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DECELLULARIZED PORCINE LIVER SCAFFOLD FOR MAINTAINING THE VIABILITY AND CAPACITY OF PANCREATIC ISLETS

N.V. Baranova[1], A.S. Ponomareva[1], A.D. Belova[1], L.A. Kirsanova[1], D.D. Filin[1, 2], E.A. Nemets[1], Yu.B. Basok[1], V.I. Sevastianov[1, 3]

[1] Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation
[2] Mendeleev University of Chemical Technology of Russia, Moscow, Russian Federation
[3] Institute of Biomedical Research and Technology, Moscow, Russian Federation

Bioengineered pancreatic constructs based on scaffolds made from decellularized tissues and pancreatic islets (PIs) may be used to extend the functional activity of transplanted PIs in patients with type I diabetes. Objective: to investigate *in vitro* the effect of decellularized porcine liver scaffold (DPLS) on the viability and insulin-producing capacity of isolated human PIs. **Materials and methods.** The resulting DPLS was subjected to histological examination, DNA quantification, and cytotoxic effect testing. The PIs were isolated from human pancreas fragments using the collagenase technique. Under standard conditions, PIs were cultured in three different environments: monoculture (control group), with DPLS present (experimental group 1) or with decellularized human pancreas scaffold (DHPS) present (experimental group 2). Vital fluorescent dyes were used to evaluate the viability of PIs. Basal and glucose-loaded insulin concentrations were determined by enzyme immunoassay. **Results.** The basic composition and structure of the extracellular matrix of liver tissue in DPLS samples were preserved thanks to the selected decellularization procedure. The samples had no cytotoxic effect, and the residual amount of DNA in the scaffold did not exceed 1.0%. PIs from the experimental groups showed no significant signs of degradation and fragmentation during the 10-day incubation period compared to PIs from the control group. On day 10, the viability of PIs from experimental group 1 was 64%, that of experimental group 2 was 72%, and that of the control group was less than 20%. After the first day of culturing, insulin concentration were 29.0% higher in experimental group 1 and 39.1% higher in experimental group 2 compared to the control group. On day 10 of the experiment, insulin levels in experimental groups 1 and 2 differed by 124.8% and 150.9%, respectively, from the control group. Under a glucose load, the insulin level in experimental group 1 was 1.7 times higher than in the control group, whereas that of experimental group 2 was 2.2 times higher. **Conclusion.** The resulting DPLS has a positive effect on the viability and insulin-producing capacity of PIs. When creating a bioengineered construct of PIs, DPLS can be used as a component obtained in sufficient quantity from an available source.

Keywords: decellularization, porcine liver, scaffold, pancreatic islets.

INTRODUCTION

Transplantation of functionally active pancreatic islets (PIs) is generally considered one of the safest and least invasive transplantation methods for treating severe cases of type I diabetes mellitus (T1D) [1]. A major focus in cell therapy research in the treatment of T1D is to maintain the viability and functional activity of transplanted beta cells as long as possible. Several key challenges impact islet graft survival, including hypoxia, oxidative stress, partial portal vein thrombosis, and the immediate blood-mediated inflammatory response. In addition, the isolation process itself significantly compromises PI viability and function before transplantation even occurs. PIs vascularization and innervation are disturbed, and the signaling interaction between islet cells and components of extracellular matrix (ECM) is fundamentally changed.

Recent advances in tissue engineering have opened new possibilities for improving the long-term survival and function of transplanted PIs [2–4]. One of the most promising strategies involves bioengineered endocrine pancreas constructs that utilize decellularized scaffolds recellularized with insulin-producing cells [5]. To obtain scaffolds, organs or tissues undergo a decellularization process to remove immunogenic cellular components, while preserving the ECM (proteins and growth factors) composition as much as possible [6, 7].

Three-dimensional (3D) scaffolds, purified from DNA and retaining essential proteins, create a microenvironment that supports functional cell survival and activity. This allows optimizing conditions for their prolonged viability [3]. In addition, decellularized tissue scaffolds provide efficient repopulation by functional cells, as they contain the appropriate 3D architecture and preserved

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

spatial framework of ECM components to maintain cell adhesion and functional activity.

Decellularized pancreas scaffolds (DPS) provide a highly supportive environment for PIs by maintaining their structural integrity, prolonging viability, and enhancing insulin secretion compared to standard culture conditions. Research has demonstrated that culturing islets in the presence of DPS prolonges survival and functioning *in vivo* [10, 11].

The liver and pancreas are known to share a similar embryonic developmental pathway (arising from the same part of the foregut entoderm). The ECM of the liver and pancreas share similar components, including type I, III, and IV collagen, elastin, laminin, fibronectin, and glycosaminoglycans [4, 7]. Given these similarities, a decellularized liver scaffold (DLS) may serve as an alternative framework for bioengineered constructs, offering a supportive environment for insulin-producing cells.

Furthermore, the transplantation of islets into the liver parenchyma via the portal vein remains the primary clinically approved site for islet transplantation [12, 13]. DLS have shown great potential in preserving both liver cells [14, 15] and endocrine islet cells [16]. For instance, studies have demonstrated that 3D scaffolds obtained from decellularized whole mouse liver lobes significantly enhance the survival and function of isolated mouse PIs *in vitro* [17].

Goh et al. demonstrated that mouse DLS can be successfully repopulated with insulin-producing cell aggregates derived from differentiated pluripotent human embryonic stem cells. The extensive vascular network of DLS allowed for even distribution of cell aggregates, unlike DPS, enabling efficient nutrient and oxygen supply to the insulin-producing cells during cultivation in a bioreactor for nine days [18].

The potential of scaffolds from other decellularized organs to prolong the function of PIs has also been demonstrated. In [19], it was shown that decellularized rat spleen scaffolds can serve as a promising carrier for beta-cell transplantation. When MIN6 cells (a mouse insulinoma beta-cell line) were seeded onto these scaffolds, insulin production was significantly increased compared to traditional 2D culture. Bioartificial PIs created using decellularized pig lung scaffolds and human PIs exhibited high viability and insulin production *in vitro* comparable to freshly isolated islets. This makes them a promising platform for real-time drug screening [20].

This strategy for creating a bioengineered pancreatic construct is expected to find potential applications in preclinical trials, drug development, and diabetes therapy.

When creating a bioengineered construct based on functional human cells and decellularized allogeneic scaffold, the problem of organ shortage cannot be avoided, which makes it necessary to obtain scaffold from xenogeneic material, for example, porcine. In addition, implants derived from decellularized porcine tissues and organs, such as skin, bladder, heart valves, and small intestine, have been successfully used in clinical practice [21]. We also note that the exocrine activity of the pancreas, and, accordingly, the possibility of self-digestion of pancreatic tissue complicates the collection and transportation of the xenogeneic organ. The liver is devoid of this disadvantage, which makes it attractive for pancreatic tissue engineering.

The **objective** of our work was to obtain decellularized porcine liver scaffold (DPLS) and to study *in vitro* its effect on the viability and insulin-producing function of isolated human islets of Langerhans.

MATERIALS AND METHODS

Objects of research

Pancreatic tissue fragments obtained during pancreatic resection in patients were used to isolate PIs and obtain a decellularized human pancreas scaffold (DHPS). All manipulations were performed according to the World Medical Association's (WMA) Declaration of Helsinki "Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects", adopted by the 18th World Medical Assembly, held in Helsinki, Finland in 1964 according to the current revised text. A report (dated March 16, 2018, Protocol No. 160318-1/1/1e) approving the experimental studies was obtained from the Local Ethics Committee of Shumakov National Medical Research Center of Transplantology and Artificial Organs.

Pancreatic tissue fragments were placed in Hanks' Balanced Salt solution (+4 °C) with antibiotic/antimycotic and stored at +4 °C…+6 °C for no more than 10 hours before the islet isolation procedure.

DHPS obtained by decellularization of human pancreatic tissue fragments according to our previously developed protocol [22] was used in the experiments.

DPLS was obtained using pig liver (weight 20 kg, age 3 months, Promagro, Stary Oskol).

Obtaining and studying decellularized porcine liver scaffold

Porcine liver was decellularized (Fig. 1) guided by a protocol including treatment of mechanically chopped tissue (fragment size no larger than 2×2×2×2 mm) in three shifts (24 hours for each shift) of phosphate-buffered saline (PBS) containing 0.1% sodium dodecyl sulfate (SDS) and increasing concentrations of Triton X-100 (1%, 2%, and 3%), and treatment with deoxyribonuclease I (DNase I) (Sci-Store, Russia) [23]. Porcine liver fragments were processed at room temperature under continuous stirring on a magnetic stirrer at a speed of 300 rpm. To achieve complete removal of cellular components, measured by the residual amount of DNA, DPLS was treated in a DNase I solution. Next, DPLS were washed of residual surfactants for 72 hours

by incubating the samples in PBS containing antibiotic/antimycotic. DPLS samples were sterilized by gamma irradiation with a dose of 1.5 Mrad.

Sterile DPLS samples were stored at –20 °C and, immediately before the experiment, were crushed to a particle size of 500 ± 45 μm to reduce the degree of microheterogeneity.

DNA content in the original and decellularized porcine liver tissue was determined. For this purpose, DNA was isolated from the samples using DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. For DNA quantification, we used fluorescent dye Quant-iT PicoGreen (Thermo Fisher Scientific, USA) and microplate reader Spark 10M (Tecan Trading, Switzerland), which was used to analyze the resulting thermionic emission at 520 nm wavelength.

For histological examination, the original tissue and DPLS samples were fixed in 10% buffered formalin (Biovitrum, Russia), washed in running water, dehydrated in ethanol of ascending concentration (70%, 80%, 90% and 96%), incubated in a mixture of 96% ethanol and chloroform and embedded in paraffin. Histological sections were deparaffinized, rehydrated, and stained with Mayer's hematoxylin (Dako, Denmark) and 1% eosin solution (Biovitrum, Russia) and according to the Masson trichrome method for total collagen. The stained sections were encapsulated in Bio Maunt balm (Bio-Optica, Italy). The preparations were analyzed and photographed using a Nikon Eclipse Ti inverted microscope (Nikon, Japan).

In vitro cytotoxicity of the DPLS samples was evaluated by direct contact method in accordance with the standard GOST ISO 10993-5-2011 [24] on a culture of the NIH/3T3 mouse embryonic fibroblasts. Cells were seeded into 24-well flat-bottom culture plates and incubated at +37 °C under standard conditions until a monolayer with an 80–85% confluence was formed. The tested samples were placed on the surface of the formed cell monolayer. Cell morphology was evaluated after 24 hours of incubation. Complete growth medium for NIH/3T3 cells served as a negative control, and a standard zinc solution in nitric acid (9.95 mg Zn in 1–2 wt.% HNO_3, dilution 1:200 with 0.9% NaCl solution for injection) served as a positive control. The cell culture was visually evaluated using a Nikon Eclipse Ti light microscope (Nikon, Japan).

Isolation and identification of human pancreatic islets

Islets were isolated by focusing on the collagenase technique using collagenase NB1 (activity 20 PZ U/g tissue) and neutral protease NP (activity 1.5 DMC U/g tissue) (Serva, Germany) [9, 22].

Freshly isolated islets were identified using dithizone staining (Sigma-Aldrich, USA), which labels zinc in insulin granules. For this purpose, part of the suspension was mixed with the dye solution in a 2:1 ratio and incubated for 20–30 min at +37 °C. Staining and islet counting were performed using a Nikon Eclipse Ti light microscope (Nikon, Japan). The resulting islet suspension was resuspended in complete growth medium and used in the experiment within 24 hours of isolation.

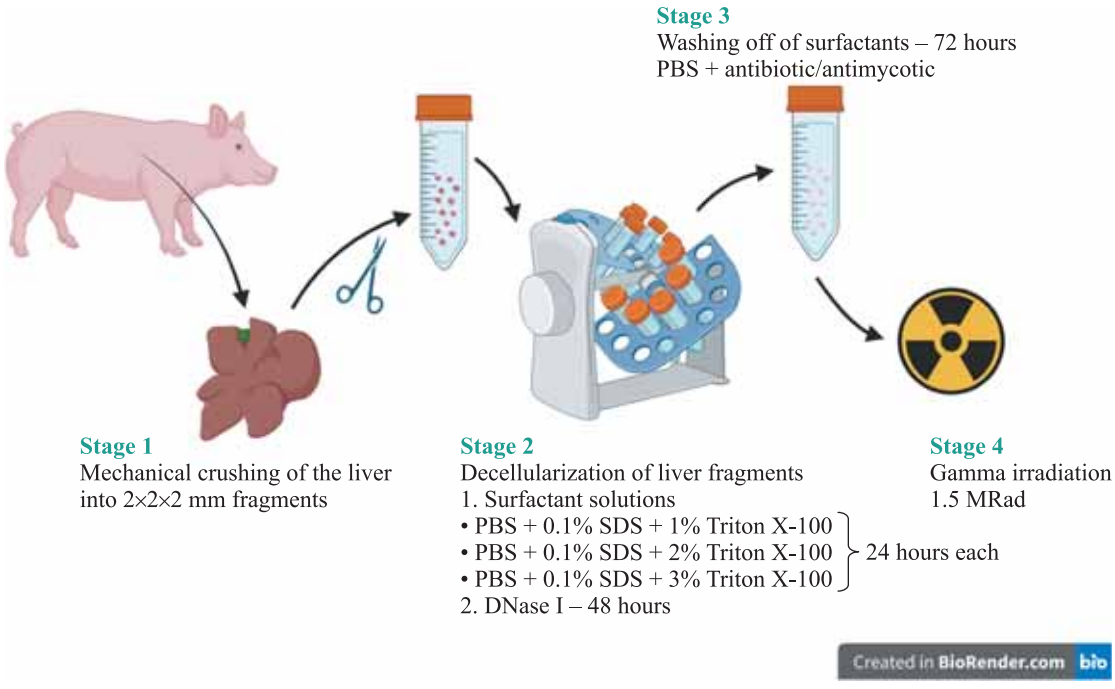

Fig. 1. Schematic representation of how a decellularized porcine liver scaffold (DPLS) is obtained

Cultivation of human pancreatic islets in monoculture and in the presence of scaffolds

Approximately equal amounts of isolated islets (n = 250 ± 10) were added to three 25 cm^2 culture vials (Greiner bio-one, Germany). No scaffolds were added to the first culture vial (control group). In the second culture vial, 20.0 ± 0.1 mg of DPLS was added (experimental group 1). In the third culture vial, 20.0 ± 0.1 mg DHPS was added (experimental group 2). Islets from control and experimental groups were cultured in complete growth medium containing DMEM (1.0 g/L glucose) (PanEco, Russia), 10% ETS (HyClone, USA), Hepes (Gibco, USA), 2 mM L-glutamine (PanEco, Russia), 1% antibiotic/antimycotic (Gibco, USA). Islets were cultured under standard conditions at +37 °C in a humidified atmosphere containing 5% CO_2, with daily visual monitoring and photography using a Nikon Eclipse Ti inverted microscope (Nikon, Japan) equipped with a digital camera. The culture medium was changed at days 1, 3, 7, and 10 in order to collect samples for further investigation of insulin content by enzyme-linked immunosorbent assay (ELISA).

Determination of pancreatic islet viability

To assess the viability of freshly isolated and cultured human PIs, fluorescent staining was performed using the LIVE/DEAD Cell Viability/Cytotoxicity Kit (Molecular Probes, USA). A portion of the islet suspension (either in monoculture or with scaffolds) was placed in a Petri dish, mixed with the prepared working dye solution at a 2:1 ratio, and incubated in the dark for 15–30 minutes. Viable islets, indicated by green fluorescence, were counted using a Nikon Eclipse 50i fluorescent microscope (Nikon, Japan).

Determination of insulin-producing function of pancreatic islets

The basal concentration of insulin in control and experimental groups at day 1, 3, 7 and 10 was determined using the ELISA Kit for insulin Human CEA448 Hu-96 (Cloud-Clone Copr., USA) according to the manufacturer's instructions. For this purpose, growth medium containing 2.8 mmol/L glucose was replaced in culture vials. After 1 hour of incubation under the same conditions (+37 °C, 5% CO_2), the culture medium was sampled.

Changes in hormone levels under glucose loading (up to 25 mmol/L) were assessed on the second day of incubation. The growth medium was first replaced with fresh medium containing a low glucose concentration (2.8 mmol/L). After a 60-minute incubation under standard conditions, culture medium samples were collected and frozen at –23 °C. The growth medium was then replaced with fresh medium containing a high glucose concentration (25 mmol/L), followed by another 60-minute incubation. Culture medium samples were again collected (two per cultivation period) and frozen at –23 °C for further ELISA analysis.

Quantification was performed by measuring optical density using a Spark 10M microplate reader (Tecan Trading, Switzerland) with Spark Control Magellan V1.2.20 software. Readings were taken at wavelengths of 450 nm and 550 nm to correct for optical defects in the microplate. Statistical analysis was conducted using Microsoft Office Excel (2016), with differences considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Decellularized porcine liver scaffold

Decellularization of porcine liver fragments was achieved using a combination of chemical (SDS and Triton X-100) and enzymatic (DNAse I) treatments. This process resulted in finely dispersed decellularized porcine liver scaffold (DPLS) with a residual DNA content of no more than 10.3 ± 1.5 ng/mg – less than 1% of the DNA present in native liver. Additionally, the fine fiber structure and essential extracellular matrix (ECM) components of the porcine liver were preserved.

Native porcine liver tissue is characterized by a pronounced division of the parenchyma into lobules bounded by connective tissue layers (Fig. 2, a). In Masson's trichrome stain, blue collagen fibers are well identified in connective tissue strands (Fig. 2, b).

DPLS specimens are characterized by preserved fibrous structure with the presence of clearly distinguishable thin ECM fibers. In the structure of the scaffold, both interlobular and intralobular stroma are determined (Fig. 2, c, d). At the same time, the samples are free of cells and cellular detritus, which is also confirmed by quantification of the content of preserved DNA.

In vitro evaluation confirms that the biocompatibility of DPLS meets biosafety criteria regarding cytotoxicity. Cytotoxicity results were assessed based on the degree of cell response after incubation with the samples. Fibroblast morphology and viability remained unchanged upon contact with DPLS samples, relative to the negative control (response degree 0) (Fig. 2, e, f). These findings indicate that the studied samples exhibit no cytotoxic effects.

According to *in vitro* evaluation, the biocompatible properties of DPLS meet the biosafety criteria for cytotoxicity. Cytotoxicity results were analyzed according to the evaluation scale of the degree of cell response after incubation with the samples. Upon contact with DPLS samples, the morphology and viability of fibroblasts relative to the negative control did not change (response degree 0) (Fig. 2, e, f). Thus, the studied samples have no cytotoxic effect.

Freshly isolated human pancreatic islets

After isolation, we observed a significant number of islets of various sizes with predominantly round shape and smooth surface. Selective dithizone red-orange staining of β-cells allowed identification of PIs (Fig. 3, a). LIVE/DEAD staining demonstrated green fluorescence in 95–98% of freshly isolated islets (Fig. 3, b).

Viability of pancreatic islets

After isolation, most PIs in the control group retained their shape and structural integrity during the first three days of incubation, with only a few exhibiting signs of fragmentation and degradation. Fluorescent microscopy analysis revealed that 78% of PIs remained viable in the control group after one day of culturing, decreasing to

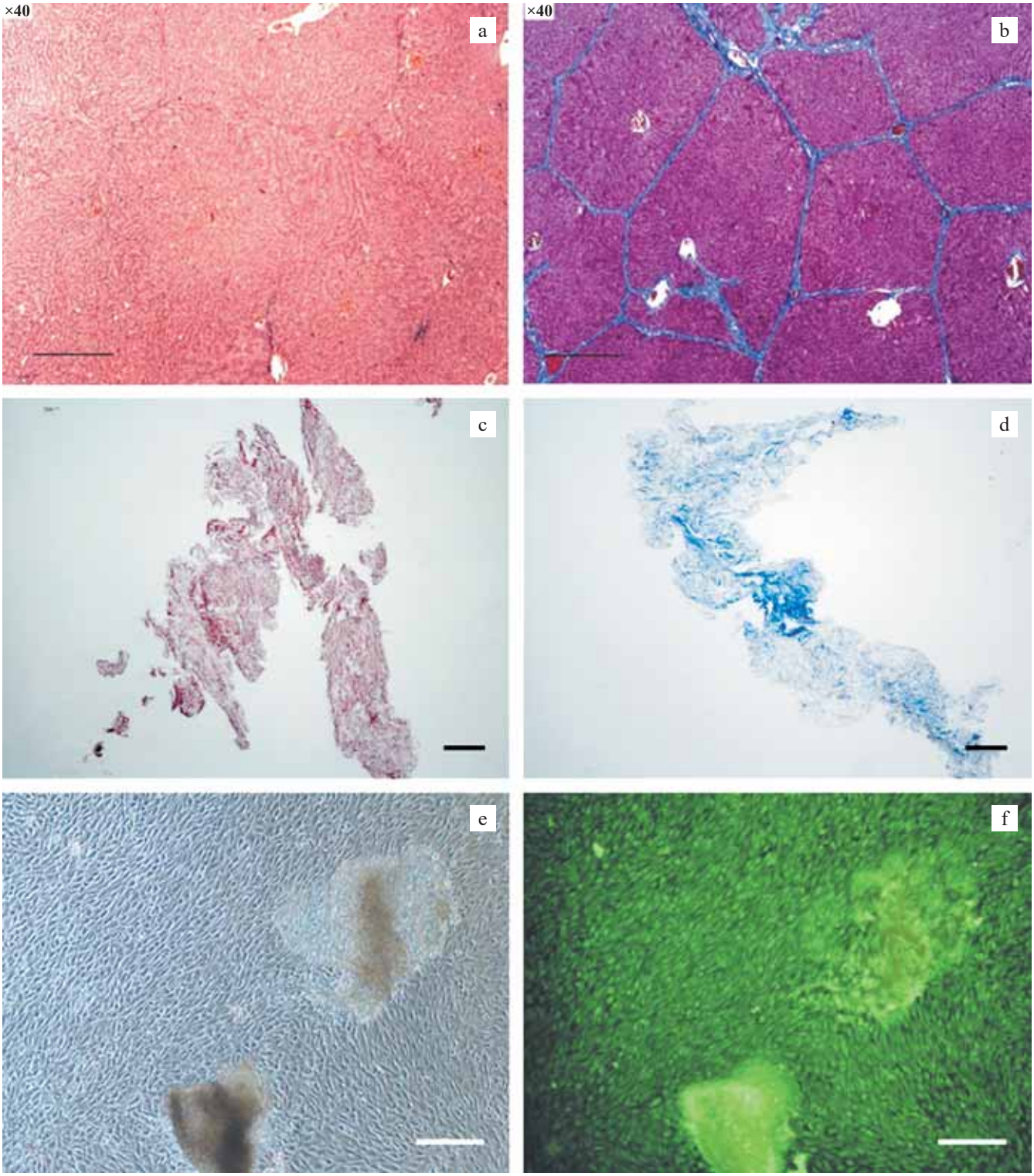

Fig. 2. Native porcine liver (a, b) and DPLS (c, d): a, c – H&E staining; b, d – Masson trichrome staining. NIH/3T3 fibroblasts cultured with DPLS: e, phase-contrast microscopy; f, LIVE/DEAD fluorescent staining. Scale bar 100 μm

approximately 60% by day three (Fig. 4). However, by the third day, noticeable morphological changes were observed, including cavity formation, fragmentation, and irregular surface contours in many PIs. By the end of the first week, significant structural deterioration had occurred, reducing PI viability to below 30%, and by day 10, viability dropped to less than 20% (Fig. 5, a, b).

Isolated islets cultured with DPLS (experimental group 1) and DHPS (experimental group 2) exhibited no signs of destruction or fragmentation throughout the 10-day observation period. Most islets showed adhesive properties, attaching to the fibrous scaffold surface, while those remaining in the culture medium continued to float. LIVE/DEAD staining on day 1 of incubation confirmed the viability of most islets, with 83% viability in experimental group 1 and 85% in experimental group 2. By day 3, islet viability was 77% and 82% for experimental groups 1 and 2, respectively (Fig. 4). A gradual decline in viability was observed by day 7, with 71% in experimental group 1 and 78% in experimental group 2. By day 10, islet viability decreased to 64% (Fig. 5, c, d) and 72% (Fig. 5, e, f), respectively. Notably, enhancing viability may be achievable through the use of a perfusion bioreactor, as the continuous circulation of culture medium could optimize nutrient and gas exchange while facilitating metabolic waste removal. This approach is

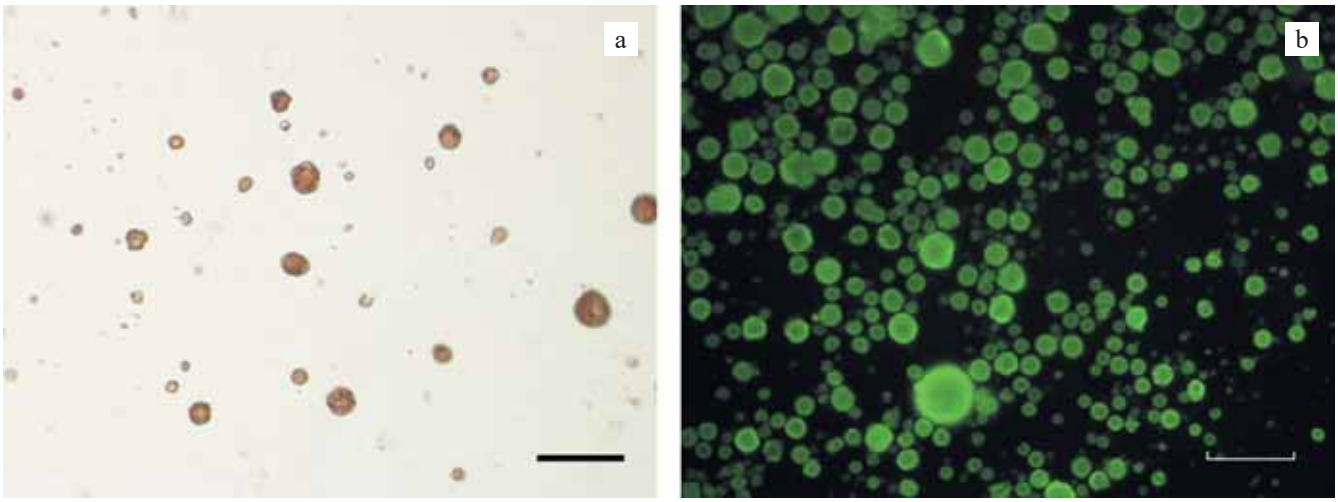

Fig. 3. Isolated human pancreatic islets: a, dithizone staining; b, LIVE/DEAD fluorescent staining. Scale bar 100 μm

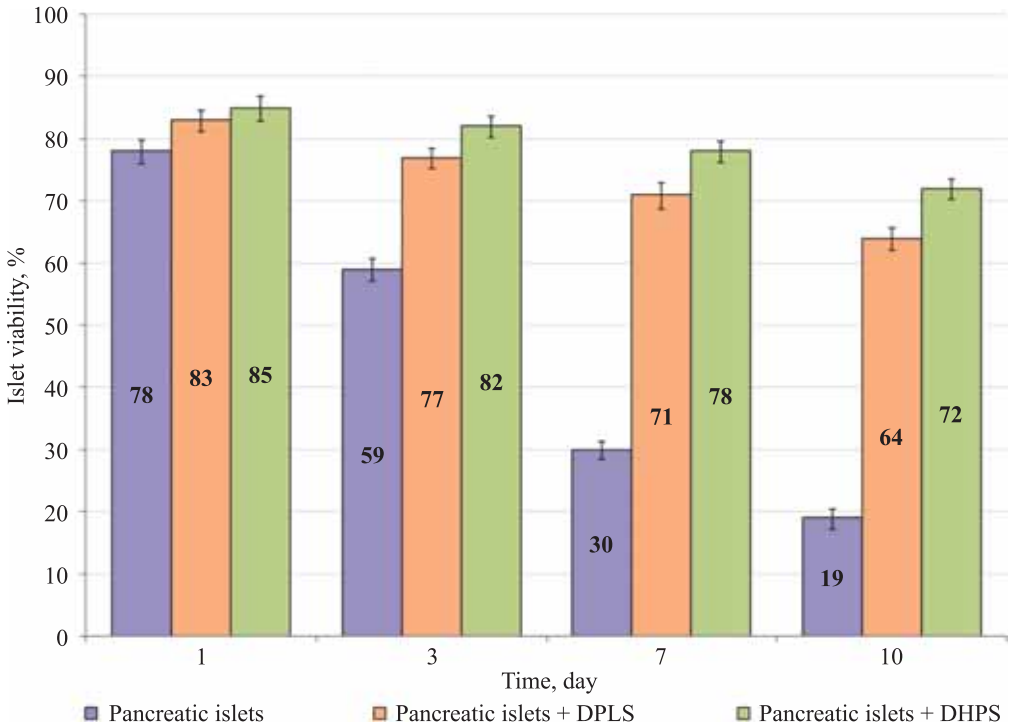

Fig. 4. Comparative analysis of the viability of pancreatic islets cultured in monoculture, with DPLS present and with DHPS present

particularly relevant for PIs embedded within the scaffold structure [25, 26].

Comparative analysis of insulin-producing function of human pancreatic islets in monoculture and in culture with scaffolds

The secretory capacity of human PIs in the control and experimental groups was determined at days 1, 3, 7 and 10, estimating the basal concentration of insulin in the culture medium.

After the first day of culturing, insulin levels were significantly higher in both experimental groups compared to the control. In experimental group 1, the insulin level increased by 29.0% (73.9 ± 8.0 pg/mL), while in experimental group 2, it rose by 39.1% (79.7 ± 7.6 pg/mL), compared to the control group (57.3 ± 6.1 pg/mL).

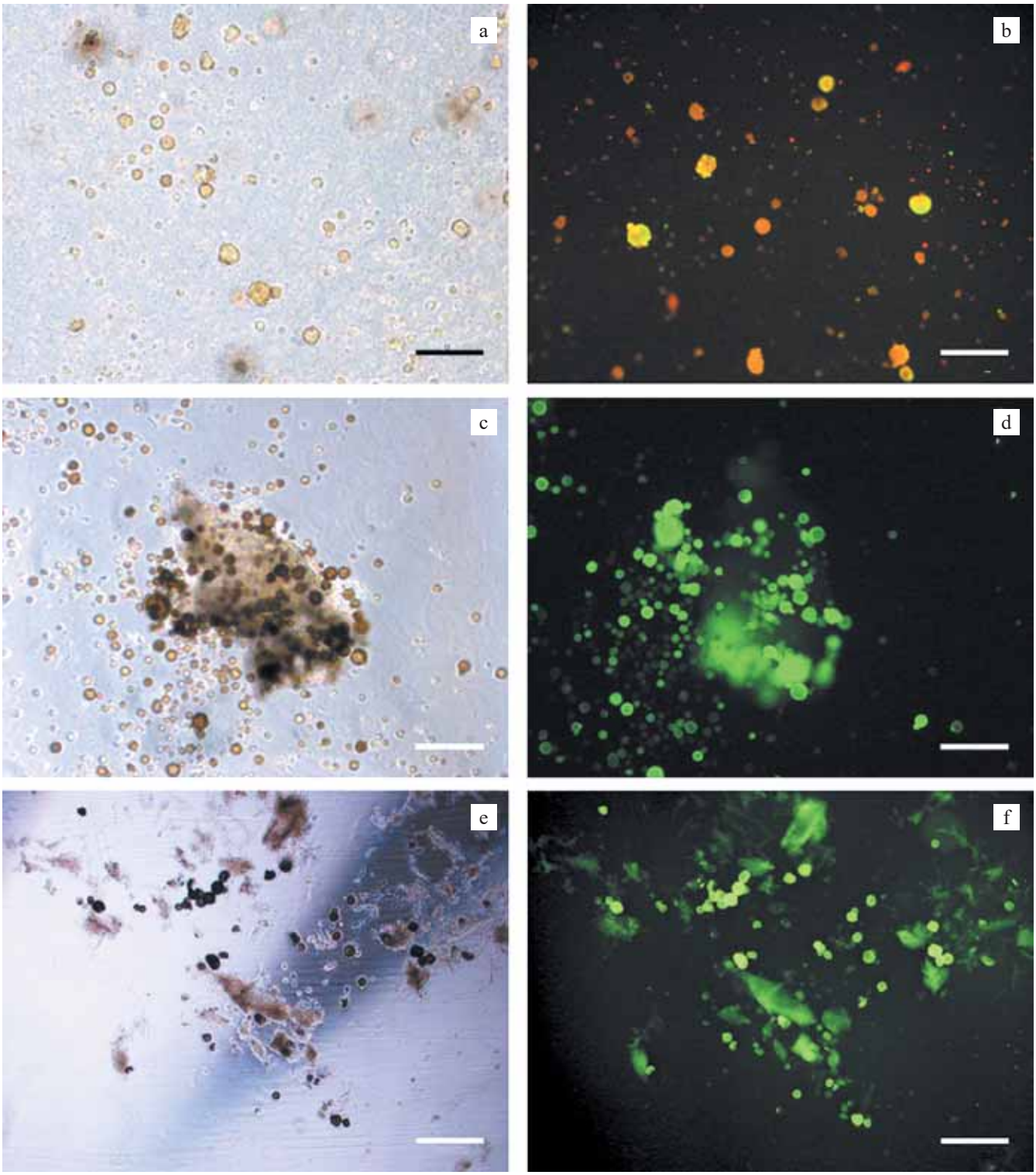

Fig. 5. Human pancreatic islets cultured in monoculture (a, b), with DPLS present (c, d) and with DHPS present (e, f). 10 days of culturing: a, c, e – inverted phase-contrast microscopy; b, d, f – LIVE/DEAD fluorescent staining. Scale bar 100 μm

By day 3 of incubation, insulin levels remained elevated, with experimental group 1 showing a 49.0% increase (62.0 ± 7.4 pg/mL) and experimental group 2 exhibiting a 69.0% increase (70.3 ± 7.0 pg/mL) relative to the control group (41.6 ± 4.9 pg/mL). By day 7, the difference in insulin levels between the experimental and control groups became even more pronounced. Insulin concentration in experimental group 1 (58.4 ± 6.9 pg/mL) was 70.8% higher than in the control group (34.2 ± 5.1 pg/mL), while experimental group 2 (63.3 ± 7.2 pg/mL) exhibited an 85.1% increase. At day 10, basal insulin production in experimental groups 1 and 2 was 124.8% and 150.9% higher than in the control group, respectively (Fig. 6, a). This corresponds to insulin secretion levels that were 2.25 and 2.51 times greater in experimental groups 1 and 2 compared to the control group. Thus, insulin secretion levels in experimental groups 1 and 2 were 2.25 and 2.51 times higher than in the control group, respectively (Fig. 6, b).

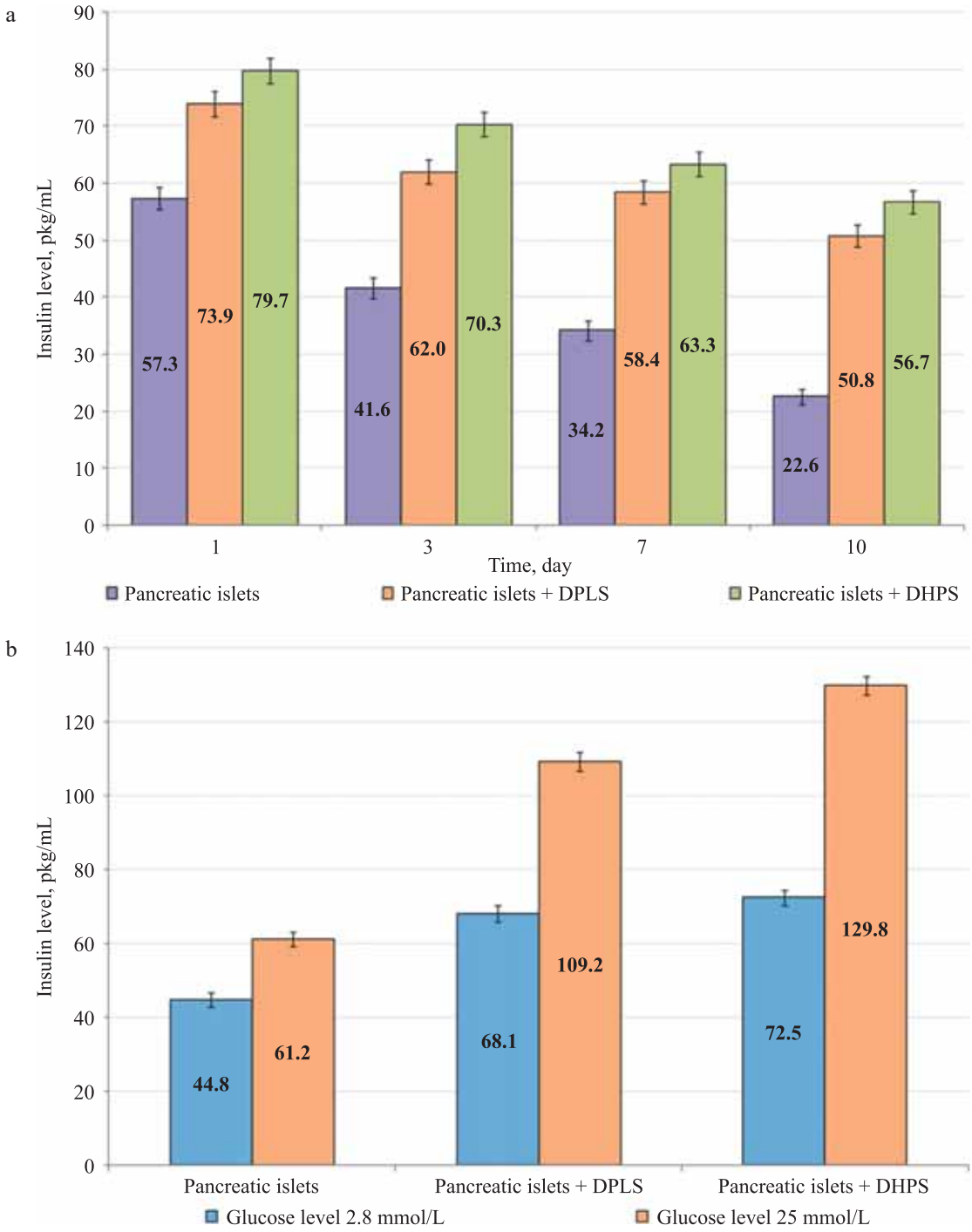

Fig. 6. Comparative analysis of the secretory capacity (a) and functional activity under glucose stimulation (b) of human pancreatic islets in monoculture (control group), cultured with DPLS (experimental group 1) and cultured with DHPS (experimental group 2)

The analysis of culture medium samples collected on day 2 of incubation, both before and after glucose stimulation (25 mmol/L), confirmed the functional activity of the cultured PIs. In the control group, insulin levels increased by 36.6% following glucose stimulation (from 44.8 ± 1.88 to 61.2 ± 1.98 pg/mL). In experimental group 1, insulin secretion rose by 60.4% (from 68.1 ± 2.13 to 109.2 ± 2.53 pg/mL), which was 1.7 times higher than in the control group. In experimental group 2, insulin levels increased by 79.0% (from 72.5 ± 2.07 to 129.8 ± 2.47 pg/mL), which was 2.2 times higher than in the control group and 1.2 times higher than in experimental group 1.

It is important to note that the relative basal insulin levels in the studied culture systems on day 2 of incubation were consistent with the values observed on days 1 and 3 when assessing islet secretory ability. This consistency confirms the reliability of the results obtained.

A comparative analysis of human PI viability and insulin secretion in the presence of DHPS or DPLS demonstrated that both scaffolds support PI survival and insulin production under standard culture conditions for 10 days, outperforming monoculture. Although DPLS had a slightly less pronounced effect on cultured PIs, its advantage lies in its availability and the ease of obtaining it in sufficient quantities compared to DHPS. The use of both DHPS and DPLS scaffolds for *in vitro* islet preservation may contribute to extending PI functionality *in vivo*.

In the future, DPLS may serve as a source for developing new biomaterials, such as macroporous sponges and hydrogels. The porous structure of these scaffolds not only enhances nutrient and gas transport but also promotes cellular colonization, vascular ingrowth, and nerve integration [27–29]. One potential approach for fabricating macroporous sponges with tailored mechanical properties is cryostructuring of DPLS hydrolysis products [30]. For instance, cryogenically structured biopolymer substrates, such as sponge agarose cryogel modified with gelatin, have demonstrated full biocompatibility and effectively supported long-term insulin secretion in cultured mouse islet cells [31, 32]. Additionally, a DPLS-based hydrogel could enable the development of injectable encapsulated cell-engineered constructs and be applied in 3D bioprinting technologies [33].

CONCLUSION

DPLS exhibits slightly lower efficacy than DHPS in supporting the viability, insulin secretion, and functional activity of human PIs. However, in the development of cell-engineered constructs, further studies exploring the use of decellularized liver scaffold – an abundant and readily available biomaterial – could contribute to advancements in both bioartificial liver and bioartificial pancreas technologies. This could enhance beta-cell maintenance and function *in vitro* and *in vivo*.

The authors declare no conflict of interest.

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