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EFFECT OF TRYPSIN ON BIOCHEMICAL AND FUNCTIONAL PROPERTIES OF DECELLULARIZED PORCINE ARTICULAR CARTILAGE

A.D. Kirillova, E.A. Nemets, A.M. Grigoriev, L.A. Kirsanova, V.A. Ryzhikova, E.A. Volkova, Yu.B. Basok, V.I. Sevastianov

Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation

Objective: to study the effect of trypsin pretreatment in the porcine articular cartilage decellularization protocol on the ability to restore the biochemical composition and functional properties of the resulting finely dispersed tissue-specific scaffold when co-cultured with human adipose-derived stem cells (hADSCs). **Materials and methods.** Porcine articular cartilage was micronized to a maximum size of 250 μm. The resulting porcine articular cartilage microparticles (CMps) were treated with trypsin (0.05, 0.25, 0.50%) / EDTA solution at +37 °C for 24 hours. Then, the CMps were successively incubated for 24 hours in three surfactant solutions containing 0.1% sodium dodecyl sulfate and increasing concentration of Triton X-100 (1, 2, 3%) at room temperature and in DNase I solution at +37 °C for 48 hours. The degree of change in the biochemical composition and the ability of decellularized CMps (DCMps) scaffolds within cell-engineered constructs (CECs) to support hADSC adhesion and proliferation, as well as their potential ability to exert a stimulatory regenerative effect, were then assessed. DNA, glycosaminoglycans (GAGs) and collagen content in the DCMps and CECs were examined. The morphology of the samples was examined using histological and immunohistochemistry staining. **Results.** Histological analysis showed that there were no cells and detritus in the DCMp samples. Pretreatment of CMps using a solution with the lowest content of trypsin (0.05%) / EDTA in the samples retained 5.14 ± 0.87 ng/mg DNA in the samples, while GAG content decreased to 5.34 ± 0.9 μg/mg and collagen to 154 ± 34 μg/mg. By day 28 of CEC cultivation, adherent cells had produced their own extracellular matrix (ECM) containing GAGs and collagen. The amount of DNA in it was 6.30 ± 0.11 μg/CEC and that of GAGs was 19.36 ± 0.73 μg/CEC. **Conclusion.** Pretreatment with trypsin allows achieving uniformly complete decellularized CMps. At the same time, onset of changes in the ECM composition indicates a decrease in the ability of hADSCs to synthesize GAGs and type II collagen during co-culturing with DCMps. The increased proliferative activity of adherent hADSCs, as well as the tissue specificity of the DCMp scaffold will allow further research towards a hydrogel matrix capable of enhancing the specific and stimulating regenerative potential when co-cultured with cells of the same phenotype.

Keywords: cartilage tissue, decellularization, trypsin, mesenchymal stromal cells, tissue engineering.

INTRODUCTION

Minimally invasive intra-articular injection of CECs, which consist of resorbable biocompatible matrices (scaffolds, carriers), loaded with stem or tissue-specific cells and bioactive molecules, represents a promising therapeutic solution that could restore the structure and functions of damaged cartilage. Decellularized tissue matrices look encouraging, they are capable not only of keeping the cells in the area around cartilage damage, but also of providing them with the necessary conditions for their vital activity.

Decellularization is the process by which cells are completely destroyed and cellular material is removed from a tissue or organ under certain influences. An important task of decellularization is to preserve ECM components as much as possible, which allows the tissue-specific scaffold to maintain cell adhesion, proliferation and differentiation [1].

Currently, tissue-specific scaffolds are obtained from decellularized whole organs (liver, kidneys, heart, lungs, pancreas) [2–6] or from decellularized organ microfragments [7, 8] using physical, chemical and enzymatic processing methods [9]. Quite often, several processing methods are used concurrently. For example, when decellularizing cartilage tissue, freeze-thaw cycles, treatment with DNase I [10] and supercritical carbon dioxide ($scCO_2$) are added to standard surfactant treatment. This facilitates diffusion of decellularizing agents into the ECM volume [11] and, thus, provides more effective cell lysis [12], reduces time and increases homogeneity of tissue processing.

Corresponding author: Aleksandra Kirillova. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Phone: (963) 633-94-34. E-mail: sashak1994@mail.ru

One method of decellularization of organs and tissues is to treat them with chelating agents such as ethylenediaminetetraacetic acid (EDTA). EDTA promotes cell dissociation from ECM proteins by binding metal ions [13, 14]. Since chelating agents alone are not sufficient to remove cells even with intensive agitation [15], they are usually used in combination with enzymes, including trypsin [16, 17].

Trypsin is commonly used as an enzymatic decellularization agent to detach cells from ECM structural proteins, destroy tissue ultrastructure and improve diffusion into the volume of subsequent decellularizing agents [16, 17]. The degree of removal of cells and ECM components under the action of trypsin depends on the incubation period. Complete decellularization by trypsin alone may require a long incubation period (at least 24 hours), even for non-cellular tissues [18]. Note that proteins have limited resistance to trypsin cleavage [19], so prolonged trypsin exposure even in 0.03–0.05% concentration can significantly reduce the concentration of ECM biomolecules (GAGs, collagen, elastin), disrupt its structure and change mechanical properties [20–22].

So, a combination of two agents – trypsin and EDTA – improves the decellularization effectiveness at the initial stage. At the same time, to minimize the negative effect of trypsin on ECM proteins, trypsin concentration is reduced by adding other methods to the decellularization process, such as treatment of tissue with surfactant or DNase. This is especially relevant for complete removal of cell nuclei from dense tissues such as cartilage [23].

Although pretreatment with trypsin has been described in a number of cartilage-decellularization protocols [24, 25], these studies are limited to analysis of biochemical and mechanical properties of the decellularized matrix, as well as evaluation of its cytotoxicity in vitro and functional efficiency in vivo. Moreover, the effect of a significant decrease in GAG and collagen content in the decellularized cartilage tissue on the ability of adherent hADSCs to synthesize GAG and type II collagen when co-cultured was not investigated.

The objective of this work was to study the effect of including trypsin pretreatment in the porcine articular cartilage decellularization protocol on the ability to restore the biochemical composition and functional properties of the resulting finely dispersed tissue-specific scaffold in the adhesion of hADSCs during their co-culturing.

MATERIALS AND METHODS

Object of study

The object of study, articular cartilage of the hips and knees of pigs, was obtained from agro-industrial holding Promagro based in Stary Oskol, after the slaughter of healthy animals. After transportation in chilled form (+4 °C), the cartilage was removed from articular surfaces with a scalpel, sliced into fragments no larger than 5 × 5 × 5 mm in size, frozen at –20 °C and stored at this temperature until cryogenic grinding began. CMps were obtained by cryogenic grinding method using cryogenic grinder CryoMill (Retsch, Germany).

Particle sizing

Size distribution of CMps in the suspension was determined by laser diffraction using the flow cell of the SALD-7101 laser diffraction analyzer (Shimadzu, Japan). A refractive index of 1.35 was used to measure the size of the microparticles. Glycerol was chosen as the dispersion medium. Data were processed using WingSald II software (Shimadzu, Japan).

Decellularization modes

A 100 mg sample of CMps was placed in a 2,500 units/mg trypsin solution (Sigma-Aldrich, USA) at 0.05%, 0.25%, and 0.50% concentrations with 0.53 mM EDTA (Sigma-Aldrich, USA) and incubated at +37 °C and periodic stirring on a magnetic stirrer (3 times daily for 1 hour at 200 rpm speed) for 24 hours.

The CMps were then treated in three changes of phosphate-buffered saline pH = 7.4 (PanEco, Russia) containing 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA) and increasing concentration of Triton X-100 (Sigma-Aldrich, USA), at room temperature and periodic stirring:

1. Solution containing 1% Triton X-100 + 0.1% SDS, 24 hours.
2. Solution containing 2% Triton X-100 + 0.1% SDS, 24 hours.
3. Solution containing 3% Triton X-100 + 0.1% SDS, 24 hours.

To achieve complete decellularization, the CMps were additionally incubated in DNase I solution (CyStorLab, Russia) for 48 hours at +37 °C.

An aqueous solution of an antibiotic (ampicillin, 20 μg/ml) and an antimycotic (amphotericin B, 2 μg/ml) was used to wash off decellularizing agents from the DCMps.

The washed DCMps matrix samples were sterilized by γ-irradiation with a 1.5 Mrad dose.

DNA quantification

DNA was isolated using the DNeasy Blood&Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. Double-stranded DNA was detected using a Quant-iT Picogreen dsDNA Assay Kit and dsDNA Reagents (ThermoFisherScientific, USA) according to the manufacturer's instructions. Further analysis was performed using a Spark 10M microplate spectrofluorometer (Tecan Trading, Switzerland) at 520 nm wavelength.

Quantification of GAGs

To analyze GAG content, the samples were preincubated in papain solution (Sigma-Aldrich, USA) at +65 °C

for 12 hours. Cationic dye 1,9-Dimethyl-Methylene blue zinc chloride double salt (DMMB) (Sigma-Aldrich, USA) was used for GAG measurement. Staining was performed in a 96-well plate: 20 μL of lysate and 200 μL of the working dye solution were added to the well, and then the GAG content was determined on a Spark 10M microplate spectrofluorometer (Tecan Trading, Switzerland) at 525 nm wavelength.

Collagen quantification

Collagen content was determined using Sircol Soluble Collagen Assay kit (Biocolor, UK) in original tissue samples and in DCMps. For collagen extraction, all samples were lysed in 0.01 M HCl solution containing 1 mg/mL pepsin (Sigma-Aldrich, USA) for 12 hours at room temperature. The resulting lysates were treated with reagents from the kit according to the manufacturer's instructions. The optical density in each sample was determined in 96-well plates at 556 nm wavelength on a Spark 10M microplate spectrofluorometer (Tecan Trading, Switzerland).

Study of the functional properties of DCMps matrix scaffold

The functional efficiency of the tissue-specific DCMps matrix is to maintain the adhesion, proliferation and functional activity of tissue-specific cells in vitro. We investigated the ability of DCMps and hADSCs to form CECs containing GAGs and collagen when co-cultured. The source of hADSCs was the subcutaneous adipose tissue of a healthy donor obtained with informed voluntary consent.

Each CEC included 5 mg of sterile DCMps and 5×10^5 hADSCs obtained under aseptic conditions. The matrix was populated with cells in tubes with complete cell culture medium (CCCM) on a MultiBio 3D orbital shaker (Biosan, Latvia) and then incubated under standard conditions for 3 days. The CCCM contained DMEM/F12 (PanEco, Russia) (1 : 1) with addition of 10% fetal bovine serum (HyClone, USA), 1% antibiotic-antimycotic (Gibco, USA) and 2 mM L-glutamine (PanEco, Russia). CCCM was then replaced with chondrogenic differentiation medium that included DMEM HG (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% transforming growth factor beta 1 (TGF-β1) (PeproTech, USA), and 1% antibiotic-antimycotic (Gibco, USA), and cultured for 28 days. The medium was replaced every second day. Third passage cells were used in the experiment.

Viability of hADSCs in CECs was assessed by in vivo staining using the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific, USA) and a Leica DMi8 Thunder inverted microscope (Leica Microsystems, Germany). Morphology of the samples was examined using histological and immunohistochemistry staining.

Histological and immunohistochemical study

The samples were fixed in 10% formalin solution, washed in running water and dehydrated in alcohols of ascending concentration, incubated in ethanol and chloroform, then in chloroform, and embedded in paraffin. Sections were deparaffinized, rehydrated, and stained with DAPI, hematoxylin and eosin, alcian blue, and Masson's trichrome. Immunohistochemical study for type II collagen was performed using Novocastra Lyophilized Rabbit Polyclonal Collagen Type II; for visualization we used Novocastra Concentrated Peroxidase Detection System (Leica Biosystems, Germany). Analysis and photography of the obtained preparations were performed using an inverted Nikon EclipseTi microscope (Nikon, Japan).

RESULTS AND DISCUSSION

Fig. 1 shows the measurement results of 5 DCMp samples. The microparticle size did not exceed 220 μm,

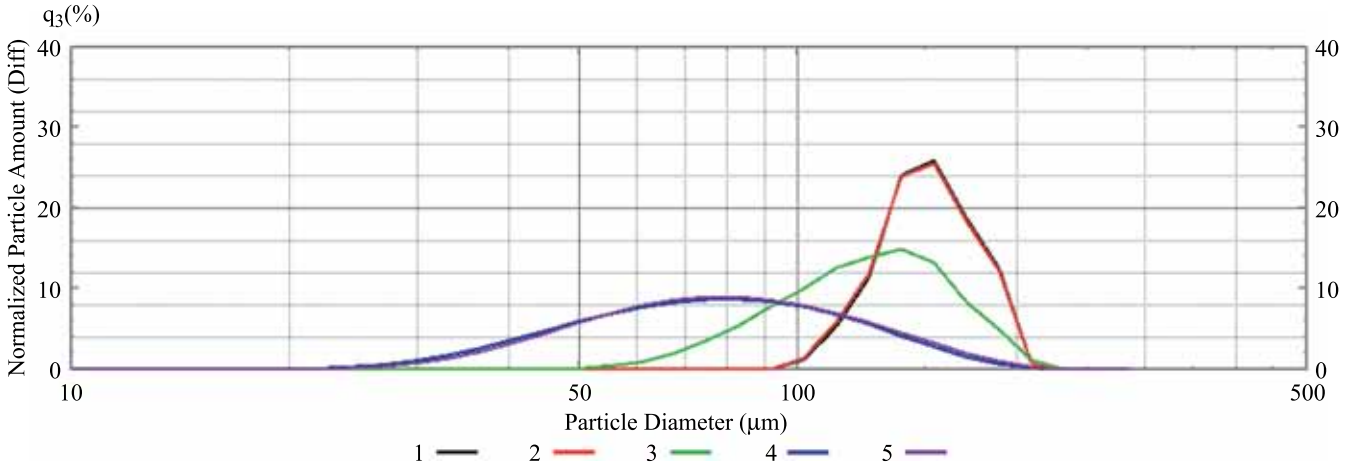

Fig. 1. Microparticle size distribution in suspension

thereby making it possible to develop a minimally invasive DCMps matrix-based injectable form of CEC for use.

Previously, we showed [10] that the use of surfactant alone does not provide an effective degree of CMp decellularization. It was suggested that introduction of a stage featuring pretreatment of CMps with trypsin/EDTA solution followed by incubation of samples in surfactant and DNase solutions would increase the completeness of decellularization.

In the present study, no cells were detected in DCMps during histological analysis, regardless of the trypsin concentration used (Fig. 2). Nuclear material and cell detritus were also not visualized, indicating uniform decellularization of CMps (Figs. 2, d, i, m). The absence of nuclear material in all DCMps matrices (Fig. 2, h, l, p), in contrast to the initial tissue state (after cryodestruction) (Fig. 2, d), was confirmed when samples were stained with DAPI fluorescent dye.

When subjected to Masson's trichrome staining, the ECM of the original tissue was homogeneously stained exclusively blue due to the presence of collagen [26]. At the same time, when DCMps matrix was stained in the same way, metachromasia was detected, which increased with an increase in trypsin concentration (Fig. 2, f, j, n), indicating biochemical changes in the decellularized ECM.

The native ECM of articular cartilage was stained intensively with alcian blue for GAGs (Fig. 2, c), in contrast to DCMps matrix samples, in which no GAGs were detected by the qualitative method (Fig. 2, g, k, o).

Data from histological analysis confirmed the high efficiency of decellularization of articular cartilage tissue – absence of cells and cellular detritus, regardless of trypsin concentration in the range from 0.05% to 0.50%. Therefore, taking into account that enzymatic methods of decellularization, in particular trypsin, reduce the residual number of GAGs in decellularized tissues [27], we performed further studies only with DCMps matrix that was treated with the minimum trypsin concentration

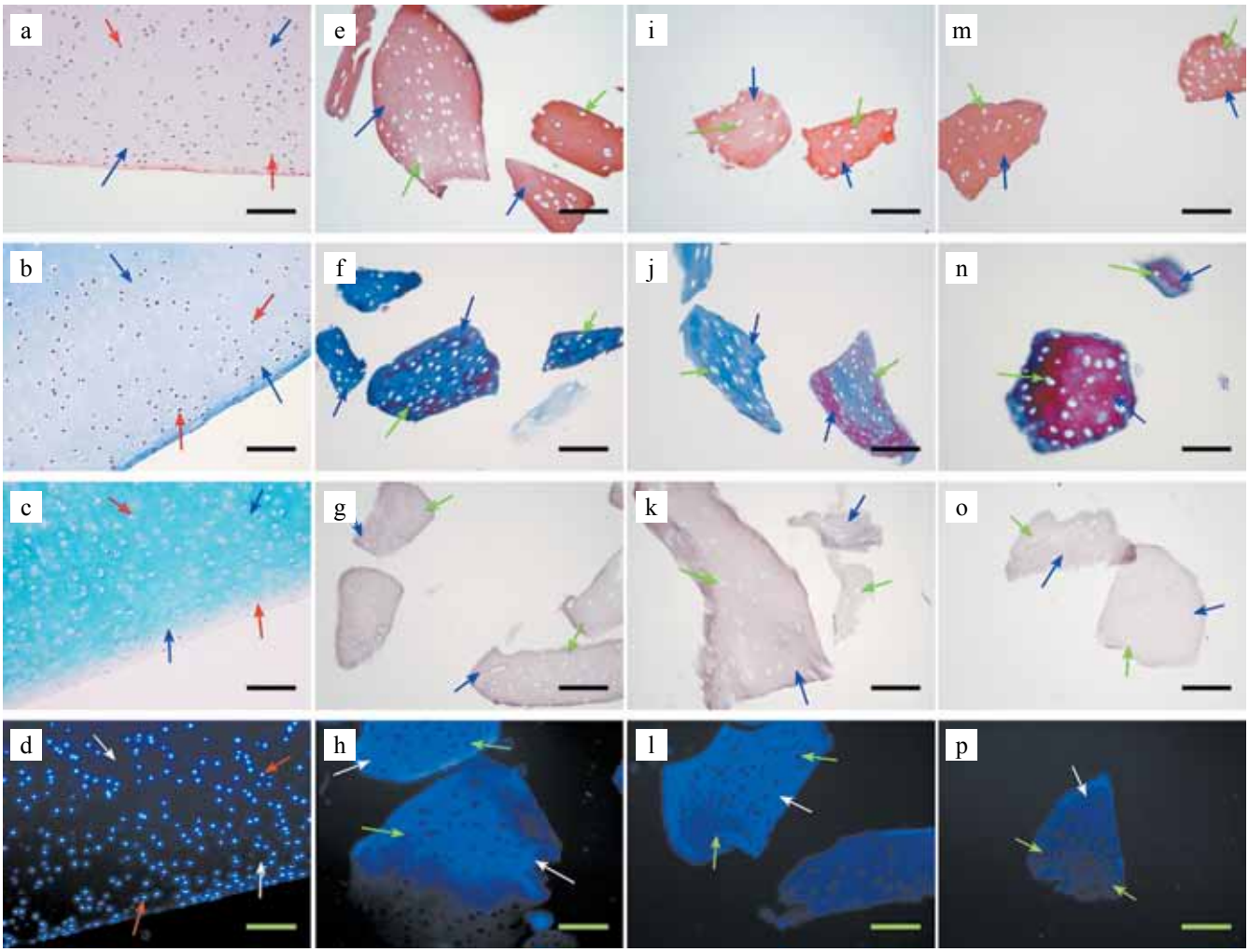

Fig. 2. Histological examination of original and decellularized cartilage tissue. a, b, c, d, original tissue; e, f, g, h, decellularization using 0.05% trypsin; i, j, k, l, decellularization using 0.25% trypsin; m, n, o, p, decellularization using 0.50% trypsin. a, e, i, m, H&E stain; b, f, j, n, Masson's trichrome stain; c, g, k, o, Alcian blue stain; d, h, l, p, DAPI stain. Blue and white arrows indicate microparticles, red arrows show cells, and green arrows indicate empty lacunas. Scale bar = 100 μm

(0.05%), allowing to minimize the negative effect of trypsin on the ECM composition.

Another indicator of the effectiveness of the chosen decellularization method is the results of determining the concentration of residual DNA in DCMps matrix samples compared to the original articular cartilage tissue. After pretreatment with trypsin (0.05% concentration)/EDTA, the DNA content in DCMps matrix decreased significantly from 366.85 ± 53.03 ng/mg of tissue in the original samples to 5.14 ± 0.87 ng/mg of tissue after decellularization.

We have previously explored the effect of including various additional treatment steps in the CMp decellularization protocol. It was found that the optimal, in terms of removing DNA, cells, and cellular detritus, as well as preserving the biochemical composition of DCMps, is a protocol that includes, in addition to surfactant and DNAase, additional ultrasound (US) treatment. With this method of CMp treatment, the residual DNA amount was 1.8 ± 0.6 ng/mg of tissue [28].

DNA residual amount in DCMps matrices that were additionally treated with both trypsin and US [28] was less than 50 ng/mg of tissue, which is the minimum criterion for satisfying the decellularization goal [13]. In addition, visible nuclear material on histological sections of DCMps matrices stained with H&E and DAPI was absent, indicating the low immunogenicity of DCMps matrices.

In the present study, the sharp decrease (almost complete absence) of GAGs in DCMps was confirmed by quantification results: GAG content was found to be 5.34 ± 0.9 μg/mg and 154 ± 22 μg/mg in DCMps and original tissue, respectively.

The content of fibrillar collagen after cartilage decellularization with trypsin also decreased to 154 ± 34 μg/mg compared to its amount in the original cartilage tissue – 508 ± 103 μg/mg. This indicates significant enzymatic hydrolysis of collagen in DCMps when treated with trypsin for a day at +37 °C [29]. It can be assumed that reducing the DCMps incubation time and/or temperature will increase preservation of fibrillar collagen structure in DCMps.

Application of additional US treatment of microparticles allowed us earlier [12] to minimize the loss of GAGs and fibrillar collagen in DCMps matrix: their content was 58 ± 12 μg/mg and 417 ± 47 μg/mg, respectively.

Thus, introduction of enzymatic pretreatment with trypsin at a minimum concentration of 0.05% in the cartilage decellularization process allows us to remove cells and their fragments, as well as to significantly reduce DNA content in DCMps to 5.14 ± 0.87 ng/mg (by more than 90%), which was impossible to achieve when using surfactant without additional treatment methods [10]. However, simultaneously with the decrease in DNA in DCMps, there was a decrease in concentration of fibrillar collagen (by 70%) and GAG (by 97%), indicating a significant destructive effect of trypsin on ECM. We observed a considerable decrease in GAG when using other articular cartilage decellularization protocols as well [28].

The next stage of the work was to investigate the functional properties of the DCMps matrix. In this regard, we carried out an experiment on creation of CECs consisting of DCMps and adherent hADSCs, followed by assessment of the viability of these cells in the process of co-culturing, as well as performing biochemical and histological studies of CECs.

In a previous study, we showed that the immunophenotypic profile of marker expression in cells isolated from adipose tissue met the International Society for Cell & Gene Therapy criteria and confirmed that these cells are multipotent mesenchymal stem cells. The primary cell culture was characterized by a high level of expression of CD29, CD44, CD49b, CD73 and CD90, while no expression of CD34, CD45 or HLA-DR was observed in the culture [30].

Our in vivo fluorescence microscopy showed that by day 21 of hADSC culturing, there was higher cell count on the matrix surface (Fig. 3). We observed the fusion of DCMps and the formation of a single conglomerate. In the main mass of green-stained live cells, dead cells with a red color were also determined.

During histological examination in CECs, we observed DCMps microparticles connected by adherent and newly formed ECM cells into a single conglomerate, as well as pronounced cell proliferation (Fig. 4).

Areas of cell necrosis accompanied by the formation of karyorrhexis products were observed in the central zone by day 14 of cultivation. This is down to the insufficient supply of nutrients deep into the CECs. At the same time, the number of hyperchromic nuclei increased with increasing duration of cultivation, which indicates a disturbance in the structure of the cell nucleus and, accordingly, an increase in the count of cells that are in a state of degradation. In the surface zone of CECs, we observed natural destructive cellular processes only by day 21 of cultivation. When subjected to Masson's trichrome stain, uniformly distributed collagen fibers were seen. When subjected to alcian blue stain, the ECM containing GAG was visualized. The uniformity of ECM staining increased with increasing cultivation time, indicating an increase in GAG production by adherent and newly formed cells.

Immunohistochemical staining of ECM for type II collagen at day 28 of CECs culture revealed positive staining (Fig. 5). However, the staining was not intense, indicating that the adherent and newly formed cells poorly produced the main type of collagen of the articular cartilage.

Biochemical examination of CECs at day 28 of cultivation included determination of DNA and GAG con-

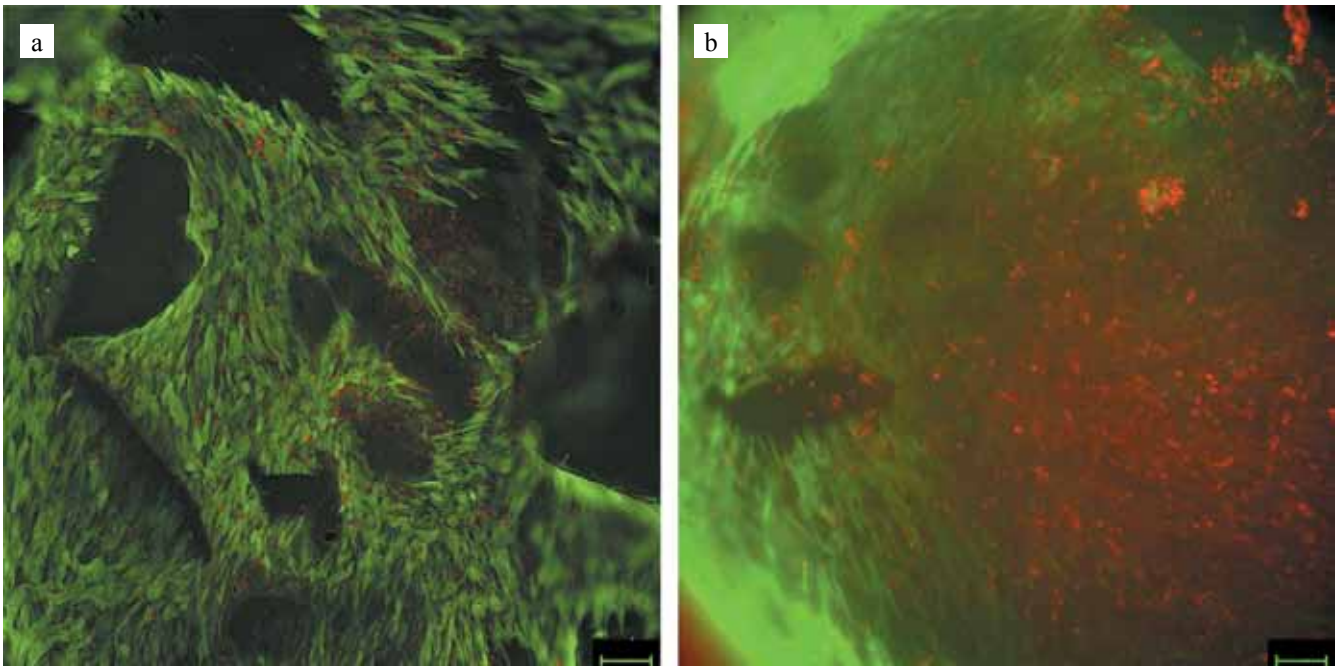

Fig. 3. Study of cell viability in CECs. a, day 14; b, day 21. Live/Dead stain. Scale bar = 100 μm

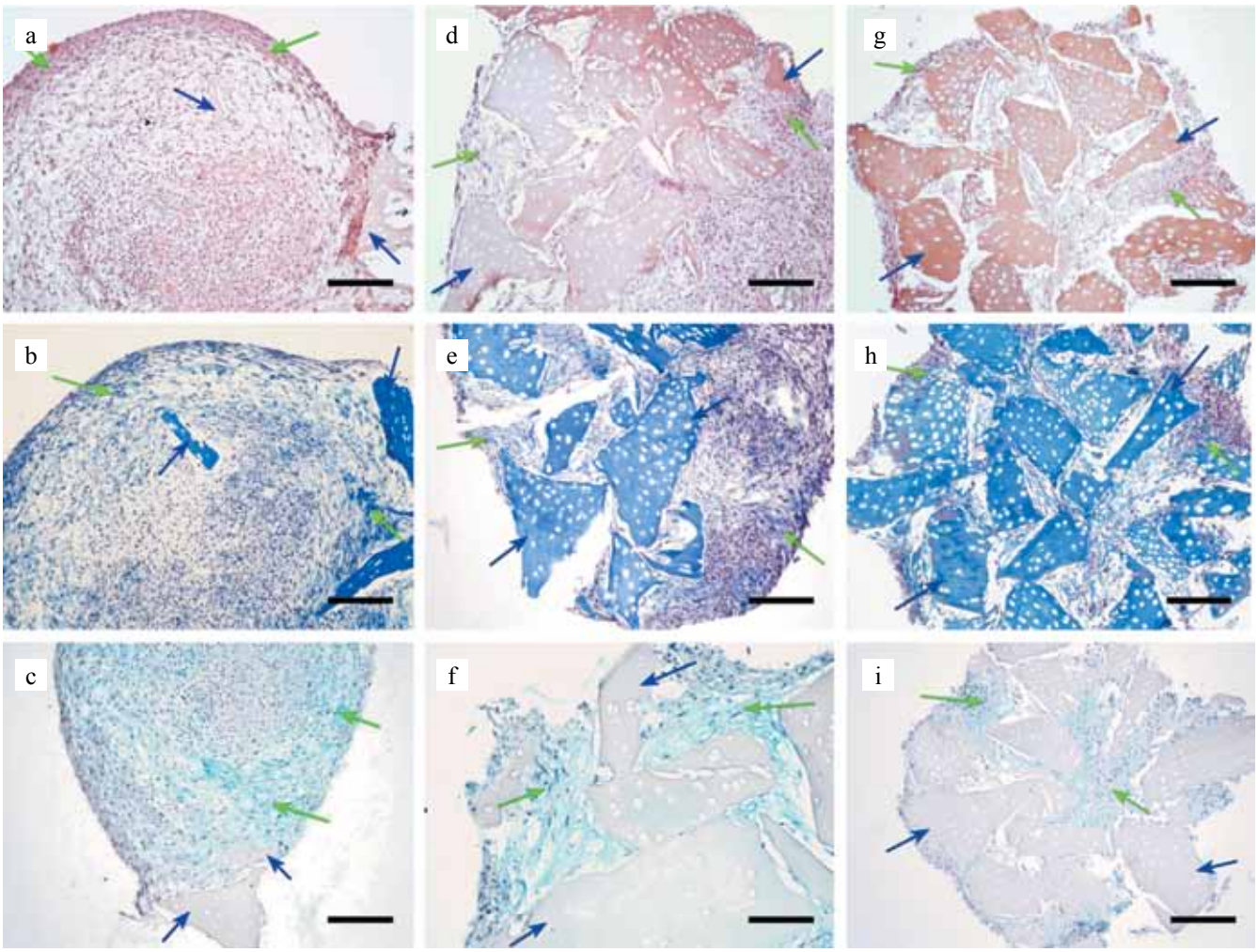

Fig. 4. Histological examination of CEC. a, b, c, day 14 of cultivation; d, e, f, day 21 of cultivation; g, h, i, day 28 of cultivation; a, d, g, H&E stain; b, e, h, Masson's trichrome stain; c, f, i, Alcian blue stain. Blue arrows indicate decellularized porcine articular cartilage microparticles, green arrows indicate ECM produced by adherent and newly formed cells. Scale bar = 100 μm

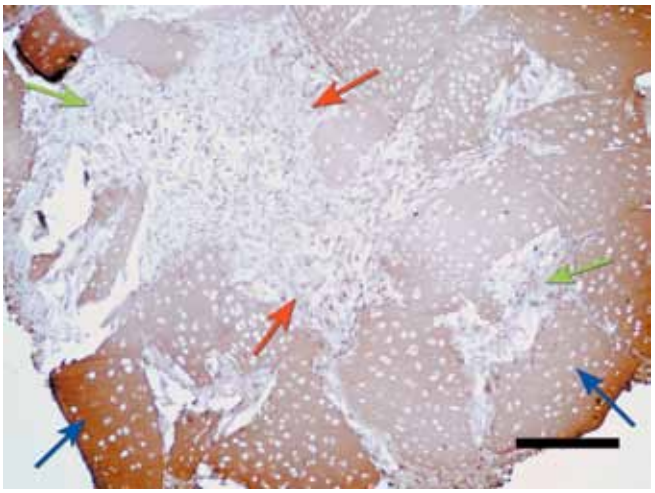

Fig. 5. Immunohistochemical study of CECs for type II collagen. On day 28 of cultivation, CECs contained little type II collagen. Blue arrows indicate decellularized porcine articular cartilage microparticles, green arrows indicate ECM produced by adherent and newly formed cells, red arrows indicate type II collagen produced by cells. Scale bar = 100 μm

centration in them. The amount of DNA was found to increase from 5.14 ± 0.87 ng/mg in DCMps to 6.30 ± 0.11 μg/CEC, and the amount of GAG also increased from 5.34 ± 0.9 μg/mg in DCMps to 19.36 ± 0.73 μg/CEC. Based on these data, the level of GAG production per unit DNA was calculated to be 3.07 ± 0.61 GAG, μg/DNA, μg.

Earlier [28], we have conducted a comparative study of the efficiency of several CMps decellularization protocols with respect to the completeness of cell and gene material removal while preserving the main ECM components as much as possible, and we evaluated the functional properties of CECs. It was found that when hADSCs were co-cultured with DCMps matrix decellularized with by pretreatment with 0.05% trypsin/EDTA, the DNA amount at day 28, reflecting the appearance of new cells, was higher than when using protocols with other additional treatments (freeze/thaw cycles, $scCO_2$, US). However, the number of GAGs produced by adherent cells on DCMps matrices was lower, indicating a negative effect of trypsin/EDTA treatment of ECM. GAG production per unit DNA was also minimal (3.07 ± 0.61 GAG, μg/DNA, μg) compared with other protocols (freeze/thaw: 13.6 ± 2.2 GAG, μg/DNA, μg, $scCO_2$: 7.1 ± 1.2 GAG, μg/DNA, μg, US: 8.4 ± 1.6 GAG, μg/DNA, μg).

These results of culturing hADSCs in CECs confirmed that when introduced trypsin/EDTA pretreatment to the decellularization protocol, DCMps matrix more effectively supports cell adhesion and growth of cell proliferation compared to other protocols but contributes to the reduction of cellular GAG production in ECM [28].

Similar data were obtained in works [31, 32, 33], where the effect of adding trypsin treatment to the bovine and porcine cartilage decellularization process on the properties of the resulting matrix was studied. Additional trypsin treatment resulted in a significant decrease in GAG content, but the decellularized tissue retained its mechanical and biocompatibility properties. In addition, decellularized costal cartilage produced a biosafe and mechanically strong matrix with great potential for clinical application in rhinoplasty.

Trypsin was also used for decellularization of other types of specialized tissues, such as porcine myocardial tissue [34], where the growth and proliferation of endothelial cells on the decellularized matrix was shown with the loss of ECM components and absence of cardiac markers expression by adherent cells.

Results obtained and analysis of literature data show that trypsin has a destructive effect on the biochemical composition of ECM during tissue decellularization – it reduces GAG and collagen content. At the same time, it was found that partial loss of the main ECM components does not negatively affect the use of trypsin-decellularized tissues as biocompatible scaffold implants in areas like rhinoplasty and tracheoplasty [32, 33].

Earlier in the development of tissue engineering and regenerative medicine technologies for the treatment of pathological changes in cartilage tissue, we have pointed out two possible ways of CEC application [35]: stimulation of physiological regeneration of damaged tissue structures and partial or complete temporary replacement of the function of damaged tissue structures.

We have shown that DCMps-based CEC (a tissue-specific ECM mimetic), obtained by a decellularization protocol that includes several treatments [28], is able to form a tissue equivalent of cartilage tissue more effectively than the hydrogel ECM mimetic, a biopolymer-based microheterogeneous collagen-containing hydrogel (BMCH). At the same time, the stimulating effect of DCMps-based CECs on the processes of physiological regeneration was lower than that of BMCH-based CECs [36].

Summarizing the results of our studies, we assume that the detected increase in proliferative activity of cells when trypsin pretreatment is involved in CMps decellularization, as well as the preserved tissue-specificity of the DCMps matrix, will allow to continue research towards creating a hydrogel matrix form with a higher specific (chondrogenic) and prolonged stimulating regenerative effect.

CONCLUSION

The results indicate that trypsin pretreatment allows achieving uniform and complete decellularization of CMps. However, changes in the biochemical composition arising in the obtained DCMps matrices reduce the ability of adherent hADSCs and newly formed cells, when co-cultured in CECs, to synthesize GAG, type II collagen and form ECM, the cell activity environment.

Meanwhile, when trypsin/EDTA pretreatment is included in the CMps decellularization protocol, the proliferative activity of adherent hADSCs increases. The increased proliferative activity as well as the tissue-specificity of the DCMps matrix will allow further research towards creating a hydrogel matrix form that can increase the specific and stimulatory regenerative potential of ECM when co-cultured with cells of the same phenotype.

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The authors declare no conflict of interest.

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