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DECELLULARIZATION OF DONOR PANCREATIC FRAGMENT TO OBTAIN A TISSUE-SPECIFIC MATRIX SCAFFOLD

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One of the pressing issues in tissue engineering is on how to obtain an artificial matrix that can simulate a biological microenvironment for cells. When creating a bioengineered pancreatic construct, a tissue-specific scaffold obtained from decellularized pancreatic tissue can serve as such matrix. **Objective:** to obtain and study the characteristic properties of a tissue-specific pancreas scaffold from decellularized human pancreatic fragments. **Materials and methods.** The decellularization protocol included 3 freeze/thaw cycles, followed by treatment with surfactants (sodium dodecyl sulfate and Triton X100). At each decellularization stage, samples were routinely stained with hematoxylin and eosin and for total collagen. In addition, immunohistochemical staining of decellularized human pancreas (DHP) for type I collagen and elastic fibers was performed. Cell nuclei in the original samples and the resulting matrix were visualized using DAPI fluorescent staining. DNA quantity in the native and decellularized pancreatic tissue was determined. The cytotoxicity of the tissue-specific matrix was evaluated *in vitro* by direct contact. The matrix properties of DHP samples were determined using mesenchymal stem cells (MSCs) of human adipose tissue. **Results.** A pancreatic decellularization method is proposed. This method allows to obtain a tissue-specific matrix in the form of a connective tissue scaffold completely free of detritus with preserved thin-fiber mesh-like structure, in which elastic and collagen fibers, including type I collagen, are identified. DAPI staining confirmed the absence of nuclear material in the decellularized matrix, while residual amount of DNA did not exceed 0.1%. Absence of matrix cytotoxicity and its ability to maintain adhesion and proliferation of human adipose tissue-derived MSCs was proved. **Conclusion.** As one of the stages in creating a bioengineered pancreatic construct, a method has been developed for producing a biocompatible (lack of cytotoxicity and immunogenicity) tissue-specific scaffold from decellularized human pancreatic tissue. In the scaffold, the morphofunctional properties of the native extracellular matrix-based scaffolds of the pancreas are preserved. Adhesion and proliferation of cell cultures are ensured.

Keywords: pancreas, decellularization, tissue-specific scaffold, tissue engineering.

INTRODUCTION

Insulin-dependent diabetes mellitus is a chronic disease resulting from the depletion of a population of β-cells due to autoimmune damage. Current research aims to replenish the population of β-cells by creating bioengineered equivalents of the pancreas consisting of β-cells, stem cells or isolated Langerhans islets and a carrier matrix that ensures longer survival and efficient functioning of the transplanted cells. The materials of various nature are used as matrices for their specific physicomechanical, biological, and functional properties, such as biocompatibility, lack of immunogenicity, mechanical strength and elasticity, biodegradability, etc. [1]. The imitation of signals from the native microenvironment, i.e. tissue specificity is an important property of matrices for tissue engineering.

There is no doubt that the "native" matrix with the characteristic features of structure and composition is the most suitable for cells. To obtain a tissue-specific matrix, organs and tissues are decellularized to remove DNA, cellular material and cellular surface antigens through various methods with chemical, enzymatic or mechanical treatment [2]. Decellularization protocols should be developed considering such factors as density and thickness of the initial tissue, cell number, and lipid content [2, 3].

Extracellular matrix (ECM) is a polypeptide chain of collagen, laminin, fibronectin, and elastin intertwined with polysaccharide chains, glycosaminoglycans [4]. Typically, collagens provide the structural rigidity and tissue adhesion, supporting the integrity and shape, structure of the organ, while elastin provides strength, elasticity, and extensibility of tissue. Besides, fibronectin, fibrillin, and laminin are involved in remodeling the cytoskeleton, contractility, and differential cell adhesion [4].

The composition and organization of ECMs varies from tissue to tissue, but the main function of all ECMs

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is to provide mechanical support for the cells and maintain a number of such biological functions as cell viability and growth. In the pancreas, ECM, which contains collagen type I, III, IV, V, and VI, elastin, laminin, and fibronectin regulates the main aspects of islets biology, including development, morphology and differentiation, intracellular signal transmission, gene expression, adhesion and migration, proliferation, secretion, and survival [4, 5]. The cell-matrix interactions are important in order for mature β-cells to remain functional and avoid apoptosis, as well as to maintain the functional mass of β-cells [6]. During the isolation process, the islets often lose a substantial portion of ECM and the vascular network, subsequently negatively affecting the viability of isolated Langerhans islets. It was shown that islets that partially retain ECM after isolation demonstrate a decrease in apoptosis rate and significantly better support insulin secretion than more aggressively purified islets [4].

Considering the importance of the ECM in ensuring the viability and functioning of Langerhans islets, in the process of decellularization it is important not only to free the matrix from the cellular component, but also, if possible, to keep the structure and composition of ECM as constant as possible. In the future, such a matrix can be recellularized with the corresponding cell types with the prospect of obtaining tissue equivalent of the pancreas with certain functional properties [6].

The pancreas matrix as a total organ was obtained from mice [7, 8], rats [9], pigs [6, 10], and humans [11]. However, the restoration of the vasculature of such intact decellularized organ scaffolds is a complex task. An alternative approach, which is especially relevant for the endocrine transplant of islets, is to combine cells with a decellularized matrix obtained from a pancreatic fragment [3]. Such a strategy may be promising for tissue engineering applications because of its ease of use.

In the present study, we researched the possibility of obtaining tissue-specific pancreatic matrix using small fragments of pancreatic tissue for decellularization. This approach makes it possible to increase the decellularization efficiency, to fully populate with cells the entire volume of the decellularized matrix, to simplify oxygen and nutrients delivery to donor cells, and specifically deep into the matrix, and to significantly reduce the cost of the dellularization procedure.

MATERIALS AND METHODS

Starting material

In the study, the tail part of pancreas obtained as a result of multi-organ retrieval of cadaver organs (n = 6) and unsuitable for transplantation (donor of 34–63 years of age) was used.

Pancreas decellularization

To increase the decellularization effectiveness, a freeze-thaw cycle was introduced into the study protocol to destroy tissue cells at a preliminary stage. Fragments of the donor pancreas were subjected to 1, 2 or 3 cycles of freezing at –80 °C and thawing to +37 °C with the subsequent mechanical grinding of the tissue to 1 × 1 × 1 mm. After that, tissue fragments were processed in three shifts in a buffer solution (pH = 7.4) containing sodium dodecyl sulfate 0.1% solution and an increasing concentration of Triton X100 (1%, 2% and 3%, respectively) (Sigma, USA). In each solution, the sample was kept at room temperature for 24 hours and was constantly stirred at 2 rpm with the CellRoll roller system (INTEGRABiosciencesAG, Switzerland). When changing solutions, the finely dispersed matrix was filtered using a metal sieve (0.4–0.6 mm cell diameter) and returned to the solution with a higher Triton X100 concentration.

At the end of the decellularization process, the pancreas fragments were thoroughly washed off the surfactant residues for 72 hours in phosphate buffer with the addition of an antibiotic / antimycotic. The washed pancreas fragments were out in cryovials, frozen and subjected to γ-sterilization (1.5 Mrad).

Histology

At each stage of decellularization, to control the efficiency of the process, part of the material was fixed in 10% buffered formalin, dehydrated in alcohols of increasing concentration, kept in a mixture of chloroform and ethanol, chloroform and poured into paraffin. 4–5 μm thick sections were obtained with the RM2245 microtome (Leica, Germany) and subsequently stained with hematoxylin and eosin, total collagen (Masson's method) and elastic fibers (Unna–Tenzer method). The cell nuclei were visualized with DAPI fluorescence staining (Sigma, USA). Besides, an immunohistochemical reaction was performed on type I collagen with the anti-collagen I antibody (Abcam, UK) and the Rabbit Specific HRP | DAB (ABC) Detection IHCkit imaging system (Abcam, UK).

Total DNA detection in matrix

The residual amount of DNA serves as an indicator of the cellular components preserved in the decellularized matrix carrying the bulk of the antigens that provide the transplant rejection reaction [12].

Before the study, the samples were stored at –20 °C. DNA was isolated with the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions.

The quantification of double-stranded DNA was performed with the Picogreen Quant-iT fluorescent dye™ (Invitrogen, USA) in accordance with the manufacturer's instructions. Briefly, 50 μl of the lysate of the test sample was diluted 1:1 with TE buffer solution, and then

added to 100 μl of the dye solution. For 5 minutes, the resulting solution was incubated at room temperature without access of light, then the samples were activated by radiation with 480 nm wavelength and then analyzed with the Spark 10M microplate reader (Tecan Trading AG, Switzerland) at 520 nm wavelength. To determine the absolute DNA amount, the bacteriophage λ DNA (Invitrogen, USA) calibration curve was used (0 ng/ml – 1000 ng/ml).

Matrix cytotoxicity study

The cytotoxicity of matrix samples in the form of fragments of decellularized human pancreatic tissue *in vitro* was evaluated by direct contact according to the GOST ISO 10993-5-2011 international standard on the mouse fibroblast culture line L929 [13]. The culture medium with 10% fetal calf serum (ETS, HyClone, SV30160.03, USA) served as the negative control. The positive control sample was a single-element aqueous standard of 10,000 μg/ml (Sigma-Aldrich, USA). All procedures were performed under aseptic conditions. Culture monitoring was performed with the Eclipse TS100 (Nikon, Japan) inverted microscope.

The metabolic activity of fibroblasts after contact with matrix samples was determined after 24 hours with the prestoBlue™ CellViabilityReagent (Invitrogen™, USA) according to the protocol recommended by the manufacturer. Changes in media absorption were recorded with the Spark 10M microplate reader with SparkControl™ Magellan V1.2.20 software at 570 nm and 600 nm.

The data quantitative and statistical processing was performed with MicrosoftExcel 2007. All results are presented as mean ± standard deviation. Differences were considered significant at $p < 0.05$.

Study of the functional properties of the matrix

The functional properties of the decellularized human pancreas (DHP) matrix with respect to its ability to maintain cell adhesion, proliferation, and differentiation were studied in a culture of human fat tissue mesenchymal stromal cells (HFTMSC). HFTMSCs were cultured (2^{nd} passage) at 15 × 104 cells / 10 mg of the matrix. After 6 days of cultivation, several samples were taken to assess the HFTMSC viability with the LIVE/DEAD® Cell Viability / Cytotoxicity Kit (Molecular probes® by Life technologies™, USA). After 15 days of cultivation, the remaining matrix samples with cells were fixed in 10% buffered formalin for further histological analysis.

RESULTS

Histology of native pancreas

A morphological study of native pancreas showed the three studied glands bearing signs of expressed lipomatosis, while signs of diffuse fibrosis of pancreatic tissue were found in three other glands (Fig. 1).

Histological analysis of tissue with lipomatosis at different stages of pancreas fragment decellularization

We studied 3 series of samples of native pancreas with signs of lipomatosis, which differ in the number of freezing and thawing cycles. Histology showed that at the initial stage of treatment, after single freezing, in pancreas tissue with lipomatosis, partial damage to the pancreatic parenchyma cells was observed, expressed in the destruction, primarily, of acinar tissue cells. At this, the borders between the cells directly in the acini looked blurred, blurred, and the cell nuclei were not visualized or were pycnotic. However, the contours of the acini were still clearly defined. The Langerhans islets, on the whole, looked structurally more intact, although a significant part of the islet cells nuclei was hyperchromic. It should be noted that at this stage, the process of cell destruction was not global in nature and involved no more than 50% of the studied fragment of pancreatic tissue (Fig. 2, a, b).

The fragments of pancreas with lipomatosis which underwent two freezing / thawing cycles significantly differed in morphological features from the previous series of samples. First of all, this concerned the total spread of the process of cell destruction to the entire tissue fragment. Not even a partial preservation of morphological signs of the initial structure of pancreatic tissue was observed: acini, islets, their fragments were not detected. In the samples, only single preserved cells scattered in the thickness or individual pyknotic nuclei were determined. At this, the stroma was abundantly masked by small grains of cellular detritus (Fig. 2, c, d). It was decided to use the samples obtained in this way for further processing with surface-active agents in order to obtain purified ECM pancreas. As a result of the decellularization, the connective tissue framework was able to get rid of the bulk of the detritus and to obtain samples characterized by a fine-fiber openwork structure, where, however, the minimum inclusion of cell detritus grains was locally revealed (Fig. 3, a, b).

In the samples of pancreas fragments with lipomatosis, after three consecutive freezing-thawing cycles, unlike previously studied samples, preserved cells, cell nuclei, and karyorhexis products were not found. At this, the stroma was disguised by finely divided detritus fragments (Fig. 2, d, e).

At the subsequent decellularization of tissue with lipomatosis, after three freezing-thawing cycles, almost complete removal of detritus grains was observed (Fig. 3, d) and, as a result, a purified fine-fiber matrix was obtai-

ned where blue collagen fibers were distinctly detected by Masson staining (Fig. 3, b). The performed immunohistochemical staining also confirmed the presence of type I collagen in the ECM, which is the most important component of the pancreatic tissue matrix (Fig. 3, a). At orcein staining, red-brown elastic fibers were noted (Fig. 4, a, b). These results indicate the preservation of the main fibrillar proteins of the pancreatic tissue matrix.

Histology of fibrous tissue at different stages of pancreatic fragment decellularization

Already at the first stages of treatment of the pancreas with fibrosis, a significant difference was revealed in the histological pattern from pancreas with lipomatosis, even after three freezing / thawing cycles, a significant number of cells with pycnotic nuclei remained in the tissue fragments (Fig. 2, g, h).

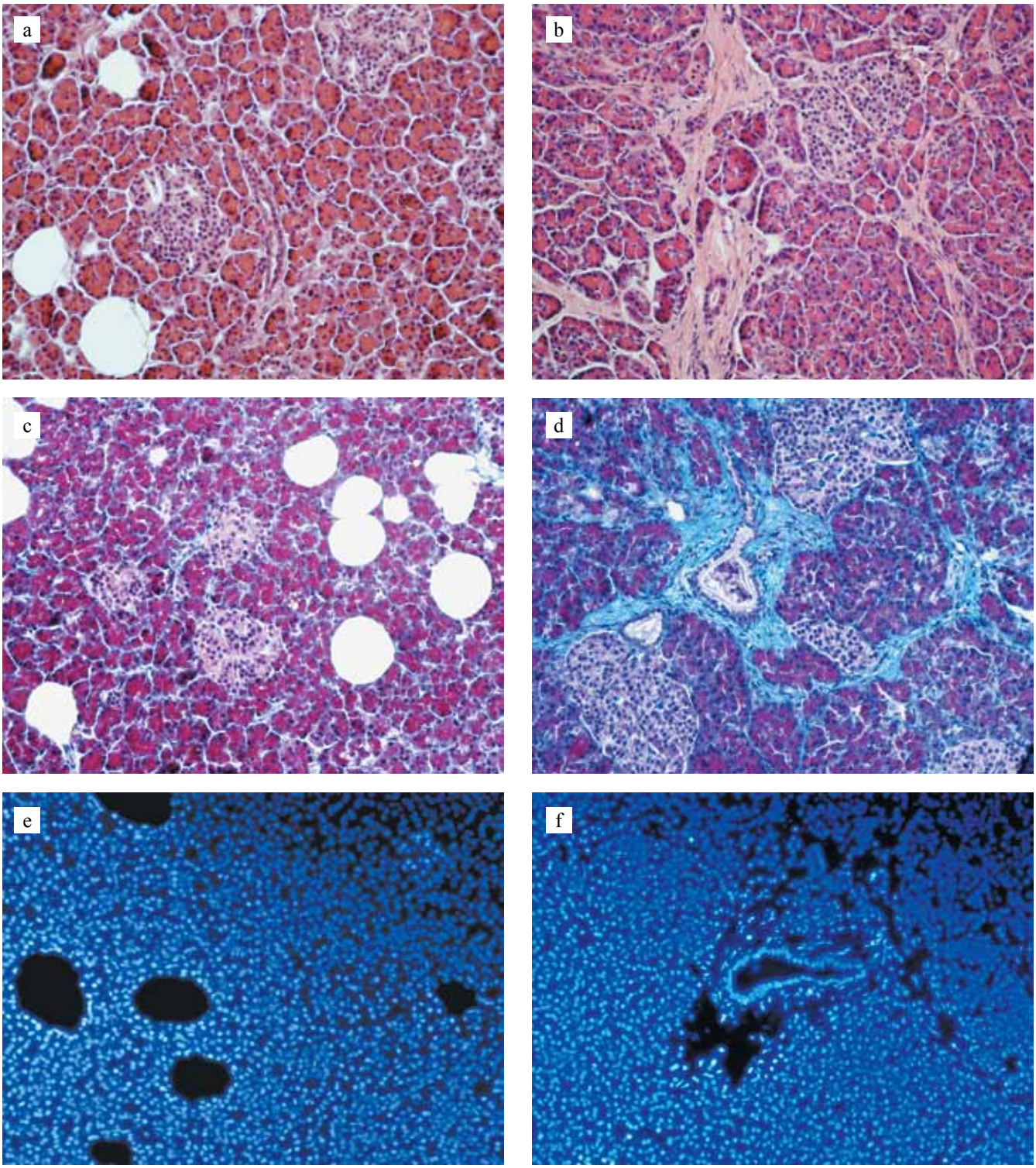

Fig. 1. The histological pattern of human pancreas: a, b, e – native donor pancreas with lipomatosis features; b, d, f – native donor pancreas with fibrosis features; a, b – hematoxylin and eosin; c, d – Masson's method; e, f – nuclear DAPI staining. ×200

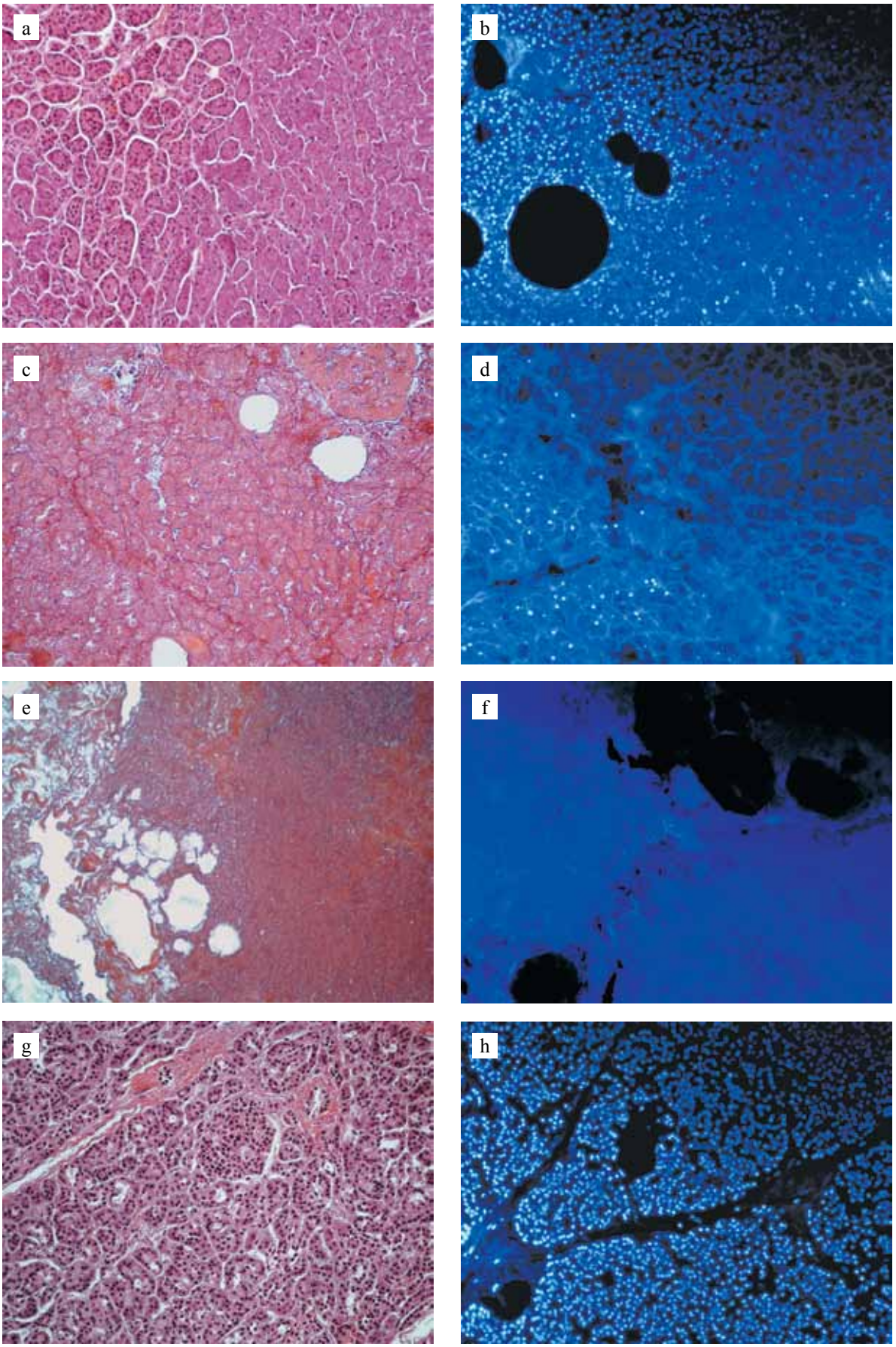

Fig. 2. The histological presentation of pancreatic tissue after successive cycles of freezing and thawing: a, b – 1 cycle of freezing up to –80 °C and thawing up to +37 °C; c, d – 2 cycles; e, f – 3 cycles of freezing and thawing of pancreas with lipomatosis; g, h – 3 cycles of freezing and thawing of pancreas with fibrosis; a, b, e, f – hematoxylin and eosin; b, d, e, h – nuclear DAPI staining. ×200

Nevertheless, such samples were further processed with surface-active agents to confirm the assumption that the developed protocol of decellularization is unsuitable for pancreas with diffuse fibrosis. Indeed, in the obtained samples there were areas with a large number of preserved cells and nuclei (Fig. 3, e, f), while with a similar treatment of the pancreas with lipomatosis, it is possible to obtain a well-purified fine-fiber matrix.

Total DNA quantification in DP matrix

Quantitative analysis showed that in case of pancreatic decellularization with diffuse fibrosis, according to

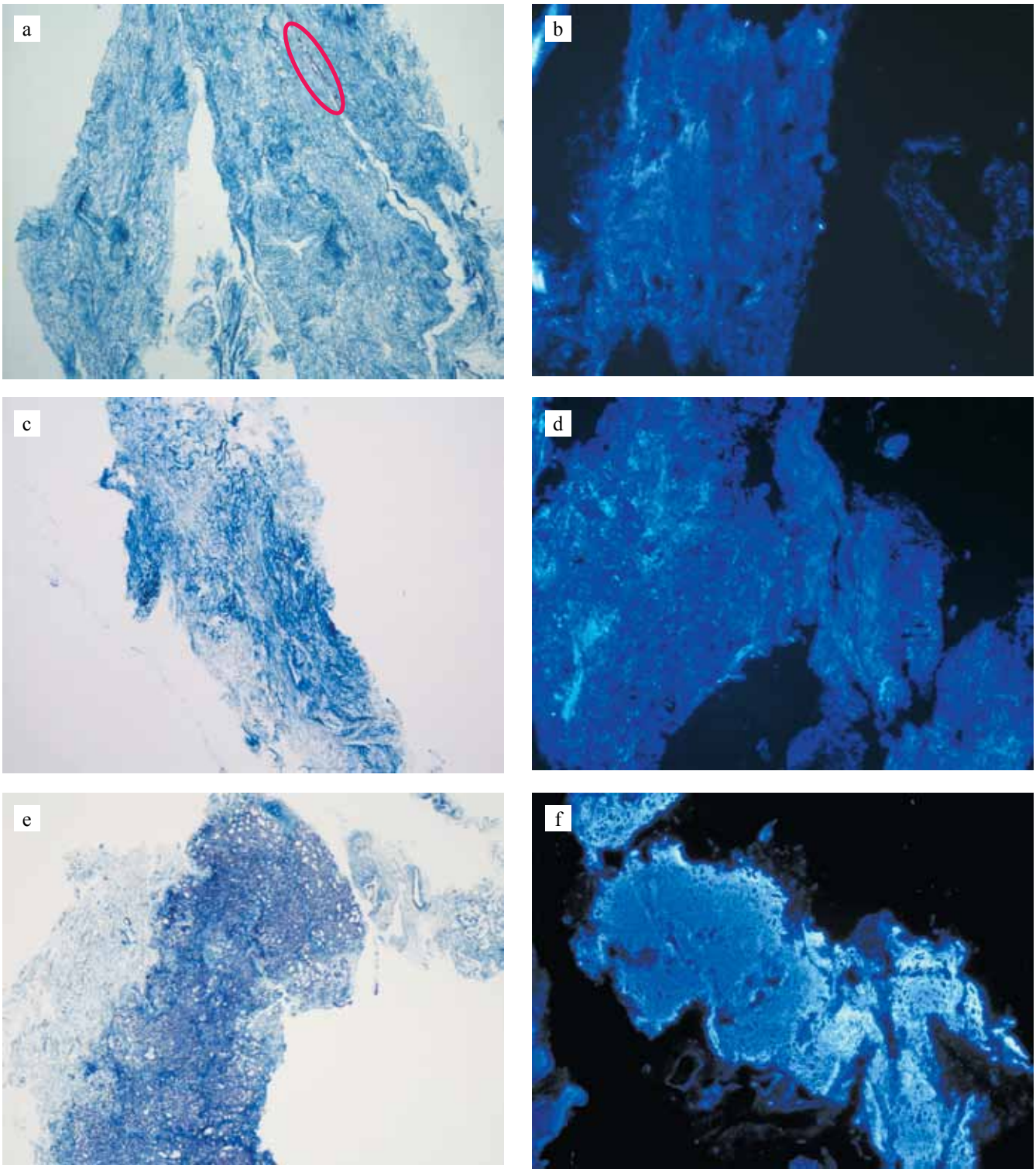

Fig. 3. The histological presentation of decellularized pancreas (DP): a, b – DP with lipomatosis after 2 cycles of freezing and thawing, the red oval marks the area with microfragments of cellular detritus; c, d – DP with lipomatosis after 3 cycles of freezing and thawing; e, f – DP with fibrosis after 3 cycles of freezing and thawing; a, c, e – Masson method; b, d, f – nuclear DAPI staining. ×100

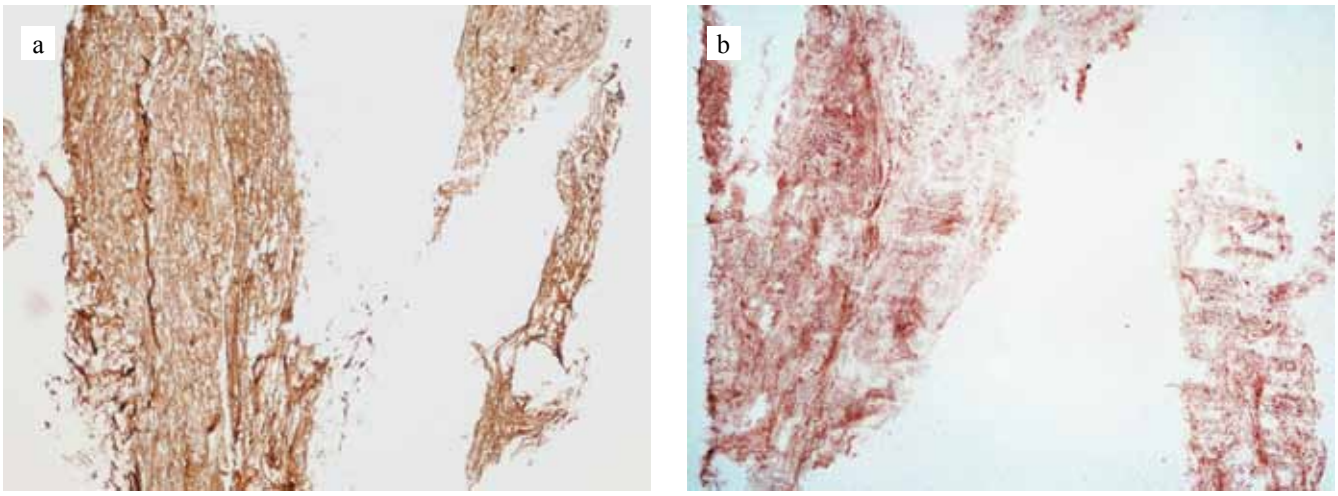

Fig. 4. The histological presentation of decellularized pancreas with lipomatosis: a – immunohistochemical staining demonstrates the presence of type I collagen in the matrix; b – Unna–Tenzer staining reveals the presence of elastic fibers in the matrix. ×100

the developed protocol, 6128.3 ± 718.0 ng DNA/mg of tissue (41.5% DNA) were preserved in the tissue. In this, after the pancreatic pancreatic gland with lipomatosis, we managed to clear the tissue off DNA to a significant extent ($p < 0.05$): the DNA content decreased from 14782.2 ± 319.9 ng/mg of tissue to 12.6 ± 0.9 ng/mg of tissue (Fig. 5), which is 0.1% DNA indicating the high efficiency of the developed protocol of decellularization and low immunogenicity of the resulting matrix, respectively.

Based on the results obtained, for further studies, we used the DP matrix obtained according to the proposed decellularization protocol only for pancreas fragments with detected signs of lipomatosis.

Cytotoxicity of DP matrix

The results were analyzed according to an evaluation scale of the degree of response of cells after incubation with matrix samples in accordance with GOST ISO 10993-5-2011 (Table 1). The negative control corresponds to reaction degree 0, the positive control – to 3 or 4. The degree of response of the test sample should not exceed 2.

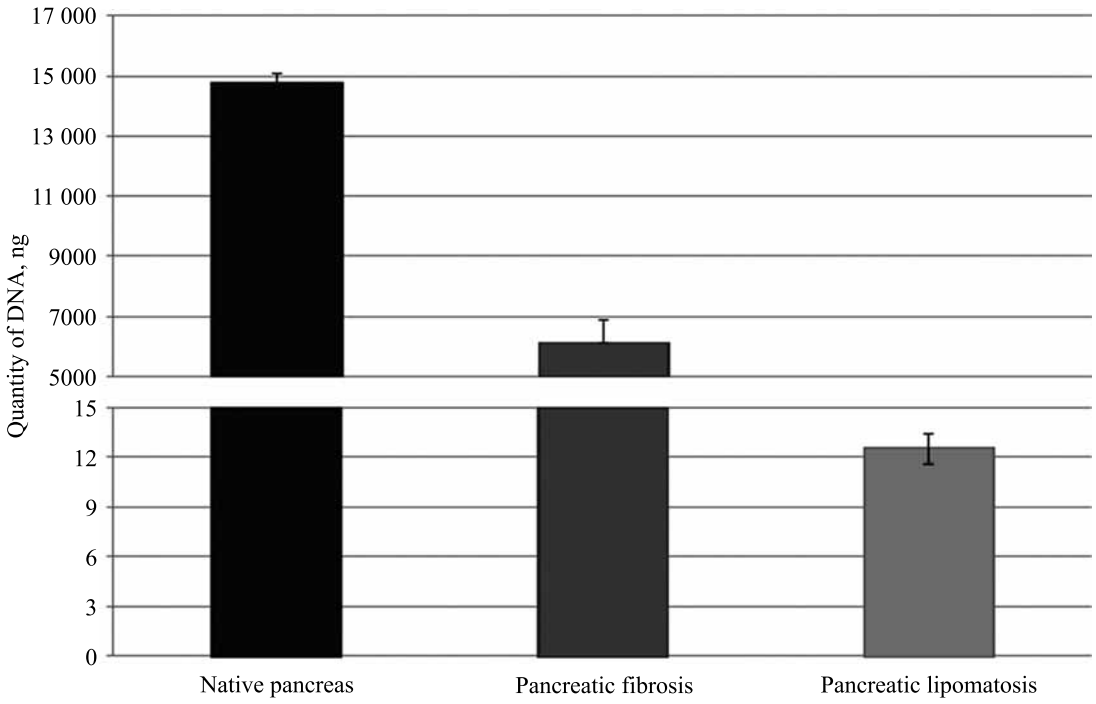

Fig. 5. Quantification of DNA content in native tissue, in the pancreas with fibrosis and with lipomatosis after decellularization

Table 1

Degrees of cell response

Degree	Response	Observed	
0	Absent	Single intracytoplasmic granules Over 90% of proliferating cells	No lysis
1	Insignificant	Not more than 20% of the cells are round, loosely attached, without intracytoplasmic granules Over 80% but under 90% of proliferating cells	Lysis not over 20%
2	Indistinct	Not more than 50% of the cells are round, without intracytoplasmic granules Over 50% but under 80% of proliferating cells	Lysis not over 50%
3	Moderate	Not more than 70% of the monolayer contains round cells Over 30% but under 50% of proliferating cells	Lysis not over 70%
4	Distinct	Almost completely destroyed monolayer Under 30% of proliferating cells	Lysis over 70%

Table 2

Percentage of viable fibroblasts of L929 line relative to the negative control

Sample No.	Sample name	% viable cells relative to the negative control ± sigma	Degree of cell response
1	DP, donor 1	97.3 ± 8.9	0
2	DP, donor 2	90.5 ± 3.9	0
3	Positive control	7.8 ± 2.3	4

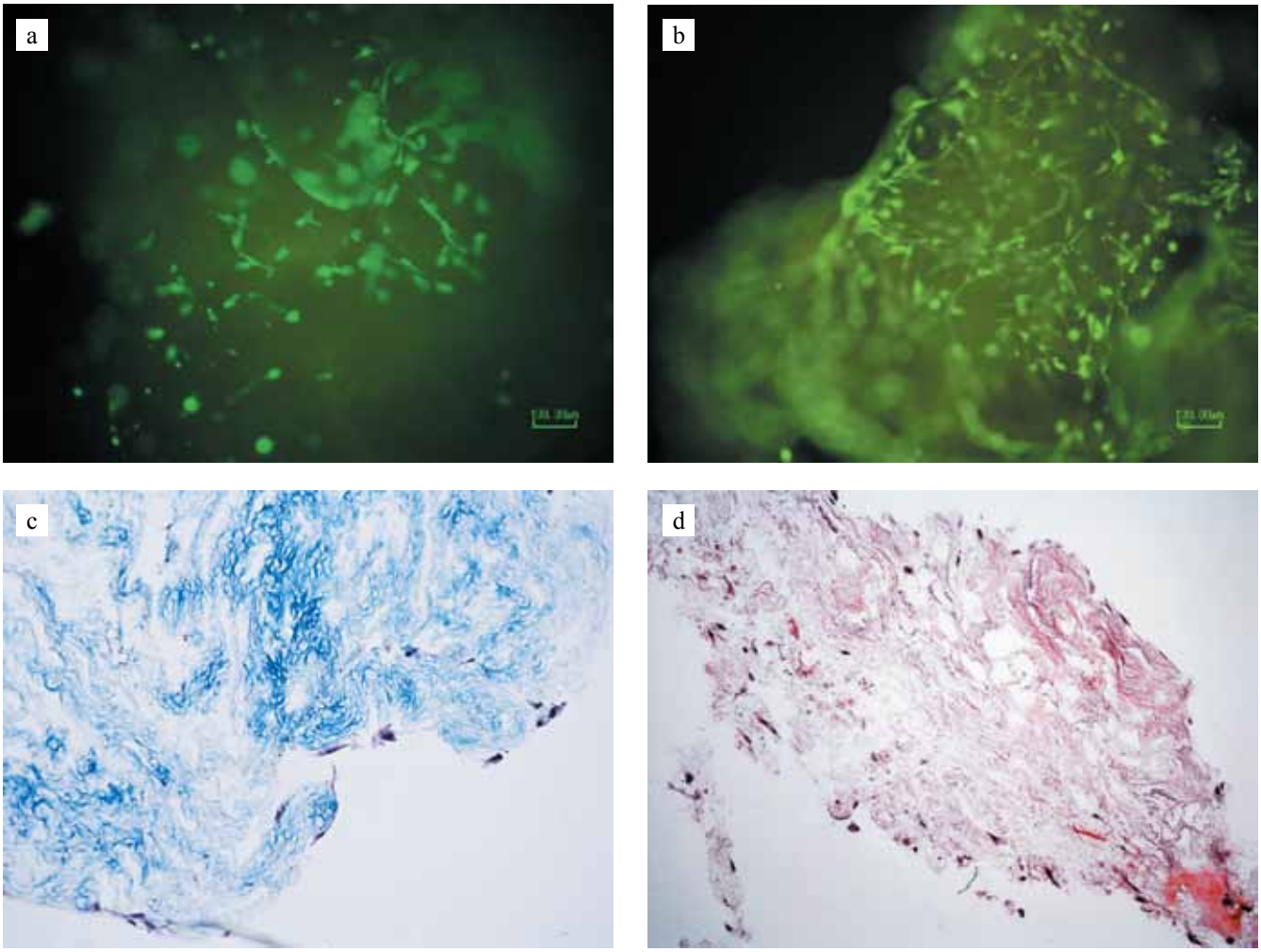

Fig. 6. Culturing of hADMSCs on tissue-specific pancreas scaffold: a – intravital staining of hADMSCs with LIVE/DEAD® vital stain after 1 day of culturing; b – intravital staining of cells with LIVE/DEAD® after 6 days of culturing; c – hADMSCs after 15 days of culturing on DP-matrix, the Masson method. ×200; d – hADMSCs after 15 days of culturing on DP-matrix, hematoxylin and eosin. ×100

Table 2 shows the values characterizing the viability of L929 fibroblasts relative to the negative control, the culture medium with 10% ETS. After contacting, the fibroblast viability remains above 90% which corresponds to the response degree of 0 and indicates the absence of the cytotoxic effect of the samples of the studied matrices. The positive control showed sharp cytotoxicity with the response degree of 4.

Functional properties of DP matrix

Already on the first day of cultivation (Fig. 6, a), HFTMSC on the DP matrices showed cell adhesion and spreading. By the 6th day, the HFTMSC number increased, there were no non-viable cells with their nuclei stained red (Fig. 6, b).

Histology of the samples showed that after 15 days of cultivation, the HFTMSC matrix was intensively populated. The cells are flattened, have a fibroblast-like shape characteristic of this type of cells, are not located only on the surface, but also actively penetrate into the deep layers of the matrix (Fig. 6, c, d).

CONCLUSION

Thus, the criterion for the selection of source material for the effective decellularization of human pancreas is defined. The advantage of using pancreas with lipomatosis was revealed compared with fibrosed pancreas. The proposed protocol for the decellularization of pancreatic donor fragments with lipomatosis is effective and allows one to obtain a tissue-specific matrix / framework free of cells and cell fragments, with a low DNA content and preservation of the morphofunctional properties of ECM pancreas. The resulting matrix does not show signs of cytotoxicity, supports HFTMSC adhesion and proliferation, and can be further used for iscellularization by islet cells (insulocytes, endocrine cells of Langerhans islets) when creating the bioengineered pancreas construct.

The authors declare no conflict of interest.

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