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Pancreatic islets implanted in an irreversible electroporation generated extracellular matrix in the liver

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Background. Pancreatic islet transplantation via infusion through the portal vein, has become an established clinical treatment for patients with type 1 diabetes. Because the engraftment efficiency is low, new approaches for pancreatic islets implantation are sought. The goal of this study is to explore the possibility that a non-thermal irreversible electroporation (NTIRE) decellularized matrix in the liver could be used as an engraftment site for pancreatic islets.

Materials and methods. Pancreatic islets or saline controls were injected at sites pre-treated with NTIRE in the livers of 7 rats, 16 hours after NTIRE treatment. Seven days after the NTIRE treatment, islet graft function was assessed by detecting insulin and glucagon in the liver with immunohistochemistry.

Results. Pancreatic islets implanted into a NTIRE-treated volume of liver became incorporated into the liver parenchyma and produced insulin and glucagon in 2 of the 7 rat livers. Potential reasons for the failure to observe pancreatic islets in the remaining 5/7 rats may include local inflammatory reaction, graft rejection, low numbers of starting islets, timing of implantation.

Conclusions. This study shows that pancreatic islets can become incorporated and function in an NTIRE-generated extracellular matrix niche, albeit the success rate is low. Advances in the field could be achieved by developing a better understanding of the mechanisms of failure and ways to combat these mechanisms.

Key words: liver cancer; pancreatic islet transplantation; non-thermal irreversible electroporation; tissue engineering; diabetes

Introduction

The experimental work of Ballinger and Lacy was the first to demonstrate the feasibility of using pancreatic islets transplants in the treatment of diabetes.[1] Intraperitoneal and intramuscular injections of pancreatic islets reduced hyperglycemia, polyuria and glycosuria in rats, but consistently normal values were rarely achieved.[1] Kemp *et al.* suggested that since insulin from the pancreatic cells is normally secreted into the portal venous system, the liver may provide a more physiological environment for transplanted islets in intraperitoneal or subcutaneous locations.[2] Indeed, injection of islets into the rat portal vein resulted in normal urine volume, normal glycemia and abolition of glyco-

suria for 2 months after transplantation, suggesting that the liver was an effective site for pancreatic islets transplantation.[2]

Pancreatic islet transplantation via infusion through percutaneous transhepatic access of the portal vein, has since become a clinical treatment for patients with type 1 diabetes or undergoing total pancreatectomy.[3,4,5] Despite major advances in pancreatic islet transplantation via the portal vein, the engraftment efficiency of islets remains low and only about 10–20% of transplanted islets are estimated to survive.[5] Approximately 60% of the transplanted islets are lost in the very early stages of the post-transplantation period.[6] The causes of low islet engraftment and functional durability are multi-factorial. Major contributing factors include early islet death due to failed engraftment and the thrombotic/inflammatory reaction induced within the portal vein.[5] Instant blood-mediated inflammatory reaction triggered by the direct exposure of the islets to blood contributes to the loss of transplanted islets.[7] As the technology to develop stem cell-derived beta-cells and islets mature, demand for islet transplantation is expected to increase and the procedure become more widespread.[8]

Identification of new and improved methods for pancreatic islets implantation has become an important area of research.[9,10] In an attempt to overcome deficiencies related to the injection of pancreatic islets through the portal vein, while retaining the advantages of islet implantation into the liver, Fujita *et al.* reported on a method of direct transplant of pancreatic islets, in the form of an islet-cell-sheet, onto the liver surface.[11] They found that the implanted islet tissue consisted predominantly of insulin-positive beta cells. Glucagon-positive alpha cells were also present, but their numbers were small and they were sparsely distributed within the islet tissue.[11]

Here, we investigate a different approach to transplant pancreatic islets within the liver utilizing the special attributes of non-thermal irreversible electroporation (NTIRE). NTIRE has become a clinically useful modality for cancer treatment in the liver.[12] NTIRE is a minimally-invasive tissue ablation technique in which electric field pulses are delivered across a treatment volume to irreversibly permeabilize the cell membrane. Cell death is induced in the NTIRE-treated volume[13,14] likely through necroptosis and/or pyroptosis pathways.[15,16] The extracellular matrix within the treated volume remains intact and blood vessels remain patent.[14,17] Accordingly, NTIRE has been used to ablate solid tumors near sensitive body structures, such as large blood vessels within the liver[18] and pancreas.[19] Large blood vessels in the NTIRE-treated region remain intact; the blood vessels do not leak and the endothelial layer regenerates.[20] Our earlier research has shown that the extracellular matrix that remains after NTIRE can serve as a scaffold for native cell regeneration in the liver[14], blood vessels[21] intestines.[17] These studies also suggested the use of NTIRE to generate an extracellular matrix for regenerative medicine.[22-24]

This study was motivated by findings from our earlier studies.[25,26] In[25] it was shown that transplanted hepatocytes engraft into host liver parenchyma when directly implanted into an NTIRE pre-treated area. NTIRE improved exogeneous cell engraftment likely by killing host hepatocytes while preserving the extracellular matrix, thereby creating space and a supportive niche for new cell engraftment. Importantly, NTIRE induces a pro-reparative innate immunity milieu, which may further promote integration of transplanted cells.[26] In contrast, an earlier study has shown that in the absence of NTIRE pre-treatment, direct implantation of three-dimensional cell clusters, such as hepatocyte organoids, into the liver, has limited durability and tissue incorporation.[27] In that study, hepatic organoids were directly implanted within a hepatotomy site in the liver of immune competent mice.[27] By day 3 after the implantation, most hepatocytes within organoids were apoptotic or necrotic, and by day 7, chronic inflammatory reactions developed around the implanted organoids.[27]

The goal of this study is to evaluate if a NTIRE decellularized matrix in the liver could serve as a graft site for pancreatic islets directly implanted in the liver and if grafted pancreatic islets could survive and become functional in this type of niche.

Materials and methods

Approvals

Experiments were performed on Sprague Dawley male rats weighing 250–350 g. Seven rats received NTIRE and islet transplants, and 7 rats were used as islet donors. All animals received humane care from properly trained professionals in compliance with both the Principals of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication no. 85-23, revised 1985), and treated according to an animal protocol approved by the Animal Care and Use Committee of the University of California, Berkeley. The

University of California Berkeley Animal Care and Use Committee approved the animal protocol AUP-2017-12-10605.

NTIRE ablation protocol

After partial mobilization of the liver from adjacent tissue, the liver lobe was gently clamped between two 10-mm-diameter electrodes (Harvard Apparatus, Holliston, MA, USA), as shown in Figure 1. The measured distance between the two electrodes is 3.0 ± 0.2 mm. A sequence of 10 square pulse with an electric field of 1000 V/cm, 100 μs pulse width, separated by 100 ms was applied between the electrodes, across the liver, using an electroporator (ECM 830, Wave Electroporation System (BTX Harvard Apparatus, Holliston, MA, USA). Previous studies with the experimental configuration in this study have shown that this electroporation protocol produces minimal thermal damage, and the tissue is affected primarily by irreversible electroporation.[23] Because the electrodes are flat parallel surfaces, the voltage on the electrodes can be set to achieve a uniform electric field through most of the liver volume bound between the surface electrodes, except at the edges. This voltage was adjusted to yield an electric field of 1000 V/cm in the central area of the parallel electrodes. Two different lobes were electroporated in each animal, one lobe for the injection of pancreatic islets and the second lobe for injection of a control of a phosphate buffered solution (PBS). Seven rats were used as recipients in the pancreatic islet transplant experiment.

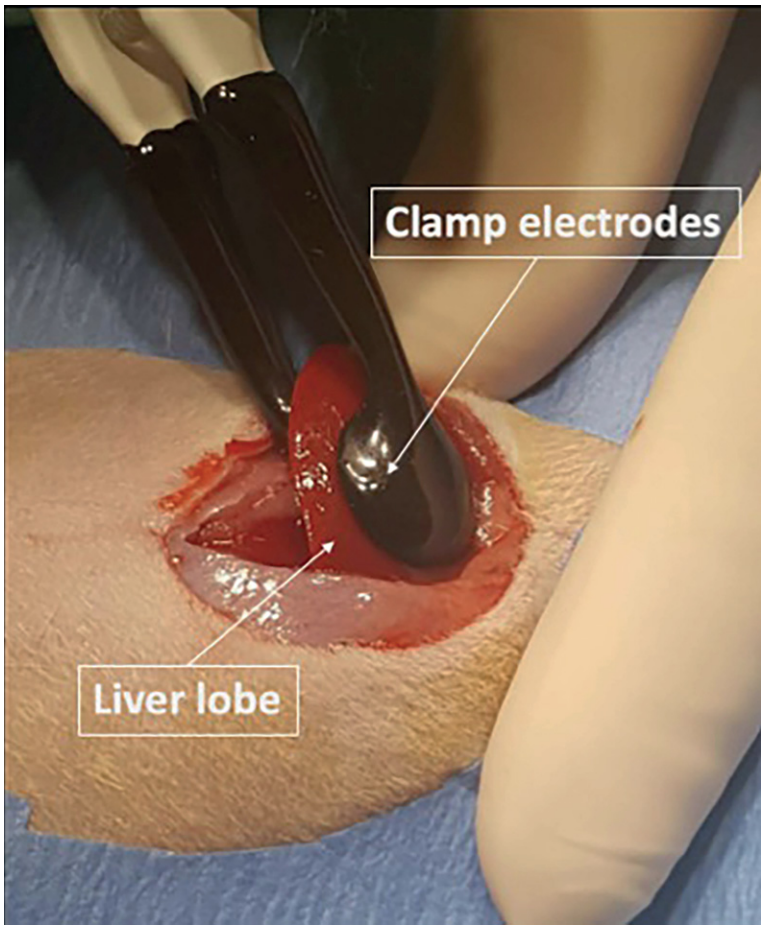

FIGURE 1. Photograph of a clamp electroporation electrode on a liver lobe.

Procurement of pancreatic islets

Pancreatic islets were prepared as described.[28] Rats were initially anesthetized with 3–5% isoflurane by placing them in an induction chamber. No more than 8 mg/kg bupivacaine was injected once subcutaneously prior to skin incision. Once anesthetized, the rat was then transferred to a nose cone respirator connected to a precision vaporizer that delivered 1–3% isoflurane for maintenance. The peritoneal cavity was entered via a midline incision of the abdomen. The common bile duct was cannulated and injected with digestion solution-containing collagenase P (Roche, #11249002001). The pancreas was carefully excised and pulled away from the intestines, stomach, mesentery and spleen, put into a 50 ml centrifuge tube, and immediately incubated in a 37°C water bath for 20 min. The rats were euthanized by a combination of an overdose of vaporized isoflurane and a bilateral chest dissection. Pancreas digestion was then terminated by adding cold washing buffer containing Hanks' Buffered Salt Solution (HBSS, Hyclone SH3058802), 1% Penicillin-Streptomycin (Pen-Strep, Invitrogen, 15070063), 1% 1M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, Invitrogen, 15630080), 0.1% Deoxyribonuclease I(DNase I), 1.7 ml 1 M $CaCl_2$/1000 ml and 1.2 ml 1 M $MgCl_2$/1000 ml. After being washed in washing buffer several times and filtered by a strainer, the digested islets were separated on a Histopaque (Sigma-Aldrich, 11191) density gradient. The islets were then hand-picked and the Histopaque was washed out by a washing buffer. The islets were resuspended in 5 ml culture media and poured into a Petri dish for hand-picking. A few sample fresh islets were stained with dithizone (DTZ) to confirm their purity. Stock solution of DTZ (0.5 mg/ml) was prepared in dimethyl sulfoxide (DMSO). The stock solution was diluted five times in Dulbecco's Phosphate

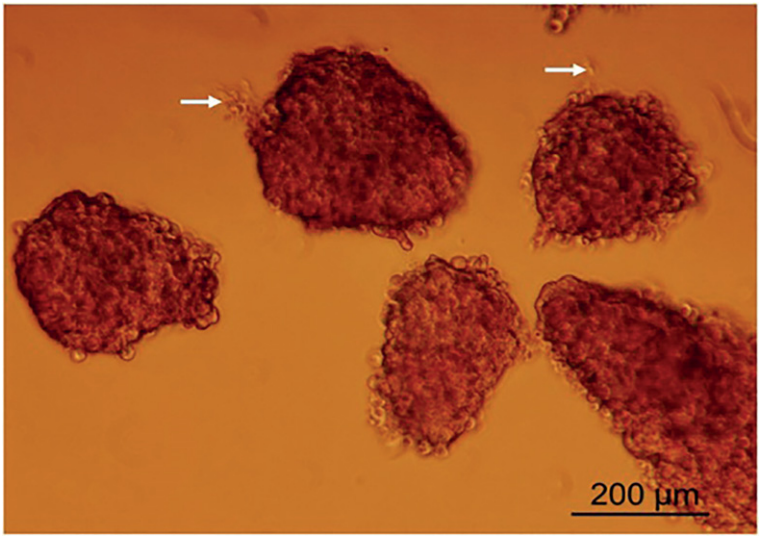

FIGURE 2. Photograph of the pancreatic islets used in this study stained by dithizone (DTZ). Arrows point to the few unstained exocrine cells left after digestion by collagenase.

Buffered Solutions, (DPBS) solution. The morphology of purified islets is shown in Figure 2. The islet cells were scarlet with DTZ staining, while exocrine cells were devoid of staining. Arrows point to the few exocrine cells left after digestion by collagenase. The pancreatic islets were generated in seven rats, with one rat providing the pancreatic islets per transplant recipient.

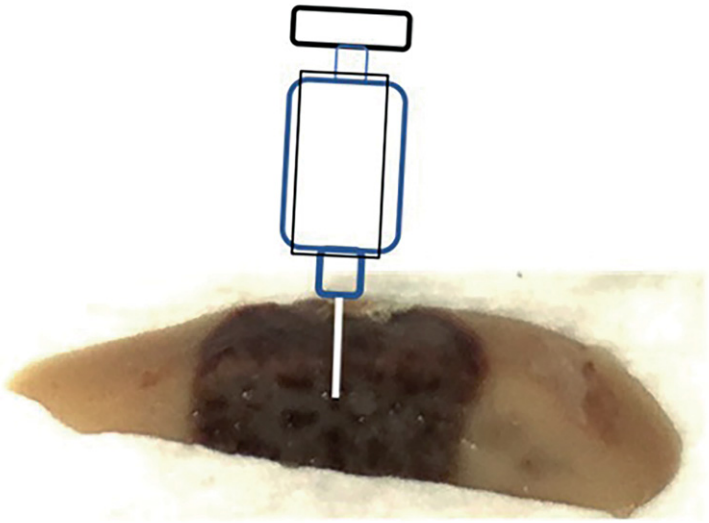

FIGURE 3. An illustration showing the implantation site in the liver. The dark area is the non-thermal irreversible electroporation (NTIRE) treated area.

Implantation of pancreatic islets

Rats pre-treated with NTIRE were anesthetized and the previously made midline incision was re-opened 16 hours later. Approximately 200 pancreatic islets were transplanted into a recipient. Islets were washed and re-suspended in 0.2 ml PBS. Using a 1 ml syringe and 26-gauge needle, islets were injected into the center of one of the IRE-treated liver lobes. The second, control lobe was injected with the same volume of PBS into the IRE- treated region in that lobe. Figure 3 illustrates the site of the injections at the center of the ablated region. At the completion of the pancreatic islet injection procedure, the abdominal wall was closed and sutured. The animals were kept two in a cage, given food and water freely and continuously monitored for well-being by a veterinarian affiliated with the animal care facility. No controls with injection of pancreatic islets in the untreated liver parenchyma was performed. A previous study has shown that in the absence of NTIRE pre-treatment, direct implantation of three-dimensional hepatocytes cell clusters into the liver had limited durability and tissue incorporation.[27] In contrast, transplanted hepatocytes engraft into host liver parenchyma when directly implanted into an NTIRE pre-treated area.[25]

Islet graft analysis

We collected and examined the livers 7 days after the NITRE procedure. Islet graft function was assessed by detecting insulin and glucagon in the liver by immunohistochemistry. The treated liver tissue samples were cut in a plane normal to the liver lobe surface, through the center of the treated lesion. The liver was fixed in formalin, embedded in paraffin, and sectioned 5 µm thick (Histo-Tec Laboratory, Hayward, CA, USA). The samples were sent to UT Health San Antonio STRL Histology/ Immunohistochemistry Laboratory Department of Pathology and Laboratory Medicine where they were stained with guinea pig anti-insulin antibodies and rabbit anti-glucagon antibodies.

Results and discussion

The livers of recipient rats were treated with 1000 V/cm NTIRE in 2 locations. Sixteen hours later, one site was injected with PBS and the other site was injected with approximately 200 donor rat pancreatic islets. We chose to analyze the livers 7 days af-

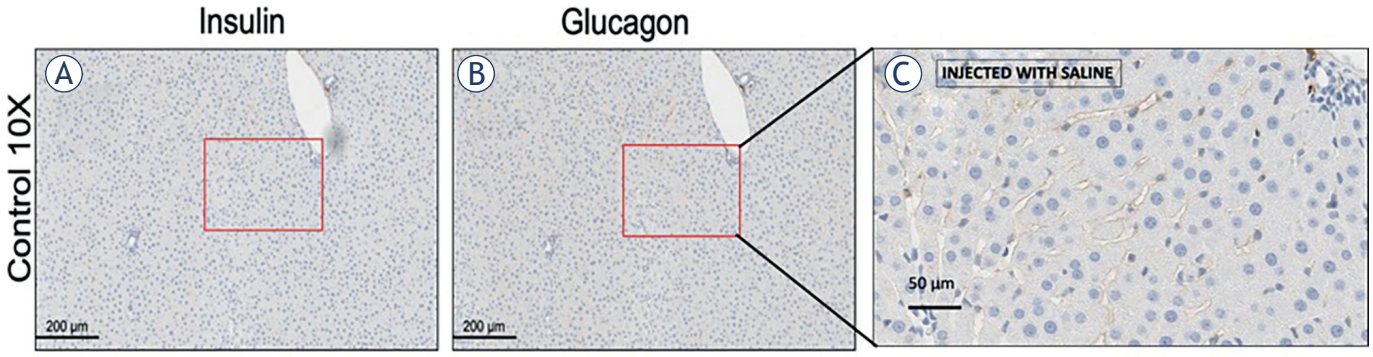

FIGURE 4. Liver tissue treated with non-thermal irreversible electroporation (NTIRE) and injected with PBS served as the control. Seven days after treatment with NTIRE and injection of PBS, immunohistochemical staining was performed on the controls using insulin and glucagon antibodies (University of Texas at Houston, USA). In the control slides, no cells were stained with insulin or glucagon antibodies, shown in **(A)** and **(B)**, respectively. The higher magnification panel 4C shows normal hepatocytes with no evidence of scar tissue.

ter the NTIRE treatment because, in rats, the liver completely regenerates 7 days after injury.[29]

Figure 4 shows the appearance of the NTIRE-treated lobe that was injected with PBS. To show that staining by insulin and glucagon antibodies was specific to the sites injected with pancreatic islets, the sites injected with PBS were stained with insulin or glucagon antibodies, Figure 4 A and B, respectively. The control NTIRE-treated liver regenerated and was not stained by the insulin or glucagon antibodies. Panel 4C is a higher magnification of the previously NTIRE treated region. It shows that 7 days after IRE ablation, the hepatocytes were intact and well-developed. Nuclei were clear, and sinusoids were seen. There was no evidence of scar tissue or fibrosis. This illustrates the regenerative capability of NTIRE-treated livers.

Within the NTIRE-treated liver parenchyma that received islet implantation, we found evidence of engrafted insulin- and glucagon-producing cells in 2 of the 7 animals. Figures 5A and 5B show the presence of engrafted pancreatic islets within the liver as demonstrated by positive insulin and glucagon staining. Insulin-stained cells (beta cells) and glucagon-stained cells (alpha cells) are marked with full arrows in the higher magnification images Fig 5C and 5D, respectively. Fully developed and normal hepatocytes were observed (dotted arrows) around the glucagon- and insulin-positive cells. These findings show that it is possible for pancreatic islets, injected in tissue treated with NTIRE, to become integrated in the liver parenchyma and become functional producing glucagon and insulin.

The fact that 7 days after the implantation the pancreatic islets have become incorporated in the liver parenchyma and generate insulin and glycogen in 2 of the 7 animals, is encouraging. By comparison, in previous experiments with direct injection of hepatocyte organoids in the liver parenchyma, most hepatocytes within implanted organoids were apoptotic or necrotic by day 3 after transplant.[27]

The goal of this study is limited to showing that pancreatic islets can become incorporated in a niche formed by NTIRE treatment of the liver. In that sense, the results are promising. Nevertheless, the incorporation was observed in only 2 of the 7 repeats, which is a low rate of success. It should be emphasized, however, that engraftment efficiency of pancreatic islet transplantation through the portal vein is not optimal, which is why alternative approaches for transplantation are explored.[5] We speculate that there are several reasons for our low rate of success.

An important aspect of the procedure is choosing the optimal time for the exogenous pancreatic islet implantation in the NTIRE treated liver. In view of the limited success in this study, this must be investigated further. In our previous study of implantation of hepatocytes in the liver, the implantation was done 3 days after the NTIRE procedure.[25] In this study, the choice for the time of implantation is based on the results of a previous study on the mechanisms of cell death in the NTIRE-treated liver.[16] That study followed the temporal events in a liver treated with the same NTIRE protocol as in this study. It was observed that the hepatocytes appear morphologically intact one hour after the NTIRE procedure. This suggested that the implantation cannot be done at the same time as the NTIRE ablation, because the hepatocytes still occupied the treated volume. At 6 hours after the procedure, dead hepatocytes are seen

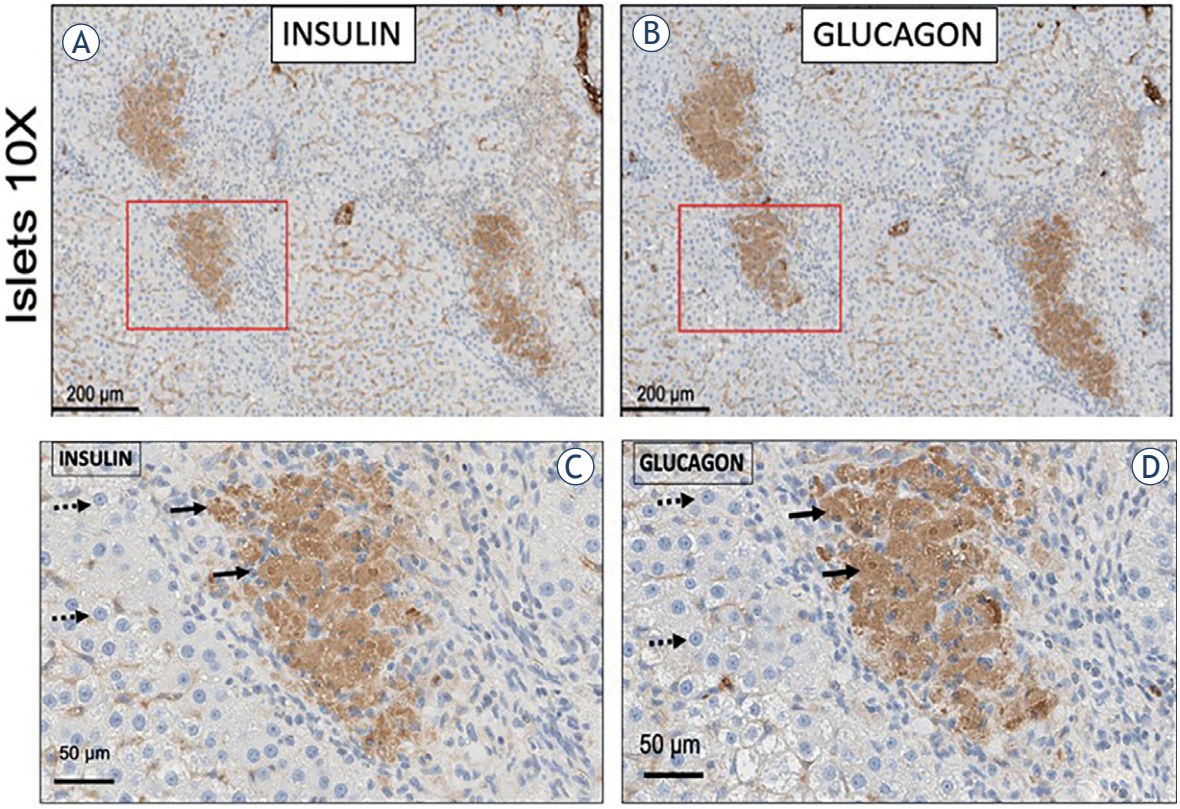

FIGURE 5. Liver tissue treated with non-thermal irreversible electroporation (NTIRE) and injected with 200 rat donor pancreatic islets. Seven days later, immunohistochemical staining was performed with anti-insulin and anti-glucagon antibodies (University of Texas at Houston, USA). In the pancreatic islets injected tissue, insulin-stained cell clusters are shown in **(A)** and **(C)** (full arrows). Normal hepatocytes were observed (dotted arrows) around the insulin-stained cells in the liver lobe. Glucagon-stained cells cluster are shown in **(B)** and **(D)** (full arrows). Normal hepatocytes were observed (dotted arrows) around the glucagon-stained cells in the liver lobe.

throughout the treated volume. By 24 hours after NTIRE, hepatocyte regenerative proliferation may be initiated.[29] On the basis of these considerations, we implanted the pancreatic islets at 16 hours after the NTIRE treatment. Ideally, one would choose a time in which the NTIRE niche is free from dying hepatocytes and from regenerating hepatocytes, so that pancreatic islets have optimized conditions to become incorporated into the extracellular matrix. However, the inflammation and repair responses within of the NTIRE-treated region are also important factors. Our group showed that pro-reparative macrophages start to replace pro-inflammatory macrophages at day 3 after NTIRE treatment and become the dominant macrophage subtype by day 7.[26] Therefore, it is quite possible that 16 hours after the NTIRE treatment is not the optimal time. A later timepoint in which pro-reparative macrophages predominant within the NTIRE-treated volume may be more conducive to islet engraftment. Determining the optimal time for implantation, in context of an immunological response coupled with hepatocyte regeneration, is a critical aspect of this procedure and requires much more research. Finally, islet graft rejection may have had an effect on the limited rate of success. No anti-rejection or immunosuppressive medications were used in this study.

Technical reasons may have also played a role in the results. The rate and mode of injection of the pancreatic islets into the liver parenchyma is important. The injection should be slow, controlled and gradual.[27] In this study, injection with a hand-held syringe may not have been the same in all the repeats. Another technical reason may be the injection of the pancreatic islets along the tract of the 26-gauge needle. As shown in Figure 5, the injection site is relatively narrow, on the order of 200 µm, and therefore, the histology section through the center of the lesion may have missed

the injected islets in some of the repeats. Another technical reason may be the small number of the injected pancreatic islets. In this study, we used 200 pancreatic islets per injection site, and this may have been too low a number.

Obviously, the low rate of success is of concern and should be further investigated.

Conclusions

This study shows that pancreatic islets injected into a niche formed in the liver by NTIRE ablation became incorporated and functional in 2 of 7 experimental animals 7 days after NTIRE treatment. While the rate of success is low, the study demonstrates that pancreatic islets can become incorporated in a tissue niche generated by NTIRE ablation. More research is needed to improve the success rate of this technique before it can have clinical value.

Author contributions

YZ designed and performed the animal experiment and aided in the analysis of the data. YL designed and performed the electroporation. YW advised on the animal experiment and edited the animal experiment section. TC, contributed to the analysis of the results, writing of the paper and with advice. BR supervised the research, designed the experiment, analyzed the data, and wrote the paper.

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