

Comparative Analysis of the Influence of Extracellular Matrix Biomimetics on the Viability and Insulin-Producing Function of Isolated Pancreatic Islets

V.I. Sevastianov^{1*,2}, N.V. Baranova¹, L.A. Kirsanova¹, A.S. Ponomareva^{1,2}, Yu.B. Basok¹, E.A. Nemets¹, S.V. Gautier^{1,3}

¹The Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, the Russian Federation

²The Institute of Biomedical Research and Technology (IBRT) autonomous non-profit organization, Moscow, the Russian Federation

³The Sechenov University, Moscow, the Russian Federation

*Corresponding author

V.I. Sevastianov, The Shumakov National Medical Research Center of Trans-plantology and Artificial Organs, Moscow, the Russian Federation.

Submitted: 19 Nov 2021; **Accepted:** 25 Nov 2021; **Published:** 30 Nov 2021

Citation: V.I. Sevastianov N.V. Baranova, L.A. Kirsanova, A.S. Ponomareva, Yu.B. Basok, E.A. Nemets, S.V. Gautier. (2021). Comparative Analysis of the Influence of Extracellular Matrix Biomimetics on the Viability and Insulin-Producing Function of Isolated Pancreatic Islets. *J Gene Engg Bio Res*, 3(2), 17-25.

Abstract

The creation of a pancreas tissue-engineered construct based on isolated pancreatic islets is hindered by problems associated with maintaining their viability and insulin-producing function. Both biopolymer and tissue-specific scaffolds can contribute to the maintenance of the structure and function of pancreatic islets in vitro and in vivo. A comparative morphofunctional analysis in vitro of isolated pancreatic islets cultured with a biopolymer collagen-containing scaffold and a tissue-specific scaffold obtained as a result of pancreatic decellularization was performed. The results showed that the use of the scaffolds contributes not only to the maintenance of the cultured islets viability, but also to the prolongation of their insulin-producing functions, compared to the islets monoculture in vitro. A significant increase was found in basal and stimulated (under glucose loading) insulin secreted by the islets cultured with the scaffolds. At the same time, the advantage of using a tissue-specific scaffold in comparison with a biopolymer collagen-containing scaffold was shown. We think that these studies will become a platform for creating a human pancreas tissue-engineered design for the treatment of type 1 diabetes.

Keywords: Biopolymer Scaffolds, Tissue-Specific Scaffold, Decellularization, Extracellular Matrix, Pancreatic Islets, Insulin Secretion

Introduction

The creation of a pancreas tissue-engineered construct (TECP) is hampered by problems associated with maintaining the viability of functionally active isolated islets of Langerhans [1-4]. It is known that during the isolation procedure the islets are exposed to a number of damaging factors, such as ischemia, oxidative stress, and the possible cytotoxic effect of the enzyme. Not only do the islets lose vascularization and innervation, but also connections with the extracellular matrix (ECM) which plays an important role in the regulation of islet physiology [5, 6]. The pancreatic extracellular matrix contains type I, III, IV, V and VI collagen, elastin, as well as laminin and fibronectin, regulates the main aspects of islet biology, including the development, morphology, differentiation, intracellular signaling, gene expression, adhesion, migration, proliferation, secretion and survival [7]. Cell-matrix interactions are important for mature β -cells to remain functional and avoid

apoptosis, as well as to maintain functional β -cell mass [8]. The islets partially retaining the ECM after isolation have been shown to exhibit a decrease in the rate of apoptosis and significantly better support insulin secretion than more thoroughly purified islets [6].

The maintenance of the structure and function of isolated islets under in vitro and in vivo conditions can be facilitated by the ECM biomimetics, both biopolymer and tissue-specific scaffolds, while providing the necessary microenvironment for the islets [9-11].

Scaffolds obtained from the ECM components are used in order to create TECP injectable forms of bioresorbable hydrogel [9]. These include three-dimensional one-, two- and multi-component non-crosslinked or cross-linked (structured) hydrogel scaffolds from collagen, gelatin, hyaluronic acid, etc., the so-called ECM biomimetics. These biomimetics imitate, to a certain degree, the

structure and bioactive properties of a native pancreas ECM. Such hydrogel biopolymer mimetics create a microenvironment for cell cultures, providing adhesion, proliferation, differentiation of cells, as well as their synthesis of a tissue-specific ECM.

In recent years, the development of cell and tissue-engineered constructs based on tissue-specific scaffolds obtained from decellularized tissues has been underway with the maximum possible preservation of the morphological, biochemical and functional properties of the native ECM [12].

The tissue-specific scaffolds most preferred for islet cells can be obtained by decellularization of the whole pancreas, as well as of the fragments of pancreatic tissue [13, 14]. When developing protocols for pancreatic decellularization, it is important to take into account the maintenance of the structural, biochemical and biomechanical properties of the native ECM, the preservation of the architectonics with the maximum complete removal of cellular material (including DNA), and cellular surface antigens to minimize the immune response during implantation [15, 16]. The presence of native ECM components in the decellularized pancreatic scaffold (the DP scaffold), such as structural proteins (various types of collagen, elastin, fibronectin, and laminin), glycoproteins, and cell adhesion factors makes it possible to create conditions for prolonged vital activity of islet cells and almost completely mimic ECM [17]. The three-dimensional ECM structure determines the topographic location of pancreatic endocrine cells that also affects the survival rate and secretory activity of the islets [18]. It was shown that the islets cultured in the presence of the DP scaffold increased insulin secretion as compared to the isolated islets in monoculture [19, 20].

In our research, we studied the possibility of obtaining a tissue-specific matrix of the rat pancreas using small fragments of pancreatic tissue for decellularization. This approach makes it possible to increase the efficiency of decellularization, almost completely populating the entire volume of the decellularized matrix with cells, simplifying the delivery of oxygen and nutrients to cells, including deep into the matrix, and significantly reducing the cost of the decellularization procedure.

The aim of our work was to compare the effects of the biopolymer collagen-containing scaffold and the tissue-specific scaffold from the decellularized rat pancreas on the morphofunctional state of cultured rat islets.

Materials and Methods

Experimental Animals

The studies were carried out on sexually mature male Wistar rats (180-220 g.). All animal manipulations were carried out in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123) Strasbourg, 1986 and approved by the Local Ethics Committee at the Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russia (28 January 2021, Protocol No. 280121-1/1e).

Biopolymer Microheterogeneous Collagen-Containing Hydrogel (The BMCH Scaffold)

As a scaffold ECM biomimetic, a biopolymer microheterogeneous collagen-containing hydrogel (BMCH scaffold) was selected. The injectable form of the BMCH scaffold (trademark Sphero®GEL, JSC Biomir Service, Russia) registered in Russia for clinical use is produced from tissue components of farm animals using the method of acetic acid extraction. The BMCH scaffold contains the main ECM components: peptides of partially hydrolyzed collagen, glycoproteins and uronic acids, as well as growth factors necessary for the vital cell activity, the synthesis of exogenous uronic acids, proteoglycans and collagen. The BMCH scaffold consists of microparticles ($145.79 \pm 0.09 \mu\text{m}$) of the collagen-based hydrogel crosslinked by a γ -radiation (1.5 Mrad) and the homogeneous hydrogel solution of the low and high molecular weight ECM components at a ratio of 1:1 (Figure 1a) [21]. The visualization of the BMCH scaffold heterogeneous component by cryo scanning probe nanotomography (AFM – image) revealed a porous structure of microparticles with a pore size of 2-4 μm which is a positive property in the processes of neovascularization and reinnervation of bioartificial structures based on it (Figure 1b) [21].

The main characteristics of the BMCH scaffold are as follows: elastic modulus $1170 \pm 12 \text{ Pa}$; viscosity modulus $62.9 \pm 7.9 \text{ Pa}$; $\text{pH} = 7.0 \pm 0.1$; resorption time – up to 9 months. Water absorption is equal to 86.6 wt.%, while the content of bound water is at least 32.8 wt.% [22-24].

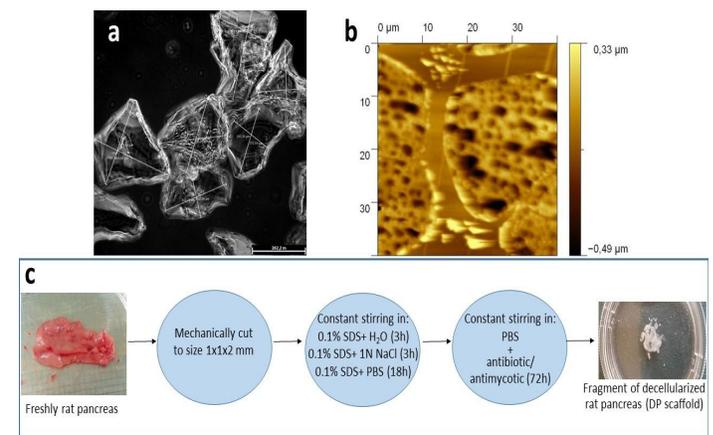


Figure 1: Phase contrast image of biopolymer collagen-containing hydrogel scaffold microparticles (the BMCH scaffold) (a). The visualization of the BMCH scaffold heterogeneous component by cryo scanning probe nanotomography (AFM - image) (b). The schematic representation of a tissue-specific scaffold (the DP scaffold) obtention process (c).

Tissue-Specific Scaffold (The DP Scaffold)

A finely dispersed tissue-specific scaffold maintained the architectonics and basic composition of the original ECM pancreas obtaining the physicochemical decellularization method of the rat pancreas using the known methods of various parenchymal organs decellularization [13]. The subtotally removed rat pancreas was

mechanically cut to 1x2 mm fragment size for the further decellularization. The pancreas fragments were processed at room temperature under continuous stirring using a MultiBio RS-24 rotary system (BioSan, Latvia).

0.1% sodium dodecyl sulphate (SDS) solution and phosphate-buffered saline (PBS) of high and low ionic strength (osmotic shock method) were used. The thorough SDS residues washing from decellularized pancreatic tissue fragments by three PBS changes containing an antibiotic (ampicillin, 10 µg/ml) and antimycotic (amphotericin, 1.5 µg/ml) followed. Then the DP scaffold samples were introduced into cryovials, frozen, sterilized (γ-sterilization, 1.5 Mrad) and stored at 4-6 °C (Figure 1c).

Scanning Electron Microscopy (SEM)

The microstructure of the DP scaffold samples was investigated with a JSM-6360LA scanning electron microscope (Jeol, Japan) at an accelerating voltage of 25 kV. The samples were fixed for 60 minutes in a 2.5% glutaraldehyde solution and dehydrated in ethyl alcohol solutions of increasing concentrations (from 50% to 100%), followed by air drying. The conductive coating was obtained by ion gold sputtering for 8-10 min at a constant current of 5-7 mA on a JFC-1100 setup (Jeol, Japan).

Biochemical DNA Study

To determine the immunogenicity of the decellularized material by the residual amount of nuclear material in the DP scaffold, DNA isolation and fluorescent staining were performed. DNA isolation from the DP scaffold samples was performed using the DNeasy Blood&Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. The quantitative DNA determination with a fluorescent dye TMPicogreen Quant - iT (Invitrogen, USA) was used according to the protocol.

Biochemical Glycosaminoglycans (GAG) Study

To determine the GAG content, an ECM component's significant biological activity, the DP-scaffold samples were preliminarily lyophilized [25]. Then the samples were lysed in a papain solution in phosphate buffer (Sigma-Aldrich, USA) for 12 hours. The resulting lysate was used for the quantitative GAG analysis using a thiazine dye (Sigma-Aldrich, USA). To determine the GAG absolute amount, a calibration curve of chondroitin sulfate (Sigma-Aldrich, USA) was used. The optical density of the samples was determined a Spark 10M microplate reader (TecanTrading AG, Switzerland) at a wavelength of 570 nm.

Cytotoxicity Study

The cytotoxicity of the DP scaffold samples in vitro was evaluated by direct contact according to the ISO 10993-5:1999 international standard on the mouse fibroblast culture line L929 [26]. The culture medium 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). The culture monitoring was performed with the Eclipse TS100 (Nikon, Japan) inverted microscope.

The metabolic activity of fibroblasts after contact with scaffold samples was determined after 24 hours with the prestoBlueTM Cell Viability Reagent (InvitrogenTM, USA) according to the protocol

recommended by the manufacturer. The proliferating cells rebuild prestoBlueTM, resulting in a color change from indigo to pink. The percentage of recovered prestoBlueTM characterizes the metabolic activity of the cells. The changes in media absorption were recorded using the Spark 10M microplate reader with Spark ControlTM Magellan V1.2.20 software at a wavelength 570 nm and 600 nm. The data quantitative and statistical processing was performed with Microsoft Excel 2007. All results are presented as mean ± standard deviation. The differences were considered significant at $p < 0.05$.

Matrix Properties Study of the Scaffolds

The matrix properties of the scaffolds in relation to their ability to maintain cell adhesion and proliferation were studied in a culture of NIH3T3 fibroblasts, seeding 5×10^4 cells / 10 mg on the BMCH scaffold and the DP scaffold. On days 1, 3, 6 and 10 of culture several samples were taken to assess the proliferative activity and viability of fibroblasts using the vital dye prestoBlueTM Cell Viability Reagent (InvitrogenTM, USA) to build the growth curves and the LIVE / DEAD[®] Cell Viability / Cytotoxicity Kit (Molecular probes[®] by Life technologiesTM, USA) for the determination of cells' viability. After 10 days of culture the scaffold samples with cells were fixed in 10% buffered formalin for further histological analysis.

Pancreatic Islets Isolation and Identification

To study the functional properties of the scaffolds, the islets were isolated from the rat pancreas and were cultured in the presence of the scaffolds. The functional properties of the scaffolds were assessed by a comparative analysis of the viability and insulin-producing function of the islets cultured with the scaffolds and islets in monoculture.

The islets were isolated from the pancreas of a mature rat based on the traditional method using type I collagenase (activity 100–110 U/ml, Sigma, USA) with some modifications in order to increase the viability and the number of isolated islets [27]. Thus, the enzyme solution was injected into the pancreatic tissue by successive intraparenchymal injections. The stretched pancreatic tissue was not cut, but carefully divided into the resulting lobules. The purification of the islets after the isolation in the Ficoll density gradient was replaced by centrifugation in the Hanks solution under the previously established regimes [24].

The dithizone staining was used to identify the islets. 0.2-0.4 ml of islet suspension were added to a Petri dish, 0.1-0.2 ml of the dithizone solution were added and incubated for 20-30 min at 37 °C. The colored islets were counted using an inverted microscope at a magnification of the objective $\times 10$. A morphological examination of freshly isolated islets was carried out. The identification of the islet β -cells was carried out by immunohistochemistry according to the classical method with horseradish peroxidase using rabbit anti-insulin antibody (Sigma, USA).

Culture of Pancreatic Islets

A comparative analysis of the morphological state, secretory capacity, and functional activity of islets cultured with the scaffolds (with the BMCH and the DP scaffolds), and islets cultured in suspension (monoculture) was carried out.

Freshly isolated islets were resuspended in DMEM/F12 medium (1:1) with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM HEPES, and 50 mg/ml gentamicin, and an approximately equal number of islets ($n = 300 \pm 25$) were added to three 25 cm² culture flasks. The islets cultured in monoculture (the flask 1) served as control. In the flasks 2 and 3, 50.0 \pm 0.1 mg of the BMCH scaffold and 50.0 \pm 0.1 mg of the DP scaffold with an average size of microfragments of 500 \pm 45 μ m, respectively, were added.

All the flasks were incubated under standard conditions at 37 °C in a CO₂ incubator in a humidified atmosphere containing 5% CO₂.

The daily monitoring and photo imaging of the cultured islets were carried out using an inverted microscope Nikon Eclipse TS 100 (Nikon, Japan) equipped with a digital camera.

Fluorescent Staining of Pancreatic Islets

The islets' viability in monoculture and cultured islets with the scaffolds was evaluated with Acridine orange and Propidium iodide fluorescent staining (AO/PI Cell Viability Kit, Sigma). Acridine orange produces an intense green fluorescence in viable cells (ex / em 502 nm / 525 nm), propidium iodide creates red fluorescence in dead cells (ex / em 535 nm / 617 nm).

0.2-0.4 ml of the islet suspension were placed in a Petri dish, 0.02-0.03 ml of the working solution were added and incubated in the dark at room temperature for 15-17 minutes. The result was evaluated using a Nikon Eclipse 50i fluorescent microscope (Nikon, Japan).

Using a fluorescent microscope, the percentage of viable islets in the stained sample (200 μ l) was calculated using the formula:

Percent viable = (total viable \times 100) / total number of islets.

An islet was considered viable if at least 50% of cells with green fluorescence were present in it.

Morphological Study

The samples of the native rat pancreas, the DP scaffold, as well as the islets, freshly isolated and cultured, were subjected to a morphological study using routine histological and specific immunohistochemical staining (IHC) methods.

Connective tissue (total collagen) was detected in the DP scaffold samples by Masson's method. Elastic fibers in the DP scaffold samples were detected by orsein staining according to the Unna-Tentzer method.

DAPI fluorescent staining (Sigma, USA) was used to qualitatively assess the completeness of the cellular (nuclear) removal material from decellularized samples.

The identification of the islet β -cells in the native pancreatic tissue and isolated islets, the determination of type I and IV collagen in the DP scaffold samples were carried out by immunohistochemistry according to the classical method with horseradish peroxidase using rabbit anti-insulin antibody (Sigma, USA), rabbit anti-collagen I antibody and rabbit anti-collagen IV antibody (Abcam, UK).

The reaction was visualized using the Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Abcam, UK) following the manufacturer's protocol. Analysis and photography of the histological preparations were carried out using a Nikon Eclipse 50i microscope (Nikon, Japan) equipped with a digital camera.

Enzyme Immunoassay (ELISA)

To determine the basal insulin concentration at different times, the growth medium was removed from the flasks containing pancreatic islets in monoculture or with the scaffolds and a fresh portion of a low glucose medium (2.8 mmol/L) was replaced. The culture medium samples were taken after 60 minutes of incubation under the same conditions and frozen (-23 °C) for ELISA.

To determine the concentration of insulin under the influence of the traditional stimulator of insulin secretion, glucose, a separate experiment was carried out on cultured islets in monoculture and in the presence of the scaffolds. On the third day of incubation, the growth medium was replaced with a fresh portion of the low glucose medium (2.8 mmol/L). The culture medium samples were taken after 60-minute incubation under the same conditions and frozen (-23 °C). Then the growth medium was removed from these flasks and replaced with a fresh high glucose medium (25 mmol/L). The samples of the growth medium after 60 minutes of incubation under the indicated conditions were also taken and frozen (-23 °C) for ELISA.

A kit for the enzyme immunoassay Rat Insulin ELISA Kit (Invitrogen, USA) was used for a quantitative analysis of the insulin concentration in a nutrient medium. The indicators were presented on the Spark 10M microplate reader (Tecan Trading AG, Switzerland) with Spark Control™ Magellan V1.2.20 software. The obtained data were statistically processed using Microsoft Excel 2007. The results of the quantitative ELISA method were calculated using a linear calibration curve. All results are presented as mean \pm standard deviation. Differences were considered significant at $p < 0.05$.

Results

Morphological Analysis of the DP Scaffold

A preliminary histological examination of the rat native pancreas revealed pancreatic tissue without signs of ischemic damage (Figure 2a, b). Morphological picture of the decellularized pancreas showed the architectonics stroma remained and an openwork fibrous structure resembling a mesh network. The surviving cells and individual cell nuclei were not found in the samples. Specific staining with DAPI confirmed the absence of cell nuclei and fragments of nuclear material in the scaffold (Figure 2c) compared to the native pancreas (Figure 2b). Masson's trichrome staining of the samples allowed visualization of intact collagen fibers in the produced scaffold (Figure 2d), and orsein staining also revealed elastic fibers (Figure 2e). Antibody immunohistochemical staining confirmed the presence of type I (Figure 2f) and type IV collagens (Figure 2g) in the decellularized samples.

SEM Analysis

The experimental sample of the DP scaffold is characterized by a porous structure with pore sizes from 36.2-304.0 μ m. The sample surface is heterogeneous: the main part of the surface looks rough, but some areas with a smooth surface are detected. Remained cells

and large cell fragments are not detected in the sample (Figure 2h).

DNA Content in the DP Scaffold

A comparative quantitative analysis of the DNA content in a sample of the original rat pancreas tissue (1268 ± 218 ng per 1 mg of tissue) and decellularized tissue 13 ± 2 ng per 1 mg of tissue showed no more than 1% of DNA remaining in the tissue. This result indicates low immunogenicity (relative to the residual amount of DNA) of the obtained tissue-specific DP scaffold (Figure 2i).

GAG content in the DP scaffold

A quantitative analysis of the GAG content in the native and decellularized tissue of the rat pancreas showed that the DP scaffold contains 43 ± 9 μ g of GAG per 1 mg of decellularized tissue, compared to the original pancreatic tissue containing 13 ± 3 μ g of GAG per 1 mg of the native tissue (Figure 2j).

The higher GAG content in the DP scaffold as compared to the native tissue is a consequence of the removal of cells and cellular components.

Cytotoxicity of the DP Scaffold

The metabolic activity of fibroblasts after a contact with the DP scaffold amounts to $96.25 \pm 1.69\%$ relative to the negative control sample (growth medium), that indicates that the DP scaffolds had no cytotoxic effect (Figure 2k).

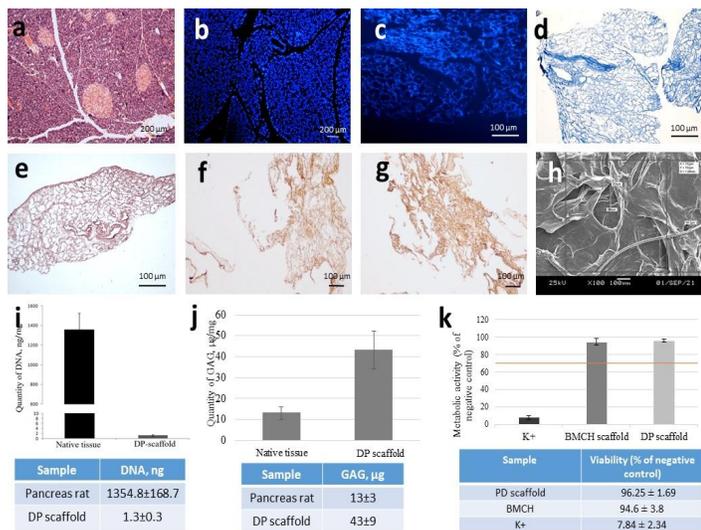


Figure 2: A histological structure of the rat native pancreas, haematoxylin and eosin (H&E) staining (a), nuclear DAPI staining (b). Nuclear DAPI staining of the DP scaffold confirmed the absence of cell nuclei and fragments of nuclear material in the scaffold (c). Masson's trichrome staining of the DP scaffold demonstrated complete absence of cells and preservation of collagen fibers (d). Unna-Tensler's staining revealed preservation of elastic fibers in the DP scaffold (e). Antibody immunohistochemical staining demonstrated the presence of type I (f) and type IV collagens (g) in the DP scaffold. A microstructure of the DP scaffold obtained by scanning electron microscopy (h). Quantification of DNA in native and decellularized (the DP scaffold) rat pancreatic tissue; $p < 0.05$ (i). Quantification of glycosaminoglycans (GAG) in native and de-

cellularized (the DP scaffold) rat pancreatic tissue; $p < 0.05$ (j). The metabolic activity of the L929 fibroblasts (cytotoxicity test) after contact the BMCH scaffold and the DP scaffold; $p < 0.05$ (k).

Adhesion and Proliferation of Fibroblasts on the Scaffolds

The matrix properties of the BMCH and the DP scaffolds for their ability to maintain adhesion and proliferation of cell cultures were investigated in a culture of mouse fibroblasts.

On the first day of fibroblasts cultured on the BMCH scaffold, partial attachment of cells to the surface of the scaffold occurred (Figure 3a). On the third and sixth days, the appearance of significant cellular growth zones was observed indicating the proliferative activity of fibroblasts (Figure 3b) under these conditions. On the 10th day of incubation, the mass cell population of the surface of the BMCH scaffold was detected (Figure 3c).

A histological examination on the 10th day of the fibroblast incubation on the BMCH scaffold showed the formation of a cell layer on the scaffold surface and the penetration of single cells (Figure 3d).

After the first day of culture, the adhesion of fibroblasts and the spreading of cells on the surface of the DP scaffold were observed (Figure 3e). From three to six days, the number of fibroblasts increased significantly which indicated their high proliferative activity (Figure 3b), and by day 10, the zones of cell growth were observed practically on the whole surface of the scaffold which was confirmed by fluorescent staining (Figure 3f).

A histological investigation of the DP scaffold with fibroblasts showed intensive cell colonization of the scaffold occurring on the 10th day; viable fibroblasts were located not only on the surface of the DP scaffold, but also as actively penetrated into its deep layers (Figure 3g). Thus, there was a gradual settlement of almost the entire volume of the decellularized scaffold.

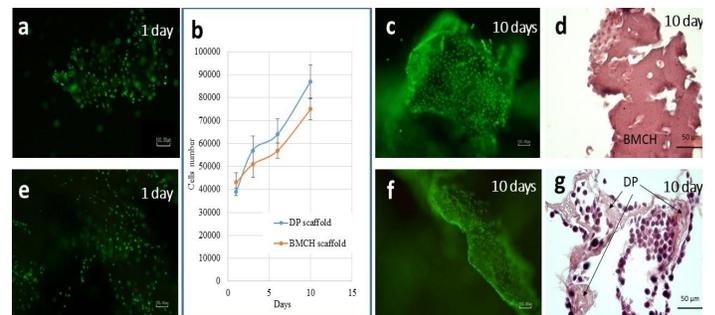


Figure 3: Adhesion and proliferation of the NIH3T3 fibroblasts on the BMCH scaffold (a, c) and on the DP scaffold (e, f). The vital staining LIVE / DEAD®. The growth curves of NIH3T3 fibroblasts on the scaffolds; $p < 0.05$ (b). Haematoxylin and eosin (H&E) staining of the BMCH scaffold (d) and the DP scaffold (g) and the NIH3T3 fibroblasts.

Freshly Isolated Islets

A preliminary morphological study confirmed the classic picture of the structure of the rat pancreas: the islets were clearly visualized among the exocrine tissue (Figure 4a). Freshly isolated islets

were generally rounded or oval, sometimes with a slight roughness formed by the remnants of the surrounding exocrine tissue. The islets remained intact, indicating that the macrostructure of the islets was not damaged during the isolation process (Figure 4b). The pancreatic islets demonstrated a terracotta-red color with the dithizone staining, while the acinar cells remained unstained (Figure 4c). Then the stained islets were counted: about 235 ± 12 islets were contained in 1 ml of the islet suspension. Thus, at least 800-900 islets from single rat pancreas were isolated. Vital staining with AO/PI confirmed their viability (Figure 4d) with almost 100% survival. The histological examination showed the maintenance of basic structural characteristics of isolated islets with a predominance of insulin-positive β -cells (Figure 4e).

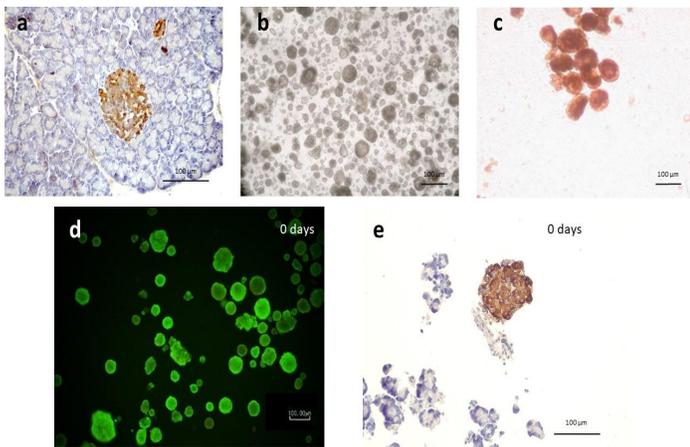


Figure 4: Immunohistochemical staining with anti-insulin antibodies of the rat native pancreas (a). Freshly isolated rat pancreatic islets: inverted microscopy (b), dithizone staining (c), fluorescent staining acridine orange and propidium-iodide (AO/PI) (d), immunohistochemical staining with anti-insulin antibodies (e).

Pancreatic Islets Cultured In Monoculture

The islets ($n = 300 \pm 25$) cultured in monoculture (control) retained their original external characteristics during the first three days of incubation. Most of these underwent destructive changes after 3 days of culture. The contours of the islets became irregular, with cavities and signs of fragmentation in some islets by the 6th day of incubation (Figure 5a). Fluorescent staining revealed the appearance of dead cells (red fluorescence) among living cells (green fluorescence) in the surviving islets (Figure 5b). A histological picture demonstrated numerous cells with pycnotic nuclei in these islets (Figure 5c).

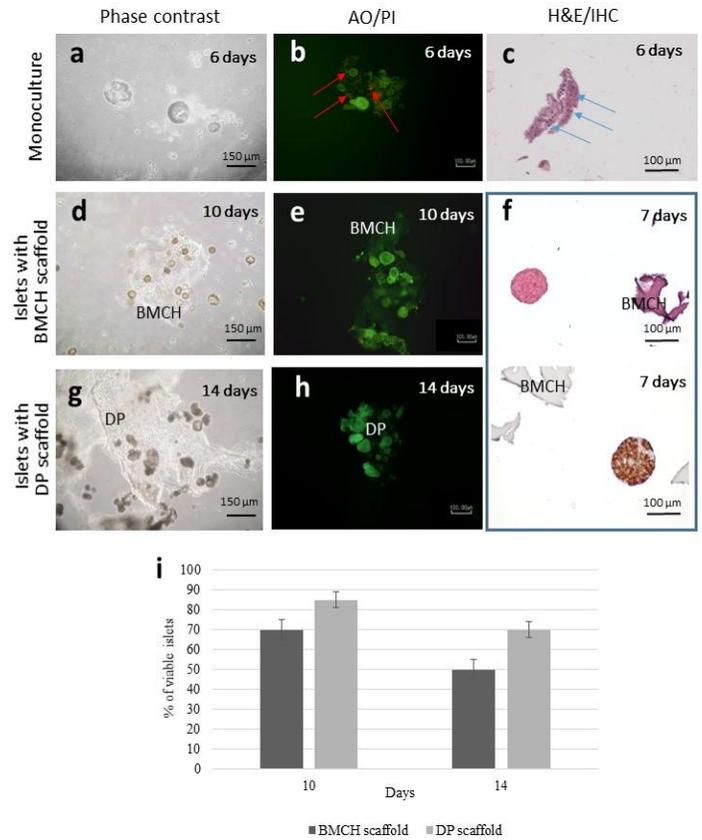


Figure 5: The destructive changes of islets in monoculture on the 6th day of culture: inverted microscopy (a), fluorescent staining with acridine orange and propidium-iodide (AO/PI) (b). Dead cells by propidium-iodide staining are in red (red arrows). Cell pycnotic nuclei in destroyed islets (blue arrows), Haematoxylin and eosin (H&E) staining (c). The islets cultured with the BMCH scaffold: inverted microscopy (d) and AO/PI on the 10th day (e), H&E and immunohistochemical staining (IHC) with anti-insulin antibodies on the 7th day (f). The islets cultured with the DP scaffold on 14th day: inverted microscopy (g) and AO/PI (h). Comparison of islets viability cultured with scaffolds on the 10th and 14th day of culture; $p < 0.05$ (i).

Pancreatic Islets Cultured with the Bmch Scaffold

A significant part (about 70%) of the islets cultured with the BMCH scaffold ($n = 300 \pm 25$), after the first day, has attached to

the surface of the scaffold microparticles (~ 210 islets), while the others islets continued to float. All the islets remained intact without the signs of structural degradation throughout the entire period (10 days of observation) (Figure 5d) compared to the islet monoculture. Fluorescent staining after 10 days of incubation (Figure 5e) confirmed the viability of the majority of the remaining islets (about 70.0%) (Figure 5i). The histological picture showed the islets with compact structure consisting of light polygonal cells with a well-defined spherical nucleus (Figure 5f). Upon immunohistochemical staining with antibodies to insulin, most of the cells in the islets were found to be immunopositive β -cells with an abundant specific granularity in the cytoplasm (Figure 5f). There was a decrease in the number of viable islets after 10 days of incubation, by 14th day there were no more than 45-50% of the survival islets (Figure 5i).

Pancreatic Islets Cultured with the DP Scaffold

The islets ($n = 300 \pm 25$) cultured with the DP scaffold did not adhere until 5 days of incubation. Subsequently, at least half (~ 150 islets) of the cultured islets were attached to the scaffold surface (Figure 5g). Fluorescent staining on the 10th and 14th day of incubation confirmed the viability of the majority of the remaining islets - 85.0% and 70.0% respectively (Figure 5h, i). These values are significantly higher than the values of the islets cultured with the BMCH scaffold in the similar conditions.

Insulin-Producing Function of Pancreatic Islet

The insulin-producing function of the islets in vitro was determined in the culture medium by basal insulin concentration and by insulin concentration upon stimulation with glucose.

A comparative analysis of the secretory capacity and functional activity of the pancreatic islets cultured with the BMCH or the DP scaffold was performed relative to islet monoculture.

The secretory activity of the islets was determined at the first, second, third, sixth, eighth and tenth days of culture. On the first day, the insulin concentration in the culture medium with the BMCH or the DP scaffolds was higher, respectively, by $26.2 \pm 3.8\%$ and $48.7 \pm 4.0\%$, compared to the secretory activity of the islets in monoculture, on the second day of culture - by $31.6 \pm 6.2\%$ and $71.6 \pm 5.7\%$, on the third day of incubation - by $62.1 \pm 8.3\%$ and $102.9 \pm 5.8\%$, respectively. The revealed difference in the concentration of the hormone in the control and experimental systems at these intervals can be explained by the positive effect of scaffolds on the secretory capacity of the islets. On the sixth day, an even greater difference ($249.6 \pm 10.4\%$ and $373.6 \pm 6.9\%$) was observed between insulin concentrations of the culture systems with scaffolds and control. This correlates with morphological data on destructive changes occurring in the islets after three days of culture in suspension.

The surviving islets in the monoculture on the 8-10th day of incubation were not found, therefore the culture medium was not examined. At the same time, the insulin concentration in experimental systems remained practically constant: $93.7 \pm 6.2 \mu\text{IE}/\text{mL}$ (with the BMCH scaffold) and $126.9 \pm 8.9 \mu\text{IE} / \text{mL}$ (with the DP scaffold). Thus, the insulin secretion level of the islets in the system with the DP scaffold was 35.5% higher compared to the

system with the BMCH scaffold (Figure 6a).

Despite the fact that the insulin concentration, expressed in absolute values, decreased with an increase in the culture period, the positive trend in the effect of the scaffolds on the secretory function of the islets in terms of the percentage remained the same throughout the entire observation period.

The functional activity of the islets cultured with the selected scaffolds was confirmed by the results of a culture medium analysis taken on the third day of incubation before and after stimulation with a hyperglycemic glucose level of 4.5 g/L (25 mmol/L). The values of insulin concentrations after glucose stimulation on the third day of islets' incubation in suspension increased by 20.8%, while for the culture system with the BMCH scaffold it increased by 33.0%, and based on the DP scaffold - by 50.7% (Figure 6b).

Note that the relative values of the increase in the basal insulin concentration in determining the functional activity of the islets cultured with the BMCH scaffold and with the DP scaffold on the 3rd day of incubation (58.1% and 107.9%, respectively) coincide with the analogous indicators obtained in detecting the secretory capacity of the islets cultured with the BMCH scaffold and the DP scaffold during the same period ($62.1 \pm 8.3\%$ and $102.9 \pm 5.8\%$, respectively), that indicates the reliability of the results. The relative nature of the quantitative assessment of the functional activity of cultured islets in monoculture and in the presence of ECM mimetics should be noted, since it is impossible to reproduce conditions similar to a native pancreas for insulin-producing cells in model experiments in vitro.

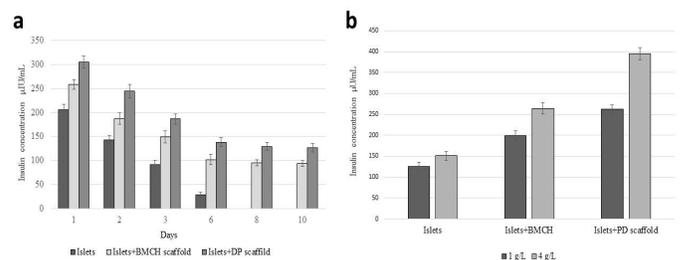


Figure 6: A comparative analysis of the secretory capacity (a) and functional activity (b) of pancreatic islets cultured in monoculture, with the BMCH scaffold and the DP scaffold; $p < 0.05$.

Discussion

Biopolymer biomimetic scaffolds are universal platforms that ensure the vital activity of cells during the formation of bioengineered structures and their subsequent implantation. The effectiveness of using the BMCH as a scaffold in cellular-engineered structures of cartilage, liver and pancreas has been shown [22-24]. However, for all their advantages, biopolymer scaffolds do not have tissue specificity with characteristic features of structure and composition. It has been shown that a decellularized scaffold from pancreatic tissue creates a microenvironment for islets similar to the native extracellular matrix which provides them with better conditions for survival and functional activity [13, 19].

There are few published works devoted to the formation of a de-

cellularized scaffold from the pancreas of a mouse, rat, pig and human, followed by their successful recellularization [13, 16, 19, 20, 28]. It should be noted that these studies were carried out using a tissue-specific scaffold obtained as a result of decellularization of a complete pancreas. However, the restoration of the vascular network in such intact decellularized scaffolds is a difficult task. A complete decellularization of the organ is a long process due to the need for penetration of all reagents into the target cells. At the same time, the longer the period of chemical and enzymatic effects, the higher the probability of a damage to the components of the extracellular matrix. An alternative approach is to obtain a decellularized scaffold from fragments of the pancreas [14, 29]. Such a strategy may be promising for tissue engineering technologies due to the minimization of a possible damage to proteins and the overall structure of the matrix, as well as the simplicity of its implementation.

In our work, we studied the possibility of obtaining a tissue-specific scaffold as a result of decellularization of small rat pancreatic tissue fragments. This approach made it possible to minimize the time of physical and chemical treatment of the tissue, when preserving the structure and composition of the extracellular matrix; significantly reducing the cost of the decellularization procedure while maintaining the efficiency of the process. A full-fledged cell colonization of the decellularized scaffold was carried out due to the simplification of the delivery of oxygen and nutrients, including into the depth of the scaffold.

The obtained scaffold is a connective tissue framework completely free of detritus with a remained fine-fibrous network-like structure, in which the protein fibrillar (elastin and type I collagen) and non-fibrillar (glycosaminoglycans and type IV collagen) components of extracellular matrix were detected. The absence of nuclear material fragments using DAPI staining was shown, and the residual amount of DNA in the scaffold was determined - 13 ± 2 ng, that meets the necessary criteria for effective decellularization [12].

The absence of cytotoxicity of the decellularized scaffold and its ability to maintain cell adhesion and proliferation were confirmed. The effect of decellularized and biopolymer scaffolds on isolated islets appeared already on the first day of culture: the basal insulin concentration in the islet monoculture was lower by 26.2% and 48.7% compared to the islets in the culture systems with the BMCH and the DP scaffolds, respectively. The islets cultured in suspension underwent destructive changes after 3 days of incubation which was confirmed by the methods of routine histological staining and fluorescence. Further studies have shown that the culture systems of isolated pancreatic islets with the BMCH scaffold or the DP scaffold not only had a better preservation of the islets compared to monoculture, but also a prolongation of their secretory capacity and an increase of functional activity.

Our results of viability and functional activity of islets in the culture systems with biopolymer and decellularized scaffolds are comparable with the data of similar studies published in the scientific literature [4, 15]. Thus, the viability of human islets cultured in collagen-containing microcapsules [6] on the 6th day of incubation was $78.3\% \pm 1.6\%$ compared to 85% of the rat islets viability cultured for the same period with the BMCH scaffold. The func-

tional activity was expressed in terms of the stimulation index: the ratio of the insulin concentration under the load of glucose to the basal insulin concentration. According to Napierala H. [13], the viability of rat islets cultured with decellularized rat pancreas was 81.96% - 84.5% as early as on the 1st day of incubation with the stimulation index - 1.2, while for the TECP based on the DP scaffold at this time, almost 100% safety of viable rat islets with a stimulation index of 1.5 was observed.

The obtained results are the foundation for the development of the human TECP formed on the basis of the scaffolds (the most promising, in our opinion, being a tissue-specific decellularized pancreas scaffold) and allogeneic islet cells obtained from donor pancreas. The development of decellularization protocols of xenogenic pancreas fragments will allow to avoid the problem of the lack of donor organs. The positive results assessing the biological safety of the human pancreas tissue-engineered design *in vitro* and *in vivo* with an emphasis on studies of its immunogenicity and immunotoxicity will serve as the proof of the possibility of using tissue-specific scaffolds from decellularized xenogenic tissues. It is presumed that the human TECP as a biomedical cell product can be used in clinical practice to compensate for the function of the damaged pancreas.

Conclusion

The proposed method of a rat pancreatic tissue decellularization allows to obtain a tissue-specific scaffold that provides the adhesion and proliferation of cell cultures due to the preservation of the morphofunctional properties of the native extracellular matrix. The viability and prolongation of the insulin-producing function of isolated islets cultured with biopolymer collagen-containing and decellularized scaffolds, compared to the islets monoculture, was established. At the same time, the advantage of a tissue-specific decellularized scaffold in creating a TECP in comparison with a biopolymer collagen-containing scaffold is shown.

These results will allow to move on to the development of the human TECP using decellularized xenogenic pancreas and allogeneic islet cells.

References

1. Kumar, N., Joisher, H., & Ganguly, A. (2017). Polymeric scaffolds for pancreatic tissue engineering: a review. The review of diabetic studies: RDS, 14(4), 334.
2. Lemos, N. E., Brondani, L. D. A., Dieter, C., Rheinheimer, J., Bouças, A. P., Leitao, C. B., ... & Bauer, A. C. (2017). Use of additives, scaffolds and extracellular matrix components for improvement of human pancreatic islet outcomes *in vitro*: A systematic review. *Islets*, 9(5), 73-86.
3. Sumi, S. (2011). Regenerative medicine for insulin deficiency: creation of pancreatic islets and bioartificial pancreas. *Journal of hepato-biliary-pancreatic sciences*, 18(1), 6-12.
4. Amer, L. D., Mahoney, M. J., & Bryant, S. J. (2014). Tissue engineering approaches to cell-based type 1 diabetes therapy. *Tissue Engineering Part B: Reviews*, 20(5), 455-467.
5. Llacua, L. A., Faas, M. M., & de Vos, P. (2018). Extracellular matrix molecules and their potential contribution to the function of transplanted pancreatic islets. *Diabetologia*, 61(6), 1261-1272.

6. Stendahl, J. C., Kaufman, D. B., & Stupp, S. I. (2009). Extracellular matrix in pancreatic islets: relevance to scaffold design and transplantation. *Cell transplantation*, 18(1), 1-12.
7. Riopel, M., & Wang, R. (2014). Collagen matrix support of pancreatic islet survival and function. *Front Biosci*, 19(1), 77-90.
8. Coronel, M. M., & Stabler, C. L. (2013). Engineering a local microenvironment for pancreatic islet replacement. *Current opinion in biotechnology*, 24(5), 900-908.
9. Fisher, S. A., Tam, R. Y., & Shoichet, M. S. (2014). Tissue mimetics: engineered hydrogel matrices provide biomimetic environments for cell growth. *Tissue Engineering Part A*, 20(5-6), 895-898.
10. Abualhassan, N., Sapozhnikov, L., Pawlick, R. L., Kahana, M., Pepper, A. R., Bruni, A., & Shapiro, A. J. (2016). Lung-derived micro scaffolds facilitate diabetes reversal after mouse and human intraperitoneal islet transplantation. *PLoS One*, 11(5), e0156053.
11. Tripathi, A., & Melo, J. S. (Eds.). (2017). *Advances in biomaterials for biomedical applications* (Vol. 66). Dordrecht, the Netherlands: Springer.
12. Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials*, 32(12), 3233-3243.
13. Napierala, H., Hillebrandt, K. H., Haep, N., Tang, P., Tintemann, M., Gassner, J., & Struecker, B. (2017). Engineering an endocrine Neo-Pancreas by repopulation of a decellularized rat pancreas with islets of Langerhans. *Scientific reports*, 7(1), 1-12.
14. Ponomareva, A.S., Kirsanova, L.A., Baranova, N.V., Surguchenko, V.A., Bubentsova, G.N., Basok, Y.B., Miloserdov, I.A., Sevastianov V.I. (2020). Decellularization of donor pancreatic fragment to obtain a tissue-specific matrix scaffold. *Russian Journal of Transplantology and Artificial Organs*, 22, 123-133.
15. Guruswamy Damodaran, R., & Vermette, P. (2018). Decellularized pancreas as a native extracellular matrix scaffold for pancreatic islet seeding and culture. *Journal of tissue engineering and regenerative medicine*, 12(5), 1230-1237.
16. Goh, S. K., Bertera, S., Olsen, P., Candiello, J. E., Halfter, W., Uechi, G., & Banerjee, I. (2013). Perfusion-decellularized pancreas as a natural 3D scaffold for pancreatic tissue and whole organ engineering. *Biomaterials*, 34(28), 6760-6772.
17. Smink, A. M., & de Vos, P. (2018). Therapeutic strategies for modulating the extracellular matrix to improve pancreatic islet function and survival after transplantation. *Current diabetes reports*, 18(7), 1-7.
18. Salvatori, M., Katari, R., Patel, T., Peloso, A., Mugweru, J., Owusu, K., & Orlando, G. (2014). Extracellular matrix scaffold technology for bioartificial pancreas engineering: state of the art and future challenges. *Journal of diabetes science and technology*, 8(1), 159-169.
19. Mirmalek-Sani, S. H., Orlando, G., McQuilling, J. P., Pareta, R., Mack, D. L., Salvatori, M., & Soker, S. (2013). Porcine pancreas extracellular matrix as a platform for endocrine pancreas bioengineering. *Biomaterials*, 34(22), 5488-5495.
20. Wu, D., Wan, J., Huang, Y., Guo, Y., Xu, T., Zhu, M., & Wang, Z. (2015). 3D culture of MIN-6 cells on decellularized pancreatic scaffold: in vitro and in vivo study. *BioMed research international*, 2015.
21. Sevastianov, V. I. (2015). Cell-engineered constructs in tissue engineering and regenerative medicine. *Russian Journal of Transplantology and Artificial Organs*, 17(2), 127-130.
22. Sevastianov, V. I. (2014). Technologies of tissue engineering and regenerative medicine. *Russian Journal of Transplantology and Artificial Organs*, 16(3), 93-108.
23. Sevastianov, V. I., Basok, Y. B., Grigor'Ev, A. M., Kirsanova, L. A., & Vasilets, V. N. (2017). Formation of tissue-engineered construct of human cartilage tissue in a flow-through bioreactor. *Bulletin of experimental biology and medicine*, 164(2), 269-273.
24. Kirsanova, L.A., Baranova, N.V., Bubentsova, G.N., Sevastianov, V.I. (2017). Morphological analysis of isolated rat pancreatic islets cultured under standard culture technique and with biopolymer microstructured collagen-containing hydrogel. *Russian Journal of Transplantology and Artificial Organs*, 19, 90-97.
25. Soares da Costa, D., Reis, R. L., & Pashkuleva, I. (2017). Sulfation of glycosaminoglycans and its implications in human health and disorders. *Annual review of biomedical engineering*, 19, 1-26.
26. ISO, E. (1999). 10993-5: 1999—Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity (ISO 10993-5: 1999). German version: DIN EN ISO, 10993-5.
27. London, N. J., Swift, S. M., & Clayton, H. A. (1998). Isolation, culture and functional evaluation of islets of Langerhans. *Diabetes & metabolism*, 24(3), 200-207.
28. Peloso, A., Citro, A., Corradetti, V., Brambilla, S., Oldani, G., Calabrese, F., & Cobianchi, L. (2017). In-Lab Manufacturing of Decellularized Rat Renal Scaffold for Kidney Bioengineering. In *Decellularized Scaffolds and Organogenesis* (pp. 103-110). Humana Press, New York, NY.
29. Sackett, S. D., Tremmel, D. M., Ma, F., Feeney, A. K., Maguire, R. M., Brown, M. E., & Odorico, J. S. (2018). Extracellular matrix scaffold and hydrogel derived from decellularized and delipidized human pancreas. *Scientific reports*, 8(1), 1-16.

Copyright: ©2021 V.I. Sevastianov, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.