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DEVELOPMENT OF APPROACHES TO ENZYME-FREE ISOLATION OF PANCREATIC ISLETS

G.N. Skaletskaya, N.N. Skaletskiy, G.N. Bubentsova, V.I. Sevastianov

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

The success of pancreatic islet allotransplantation in the treatment of patients with a difficult-to-manage type 1 diabetes depends mainly on the quantity and quality of islets isolated from the pancreas of deceased donors using enzyme preparations, primarily collagenase. Numerous studies on improvement and standardization of islet isolation techniques have reached their limits in the last decade. This has made it impossible to further boost the number and quality of clinical transplants. Taking into account the negative impact of collagenase technique on the morphofunctional properties of isolated islets, this work has studied the possibility of enzyme-free isolation of islet tissue purified of exocrine ballast. Experiments using the pancreas of newborn and young rabbits showed that developing methodological approaches to obtaining islet-like cultures without the use of exogenous enzymes is feasible.

Keywords: pancreas, exocrine tissue, islets, isolation, collagenase, rabbits, floating islet-like cultures.

INTRODUCTION

The early 21st century saw a breakthrough in effective allotransplantation of pancreatic islets (PIs) in patients with type 1 diabetes mellitus by the emergence of the Edmonton Protocol [1]. Since then, there have been certain improvements in the outcomes and safety of islet transplantation [2]. At the same time, some transplantation centers have consistently been more successful than others [3], and this difference is largely, if not to a decisive extent, due to the quality of isolated islets, which depends on the methodological level and experience of the researchers involved in this problem.

The proper use of collagenase preparations, which are the most important reagents used for islet isolation, significantly affects the quantity and quality of islets (more precisely, islet equivalents) and ultimately determines the outcomes of their transplantation in diabetic patients [3]. For a number of years, Liberase H1 (Roche) was most widely used for islets isolation from collagenase preparations, which, in fact, was considered to be the enzyme of choice [4]. In 2007, however, concerns about its use arose after attention was drawn to the fact that it used raw material derived from bovine brains, which, in theory, could potentially transmit prion-related diseases [5]. Since then, efforts have been made to substitute this preparation by modifying its components and studying the digestive activity of new enzymes [6]. As a result, enzyme mixtures including Serva collagenase NB1, a mammalian tissue-free version of Liberase (Roche), and a new Vitacyte mixture were used [7–9]. Despite the fact that Liberase HI and Collagenase NB1 were the most widely studied enzymes for human islet isolation, quantitative and qualitative results obtained in a number of centers differed significantly and were often contradictory. At the same time, determination of islet β-cell function (basal and stimulated insulin secretion) was the criterion for qualitative assessment of islet samples isolated at multiple U.S. centers from donors with varying characteristics [10].

The lack of standardization of enzymatic treatment of human pancreatic tissue can, to a certain extent, explain the high variability of islet isolation results. In order to determine a reasonable choice of the most acceptable option for pancreatic tissue treatment, a comparative meta-analysis of the results of using mixtures of different enzyme preparations in islet isolation from deceased donors was performed [11]. The effect of different enzymes on the equivalent number of islets obtained from 1 gram of pancreas was evaluated, determining the degree of their purification, viability, and glucose-stimulated insulin secretion. The meta-analysis showed that it seems that research on standardization of isolation of sufficient numbers of islets from donor pancreas has reached its limits, and after a surge in achievements in the early 2000s there was a stagnation period in this field, which made it almost impossible to significantly increase effective islet transplantation in the clinic. Numerous experiments with the use of the pancreas of laboratory animals, primarily rodents, continuing to the present time, have not allowed to significantly improve islet isolation method [12–14] and extrapolate the obtained data to the protocol for islet isolation from the pancreas of deceased donors. Experiments on islet co-culture with

Corresponding author: Galina Skaletskaya. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Phone: (499) 190-42-66; (903) 771-18-13. E-mail: Skalink@mail.ru

mesenchymal stromal cells give some hope for increasing the survival time and functional capabilities of islets [15, 16].

It is important to note that, in addition to exogenous enzyme preparations, activation of its own proteolytic enzymes produced by acinar cells can have a significant impact on the number and quality of islets isolated from donor pancreas. As is known, the exocrine secretory function of the pancreas is to secrete pancreatic juice into the duodenum, which promotes the breakdown of protein from food into amino acids. Proteolytic enzymes represented by trypsin, chymotrypsin and carboxypeptidase, are secreted into the duodenal lumen in an inactive state, and they are activated under the influence of intestinal juice enterokinase. However, intraorgan activation of intrinsic enzymes in donor pancreas is quite real and is mainly associated with impaired oxygen supply during its extraction, cold storage and during islet isolation procedure [17]. Increased lactate production as a result of anaerobic glucose breakdown under hypoxia/anoxia causes intracellular acidosis, which is one of the main inducers of premature intracellular trypsinogen autoactivation and subsequent triggering of the enzyme cascade in acinar cells [18]. Since 90% of proteins synthesized by acinar cells are digestive enzymes, the inevitable ischemia periods provide "ideal" conditions for triggering autolytic processes in the pancreas [19]. The negative effect of endogenous pancreatic proteases on functional ability of islets was noted, in particular, in ischemia/reperfusion-induced pancreatitis developing during preservation of donor pancreas [20]. Islet survival during ischemia can also be hindered by the fact that islets are directly surrounded by acinar cells, which are characterized by higher density of zymogen granules compared to teleinsular cells. This histological feature makes islets particularly vulnerable to proteolytic damage [21].

Lack of progress in the development of islet isolation methods has made it necessary to search for new, more effective approaches to obtaining islet tissue in quantities sufficient for successful transplantation treatment of patients with diabetes mellitus. In this work, we have studied the possibility of obtaining islet cell cultures purified of exocrine ballast without using standard enzyme preparations, which, as mentioned above, significantly reduce survival rate and functional capabilities of isolated islets.

MATERIALS AND METHODS

Laboratory Soviet chinchilla rabbits of different age and body weight were used as pancreas donors. There were 60 newborns (1–2 days old) weighing 60–70 g and 12 one-month old weighing 600–700 g, i.e. much higher than that of newborn rabbits. The animals were obtained from a laboratory animal nursery belonging to KrolInfo LLC with presentation of a veterinary certificate.

Given the above-described deleterious effect of proteolytic enzymes released during prolonged manipulations with donor pancreas on islets, we used techniques that reduce this effect, minimizing, in particular, organ ischemia time. We assumed that moderate proteolysis by endogenous enzymes, inevitable even at the shortest treatment of pancreatic tissue, would lead to death of acinar cells only, but would not have a damaging effect on islets. Therefore, in order to prevent excessive autolysis of pancreatic tissue, immediately after euthanasia of the animals and extraction of the pancreas, the latter was placed in a cold (4 °C) Hanks' balanced salt solution (PanEco), then quickly, using ophthalmic forceps, the capsule, visible blood vessels and the excretory ducts were removed and the organ was cut into about 2 mm fragments, which were washed twice with cold Hanks' solution, then carefully crushed with sharp ophthalmic scissors for 7–10 minutes. The duration of microdissection depended on the visible features of the treated gland and was determined by the researcher in each particular case. The resulting thick tissue suspension was washed with cold Hanks' solution at least three times. As a result of these manipulations, the treated pancreatic tissue at room temperature (20–24 °C) was presumably exposed only to sparing proteolytic effect of endogenous pancreatic enzymes, which, after abundant washing of the resulting tissue suspension, were removed together with fragments of autolysed exocrine tissue. The tissue suspension obtained after treatment of 1 pancreas of a one-month-old rabbit, or 5 pancreases of newborn rabbits consisted mainly of microfragments smaller than 1 mm^3, which were transferred to a 25 cm^3 culture tube (Corning), where 10–12 mL of RPMI-1640 HEPES medium without glutamine (PanEco) was immediately added and 1 mL of fetal calf serum (HyClone) was added. The tubes were placed in an incubator and cultured at 37 °C. The growth medium was replaced with fresh medium every 2–3 days. Changes occurring during incubation were monitored through a Nikon Eclipse TS 100 inverted microscope by daily monitoring; significant changes were recorded using a digital camera.

Histological examination of native pancreas of the newborn and one-month-old rabbits, as well as samples of the resulting cultures, at different periods of incubation of pancreatic microfragments, was carried out. The studied material was fixed in formalin. After routine dehydration, the samples were embedded in paraffin. The 4 μm thick media were stained with hematoxylin and eosin, and subjected to immunohistochemical staining according to the horseradish peroxidase standard technique to detect the main types of islet cells using appropriate monoclonal antibodies: antinsulin and antiglucagon (Sigma).

RESULTS AND DISCUSSION

Histological examination of the pancreas of 1–2-day-old rabbits and the pancreas of one-month-old rabbits revealed a significant difference in the ratio of endocrine (islet) and exocrine (acinar) tissues in these animals of different ages. The proportion of exocrine tissue in young animals was significantly higher than in the newborns. At the same time, oval-shaped islets were clearly separated from the surrounding exocrine tissue by interlayers of connective tissue (Fig. 1, a). At the same time, in newborn animals, due to the natural absence of active digestion, the exocrine pancreas is poorly developed; islets in the neonatal pancreas are markedly smaller, irregular, jagged and have no pronounced connective-tissue interlayers at the border with exocrine cells (Fig. 1, b).

Such histological features made the treatment of pancreatic tissue of newborn rabbits with collagenase preparations inappropriate because of the practical absence of a point of action of such enzymes (collagen fibers of connective-tissue interlayers). Therefore, it was decided to study the changes occurring during cultivation of the pancreas of newborn rabbits subjected only to mechanical crushing without enzyme treatment.

Observations using an inverted microscope revealed a significant decrease in the mass of exocrine tissue already on day 2–3 of incubation of neonatal pancreatic microfragments and their compaction and "growing" against the background of final death and elimination of acinar cells by the end of day 5–7 (Fig. 2).

The detritus formed during acinar cell destruction was safely removed during the next replacement of the culture medium. As a result, a culture was formed consisting almost entirely of free-floating dense globular or ovoid structures (Fig. 3).

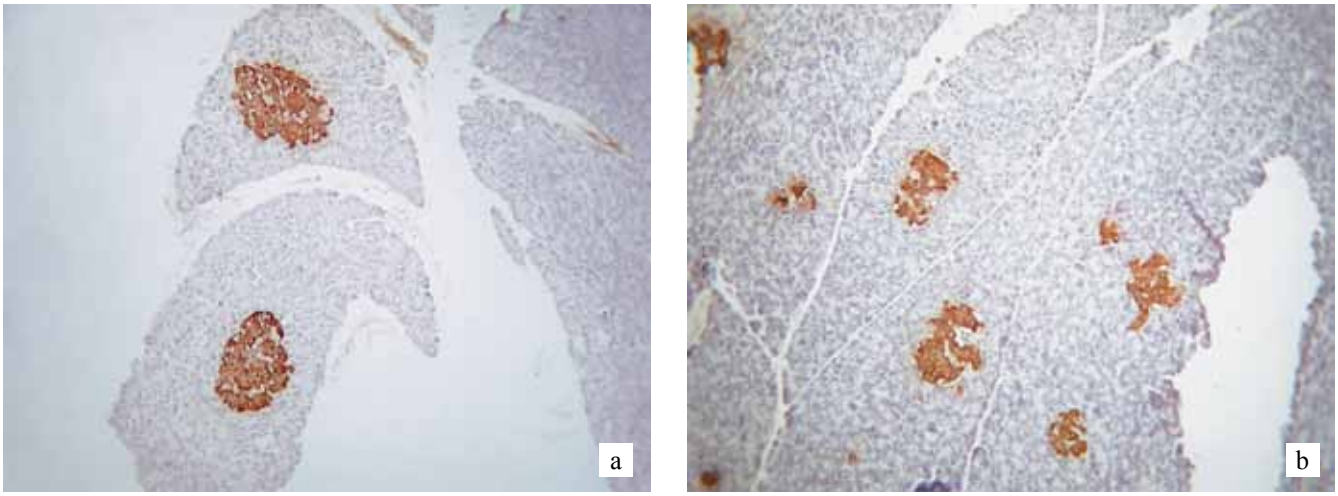

Fig. 1. a, pancreas of a one-month-old rabbit; b, pancreas of a one-day-old rabbit. Immunohistochemical staining of beta-cells of islets with insulin antibodies. 200×

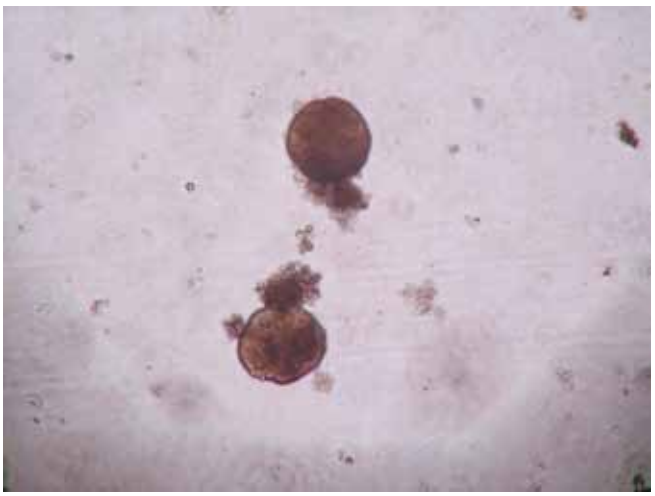

Fig. 2. Completion of spontaneous purification of newborn rabbit pancreatic microfragments from exocrine tissue. Inverted microscope. 100×

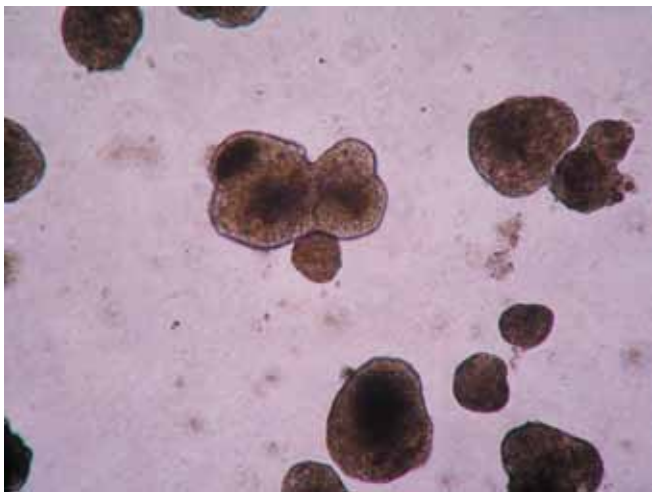

Fig. 3. Formation of floating cultures after 7-day incubation of pancreatic microfragments of newborn rabbits. Inverted microscope. 100×

Histological analysis of the cultures showed that they consisted of epithelium and were surrounded at the periphery by a layer of epithelium-like or fibroblast-like cells (Fig. 4).

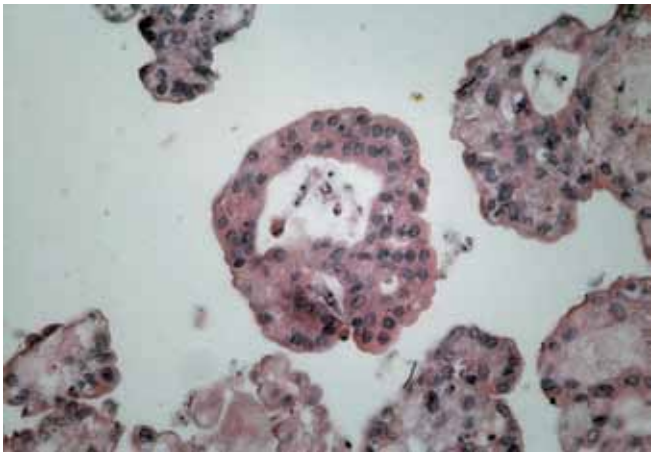

Fig. 4. Floating cultures obtained from the pancreas of newborn rabbits. H&E stain. 200×

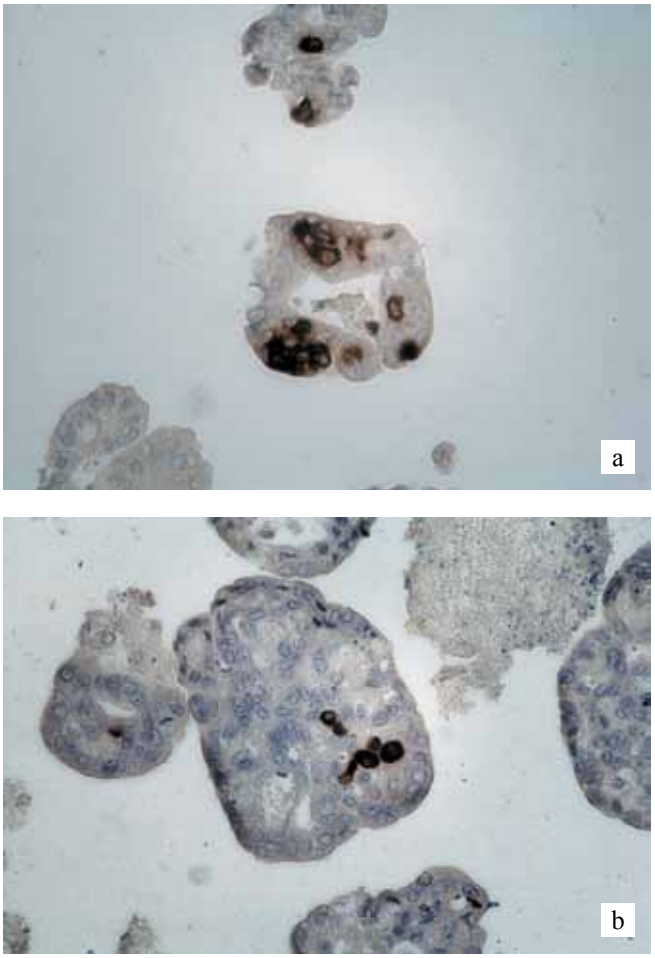

Fig. 5. Immunohistochemical staining of floating cultures obtained from the pancreas of newborn rabbits using insulin (a) and glucagon (b) antibodies. 200×

Immunohistochemical staining made it possible to identify the epithelium contained in the cultures as insulin-positive cells (to a greater extent) and glucagon-positive cells (Fig. 5).

The characteristic spherical and/or ovoid shape of the resulting free-floating cultures and detection of islet β- and α-cells in them gave grounds to call them floating islet-like cultures (FICs).

In contrast to obtaining cultures from the pancreas of newborn rabbits, the use of similar conditions when incubating pancreatic microfragments of one-month-old rabbits did not lead to pronounced elimination of exocrine tissue. Apparently, the reason for this failure was the presence of a significantly higher proportion of exocrine pancreatic tissue in young rabbits compared with the neonatal pancreas. Degradation by pancreatic acini was slow, and a significant amount of them persisted even after 8–10 days of incubation (Fig. 6).

At the same time, the prolonged effect of proteolytic enzymes released from acinar cells on the islet tissue apparently had a negative effect on its morphofunctional state, which prevented FICs formation. Therefore, we decided to increase the incubation temperature, which, presumably, could accelerate the death of exocrine tissue and provide more favorable competitive conditions for endocrine tissue survival.

In spite of the fact that the classical incubation condition is normothermia (temperature not higher than 37 °C), taking into account the natural resistance of pancreatic islets to unfavorable conditions and absence of such in exocrine tissue, we decided for the first time to incubate pancreatic microfragments at 38 °C, especially since normally the temperature inside the human and mammalian body can reach 38 °C.

As observations using an inverted microscope showed, such a regime (formally hyperthermic) is able to

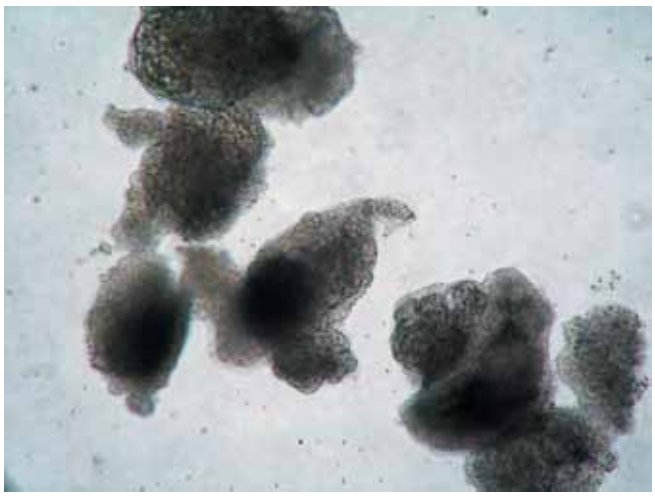

Fig. 6. Preserved exocrine tissue in the pancreatic microfragments of one-month-old rabbit after 10-day incubation. Inverted microscope. 100×

accelerate death and elimination of exocrine pancreatic tissue, which significantly reduces possible proteolytic effect on islets, contributing to their rapid cleansing and survival. Due to creation of such temperature conditions, a distinct degradation of exocrine tissue was observed already by day 3–4 of incubation (Fig. 7), and by day 8–10 ballast-cleared cultures were formed (Fig. 8).

Histological examination of the obtained floating cultures showed that they consisted mainly of viable epithelial cells (Fig. 9).

Using immunohistochemical staining, insulin granules were detected in their central part, indicating secretory activity of islet β-cells (Fig. 10). Thus, it was confirmed that islet-like cultures were obtained from the pancreas of one-month-old rabbits.

CONCLUSION

In our opinion, the very natural isolation, closeness of islets from the surrounding exocrine tissue, this "enzyme-boiling cauldron", allows us to hope that the native enzyme system of the pancreas is less dangerous for islets than the aggressive enzyme mixture that is unnaturally introduced into pancreatic tissue with the only purpose of "knocking out" islets, isolating them from the exocrine tissue. This consideration is confirmed by the fact that in acute pancreatitis, the endocrine (islet) tissue is very rarely affected, and only in cases of repeated

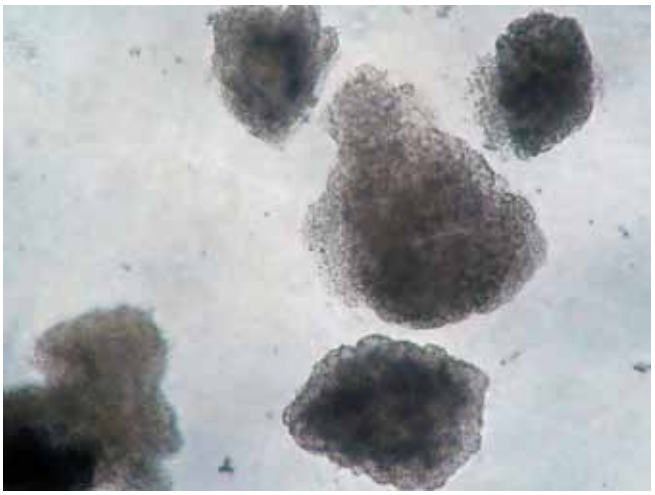

Fig. 7. Beginning of spontaneous purification against exocrine tissue at day 4 of incubation of pancreatic microfragments of one-month-old rabbits under hyperthermic conditions. Inverted microscope. 100×

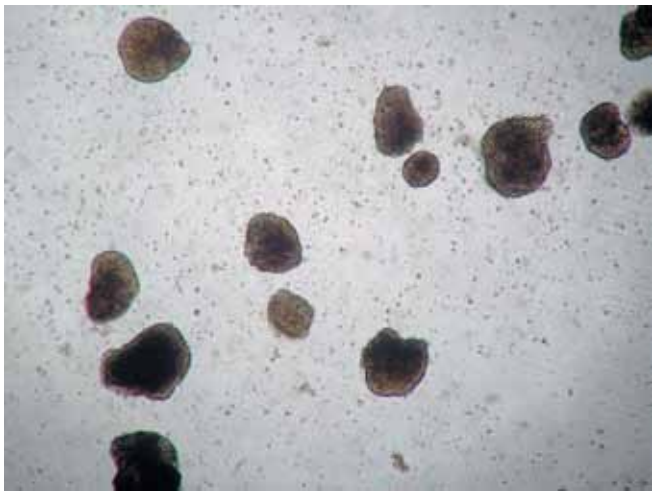

Fig. 8. Formation of floating cultures at day 10 of incubation of pancreatic microfragments of one-month-old rabbits under hyperthermic conditions. Inverted microscope. 40×

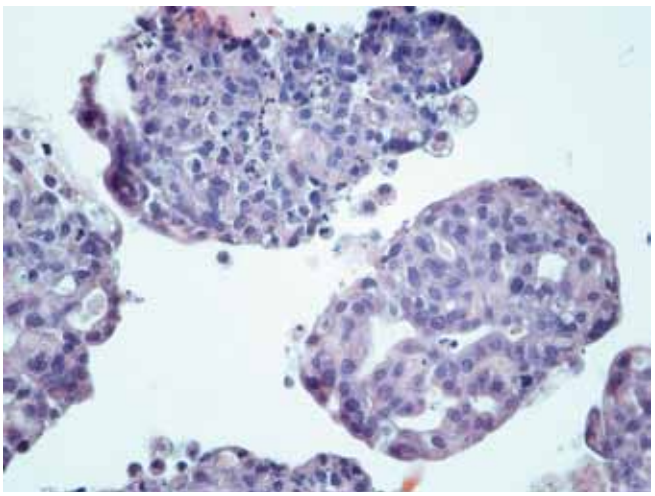

Fig. 9. Floating cultures obtained from the pancreas of one-month-old rabbits, day 8 of incubation under hyperthermic conditions. H&E stain. 400×

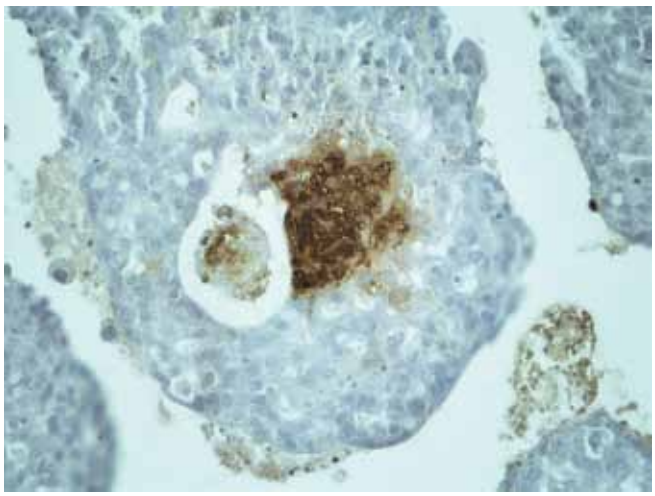

Fig. 10. Floating cultures obtained from the pancreas of one-month-old rabbits, day 8 of incubation under hyperthermic conditions. Immunohistochemical staining with insulin antibodies. 400×

episodes of recurrent pancreatitis or, more often, as a result of extensive pancreonecrosis, significant number of islets die, which leads to a pronounced deficit of insulin-producing β-cells, development of insulin deficiency and, naturally, to the clinical manifestation of insulin-dependent diabetes mellitus.

Results obtained in this study suggest that the use of exogenous enzymes for the treatment of donor pancreas tissue in the process of obtaining cultures consisting mainly of endocrine (islet) cells can be abandoned. Numerous observations using an inverted microscope showed that under standard conditions of incubation of the microfragments of mechanically crushed pancreas, its exocrine tissue dies spontaneously. This destructive process seems to be caused by autolysis of acinar cells under the influence of digestive enzymes contained in them during normothermic cultivation. The resulting detritus is removed in a timely manner during the next replacement of the culture medium. At the same time, naturally protected islets are not significantly damaged, which is due to the presence of islet-surrounding basement membranes consisting of various extracellular matrix proteins [22, 23]. The basement membrane serves as a peculiar interface between islets, endothelial cells and acinar cells through integrins and other cell receptors [24, 25], forming a protective barrier that ensures morphological integrity of islets during non-intensive self-digestion of the pancreas. As a result, the endocrine (islet) tissue seems to spontaneously cleanse itself from the, unnecessary ballast but highly immunogenic, exocrine tissue, while preserving the extracellular matrix necessary for survival and functioning of islet cells, represented, in particular, by the periosteal membrane mentioned above.

The most rapid process of getting rid of exocrine tissue occurs at the standard incubation temperature (37 °C) of microfragments of newborn rabbits. However, this mode turned out to be unsuitable for the pancreas of young (one-month-old) animals, which is due to the presence of a much higher percentage of exocrine tissue. It was decided to apply non-standard hyperthermic (38 °C) incubation for the first time in order to intensify autolytic processes. As expected, under the new temperature conditions, there was a significantly greater loss of exocrine tissue contained in the crushed pancreatic microfragments already at day 5–7, as well as their gradual compaction and scalding. These processes led to the formation of FICs similar to those we obtained from the pancreas of newborn rabbits.

Therefore, the rational methodological approaches developed in this study make it possible to obtain islet-like cultures purified of exocrine tissue without the use of the expensive and ambiguously effective enzyme preparations. In our opinion, further experiments on modification of the enzyme-free method of obtaining islet cell cultures from adult rabbits will provide data that can be extrapolated to develop a more rational and productive method of pancreatic islet isolation from deceased human donors.

The authors declare no conflict of interest.

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