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CRYOGENICALLY STRUCTURED GELATIN-BASED HYDROGEL AS A RESORBABLE MACROPOROUS MATRIX FOR BIOMEDICAL TECHNOLOGIES

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Objective: to investigate the biological properties of a matrix made of cryogenically structured hydrogel in the form of a macroporous gelatin sponge, as well as the possibility of creating cell-engineered constructs (CECs) on its basis. **Materials and methods.** The main components of the cryogenically structured hydrogel were gelatin (type A) obtained from porcine skin collagen, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, (EDC) and urea (all from Sigma-Aldrich, USA). Surface morphology was examined using scanning electron microscopy (SEM). The degree of swelling in water of the samples was determined by gravimetric method. Cytotoxicity was studied on NIH3T3, a fibroblast cell line isolated from a mouse, and on human adipose-derived mesenchymal stem/stromal cells (hAMSCs) using IncuCyte ZOOM (EssenBioscience, USA). The metabolic activity of hAMSCs was assessed using PrestoBlue™ reagents (Invitrogen™, USA). To create CECs, we used hAMSCs, human hepatocellular carcinoma cell line HepG2 or human umbilical vein endothelial cell lines EA.hy926. Albumin content in the culture medium was determined by enzyme immunoassay. Ammonia metabolism rate was assessed after 90 minutes of incubation with 1 mM ammonium chloride (Sigma-Aldrich, USA) diluted in a culture medium on day 15 of the experiment. **Results.** Obtaining a cryogenically structured hydrogel scaffold in the form of macroporous gelatin sponge included freezing an aqueous solution of a gelatin+urea mixture, removal of polycrystals of frozen solvent by lyophilization, extraction of urea with ethanol and treatment of the cryostructurate with an ethanol solution of EDC. Scanning electron microscopy identified three types of pores on the carrier surface: large (109 ± 17 μm), medium (39 ± 10 μm), and small (16 ± 6 μm). The degree of swelling in water of the matrix samples was 3.8 ± 0.2 g H_2O per 1 g of dry polymer. The macroporous gelatin sponge as a part of CEC was found to have the ability to support adhesion and proliferation of hAMSCs, EA.hy926 and HepG2 for 28, 15 and 9 days, respectively. Albumin secretion and ammonia metabolism when HepG2 cells were cultured on the gelatin sponge were detected. **Conclusion.** The use of a matrix made from macroporous cryogenically structured gelatin-based hydrogel for tissue engineering products is shown to be promising using a cell-engineered liver construct as a case.

Keywords: cryogenically structured hydrogel, gelatin, macroporous sponge, tissue engineering, liver.

INTRODUCTION

According to projections for the coming years, acute shortages in donor organs will only worsen. This is already stimulating a search for alternative ways to compensate or replace the functions of damaged vital organs. For these purposes, along with the use of medical methods and artificial organs, technologies based on implantation of cell-engineered constructs (CECs), including matrix carriers loaded with stem and/or specialized cells, appear promising [1].

Independent studies by a number of scientific groups have shown that the creation of CECs based on resorbable biopolymer matrices makes it possible to provide a microenvironment close to the natural extracellular matrix to facilitate cell adhesion, proliferation, differentiation and functional activity [2, 3]. Gelatin, a denaturation product of collagen, is most commonly used to form matrices in the form of sponges, meshes, and hydrogels [4, 5], is not only less immunogenic, but also, like collagen, contains the Arg-Gly-Asp (RGD) amino acid sequence, which determines its adhesive properties [6, 7]. Drug and cell carriers based on gelatin or in combination with other natural or synthetic polymers are widely used in various forms, including capsules and microcapsules, micro- and nanoparticles, micro- and nanofibers, and hydrogels [8–10].

When creating CECs, the preferred matrix forms are macroporous systems, including solid polymer scaffolds

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and sponges [11]. An open network of interconnected macropores (100 to 350 μm) ensures unhindered cell penetration and transport of oxygen, vital products and nutrients [12]. The functional properties of such matrices in CECs depend not only on the porosity, size and degree of interconnectedness of the pores [13], but also on the type of cells seeded on them. For example, for chondrocytes [14] and osteoblasts [15], the best results in adhesion and migration for the inner surface of the hydrogel matrix were achieved in the presence of open 250–325 μm pores, and for fibroblasts – with open pores of no more than 100–160 μm [16, 17].

One of the technological approaches to the formation of 3D carriers in the form of sponges is cryogenic structuring of polymer systems [18–21]. When the formation of covalent or non-covalent knots of three-dimensional mesh occurs in a frozen sample, this process itself is called cryotropic gelation, and the resulting polymeric objects are called cryogels; if there is no gelation, the final products (usually after removal of the frozen solvent) are polymeric objects called cryostructurates [22]. Macroporosity of both cryogels and cryostructurates is their characteristic morphological feature; it is formed by polycrystals of the frozen solvent acting as a porogen [23].

The objective of this work was to investigate the biological properties of a scaffold made of cryogenically structured hydrogel in the form of a macroporous gelatin sponge, as well as the possibility of creating CECs on its basis.

MATERIALS AND METHODS

Obtaining a gelatin-based cryogenically structured hydrogel

Gelatin (type A) obtained from porcine skin collagen, EDC (all from Sigma-Aldrich Inc., USA), urea (high purity) and 96% ethanol (Reachem, Russia) were used without additional purification to obtain macroporous gelatin sponges [24].

Dry gelatin was dispersed in a calculated deionized water volume and then dissolved with stirring at 60 °C. Urea was dissolved in a prepared 6% polymer solution to obtain a 1 mol/L concentration. This solution was then poured into 2 mL plastic Petri dishes (40 mm diameter), which were placed on a strictly horizontal metal plate in liquid cryostat chamber F-32 (Julabo, Germany) with a predetermined negative temperature of –20 °C. Samples were frozen and incubated for 18 hours and then freeze-dried using an ALPHA 1-2 LD plus freeze-drying machine (Martin Christ, Germany). Dry discs were washed with ethanol to dissolve and remove urea until urea was absent in the washing liquid, and then transferred to 0.05 M ethanol solution of EDC, where they were incubated with periodic stirring for 48 hours, and then discs were washed 3 times 30 minutes each with pure ethanol, under which layer obtained samples were stored at 4 °C.

Physicochemical properties and microstructure of cryogenically structured gelatin carrier

The degree of swelling in water of samples of macroporous gelatin matrices was determined by gravimetric method. For this purpose, free liquid was removed from the spongy sample swollen in water under a 145 g load on a glass filter under vacuum (water-jet pump) for 5 minutes. The resulting sample was weighed and then dried (air thermostat SNOL 24/200, AB Utenos Elektrotechnika, Lithuania) at 105 °C until constant weight was achieved.

The degree of swelling (S – swelling), which is an indicator of cross-linking density of 3D polymer mesh of the material, was calculated by the formula:

$$S = \frac{m_{wt} - m_{dr}}{m_{dr}} \text{ (g } H_2O\text{/g polymer)},$$

where m_{wt} is the mass of wet sample, and m_{dr} is the mass of dried sample.

The morphology of the surface and the nearest subsurface of the samples was studied by SEM using lanthanide staining. The processing protocol included an initial wash, exposure in BioREE-A contrasting solution (Glaucon LLC, Russia) for 45 minutes, and a final wash with distilled water. After that, excess moisture was removed from the sample surface using an air brush 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 20–25 kV. The images were captured with a backscattered electron detector (BSE mode). The pore size of the cryostructured carrier was determined by measuring 90 randomly selected pores on SEM images using Image J software (National Institutes of Health, USA).

Cell cultures

Cultures of NIH3T3 (ATCC®CRL-1658™) EA.hy926 (ATCC®CRL-2922™) from the American Type Culture Collection (ATCC) were stored in liquid nitrogen at –196 °C before use. After thawing, NIH3T3 and EA.hy926 were seeded into 25 cm^2 standard culture vials (CELLSTAR® Greiner Bio-One, Germany) and cultured in appropriate complete cell culture medium DMEM with high glucose content (PanEco, Russia) supplemented with 10% bovine serum (CS, Biosera, Germany) or fetal bovine serum (HyClone, USA), antibiotic-antimycotic Anti-Anti (Gibco® by Life Technologies™, USA) and 2 mM alanyl-glutamine (PanEco, Russia), respectively, in a CO_2 incubator under standard conditions: 37 °C, in a humid atmosphere containing (5 ± 1)% CO_2.

A hAMSCs culture was obtained at Shumakov National Medical Research Center of Transplantology and Artificial Organs according to the previously developed technique [25]. The HepG2 cell culture was taken from the collection of cell cultures of Shumakov National Medical Research Center of Transplantology and Artificial Organs. Prior to use, hAMSCs and HepG2 were stored in liquid nitrogen at –196 °C. After thawing, hAMSCs and HepG2 were seeded into standard 25 cm^2 culture vials (CELLSTAR® Greiner Bio-One, Germany) and cultured in complete cell culture medium DMEM/F12 (PanEco, Russia) supplemented with 10% fetal bovine serum (HyClone, USA), 10 μg/mL human basic fibroblast growth factor (FGF-2, Peprotech, AF-100-18B, USA), antibiotic-antimycotic Anti-Anti (Gibco® by Life Technologies™, USA), 1 mM HEPES (Gibco® by Life Technologies™, SC) and 2 mM alanyl-glutamine (PanEco, Russia) in a CO_2 incubator under standard conditions: 37 °C, in a humid atmosphere containing (5 ± 1) % CO_2; hAMSCs (passages 5–6) were used in the experiments.

Before the experiment, cells were removed from the surface of the culture plate using dissociation reagent TrypLE™ Express Enzyme (Gibco® by Life Technologies™, UK) and a suspension with the required cell concentration was prepared.

The initial number of cells in the suspension was determined on an automated cell counter (TC20™ Automated Cell Counter, BIORAD, Singapore) with simultaneous viability analysis by trypan blue dye exclusion (BIORAD, # 145-0013, Singapore).

Medium cytotoxicity assessment

To determine the cytotoxicity of gelatin sponge samples, NIH3T3 mouse fibroblasts line were seeded into flat-bottomed 6-well culture plates (CELLSTAR® Greiner Bio-One, Germany) at a concentration of 5 × 10^5 cells per well and incubated for 24 hours at 37 °C in a humid atmosphere containing (5 ± 1)% CO_2 until a (80 ± 10) % monolayer was formed. Then gelatin sponge samples were placed on the surface of the cell monolayer in the form of disks 6 mm in diameter and 2 mm thick, thoroughly washed from ethanol residues with two portions of sterile distilled water and left for 24 hours in complete cell culture medium (CCCM) at 37 °C. The CCCM served as the negative control sample, while single-element aqueous zinc standard 10 mg/mL (Sigma-Aldrich, USA) served as a positive control sample.

For a more detailed assessment of growth dynamics, additional plates in which cells were incubated from the moment of introduction in the presence of sponge samples using IncuCyte ZOOM system (EssenBioscience, USA), which makes it possible to automatically estimate the monolayer density in automatic mode every 2 hours throughout the experiment with simultaneous construction of growth curves. The experiment lasted for 90 hours.

Assessment of cell adhesion and proliferation support

For a comparative study of the influence of hydrogel matrices on hAMSCs growth parameters, we used gelatin sponge samples (cylinders 6 mm in diameter and 2 mm thick) and biopolymer-based microheterogeneous collagen-containing hydrogel (BMCH, BIOMIR Service JSC, Russia) (0.2 mL) with the following characteristics: average microparticle size 145.79 ± 0.09 μm; elastic modulus 1170 ± 12 Pa; viscosity modulus 62.9 ± 7.9 Pa; resorption time – up to 9 months. BMCH has been shown to be effective as a matrix for creating various medical and biological products [1, 26].

To assess the ability of the test samples to support adhesion and proliferation of hAMSCs cultures, 1 mL of cell suspension with a 1 × 10^5 cells/ml concentration was dropwise applied to the surface of the sample presaturated with CCCM for 24 hours at 37 °C. The samples were placed in 50 mL centrifuge tubes and left in a CO_2 incubator for cell attachment for 1 hour, after which the level of CCCM in the tubes was brought to 5 mL and cultivation was continued under standard conditions. Test tube lids were loosely closed to maintain gas exchange. On days 1, 3, 6, 9, and 14, three portions of CCCM were taken for the metabolic activity test with PrestoBlue™ HS Cell Viability Reagent (Invitrogen™ by Thermo Fisher Scientific, USA) according to the protocol recommended by the manufacturer. Spectrophotometric analysis was performed using a Spark 10 M microplate reader (Tecan, Austria) with Spark Control™ Magellan V1.2.20 software at 570 nm and 600 nm wavelengths. Optical 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} is the optical absorption at 570 nm, and Abs_{600} is the optical absorption at 600 nm.

The number of cells corresponding to the value of the obtained coefficient K was determined from the calibration graph, which was plotted using the values of metabolic activity coefficients corresponding to the known numbers of cells.

CECs based on cryogenically structured gelatin matrix and different cell types

To create CECs based on macroporous gelatin sponges and hAMSCs, HepG2 or EA.hy926 cells, suspensions of corresponding cultures with a 1 × 10^6 kl/ml concentration were prepared. Sponge samples in the form of discs, 1 cm^2 in area and 2 mm thick, were immersed in the suspension and processed for 1 hour using a laboratory

shaker in orbital stirring mode at 40 rpm to improve cell penetration deep into the spongy structure of the sample. The resulting CECs were cultured under standard conditions for 9, 15, and 28 days using HepG2, EA.hy926, and hAMSCs, respectively.

The pattern of cell distribution over the sample volume, viability, morphology, and proliferative activity were assessed by in vivo microscopy with fluorescent dyes Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes® by Life Technologies™, USA) according to the protocol recommended by the manufacturer.

Functional properties of HepG2 cells when cultured on macroporous gelatin sponge

HepG2 cells (5 × 105 kl) were plated on a 10 × 10 × 2 mm fragment of gelatin sponge. The resulting CECs were cultured in CCCM under standard conditions for 15 days. On day 15, albumin content in the culture medium was determined by enzyme immunoassay using Human Albumin ELISA Kit (Invitrogen™ by Thermo Fisher Scientific, USA). As a control, we used culture medium from cells that were cultured on plastic in the same quantity.

Ammonia metabolism rate was determined after 90 minutes of incubation with 1 mM ammonium chloride (Sigma-Aldrich, USA) diluted in culture medium on day 15 of the experiment. The amount of urea in the medium was estimated on a KonelabPrime 60i biochemical analyzer (ThermoFisher Scientific, Finland).

Significance of differences was determined by Student's t-test (standard software package Microsoft Excel 2007). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Fig. 1, a shows the appearance of a sample of gelatin-based cryogenically structured hydrogel in the form of a macroporous sponge. Scanning electron microscopy allowed us to distinguish three types of pores on the surface of the carrier: large (109 ± 17 μm), medium (39 ± 10 μm), and small (16 ± 6 μm) (Fig. 1, b).

Note that large pores are able to ensure migration of cells into the sponge thickness, while the importance of medium- and small-sized pores lies in supporting the efficient mass transfer of nutrients and gases.

Cytotoxicity of cryogenically structured gelatin matrix

Assessment of the cytotoxicity of the gelatin sponges obtained in this work by direct contact revealed no negative effect on the development of NIH3T3 cells. No changes in cell morphology or a decrease in cell proliferation were found both during the first hours and after 72 hours of cell-carrier contact.

The obtained data confirm the cell growth curves when cultured on the culture plate in the presence of gelatin sponge, demonstrating an increase in confluence of cell monolayer in all variants of the experiment with the dynamics characteristic of this cell culture (Fig. 2). Note that to correctly compare the data of two curves shown in Fig. 2, we need to introduce a correction factor of 1.12 for the experimental variant that takes into account the well area (9.6 cm^2) occupied by the sample (1 cm^2) and excluded in the automatic analysis of images. Taking into account the correction, there were no significant differences in the confluence of the cell monolayer on the plateau in the experiment (the confluence of the monolayer was 94 ± 5% without sample and 85 ± 6% with sample).

The ability of the cryogenically structured gelatin sponge to support cell adhesion and proliferation, confirmed on NIH3T3 cells, allowed us to proceed to creation of CEC – cultivation of human cells (hAMSCs, EA.hy926 and HepG2) on the cryogenically structured gelatin sponge.

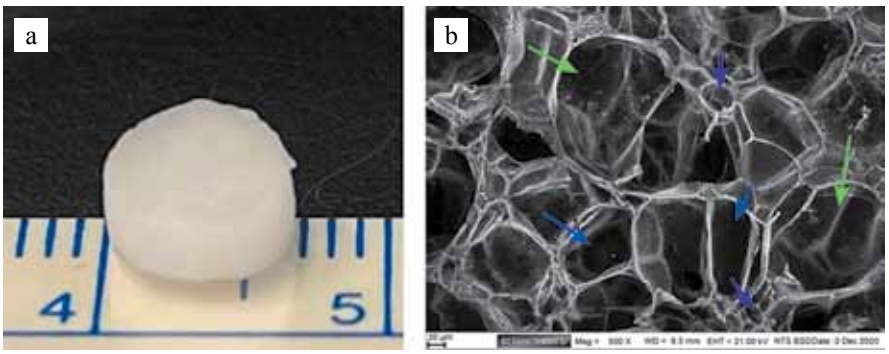

Fig. 1. Gelatin-based macroporous sponge morphology. a, View of the carrier; b, Microphotograph of the surface structure. SEM using BioREE lanthanoid contrasting. Scale bar 20 μm. Green arrows, large pores; blue arrows, medium-sized pores; purple arrows, small pores

Metabolic activity of hAMSCs during culturing

Mesenchymal stem cells (MSCs) perform various biological functions, which determines their relevance in tissue engineering. Firstly, MSCs can differentiate into various directions, including chondrogenic, osteogenic, adipogenic, myogenic and neurogenic differentiations [27]. Secondly, MSCs secretion has a positive effect on the therapy of various diseases [28].

The number of hAMSCs for the study of metabolic activity when cultured on a cryogenically structured gelatin sponge and BMCH was 100,000 cells/mL. The growth curves show that cell adhesion on the surface is only 20–25% of the applied amount (Fig. 3). In the case of the gelatin matrix, a lag phase was observed, which was necessary for cell adaptation and plating, after which active proliferation began after 3 days, and by day 6 of the experiment, the number of proliferating cells had increased 4–5-fold to 100,000 cells. Then, after a slight plateau up to 9 days, there was further logarithmic growth of the cell population up to the end of the experiment – 14 days. The absence of an increase in proliferative activity during 6–9 days on the cryogenically structured gelatin matrix and continuation of the logarithmic growth of the population afterwards, apparently, are related to cell colonization of the most accessible macropore surface and cell migration into the sample volume. When cultured with BMCH, cell adaptation is much faster, as evidenced by the absence of a pronounced lag phase. Intensive proliferation is observed almost from the beginning of the experiment and already by day 3, the number of cells is twice as high as that of gelatin sponge. Also, more rapid and active cell growth was observed, a plateau being reached by day 9. The maximum cell number per sample reached about 280,000 cells for BMCH on day 9 and about 220,000 cells for the cryogenically structured gelatin hydrogel on day 14 of the experiment. The decrease in the number of cells by day 14 of cultivation with BMCH as compared to day 9 indicates the onset of the cell death phase, probably related to the lack of a free carrier surface for cell colonization. In general, hAMSCs, when cultured on the investigated hydrogels, showed growth dynamics typical of this cell type on the culture plate [29].

Thus, a more intensive proliferative activity of hAMSCs is observed when they are cultured on BMCH, while cell mass growth is slower in the presence of a gelatin matrix. However, at day 14, the number of hAMSCs with metabolic activity is higher in the case of gelatin sponge than for BMCH.

Cultivation of cells of different types on a cryogenically structured gelatin sponge

It was shown that mesenchymal, epithelial and endothelial cells exhibit a high level of adhesion to the matrix surface, actively proliferate and repopulate the carrier surface when applied in an amount of 500,000 per 1 cm^2. The use of "stacking", i.e., shifting the focus point of the microscope objective deep into the sample at a depth of about 100 μm, followed by software image processing, showed that the cells spread into the internal volume of the sponge as well.

The most prolonged cell growth was observed in the case of culturing hAMSCs on a gelatin sponge (Fig. 4), and by day 28, dense 3D structures with a high cell density were formed in the matrix volume.

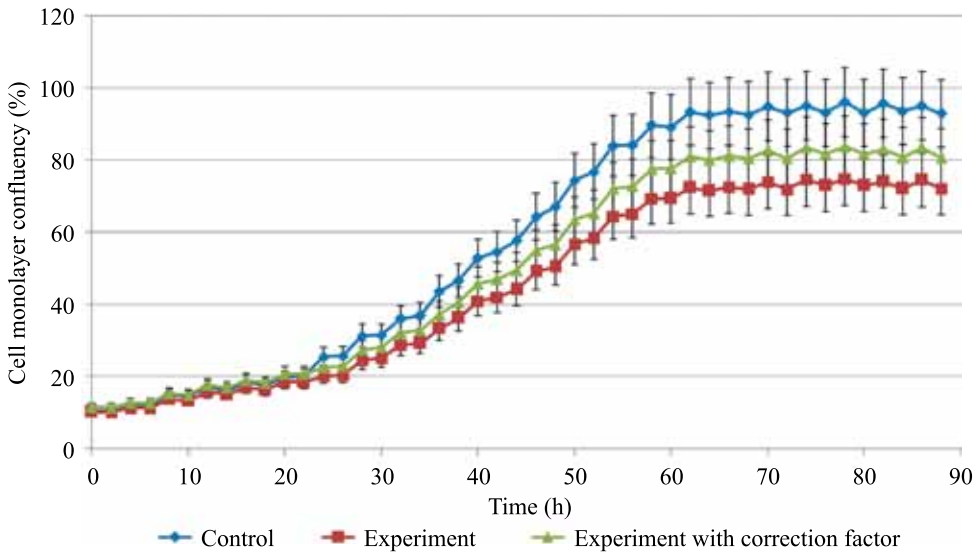

Fig. 2. Growth curve of NIH3T3 on culture plate in the presence of cryogenically structured gelatin sponge (experiment) and without cryogenically structured gelatin sponge (control)

As seen in Fig. 4, b, ethidium homodimer-1, in addition to dead cells, stained the matrix red, allowing the pore walls to be visualized.

HepG2 cells, which are usually used as an in vitro model of hepatocytes, also actively proliferated on the matrix (Fig. 5).

On day 3 of the experiment, there was active cell proliferation and spread over the area of the carrier. On day 7, formation of cell clusters occurred in the samples, and by day 9, the surface of the gelatin sponge macropores was almost completely populated with cells (see Fig. 5).

One of the conditions for creation of tissue equivalents is matrix vascularization [30]. Immortalized cell lines, including EA.hy926 line, demonstrating similarity to primary endothelial cells, are widely used to create models of capillary system in tissue-engineered constructs in vitro [31]. When EA.hy926 was cultured on a macroporous gelatin matrix, it was rapidly and uniformly populated with cells (Fig. 6).

By day 15 of cultivation, dense cell structures were formed on the surface with cells sprouting into the sponge volume. At the same time, the proportion of living cells prevailed over dead ones.

Assessment of the functional properties of HepG2 when cultured on a cryogenically structured gelatin carrier

The presence of functional properties of the created CEC were analyzed by albumin synthesis and urea production. The Table shows the results of the assessment

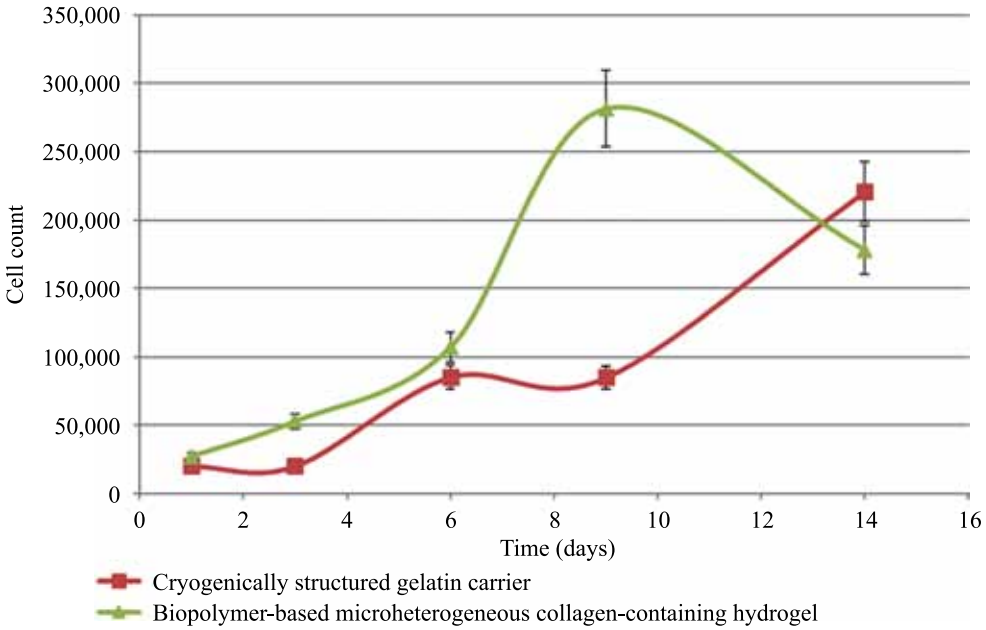

Fig. 3. Growth curves of hAMSCs in a cryogenically structured gelatin sponge and a collagen-containing biopolymer-based hydrogel

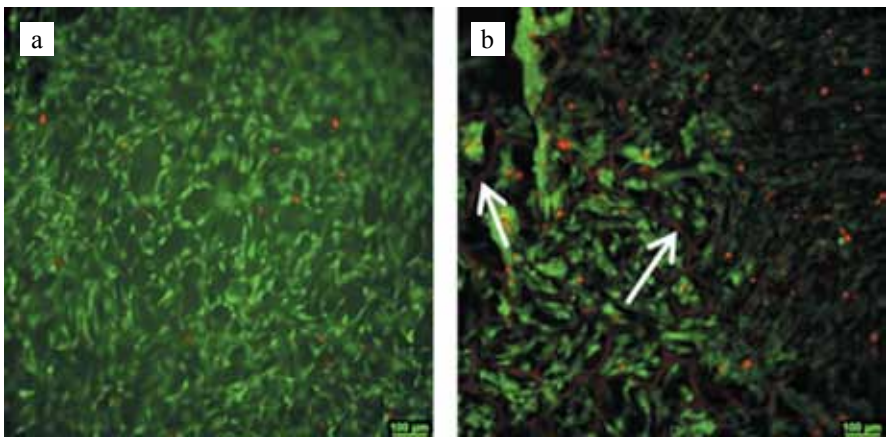

Fig. 4. Growth of hAMSCs in a cryogenically structured gelatin sponge: a, 9 days in culture; b, 28 days. Live/Dead™ staining, live cells are stained green, dead cells are stained red. Arrows show the walls of the sponge pores. Scale bar 100 μm

of albumin synthesis by HepG2 cells in suspension and in the CEC.

The data obtained indicate that HepG2 seeded on a gelatin sponge can maintain its secretory function and ammonia metabolism at 15 days of cultivation at a higher level than as a cell suspension.

CONCLUSION

The biological properties of a gelatin-based cryogenically structured hydrogel as a resorbable macroporous sponge were studied.

The absence of cytotoxicity and the presence of functional properties of the samples in vitro were proved on NIH3T3, hAMSCs, EA.hy926 and HepG2 cultures. Using the example of a liver CEC, creation of tissue-engineered products using a matrix of macroporous gelatin-based cryogenically structured hydrogel were shown to have some prospects.

The works carried out in terms of obtaining samples of gelatin-based cryogenically structured hydrogel were partially funded by the Ministry of Science and Education of the Russian Federation.

The authors declare no conflict of interest.

Table

Albumin content and urea level in the culture medium samples on day 15 of HepG2 culturing in a suspension (control) and in cryogenically structured gelatin sponge (experiment)

	Albumin, mmol/mL	Urea, mmol/L
Cultivation in suspension	997 ± 139	1.1 ± 0.1
Cultivation in a cryogenically structured gelatin sponge	1560 ± 312	1.8 ± 0.4

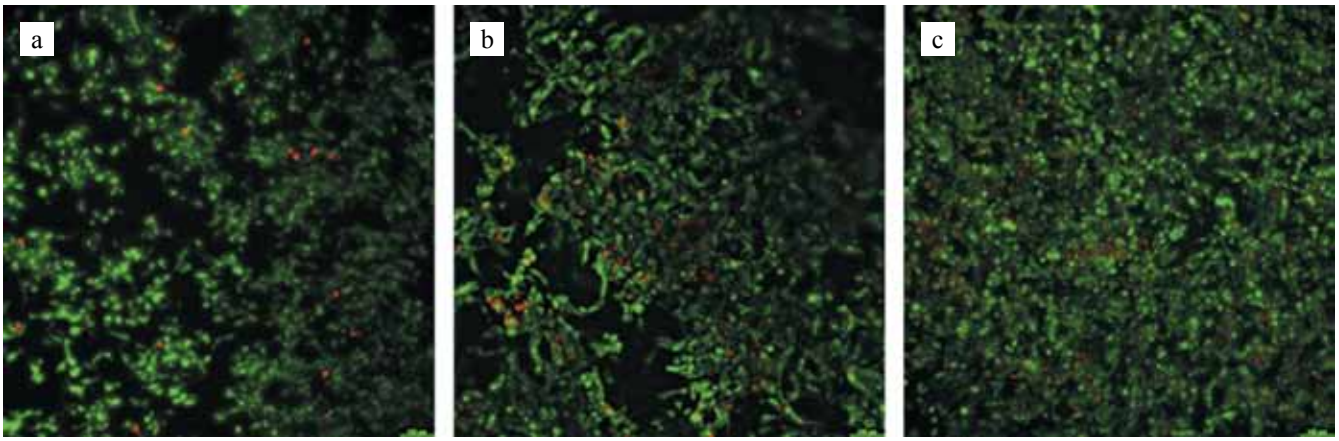

Fig. 5. Growth of HepG2 in a cryogenically structured gelatin sponge: a, 3 days in culture; b, 7 days; C, 9 days. Live/Dead™ staining, live cells are stained green, dead cells are stained red. Scale bar 100 μm

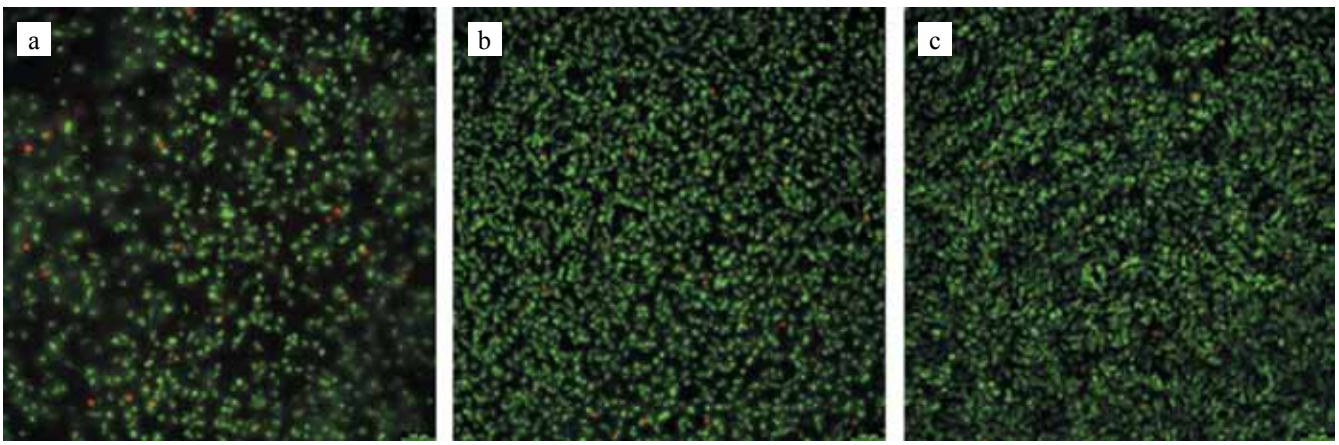

Fig. 6. Growth of EA.hy926 in a cryogenically structured gelatin sponge. a, 2 days in culture; b, 7 days; c, 15 days. Live/Dead™ staining, live cells are stained green, dead cells are stained red. Scale bar 100 μm

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