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EXPERIMENTAL APPROACHES TO CREATING A TISSUE-SPECIFIC MATRIX FOR A BIOARTIFICIAL LIVER

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Shortage of donor organs for liver transplantation in the treatment of end-stage liver disease dictates the need to develop alternative methods that include technologies on tissue engineering and regenerative medicine. **Objective:** to study the ability of a tissue-specific matrix from decellularized human liver fragments (DHLF) to maintain adhesion and proliferation of human adipose tissue-derived mesenchymal stem cells (hAT-MSCs) and HepG2 under static conditions and in a flow-through bioreactor. **Materials and methods**. Treatment with surfactants (SAS) – sodium dodecyl sulfate, Triton X-100 – followed by exposure to DNase was used for decellularization of human liver fragments (no more than 8 mm^3). Biochemical screening included the determination of DNA quantity in the test samples. Efficiency of surfactant washing was assessed by the cytotoxicity of the matrix in the NIH 3T3 fibroblast culture. Viability and metabolic activity of cells were assessed via vital staining with a complex of fluorescent dyes LIVE/DEAD® and PrestoBlue™ (Invitrogen, USA). Morphological examination of the liver cell-engineered constructs was carried out through histological staining and scanning electron microscopy with lanthanide contrast. **Results.** It was shown that the liver decellularization method used allows to obtain a biocompatible matrix with a residual DNA quantity <1%, which is capable of maintaining adhesion and proliferation of hAT-MSCs and HepG2. On day 7 of cultivation in the bioreactor, there was formation of a single conglomerate of the DHLF matrix with numerous groups of viable cells with a high nuclear-cytoplasmic ratio. The urea content in the culture medium is 1.5 ± 0.1 mmol/L, exceeding that of samples obtained under static conditions. This indicates the metabolic activity of HepG2 in the composition of the obtained culture systems. It was shown that constant flow of the culture medium in the perfusion bioreactor increased the proliferative activity of HepG2 and allowed to provide a more uniform colonization by matrix cells in comparison with static cultivation conditions. **Conclusion.** The conditions for uniform colonization of DHLFs in a flow-through bioreactor with cell cultures were established. The ability of the matrix to maintain adhesion and proliferation of hADSCs and HepG2 for 11 days indicates that it could be used in liver tissue engineering.

Keywords: liver, decellularization, mesenchymal stem cells, HepG2, cell-engineered construct, tissue engineering, bioreactor.

INTRODUCTION

Shortage of donor organs for liver transplantation in the treatment of terminal stages of liver failure dictates the need to develop alternative methods, which include technologies of tissue engineering and regenerative medicine [1, 2].

Tissue decellularization is a promising method for creating matrices for regenerative medicine due to the removal of immunogenic factors (DNA, galactose-α-1,3-galactose) and preservation of morphology that is largely specific for organs and tissues and the natural extracellular matrix (ECM), which includes the necessary sites for adhesion, migration and proliferation of cells [3, 4].

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Significant progress has now been achieved in decellularization of the whole liver by perfusion with surface acting agent (SAA) solutions [5, 6]. The attractiveness of this method of decellularization lies in the potential for obtaining whole organs when populated with cells using perfusion and transplantation of the obtained tissue-engineered structures in vivo by creating anastomoses with preserved vascular structures [7, 8]. However, decellularization of the whole liver has a number of disadvantages: low efficiency of removal of cells and

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their fragments and recellularization due to microcirculation disorders and the difficulty of transporting sufficient amounts of oxygen and nutrients to cells in the thickness of the organ. In this regard, the most rational approach seems to be the decellularization of not the whole organ, but its fragments. Earlier, we developed a method for decellularization of human liver fragments, which made it possible to obtain a matrix with a complete absence of cellular debris and a preserved tissue structure [9]. Note that the interaction of cells with the resulting tissue-specific matrix has not been studied.

The inherent immunomodulatory properties of mesenchymal stromal cells (MSC), the ability to stimulate regeneration, and the ability to differentiate into hepatocyte-like cells indicate the possibility of their use for creating cellular engineering structures (CES) of the liver for the treatment of various liver diseases [10]. Due to the complexity of expansion and low viability of primary hepatocytes during in vitro cultivation, it is advisable to use cell lines capable of performing functions characteristic of liver cells to study the interaction of specialized cells with fragments of decellularized liver [11]. Note that although the metabolic functions of hepatocellular carcinoma HepG2 cells are limited compared to primary hepatocytes, their use as an in vitro hepatocyte model is due to the availability of HepG2, ease of handling, almost unlimited lifespan and phenotype stability [12].

It is known that to create the conditions closest to natural during *in vitro* cultivation, bioreactors are used, which make it possible to improve cell nutrition, transport gases to them, and excretion of metabolic products due to constant circulation of the culture medium [13]. Nevertheless, the cultivation of cells in a flow is not without drawbacks associated with mechanical damage to cells, high consumption of culture media, and the difficulty of maintaining aseptic conditions during long-term experiments.

The purpose of the present study was to study the ability of tissue-specific matrix from decellularized fragments of human liver (DFHL) to maintain adhesion and proliferation of MSC HGT and HepG2 under static conditions and in a flow-through bioreactor.

MATERIALS AND METHODS

A donor human liver wasused, washed according to the traditional method from blood elements with a Custodiol solution (Dr. F. Koehler Chemie GmbH, Germany). The liver was not suitable for transplantation due to severe fatty hepatosis (more than 40%). The washed liver or part of it was placed in sterile saline, if steatosis was detected before washing the vascular bed, or in the preservative Custodiol, if the presence of pronounced fatty infiltration was determined after removing blood elements from the liver.

Fragments of the liver (no more than 2 × 2 × 2 mm) were obtained using a scalpel and scissors. Decellularization of human liver fragments was carried out in three changes of phosphate-buffered saline (PBS) (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 (SDS) and an increasing concentration of Triton X-100: 1%, 2% and 3% [9]. The total time of decellularization was 72 hours – 24 hours for each change of SAA solution with stirring on a magnetic stirrer.

Then, fragments of human liver were treated in a solution of DNase I type (New England Biolabs Inc., USA). Matrix samples with a volume of 0.5 ml were placed in 1.0 ml of 10 mM Tris-HCl buffer solution (pH 7.6) containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 50 U/ml DNase I and incubated for 48 h at 37 °C.

Washing DFHL from SAA included an exposure of the matrix in PBS containing an antibiotic (ampicillin, 20 μg/ml) and an antimycotic (amphotericin B, 2.0 μg/ml) for 96 h, followed by sterilization of the samples by γ-irradiation (1.5 Mrad).

The residual DNA content was an indicator of the cellular components preserved in the DFHL samples, which carry the bulk of the antigens that cause the reaction of the xenogenic matrix rejection. The DNeasy Blood & Tissue Kit (QIAGEN, Germany) was used to isolate DNA from the original and decellularized tissue according to the manufacturer's protocol. Samples of the original human liver (n = 3) and DFHL (n = 3) weighing 10 mg were lysed using lysis buffer and proteinase K for 16 hours at +56 °C. For the quantitative determination of DNA in the samples, the fluorescent dye ™Picogreen Quant-iT (Invitrogen, USA) was chosen.

The source of MSC HGT was the subcutaneous adipose tissue of a healthy donor taken from him with informed voluntary consent. A sample of subcutaneous adipose tissue was crushed with a scalpel, washed twice with cold (+4 … +6 °C) Hanks solution, and then incubated in a 0.1% type I collagenase solution (Gibco, USA) at 37 °C for 20 min. Then the resulting suspension was sequentially passed through cell sieves with a pore diameter of 100 and 70 μm.

All cells were precipitated by centrifugation, resuspended in complete growth medium (ORS) of DMEM/F12 composition (1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Gibco, USA) and cultured until the formation of a monolayer, changing the medium 2 times a week. The cells were transferred into suspension by treatment with a Versene solution at 37 °C for 1 min, followed by the addition of TrypLe™ dissociating agent (Invitrogen, USA). For experiments, cells of the 3rd passage were taken.

Human hepatocellular carcinoma cell culture HepG2 was obtained from the laboratory collection of cell cultures of the Department of Biomedical Technologies and Tissue Engineering of the Shumakov National Me-

dical Research Center of Transplantology and Artificial Organs.

The cytotoxicity of the matrix samples was studied in accordance with GOST ISO 10993-5-2011 [14] by the method of direct contact of the samples with the culture of mouse fibroblasts of the NIH 3T3 line obtained from the collection of transplanted somatic vertebrate cells of the D.I. Ivanovsky Research Institute of Virology.

Mouse fibroblasts were seeded in cultured flat-bottom 6-well plates and incubated for 24 hours at 37 °C under standard conditions in a humid atmosphere containing (5 ± 1)% CO_2. The samples under study were placed on the surface of the formed (80 ± 10)% monolayer of cells. After a day of incubation, the morphology and lysis of cells were visually assessed according to a standard technique using a Nikon Eclipse TS100 biological microscope (Nikon, Japan). The negative control was general nutrient medium containing fetal bovine serum, the positive control was a standard solution of zinc in nitric acid (9.95 mg Zn in 1–2 wt.% HNO_3, dilution 1:200 with 0.9% NaCl solution for injection).

HepG2 and MSC HGT were cultured according to the standard method to a monolayer confluence of 70–80%, after which they were washed off the plastic using Versene solution and TrypLe™ reagent (Invitrogen, USA) and a working cell suspension was prepared in general nutrient medium with a concentration of 1 × 105 cells/ml. Cell counting and assessment of their viability were performed on a TS20 cell counter (BioRad, USA) according to the manufacturer's method.

Matrix pellets (30 mg) were thawed and placed in ORS for a day at room temperature. DFHL cells were additionally washed twice with fresh ORS before plating. An aliquot of the cell suspension was added to each DFHL tube to populate the matrix. The tubes were placed in a rack and shaken on a MultiBio 3D laboratory shaker (Biosan, Latvia) in a reciprocal platform rotation mode at 80 rpm for 2 hours to evenly distribute the cells over the matrix surface. Then the tubes were placed in an incubator and cultured under standard conditions.

The viability and adhesion of cells were assessed by the method of in vivo staining with a complex of LIVE/DEAD® fluorescent dyes (Invitrogen, USA). This complex includes two components: calcein AM gives green fluorescence of living cells, recorded at a wavelength of 515 nm, ethidium homodimer-1, penetrating through the damaged cell membrane and binding to DNA, gives red fluorescence at 635 nm. Microscopy was performed on a Nikon Ti fluorescence microscope (Nikon, Japan).

Determination of the metabolic activity of cells was performed by the test with a PrestoBlue™ reagent (Invitrogen, USA) according to the manufacturer's instructions. Spectrophotometric analysis was performed on a Tecan Spark10 plate reader (Tecan, Austria). In the study of metabolic activity, 5 × 104 HepG2 cells or 2 × 104 MSC HGT were applied to each DFHL pellet. The absorbance data were used to calculate the metabolic activity coefficient (K) by 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} – absorption at 570 nm wavelength; Abs_{600} – absorption at 600 nm wavelength.

When creating CES, 1 × 105 HepG2 cells or 1 × 105 MSC HGT were applied to 1 DFHL pellet. To carry out experiments on the cultivation of HepG2 on DFHL under flow conditions, a modified version of the perfusion bioreactor was used [13]. The flow rate was 0.02 ml/min. On the 7th day, the culture chamber with CES was removed from the bioreactor.

The following types of samples were studied: a fragment of a native human liver, a fragment of a decellularized human liver, CES – MSC HGT, cultured on a decellularized liver fragment.

The morphology of the surface and the nearest subsurface layer of the samples was studied jointly with the staff of the Laboratory of Basic Research in Ophthalmology of the Research Institute of Eye Diseases by scanning electron microscopy (SEM) using lanthanide contrasting.

The preparation of water-containing samples for SEM with deposition of a conductive layer requires their dehydration, which leads not only to structural changes in such objects, but also poor visualization of cellular elements. The lanthanide contrasting method makes it possible to observe non-fixed biological samples in a low vacuum mode after holding them in a saturated solution of a rare earth metal. At the same time, the maximum native state of the object under study is preserved, and the image obtained in the backscattered electron detection mode carries extended information on the cellular structures [15].

The processing protocol included initial washing, holding for 45 min in a BioREE contrast solution (Glaucon LLC, Russia) and final washing with distilled water. After contrasting, excess moisture was removed from the sample surface with an air brush and placed on the stage of an EVO LS10 microscope (Zeiss, Germany). The observations were carried out in a low vacuum mode (EP, 70 Pa), at an accelerating voltage of 20 kV.

For morphohistological studies, the initial and decellularized samples were prepared according to the standard technique, stained with hematoxylin and eosin, and according to Masson's method. The analysis and photography of the obtained preparations were performed using a Nikon eclipse microscope (Nikon, Japan) equipped with a digital camera.

The concentration of glucose and urea was determined using a KonelabPrime 60i biochemical analyzer (Thermo Fisher Scientific, Finland).

Statistical data processing was performed with a standard Microsoft Excel software.

RESULTS AND DISCUSSION

We have previously shown that decellularization of human liver fragments using SAA allows removing cellular debris and preserving the tissue structure [9]. However, when determining the amount of DNA in DFHL, it was found that the process of chemical decellularization led to the removal of only 69.5 ± 3.6% of the DNA (Fig. 1). Note that during liver decellularization, it is recommended to keep no more than 10% of the amount of DNA in the original tissue [16]. In this regard, an additional stage was introduced into the decellularization protocol after SAA treatment, which included exposure of DFHL in DNase I solution. As can be seen from Fig. 6, DNase treatment made it possible to reduce the residual amount of DNA in the decellularized sample to 3.6 ± 0.6 ng/mg tissue (less than 1%), which indicates good decellularization, and, accordingly, low immunogenicity of the resulting matrix [17].

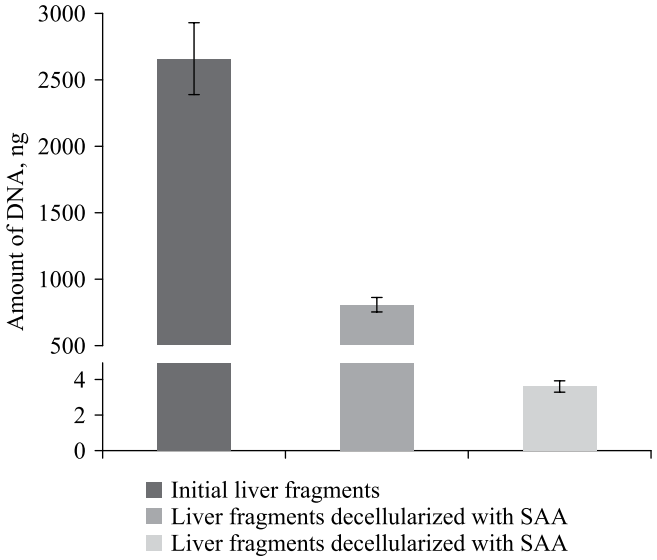

Fig. 1. Amount of DNA in the original and decellularized fragments of the human liver

The study of cytotoxicity by the direct contact method on mouse fibroblasts of the NIH/3T3 line did not reveal manifestations of the cytotoxic effect of the hepatic matrix during the study time of 24 hours. The dynamics of cell growth did not practically differ in the experimental and control variants. No areas of cell lysis were observed under the sample or in the contact area of the sample with cells. A trypan blue viability test performed after 24 hours of cell contact with samples also showed no cytotoxic effect. Based on the data obtained, it was concluded that the protocol for washing the matrix from SAA was effective and that DFHL was not cytotoxic.

The absence of matrix cytotoxicity made it possible to proceed to the study of the viability and proliferative activity of MSC HGT on the DFHL surface. It was shown by the method of intravital fluorescence microscopy that although some of the cells are in a suspension state in the culture medium, most of the cells successfully attach to the matrix surface and proliferate already on the 3rd day of the experiment (Fig. 2, a). At the beginning of the experiment, cells are unevenly distributed over the surface of the matrix – first of all, active collonization of the DFHL marginal zones occurs. Then the cells migrate into the volume of the matrix, forming a cellular network, and then continuous sections of dense cell layers (Fig. 2, b).

Fig. 3 shows the results of SEM studies of native and decellularized human liver, as well as CES, including MSC HGT, cultured on decellularized fragments of human liver for 7 days.

As seen in micrographs (Fig. 3, a, b), the native tissue significantly differed in surface structure from the decellularized one. The original fabric had a continuous relief surface. After removing the cells, the matrix acquired a porous structure. The pore boundaries were an interweaving of numerous micro- and nanofibers. The pore size was heterogeneous: there were both small pores

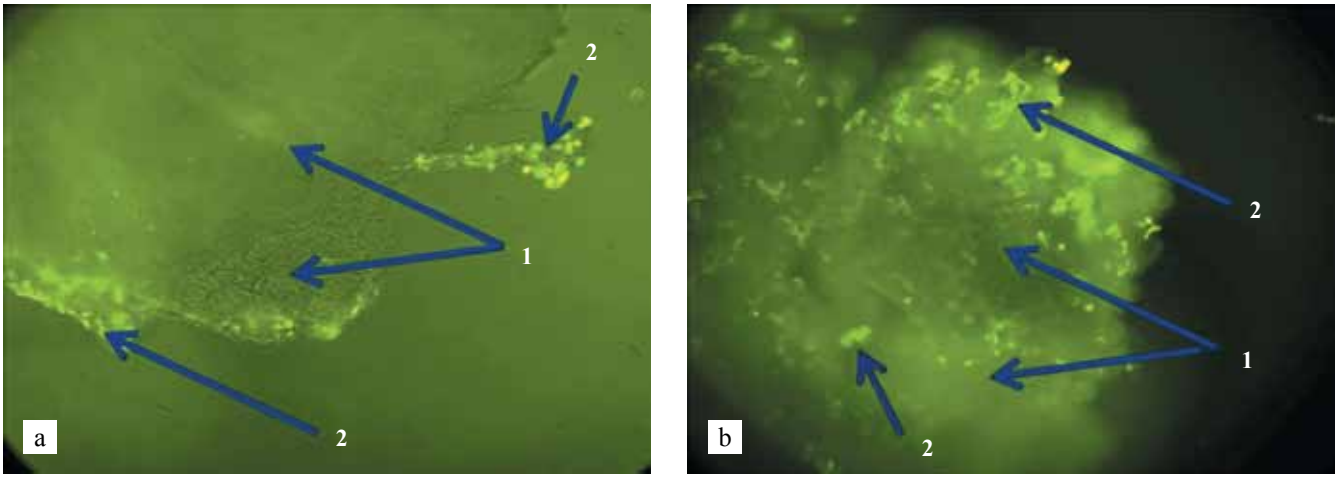

Fig. 2. Viability of MSC HAT on the surface of the: a – 3 days of cultivation; b – 7 days of cultivation; 1 – the surface of the DPHL; 2 – the MSC HAT. Coloring with Live/Dead fluorescent dyes. ×40

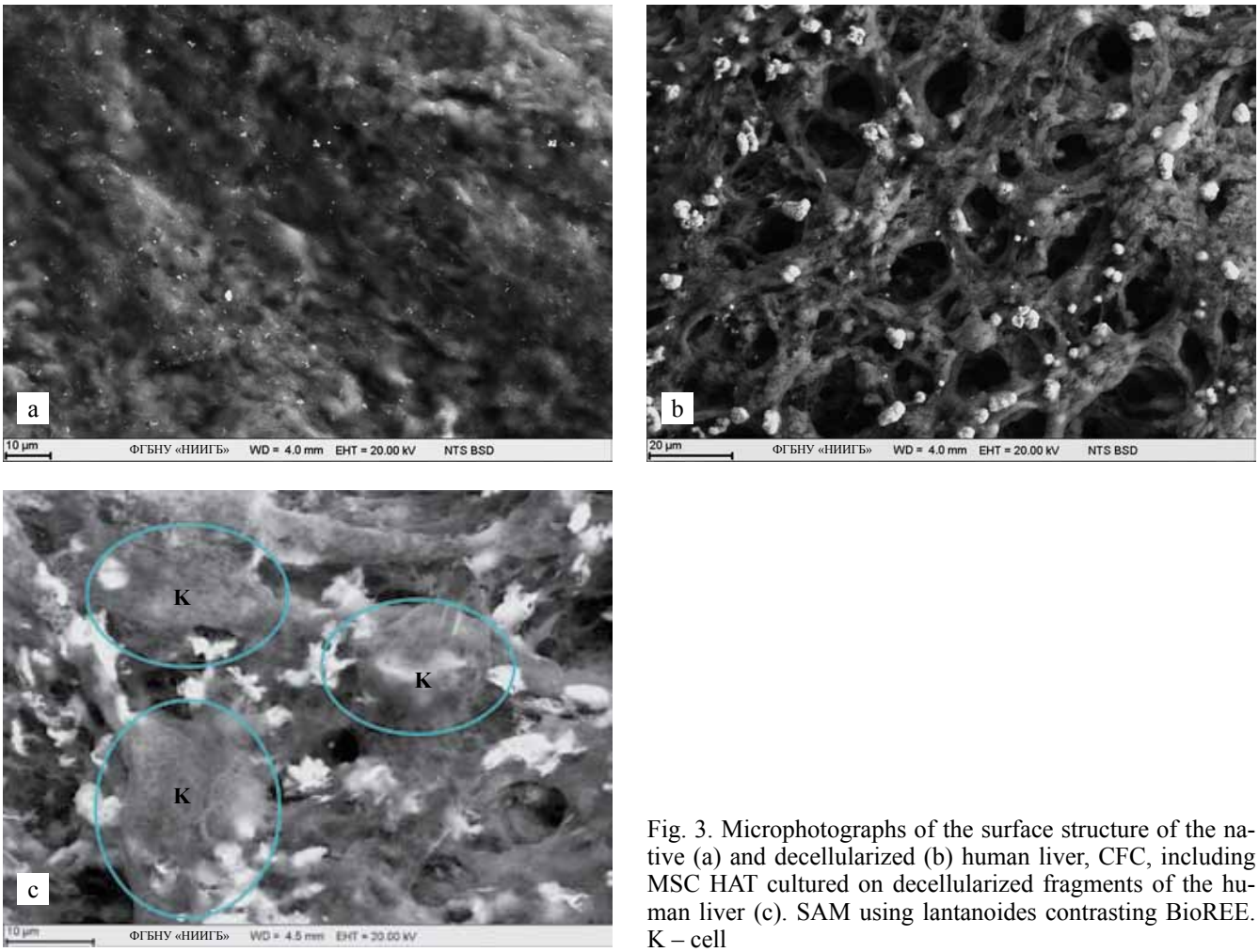

Fig. 3. Microphotographs of the surface structure of the native (a) and decellularized (b) human liver, CFC, including MSC HAT cultured on decellularized fragments of the human liver (c). SAM using lantanoides contrasting BioREE. K – cell

about 1–2 µm in size and large pores up to 20 µm in size. Light formations, which are determined on the surface of decellularized tissue and CES, most likely represent matrix-bound phosphorus-containing components of the culture medium adsorbed on its surface. MSC HGT, attached to the surface of the carrier, had a spread shape, which indicates the biocompatibility of the DFHL matrix with respect to interaction with cells (Fig. 3, c).

The obtained SEM data were confirmed by histological analysis. As seen in Fig. 4, cell morphology is spread out, fibroblast-like, which is characteristic of MSC HGT in the phase of active proliferation.

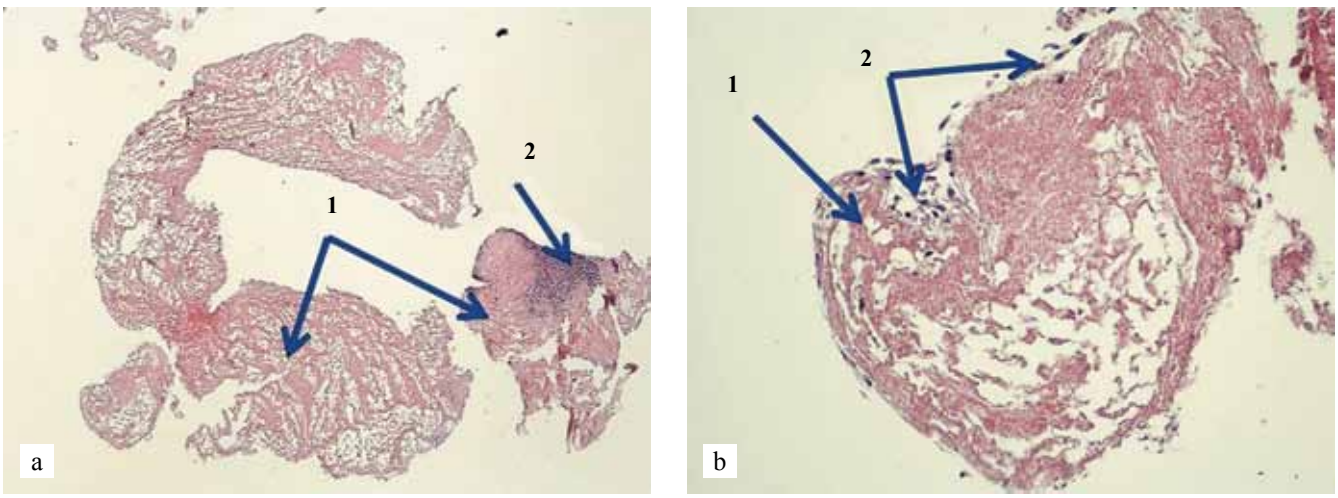

Fig. 4. Growth of MSC HAT on the tissue-specific matrix of the decellularized human liver. 1 – the matrix; 2 – MSC HAT. Staining with hematoxylin and eosin; a – ×40, b – ×200

The growth of fibroblast-like cells was observed at the periphery of all matrix particles. Note that single cells penetrated deep into the carrier, and pronounced proliferation was observed on one of the fragments.

The obtained positive results on the cultivation of MSC on the DFHL surface allowed us to switch to the DFHL – HepG2 culture system.

Analysis of the metabolic activity of cells showed that its growth occurs starting from the 3rd day of the experiment and reaches a maximum by the 7th day (Fig. 5). The data of the metabolic activity indicator are confirmed by intravital fluorescence microscopy (Fig. 6, a, b).

When studying the colonization of DFHL by cells of hepatocellular carcinoma HepG2, it was found that cells

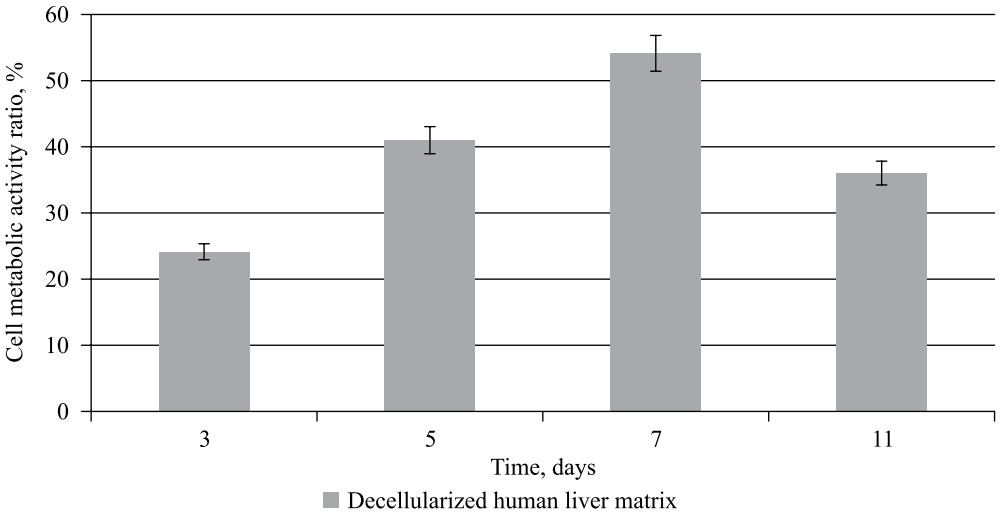

Fig. 5. Metabolic activity of HepG2 cells on a tissue-specific matrix from decellularized human liver

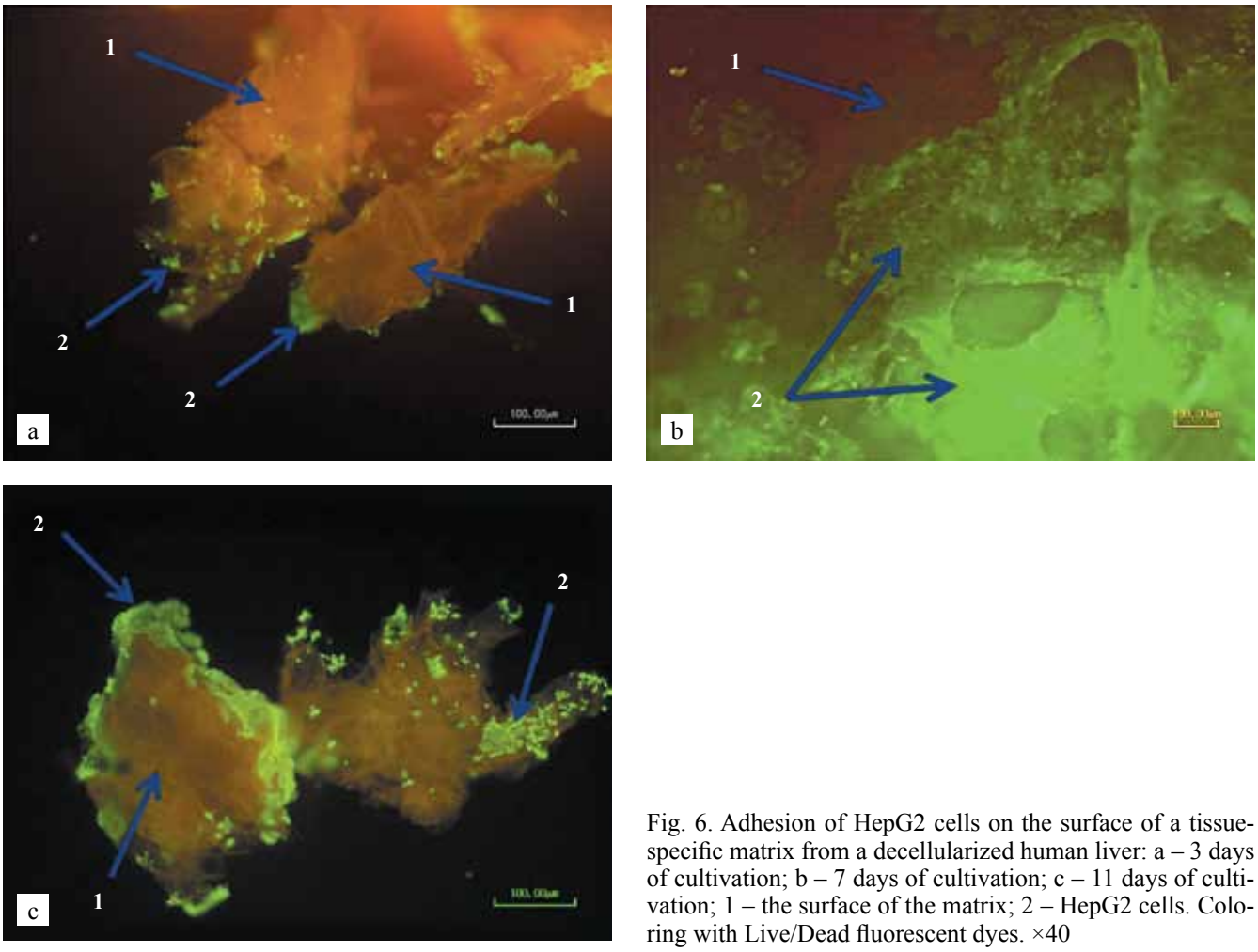

Fig. 6. Adhesion of HepG2 cells on the surface of a tissue-specific matrix from a decellularized human liver: a – 3 days of cultivation; b – 7 days of cultivation; c – 11 days of cultivation; 1 – the surface of the matrix; 2 – HepG2 cells. Coloring with Live/Dead fluorescent dyes. ×40

selectively attach to the matrix surface. The micrographs (Fig. 6, a, b) show that the cells mainly adhere to the marginal regions of the matrix, followed by their active proliferation. The central areas of the surface of the matrix fragments are very poorly populated by cells. According to microscopy and assessment of the metabolic activity of cells, its maximum falls on the 7th day. This trend persists throughout the experiment for 11 days. By the 11th day, proliferative activity decreases, which is associated with aging of the culture. In Fig. 6, c, it can be seen that by this time the marginal areas of the matrix are completely covered in places by the formed cell clusters and layers, while in the central zones the colonization of the host cells did not occur.

Thus, the intravital microscopy of the samples demonstrates active proliferation of HepG2 cells and uneven DFHL adhesion (Fig. 6).

On histological analysis, as seen in Fig. 7, the predominant growth of epithelial-like cells with a high nuclear-cytoplasmic ratio was observed on the surface of the matrix by the third day of cultivation. In this case, the cells united into numerous groups, and some of the cells formed small clusters in the volume of the matrix. Note the presence of collagen in DFHL, which indicates the preservation of the main structural component of ECM. By the seventh day, a significant increase in cell mass was observed, which was associated with active cell proliferation. On the surface of the carrier, multi-layered dense cell layers were found, and in the thickness of the matrix, the formation of larger cell clusters than in the previous period was observed.

The cells show a fine-grained cytoplasm with a few small vacuoles, and also observed an atypical mitosis characteristic of this cell line (Fig. 8).

Biochemical analysis of samples of the culture medium on the third day of the experiment did not reveal in the samples of urea at a level exceeding the detection limit of 1.1 mmol/L. However, by the 7th day, the urea content in the culture medium was 1.2 ± 0.1 mmol/L, which proves the presence of functional activity of cells in the CES composition.

It was suggested that the detected uneven colonization of matrix cells and insignificant cell penetration into its volume during cultivation under static conditions can

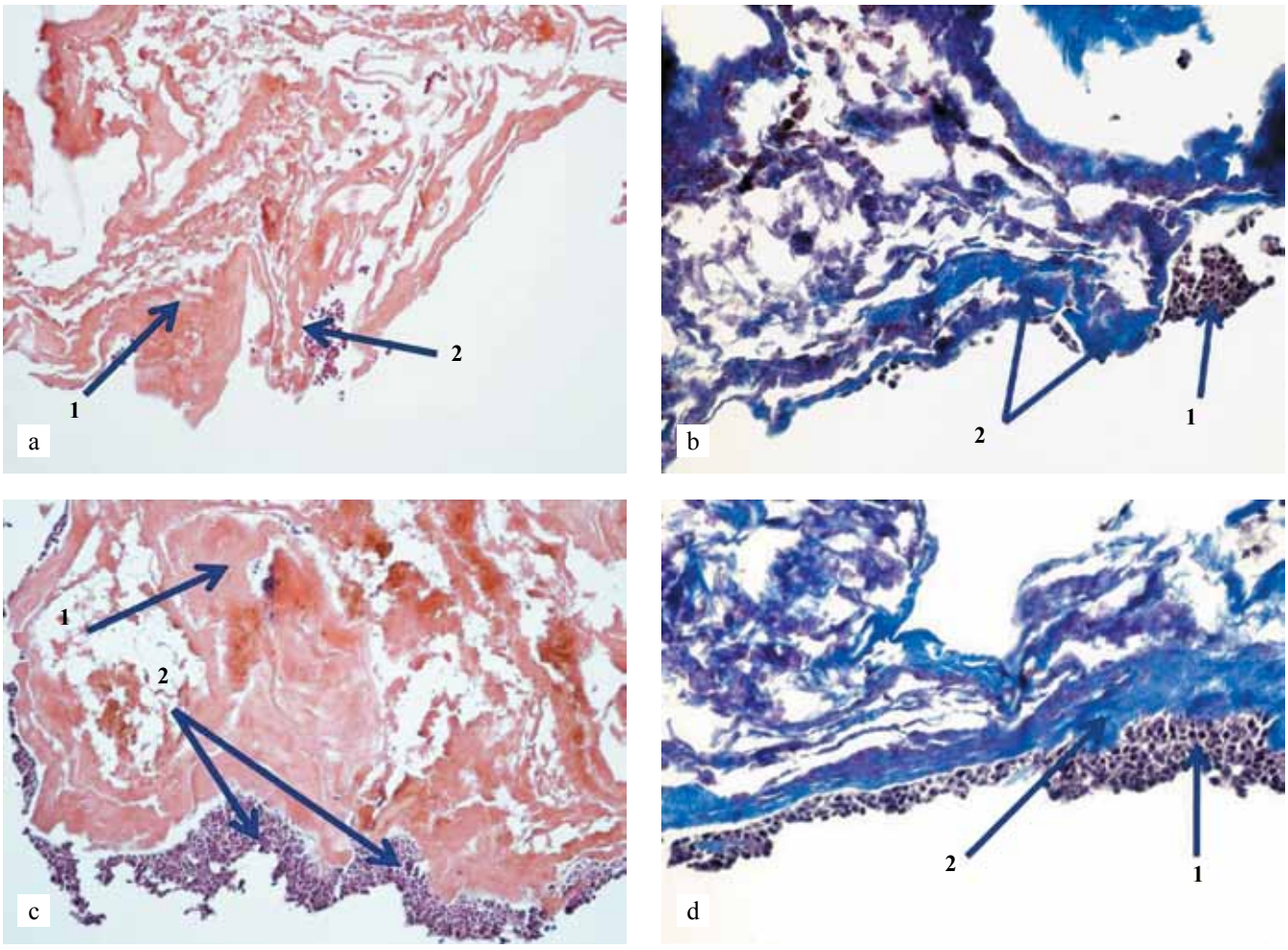

Fig. 7. Growth of human hepatocellular carcinoma cells HepG2 on a tissue-specific matrix from a decellularized human liver. ×100. Staining with hematoxylin and eosin: a – 3 days of cultivation; b – 7 days of cultivation; 1 – matrix; 2 – HepG2 cells. Masson staining for collagen. c – 3 days of cultivation; d – 7 days of cultivation; 1 – HepG2 cells; 2 – collagen. ×200

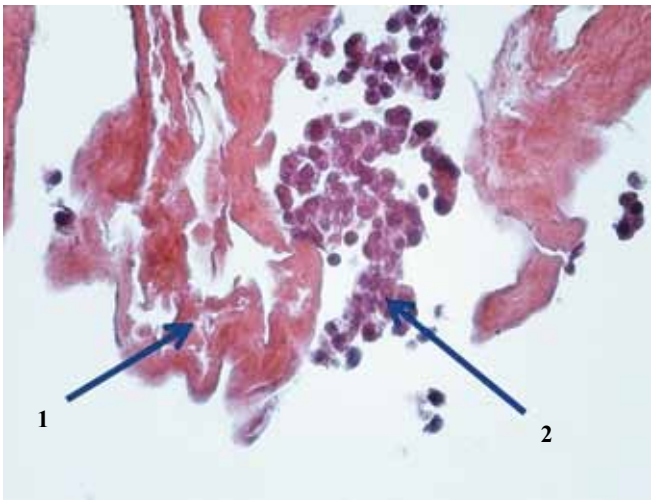

Fig. 8. Morphology of human hepatocellular carcinoma cells HepG2 when cultured on a tissue-specific matrix from a decellularized human liver. 3 days of cultivation: 1 – matrix; 2 – HepG2 cells. ×400. Staining with hematoxylin and eosin

be avoided by cultivating CES DFHL – HepG2 in a flow bioreactor [13].

Indeed, on the 7th day of cultivation in the bioreactor, the DFHL matrix with HepG2 formed a single conglomerate (Fig. 9). Most of the numerous groups of adhered epithelial-like cells with a high nuclear-cytoplasmic ratio of cells were viable and stained green. The bulk of cells was concentrated on the surface of the matrix, but cells forming small clusters were also detected in the volume of the carrier. The morphology of the cells changed from rounded to more elongated; some cells were in a state of destruction.

The metabolic activity of cells during cultivation in a bioreactor was also higher than in statics. In the cells, reactions of energy metabolism were actively taking place: the glucose content in the culture medium by the seventh day of the experiment significantly decreased – from 7.69 ± 0.38 to 4.69 ± 0.23 mmol/L. The urea content in the culture medium (1.5 ± 0.1 mmol/L) exceeded the

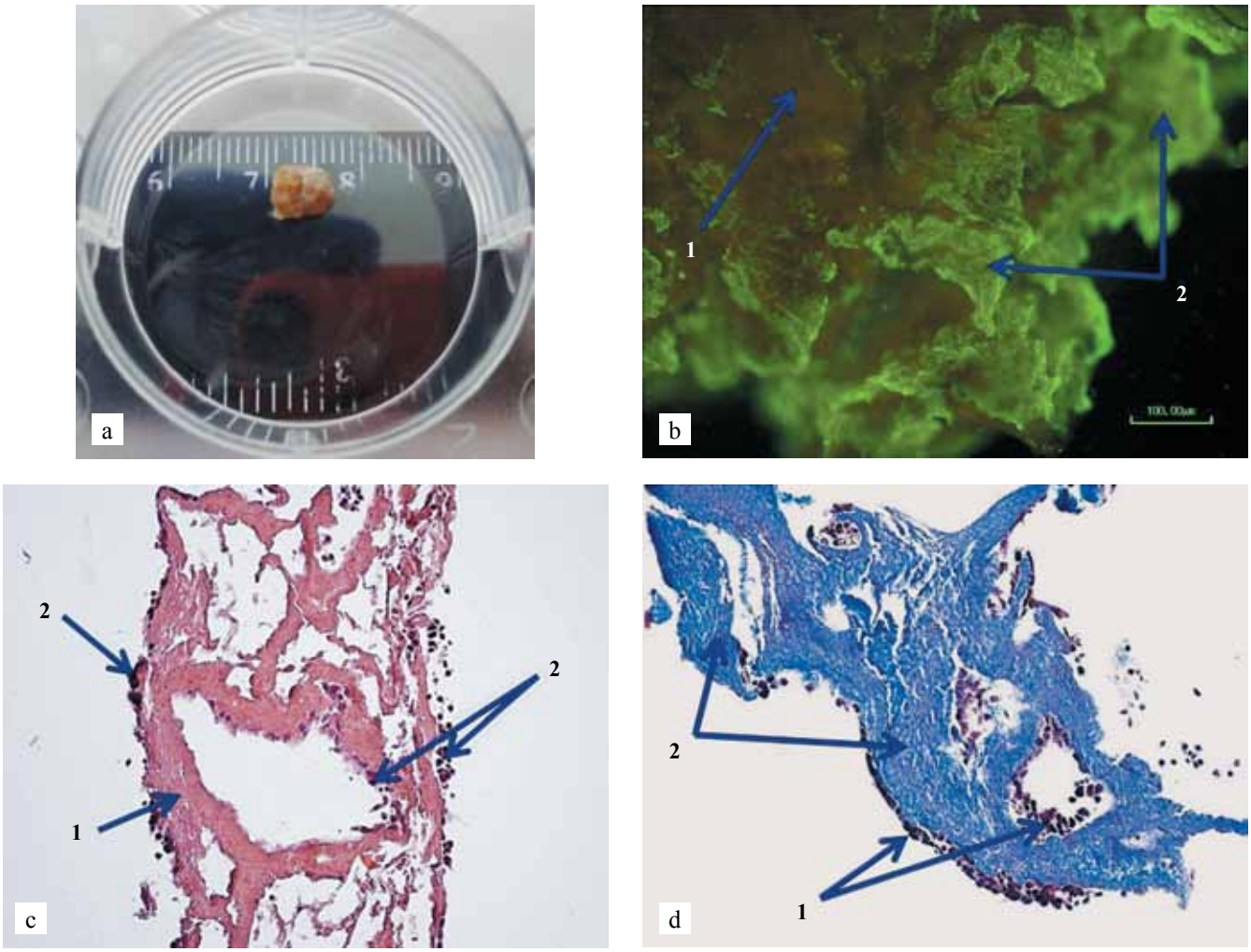

Fig. 9. Growth of human hepatocellular carcinoma cells HepG2 on a tissue-specific matrix from a decellularized human liver in a flow bioreactor at a flow rate of 0.02 ml/min: a – overview photo; b – Live/Dead vital dye staining; 1 – matrix; 2 – HepG2 cells. ×100; c – staining with hematoxylin and eosin; 1 – matrix; 2 – HepG2 cells. ×200; d – Masson staining for collagen; 1 – HepG2 cells; 2 – collagen. ×200

value for the samples obtained under static conditions (1.2 ± 0.1 mmol/L).

Thus, it has been shown that the cultivation of tissue-specific matrix DFHL with hepatic HepG2 cells in a bioreactor makes it possible to achieve a more uniform recellularization of the matrix volume, increase metabolic activity, and provide favorable conditions for cell proliferation.

CONCLUSION

A protocol has been proposed for decellularization of donor liver fragments, which makes it possible to obtain a tissue-specific matrix free of cells and detritus, with a low DNA content and preservation of the ECM structure. The lack of cytotoxicity of DFHL and their ability to maintain the adhesion and proliferation of MSC HGT and tissue-specific HepG2 cells indicates the possibility of using the matrix in liver tissue engineering. The advantage of DFHL matrix recellularization in a flow-through bioreactor compared to DFHL cultivation with HepG2 was shown.

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The authors declare no conflict of interest.

REFERENCES

1. *Croce S, Peloso A, Zoro T, Avanzini MA, Cobianchi L.* A hepatic scaffold from decellularized liver tissue: food for thought. *Biomolecules*. 2019; 9 (12): 813. doi: 10.3390/biom9120813. PMID: 31810291.
2. *Yang W, Xia R, Zhang Y, Zhang H, Bai L.* Decellularized liver scaffold for liver regeneration. *Methods Mol Biol*. 2018; 1577: 11–23. doi: 10.1007/7651_2017_53. PMID: 28856614.
3. *Bilodeau C, Goltsis O, Rogers IM, Post M.* Limitations of recellularized biological scaffolds for human transplantation. *J Tissue Eng Regen Med*. 2020; 14 (3): 521–538. doi: 10.1002/term.3004. PMID: 31826325.
4. *Porzionato A, Stocco E, Barbon S, Grandi F, Macchi V, De Caro R.* Tissue-engineered grafts from human decellularized extracellular matrices: a systematic review and future perspectives. *Int J Mol Sci*. 2018; 19 (12): 4117. doi: 10.3390/ijms19124117. PMID: 30567407.
5. *Ahmed E, Saleh T, Yu L, Kwak HH, Kim BM, Park KM et al.* Micro and ultrastructural changes monitoring during decellularization for the generation of a biocompatible liver. *J Biosci Bioeng*. 2019; 128 (2): 218–225. doi: 10.1016/j.jbiosc.2019.02.007. PMID: 3090445.
6. *Willemse J, Verstegen MMA, Vermeulen A, Schurink IJ, Roest HP, van der Laan LJW et al.* Fast, robust and effective decellularization of whole human livers using mild detergents and pressure controlled perfusion. *Mater Sci Eng C Mater Biol Appl*. 2020; 108: 110200. doi: 10.1016/j.msec.2019.110200. PMID: 31923991.
7. *Butter A, Aliyev K, Hillebrandt KH, Raschzok N, Kluge M, Seiffert N et al.* Evolution of graft morphology and function after recellularization of decellularized rat livers. *J Tissue Eng Regen Med*. 2018; 12 (2): e807–e816. doi: 10.1002/term.2383. PMID: 27957815.
8. *Naeem EM, Sajad D, Talaei-Khozani T, Khajeh S, Azarpira N, Alaei S et al.* Decellularized liver transplant could be recellularized in rat partial hepatectomy model. *J Biomed Mater Res A*. 2019; 107 (11): 2576–2588. doi: 10.1002/jbm.a.36763. PMID: 31361939.
9. *Gautier SV, Sevastyanov VI, Shagidulin MYu, Nemets EA, Basok YuB.* Tkanespetsificheskiy matriks dlya tkanevoy inzhenerii parenkhimatoznogo organa i sposob ego polucheniya. Patent na izobretenie RU 2693432 C2, 02.07.2019.
10. *Elchaninov A, Fatkhudinov T, Usman N, Arutyunyan I, Makarov A, Lokhonina A et al.* Multipotent stromal cells stimulate liver regeneration by influencing the macrophage polarization in rat. *World J Hepatol*. 2018 Feb 27; 10 (2): 287–296.; *Zhou Q, Li L, Li J.* Stem cells with decellularized liver scaffolds in liver regeneration and their potential clinical applications. *Liver Int*. 2015 Mar; 35 (3): 687–694. doi: 10.1111/liv.12581.
11. *Shimoda H, Yagi H, Higashi H, Tajima K, Kuroda K, Abe Y et al.* Decellularized liver scaffolds promote liver regeneration after partial hepatectomy. *Sci Rep*. 2019; 9 (1): 12543. doi: 10.1038/s41598-019-48948-x. PMID: 31467359.
12. *Donato MT, Tolosa L, Gómez-Lechón MJ.* Culture and Functional Characterization of Human Hepatoma HepG2 Cells. *Methods Mol Biol*. 2015; 1250: 77–93. doi: 10.1007/978-1-4939-2074-7_5.
13. *Sevastyanov VI, Basok YB, Grigoryev AM, Kirsanova LA, Vasilets VN.* A perfusion bioreactor for making tissue-engineered constructs. *Biomedical Engineering*. 2017; 51 (3): 162–165.
14. GOST ISO 10993-1-2011 "Izdeliya meditsinskie. Otsenka biologicheskogo deystviya meditsinskikh izdeliy. Chast' 5. Issledovanie na tsitotoksichnost': metody *in vitro*". M.: Standartinform, 2014.
15. *Novikov I, Subbot A, Turenok A, Mayanskiy N, Chebotar I.* A rapid method of whole cell sample preparation for scanning electron microscopy using neodymium chloride. *Micron*. 2019; 124: 102687. doi: 10.1016/j.micron.2019.102687. PMID: 31302532.
16. *Zhang L, Guan Z, Ye J-S, Yin Y-F, Stoltz J-F, de Isla N.* Research progress in liver tissue engineering. *Biomed Mater Eng*. 2017; 29 (s1): S113–S119.
17. *Crapo PM, Gilbert TW, Badylak SF.* An overview of tissue and whole organ decellularization processes. *Biomaterials*. 2011; 32 (12): 3233–3243. doi: 10.1016/j.biomaterials.2011.01.057.
18. *Nibourg GA, Hoekstra R, van der Hoeven TV, Ackermans MT, Hakvoort TB, van Gulik TM, Chamuleau RA.* Increased hepatic functionality of the human hepatoma cell line HepaRG cultured in the AMC bioreactor. *Int J Biochem Cell Biol.* 2013 Aug; 45 (8): 1860–1868.

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