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EXPERIMENTAL BIOLOGY

Relationship between Gelatin Concentrations in Silk Fibroin-Based Composite Scaffolds and Adhesion and Proliferation of Mouse Embryo Fibroblasts

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Porous scaffolds of silk fibroin and composite porous scaffolds with 10, 20, 30, 40, and 50% gelatin were made by the freezing–thawing method. The relationship between adhesion and proliferation rate mouse embryo fibroblast and the scaffold composition was studied by laser confocal scanning microscopy. Addition of gelatin to the scaffold structure stimulated adhesion and proliferation of mouse embryo fibroblasts; the optimal content of gelatin was 30%.

Key Words: *fibroin; gelatin; composite biodegraded scaffolds; adhesion; proliferation*

Development of technologies for replacement or restoration of lost or damaged organs and tissues is a priority goal of modern regenerative medicine. The search for appropriate materials for biological man-made analogs *in vitro* is in progress. Biodegraded polymers of natural origin, derived from plants, animal or human tissues are now more preferable for creation of materials for tissue engineering constructions. Ceramic composites and synthetic polymers, such as aliphatic polyesters, are also used [1]. Natural polymers are characterized by high biocompatibility and are nontoxic [3,5,8]. One of them is silk fibroin from *Bombyx mori* cocoon, with characteristics essential for creation of tissue engineering constructions. Articles made from fibroin are nontoxic, do not provoke infection development, retain their functional characteristics during the intended length of service, and are resistant to environmental conditions. By its chemical characteristics silk fibroin is an amphiphilic protein with a hydrophobic trend [10], its isoelectric point pI=4.2, and hence, fibroin is not soluble in water or in many of diluted acids and alkali. Normally, silk fibroin acquires a negative charge at the medium pH of 7.0. This promotes inhibition of cell adhesion and stimulates proliferation [2,4,9].

Natural polymer gelatin, a collagen denaturation product, was used as an additive to composite articles. Gelatin is the major component of extracellular matrix, it forms numerous fibrils, meshes, and filaments, constituting its structural and mechanical base. The main function of collagen is to maintain mechanical strength of tissues. This protein is involved in cell adhesion and migration, angiogenesis, tumor formation, tissue morphogenesis and regeneration. A total of 29 collagens and numerous collagen-like proteins are described for vertebrates [6]. A specific feature of collagen and gelatin is the presence of arginine, glycin, and aspartate triamino acid sequences (RGD) in their structure. Cell receptors integrins are RGD-specific, which improves the efficiency of mechanisms of eukaryotic cell adhesion to the surface of

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scaffolds from materials with this sequence in their structures [7].

We attempted creation of experimental scaffold specimens based on fibroin and composite scaffolds with 10, 20, 30, 40, and 50% gelatin and studied the relationship between adhesion and proliferation of mouse embryonic fibroblast (MEF) primary culture and the level of composite polymer.

MATERIALS AND METHODS

Bombyx mori cocoons were a kind gift from V. V. Bogoslovskii, Head of Republican Research Station for Silkworm Breeding, the Russian Academy of Agricultural Sciences (Stavropolye Territory, Zheleznovodsk). The cocoon silk was cleansed from minor component sericin (desericinization).

Sericin-free silk was dissolved in a mixture of $CaCl_2$:C_2H_5OH:H_2O (1:2:8 molar proportion) in water bath for 5 h. The resultant solution was centrifugated and dialyzed against distilled water. After 4 dialysis cycles the solution was centrifugated and its concentration was brought to 20 mg/ml by adding distilled water.

In order to make experimental fibroin-based scaffold and composite fibroin scaffold specimens with 10, 20, 30, 40, and 50% gelatin by the freezing–thawing method, 1% DMSO was added to polymer solution with a concentration of 20 mg/ml. Scaffolds were formed in the well of 24-well plates; 700 μl solution was added to each well. The plates were put into a freezer and left there for 7 days at -20°C. Frozen scaffolds were processed with 96% ethanol for 1 h, after which the samples were removed from the wells and transferred to 70% ethanol for storage.

The structure of scaffolds was studied by scanning electron microscopy and the pore size was evaluated. The samples were prepared as follows: the preparations were dehydrated in 96% ethanol and acetone and dried by the critical point transition method on the HCP-2 critical point dryer (Hitachi Ltd.). The inner structure of the scaffolds was studied on transverse sections. The preparations were covered with a layer of gold (20 nm) in argon at ionic current of 6 mA and 0.1 mm Hg pressure on an Ion Coater IB-3 device (Eiko Engineering Co., Ltd.). Experimental studies were carried out in a Camscan Series II electron microscope (Cambridge Instruments) in the SEI mode at microscope resolution of 10 nm and working voltage of 20 kV. The images were obtained by MicroCapture software (SMA).

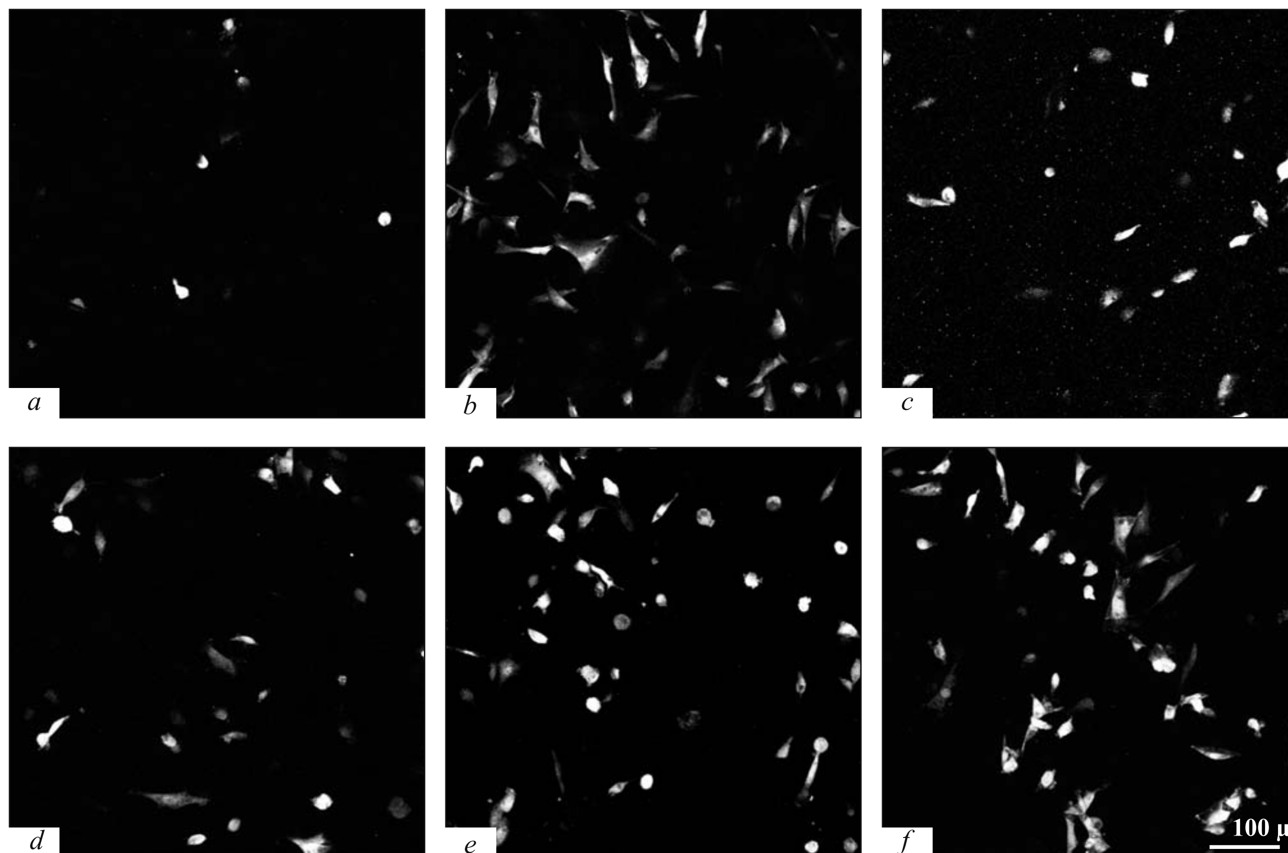

Fig. 1. Day 1 of MEF culturing in fibroin-based scaffolds (*a*) and composite scaffolds with 10% (*b*), 20% (*c*), 30% (*d*), 40% (*e*), and 50% (*f*) gelatin.

Scaffold porosity was studied by plunging the samples in 96% ethanol for 10 min, after which they were removed from the fluid, ethanol was wiped from the scaffold surface by filter paper, and the scaffold weight (W_w) and volume (V) were measured. Ethanol penetrated into the scaffold pores and filled them completely without causing the sample swelling or shrinkage, and hence, the ethanol volume absorbed by the scaffold could be considered equivalent to the pore size. Experimental samples were dried at ambient temperature for 12 h and the weight of dry scaffold (W_d) was then measured. The scaffold porosity was estimated by the formula:

$$\varepsilon(\%)=\frac{W_w-W_d}{\rho V}\times 100\%$$

MEF were isolated from GFP^+ embryos on day 13.5 of intrauterine development. In order to verify the date of pregnancy, two C57BL/6 females were placed together with a GFP^+ male overnight, after which the females were tested for the copulative plug. The moment of the copulative plug detection was assumed to be day 0.5 of pregnancy. On pregnancy day 13.5, the female was sacrificed, the uterus was removed, the embryo heads and viscera were removed, GFP expression in the embryos was verified on the UV transilluminator, the remaining tissues were fragmented with fine scissors under sterile conditions, dissociated in 0.05% trypsin/EDTA solution, and centrifugated (5 min, 1000 rpm), after which the cell suspension was transferred into a flask with 25 cm^2 bottom for adhesive cultures (Greiner). The cells were then cultured in DMEM with 4.5 g/liter glucose (HyClone) and 10% fetal calf serum (FCS; HyClone) at 37°C, 5% CO_2, and 95% humidity. The cells were reinoculated (1:3) every 3 days after formation of an 80-85% monolayer.

C57BL/6 females were bred at Pushchino Breeding Center, transgenic GFP^+ males were a kind gift from Dr. N. V. Logunova (Central Research Institute of Tuberculosis, the Russian Academy of Medical Sciences).

The diameters of scaffold pores were evaluated and MEF adhesion and proliferation were studied by confocal laser scanning system (Nikon Corporation) under an Eclipse biomedical inverted microscope for laboratory studies with A1 confocal modulus. High resolution images were obtained by making photographs of a series of optical sections with pinhole size, set according to the instruction. The diameter was 1 Airy unit, which allowed the optimal signal/murmur

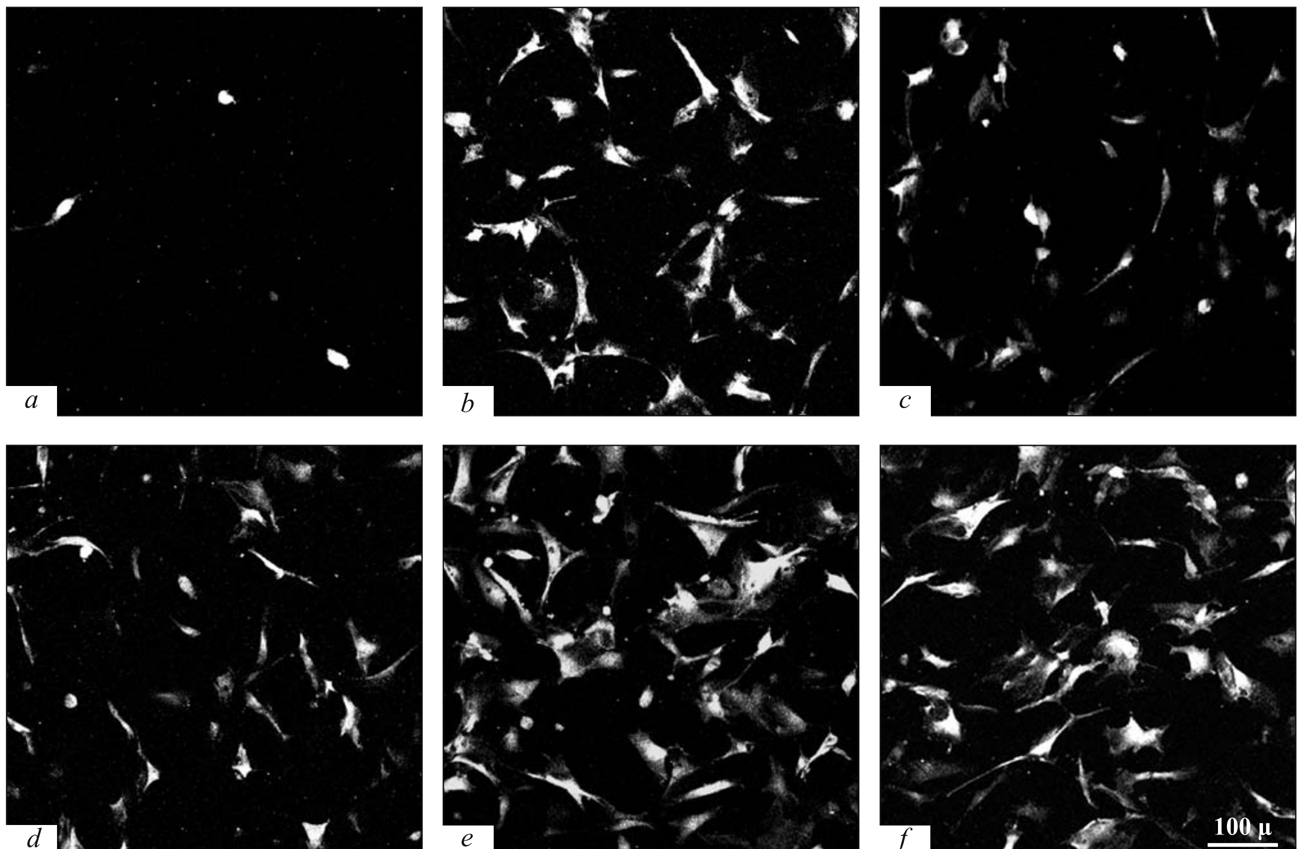

Fig. 2. Day 4 of MEF culturing on fibroin-based scaffolds (*a*) and composite scaffolds with 10% (*b*), 20% (*c*), 30% (*d*), 40% (*e*), and 50% (*f*) gelatin.

proportion. Laser and analyzing filters were adjusted according to the instruction.

After statistical processing the results were presented as the histogram of the results of one of the three analogous experiments. The common mean and the standard deviation were calculated.

RESULTS

Experimental samples of silk fibroin-based scaffolds and composite scaffolds retained the preset shape and were not destroyed in 70 or 90% ethanol. However, the scaffolds containing 40 and 50% gelatin lost their integrity after 1-week incubation in water. All the articles were elastically deformed in response to direct mechanical pressure; however, the elasticity was lost with increase of gelatin concentration. Composite scaffolds with gelatin concentrations higher than 30% restored their shape after deformation slower than fibroin-based samples and composite scaffolds with lesser gelatin concentrations.

The relationship between gelatin content in composite scaffolds and MEF proliferation and adhesion was studied. Cells were counted in images obtained by confocal laser scanning microscopy (Figs. 1 and 2). Analysis showed that addition of gelatin to the scaffold structure stimulated adhesion and proliferation of MEF (Fig. 3).

MEF migrated into the inner layers of composite scaffolds with high gelatin content worse than in scaffolds with lower levels of gelatin (Fig. 3).

Elements of the scaffold structure and cells at a depth of up to 300 μ could be observed; cell counts were analyzed for an area of 1 mm^2. Addition of gelatin into the scaffold structure increased the adhesion and accelerated the proliferation of MEF (Fig. 3). The counts of cells on the surfaces of composite scaffolds with 10% gelatin were 2-fold higher than on the surfaces of fibroin scaffolds after 24 h of culturing. Fibroin-based scaffolds supported cell migration, due to which the count of cells on the scaffold surface decreased. Addition of 10, 20, and 30% gelatin to the structure of composite scaffolds caused no changes in its mechanical characteristics, increased cell adhesion to its surface, and promoted cell migration to the inner layers of the scaffold. The content of cells on the surface of composite scaffolds with 10% gelatin did not increase, as cell migration was high. Cell adhesion increased on scaffolds with 20 and 30% polymer content, while the migration decreased, as a result of which cell counts increased 1.3-1.4 times on day 4. Hence, the data indicated that the optimal level of gelatin in fibroin-based composite scaffolds was 30%.

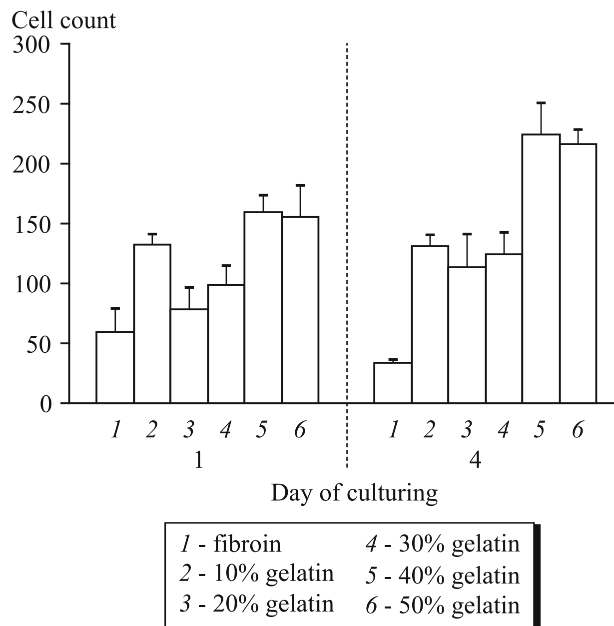

Fig. 3. Total count of MEF after culturing in 3D porous scaffolds based on silk fibroin and gelatin.

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REFERENCES

1. V. I. Sevast'yanov and M. P. Kirpichnikov, *Biocompatible Materials* [in Russian], Moscow (2011).
2. I. I. Agapov, M. M. Moisenovich, T. V. Druzhinina, *et al.*, *Dokl. Biochem. Biophys.*, **440**, 228-230 (2011).
3. I. I. Agapov, M. M. Moisenovich, T. V. Vasilyeva, *et al.*, *Dokl. Biochem. Biophys.*, **433**, 201-204 (2010).
4. I. I. Agapov, O. L. Pustovalova, M. M. Moisenovich, *et al.*, *Dokl. Biochem. Biophys.*, **426**, 127-139 (2009).
5. A. Bonartsev, S. Yakovlev, A. Boskhomdzhiev, *et al.*, *PLoS One*, **8**, No. 2, doi:10.1371/journal.pone.0057200 (2013).
6. K. E. Kadler, C. Baldock, J. Bella, and R. P. Boot-Handford, *J. Cell. Sci.*, **120**, Pt. 12, 1955-1958 (2007).
7. A. W. Morgan, K. E. Roskov, S. Lin-Gibson, *et al.*, *Biomaterials*, **29**, No. 16, 2556-2563 (2008).
8. M. M. Moisenovich, O. L. Pustovalova, A. Y. Arkhipova, *et al.*, *J. Biomed. Mater. Res.*, **96**, No. 1, 125-131 (2011).
9. M. M. Moisenovich, O. L. Pustovalova, J. Shackelford, *et al.*, *Biomaterials*, **33**, No. 15, 3887-3898 (2012).
10. E. Wenke, H. P. Merkle, and L. Meinel, *J. Control. Release*, **150**, No. 2, 128-141 (2010).