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A TECHNIQUE FOR SEPARATING VIABLE ISLETS OF LANGERHANS FROM A FRAGMENT OF HUMAN PANCREATIC TAIL

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Introduction. Modern techniques of tissue engineering in the treatment of some degenerative diseases suggest the prospective viability of the biomedical technologies based on the creation of the equivalent of the damaged tissue (organ), including the tissue-engineered construct (TEC) of the endocrine pancreas (EP). Obtaining viable islets of Langerhans (IL) from the pancreas is a decisive step towards the creation of a TEC EP. The classic method of IL separation is based on enzymatic digestion of pancreatic tissue and further islet purification in ficoll density gradient during centrifugation, which adversely affects the morphofunctional state of IL. **The aim** of the study was the development of a method for separating viable pancreatic islets from a fragment of human pancreatic tail with different cold ischemia times. **Materials and methods.** A procedure of IL separation is proposed to be conducted without the use of EP tissue collagenase perfusion in the Ricordi chamber at the stage of IL separation and without ficoll solution with a varying density gradient at the stage of IL purification. Identification of IL obtained was performed by dithizone staining. The IL viability was evaluated using the LIVE/DEAD® Cell Viability Kit. Histological analysis of the initial material included routine staining methods as well as immunohistochemical staining of the main types of islet cells. **Results.** The morphological study of the EP fragments at different times of cold ischemia did not reveal significant differences in the histological presentation of the organ parenchyma; the islet structure appeared intact. Vital staining confirmed the separated IL viability *in vitro* for at least 1–3 days. **Conclusion.** The proposed method of pancreatic tissue treatment allowed to reduce the number of stages, thereby minimizing the adverse effects of centrifugation and ficoll on the integrity of IL. It is possible to obtain the necessary amount of viable IL from a small EP fragment with the cold ischemia time of up to 19 hours, which can be used to create a TEC of a pancreas.

Key words: islets of Langerhans, human pancreas, tissue engineering.

МЕТОДИКА ВЫДЕЛЕНИЯ ЖИЗНЕСПОСОБНЫХ ОСТРОВКОВ ЛАНГЕРГАНСА ИЗ ФРАГМЕНТА ХВОСТОВОЙ ЧАСТИ ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ ЧЕЛОВЕКА

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Введение. Современные методы тканевой инженерии в лечении ряда дегенеративных заболеваний позволяют надеяться на перспективность биомедицинских технологий, основанных на создании эквивалента поврежденной ткани (органа), в том числе тканеинженерной конструкции (ТИК) эндокринного отдела поджелудочной железы (ПЖ). Получение жизнеспособных островков Лангерганса (ОЛ) из поджелудочной железы является определяющим шагом на пути создания ТИК ПЖ. Классическая методика выделения ОЛ базируется на ферментативном переваривании панкреатической ткани и дальнейшей очистке островков в градиенте плотности фиколла при центрифугировании, что неблагоприятно сказывается на морфофункциональном состоянии ОЛ. **Цель работы** состояла в разработке методики выделения жизнеспособных

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панкреатических островков из фрагмента хвостовой части ПЖ человека с учетом сроков холодовой ишемии органа. **Материалы и методы.** Опробована методика выделения ОЛ без применения перфузии коллагеназой ткани ПЖ в камере Рикорди на стадии выделения ОЛ и без фиколла с разным градиентом плотности на стадии очистки ОЛ. Идентификацию полученных ОЛ проводили с помощью окрашивания дитизоном. Жизнеспособность ОЛ определяли с помощью набора LIVE/DEAD® Cell Viability Kit. Гистологическое исследование исходного материала включало рутинные методы окрашивания, а также иммуногистохимическое окрашивание основных типов островковых клеток. **Результаты.** Морфологическое исследование фрагментов ПЖ на разных сроках холодовой ишемии не обнаружило существенных различий гистологической картины паренхимы органа; островковый аппарат при этом выглядел сохранным. Прижизненное окрашивание подтверждает жизнеспособность выделенных ОЛ *in vitro*, по меньшей мере, в течение 1–3 суток. **Заключение.** Предложенный способ дал возможность сократить количество стадий обработки панкреатической ткани человека, тем самым минимизировать неблагоприятное воздействие центрифугирования и фиколла на сохранность ОЛ. Из небольшого фрагмента ПЖ человека на сроках холодовой ишемии до 19 ч удается получить необходимое количество жизнеспособных ОЛ, которые могут быть использованы при создании ТИК поджелудочной железы.

Ключевые слова: островки Лангерганса, поджелудочная железа человека, тканевая инженерия.

INTRODUCTION

Modern techniques of tissue engineering in the treatment of some degenerative diseases [1] suggest the prospective viability of the biomedical technologies based on the creation of the equivalent of the damaged tissue (organ), including the tissue-engineered construct (TEC) of the endocrine pancreas (EP). The significance of this issue is indubitable since the worldwide incidence of insulin-dependent diabetes mellitus (IDDM) is growing annually [2, 3]. The perfection of the traditional treatment method – insulin therapy (the application of recombinant monocomponent insulin, the use of insulin pump, transdermal hormone delivery, etc.) [4] – does not prevent the development of serious diabetes mellitus complications, such as diabetic angiopathies and neuropathy [5].

It is known that the cellular basis of IDDM development is the autoimmune damage to the β-cells of islets of Langerhans (IL) of EP, which leads to the depletion of pool of those cells and the gradual progressive endogenous insulin deficiency [6, 7]. However, the β-cell secretory product is heterogenous and β-cell destruction deprives the body not only of endogenous insulin but also of biologically active polypeptides such as C-peptide and amylin. In a healthy body those polypeptides, secreted by β-cells, circulate in blood in concentrations typical for hormones. The importance of C-peptide in the treatment of IDDM complications is noted in several articles [8]. Thus, the animal studies of the diabetes mellitus model and clinical trials of IDDM patients show that C-peptide is effective at early stages of diabetic nephropathy, retinopathy, and neuropathy. It is thought that the basis of the C-peptide positive influence is the stimulation of Na-K-ATPase. The role of amylin is less studied, although it is known that, modulating the insulin secretion, it also participates in regulation of blood glucose levels [9]. The replacement of defective β-cells via transplantation of normal islet cells, secreting the entire spectrum of biologically active peptides, allows to achieve the effect not observed with the standard insulin therapy.

IL allotransplantation, considered as an alternative to EP transplant in the treatment of IDDM [10, 11], is capable to ensure the insulin independence of patients without serious surgical intrusion [12, 13]. However, the major drawback of pancreatic islet transplantation is the low functional activity in many ways related to a number of damaging factors during separation and cultivation [14], particularly the breach in interaction of islets cells with extracellular matrix (ECM) which plays an important part in islet functioning. This task may be resolved by means of creation of a pancreatic tissue-engineered construct (TEC EP) [15], consisting of separated IL and the carrier matrix providing the conditions typical of native IL microenvironment.

The obtainment of viable islets from the pancreas is a decisive step towards the creation of TEC. It is known that human IL are very sensitive to separation and are easily destroyed [16], while the preservation of islet structure is a necessary condition for their functionality. A pancreatic islet may be viewed as a microorgan which contains at least five types of endocrine cells [17] with strong paracrine interactions [18] necessary for the effective secretory cell activity. Thus it is important to minimize the influence of specific processing stages in order to avoid the islet fragmentation during separation. The classic method of IL separation is based on enzymatic digestion of pancreatic tissue and further islet purification in ficoll density gradient during centrifugation [19, 20, 21]. However, certain properties of ficoll such as hypertonicity, high viscosity, and possible endotoxin presence [22] may adversely affect the morphofunctional state of IL.

The aim of the study was the development of a method for separating viable pancreatic islets from a fragment of human pancreatic tail with different cold ischemia times.

STUDY MATERIALS AND METHODS

Source material

EP obtained as a result of multiorgan harvesting from post-mortem donors (n = 4) and unsuitable for transplantation was used as a source of IL. The donors were men aged 47–64 years, the cold ischemia times after harvesting were from 6 to 19 hours (Table 1).

Table 1

Data on pancreas donors

Donor	Sex	Age, yrs	Cold ischemia times, hrs
1	♂	58	12
2	♂	55	4
3	♂	63	6
4	♂	47	19

Histological and immunohistochemical study of human EP

EP samples were studied morphologically using the routine histological and immunohistochemical staining methods. The material was fixed in 10% buffered formalin solution, dehydrated in alcohols of ascending concentrations, xylene, and embedded in paraffin wax. 4–5 μm sections were obtained using microtome RM 2245 (Leica, Germany) with subsequent deparaffinization, rehydration and hematoxylin and eosin staining, as well as by Masson's method.

In order to detect the main types of endocrine cells, the IL staining for insulin and glucagon antibodies (Cell Marque, USA) was conducted per the standard method with horseradish peroxidase, using the visualization system Reveal-Biotin-Free Polyvalent DAB (Spring, USA).

The visual control of the degree of purification, identification, and the monitoring of the cultivation process and IL viability were performed using luminescent inverted microscope TS-100 (Nikon, Japan), equipped with digital camera Digital Sight DS-Vi1 (Nikon, Japan).

Separation of islets of Langerhans

The modification of a method of IL separation from a fragment of human pancreatic tail, based on classic protocols with the use of collagenase [19, 20], consists in the omission of the EP tissue sample collagenase perfusion in the Ricordi chamber at the stage of IL separation and of the ficoll solution at the stage of IL purification.

A small fragment (~2.0 g) of human pancreatic tail was placed in a Petri dish under sterile conditions with the intraparenchymal injections of collagenase solution of type 1A (Sigma-Aldrich, USA) with the dosage of 225 units/g of pancreatic tissue. The tissue was mechanically grinded and incubated for 40 min at 37 °C in a thermostat. The action of collagenase was stopped by adding the triple volume of cold (4 °C) Hanks' solution (PanEko, Russia). The flask with the disaggregated pancreatic tissue was shaken manually during several seconds. The resulting EP fragments were filtered through a metal sieve with the mesh diameter of 0.4–0.6 mm. A certain centrifugation regimen was selected for IL purification.

Identification of islets of Langerhans

IL were identified by dithizone staining (Sigma-Aldrich, USA) immediately after separation. To this end, part of the suspension was mixed with dithizone solution at the ratio of 2:1 and incubated for 20–30 min at the temperature of 37 °C. Dithizone selectively stained pancreatic islets, while acinar cells remained unstained.

Cultivation of islets of Langerhans

The IL suspension obtained was resuspended in complete growth medium, containing DMEM/F-12 (PanEko, Russia), 10% fetal calf serum (HyClone, USA), Hepes (Gibco by Life technologies™, USA), 2 mM of L-glutamine (PanEko, Russia), 1% of antibiotic/antimycotic (Gibco by Life technologies™, USA) and introduced into 25 cm^2 culture flasks (Greiner bio-one, Germany). Cultivation was conducted at 37 °C in moist atmosphere containing 5% CO_2 with daily visual observation and IL photography. The culture medium was changed after 2 days of incubation.

Live staining of islets of Langerhans

The IL viability was evaluated on Days 1 and 3 using the LIVE/DEAD® Cell Viability/Cytotoxicity Kit. (Molecular probes® by Life technologies™, USA) per the manufacturer's instructions. The LIVE/DEAD® kit contains calcein and ethidium homodimer and allows to identify simultaneously live and dead cells by means of double fluorescent staining. Calcein easily permeates live cells and creates intensive homogenous green fluorescence (ex/em ~495 nm/~515 nm). Ethidium homodimer permeates cells with damaged membranes and while linking with nucleic acids results in bright-red fluorescence in dead cells (ex/em ~495 nm/~635 nm), while being excluded by the intact plasma membrane of a live cell.

RESULTS AND DISCUSSION

Morphological study of a human EP

A histological study of EP fragments with different times of cold ischemia (2 samples had ischemia times over 10 hours) did not detect pronounced morphological differences in the state of parenchyma on the light-optical level, which indicated good organ preservation in storage solution Custodiol (Dr. Franz Köhler Chemie GmbH, Germany). Signs of moderate lipomatosis were detected in the majority of samples (Fig. 1, a; 2, a). Sclerosis of major interlobular ducts, medium and small intralobular ducts was observed in all samples studied (Fig. 1, b; 2, b).

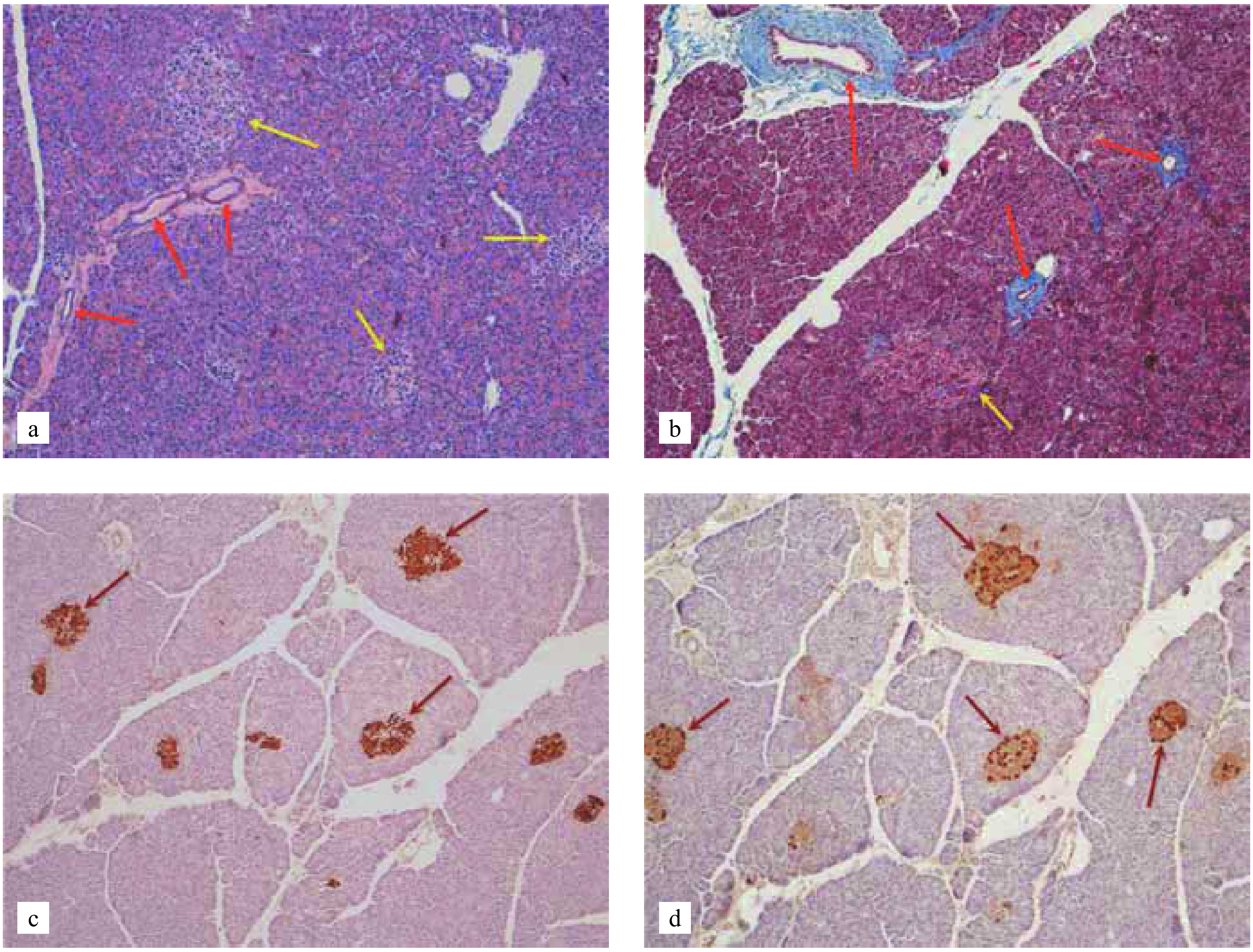

Fig. 1. A histological presentation of human pancreas, the cold ischemia time is less than 10 hours. Red arrows indicate sclerotic interlobular and intralobular ducts; yellow arrows indicate islets of Langerhans: a – hematoxylin and eosin staining; b – Masson's method; c – immunohistochemical staining with anti-insulin human antibodies. Numerous well-granulated β-cells in islets of Langerhans are shown (brown arrows); d – immunohistochemical staining with anti-glucagon antibodies. Glucagon-positive α-cells in islets of Langerhans (brown arrows). ×100

The abundance of islets of Langerhans in gland parenchyma confirmed the assumption that the majority of islets in human EP concentrate in the tail part (Fig. 1, c; 2, c). The islets usually had a rounded (rarely oblong) shape and compact (sometimes lobulated) formation. Compact formation was more characteristic of smaller-sized islets, while lobulation was detected in some larger islets. Immunohistochemical staining of main types of islet cells (β- and α-cells) demonstrated a positive reaction in all samples studied regardless of source material ischemia time (Fig. 1, c, d; 2, c, d). Brown precipitate granules abundantly filled insulin-positive β-cells which composed the main cellular mass of the islet (Fig. 1, c; 2, c). The less numerous glucagon-positive α-cells were spread mosaically in the islet (Fig. 1, d; 2, d). The data obtained indicates the integrity of the islet apparatus and the potential for the use of the gland for IL separation even with the lengthy time of cold ischemia.

Freshly separated islets of Langerhans

The proposed modified method of IL separation allowed to obtain a significant amount of islets of various sizes which correlates with the morphological presentation of the source tissue. The IL observed via the inverted microscope had predominantly a rounded shape with a smooth surface. Certain roughness created by the remnants of surrounding exocrine tissue was observed on the surface of some islets (Fig. 3, a).

Dithizone staining resulted in the orange-red color of the islets, which allowed to identify IL, while the remnants of acinar tissue remained unstained (Fig. 3, b).

Culturing and live staining of islets of Langerhans

Observation via the inverted microscope demonstrated that during the first three days of culturing IL preserved the initial external characteristics.

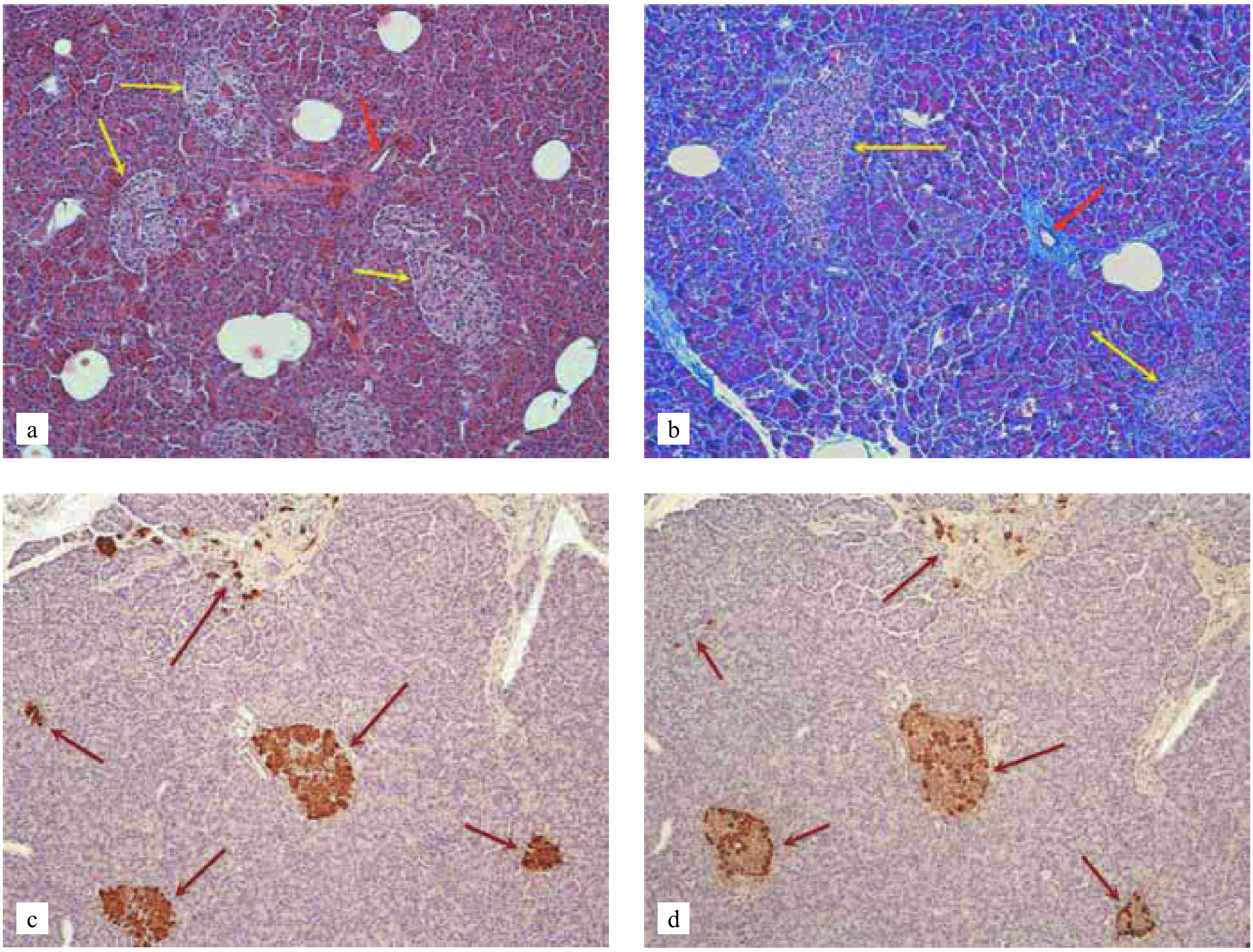

Fig. 2. A histological presentation of human pancreas, the cold ischemia time is more than 10 hours. Red arrows indicate sclerotic intralobular ducts; yellow arrows indicate islets of Langerhans: a – hematoxylin and eosin staining; b – Masson's method; c – immunohistochemical staining with anti-insulin human antibodies. Numerous well-granulated β-cells in islets of Langerhans are shown (brown arrows); d – immunohistochemical staining with anti-glucagon antibodies. Glucagon-positive α-cells in islets of Langerhans (brown arrows). ×100

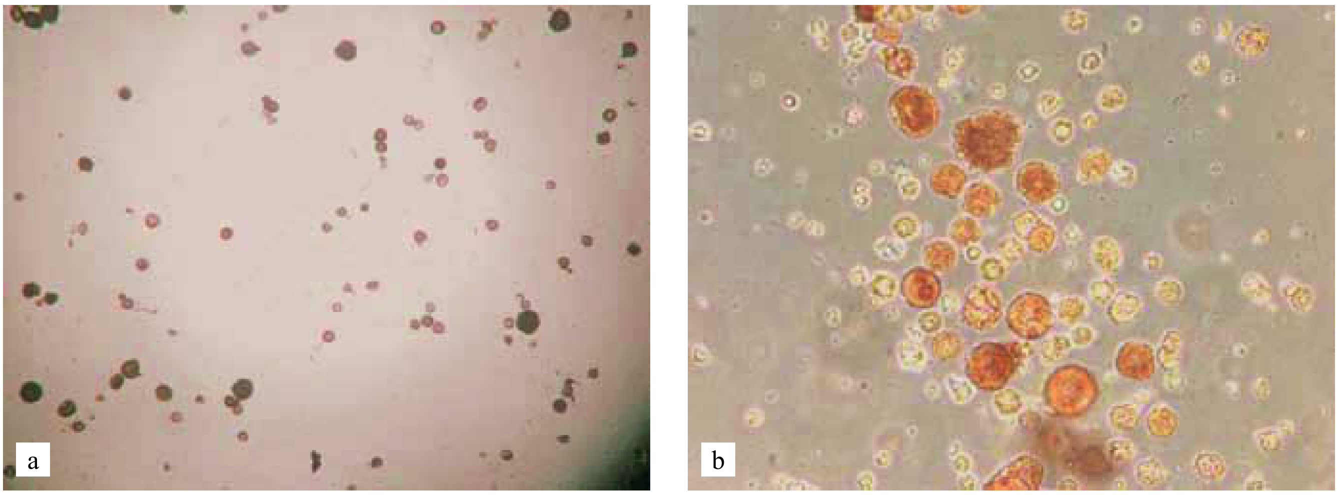

Fig. 3. Freshly separated human islets of Langerhans: a – without staining. ×100; b – dithizone staining. ×200

On the first day of culturing, LIVE/DEAD® staining was complicated by the presence of a strong background luminance due to the large amount of the acinar tissue remnants. Nevertheless, separate live cells in the IL structure were clearly visualized (Fig. 4). Red fluorescence was demonstrated predominantly by dead acinar

Fig. 4. 1-day culture of islets. Fluorescent staining with LIVE/DEAD®. ×100

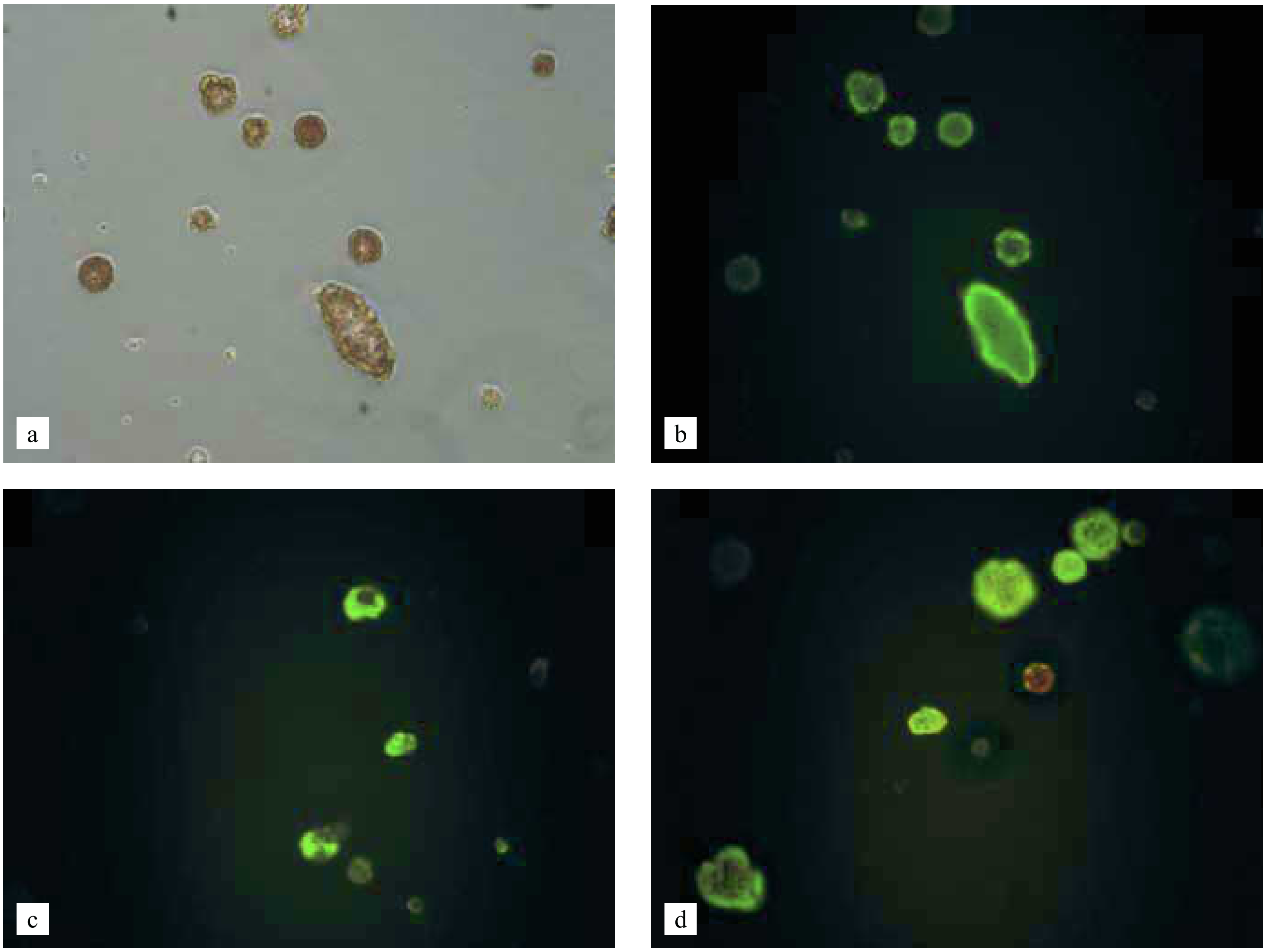

Fig. 5. 3-day culture of islets: a – without staining; b, c, d – fluorescent staining with LIVE/DEAD®. ×200

cells around islets or in culture medium. On Day 3 of culturing the preservation of shape and integrity of the majority of the islets was observed via light microscope (Fig. 5, a). Only a few of them underwent destruction. However, LIVE/DEAD® staining in some IL detected the emergence of cavities and signs of fragmentation (Fig. 5, b, c); the emergence of dead cells with red fluorescence along the live ones (Fig. 5, d).

CONCLUSION

The proposed method of treatment of human pancreatic tissue even after a significant time of organ cold ischemia allows to separate islets of Langerhans from a small EP fragment without using the EP tissue sample collagenase perfusion in the Ricordi chamber nor purifying the islets with the ficoll density gradient. This allowed to simplify the method, reduce the number of stages of tissue treatment, thereby minimizing the adverse effect of centrifugation and ficoll on the IL integrity. Live staining confirms viability of IL separated *in vitro* for at least 1–3 days. Thus obtained IL may be used in further experimental studies in creation of a human TEC EP.

The authors declare no conflict of interest.

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