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BIODEGRADABLE MATERIALS BASED ON NATURAL SILK FABRIC AS PROMISING SCAFFOLDS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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Objective: to develop a method for obtaining scaffolds based on natural silk fabric and to study their biocompatibility *in vitro*. **Materials and methods.** To obtain biodegradable scaffolds based on natural silk fabric, we propose treating natural silk fabric with a water-ethanol solution of calcium chloride. Differences in the structure of the resulting scaffolds were identified via scanning electron microscopy. **Conclusion.** The resulting scaffolds are non-toxic to cells and support cell adhesion and proliferation. Our studies make it possible to consider the resulting biodegradable scaffolds as promising constructs for tissue engineering and regenerative medicine.

Keywords: silk, natural silk fabric, biodegradable scaffolds, biodegradation rate.

INTRODUCTION

Development of biodegradable constructs (scaffolds) for regeneration of damaged organs and tissues is one of the most important and urgent problems in tissue engineering and regenerative medicine. Such constructs should be biocompatible, have high mechanical properties for surgical manipulations, and their biodegradation rate should be the same with the rate of regeneration of damaged tissue.

One of the most promising natural polymers for creating such constructs is silk fibroin produced by the Bombyx mori silkworm. Various groups of researchers have shown the successful application of silk fibroin-based constructs in many areas of tissue engineering, such as cultivation of cells of various species, regeneration of bone and cartilage tissue, regeneration of the skin, cornea, nervous tissue, etc [1–4].

The source of silk fibroin can be both silkworm cocoons and commercial products made from natural silk – sutures and woven materials. The use of suture and woven materials does not require multistage silk processing and production of regenerated silk fibroin solutions. This can be an advantage for solving a number of problems in tissue engineering and regenerative medicine.

The unique combination of high mechanical characteristics (such as strength and elasticity) with biocompatibility makes silk fibroin very suitable for reinforcing structures based on brittle and fragile materials with higher biological activity.

Silk fibroin-based constructs are positioned in tissue engineering and regenerative medicine as biodegradable. However, in some cases, the degradation period of these constructs can reach 1 year [5]. Many factors, such as the secondary structure of the polymer, polymer processing technology during production of the structure, source of silk, etc., can influence the value of the biodegradation rate of the resulting construct. The structure of silk fibroin provides the ability to control the rate of degradation of silk fibroin-based constructs, which can significantly expand the scope of application of silk fibroin in tissue engineering and regenerative medicine [6–8]. Therefore, development of methods for monitoring and regulating the degradation rate of silk fibroin-based constructs to increase the efficiency and expand the scope of their application is an urgent task.

One of the strategies for working in this direction is to use free chemical groups present in the structure of silk fibroin molecule. These groups can be linked by various crosslinking agents, such as glutaraldehyde and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [9, 10]. By adjusting the silk fibroin crosslinking degree, the degradation rate of the resulting material can be changed. However, crosslinking agents can reduce the biocompatibility of a scaffold due to its toxicity. Moreover, the use of amino acid sequences does not in all cases have a significant effect on the rate of its biodegradation. At the same time, both methods can only reduce the degradation rate of silk fibroin-based constructs. However, in tissue engineering and regenerative medicine, rapid biodegradation of the material is often required for more efficient cell proliferation.

Another strategy is to create silk fibroin-based composite materials. Silk fibroin solutions are mixed with solutions of synthetic and natural materials used in tissue engineering and regenerative medicine, such as alginates, hyaluronic acid, gelatin, collagen, chitosan, polylactides,

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polycaprolactone, etc. [11–16]. This makes it possible to create composite materials and composite constructs, whose degradation rate depends on the quantitative ratio of various substances that make up the construct. In this case, it becomes possible to both increase and decrease the biodegradation rate of the construct. However, introduction of composite additives is accompanied by a change in the structure of the construct and its mechanical properties, which limits the scope of application of such constructs in tissue engineering [17]. In addition, the degradation of some substances (for example, polylactides) introduced into the construct is accompanied by formation of products that cannot be incorporated into cell metabolism and create unfavorable conditions for their proliferation, which reduces the biocompatibility of the constructs [18].

We have previously proposed a method for regulating the biodegradation rate of natural silk tissues to obtain natural silk biodegradable scaffolds [19]. Within the framework of this study, various approaches to the use of natural silk fabrics for production of biodegradable scaffolds are shown, their structure and biocompatibility *in vitro* are studied.

MATERIALS AND METHODS

Obtaining biodegradable scaffolds

Natural silk fabrics were used to obtain biodegradable scaffolds. In this study, two types of fabrics of different densities were used: gas-chiffon (15 g/m^2 density) and double-sided satin (155 g/m^2 density). Fabric samples for research were obtained according to the method previously described by the authors [19].

The fabrics were preliminarily washed from impurities according to the following procedure. Fabric flaps were boiled in a water bath in a sodium bicarbonate solution prepared at 500 mL of double-distilled water, 1260 mg of sodium bicarbonate per 1 g of tissue for 40 minutes, then washed with 3.6 L of distilled water. Then it was boiled in 500 mL of double-distilled water for 30 minutes and washed with 3.6 L of distilled water. The last procedure was repeated 3 times. The fabric flaps were dried in air at room temperature. The obtained flaps were cut into 5 cm × 5 cm fragments. Group 1 and 4 samples were obtained in this manner (Table).

Next, the fabric fragments were processed according to the following procedure. At the first stage, the fabrics were incubated in a water-alcohol solution of calcium chloride containing 389 mg of $CaCl_2$, 388 μL of C_2H_5OH, and 544 μL of H_2O per 1 mL of solution for 45 minutes at 37 °C temperature. Then 16 washes were carried out with double-distilled water, 30 minutes each, and air-dried at room temperature. Group 2 and 5 samples were obtained in this manner (Table).

The fabric fragments were then incubated in type A gelatin solution with 20 mg/mL concentration for 1 hour and air-dried on the surface of polished Teflon at room temperature. The dried fabric fragments, treated with gelatin solution, were additionally put in a solution containing 30 mM EDC, 8 mM N-hydroxysuccinimide (NHS), 50 mM sodium dihydrogen phosphate, and 100 mM sodium chloride (pH = 6) for 2 hours at room temperature. Then 16 washes were carried out with double-distilled water, 30 minutes each, and air-dried at room temperature. Group 3 and 6 samples were obtained in this manner (Table).

Analysis of the surface structure of biodegradable scaffolds by scanning electron microscopy (SEM)

Samples of 8 mm × 8 mm biodegradable scaffolds were fixed with 2.5% glutaraldehyde solution in phosphate-buffered saline with pH = 7.4 for 2 hours in the dark at 4 °C, after which the samples were washed five times from the fixing solution with phosphate-buffered saline with pH = 7.4 for 10 minutes. Then the samples were dehydrated with increasing ethanol concentrations. Ethanol concentrations of 10%, 20%, 50%, 70% and 96% were used, incubating the samples for 1 hour in ethanol of each concentration. Samples were then transferred to acetone for 30 minutes, and then the acetone was changed.

The prepared samples were dried by critical point transition ($T_{cr.CO_2}$ = 31 °C, $p_{cr.CO_2}$ = 72.8 kg/cm^2) using a K850 unit (Quorum Technologies, UK). The dried samples were coated with a 10 nm thick gold layer in argon atmosphere at 20 mA ion current and 1 mbar pressure using the Q150R ES vacuum sputtering machine (Quorum Technologies, Great Britain). The obtained samples were analyzed using a Tescan Vega3 SBU scanning electron microscope (Tescan, Czech Republic) at 15 kV operating voltage. Images were obtained and analyzed using the VegaTC software (Tescan, Czech Republic).

Analysis of cytotoxicity of the obtained constructs

Mouse 3T3 fibroblast cell line was used in the experiments. Cells were cultured in plastic vials in DMEM low glucose medium containing 10% fetal bovine serum, 0.324 mg/mL glutamine and 10 mg/mL gentamicin at 37 °C, 5% CO_2. The culture medium was changed every 48 hours. Cell monolayer was disaggregated using a trypsin-Versene solution. Cells were counted in the Goryaev chamber and seeded in a 1 : 3 ratio.

Cytotoxicity of all obtained samples was analyzed in accordance with the GOST ISO 10993-5-2011 standard "Medical devices. Assessment of the biological effect of medical devices. Part 5. Cytotoxicity studies: *in vitro* methods" using the MTT test [20] in the mouse 3T3 fibroblast cell line model.

For this purpose, mouse 3T3 fibroblasts were cultured in 300 μL of culture medium in a 96-well plate in a thermostat at 37 °C and 5% CO_2 for 3 days. Samples of 3 mm × 3 mm biodegradable scaffolds were sterilized in an MLS-3020U autoclave (Sanyo, Japan) at 121 °C for 15 minutes. Before the test, the culture medium was changed, and sterile samples of biodegradable scaffolds were introduced into the plate wells. The culture plate was used as a control. The plates were incubated in a thermostat at 37 °C, 5% CO_2. Cytotoxicity was assessed on days 3, 5 and 7 of the experiment. For this purpose, 60 μL of MTT solution with 5 mg/mL concentration was added to each well of the plate. It was incubated in a thermostat at 37 °C and 5% CO_2 for 4 hours until dark blue crystals of formazan precipitated out. The biodegradable scaffold samples were then removed from the wells, and the plate was centrifuged for 5 minutes at 885 g. The supernatant was removed, the formazan precipitate was dissolved in 300 μL of dimethyl sulfoxide for 20 minutes, and the optical density of the solution was measured at 540 nm wavelength.

Analysis of cell adhesion and proliferative activity on the obtained constructs

Human liver cancer cell line Hep-G2 was used in the experiments. The cells were cultured in plastic vials in a 1 : 1 mixture in DMEM high glucose medium and in Ham's F-12 medium containing 10% fetal bovine serum, 0.324 mg/mL glutamine and 10 mg/mL gentamicin at 37 °C, 5% CO_2. The culture medium was changed every 48 hours. Cell monolayer was disaggregated using a trypsin-Versene solution. Cells were counted in a Goryaev chamber and seeded in a 1 : 3 ratio.

Samples of 4 mm × 4 mm biodegradable scaffolds were sterilized in an MLS-3020U autoclave (Sanyo, Japan) at 121 °C for 15 minutes. Sterile 4 mm × 4 mm biodegradable scaffold samples were positioned in the wells of sterile 96-well culture plates. The culture plate was used as a control. Sterile phosphate-salt buffer solution was added to the wells of the plate and incubated for 15 minutes, after which the phosphate-buffered saline was changed. This procedure was repeated three times. Next, 300 μL of incubation medium was added to the plate wells for 30 minutes.

Cell suspension in the incubation medium was transferred to 96-well plates at 1000 cells per well in 300 μL of the incubation medium. The plates were incubated in a thermostat at 37 °C and 5% CO_2.

Cell adhesion was assessed visually using a Carl Zeiss Axio Vert.A1 microscope (Zeiss, Germany). Cell proliferative activity was assessed on days 3, 5 and 7 of the experiment using MTT [20]. To this end, 50 μL of a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution with a 200 μg/mL concentration was added to each well of the plate. The plates were incubated in a thermostat at 37 °C and 5% CO_2 for 4 hours until dark blue formazan crystals precipitated, and then centrifuged for 5 minutes at 885 g. The supernatant was removed, the formazan precipitate was dissolved in dimethyl sulfoxide at 300 μL of dimethyl sulfoxide per well. The optical density of the solution was measured at 540 nm wavelength.

Statistical data processing

The statistical significance of the results was assessed using the Mann–Whitney U test. The statistical significance level α was taken to be 0.05.

RESULTS

In this study, 6 groups of scaffold samples were obtained on the basis of two varieties of natural silk fabrics with different densities (Fig. 1). The composition of the samples of each group is described in Table.

Table

Samples of natural silk fabric-reinforced scaffolds obtained during the study

Group	Natural silk fabric used as the base for scaffold	Natural silk fabric processing method
1	Gas-chiffon (density 15 g/m^2)	1) Washing the fabric of impurities
2	Gas-chiffon (density 15 g/m^2)	1) Washing the fabric of impurities 2) Treatment with a water-ethanol solution of calcium chloride
3	Gas-chiffon (density 15 g/m^2)	1) Washing the fabric of impurities 2) Treatment with a water-ethanol solution of calcium chloride 3) Treatment with gelatin solution, covalent crosslinking with an EDC/NHS solution
4	Double-sided satin (density 155 g/m^2)	1) Washing the fabric of impurities
5	Double-sided satin (density 155 g/m^2)	1) Washing the fabric of impurities 2) Treatment with a water-ethanol solution of calcium chloride
6	Double-sided satin (density 155 g/m^2)	1) Washing the fabric of impurities 2) Treatment with a water-ethanol solution of calcium chloride 3) Treatment with gelatin solution, covalent crosslinking with an EDC/NHS solution

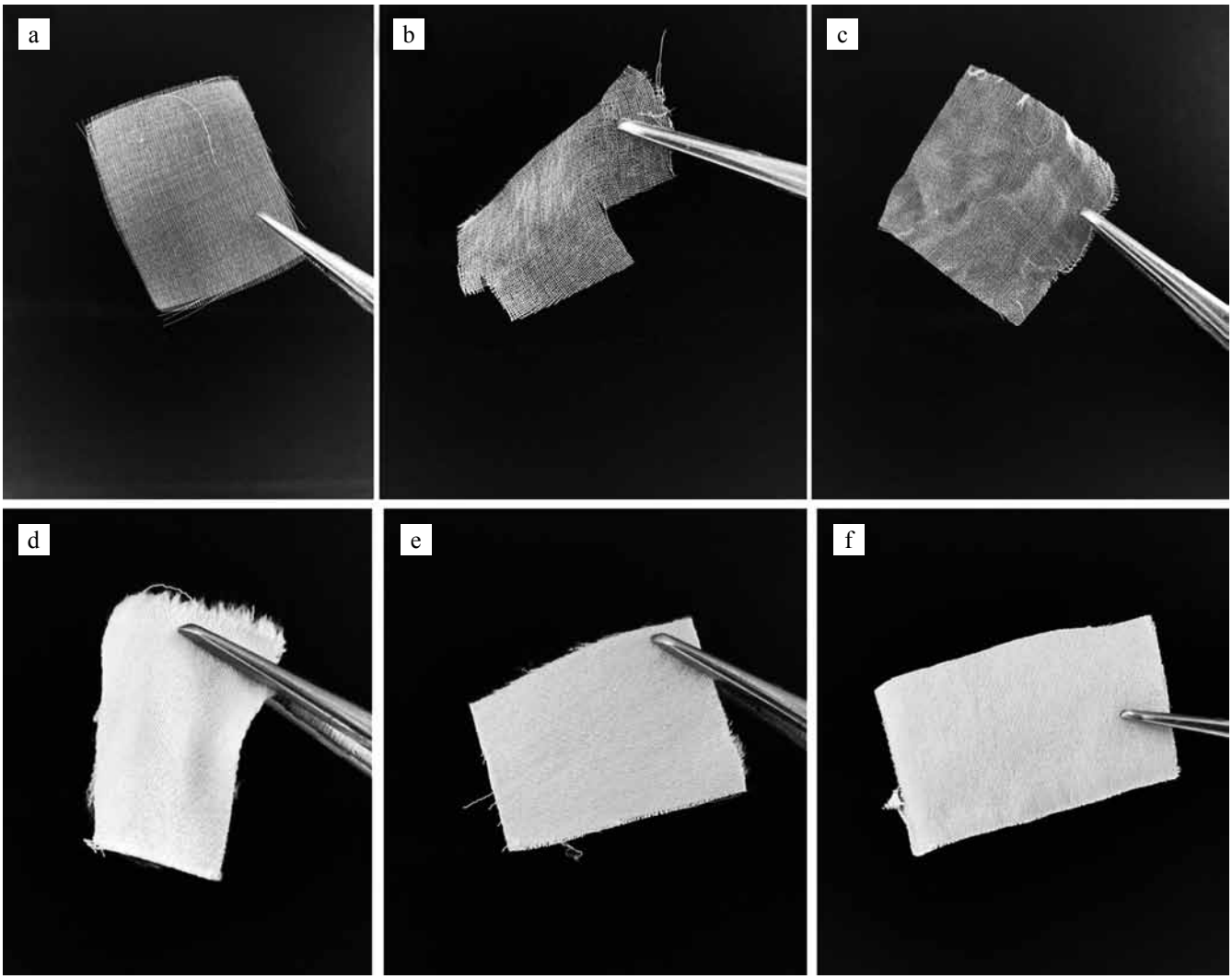

Fig. 1. Samples of natural silk fabric-reinforced scaffolds obtained during the study: a) Group 1, b) Group 2, c) Group 3, d) Group 4, e) Group 5, f) Group 6

The structure of the resulting scaffolds was studied by SEM (Fig. 2, 3). According to SEM data, samples of scaffolds based on textiles with a lower density (groups 1–3) were characterized by regular arrangement of fabric fibers (Fig. 2). Group 1 and 2 scaffolds were a mesh with an approximate mesh size of 300 μm × 300 μm, consisting of a single layer of fabric fibers with 100–150 μm average thickness. According to SEM data, no differences in surface microstructure were found between the samples of groups 1 and 2 (Fig. 2a, 2b).

Group 3 scaffolds were fiber-reinforced gelatin films. In this case, the gelatin in the scaffold composition evenly covers the fabric fibers, which leads to alignment of the microrelief of the fibers of the fabric.

Samples of scaffolds based on natural silk fabric with a higher density (groups 4–6) were characterized by dense arrangement of fabric fibers with 150–200 μm thickness. Group 5 scaffolds had a more pronounced surface microrelief compared to group 4 scaffolds. Gelatin forms a uniform coating on the surface of group 3 scaffolds, which, as in the case of textile-based scaffolds with a lower density, led to alignment of textile microrelief.

The samples of all the resulting scaffolds were then examined for cytotoxicity (Fig. 4a, 5a), which revealed that the obtained samples had no toxic effect on cells, which made it possible to conduct further studies on the biocompatibility of the resulting scaffolds.

Cell adhesion on the obtained scaffolds was assessed on day 1 of the experiment. Cell adhesion was detected on all scaffold samples; the morphology of adhered cells on different samples did not differ.

The proliferative activity of cells on the obtained scaffolds was assessed on days 3, 5 and 7 of the experiment (Fig. 4b, 5b).

The proliferative activity of cells on scaffold samples based on textiles with a lower density (groups 1–3, Fig. 4b) steadily increased during the experiment. At the same time, there were no differences in the proliferative activity of cells in the samples of the different groups.

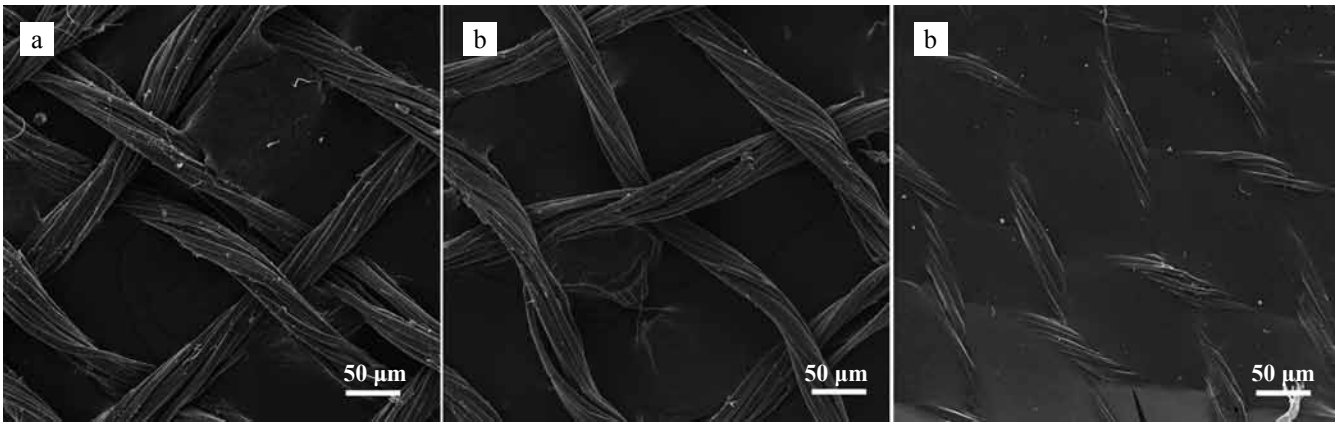

Fig. 2. Images of scaffold samples based on natural silk fabric with 15 g/m^2 density (gas-chiffon): a) Group 1, b) Group 2, c) Group 3. 50 μm

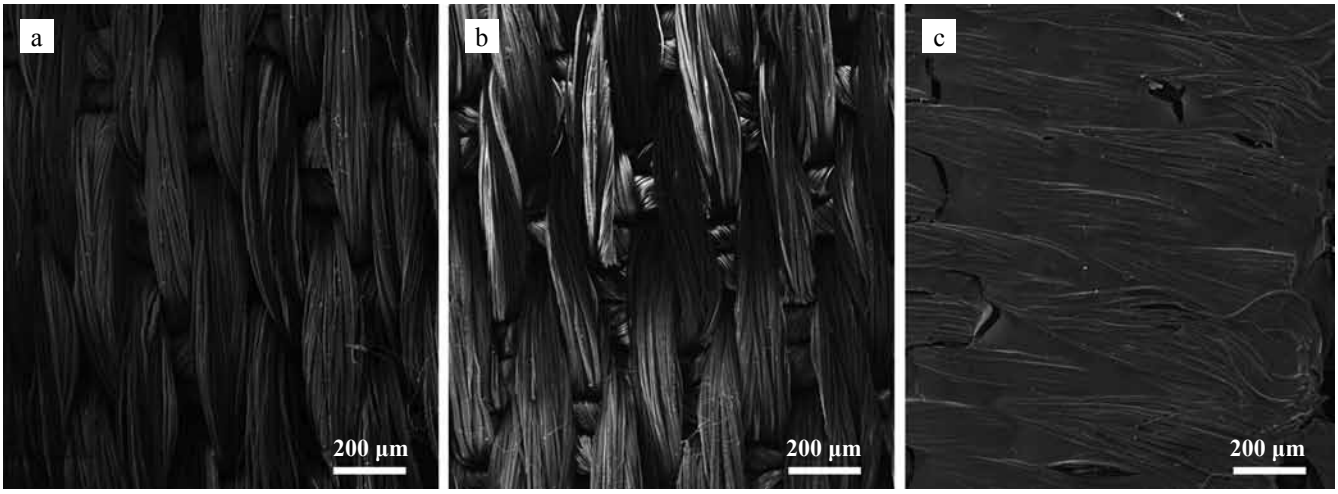

Fig. 3. Images of scaffold samples based on natural silk fabric with 155 g/m^2 density (double-sided satin): a) Group 4, b) Group 5, c) Group 6. 200 μm

Similar results were obtained for samples of scaffolds based on tissue with a higher density. However, from days 5 to 7 of the experiment, the proliferative activity of cells on the scaffold samples did not change and remained at a high level.

DISCUSSION

Six groups of natural silk fabric-reinforced scaffold samples were obtained for the study (Fig. 1). We developed a technique for regulating the rate of biodegradation of silk fabric-reinforced scaffold. The technique involves treating the natural silk fabric with an aqueous-alcoholic solution of calcium chloride [19]. In this study, the fabrics were treated with the solution for 45 minutes at 37 °C. Treatment with an aqueous-alcoholic solution of calcium chloride in a water bath is used by various scientific groups as one of the stages of obtaining an aqueous solution of silk fibroin for transition of silk fibroin molecules to an α-conformation state, which promotes protein dissolution [13, 21, 22]. It is also known that the rate of degradation of silk fibroin-reinforced constructs depends on conformation of protein molecules within the constructs. It was shown that the degradation rate of silk fibroin-based constructs increases when the proportion of molecules in the α-helix state increases [23]. Thus, the development considered in the study is based on implementation of the phase transition of silk fibroin without its dissolution due to the mild conditions in which the treatment is carried out.

Changes in the surface microstructure of the obtained scaffold samples as a result of treatment were shown by SEM. The study revealed an increase in the roughness of a scaffold based on a denser fabric after treatment. A similar effect after treatment of lower-density fabric was not detected by this method; so, it is necessary to study the structure of these samples with high resolution to reveal the features of micro- and nanostructure using methods that provide higher resolution, for example, scanning probe nanotomography [24].

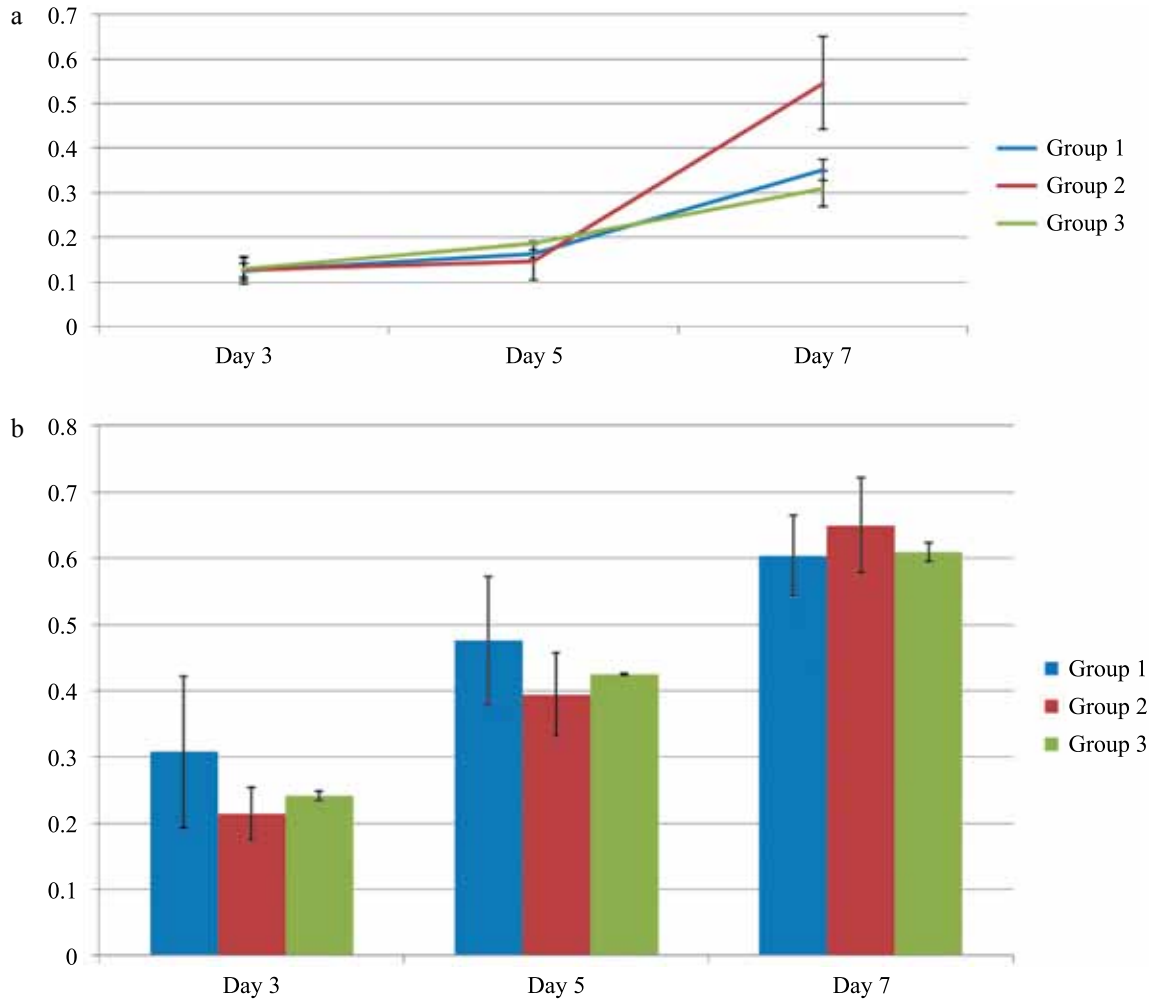

Fig. 4. Results of biocompatibility studies of scaffold samples based on natural silk fabric with 15 g/m^2 density (gas-chiffon, Groups 1–3): a) cytotoxicity, b) proliferative activity of Hep-G2 cells. Standard deviation values are shown for 5 independent measurements

The protocol of treatment with an aqueous-alcoholic solution of calcium chloride used in this study does not lead to fabric destruction and significant changes in its organoleptic properties. This allows for further manipulations with the treated fabric.

In this study, samples of biodegradable natural silk fabric-based scaffolds were treated with a gelatin type A solution. Gelatin type A is widely used in the field of cell technologies and tissue engineering to improve the biocompatible properties of constructs made of other polymers, as well as to ensure hemocompatibility [25, 26]. Treatment of biodegradable natural silk fabric-reinforced scaffolds with gelatin illustrates several possible ways of using such a construct.

Today, a wide range of naturally occurring polymers such as chitosan, alginate, gelatin, collagen, etc. are used for various tasks of tissue engineering and regenerative medicine. The main drawback is that they have low mechanical characteristics, such as strength and elasticity, which leads to fragility of the constructs obtained on their basis [27]. The use of natural silk fabrics as a reinforcing component of such constructs can provide the necessary mechanical characteristics and expand the scope of these polymers. Thus, a fabric with a lower density within the framework of this study can be considered as a reinforcing component of a gelatin film.

Another promising area of application of natural silk fabrics is their use as carriers for targeted drug delivery for gradual drug release. Thus, it is possible to obtain a scaffold with a predetermined degradation period, containing various bioactive substances or drugs that will be released from the scaffold as it degrades and provides effective organ and tissue regeneration.

Samples of the obtained biodegradable scaffolds do not exhibit a cytotoxic effect and are biocompatible, maintaining a high level of cell proliferative activity. The experiments did not reveal any differences in cell proliferative activity between the scaffolds of different groups. This is due to the fact that gelatin reduces the roughness of the scaffold surface, thereby hindering cell adhesion, which affects proliferative activity. At the same time, gelatin dissolution in the scaffold does not occur

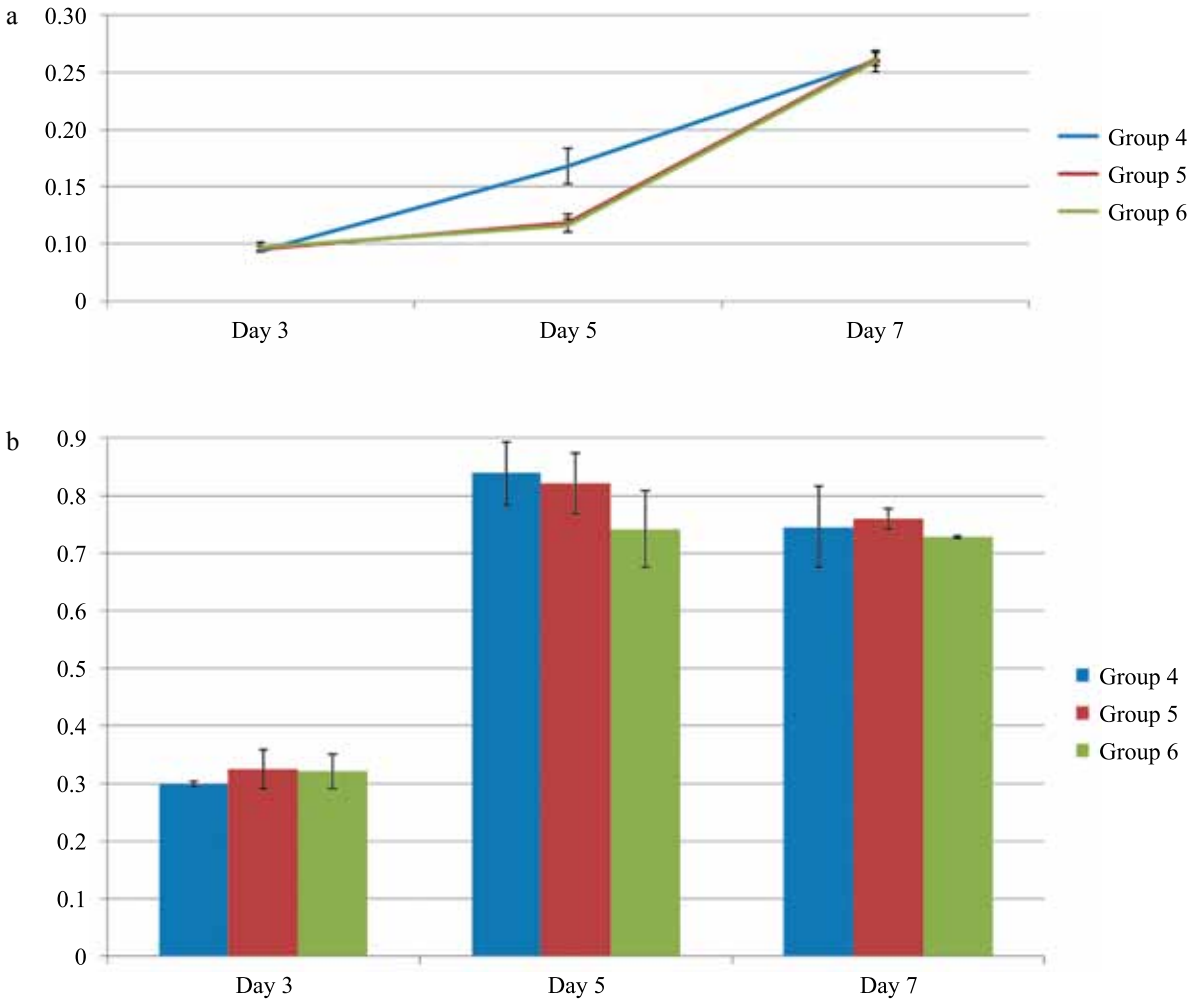

Fig. 5. Results of biocompatibility studies of scaffold samples based on natural silk fabric with 155 g/m^2 density (double-sided satin, groups 4–6): a) cytotoxicity, b) proliferative activity of Hep-G2 cells. Standard deviation values are shown for 5 independent measurements

due to treatment with crosslinking agents – EDC and NHS, which reduce the rate of degradation of the gelatin coating.

Thus, the approach to the use of natural silk fabrics used in the study makes it possible to obtain biocompatible silk fabric-reinforced scaffolds. The use of natural silk fabrics is a promising technological solution for obtaining biocompatible biodegradable scaffolds and their use in various fields of tissue engineering and regenerative medicine.

CONCLUSION

The developed technique facilitates production of silk fibroin-based scaffolds. The scaffolds obtained are biocompatible, which opens up the possibility of them being used in various fields of tissue engineering and regenerative medicine. The structure of the obtained scaffolds, as well as the dynamics of degradation *in vitro* and *in vivo*, require further detailed analysis.

The authors declare no conflict of interest.

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