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SUPERCRITICAL CARBON DIOXIDE AS A TOOL FOR IMPROVING THE BIOCOMPATIBLE PROPERTIES OF BIOPOLYMER AND TISSUE-SPECIFIC SCAFFOLDS FOR TISSUE ENGINEERING

E.A. Nemets[1, 2], A.E. Lazhko[3], A.M. Grigoryev[1], V.Yu. Belov[1, 2], V.A. Surguchenko[1], Yu.B. Basok[1, 2], A.D. Kirillova[1], V.I. Sevastianov[1, 2]

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

[2] Institute for Biomedical Research and Technology, Moscow, Russian Federation

[3] Kurnakov Institute of General and Inorganic Chemistry, Moscow, Russian Federation

Objective: to investigate the efficacy of supercritical carbon dioxide (sc-CO_2) for enhancшtп the biocompatibility of biopolymer scaffolds from biodegradable materials and tissue-specific scaffolds from decellularized porcine liver slices (PLSs) or fine porcine cartilage particles (FPCPs). **Materials and methods.** Biopolymer scaffolds of a polyoxy(butyrate-co-valerate) and gelatin copolymer composition, 4 mm in diameter and 80 mm in length, were formed by electrospinning (NANON-01A, MECC CO, Japan) and stabilized by incubation in glutaraldehyde vapor for 48 hours at room temperature. For decellularization, PLSs and FPCPs were incubated under periodic stirring in buffer (pH = 7.4) solutions of sodium dodecyl sulfate (0.1%) and Triton X-100 with increasing concentrations (1, 2, and 3%). Treatment in a sc-CO_2 atmosphere was done at 150–300 bar pressure, 35 °C temperature, and 0.25–2.5 mL/min flow rate of sc-CO_2 for 8–24 hours. 10% ethanol was introduced as a polarity modifier. Cytotoxicity was studied according to GOST ISO 10993-5-2011. The growth of NIH/3T3 in the presence of samples was studied using an interactive optical system IncuCyte Zoom. **Results.** The effect of the sc-CO_2 flow rate and pressure, and the effect of addition of ethanol, on the biocompatibility of scaffolds was investigated. It was found that treatment at a low sc-CO_2 flow rate (0.25 mL/min) does not achieve the required cytotoxicity. Complete absence of cytotoxicity in biopolymer scaffolds was achieved in the presence of 10% ethanol, at a sc-CO_2 flow rate of 2.5 mL/min, 300 bar pressure and 35 °C temperature after 8 hours of treatment. Effective removal of cytotoxic detergents from decellularized liver occurs already at a 150-bar pressure and does not require the addition of ethanol. Adding ethanol to sc-CO_2 eliminates not only the cytotoxic, but also the cytostatic effect of tissue-specific scaffolds. **Conclusion.** Sc-CO_2 treatment is an effective way to enhance the biocompatibility of three-dimensional porous matrices produced using cytotoxic substances: bifunctional crosslinking agents for biopolymer scaffolds and surfactants in the case of tissue-specific matrices. Addition of ethanol as a polarity modifier improves the treatment efficiency by eliminating both cytotoxic and cytostatic effects.

Keywords: pig liver, pig cartilage, decellularization, biopolymer scaffolds, supercritical CO_2, polarity modifier, cytotoxicity, biocompatibility.

INTRODUCTION

The key challenge in creating tissue-engineered constructs for use in tissue and regenerative medicine is the development of biodegradable highly porous scaffolds (*synonyms:* matrices, scaffolds) that could enable specific cells to be delivered to an organ requiring correction and treatment, and ensure their long-term functioning in that organ.

Today, so many scaffolds possessing the necessary complex of physicomechanical and biological properties have been developed. In their manufacture, preference is given to high-molecular-weight natural materials. Collagen, which forms the main component of the extracellular matrix (ECM) and can stimulate reparative processes, and its partially denatured derivatives, such as gelatin, are most commonly used for this purpose [1, 2]. However, collagen scaffolds undergo extremely rapid (less than 1 month) resorption when injected into the body [3, 4]. In order to increase resorption time of biopolymer-based materials, a wide range of physical and chemical cross-linking methods have been developed [5–7]. Collagen crosslinking methods using bifunctional crosslinking agent, glutaraldehyde (GA), are the most widely used. Along with the high efficiency of biopolymer structure stabilization, crosslinking with GA comes with a number of side effects including cytotoxicity of the final product [8, 9].

In natural tissues, ECM is able to provide not only physical support to cells, but also send biological signals

Corresponding author: Evgeny Nemets. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Phone: (903) 579-23-79. E-mail: evgnemets@yandex.ru

selectively supporting adhesion, proliferation, and differentiation of cells of a particular tissue or organ [10, 11]. In this regard, there is great interest in scaffolds made by decellularization, a process aimed at removing cells and genetic material from the tissue while preserving not only the structural but also tissue-specific properties of ECM. Currently, a wide range of different physical, chemical and biological methods are used for decellularization of organs and tissues, among which treatment with surface-active agents (surfactants) of ionic or non-ionic nature is the most common [12–14]. One of the significant disadvantages of surfactant use for organ and tissue decellularization is the need to wash them thoroughly in buffer solutions from residual detergents for a long time (at least 72 h) [12–15]. This increases the risk of washing out significant amounts of glycans and cytokines, and can lead to disruption of matrix recellularization processes [16]. Consequently, reducing the time of contact with the aqueous phase is necessary in terms of minimizing the risk of such complications.

Recently, there has been increased interest in the use of supercritical fluids (SCF) in the creation of scaffolds for tissue engineering and regenerative medicine [17–24]. Any substance at a temperature and pressure above the critical point goes into the SCF state (Fig. 1), at which the difference between the liquid and gas phases disappears. One of the most important properties of SCF is its ability to dissolve substances, and the dissolving power increases with increasing density. Since the SCF density increases with increasing pressure at a constant temperature, changing the pressure can affect its dissolving ability [25].

Of the known SCFs, supercritical carbon dioxide (sc-CO_2) is used most often in biomedical technologies (sc-CO_2). Sc-CO_2 can be treated at temperatures close to physiological values (35–40 °C), and does not require the use of additional organic solvents. At the end of treatment, the carbon dioxide is easily and practically

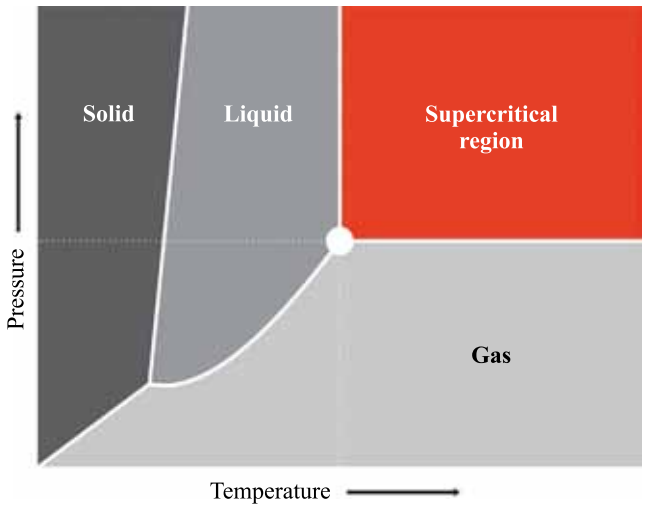

Fig. 1. Transition of substance into supercritical state

without residue removed by simple depressurization. At the same time, removal of toxic compounds soluble in sc-CO_2 (unreacted monomers and oligomers, surfactants, plasticizers, etc.) can occur, which leads to significant improvement in the biocompatible properties of the obtained materials [26].

Since carbon dioxide is a non-polar compound, to increase the efficiency of removal of polar phospholipid components of cell membranes, treatment with sc-CO_2 is performed in a hydrophilic agent, usually ethanol [27]. The addition of ethanol also makes it possible to increase the preservation of such important ECM components as collagens, glycosaminoglycans, adhesive proteins (fibronectin, laminin, etc.), as well as angiogenic factors during decellularization [28].

All this suggests that treatment with sc-CO_2 can facilitate the effective removal of cytotoxic surfactant residues and unreacted GA used in the matrix formation process for tissue engineering.

The **aim** of this work is to investigate the efficacy of sc-CO_2 to improve the biocompatibility of tubular porous scaffolds made of a copolymer composition of polyoxy(butyrate-co-valerate) and polyhydroxybutyrate-valerate-gelatin (PHBV-G) stabilized by GA and tissue-specific scaffolds made of porcine liver slices (PLSs).

MATERIALS AND METHODS

Obtaining scaffolds

Biopolymer porous tubular scaffolds (Fig. 2) from 10% (by weight) solutions of polyhydroxybutyrate-valerate (PHBV) and gelatin (type A) in hexafluoroisopropanol (all Sigma-Aldrich, USA), mixed in a 1:2 ratio (by volume), formed using electrospinning, NANON-01A unit (MECC CO, Japan). The voltage between the electrodes was 25 kV, the distance between the electrodes was 10 cm, the rod diameter was 4 mm, and the rod rotation speed was 500 rpm. The samples obtained from the composition (PHBV-G) were mechanically removed from the substrate, dried at 37 °C for 4–6 h, and vacuum-treated at 37 °C and residual pressure of 10–20 mm Hg within 18–24 hours.

Additional stabilization of the biopolymer scaffold structure by GA vapor (Serva, Germany) was performed by placing pre-swollen samples (distilled water, 24 h at room temperature) in a closed container (desiccator) containing a 25% GA solution, without direct contact of the sample with the GA solution, and incubated at room temperature for 48 h.

Decellularization of porcine liver and cartilage

Porcine liver was obtained at a slaughterhouse (Promagro Ltd, Russia) after slaughtering healthy animals (weight about 120 kg) in accordance with European Directive 64/433/EEC. After refrigerated transportation

(4 °C), the porcine liver was cut into PLSs 0.1 × 0.3 cm in size, frozen at –80 °C and stored at this temperature until decellularization began.

The hip and knee joints of the pigs were obtained at the slaughterhouse (Promagro Ltd, Russia) 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, cut into 0.5 × 0.5 cm slices, frozen at –80 °C, and crushed under continuous liquid nitrogen cooling for 4 minutes at 25 Hz shaking frequency of the grinding jar using a CryoMill (Retch GmBH, Germany). Fractions of finely minced porcine cartilage particles (FMPCP) sized 30–100 μm were isolated by sifting the contents of the grinding jar through a set of sieves with appropriate cell size.

Decellularization of FMPCPs and PLSs was performed in three buffer solutions (phosphate-buffered saline (PBS), pH = 7.4) of 0.1% sodium dodecyl sulfate (SDS) and with increasing concentration (1%, 2%, and 3%) of Triton X-100 (all Sigma-Aldrich, USA) with periodic stirring (200 rpm, 3 times daily, 1 hour, at room temperature). After thorough washing from surfactants in three changes of PBS and subsequent incubation in buffer solution for 24 hours at room temperature, decellularized porcine liver slices (DPLSs) were transferred to cryotubes and stored at –80 °C until treatment with sc-CO_2.

Treatment with supercritical CO_2

Treatment in a sc-CO_2 atmosphere was done on a RESS-SAS unit (Waters corporation, USA) at T = 35 °C, 150 and 300 bar pressure, 0.25 and 2.5 mL/min sc-CO_2 feed rate for 8 or 24 hours. Ethanol at 10% concentration (by volume) was chosen as the polarity modifier.

Cytotoxicity study

Cytotoxicity of the test samples was assessed in vitro according to interstate standard GOST ISO 10993-5-2011 [29]. All procedures were performed under aseptic conditions. We used the NIH 3T3 mouse fibroblast cell line obtained from a collection of transplanted vertebrate somatic cells at Ivanovsky Institute of Virology, Moscow, Russia. The cells were seeded into 24- and 96-well flat-bottomed culture plates (Corning-Costar, USA) at a concentration of 8 × 10^4 cells/well and 2 × 10^4 cells/well, respectively, and incubated for 24 hours at 37 °C in a humid atmosphere containing 5 ± 1% CO_2 before an 80 ± 10% monolayer was formed.

At least three extracts and samples were prepared for each scaffold sample. Sodium chloride (NPK, Russia) was used as the extraction solution. The ratio of sample surface area to extraction solution volume was 3:1 cm^2/mL. Extraction time was 24 hours and temperature was 37 °C. The obtained extract was introduced into a 96-well plate with a formed cell monolayer in the volume of 100 μl/well.

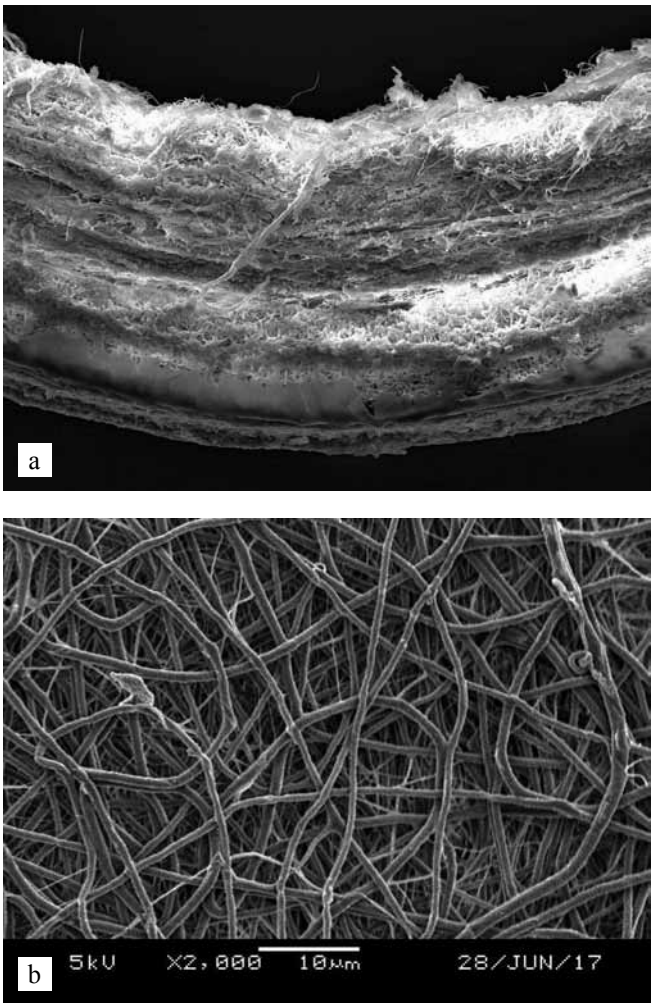

Fig. 2. Structure of a biopolymer porous tubular scaffold, d = 4 mm. a) a cross section (100× magnification); b) inner surface (2000× magnification)

For the cytotoxicity study by direct contact, the biopolymer scaffold samples (1 × 1 cm^2 and 0.1 cm thick) and tissue-specific matrices (5 mg weighed portion) were placed directly into a 24-well plate on the cell monolayer surface. The plates were incubated for 24 hours at 37 °C in a humid atmosphere containing 5 ± 1% CO_2.

On day 2 of incubation, we evaluated monolayer confluence (the degree of cell coverage of the substrate), as well as the degree of cell lysis using inverted binocular microscope MC 700 (Micros, Austria).

Negative control (K–) was DMEM culture medium with or without fetal calf serum, and the positive control (K+) was zinc solution in nitric acid: Zn 1–2 wt% in HNO_3 (Sigma-Aldrich, USA), diluted 1:100 with saline.

The results were analyzed according to the grading scale of the degree of cell reactivity:

Grade 0: No reaction, discrete intracytoplasmic granules observed, no cell lysis;

Grade 1: slight reaction, no more than 20% of cells are rounded (round, loosely attached, no intracytoplasmic granules);

Grade 2: mild reaction, no more than 50% of cells are lysed (round, loosely attached, no intracytoplasmic granules);

Grade 3: moderate reaction, no more than 70% of cells are lysed (round, loosely attached, no intracytoplasmic granules);

Grade 4: strong reaction, more than 70% of cells are lysed (almost completely destroyed monolayer).

Negative control – the degree of cell reaction is 0, cell reaction is None. Positive control – the degree of cell reaction is 3 or 4. The reactivity of the extract under study must not exceed grade 0 (reaction None).

The study of prolonged (up to 72 hours) cytotoxic effect under direct contact of the cells with the samples under study was performed using the IncuCyte Zoom interactive optical system for long-term cell research (Essen BioScience, USA). This complex allows you to incubate cells under standard conditions and microscopy the culture plate at specified time intervals with image photofixation. Analysis of the obtained images using the built-in software makes it possible to calculate the change in the cell monolayer confluence for each sample as a function of time.

The effect of cytotoxic residues on the proliferative activity of NIH 3T3 fibroblasts was studied using an interactive optical system IncuCyte Zoom, which allows real-time recording of cell growth curves on the surface of the culture plate during direct contact with the test sample.

Statistical data processing was performed using the standard Microsoft Excel software package. The level of statistical significance was $p < 0.05$.

RESULTS AND DISCUSSION

Before treatment with sc-CO_2, the cytotoxicity of the extracts was "moderate" (grade 3) in the case of biopolymer scaffolds and at grade 4 (acute cytotoxic reaction) for tissue-specific scaffolds.

After exposure to sc-CO_2, regardless of the choice of treatment mode, the extracts from biopolymer scaffolds as well as FMPCP and DPLS matrices were not cytotoxic (grade 0). At the same time, under conditions of direct contact with the NIH 3T3 monolayer, the samples showed cytotoxicity, whose severity depended on treatment conditions.

Treatment of scaffolds with sc-CO_2 at a low feed rate (0.25 ml/min) turned out to be little effective. The greatest reduction in the level of cytotoxicity to "mild" (level 2) was achieved after prolonged treatment (24 hours) of DPLS samples in sc-CO_2 medium with the addition of ethanol. However, this result does not meet the GOST ISO 10993-5-2011 standards.

Increasing the sc-CO_2 feed rate to 2.5 mL/min had a positive effect on the treatment efficiency of both biopolymer and tissue-specific scaffolds (Tables 1 and 2).

Treatment of biopolymer scaffolds with sc-CO_2 at 2.5 mL/min feed rate and 150 bar pressure was marginally effective even when ethanol was added (Table 1). Increasing the sc-CO_2 pressure to 300 bar was accompanied by a marked decrease in cytotoxicity to "insignificant" (level 1) after 24 hours of exposure (Table 1). Only the addition of ethanol into the sc-CO_2 composition makes it possible to achieve a complete absence of cytotoxic effect of biopolymer scaffolds at 300 bar pressure after 8 hours of treatment (Table 1).

In the case of DPLS scaffolds, increasing the sc-CO_2 feed rate has a more pronounced effect on reducing cytotoxicity (Table 2). In the case of samples treated for 24 hours with individual sc-CO_2 at 150 bar pressure, cytotoxicity decreases to "insignificant" (level 1). The addition of ethanol makes it possible to completely suppress the cytotoxicity of scaffolds already after 8 hours of sc-CO_2 treatment.

Increasing the pressure from 150 to 300 bar enhances the effect of treating DPLSs with sc-CO_2 (Table 2). Complete absence of cytotoxicity (level 0) was obtained after 8 hours of exposure to sc-CO_2 without adding ethanol.

It should be particularly noted that the samples treated with regimens that provided effective surfactant removal demonstrated the absence of cytotoxicity not

Table 1

Cytotoxicity of porous biopolymer scaffolds (sc-CO_2 feed rate 2.5 mL/min)

	Ethanol, %	Pressure, bar	Time, hour	Cytotoxicity
1	Control (without sc-CO_2 treatment)			Moderate (3)
2	–	150	8	Moderate (3)
3	–	150	24	Moderate (3)
4	10	150	8	Mild (2)
5	10	150	24	Mild (2)
6	–	300	8	Mild (2)
7	–	300	24	Insignificant (1)
8	10	300	8	None (0)
9	10	300	24	None (0)

only after 24 hours of direct contact with cells, but also under prolonged direct contact with cells for 72 hours.

The obtained results suggest that for effective washing from cytotoxic surfactant residues, DPLS treatment should be performed for 8 hours at 35 °C temperature, sc-CO_2 feed rate of 2.5 ml/min and a pressure of 300 bar in an atmosphere of pure carbon dioxide. A similar result can be achieved by treatment at a pressure of 150 bar with the addition of a polarity modifier (ethanol) to the supercritical fluid.

Methods of decellularization under the influence of detergents [12–15] involve a long (at least 72 hours) washing of obtained scaffolds from cytotoxic surfactants, which increases the risk of washing out significant amounts of bioactive molecules (glycosaminoglycans and cytokines) that play an important role in scaffold recellularization.

Table 2

Cytotoxicity of tissue-specific scaffold (sc-CO_2 feed rate 2.5 mL/min)

	Ethanol	Pressure, bar	Time, hour	Cytotoxicity
1	Control (without sc-CO_2 treatment)			Severes (4)
2	–	150	8	Mild (2)
3	–	150	24	Insignificant (1)
4	10%	150	8	None (0)
5	10%	150	24	None (0)
6	–	300	8	None (0)
7	–	300	24	None (0)
8	10%	300	8	None (0)
9	10%	300	24	None (0)

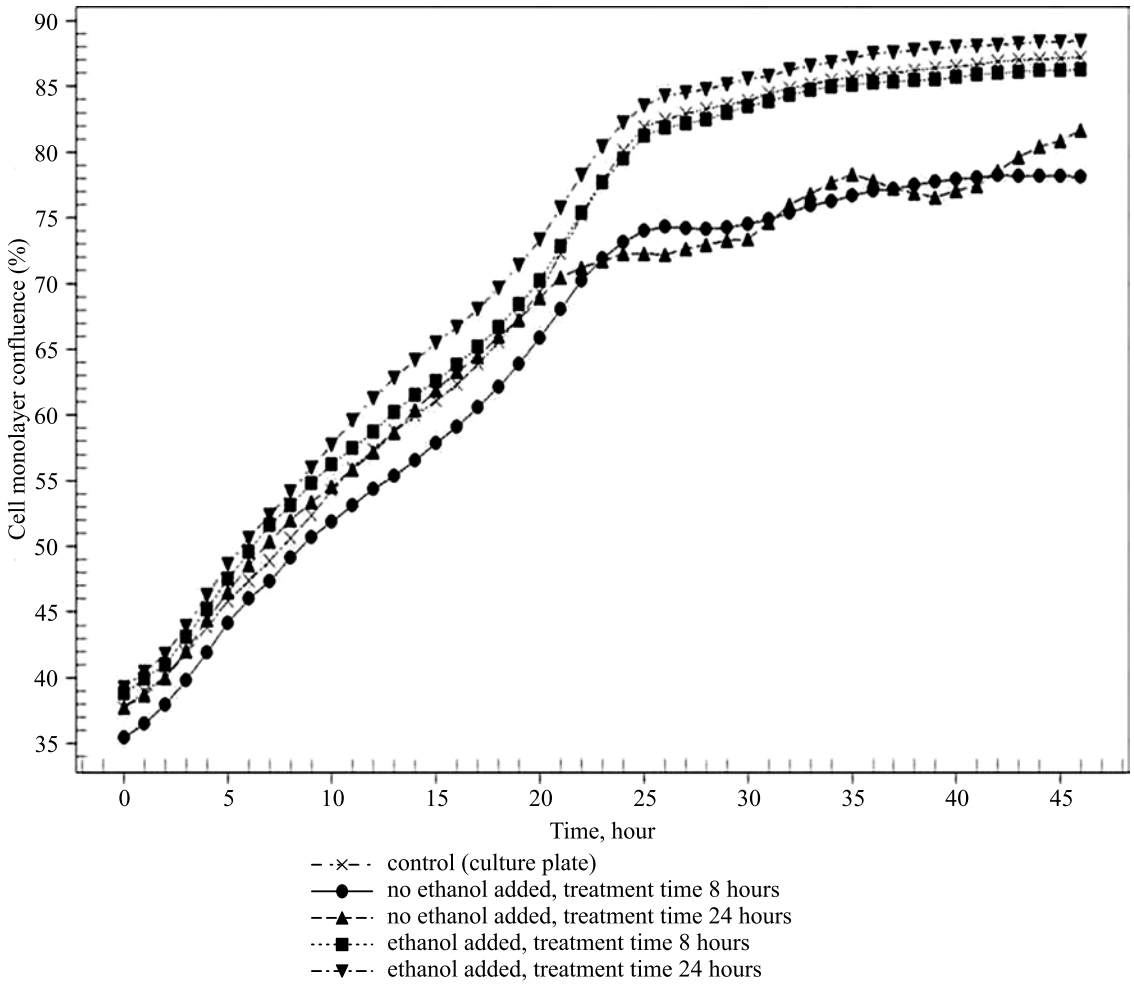

Fig. 3. Growth curve of NIH 3T3 cells in the presence of DPLS matrices, treated with sc-CO_2 (300 bar, 35 °C, sc-CO_2 feed rate 2.5 mL/min)

The use of SCF treatment can reduce to 24 hours the time of surfactant removal in an aqueous medium.

To reveal the effect of cytotoxic residues on cell proliferative activity, an additional study was performed using interactive optical system IncuCyte Zoom, which allows real-time recording of changes in cell proliferative activity in the presence of scaffolds.

For this study, samples were selected that demonstrated the absence of cytotoxic effect using the evaluation scale and were obtained under the same sc-CO_2 treatment conditions: feed rate 2.5 ml/min, pressure 300 bar (Table 2, samples 6–9).

A marked decrease in NIH 3T3 proliferative activity was observed immediately after the introduction of samples (20 hours after seeding the cells on the culture plate) in the case of sc-CO_2-treated samples without the addition of ethanol (Fig. 3). At the same time, ethanol-treated scaffolds did not induce a decrease in cell proliferation compared to the control (cells on culture plate.

Microscopic examination carried out after the end of the experiment to study the growth of NIH 3T3 in the presence of sc-CO_2-treated DPLS scaffolds in different regimens (Fig. 4) confirmed the absence of any signs of negative effects on cells (lysis, scoring), which once again confirms the conclusion made earlier that the samples studied have no cytotoxicity. However, the decrease in the proliferative activity of cells detected in contact with sc-CO_2-treated scaffolds without the addition of ethanol suggests the presence of a cytostatic effect in this case.

FMPCP scaffolds treated under conditions that are optimal for enhancing the biocompatibility of porcine liver scaffolds (sc-CO_2 2.5 ml/min with the addition of ethanol; 35 °C; 300 bar; 8 hours) also showed no cytotoxicity (level 0). Meanwhile, the presence of decellu-

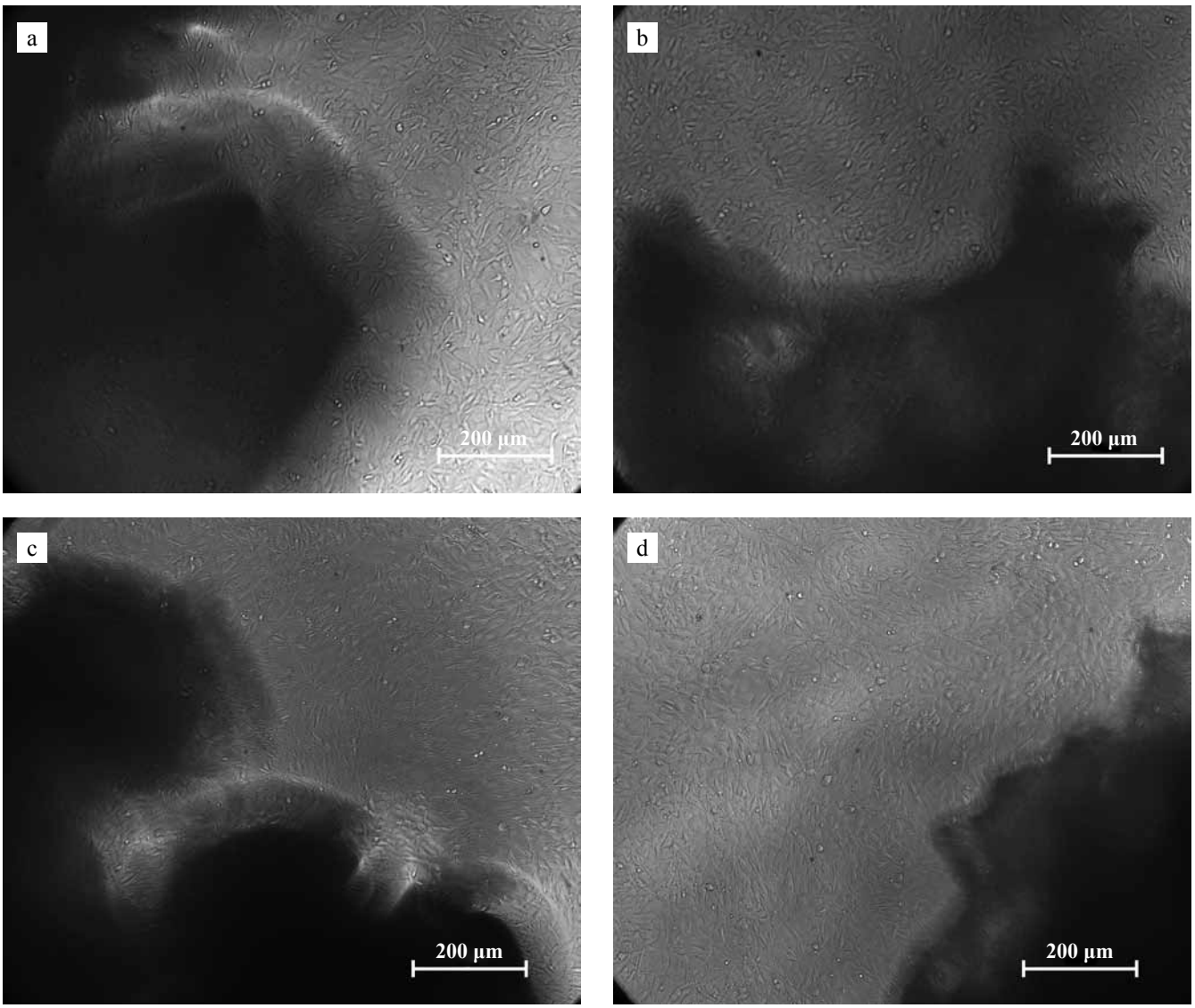

Fig. 4. Cell populations after 48 hours of culturing NIH 3T3 in the presence of sc-CO_2-treated PLS matrices (300 bar, 35 °C, 2.5 mL/min). a) sc-CO_2, 8 hours; b) sc-CO_2, 24 hours; c) sc-CO_2 + ethanol, 8 hours; d) sc-CO_2 + ethanol, 24 hours. 100× magnification

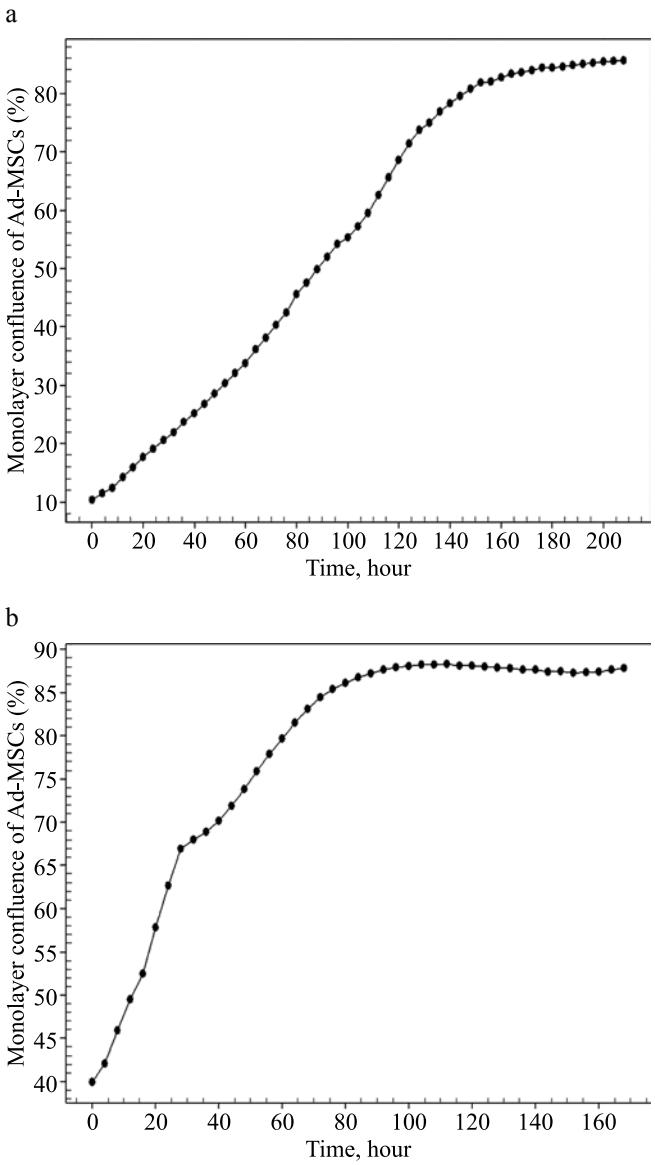

Fig. 5. Growth curve of NIH 3T3 cells on the surface of culture plate in the absence (a) and presence (b) of FMPCP matrices treated with supercritical fluids in the optimal mode (sc-CO_2 2.5 mL/min + ethanol, 300 bar, 35 °C, 8 hours)

larized fine cartilage particles not only has no cytostatic effect on human adipose tissue-derived mesenchymal stem cells (Ad-MSCs), but also demonstrates a stimulating effect on cell proliferation (Fig. 5): the time for the confluent monolayer level to rise to 80% in the presence of decellularized cartilage particles is noticeably shorter. Probably, the reason for this is the presence of signaling molecules in the decellularized cartilage matrix that accelerate Ad-MSC adhesion and proliferation, which coincides with experimental results described in reports [30].

Thus, from the point of view of suppression of both cytotoxic and cytostatic effect of tissue-specific matrices after decellularization using detergents, SCF treatment regimens based on carbon dioxide containing ethanol additives are therefore optimal. In our opinion, the reason for this is that both non-ionic (Triton X-100) and ionic (SDS) detergents are present in the composition of the decellularizing solution. Supercritical carbon dioxide is a nonpolar solvent whose efficiency, in terms of removing polar surfactant, is sufficient to eliminate cytotoxic effect, but not sufficient to suppress cytostatic effect. Addition of polar ethanol (polarity modifier) can reduce the SDS content to levels that are not capable of exerting a cytostatic effect.

CONCLUSION

Sc-CO_2 treatment is an effective way of enhancing the biocompatibility of three-dimensional porous matrices obtained using cytotoxic substances: bifunctional cross-linking agents for biopolymer scaffolds and surfactants, in the case of tissue-specific matrices FMPCP and DPLSs. Adding ethanol as a polarity modifier improves the treatment efficiency by eliminating both cytotoxic and cytostatic effects.

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The authors declare no conflict of interest.

REFERENCES

1. *Surguchenko VA*. The matrices for tissue engineering and hybrid organs. Biocompatible materials (textbook). Ed. by: V.I. Sevastianov, M.P. Kirpichnikov. M.: MIA, 2011. Chast' II: 199–228.
2. *Sevastianov VI, Basok YB, Grigor'ev AM, Kirsanova LA, Vasilets VN.* Formation of tissue-engineered construct of human cartilage tissue in a flow-through bioreactor. *Bull Exp Biol Med*. 2017; 164 (2): 269–273. doi: 10.1007/s10517-017-3971-z.
3. *Goissis G, Suzigan S, Parreira DR, Maniglia JV, Braile DM, Raymundo S.* Preparation and characterization of collagen-elastin matrices from blood vessels intended as small diameter vascular grafts. *Artif Organs*. 2000; 24: 217–223. doi: 10.1046/j.1525-1594.2000.06537.x. PMID: 10759645.
4. *Busra MFM, Lokanathan Y*. Recent development in the fabrication of collagen scaffolds for tissue engineering applications: A review. *Curr Pharm Biotechnol.* 2019; 20 (12): 992–1003. doi: 10.2174/1389201020666190731121016. PMID: 31364511.
5. *Oryan A, Kamali A, Moshiri A, Baharvand H, Daemi H.* Chemical crosslinking of biopolymeric scaffolds: Current knowledge and future directions of crosslinked engineered bone scaffolds. *Int J Biol Macromol*. 2018; 107 (Pt A): 678–688. doi: 10.1016/j.ijbiomac.2017.08.184.
6. *Kawecki M, Łabuś W, Klama-Baryla A, Kitala D, Kraut M, Glik J et al*. A review of decellurization methods caused by an urgent need for quality control of cell-free extracellular matrix' scaffolds and their role in regenerative medicine. *J Biomed Mater Res B Appl Biomater*. 2018; 106 (2): 909–923. doi: 10.1002/jbm.b.33865. PMID: 28194860.

7. *Rose JB, Pacelli S, Haj AJE, Dua HS, Hopkinson A, White LJ et al*. Gelatin-based materials in ocular tissue engineering. *Materials (Basel)*. 2014; 7 (4): 3106–3135. doi: 10.3390/ma7043106. PMID: 28788609.
8. *Nemets EA, Pankina AP, Sevastianov VI*. Comparative analysis of methods for increasing of biostability of collagen films. *Inorganic Materials: Applied Research*. 2017; 5: 718–722.
9. *Umashankar PR, Arun T, Kumari TV*. Short duration gluteraldehyde cross linking of decellularized bovine pericardium improves biological response. *J Biomed Mater Res*. 2011; 97 (3): 311–320. doi: 10.1002/jbm.a.33061. PMID: 21448995.
10. *Gattazzo F, Urciuolo A, Bonaldo P*. Extracellular matrix: a dynamic microenvironment for stem cell niche. *Biochim Biophys Acta*. 2014; 1840 (8): 2506–2519. doi: 10.1016/j.bbagen.2014.01.010. PMID: 24418517.
11. *Sun Y, Wang TL, Toh WS, Pei M*. The role of laminins in cartilaginous tissues: from development to regeneration. *Eur Cell Mater*. 2017; 34: 40–54. doi: 10.22203/eCM.v034a0.
12. *Shirakigawa N, Ijima H*. Decellularized tissue engineering. *Advanced Structured Materials*. 2017; 66: 185–226. doi: 10.1007/978-981-10-3328-5_5.
13. *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. PMID: 21296410.
14. *Gilpin A, Yang Y.* Decellularization strategies for regenerative medicine: From processing techniques to applications. *Biomed Res Int*. 2017; 2017: 9831534. doi: 10.1155/2017/9831534. PMID: 28540307.
15. *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.
16. *Kawasaki T, Kirita Y, Kami D, Kitani T, Ozaki C, Itakura Y et al*. Novel detergent for whole organ tissue engineering. *J Biomed Mater Res A*. 2015; 103 (10): 3364–3373. doi: 10.1002/jbm.a.35474. PMID: 25850947.
17. *Song C, Luo Y, Liu Y, Li S, Xi Z, Zhao L et al*. Fabrication of PCL scaffolds by supercritical CO_2 foaming based on the combined effects of rheological and crystallization properties. *Polymers (Basel)*. 2020; 12 (4): 780. doi: 10.3390/polym12040780. PMID: 32252222.
18. *Nemets EA, Belov VJu, Ilina TS, Surguchenko VA, Pankina AP, Sevastianov VI*. Composite porous tubular biopolymer matrix of small diameter. *Perspektivnye materialy*. 2018; 9: 49–59. [In Russ, English abstract]. doi: 10.30791/1028-978X-2018-9-49-59.
19. *White LJ, Hutter V, Tai H, Howdle SM, Shakesheff KM*. The effect of processing variables on morphological and mechanical properties of supercritical CO_2 foamed scaffolds for tissue engineering. *Acta Biomater*. 2012; 8 (1): 61–71. doi: 10.1016/j.actbio.2011.07.032. PMID: 21855663.
20. *Antons J, Marascio MG, Aeberhard P, Weissenberger G, Hirt-Burri N, Applegate LA et al*. Decellularised tissues obtained by a CO_2-philic detergent and supercritical CO_2. *Eur Cell Mater*. 2018, 36: 81–95. doi: 10.22203/eCM.v036a07. PMID: 30178445.
21. *Casali DM, Handleton RM, Shazly T, Matthews MA*. A novel supercritical CO_2-based decellularization method for maintaining scaffold hydration and mechanical properties. *J Supercrit Fluids*. 2018; 131: 72–81. doi: 10.1016/j.supflu.2017.07.021.
22. *Huang YH, Tseng FW, Chang WH, Peng IC, Hsieh DJ, Wu SW et al*. Preparation of acellular scaffold for corneal tissue engineering by supercritical carbon dioxide extraction technology. *Acta Biomater*. 2017; 58: 238–243. doi: 10.1016/j.actbio.2017.05.060. PMID: 28579539.
23. *Gil-Ramírez A, Rosmark O, Spégel P, Swärd K, Westergren-Thorsson G, Larsson-Callerfelt AK et al*. Pressurized carbon dioxide as a potential tool for decellularization of pulmonary arteries for transplant purposes. *Sci Rep*. 2020; 10 (1): 4031. doi: 10.1038/s41598-020-60827-4. PMID: 32132596.
24. *Razgonova MP, Zaharenko AM, Sergievich AA, Kalenik TK, Golohvast KS*. Sverhkriticheskie fljuidy: teorija, jetapy stanovlenija, sovremennoe primenenie: uchebnoe posobie. SPb.: Lan', 2019. 192.
25. *Alekseev ES, Alent`ev AYu, Belova AS, Bogdan VI et al*. Supercritical fluids in chemistry. *Rus Chem Rev.* 2020; 89: 1337–1427. [In Russ, English abstract]. doi: 10.1070/RCR4932.
26. *Popov VK*. Implantaty v zamestitel'noj i regenerativnoj medicine kostnyh tkanej. *Biosovmestimye materialy (uchebnoe posobie)*. Pod red. V.I. Sevast'janova, M.P. Kirpichnikova. M.: MIA, 2011. Chast' II: 271–294.
27. *Ingrosso F, Ruiz-López MF.* Modeling Solvation in Supercritical CO_2. *Chemphyschem.* 2017; 18: 2560–2572. doi: 10.1002/cphc.201700434.
28. *Seo Y, Jung Y, Kim SH*. Decellularized heart ECM hydrogel using supercritical carbon dioxide for improved angiogenesis. *Acta Biomater*. 2018; 67: 270–281. doi: 10.1016/j.actbio.2017.11.046. PMID: 29223704.
29. GOST ISO 10993-5-2011 "Izdelija medicinskie. Ocenka biologicheskogo dejstvija medicinskih izdelij. Chast' 5. Issledovanija na citotoksichnost': metody *in vitro*".
30. *Sun Y, Yan L, Chen S, Pei M*. Functionality of decellularized matrix in cartilage regeneration: A comparison of tissue versus cell sources. *Acta Biomater.* 2018; 74: 56–73. doi: 10.1016/j.actbio.2018.04.048. PMID: 29702288.

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