Comparative Study of Two Different Islet Transplantation Sites in Mice: Hepatic Sinus Tract vs Splenic Parenchyma

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Abstract
Although 90% of clinical islet transplantations are performed via the portal vein approach, it is still far from the ideal transplant site. Alternative islet transplant sites are promising to reduce the islet dose required to reverse hyperglycemia, thereby improving the efficiency of islet transplantation. The aim of this study was to compare the differences in survival and metabolic function of islet grafts transplanted into the hepatic sinus tract (HST) and the splenic parenchyma (SP). Approximately 300 syngeneic mouse islets were transplanted into the HST (n = 6) and the SP (n = 6) of recipient diabetic mice, respectively. After transplantation, the glycemic control, glucose tolerance, and morphology of islet grafts were evaluated and compared in each group. The nonfasting blood glucose of the two groups of mice receiving islet transplantation gradually decreased to the normal range and sustained for more than 100 d. There is no significant difference in the time required to restore normoglycemia (P > 0.05). The results of the glucose tolerance test showed that the SP group presented a smaller area under the curve than the HST group (P < 0.05). Histopathological results showed that islet grafts in the HST and the SP were characterized with normal islet morphology and robust insulin production. Compared with the HST, islet transplantation in the SP presents better blood glucose regulation, although there is no significant difference in the time required to restore normoglycemia.

Keywords
islet transplantation, diabetes, transplantation site, hepatic sinus tract, spleen

Introduction
Currently, diabetes has become a worldwide public health problem that seriously threatens human health and imposes substantial economic burden on society. According to World Health Organization statistics¹, there are currently approximately 425 million diabetic patients worldwide, and it is estimated to increase further to 629 million by 2045. Islet transplantation is currently considered to be an effective method for the treatment of type 1 diabetes and certain insulin-dependent type 2 diabetes. Islet transplantation sites reported by literature mainly include the portal vein², subrenal capsule³, splenic parenchyma (SP)⁴,⁵, omentum⁶, muscle⁷,⁸, subcutaneous⁹,¹⁰, gastrointestinal tract¹¹, bone marrow¹², and so on. These transplantation sites each have their own characteristics and show more or less advantages, but the optimal islet transplantation site is still controversial. The search for the ideal transplantation site has never stopped, because a more optimized transplantation site is expected to significantly improve the efficiency of islet transplantation and make limited organ resources benefit more patients. In the previous study¹³, our team successfully established a model of islet transplantation in the hepatic sinus tract (HST), and achieved a therapeutic effect comparable to that of subrenal islet transplantation. Recent studies have reported that the spleen is an ideal islet transplantation site due to its reduced inflammation and expansion of the islet graft¹⁴,¹⁵. However, we have no idea of which islet transplantation site is better between the HST and SP.
Therefore, it is necessary to conduct a direct comparative study of the two clinical promising islet transplantation sites.

**Materials and Methods**

**Animals**

Specific pathogen-free (SPF) grade, male C57bl/6 mice, weighing 20 to 25 g, were purchased from the Experimental Animal Resource Center of Liaoning Province and used as recipients and donors. Recipient mice were randomly divided into four groups and exposed to different treatments. Schematic representation of the experimental protocol is available in the Supplemental Fig. 1. All animals were raised under SPF conditions. All experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of China Medical University (No. 2019014).

**Establishment of Diabetes Model**

The mice were intraperitoneally injected with Streptozotocin (Sigma-Aldrich, Shanghai, China) at 180 mg/kg 1 wk before transplantation. Then, mice were considered to be diabetic when their nonfasting blood glucose levels reached at least 19.4 mmol/l for two consecutive daily readings. Mice with nonfasting blood glucose in the range of 20 to 30 mmol/l were screened for subsequent studies.

**Isolation and Purification of Islets**

Isolation and purification of islets were performed according to the previous method\(^{13}\). Briefly, mice were sacrificed by cervical dislocation and then immersed in 75\% alcohol to sterilize. Then V-shaped laparotomy was performed, and the end of the common bile duct was ligated with 6-0 silk thread. Thereafter, collagenase V (1 mg/ml) (Sigma-Aldrich) was injected through the common bile duct by a 5-ml syringe connected to a 31G needle. After the pancreas was fully swelled, the pancreas was completely removed and placed in a 37°C water bath for 10 to 20 min. Immediately after the digestion was completed, precooled Hank’s solution (Beyotime, Shanghai, China) containing 10\% fetal bovine serum (Clark, China) was added to discontinue digestion. After filtering digested pancreas through a 30-mesh filter, the islets were washed twice and then purified by Ficoll (Sigma-Aldrich) density gradient centrifugation. About 300 islets were hand-picked and cultured in the precooled 199 medium (Gibco, USA) for subsequent experiments.

**Dithizone Staining**

The islet suspension was transferred to a 15-ml centrifuge tube for centrifugation (300 × g for 3 min), and the supernatant was discarded after centrifugation. Then, the pellet was resuspended and transferred to a six-well plate with 2 ml Hank’s solution (Beyotime). About 20 μl of dithizone staining solution (0.67 mg/ml) (Solarbio, Beijing, China) was added in each well and incubated in dark for 10 min at room temperature, and then images were captured under an inverted microscope (Nikon, Japan).

**AO-EB Staining**

The collected islets were transferred to the six-well plate as previously described. Then, the prepared AO-EB dyeing working solution (Solarbio) was added according to the instructions. After incubating at room temperature for 2 min,
the photos were recorded after excitation with 490 and 510 nm fluorescence. The living cells emitted green fluorescence, while the dead cells emitted red fluorescence. After capturing images, the islet viability was analyzed and calculated by Image J software.

**Islet Transplantation**

The islet suspension was centrifuged, resuspended with a small amount of 199 medium (Gibco), transferred to a PE50 tube, and kept on ice for subsequent experiments.

Procedures of islet transplantation in the SP group: The mice were anesthetized with continuous isoflurane (Yimeining, Shandong, China) inhalation through an animal anesthesia machine (MIDMARK, USA) and fixed in a lateral position. The spleen was exposed through the left inferior costal incision, and the prepared islet suspension was slowly pushed into the SP with a PE50 tube from the lower pole of the spleen. After the injection was completed, the spleen tail was ligated with 3-0 silk (ETHICON, Shanghai, China) circularly, and the abdomen was closed when no active bleeding was seen. Tramadol hydrochloride (30 mg/kg) (Qimaite, Hebei, China) and cefazolin sodium (90 mg/kg) (Lukang, Shandong, China) were given subcutaneously in the first 3 d after the operation.

Procedures for islet transplantation in the HST group: According to the previous method, the HST was created by temporarily placing a nylon material in the liver parenchyma for 4 wk, then the prepared islet suspension was slowly injected into the HST with a micro-syringe (Gaoge, Shanghai, China). After injection, the liver capsule near the entrance of the HST was sutured with 11-0 suture (Chenghe, Hebei, China) and cefazolin sodium (50 mg/kg) (Lukang, Shandong, China) were given subcutaneously in the first 3 d after the operation.

As shown in Fig. 3A, the initial blood glucose levels of the mice in the HST group was comparable (15.0 ± 3.1, 25.0 ± 3.1, and 26.1 ± 2.4 vs 25.2 ± 3.2 mmol/l, respectively). There was no significant difference among these three groups (P > 0.05). Furthermore, the mean time required to restore normoglycemia for the islets in the HST and SP groups gradually decreased to below 11.1 mmol/l. The AUC of the two groups was 505.3 ± 53.2 and 508.7 ± 37.5 mmol/l, respectively (Fig. 3B). There was no significant difference between the two groups (P > 0.05). Furthermore, the mean time required to restore normoglycemia for mice in the HST group was comparable (15.0 ± 2.4 d vs 14.5 ± 3.1 d, P > 0.05) with the SP group (Fig. 3C).
Glucose Tolerance Test

The IPGTT was performed on the 60th day after transplantation. Both the mice in the HST (n = 6) and the SP (n = 6) groups showed pretty glucose tolerance curves (Fig. 4A), but the AUC in the SP group (1143.0 ± 55.3 mmol/l/120 min) was smaller than the HST group (1355.3 ± 156.6 mmol/l/120 min, P < 0.05, Fig. 4B). Actually, the SP group displayed a similar glucose tolerance pattern to the Naive group (1143.0 ± 55.3 mmol/l/120 min vs 1042.0 ± 21.9 mmol/l/120 min, P > 0.05). In contrast, the mice in the DM group were intolerant to glucose stimulation, and the AUC was 3250.7 ± 128.5 mmol/l/120 min.

Long-Term Islet Graft Retrieval

The results of long-term observation of the islet graft function are shown in Fig. 5. Both the mice in the HST (n = 4) and SP (n = 4) groups sustained normoglycemia for more than 100 d. After the grafts were completely excised on day 120 after transplantation, the blood glucose levels in both groups returned to the pretransplant levels in a week.

Histopathological Staining of the Islet Grafts

On the 120th day after islet transplantation, the left lobe of the liver where the implanted graft was located was completely excised, and the sections of the islet graft were stained with H&E (Fig. 6A). In the normal form of hepatocytes and hepatic sinusoids, no obvious inflammatory cells infiltration was seen in or around the islet graft. Similarly, the sections of the spleen were stained with H&E (Fig. 6C). It was found that the surviving islet cell clusters were located in the parenchyma of the spleen. There was no significant inflammatory cell infiltration within the grafts. Immunohistochemical staining of the islet graft sections for insulin in both groups (Fig. 6B,D) showed that insulin was expressed uniformly in the cytoplasm of the islet cell mass.
Discussion

Since the Edmonton protocol proposed in 2000\(^1\), more than 1,000 patients worldwide have been treated with islet transplantation.\(^{17}\) Currently, 90% of islet transplantations are clinically performed through the portal vein, but a large amount of literature indicates many defects associated with this approach, including instant blood-mediated inflammatory reaction,\(^{20,21}\) the risk of hemorrhage, embolism,\(^{22,23}\) and the hepatic tissue necrosis.\(^{24}\) Besides, due to the scattered distribution of islets, graft biopsy is rarely performed,\(^ {11}\) which is not conducive to the early detection of graft rejection. At present, it is believed that an ideal transplantation site should have convenient operation, less complications, lower risk, repeatable operation and biopsy, rich blood flow, and relatively small rejection.\(^ {25}\) Obviously, the portal vein route is not an ideal site for islet transplantation.

Due to its abundant blood supply, draining into the portal vein, spleen may become a potential transplantation site. Previous studies have suggested that the graft is more likely to be rejected by the recipient when it directly contacts with lymphoid tissue or organs such as spleen, and on the other hand, no special advantages of islet grafts in the SP over the liver were found.\(^ {26,27}\) However, recent studies\(^ {14,15}\) have found that intrasplenic islet transplantation requires less marginal islet mass (50 syngeneic islets) than the portal vein and subrenal capsule route, suggesting that the spleen may have a better survival environment for islet grafts. In other words, the efficiency of intrasplenic islet transplantation remains controversial.\(^ {28-30}\)

The purpose of this experiment is to compare the effects of syngeneic islet transplantation into the HST and the SP, thus providing a reference for the selection of laboratory and clinical islet transplantation sites. It can be seen that glycemic control and graft morphology, including the immunohistochemical staining, showed similar results in the HST and the SP groups. However, in terms of glucose tolerance test, the islet grafts in the SP group showed better blood glucose regulation than the HST group. The reason for this difference may be due to the characteristics of SP. First, the spleen favors the expansion of the islet graft.\(^ {14}\) Secondly, the spleen acts as a stem cell reservoir, which accelerates the repair of the damage caused by the transplantation procedure.\(^ {15}\) Furthermore, it has been reported that these splenic mesenchymal stem cells may differentiate directly into islets.\(^ {31}\)

Although intrasplenic islet transplantation achieved improved results, we still cannot ignore its inherent defects, such as postoperative spleen infarction\(^ {5,32}\) and bleeding. Frankly, no perfect islet transplantation site has been found so far. But it is believed that spleen may prove to be superior than other sites when further researches were done. Continuous exploration of the ideal islet transplantation site will help clarify the necessary conditions for the survival of islet...
grafts, and obtain new ideas for improving the transplantation site.

**Conclusion**

Compared with the HST, islet transplantation in the SP presents better blood glucose regulation, although there is no significant difference in the time required to restore normoglycemia.

**Author Contributions**

FL participated in the research design, performance of the research, data analysis, and writing of the manuscript. YL, ZY, and TG participated in the performance of the research and writing of the manuscript. XL and JZ participated in research design and writing of the manuscript.

**Ethical Approval**

This study was approved by the IACUC of China Medical University, Liaoning Province, China (No. 2019014).

**Statement of Human and Animal Rights**

All experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of China Medical University, China. No human rights were involved in this study.

**Statement of Informed Consent**

There are no human subjects in this article and informed consent is not applicable.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Fig. 6.** Histopathological staining of the islet grafts transplanted into the HST or the SP, 120 d posttransplantation. (A) H&E staining of the horizontal section of the left lobe of the liver revealed that the islet grafts in the HST were surrounded by normal liver cells and hepatic blood sinuses, with no obvious inflammatory cell infiltration. (C) H&E staining of horizontal section of the spleen showed that the islet graft was located in the splenic parenchyma, and no inflammatory cells infiltrated into the islet graft. (B, D) Immunohistochemical staining of the islet graft sections for insulin in both groups showed that insulin was expressed uniformly in the cytoplasm of the islet cell mass. Scale bars, 100 μm. H&E: hematoxylin and eosin; HST: hepatic sinus tract; SP: splenic parenchyma.
Supplemental Material
Supplemental material for this article is available online.

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