DOI: 10.15825/1995-1191-2022-1-64-71

DETERMINING THE OPTIMAL PANCREATIC DECELLULARIZATION PROTOCOL, TAKING INTO ACCOUNT TISSUE MORPHOLOGICAL FEATURES

A.S. Ponomareva, N.V. Baranova, L.A. Kirsanova, G.N. Bubentsova, E.A. Nemets, I.A. Miloserdov, V.I. Sevastianov

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

Introduction. Developing a tissue-engineered pancreatic construct (TEPC) involves a search for matrices/scaffolds capable of mimicking the structure and composition of the natural extracellular matrix (ECM), which is an important component of the tissue microenvironment. A cell-free, tissue-specific matrix obtained from pancreas decellularization seems to be the most suitable for creation of a TEPC. The choice of pancreatic tissue decellularization protocol should take into account the morphological characteristics of the original pancreas. Preservation of the architectonics and composition of the native tissue in the decellularized pancreas matrix (DPM), and the presence of native ECM components allow for creation of conditions for prolonged vital activity of functionally active islet (insulin-producing) cells when creating TEPC. **Objective:** to determine the optimal parameters for decellularization of deceased donor pancreas with fibrosis, lipomatosis, and without pronounced signs of fibrosis and lipomatosis. **Materials and methods.** We used the caudal part of the pancreas obtained after multiorgan procurement from deceased donors, which was unsuitable for transplantation. Tissue-specific matrix was obtained by a combination of physical and chemical methods of pancreatic decellularization. A freeze-thaw cycle protocol and two protocols using osmotic shock were used. Samples of initial pancreatic tissue and decellularized fragments were subjected to histological analysis. **Results.** It was shown that a physico-chemical method with freeze-thaw cycles is suitable for effective pancreatic decellularization in severe lipomatosis; a physico-chemical method using osmotic shock, but different protocol variants, is suitable for pancreas with diffuse fibrosis and for pancreas without pronounced signs of fibrosis and lipomatosis. **Conclusion.** For complete human pancreatic decellularization, the protocol should be correlated with histological features of the original tissue.

Keywords: pancreas, lipomatosis, fibrosis, decellularization, tissue-specific scaffold.

INTRODUCTION

Developing tissue-engineered constructs of tissues and organs, including the pancreas, includes the search for matrices (scaffolds, frameworks) capable of mimicking the structure and composition of the natural extracellular matrix (ECM). ECM secreted by cells is a network of macromolecules, including polysaccharide glycosaminoglycans (GAG) and proteins (collagens, laminins, fibronectin), and is an important component of the tissue microenvironment [1]. ECM performs many functions in the tissue, such as provision of structural integrity, mechanical properties and tissue organization, cell-matrix and signaling interactions such as cell attachment sites, regulation of cell adhesion, proliferation, migration, differentiation and death [2]. A tissue-specific matrix, in which the features of the native tissue architectonics and composition are preserved, seems to be the most suitable for TEPC creation [3–5].

One of the promising methods for obtaining tissue-specific matrices is decellularization of tissues and organs. An efficient decellularization process ensures removal of cellular material, including DNA, and cell surface antigens from the native tissue, while keeping as much as possible the structural, biochemical and biomechanical properties of the native ECM using different tissue processing techniques [1].

Most protocols describe the combined and sequential application of various physical, chemical and enzymatic methods to achieve effective decellularization. Decellularization of a large tissue fragment is quite a long process due to the need for penetration of all reagents into the target cells [4, 6]. In this case, physical exposure can disrupt the matrix structure, while chemical and enzymatic methods can cause reactions that will damage ECM components and even change ECM chemical composition [1]. Decellularization protocols must also take into account the characteristics of the original

Corresponding author: Anna Ponomareva. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Phone: (499) 196-26-61; (926) 585-23-73. E-mail: a.s.ponomareva@gmail.com

tissue, such as density and thickness, and the presence of lipids. Identical tissue may have different characteristics of structure and composition depending on the characteristics of the donor. For these reasons, optimization of the decellularization protocol is of paramount importance on a case-by-case basis.

Among the physical methods of decellularization, freeze-thaw cycles, osmotic shock method, mechanical agitation, perfusion, ultrasound and others are widespread. When tissue is frozen, intracellular ice crystals are formed, resulting in destruction of cell membranes and cell lysis. However, ECM protein structures can also be destroyed, so it is necessary to monitor the rate of temperature change in order to control the size of ice crystals formed [7]. Hypotonic and hypertonic solutions (osmotic shock method) [1] promote cell lysis but do not remove cell fragments from the matrix. Moreover, removal of DNA residues is of paramount importance in all decellularization protocols because of the tendency of nuclear material to attach to ECM proteins. Removal of cellular detritus is facilitated by a mechanical stirring process carried out with a magnetic stirrer, an orbital shaker, or a roller system [5, 8].

Physical methods alone are not sufficient for complete tissue decellularization. However, they are effective when combined with chemical and enzymatic processes. Surfactants are used as the chemicals (detergents) for dissolution of cell membranes and detritus dissociation in the decellularization process. Triton X100 detergent, targeting lipid-lipid and lipid-protein interactions, is often used to treat tissues with high protein content in ECM, while it is used with caution to treat tissues with high GAG content [9]. Sodium dodecyl sulfate (SDS) is used to effectively remove nuclear and cytoplasmic fragments. SDS dissolves both cellular and nuclear membranes but tends to denature proteins and can alter the natural structure of matrix [1]. For this reason, short-term SDS treatment is the most common – in order to minimize possible damage to proteins and to the overall matrix structure.

When creating TEPCs, the presence of native ECM components in the decellularized pancreatic matrix (DPM) allows creating conditions for prolonged life activity of functionally active islet (insulin-producing) cells [3–6, 10]. Maximum complete removal of cellular material from DPM minimizes immune response during further TEPC implantation [6]. Pancreatic islets cultured in the presence of DPM have been shown to increase insulin secretion compared to isolated islets in monoculture [11].

The aim of our study was to determine the optimal parameters for decellularization of deceased donor pancreas with fibrosis, lipomatosis and without pronounced signs of fibrosis and lipomatosis.

MATERIALS AND METHODS

Baseline

A significant number of deceased donor pancreas cannot be used for transplantation because organ transplantation requires strict pancreas selection criteria [12]. As a result of strict requirements for the quality of donor pancreas [13], many organs are rejected on the basis of medical history, fibrosis, lipomatosis, anthropometric characteristics and other parameters, even if the pancreas is healthy and functioning. Such organs can be reprocessed (including undergoing a decellularization process) for creation of biomaterials and can be used for tissue engineering rather than disposed of.

For the study, we used the caudal portion of the pancreas obtained as a result of multiorgan harvesting from deceased donors (n = 10, age of donors 34–63 years) and unsuitable for transplantation. Pancreatic tissue was stored at –80 °C until decellularization.

Decellularization of pancreatic fragments using freeze-thaw cycles

We have previously proposed a protocol for decellularization of donor pancreatic fragments with lipomatosis, which allows to obtain a tissue-specific matrix/scaffold free of cells and cell fragments, with low DNA content and preserved morphofunctional properties of the pancreatic ECM [14]. Pancreatic tissue was subjected to three freeze-thaw cycles to –80 °C and thawing to +37 °C followed by mechanical grinding (2 × 1 × 1 mm) and processing at room temperature under constant stirring in a CellRoll rotary system (INTEGRA Biosciences AG, Switzerland), with the buffer solution (pH = 7.4) containing 0.1% SDS solutions changed three times and increasing Triton X100 concentration (1, 2, and 3%, respectively) (Sigma, USA). Decellularized pancreatic fragments were then thoroughly washed of surface-active agent residues for 72 hours in phosphate-buffered saline with addition of an antibiotic/antimycotic.

In our work, we used this protocol for decellularization of pancreatic tissue that had no signs of fibrosis and lipomatosis.

Decellularization of pancreatic fragments using osmotic shock (two protocol variants)

Focusing on known methods of decellularization of different parenchymatous organs [1, 3–6, 15], we used physicochemical method with osmotic shock (exposure to ionic strength) in two versions for pancreas with fibrosis and for pancreas without fibrosis and lipomatosis symptoms.

Mechanically crushed pancreatic tissue (to a size of no more than 2 × 1 × 1 mm) was treated with detergents

at room temperature under continuous stirring in a Cell-Roll rotary system (INTEGRA Biosciences AG, Switzerland). In the first variant, 0.1% SDS solution and low and high ionic strength phosphate-buffered saline were used (variant I), and in variant II, 0.1% SDS solution and high and low ionic strength phosphate-buffered saline were used (variant II). Both variants were followed by thorough washing off of residual detergents from decellularized pancreatic tissue fragments – matrix (DPM) in three changes of the phosphate-buffered saline containing antibiotic and antimycotic.

Histological examination

Samples of the original pancreatic tissue and decellularized fragments were subjected to histological analysis. The material was fixed in 10% buffered formalin, dehydrated in ascending alcohols, incubated in a mixture of chloroform and ethanol, chloroform, and embedded in paraffin. Sections, 4–5 μm thick, were obtained using an RM2245 microtome (Leica, Germany) and further subjected to hematoxylin and eosin staining and Masson's trichrome stain; cell nuclei were visualized by DAPI staining (Sigma, USA). Obtained histological preparations were analyzed using a Nikon Eclipse 50i microscope (Nikon, Japan) equipped with a digital camera.

RESEARCH RESULTS

Histological analysis of the original pancreas

The morphological study of the original material revealed three types of pancreatic tissue samples: a pancreas with pronounced signs of lipomatosis (Fig. 1, a–c), a pancreas with diffuse fibrosis (Fig. 1, d–f) and a pancreas without pronounced morphological signs of pathology (Fig. 1, g–i). In spite of the identified differences, all samples showed preserved islets, which, as a rule, had a round (less often, elongated) shape and compact, sometimes lobular, structure. The compact structure was characteristic of smaller islets, while lobularity was determined in some larger islets. Specific DAPI staining confirmed the presence of cell nuclei both in the islet and in the surrounding acinar tissue.

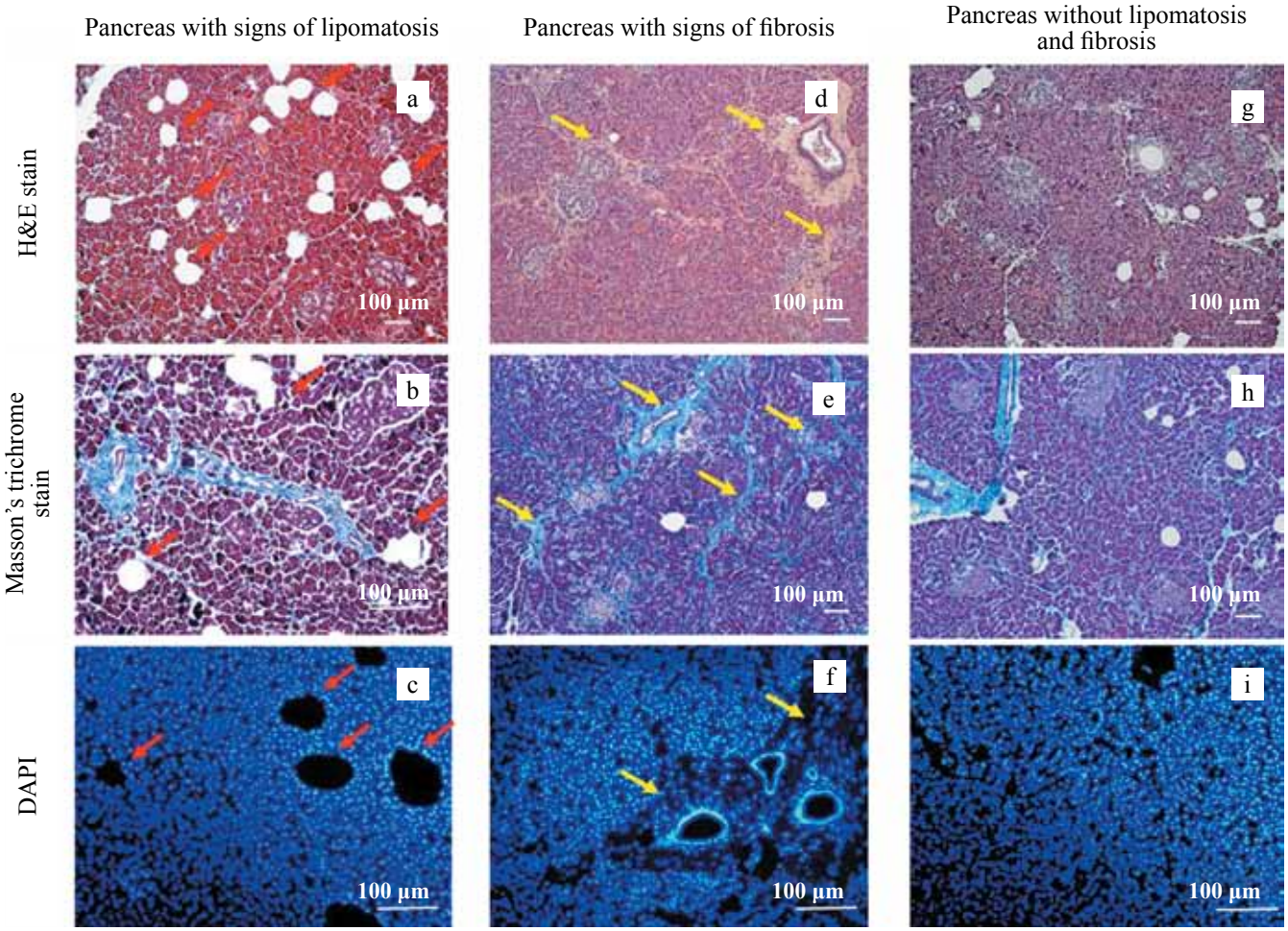

Fig. 1. Histological picture of deceased donor pancreas: a, d, g, H&E staining; b, e, h, Masson's trichrome staining; c, f, i, DAPI staining. Red arrows indicate lipomatosis features, yellow arrows indicate fibrous cords

Histological analysis of pancreas decellularized using freeze-thaw cycles

In samples of pancreatic fragments with lipomatosis after three consecutive freeze-thaw cycles and treatment with detergents, we observed a complete absence of preserved cells, complete absence of individual cell nuclei, and complete absence of small fragments of cellular detritus in the obtained connective tissue scaffold (Fig. 2, b). The morphological picture showed that a purified thin-fibrous matrix was obtained (Fig. 2, a).

After full protocol decellularization of a pancreas with diffuse fibrosis, the histological picture, in general, was different from that obtained when treating a pancreas with lipomatosis. Along with separate, well-purified openwork thin-fiber fragments, the samples showed dense areas (Fig. 2, c) in which a significant number of cells and nuclei were preserved, detected by DAPI staining (Fig. 2, d). This led to the conclusion that the tissue was incompletely decellularized, while a similar treatment of pancreas with lipomatosis allowed to obtain a completely purified matrix.

After decellularization of pancreatic tissue without pronounced signs of fibrosis and lipomatosis, we observed, in the obtained samples, preserved thin collagen fibers in the stroma and absence of preserved cells and cell nuclei (Fig. 2, e). However, numerous small grains of cellular detritus were detected in the thickness of the sample (Fig. 2, f), indicating that the decellularization procedure was ineffective.

Thus, the studies showed that the proposed protocol is suitable only for pancreas with lipomatosis, which seems to be related to the morphological features of the tissue.

Histological analysis of pancreas decellularized using osmotic shock (I and II versions of the protocol)

To determine optimal conditions of effective decellularization of pancreatic tissue with diffuse fibrosis and without marked signs of fibrosis and lipomatosis, we tested a physico-chemical pancreas decellularization method using osmotic shock – variant I of the protocol.

Decellularization of fibrotic pancreatic tissue by osmotic shock method (variant I of the protocol) resulted in samples in which the main part looked dense, non-porous due to the collapsed framework. At the same time, the preparations stained by Masson's method showed rough, densely packed collagenous strands (Fig. 3, a). The presence of cellular detritus (mainly in the peripheral zone), including nuclear material, was confirmed by DAPI staining (Fig. 3, b). Based on these results, we can conclude that the proposed decellularization protocol does not allow to obtain a purified, porous connective tissue scaffold and cannot be further recommended for decellularization of a pancreas with diffuse fibrosis.

Samples of decellularized matrix from the pancreas without evident signs of fibrosis and lipomatosis, obtained by the osmotic shock method (variant I of the protocol) demonstrated complete absence of preserved cells and cell nuclei (confirmed by DAPI staining) (Fig. 3, d)

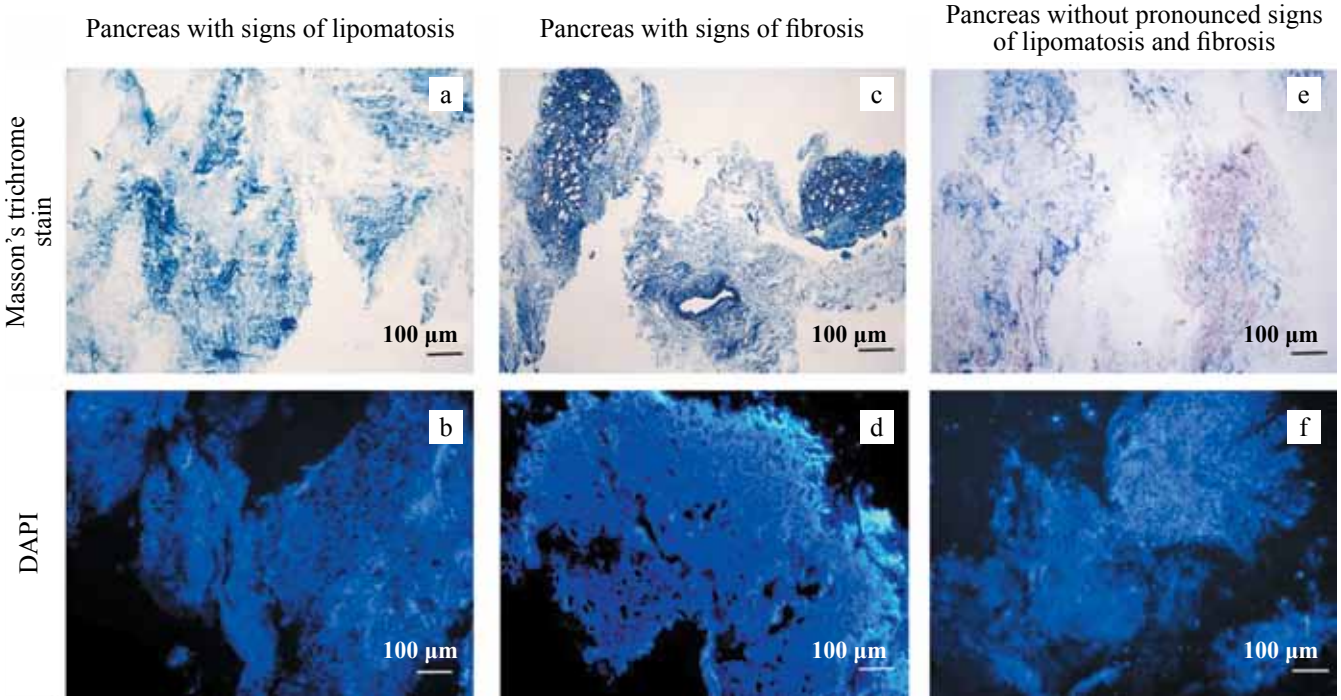

Fig. 2. Histological picture of pancreas, decellularized using freeze-thaw cycles: a, c, e, Masson's trichrome staining; b, d, f, DAPI staining

and a well-defined fine-cell thin-fiber structure tissue scaffold with blue collagen fibers (Fig. 3, c). Thus, the proposed protocol was effective in obtaining a matrix as a result of decellularization of pancreas without pronounced signs of fibrosis and lipomatosis.

Decellularization of a pancreas with diffuse fibrosis using the osmotic shock method according to variant II of the protocol was successful. In contrast to the samples obtained using variant I, the obtained matrix was characterized by a porous, fine-cell, thin-fiber structure with preserved collagen fibers (Fig. 4, a). At the same time, preserved cells, cell nuclei, and fragments of cellular detritus were not visualized in the samples when stained with DAPI (Fig. 4, b). The use of this protocol for decellularization of a pancreas with diffuse fibrosis allows to obtain tissue-specific matrix/framework free of cells and cellular fragments.

Thus, histological analysis of deceased donor pancreas revealed morphological features of the samples associated with the presence of signs of lipomatosis and diffuse fibrosis, which requires the use of different processing regimens for effective decellularization. The study of physical and mechanical properties of pancreatic tissue, such as density, stiffness, elasticity, is also an important aspect of decellularization protocol optimization [5]. It can be assumed that physico-mechanical properties will correlate to a certain extent with histological features of pancreas. Confirmation of this hypothesis needs further investigation.

We have not found any information in the published domestic and foreign scientific literature on the influence of human pancreas morphological features on the choice of decellularization protocol.

CONCLUSION

On the basis of the obtained data, we can state that in order to perform full decellularization of human pancreatic fragments, the treatment protocol should correlate with the histological features of the original tissue. It has been shown that physico-chemical method with freeze-thaw cycles is suitable for effective decellularization; for a pancreas with severe lipomatosis and for a pancreas

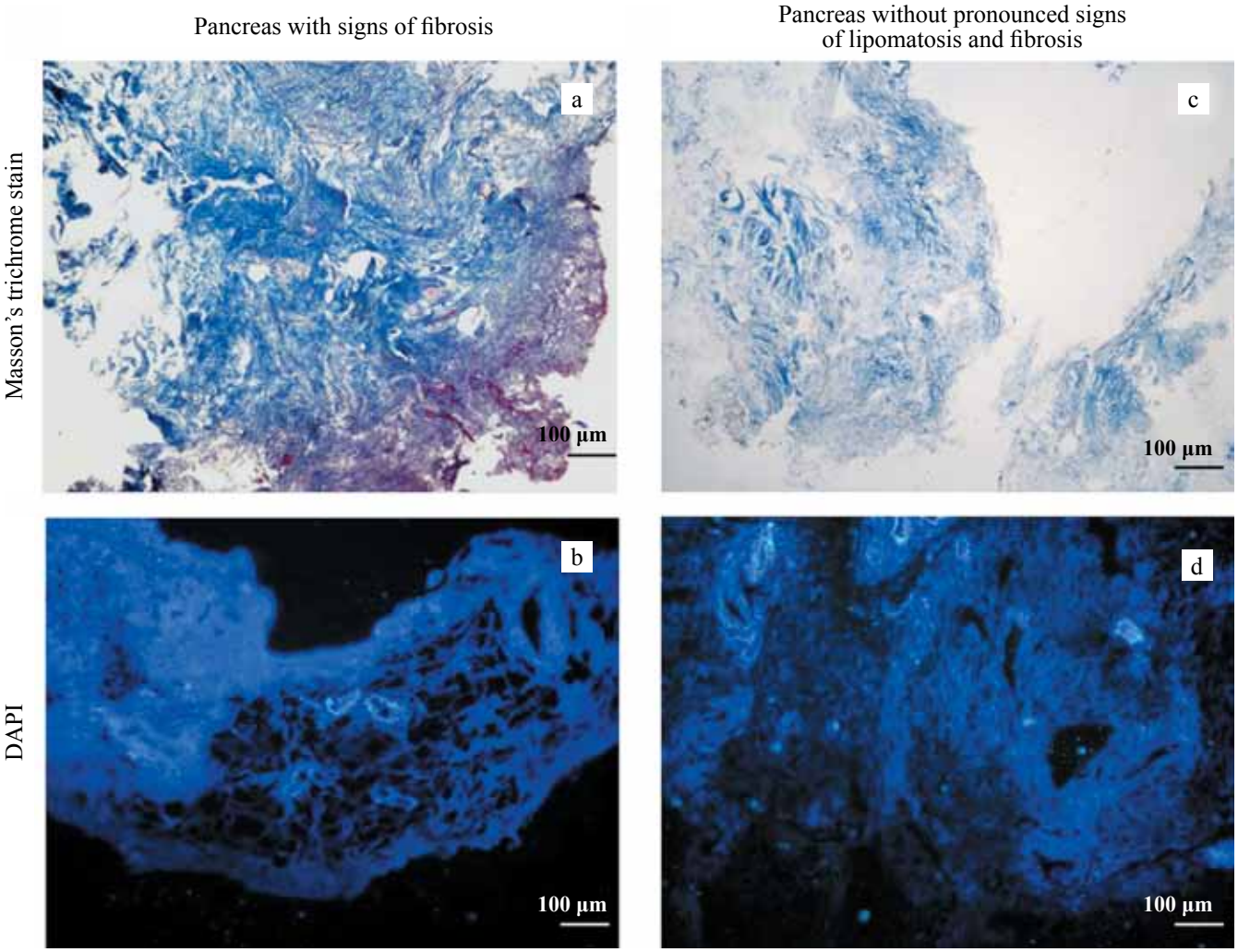

Fig. 3. Histological picture of the pancreas, decellularized using osmotic shock (protocol option I): a, c, Masson's trichrome staining; b, d, DAPI staining

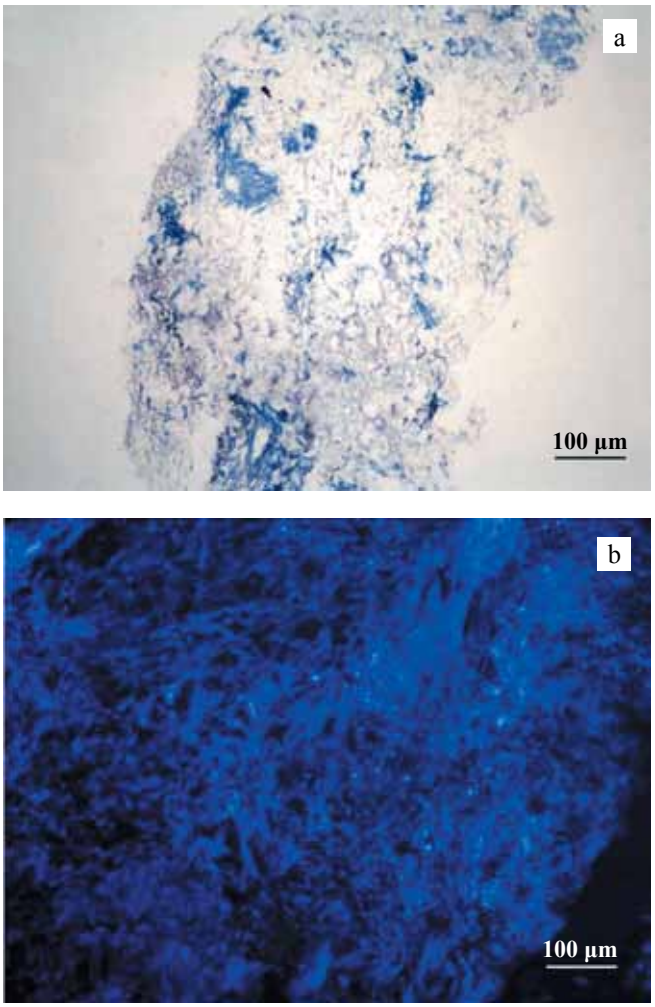

Fig. 4. Histological picture of the pancreas with diffuse fibrosis, decellularized using osmotic shock (protocol option II): a, Masson's trichrome staining; b, DAPI staining

without pronounced signs of fibrosis and lipomatosis, physico-chemical method using osmotic shock, but different variants of the protocol, is suitable.

The authors declare no conflict of interest.

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The article was submitted to the journal on 12.11.2021