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INTRASPLENIC IMPLANTATION OF TISSUE-ENGINEERED PANCREATIC CONSTRUCT IN EXPERIMENTAL DIABETIC RATS

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Objective: to study the effect of intrasplenic implantation of a tissue-engineered pancreatic construct (TEPC) on experimental diabetes mellitus. **Materials and methods.** Floating islet-like cultures (FICs) were obtained from the pancreas of newborn rabbits. To form TEPC, FICs were incubated with biopolymer microheterogeneous collagen-containing hydrogel (BMCH). TEPC samples were injected into the splenic pulp of rats with streptozotocin-induced diabetes. **Results.** TEPC with insulin-producing activity was formed on the 7–10th day of incubation of FICs with BMCH. After TEPC implantation in recipient rats, persistent decrease in hyperglycemia and disappearance of clinical signs of diabetes were noted. Histological analysis revealed the presence of groups of islet cells without signs of immune cell response at the TEPC implantation site. **Conclusion.** Our findings indicate that xenogeneic islet cells that were part of the TEPC of the pancreas can survive and actively function after implantation in the splenic pulp of diabetic rat.

Keywords: pancreas, newborn rabbits, floating islet-like cultures, biopolymer microheterogenous collagen-containing hydrogel, tissue-engineered construct, rats, streptozotocin-induced diabetes, splenic implantation, glycemia.

INTRODUCTION

According to the International Diabetes Federation (IDF), 415 million people worldwide have diabetes. This number is predicted to rise to 642 million by 2040 (http://www.diabetesatlas.org). Sources estimate that by the year 2050, as many as one in three adults in the US could have diabetes. The treatment of patients with type 1 diabetes (T1D), who constitute about 10% of all people with diabetes, has fundamentally changed over the past century – daily insulin injection remains the only way to save life. Moreover, the use of intensive insulin therapy with various synthesized hormone drugs cannot protect against development and steady progress of late diabetic complications, such as retinopathy, nephropathy and neuropathy, which are the main causes of disability and premature death in diabetic patients [1, 2]. A natural direction in medicine is the development of methods capable of compensating for β-cells absent in T1D (due to autoimmune destruction) in the pancreatic islets by administering donor islet cells, whose active function, in addition to reducing the need for insulin drugs (up to complete withdrawal for a certain period of time), can lead to regression of diabetic angiopathies. However, islet allotransplantation, which is considered the most effective substitution treatment method, can hardly be called a promising option because of the insurmountable shortage of islet sources – pancreas donated after death. Moreover, in order to attain insulin independence, it is usually necessary to use from two to four donor organs [3], since a significant number of islets are damaged during multi-stage isolation from exocrine pancreatic tissue, as well as in the first 3 days after infusion into the portal vein, during which up to 60% of the transplanted islets are lost. The causes of such losses are, first of all, inflammatory reaction at implantation sites and lack of adequate vascularization. In addition, intraportal administration remains an unsafe infusion method and requires limiting the injected cellular material mass due to the risk of massive portal vein embolization. In this regard, it is expedient to search for safer ways of introducing islets (islet cells), provided there is a favorable microenvironment and sufficient blood supply to the implant. In this connection, there are some hope on creation of a tissue-engineered pancreatic construct (TEPC), which can solve the above problems to some extent.

Earlier, we published some materials on TEPC experimental model [4] and the outcomes of its intraperitoneal implantation. In the present study, we have investigated the effect of intrasplenic implantation of TEPC on experimental diabetes mellitus in rats. Observation of animal recipients was limited to 4 weeks, since persistence of transplant functioning signs during this period allows us to consider its persistent engraftment in the recipient's body to be a fait accompli. The choice of this administration option was primarily due to the possibility of de-

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termining the fate of the introduced TEPC by histologic examination of the implantation site (spleen pulp).

MATERIALS AND METHODS

Obtaining tissue-engineered pancreatic construct

Donor animals (1–3-day-old newborn rabbits) were brought from a specialized nursery owned by the Research Center for Biomedical Technologies, Federal Medical and Biological Agency. TEPC was prepared by joint incubation of floating islet-like cultures (FICs) obtained from the pancreas of newborn rabbits using the method developed by us [4] and through biopolymer microheterogeneous collagen-containing hydrogel (BMCH). Home-made *Sfero*®GEL [5] was used as BMCH.

Formation of tissue-engineered construct was monitored using a Nikon Eclipse TS100 inverted microscope by almost daily monitoring. Significant changes were recorded using a digital camera.

A culture scraper was used to collect TEPC immediately prior to implantation.

Preparation of animals with experimental diabetes mellitus

Male Wistar rats weighing 200–240 g body weight were brought from a laboratory animal nursery owned by Manikhino Experimental Production Facility. Experimental T1D was induced by fractional administration of streptozotocin (70 mg/kg – 12 mg/kg for 5 consecutive days), which, according to our data [6], ensures a stable diabetic status. All manipulations with animals were carried out according to the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123) Strasbourg, 1986. Postprandial glycemia in rat capillary blood was determined using a One Touch Ultra glucometer (Life Scan Johnson & Johnson, USA).

Of 16 rats with streptozotocin-induced diabetes, two groups were formed: Group 1 (control) – 8 rats that did not undergo any treatment, and Group 2 (experimental) – 8 rats that underwent TEPC intrasplenic implantation.

TEPC implantation technique

Each of the Group 2 rats was injected with a TEPC sample. The tissue component contained FICs obtained from 10 pancreas of newborn rabbits.

A TEPC sample was injected into the experimental anesthetized animals (Zoletil, intraperitoneally at a dose of 20 mg per 1 kg of body weight) as follows. After midline laparotomy, the spleen was gently removed into the surgical wound area and placed on a sterile gauze napkin. Using TEPC silicone catheter in the form of a cellular gel composition suspended in 1.0–1.5 ml of medium 199, it was taken ex tempore into a 2 mL syringe. After disconnecting the catheter, an injection needle with a diameter of at least 1 mm was put in its place. The needle was gently pierced into the spleen surface, and a TEPC suspension was slowly injected into the pulp of the organ. To stop bleeding and prevent release of introduced cells, the injection site was tightly closed with a sterile gauze swab for 2–3 minutes. The spleen with the implant was gently returned to the peritoneal cavity. Then the abdominal wall was sutured in layers and the surgical wound was treated with iodine solution.

Histological examination

Control examination of paraffin slices of FICs samples was performed by haematoxylin and eosin staining, as well as by immunohistochemical and immunofluorescence staining with insulin antibodies.

Excised spleen fragments of recipient rats, presumably corresponding to the TEPC injection site, were fixed in formalin. After appropriate treatment of this material and preparation of paraffin blocks, the slices were prepared, which were stained with classic dyes (hematoxylin and eosin), as well as with Mallory's trichrome stain.

RESULTS AND DISCUSSION

TEPC formation

Data from morphological examination of FICs as a tissue component of TEPC showed good morphological integrity with the presence of hormone-secreting beta cells (Fig. 1–3).

After 8–10 days of formation of FICs, they were incubated with biomatrix (BMCH). During co-incubation, the FICs settled to the bottom of a culture vial evenly coated with the biomatrix. Contact with the latter had a beneficial effect on the cultures. They were successfully attached to the BMCH (Fig. 4) with subsequent formation of single-layer growth zones around the FICs

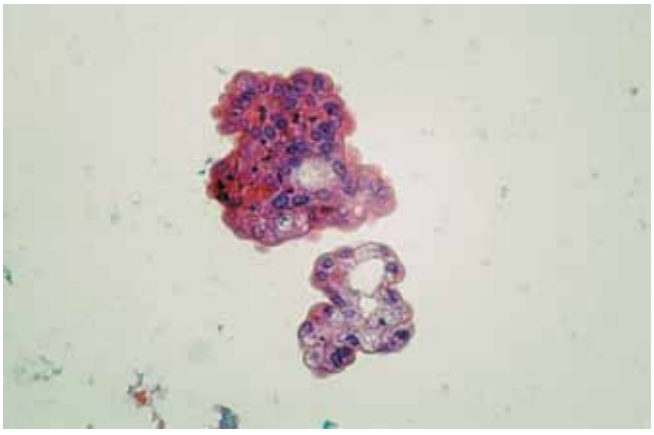

Fig. 1. Floating islet-like cultures, obtained from the pancreas of newborn rabbits, 10th day of incubation. Hematoxylin and eosin staining. ×200

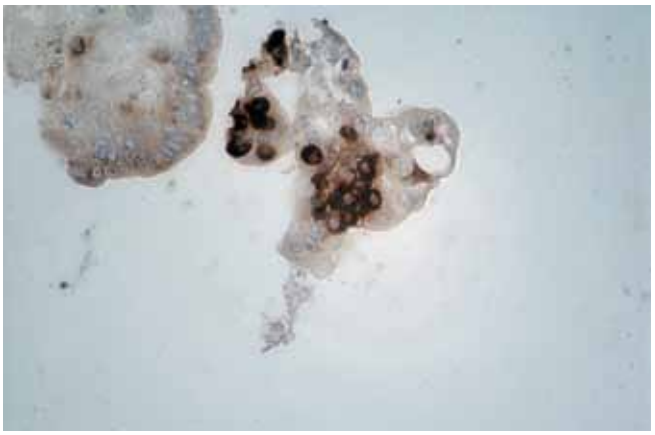

Fig. 2. Floating islet-like cultures, obtained from the pancreas of newborn rabbits, 10th day of incubation. Immunohistochemical staining with insulin antibodies. ×200

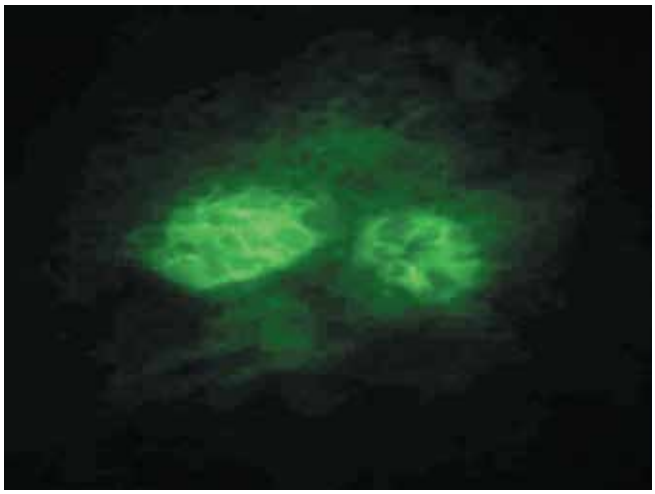

Fig. 3. Glow of insulin granules in floating islet-like pancreatic cultures. Immunofluorescence staining with insulin antibodies. ×200

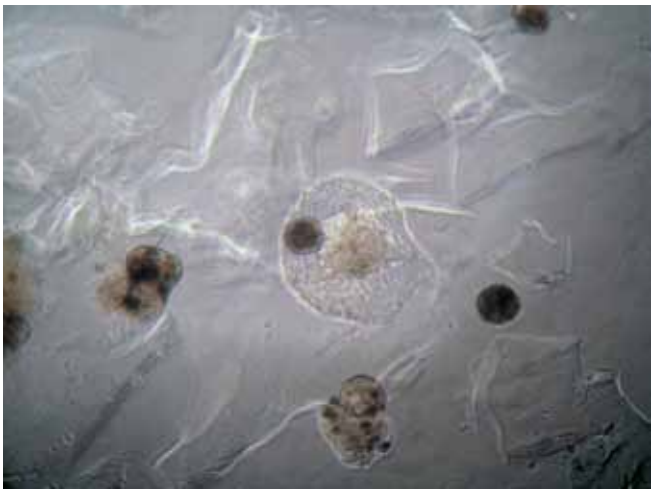

Fig. 4. Attachment of flotation islet-like cultures to matrix. The inverted microscope. ×40

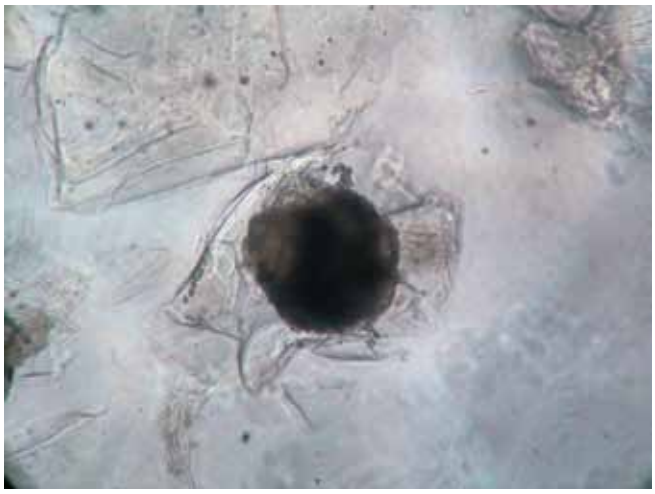

Fig. 5. Formation of pancreatic tissue engineering construct. The inverted microscope. ×100

adhesion site, which indicated good matrix properties of BMCH relative to FICs. Co-cultivation of FICs and biomatrix *in vitro* resulted in TEPC formation (Fig. 5).

Animal condition and blood sugar test results

In group 1 (control diabetes), 2 weeks after streptozotocin administration, all 8 animals showed characteristic clinical signs of diabetes: weight loss, polydipsia, polyuria, lethargy, hair loss and hair yellowing. Such pronounced diabetic status corresponded to high blood sugar levels – from 22.5 to 32.4 mmol/L (Table 1). None of the rats in this group showed a tendency to significantly reduced hyperglycemia, which testified to the reliability of the T1D model used in this study. At the same time, two rats (#17 and #24) with the highest levels of hyperglycemia during the observation died on the background of extreme exhaustion.

In Group 2 (experimental), the pre-transplan blood sugar level 2 weeks after the last streptozotocin injection was almost the same as in the control diabetic animals at the same time (Table 2). However, a week after TEPC implantation, there was a clear tendency toward decreased hyperglycemia. A week later, the average blood sugar level in the recipient rats was significantly reduced ($p < 0.05$), dropping to 17.1 mmol/L. During this period, three animals with relatively moderate reduction in hyperglycemia (#13, #19 and #21) were euthanized to histologically examine the spleen fragment, where the TEPC sample was supposedly injected. The remaining recipient rats were followed up for 4 weeks after implantation. Although none of the experimental rats had normalized blood glucose concentrations, glycemia did not return to its high pre-transplant levels. Moreover, in all Group 2 animals, the pronounced clinical signs of diabetes practically disappeared, a slow but steady in-

Table 1

Changes in blood sugar levels (mmol/L) in control group rats (#1) (streptozotocin-induced diabetes without treatment)

S/n of rat	Weeks before (–) and after (+) implantation in Group 2						
	–2	–1	**0**	+1	+2	+3	+4
2	25.1	23.3	**22.0**	24.5	23.9	22.5	25.4
5	26.2	24.4	**26.3**	25.1	24.8	25.0	25.4
6	22.5	23.7	**24.1**	23.2	23.4	22.8	23.6
9	26.9	27.4	**26.0**	28.5	27.9	27.5	26.4
17	32.4	>33.3	**>33.3**	death			
23	27.7	30.0	**28.7**	27.3	28.1	25.5	26.4
24	31.8	32.5	**›33.3**	>33.3	>33.3	death	
29	28.1	28.5	**26.1**	27.2	27.9	27.5	28.4
M	**27.6**	**27.1**	**25.9**	**25.8**	**25.4**	**25.5**	**26.2**

Table 2

Changes in blood sugar levels (mmol/L) in Group 2 rats after implantation of tissue-engineered pancreatic construct into spleen pulp

S/n of rat	Weeks before (–) and after (+) implantation						
	–2	–1	**0**	+1	+2	+3	+4
3	25.2	23.9	**24.3**	19.8	16.4	17.7	14.0
4	29.6	25.8	**25.7**	19.2	15.3	13.9	14. 1
11	20.3	21.6	**22.0**	19.9	12.8	14.9	12.5
13	26.9	23.2	**26.5**	24.9	18.4	euth.	
19	27.7	23.1	**26.0**	21.4	16.1	euth.	
20	24.4	28.6	**31.1**	26.6	14.7	15.8	16.4
21	25.3	24.7	**27.2**	22.1	21.9	euth.	
28	33.1	32.2	**31.1**	28.2	21.1	21.4	21.0
M	**26.5**	**24.9**	**25.5**	**22.8**	**17.1**	**16.7**	**15.6**

crease in body weight began, including in rat #28, which had the highest glycemia level before transplantation (31.1 mmol/L) Apparently, a 1/3 decrease in blood glucose concentration allowed the animal to adapt to daily relatively high glycemia and maintain viability. Clinical remission of the diabetic status in Group 2 animals remained until the end of post-implantation observation.

Data from histological examination of the spleen of recipient rats

Results from morphological examination of the spleen of recipient rats were of particular interest, as it was possible to determine to some extent the fate of the TEPC implanted in rats with experimental T1D. At the same time, 3 rats from Group the 2 were first euthanized for subsequent histological examination of the spleen. This was to first identify the post-implantation state of TEPC in the short-term period after administration (2 weeks). In one of these rats (#21), in which hyperglycemia decreased slightly (see Table 2), implant-like structures were not detected, but signs of spleen pulp trauma in the form of spleen pulp ruptures were revealed. Apparently, during administration of the TEPC-containing suspension through the ruptures made by the injection needle, a part of the implant went beyond the spleen and got into adverse conditions. This did not allow the introduced islet cells to manifest themselves sufficiently.

In rat #19 with more pronounced hyperglycemia reduction, structural formations were identified, which can be attributed to fragments of the implanted TEPC (Fig. 6), as they contained both epithelial (islet) cell groups and biomatrix residues surrounded by white blood cell groups, which, apparently, were actively involved in BMCH resorption. In rat #21, which was euthanized also 2 weeks after TEPC administration, spleen pulp sections were found with moderate leukocyte infiltration around a group of islet-like structures and with formation of a soft connective tissue capsule (Fig. 9, 10), which may have been formed during resorption of implanted biomatrix residues.

At the end of the experiment – 4 weeks after TEPC implantation – islet-like implants of epithelial cells were found in two recipient rats (#4 and #11) (Fig. 9, 10). In both animals, glycemia levels by the time of euthanasia decreased (almost twice) when compared to pre-implantation levels, in the absence of characteristic clinical manifestations.

It is important to note that there were no histological signs of cellular immune response to cellular xenograft

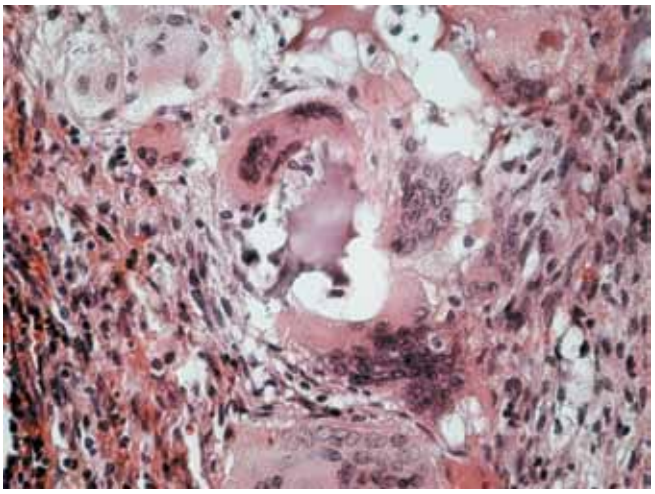

Fig. 6. Spleen from rat #19 two weeks after intrapulpal implantation of TEPC. In the center is a resorbable BMCH fragment with scalloped edges, surrounded by white blood cell groups. ×200

Fig. 7. Spleen from rat #21 two weeks after intrapulpal implantation of TEPC. In the center are groups of islet-like structures and biomatrix residues. Hematoxylin and eosin staining. ×100

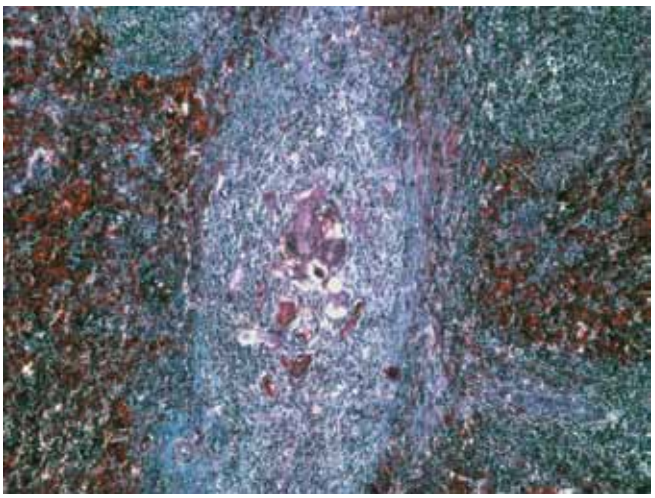

Fig. 8. The same. Mallory's trichrome stain. ×200

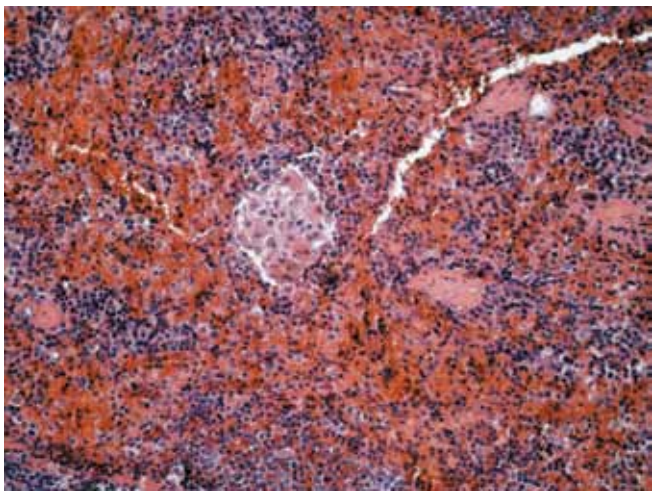

Fig. 9. Spleen from rat #4 four weeks after intrapulpal implantation of TEPC. In the center is an epithelial islet-like structure without signs of destruction and cellular immune response in the absence of BMCH residues. Hematoxylin and eosin staining. ×200

implanted as part of TEPC in the complete absence of biomatrix residues. Apparently, by the end of the 4th week after TEPC implantation in the spleen pulp, BMCH resorption was completely completed and the accompanying leukocyte reaction disappeared. It is possible that the biomatrix that remained for a certain time, to some extent, switched the immune response of the spleen to itself and allowed xenogenic islet-like structures to survive and function for a long time in the body of a foreign recipient. At the same time, islet cell cultures that were part of TEPC themselves, as was shown earlier, have significantly reduced immunogenicity [7].

Thus, experiments on intra-splenic implantation of a TEPC, consisting of islet-like cultures and biodegradable microheterogenous collagen-containing matrix in rats with streptozotocin-induced diabetes, confirmed the morphological integrity and functional activity of TEPC *in vivo*. However, the hypoglycemic effect of TEPC implantation in the spleen was less pronounced compared to intraperitoneal injection of similar TEPC samples, which we had previously performed [4]. Apparently, this difference can be explained by the fact that when a TEPC sample is implanted into the peritoneal cavity, its entire quantity reaches its destination, which is ensured by additional washing of the syringe and injection needle with the aim of introducing the remnants of the cellular gel suspension. However, when spleen is introduced into the pulp, the quantity of TEPC sample introduced is naturally limited, and attempts to increase the implant volume are fraught with organ ruptures and

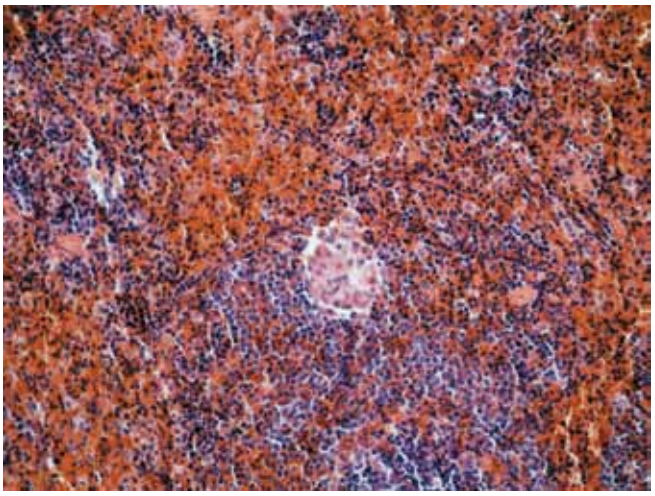

Fig. 10. Spleen from rat #11 four weeks after intrapulpal implantation of TEPC. In the center is an islet-like structure with no signs of destruction and cellular immune response. Hematoxylin and eosin staining. ×200

suspension backflow. Therefore, the mass and functionality of intrasplenic and intraperitoneal implants can vary significantly. At the same time, this study has to some extent achieved its main goal, which could not be achieved with intraperitoneal TEPC administration. If the implant is not found in the peritoneal cavity several weeks before euthanasia, it can be found in the spleen pulp of the rat recipient. Histological analysis conducted showed that two weeks after intrasplenic implantation of TEPC, there was active biomatrix resorption, and two weeks later it was completely dissolved, and the implant was preserved in the form of islet-like structures. Their morphological integrity and absence of signs of cellular immune response made it possible to explain the obtained anti-diabetic effect via the functioning of islet cells that were part of TEPC.

The authors declare no conflict of interest.

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