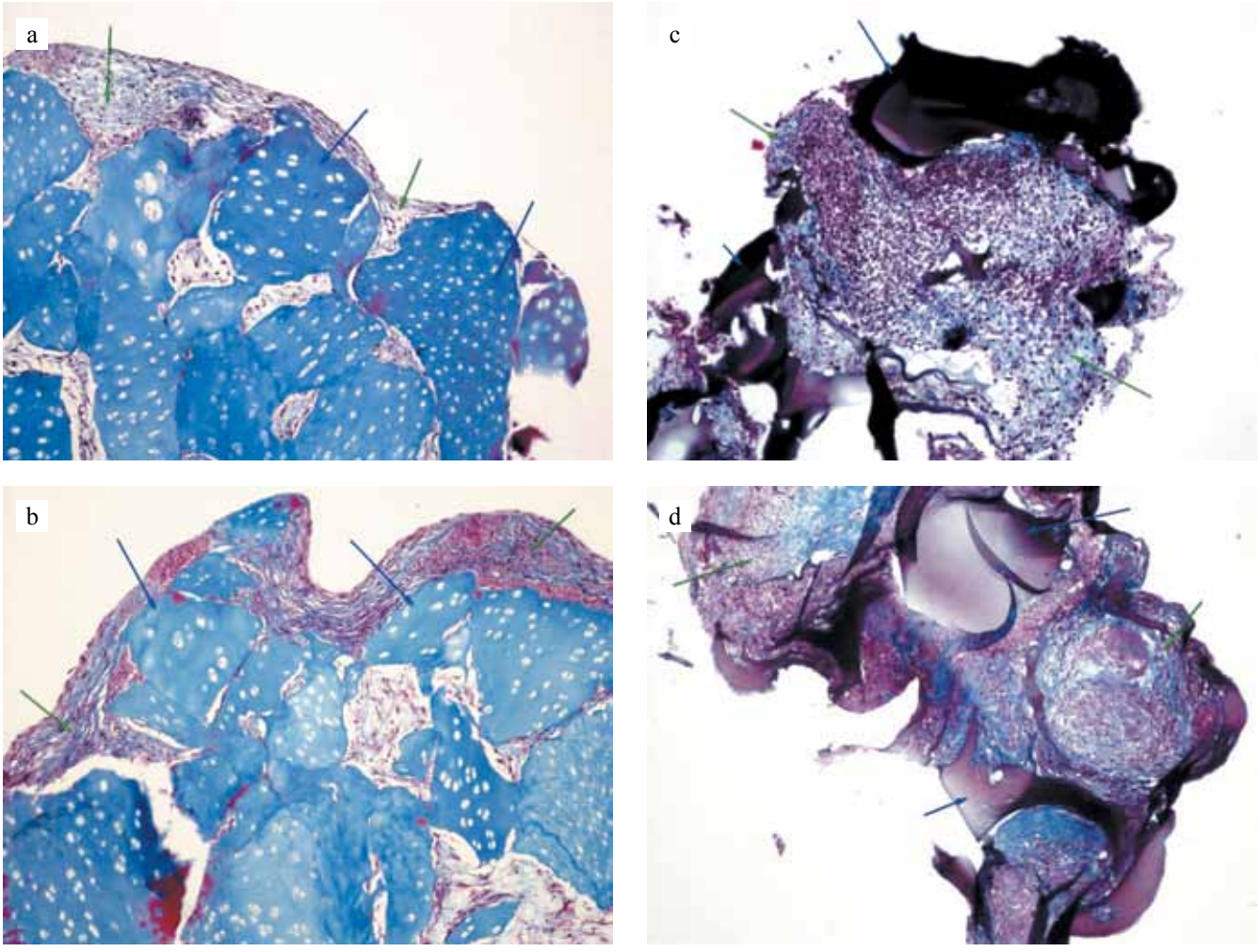

Fig. 6. Histological picture of CEC formation dynamics. H&E staining. 100× magnification. a, b – DPAC-based CEC, c, d – BMCH-based CECs. a, c – 14 days of cultivation in chondrogenic differentiation medium, b, d – 42 days of cultivation in chondrogenic differentiation medium. Blue arrows – cell carrier, green arrows – cells with the obtained ECM

for TGF β1 signaling and improving ECM synthesis [18, 19]. In addition, it has been shown that collagen type II, which is the main protein component of hyaline cartilage ECM from which DC was derived, can promote preservation of chondrocyte morphology and synthesis of more GAGs than type I collagen [20]. Type II collagen has also been shown to enhance chondrogenic differentiation of MSCs when added to agarose matrices [21]. Note that in CEC with DC, the cells were more evenly distributed, whereas in the CEC with BMCH, the cells adhered and proliferated only in certain areas of the matrix surface. This is probably due to preservation of cell adhesion sites on the surface of DC microparticles due to a gentler decellularization procedure compared to the acetic acid hydrolysis to which farm animal tissues are subjected during BMCH fabrication.

CONCLUSION

The established ability of decellularized cartilage (obtained using the developed protocol) to form cell-engineered constructs with hAMSCs with uniform cell distribution and the cell-derived specific extracellular matrix containing collagen and glycosaminoglycans indicates its potential for regeneration of damaged cartilage. Biopolymer microheterogeneous collagen-containing hydrogel and tissue-specific decellularized cartilage showed they can support chondrogenic differentiation of hAMSCs. The high ability of the obtained decellularized cartilage matrix to support adhesion, proliferation and chondrogenic differentiation of hAMSCs in comparison to the clinically applied equivalent indicates that it has prospects for application in tissue engineering.

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The authors declare no conflict of interest.

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