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IN VITRO EFFECT OF BIOSCAFFOLDS ON VIABILITY AND INSULIN-PRODUCING FUNCTION OF HUMAN ISLETS OF LANGERHANS

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Introduction. The culture of islets of Langerhans with bioscaffolds – extracellular matrix (ECM) mimetics – can provide a native microenvironment suitable for islets. This is one of the main conditions for creating a pancreatic tissue equivalent. **Objective:** to compare the secretory capacity of viable human pancreatic islets in monoculture (control group) and cultured in the presence of two bioscaffolds: biopolymer collagen-based hydrogel scaffold (experimental group 1) and tissue-specific scaffold from decellularized deceased donor pancreas (experimental group 2). **Materials and methods.** Islets of Langerhans were isolated from the caudal pancreas using a collagenase technique. The viability of cultured islets was accessed by vital fluorescence staining, while secretory capacity was evaluated by enzyme-linked immunosorbent assay (ELISA). **Results.** Pancreatic islets cultured with bioscaffolds showed no signs of degradation and fragmentation, they remained viable throughout the entire period of observation (7 days). The monoculture of islets showed significant destructive changes during this period. Basal insulin levels in experimental groups 1 and 2 increased by 18.8% and 39.5% on day 1 of culture compared to the control group, by 72.8% and 102.7% on day 4 of incubation, and by 146.4% and 174.6% on day 7, respectively. The insulin secretion level of islets with tissue-specific scaffolds was 17.4% higher than that when cultured with biopolymer collagen-based scaffolds. **Conclusion.** Biopolymer and tissue-specific ECM mimetics contribute not only to preservation of the viability of isolated islets of Langerhans but also maintain their insulin secretion capacity for 7 days at a higher level in comparison with monoculture. The experiments revealed that the use of a tissue-specific scaffold for the creation of a pancreatic tissue equivalent has slight potential advantage over biopolymer scaffold.

Keywords: pancreas, culture of the islets of Langerhans, insulin-producing function, tissue-specific scaffold, biopolymer scaffold.

INTRODUCTION

One of the directions of tissue engineering and regenerative medicine technologies includes creation of a tissue equivalent of the endocrine pancreas [1]. The incidence of type 1 diabetes (T1D) in the world is increasing from year to year [2], and improvement in the traditional method of treatment – insulin therapy [3] – does not protect against developing severe complications, such as diabetic angiopathy and neuropathy [4, 5]. Allotransplantation of pancreatic islets is able to provide insulin independence in patients for a certain period of time, without subjecting patients to serious surgical intervention, as in pancreas transplantation [6–8]. However, a significant drawback in this treatment method is the low functional activity of islets due to the action of a number of damaging factors during isolation and culturing procedures.

It is known that the T1D development mechanism is based on autoimmune destruction of insulin-producing pancreatic beta cells of islets of Langerhans, which leads to depletion of the pool of these cells and gradually increasing, progressive loss of endogenous insulin synthesis [9].

During isolation, pancreatic islets are exposed to a number of damaging factors, such as ischemia, oxidative stress, and possible cytotoxic enzyme action. In the process of culturing, islets are fragmented and degraded due to impaired innervation and vascularization provided in the body by ECM [10, 11]. By participating in morphogenesis, differentiation, intracellular signaling, gene expression, adhesion, migration, proliferation, secretion and survival of pancreatic islets [12], ECM contributes to the integrity of islet structure, which is a necessary condition for their functioning.

Previously, we studied the possibility of obtaining viable isolated pancreatic islets from a fragment of the caudal part of donor human pancreas using the collagenase technique [13]. In the post-isolation period, it seems essential to provide the islets with a microenvironment characteristic of native ECM in situ. This problem can be solved by creating a tissue equivalent of the pancreas

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consisting of islet cells and a bioscaffold, which can most of all mimics the structure and composition of ECM to preserve the viability and functional activity of isolated islets in vitro and in vivo [14].

Such bioscaffolds include a commercially available biopolymer-based microheterogeneous collagen-containing hydrogel (BMCH scaffold), registered in Russia for clinical use as a bioimplant – "Composition of microheterogeneous collagen-containing gel Sphero®GEL" (BIOMIR Service, Krasnoznamensk). Sphero®GEL, produced from components of farm animal tissues by acetic acid extraction, contains the main components of ECM: peptides of partially hydrolyzed collagen, glycoproteins, uronic acids and growth factors required for cell life-sustaining activity, synthesis of exogenous uronic acids, proteoglycans and collagen [15].

Tissue-specific bioscaffolds made from decellularized pancreas or its fragments (DP scaffold) appear to be the most promising components of tissue equivalents of pancreas [16–18]. All pancreas decellularization protocols are aimed at preserving the structural, biochemical and biomechanical properties of native ECM with maximum complete removal of cellular material (including antigenic) to minimize immune response to implantation of DP scaffold [19–21]. Presence of the main ECM components in the decellularized pancreatic scaffold, such as structural proteins (type I, III, IV, V and VI collagens, elastin, fibronectin and laminin), glycoproteins and cell adhesion factors, allows creating conditions for prolonged life-sustaining activity of islet cells and maximum imitation of ECM properties [22]. Preservation of ECM architectonics in DP scaffold also affects the survival and secretory function of islets [23, 24]. Scientific literature data and our studies demonstrate increased insulin secretion by porcine pancreatic islets [25] and rat islets [18] cultured in the presence of allogeneic tissue-specific scaffolds compared to monoculture of islets.

The **objective** of our work was to compare the insulin-producing function of viable human pancreatic islets in monoculture and cultured in the presence of two bioscaffolds: biopolymer-based microheterogeneous collagen-containing hydrogel (BMCH) and tissue-specific scaffold from decellularized human pancreas (DP-TSS).

MATERIALS AND METHODS

Baseline

To isolate pancreatic islets and obtain tissue-specific decellularized matrices, we used the caudal part of the pancreas obtained from multiorgan procurement of organs (not suitable for transplantation) from deceased donors.

Isolation and identification of islets of Langerhans

To obtain islets of Langerhans, a small fragment (~2.0 g) of the caudal part of the pancreas was mechanically crushed (1.5 × 1.5 × 1.5 mm) and incubated in collagenase NB1 solution (activity 20 PZ U/g tissue) with neutral protease NP (activity 1.5 DMC U/g tissue) (Serva, Germany) for 10–15 minutes at 37 °C. The action of the enzymes was stopped by adding a threefold volume of cold (4 °C) Hanks' balanced salt solution (PanEco, Russia), followed by filtration through a metal sieve with 0.4–0.6 mm mesh diameter. To purify the islets, a centrifugation mode was selected to avoid using a ficoll density gradient (1 minute at 900 rpm, then 2 minutes at 1300 rpm).

Islets were identified by dithizone staining (Sigma-Aldrich, USA) immediately after isolation. For this purpose, part of the suspension was mixed with a 2 : 1 dithizone solution and incubated for 20–30 minutes at 37 °C. The dithizone selectively stained the pancreatic islets red-orange, while the acinar cells remained unstained. Freshly isolated islets were resuspended in growth medium and used in the experiment no later than 24 hours after isolation.

Biopolymer-based microheterogeneous collagen-containing hydrogel (BMCH scaffold)

One of the bioscaffolds chosen was an injectable form of BMCH (trade name Sphero®GEL, manufactured by Biomir Service, Russia). BMCH scaffold consists of microparticles (145.79 ± 0.09 μm) of scleral collagen type 1, cross-linked by gamma radiation (1.5 Mrad), and a homogeneous hydrogel containing low- and high-molecular ECM components in a 1 : 1 ratio [15]. The heterogeneous component of BMCH scaffold has a porous structure of microparticles with 2–4 μm pore size, which is a positive property in the processes of neovascularization and neoinnervation of tissue-engineered constructs based on it [18].

Tissue-specific matrix from decellularized pancreas (DP scaffold)

As a tissue-specific biomimetic ECM, we used a bioscaffold representing finely dispersed fragments of decellularized human pancreatic tissue. The technique of obtaining DP scaffold (Fig. 1) was developed earlier [21].

The decellularization protocol included three freeze (–80 °C) and thaw (+37 °C) cycles of prostate fragments followed by mechanical grinding of the tissue to a size ≤1 × 1 × 2 mm. The crushed fragments were treated at room temperature in three changes of phosphate-buffered saline (PBS) (pH = 7.4) containing 0.1% sodium dodecyl sulfate and increasing concentration of Triton X100 (1%,

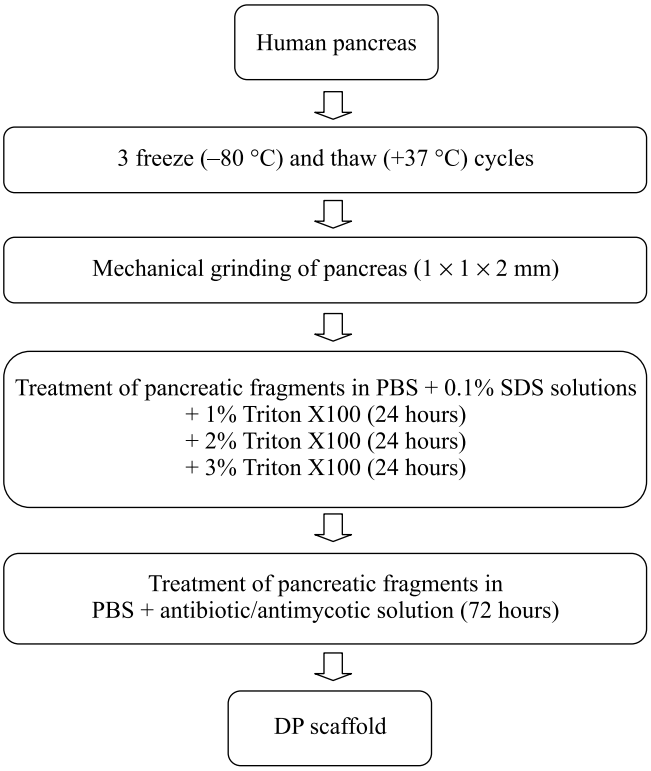

Fig. 1. Schematic representation of the stages for obtaining tissue-specific scaffold from pancreatic tissue

2% and 3%, respectively) (Sigma, USA) under constant stirring for 24 hours. At the final stage of the decellularization process, the fine DP scaffold fragments were thoroughly washed of surfactant residues for 72 hours in three changes of antibiotic-antimycotic solution in PBS.

Fine DP scaffold samples were dehydrated using filter paper, weighed, and added to cryovials at 20.0 ± 0.1 mg each, sterilized with gamma radiation (1.5 Mrad), and frozen at –80 °C. The shelf life of sterile frozen DP scaffold samples with preservation of biochemical and structural properties was not more than 1 year.

DP scaffold contains type I collagen and elastin, has low immunogenicity (no more than 0.1% DNA), is not cytotoxic and retains the morphofunctional properties of native pancreatic tissue ECM regarding adhesion and proliferation of cell cultures [21].

Culture of islets of Langerhans

Equal amounts of isolated islets (~200) were added to three 25 cm^2 culture vials (Greiner bio-one, Germany). No scaffold was added to the first culture vial (control). In the second and third culture vials, 20.0 ± 0.1 mg BMCH scaffold (experimental group 1) and 20.0 ± 0.1 mg DP scaffold (experimental group 2) were added, respectively. All islets were cultured in complete growth medium containing DMEM (1.0 g/L glucose) (PanEco, Russia), 10% fetal calf serum (HyClone, USA), Hepes (Gibco by Life technologies™, USA), 2 mM L-glutamine (PanEco, Russia), and 1% antibiotic/antimycotic (Gibco by Life technologies™, USA). Islets were cultured under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. They were subjected to daily visual monitoring and photography using an inverted microscope (Nikon, Japan) equipped with a digital camera. The culture medium was changed at 1, 4, and 7 days to allow sampling for subsequent testing for insulin content.

Viability determination

The viability of freshly isolated islets, as well as islets cultured in control and experimental groups, was assessed on days 1, 4, and 7 using the LIVE/DEAD® Cell Viability/Cytotoxicity Kit (Molecular probes® by Life technologies™, USA). For LIVE/DEAD® staining, a part of islets suspension (monoculture or with matrices) was placed in a Petri dish, mixed with the prepared working dye solution in a 1 : 2 ratio, and incubated in the dark for 15–30 minutes. The results were evaluated using a luminescence microscope (Nikon, Japan).

Determination of insulin-producing function

To determine the insulin-producing function of islets in control and experimental groups, the growth medium was replaced in culture vials on days 1, 4 and 7. After 1 hour of incubation under the same conditions (37 °C, 5% CO_2), samples of growth medium were taken from all vials. The samples were stored frozen (–23 °C) for subsequent ELISA analysis.

Basal concentration of insulin in the culture medium of control and experimental groups was determined using ELISA Kit for insulin human CEA448 Hu-96 (Cloud-Clone Copr., USA) according to the manufacturer's instructions.

This ELISA variant uses the competitive inhibition method. An insulin-specific monoclonal antibody is pre-applied on a microtiter plate. A competitive inhibition reaction is initiated between biotinylated enzyme-labeled insulin and unlabeled insulin (standard and culture medium samples) with the pre-applied insulin-specific antibody. After incubation with all kit reagents, a substrate solution was added that reacted with the complex to produce a signal as a stained product. The intensity of this signal was expressed through optical density, which is inversely proportional to the insulin levels in the samples tested. The inverse correlation between the insulin levels in the sample and the signal intensity is plotted as a standard curve with a logarithmic function.

Results of quantitative ELISA were calculated by measuring the optical density on a Spark 10M microplate reader (TecanTrading AG, Switzerland) with Spark Control™ Magellan V1.2.20 software at 450 nm and 550 nm wavelengths to account for optical defects in the microplate. The data obtained were processed using SPSS26.0 software. The results presented are shown as

mean ± standard deviation. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Freshly isolated islets of Langerhans

Using an inverted microscope, we observed a significant number of freshly isolated islets of various sizes with a predominantly round shape and smooth surface (Fig. 2, a). The remains of exocrine tissue were revealed as follows: acinar cells created a certain roughness on the surface of some islets and were not stained with dithizone. At the same time, dithizone stained pancreatic islets in orange-red color, which made it easy to identify them (Fig. 2, b).

LIVE/DEAD® staining of freshly isolated islets was complicated by the presence of strong background brightness due to the presence of acinar cells around the islets or in the culture medium. Nevertheless, individual living cells in the islet structure were clearly visualized (Fig. 2, c).

Viability of islets of Langerhans

Islet monoculture

Observation under an inverted microscope showed that most islets cultured without bioscaffolds (control group) retained their shape and integrity within the first three days of incubation. Few of them showed signs of fragmentation or were destroyed.

LIVE/DEAD® staining with a fluorescence microscope showed green fluorescence of the islets, confirming their viability (Fig. 3, a). Some dead acinar cells, stained red with ethidium homodimer, were detected in the culture medium surrounding the islets.

After 3 days of culture, the morphology of islets in the control group changed. Some islets showed cavities, signs of fragmentation, the surface of a significant number of islets acquired uneven outlines and became lumpy (Fig. 3, b). Live staining with LIVE/DEAD® at 4–7 days of culture revealed dead cells with red fluorescence in the preserved islets (Fig. 3, c). Thus, by one week of cultivation without bioscaffolds, the islets had undergone significant destructive changes.

Islets of Langerhans in the presence of BMCH scaffold

Islets cultured with BMCH scaffold (experimental group 1) remained intact and underwent no fragmentation or degradation throughout the entire observation period (7 days). On day 2 of incubation, the islets were found to have adhered to the smooth surface of the BMCH scaffold (Fig. 4, a); the unattached islets floated freely in the culture medium. LIVE/DEAD® fluorescence staining performed on days 1, 4, and 7 of incubation confirmed the viability of the islets in the experimental group 1 (Fig. 4, b).

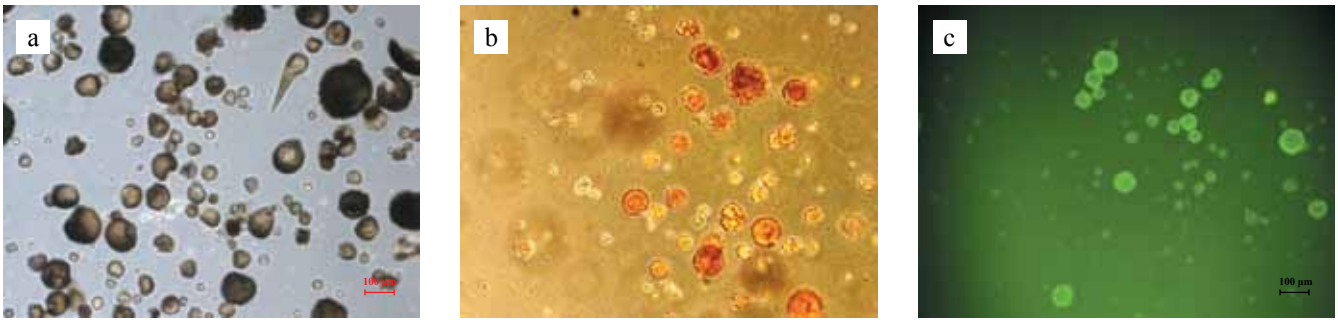

Fig. 2. Freshly isolated human islets of Langerhans. a, inverted phase-contrast microscopy; b, dithizone staining; c, LIVE/DEAD® fluorescence staining. Bar 100 µm

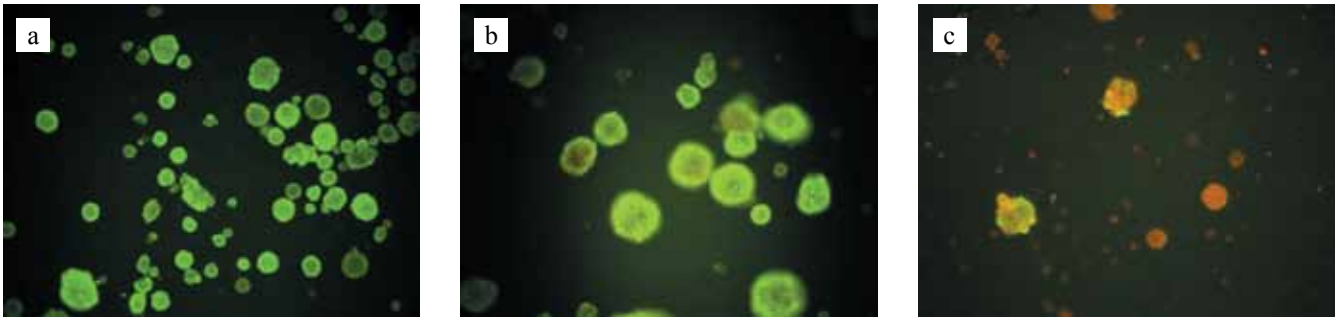

Fig. 3. Monoculture of human islets of Langerhans (control group). LIVE/DEAD® fluorescence staining. a, day 3 of culture. Bar 100 µm; b, day 4 of culture. Bar 200 µm; c, day 7 of culture. Bar 100 µm

Islets of Langerhans in the presence of DP scaffold

Islets cultured with DP scaffold (experimental group 2), just as in experimental group 1, showed no signs of destruction and fragmentation throughout the entire observation period (7 days). On day 2 of incubation with DP scaffold, most of the islets showed adhesive qualities and settled on the fibrous surface of the matrix (Fig. 5, a); the islets remaining in the culture medium continued to float. Live staining of islets in experimental group 2 with LIVE/DEAD® performed at 1, 4, and 7 days of incubation confirmed the viability of the preserved islets (Fig. 5, b).

Insulin-producing function of pancreatic islets

The insulin-producing function of the studied islets was determined at days 1, 4, and 7. Comparative analysis of insulin secretion in experimental groups 1 and 2 was performed in relation to the control group.

After the first day of culture, insulin levels in experimental groups 1 and 2 was 18.8% (46.78 ± 1.29 pg/ml) and 39.5% (54.93 ± 1.58 pg/ml) higher than in the control group (39.37 ± 1.25 pg/ml); on day 4 of incubation, by 72.8% (41.65 ± 0.81 pg/ml) and 102.7% (48.88 ± 1.32 pg/ml), respectively, compared with the control group (24.11 ± 0.58 pg/ml). At day 7, an even more significant difference was observed between the insulin levels in experimental groups 1 (32.9 ± 1.08 pg/ml) and 2 (36.66 ± 1.38 pg/ml) and the control group (13.35 ± 0.55 pg/ml) (Table).

The positive effect of bioscaffolds on the insulin-producing function of islets is manifested by the difference in hormone concentrations in the control and experimental groups at all periods of the study (Fig. 6). A significant difference between the insulin levels

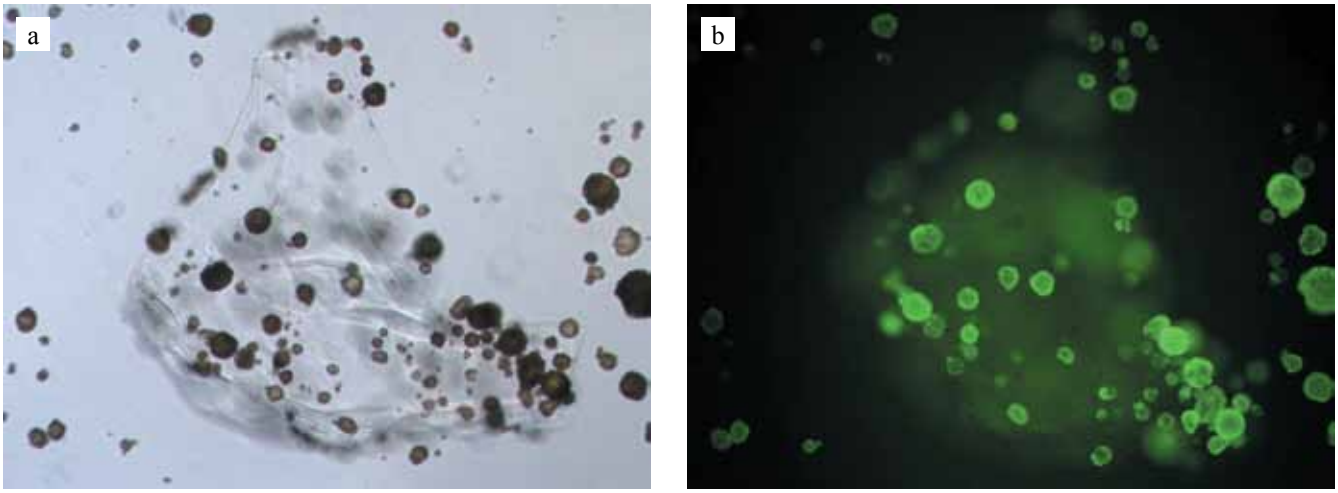

Fig. 4. Human islets of Langerhans cultured with biopolymer collagen-based scaffold (BMCH scaffold). Day 7 of culture: a, inverted phase-contrast microscopy; b, LIVE/DEAD® fluorescence staining. Bar 100 μm

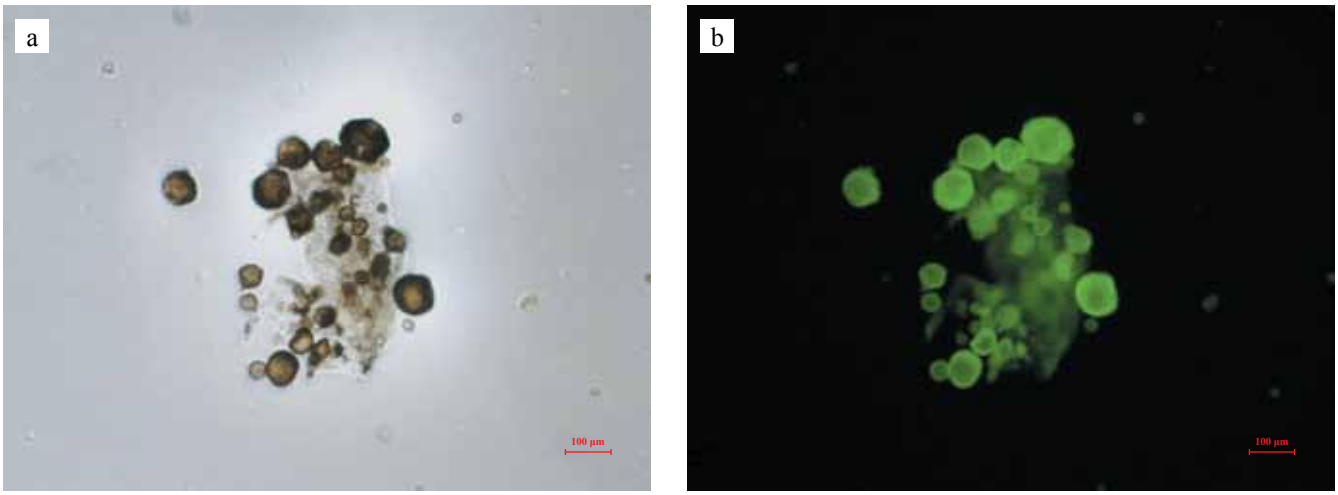

Fig. 5. Human islets of Langerhans cultured with tissue-specific scaffold from decellularized human pancreas (DP scaffold). Day 7 of culture. a, inverted phase-contrast microscopy; b, LIVE/DEAD® fluorescence staining. Bar 100 μm

Table

Comparative analysis of insulin levels (%) in the experimental groups relative to the control group (monoculture of islets)

Day	Islets + BMCH scaffold (experimental group 1), %	Islets + decellularized pancreas scaffold (experimental group 2), %
1	18.8 ± 3.3	39.5 ± 4.0
4	72.8 ± 3.4	102.7 ± 5.5
7	146.4 ± 8.1	174.6 ± 10.3

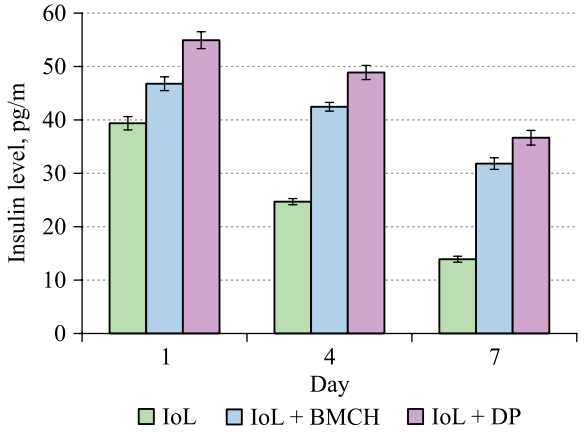

Fig. 6. Insulin-producing function of human islets of Langerhans in control and experimental groups. IoL, islets of Langerhans; BMCH, biopolymer-based microheterogeneous collagen-containing hydrogel; DP, decellularized pancreas. $p < 0.05$

in experimental groups 1 and 2 and the control group may be due to the destructive changes in the monoculture of islets after three days of culture, which is confirmed by fluorescent staining of islets at different time points. The positive trend of the effect of biopolymer and tissue-specific ECM mimetics on the secretory function of islets in percentage terms was maintained throughout the whole period of observation, despite the fact that insulin level, expressed in absolute values, decreased with increasing culture period.

The level of insulin secretion on days 1 and 4 of culture of islets in group 2 was 17.37 ± 0.05% higher than in group 1; 11.43% higher on day 7. Thus, we revealed a slight advantage of using tissue-specific DP scaffold over BMCH scaffold when culturing human pancreatic islets. Earlier we have studied the insulin-producing function of rat islets cultured in the presence of BMCH scaffold and scaffold from decellularized rat prostate [18]. A comparative analysis of insulin secretion showed a more pronounced effect of the studied bioscaffolds on rat islets than on human islets, and the level of insulin secretion in rat islets cultured in DP scaffold was 35.5% higher than that of rat islets cultured in BMCH scaffold.

This study showed that isolated human islets cultured in the presence of bioscaffolds can be preserved for 7 days under standard incubation conditions without significant loss of morphofunctional properties and viability. This method of preserving islet potential in vitro before implantation in patients with T1D is likely to increase the duration of islet function in vivo and in the post-implantation period.

CONCLUSION

Culture of isolated islets of Langerhans with biopolymer and tissue-specific ECM mimetics contributes not only to preservation of islets viability, but also to maintenance of their secretory function at a higher level for 7 days in comparison with the culture of islets without bioscaffolds. The experiments revealed that using tissue-specific DP scaffold has a slight potential advantage over BMCH scaffold when creating a tissue equivalent of the pancreas.

The authors declare no conflict of interest.

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