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Minimizing Post-Infusion Portal Vein Bleeding during Intrahepatic Islet Transplantation in Mice

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TITLE:

Minimizing Post-Infusion Portal Vein Bleeding during Intrahepatic Islet Transplantation in Mice

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SUMMARY:

Here we present refined surgical procedures on successfully performing intraportal islet transplantation, a clinically relevant but technically challenging surgical procedure, in mice.

ABSTRACT:

Although the liver is currently accepted as the primary transplantation site for human islets in clinical settings, researchers in most rodent preclinical islet transplantation studies transplanted islets under the kidney capsule. This model is commonly used because murine intrahepatic islet transplantation is technically challenging, and a high percentage of mice could die from surgical complications, especially bleeding from the injection site post-transplantation. In this study, two procedures that can minimize the incidence of post-infusion portal vein bleeding are demonstrated. The first method applies an absorbable hemostatic gelatin sponge to the injection site, and the second method involves penetrating the islet injection needle through the fat tissue first and then into the portal vein by using the fat tissue as a physical barrier to stop bleeding. Both methods could effectively prevent bleeding-induced mouse death. Whole liver section showing islet distribution and evidence of islet thrombosis post-transplantation, a typical feature for intrahepatic islet transplantation, were presented. These improved protocols refine the intrahepatic islet transplantation procedures and may help laboratories set up the procedure to study islet survival and function in pre-clinical settings.

INTRODUCTION:

Intraportal islet transplantation (IIT) via the portal vein is the most commonly used method for human islet transplantation in clinical settings. The mouse IIT model offers a great opportunity to study islet transplantation and test promising interventional approaches that can enhance the efficacy of islet transplantation[1]. IIT was first described in the 1970s and used by several groups[1-5]. It regained popularity after the breakthrough in human islet transplantation in the year 2000[6,7]**.** However, most islet transplant studies used the kidney capsule as a preferred site for experimental islet transplantation due to its easy success. On the contrary, IIT is more technically challenging and less frequently used for islet transplantation studies[8,9]. Unlike IIT, however, islets transplanted under the kidney capsule do not suffer from the immediate blood-mediated inflammatory reaction characterized by thrombosis, inflammation, and hepatic tissue ischemia, and thus have better function than islets transplanted into the liver. The kidney capsule model, therefore, may not fully mimic the stresses encountered by islets in human islet transplantation[10-12].

One of the major complications of IIT in mice is bleeding from the injection site after transplantation, which could cause 10-30% of mortality among different mouse strains[12]. In this paper, two refined approaches have been developed to stop bleeding more rapidly and securely and to reduce mouse mortality after an IIT. Visual demonstration of these refined details will help researchers identify the key steps of this technically challenging procedure. In addition, Hematoxylin and Eosin (H&E) staining of the whole section of liver tissue bearing transplanted islets is presented to facilitate location of transplanted islet grafts.

PROTOCOL

All procedures were conducted with the approval of the Institutional Animal Care and Use Committees at the Medical University of South Carolina and the Ralph H Johnson Medical Center in Charleston.

1. Diabetes induction using streptozotocin (STZ)

1.1. Recipient mice preparation

1.1.1. Weigh all mice individually.

1.1.2. Check blood glucose levels from a tail vein blood sample using a glucometer.

1.2. STZ dose determination for three different scenarios:

1.2.1. For mice with fatty liver disease inject one dose of STZ (40 mg/kg/day, i.p.) for 5 consecutive days.

1.2.2. For NOD-SCID mice inject 125 mg/kg of STZ, single injection, i.p. after overnight fasting.

1.2.3. For C57BL/6 mice inject 225 mg/kg of STZ, single injection, i.p.

1.3. Calculations for STZ (13.5 mg/mL):

NOTE: This calculation is for five C57BL/6 mice with body weights of 30 g:

1.3.1. Total body weights: 5 mice x 30 g/mouse = 150g

1.3.2. STZ needed: 150 g x 225 mg/1000g STZ = 33.75 mg

1.4. STZ preparation:

1.4.1. Weigh the STZ following the pre-calculated dose.

1.4.2. Transfer the weighed STZ powder into a 10 mL beaker on ice.

1.4.3. Add 3 mL of sodium citrate solution to the beaker to dissolve the STZ.

1.4.4. Mix well and use the STZ solution within 10 min of preparation.

1.5. STZ injection

1.5.1. Load the desired amount of STZ solution (enough for one mouse) into 1 mL syringe.

1.5.2. Perform intraperitoneal injection at the lower right quadrant of mouse abdomen.

1.5.3. Observe mice for 5 min after injection and check for any signs of discomfort during this period of time before putting them back into the cages.

1.5.4. Monitor blood glucose level from a tail vein blood sample using a glucometer daily after the STZ injection.

NOTE: In this experiment, mice are considered diabetic when non-fasting blood glucose is > 350 mg/dL for two consecutive days.

2. Islet preparation

NOTE: Human islets were cultured in CMRL-1066 media supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S) at a density of 10,000 islet equivalent number (IEQ) per 100 mm cell culture dish[9]. Mouse islets were cultured in DMEM with 10% FBS and 1% P/S with the same density[13]. Male NOD-SCID and C57BL/7 mice between 6-10 weeks of ages were obtained from commercial sources.

2.1. Detach cultured islets from cell culture dish by gentle scratching.

2.2. Hand-pick desired numbers of islets (e.g., 300-350 islets) using a 1cc syringe and put them into sterile 1.5 mL microcentrifuge tubes on ice.

2.3. Spin the tube for 10 s using the microcentrifuge.

2.4. Remove the supernatant, leaving some liquid to avoid losing the pellet.

2.5. Resuspend the pellet in 200 μL of HBSS with 0.5% bovine serum albumin (BSA).

2.6. Aspirate the resuspended islets into a 0.5 mL insulin syringe.

2.7. Place the syringe in the upright position. Let the islets sink down for 1 min.

2.8. Push the syringe to remove all the bubbles, leaving about 100-150 μL of liquid containing islets.

2.9. Place syringe head down and gently tap the side of the syringe to let the islets equally distribute throughout the liquid. Islets are now ready for injection.

3. Islet transplantation

3.1. Induce and maintain the mouse under general anesthesia with 2% isoflurane. Check for the lack of pedal reflexes to ensure proper anesthetization of the animal.

3.2. Shave and remove the fur in the abdomen area of the mouse.

3.3. Administer a single pre-operative dose of Buprenorphine (0.1 mg/kg i.p.).

3.4. Disinfect the surgical area with three alternating wipes of 2% iodine and 75% alcohol.

3.5. Perform a laparotomy with micro scissors to generate a 1-1.5 cm incision.

3.6. Open the peritoneal cavity with a retractor. Follow with either method A or method B as detailed below.

4. Method A: (stop bleeding with gel foam, Figure 1A)[14-16]

4.1. Mouse preparation

4.1.1. Place a sterile gauze around the incision.

4.1.2. Gently pull out the intestine using a forceps and keep it on the gauze.

4.1.3. Identify the portal vein by its location and expose it well.

4.1.4. Cover the intestine with a warm saline-wet gauze during the entire surgery.

4.2. Insert the islet preloaded insulin syringe needle through the portal vein near the duodenum (**Figure 1B**). To do so, hold the needle with the hole (bevel) facing down and position the opening surface's angle parallel to the portal vein wall before penetrating through the wall.

4.2.1. Pull the plunger to draw some blood (20-50 μL) into the syringe to mix the islets first.

4.2.2. Infuse the islets into the portal vein slowly while repeatedly pulling and pushing the plunge.

4.2.3. Place a piece of gel foam (about 0.5 cm x 0.5 cm in size) to cover the injection site.

4.2.4. Press the gel foam down with a cotton tip while pulling out the needle from the portal vein.

4.2.5. Continue pressing on the gel for about 2 min to confirm there is no active bleeding.

4.2.6. Rollover from the gel foam to make sure the gel foam covers the portal vein well.

5. Method B: (stop bleeding with fat pad, Figure 1C)[17]

5.1.1. Expose the portal vein thoroughly.

5.1.2. Use two cotton tips to hold the exposed portal vein from both the left and the right sides.

5.1.3. Identify the fat tissue pad between the duodenum and the portal vein.

5.1.4. Penetrate through the fat pad before inserting the needle into the portal vein (**Figure 1D**).

5.1.5. Infuse the islets, following the similar procedure described above in part 4.2.1 of Method A.

5.1.6. Pull out the needle while pressing down on the fat with a cotton tip.

5.1.7. Continue pressing on the fat pad for 1 min after removing the needle.

5.2. After confirming that there is no bleeding from the portal vein, gently return the intestine to the peritoneal cavity in its original position.

5.3. Leave 0.5 mL of warm saline (36-37 °C) in the abdominal cavity before closure.

NOTE: Warm saline facilitates post-surgery intestine movement and recovery and prevents intestine necrosis.

5.4. Close the muscle layer with an 5-0 suture.

5.5. Close the skin layer with an 4-0 suture.

5.6. Place the mouse in a clean cage on a heating pad until fully recovered from anesthesia.

5.7. Continue to provide an analgesic (e.g., buprenorphine 0.1 mg/kg i.p.) every 12 h and supplemental heat for 48 h post-surgery.

NOTE: The islet transplantation procedure takes approximately 15-20 min to complete.

6. H&E staining and photograph of whole liver section

6.1. Liver perfusion

6.1.1. Put the mouse under anesthesia as described above in part 3.1.

6.1.2. Carefully expose the portal vein and cut the inferior vena cava.

6.1.3. Manually perfuse the liver using 20 mL of 10% paraformaldehyde via the portal vein for about 5 minutes, using a 20 mL syringe with 25G needle[18].

NOTE: Liver perfusion can remove blood from liver tissue and improve liver fixation without disturbing the islet grafts.

6.1.4. Dissect the perfused whole liver from other organs.

6.1.5. Fix the perfused liver tissue in 10% paraformaldehyde for 24 h.

6.1.6. Embed the tissue in paraffin.

6.1.7. Cut tissue sections of 5 µm thickness each and put them on a glass slide for staining.

6.1.8. Perform H&E, insulin, fibrin, and polymorphonuclear neutrophil (PMN) staining using standard methods[15,16].

6.1.9. Scan whole liver section under a microscope.

REPRESENTATIVE RESULTS

We performed syngeneic and xenogeneic islet transplantations via the portal vein. Islet graft function was observed in a dose-dependent manner in both islet transplantation models. In the

syngeneic islet transplantation model using C57BL/6 mice, transplantation of 250 islets led to transitory normoglycemia before mice returned to hyperglycemia. Mice receiving 500 islets reached and maintained normoglycemia beyond 30 days after transplantation (**Figure 2A**). Mice in both groups showed increased body weights (**Figure 2B**).

Similarly, in the human islets to diabetic NOD-SCID mouse islet transplantation model, islet graft function was compared when 45, 85, or 140 IEQs/kg of body weights were transplanted. Normoglycemia could not be achieved when 45 IEQ/g (~225-275 islets/mouse) human islets were transplanted. When the islet number increased to 85 IEQ/g (~ 400-450 islets/mouse), 35.7% (10/28) of the recipients achieved normoglycemia (p =0.02 vs. 45 IEQ/g group) at day 60 post-transplantation. Furthermore, 83.3% (5/6) of the recipients who received 140 IEQ/g (~ 600-650 islets/mouse) of human islets reached normoglycemia (**Figure 2C**). In addition, majority mice who had bleeding died after surgery while mice without bleeding survived **(Figure 2D).**

Once enough human islets are engrafted to NOD-SCID recipients, their blood glucose levels can be well-controlled at the early-stage post-transplantation and well-maintained until the end of the study. The grafted islets can be easily identified by H&E and insulin staining. At 28 days post-transplantation, transplanted human islets were distributed evenly throughout the whole liver, mostly around/close to a blood vessel (**Figure 3)**.

The intrahepatic model was used to demonstrate instant blood mediated inflammatory reaction as seen in human islet transplantation. In our tissue section, we observed expression of insulin, and presence of fibrin and PMN infiltration in transplanted islets (**Figure 4A-D**).

FIGURE LEGENDS:

Figure 1: Illustration of intrahepatic islet transplantation procedures. (**A**, **C**). Schematics of key steps used in Method A and Method B. (**B**, **D**). Islets were injected directly via the portal vein (**C**) or indirectly via fat pat (**D**).

Figure 2: Representative outcomes of intraportal islet transplantation (**A, B**). Syngeneic mouse islet intraportal transplantation. Pancreatic islets (250 or 500) from C57BL/6 mice were transplanted into male C57BL/6 mice that were rendered diabetic by STZ. (A) Serial blood glucose levels were measured. Normoglycemia was defined as glucose levels <200 mg/dL for >2 consecutive days. (**B**) Increase in the recipients' body weight was observed post islet transplantation. (**C**) Percentage of diabetic NOD-SCID mice reaching normoglycemia in mice receiving a different number of human islets at 45 IEQ/g (n=7), 85 IEQ/g (n=28), and 140 IEQ/g (n=6). (**D**) Percentage of survival after IIT in bleeding and non-bleeding mice (n=14 each).

Figure 3: H&E staining of liver sections of NOD-SCID liver bearing human islet graft at 28 days post-transplantation. Islets are marked by black circles. The diameter of each circle positively corresponds with the size of each islet. Scale bar =1,000 μm in whole liver section and 100 μm in inset.

Figure 4: Representative histological pictures of intraportal transplanted mouse islets in liver 6 h after intraportal transplantation. (**A**) H&E, (**B**) insulin (red) (**C**) Fibrin, and (**D**) PMN stains. Scale bar = 100 μm.

DISCUSSION

In this study, two improved procedures that can prevent bleeding and may reduce mouse mortality during mouse IIT have been demonstrated. This study enables researchers to visualize the islet transplantation model that is unique in studying the instant blood mediated inflammatory response after transplantation. The IIT model is a distinctive model for studying islet cell survival and hepatic ischemic injuries in response to islet transplantation[19]. Here, we refined the procedure based on previous studies and reduced early complication-induced mouse mortality. Both method A[14-16] and method B[8,9] were used in multiple studies. We showed that islets distributed among the whole liver, and neutrophil infiltration and thrombosis typically associated with IIT were prominent in graft immediately after transplantation.

There are several key steps in mouse hepatic islet transplantation. Because both human and mouse islets can be as large as 200 μm in size, a needle size of at least 27G must be used for transplantation to ensure the islet products' smooth flow. However, this would generate a large hole in the portal vein that may cause bleeding after needle removal. By injecting islets via the correct angle and using a dental sponge to block the injection site or injection through the fat tissue, the chance of bleeding can be minimized, and mice have higher survival rates after transplantation. These steps may also help avoid liver warm ischemia-reperfusion injuries caused by blockage of portal vein blood flow when performing this procedure[19]. They can also reduce the damages to the liver and the intestines that may contribute to mouse mortality post-surgery.

There are also several limitations of the mouse intrahepatic islet transplantation model compared to the human islet transplantations setting. First, we cannot monitor mouse portal vein pressure during islet infusion as we do in clinic settings. Second, the volume that can be transplanted into the mice may not reflect the high amount of islet product transplanted into a human. Therefore, the extent of thrombosis may be different. Thirdly, mouse islet grafts after transplantation will be temporarily exposed to a hyperglycemic environment since no insulin will be given to mice, while in humans[20], insulin would be given during the peri-transplantation period to reduce the stress of transplanted islets[20]. Nevertheless, the intrahepatic islet model offers a unique pre-clinical model that can be used to study human islet transplantation.

ACKNOWLEDGMENTS:

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DISCLOSURE:

All authors declare that they do not have conflict of interest.

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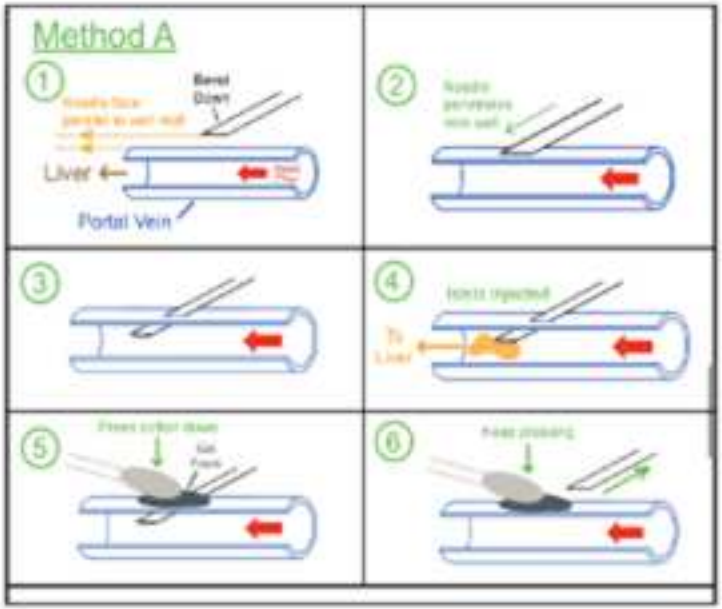
Figure 1

A



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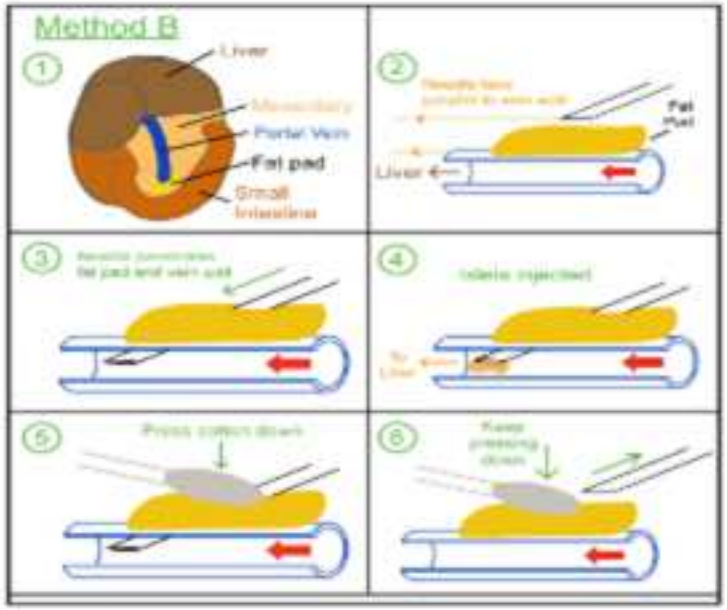

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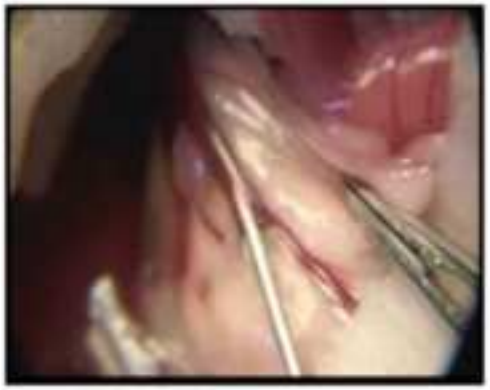

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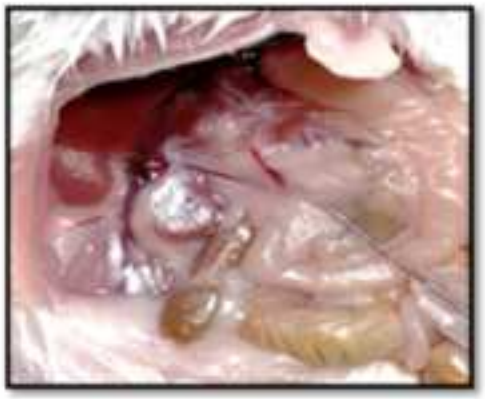

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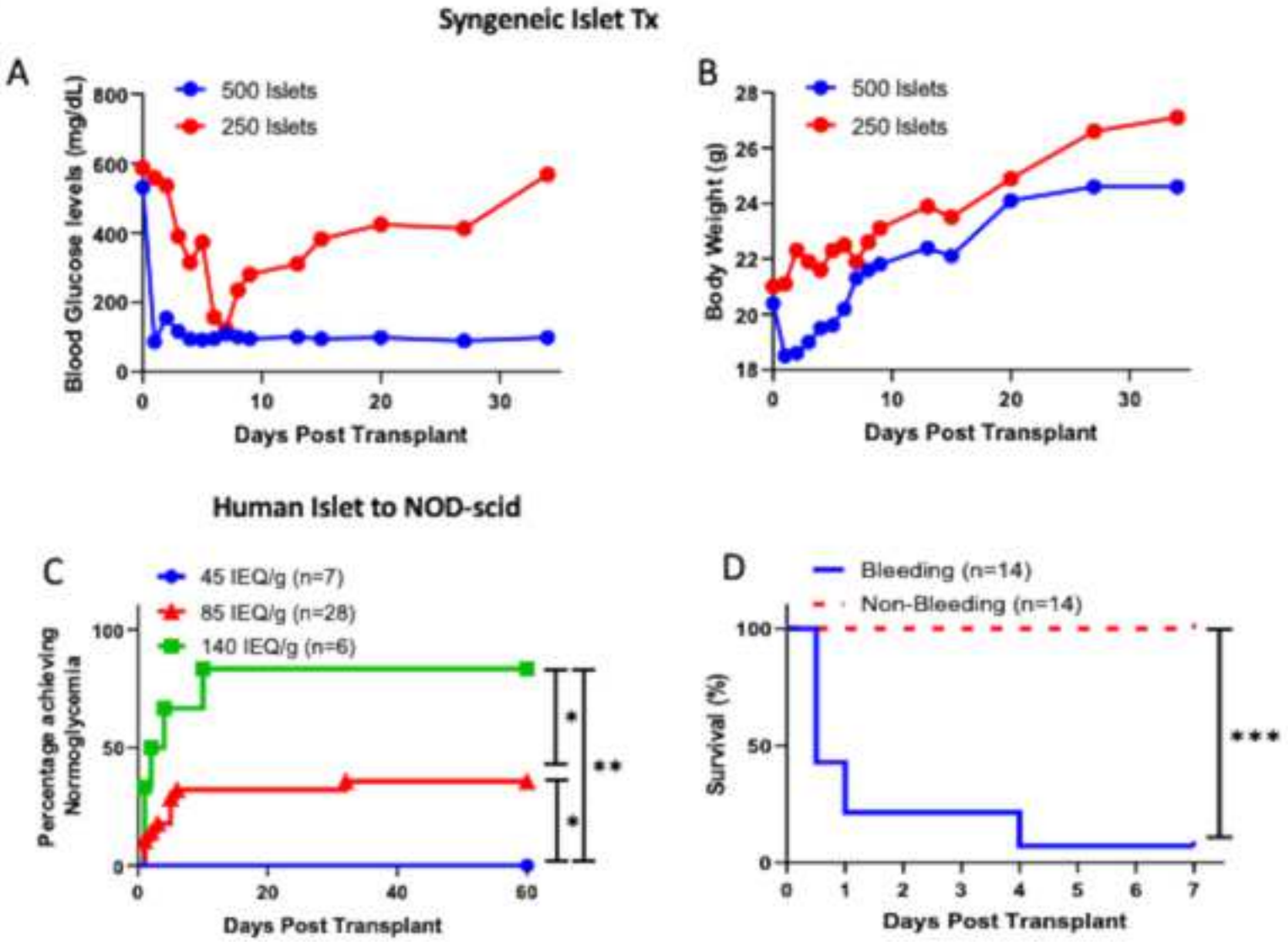


Figure 3

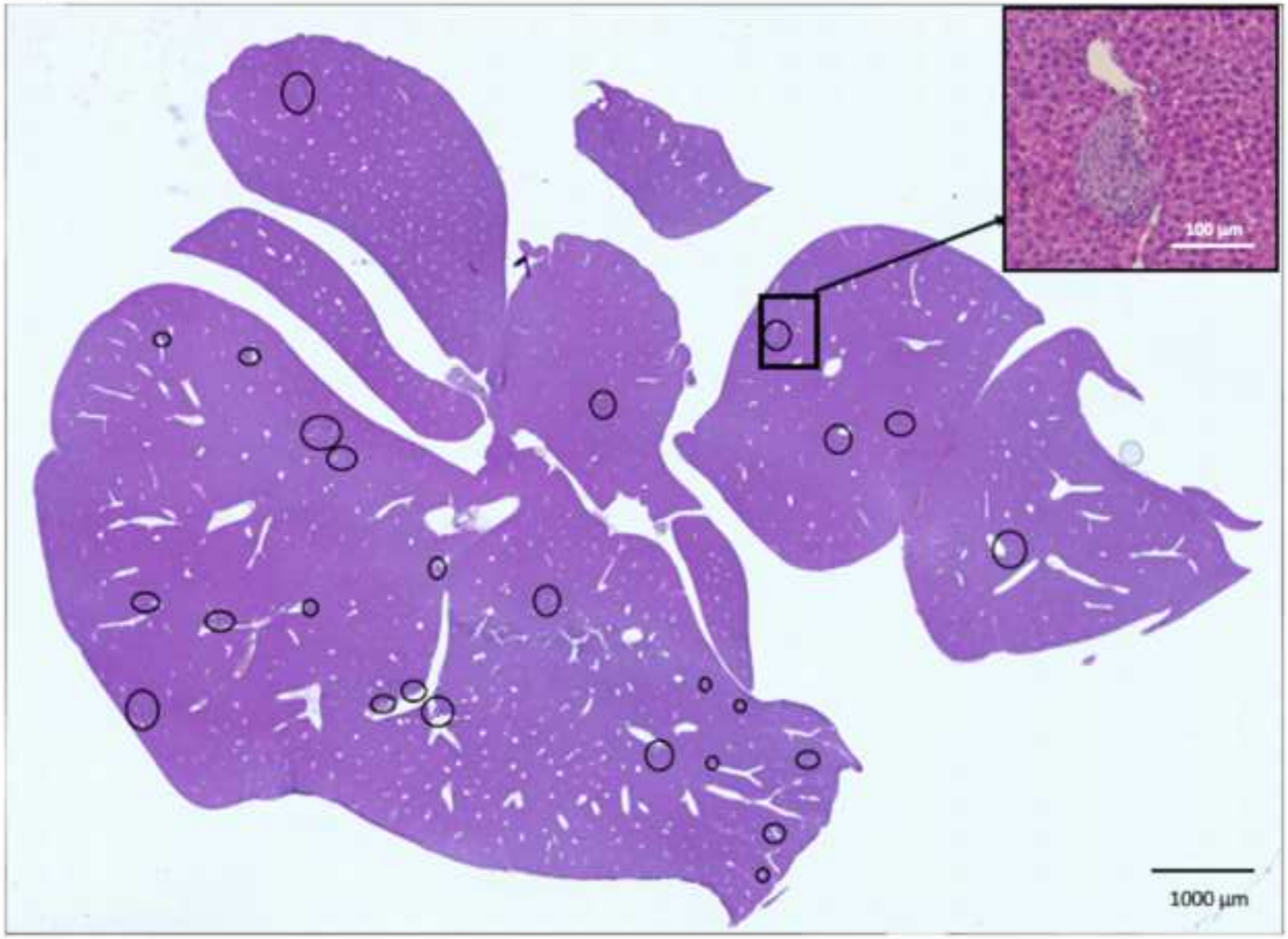
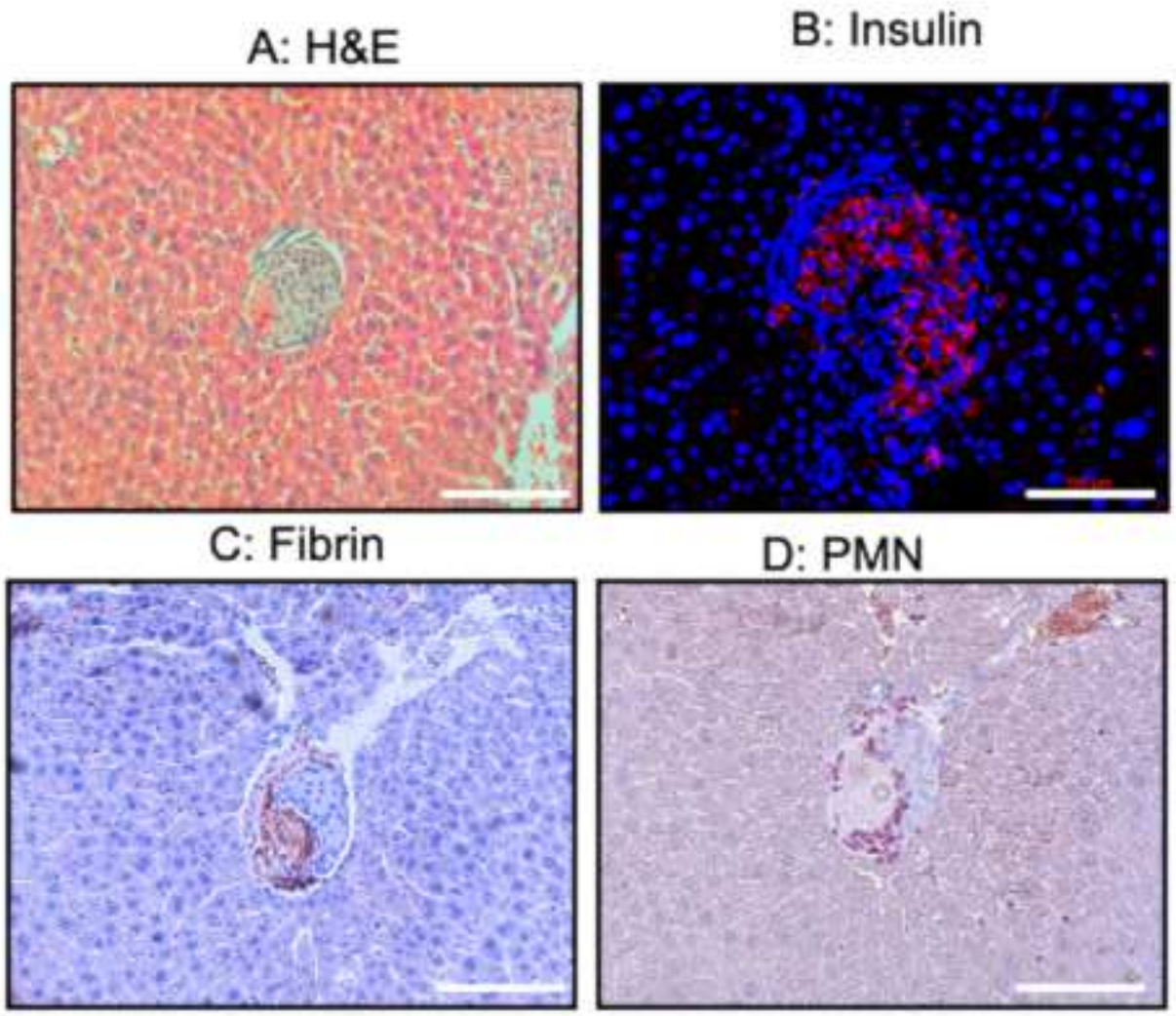


Figure 4



Materials

Click here to access/download;Table of Materials;4 JoVE_Table_of_Materials_Islet Tx 03.08.2021.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
10% Neutral buffered formalin v/v	Fisher Scientific	23426796	
1 mL Syringe with needle	AHS	AH01T	
20 mL Syringe	BD	301031	
25G x 5/8" hypodermic needles	BD	305122	
Alcohol prep pads, sterile	Fisher Scientific	22-363-750	
Animal Anesthesia system Buprenorphine hydrochloride,	VetEquip, Inc. Par Sterile	901806 NDC 42023-179-05	
Centrifuge tubes, 15 mL	Fisher Scientific	0553859A	
CMRL-1066	Corning	15110CV	
DMEM	Corning	10013CV	
Ethanol, absolute (200 proof), molecular biology grade	Fisher Scientific	BP2818500	
Extra fine Micro Dissecting scissors 4” straight sharp	Roboz Surgical Instrument Co.	RS-5882	
Fetal bovine serum (FBS)	Corning	35011CV	
FreeStyle Glucose meter FreeStyle Blood Glucose test strips	Abbott Abbott	Lite Lite	
Gelfoam (absorbable gelatin	Pharmacia &	34201	
Graefe forceps 4” extra delicate tip	Roboz Surgical Instrument Co.	RS-5136	
Heated pad	Amazon	B07HMKMBKM	
Hegar-Baumgartner Needle Holder 5.25”	Roboz Surgical Instrument Co.	RS-7850	
Insulin syringe with 27-gauge needle	BD	879588	
Iodine prep pads	Fisher Scientific	19-027048	
Isoflurane	Piramal Critical Care	NDC 66794-017-25	
Penicillin/streptomycin (P/S)	HyClone	SV30010	

Polypropylene Suture 4-0	Med-Vet International	MV-8683	
Polypropylene Suture 5-0	Med-Vet International	MV-8661	
Sodium chloride, 0.9% intravenous solution	VWR	2B1322Q	
Streptozocin (STZ)	Sigma	S0130	
Surgical drape, sterile	Med-Vet International	DR1826	
Tissue Cassette	Fisher Scientific	22-272416	

March 8, 2021

Journal of Visualized Experiments
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Answer: This has been done.

3. Please provide details about the age, sex, breed of the mice used.
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4. Line 106: Consider providing a short background about how the islet cells were obtained and cultured. Previously published literature can be cited.
Answer: This has been done.

5. Line 139: How much blood should be drawn?
Answer: During islet infusion, 20-50 μl of blood was drawn during the infusion. This information was added in the revised manuscript.

6. Line 152: Step heading is missing.
Answer: This was corrected.

7. Use "mL" instead of "ml". Add a single space between the quantity and its unit. E.g. "5 mm" instead of "5mm".
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8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
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Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Eppendorf, Keyence, etc.
Answer: Acknowledged.

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Answer: This has been done.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Do not use "&/and" in the author list of the references. Please include volume and issue numbers for all references.
Answer: The references have been formatted using endnote style for JOVE.

Reviewers' comments:
Reviewer #1:
Manuscript Summary: Learning about the procedure of intraportal islet transplantation is important for islet researchers, it reflects on the model of clinical islet transplantation. It is suitable for using in islet studies about ischemia, hypoxia and innate and acquired immunity. I agree with the validity of this model as intraportal islet transplantation and hope some opinions/answers for my comments about this protocol following,
We appreciate the positive comments.

Major Concerns:
1. I wish to know how do you collect islets in syringe for islet transplantation. I agree with using 27G needle for transplantation (larger needle may cause bleeding because of the hole size of penetrated site and smaller may break islets at passing through the needle), but I think 27G needle may be too small to collect islets completely in the syringe (especially collect larger islets).
Answer: We appreciate the critical point. We could use a 27G needle to collect islets since the majority of handpicked islets have a diameter between 100-200µm. The inner diameter of a 27G needle is 210µm (190.5-228.6µm). It is known that in mammals the typical size of an islet of Langerhans lies between 100 and 200µm, regardless of species (1). We corrected the needle size in the Table of Materials.

2. induction of diabetes: I think 125 mg/kg and 225 mg/kg injection may be done by one injection, not continuous 5 times injection. Please describe.

Answer: We agree with the comment. In our studies, 125 mg/kg and 225 mg/kg of STZ were given as one dose and 40mg/kg/day of STZ was given as multiple doses. We corrected the text as following:

- For mouse recipients with fatty liver disease: Multiple low-dose (40 mg/kg/day, i.p,) for 5 consecutive days;
- For NOD-SCID recipients: 125 mg/kg of STZ, once i.p.
- For C57BL/6 mice: 225 mg/kg of STZ, once i.p.

3. I think collecting human islets in similar size (larger islets may embolize portal vein) is difficult. Purity also affects the therapeutic effect of islet transplantation (lower purity does not contribute to the good outcome of islet transplantation). Please describe the information about the both human and mouse islets including the purity, size and how to regulate them...
Answer: To achieve high purity with desired size, the islets were handpicked under a stereo microscope, which ensures that the islets are nearly 100% pure and their size is approximately 50-200 µm in diameter. This handpicking procedure is the regulation process for purity and size selection.

Reviewer #2:
Manuscript Summary: Wenyu Gou et al. presented two methods to stop bleeding post-portal vein infusion of islet grafts in mice. Mouse islet transplantation is a technically challenging procedure. Compared to implanting islet grafts under the kidney capsule, infusion of islet grafts through portal vein is more clinically relevant. The refining procedures described in this paper could help others increase the success of intrahepatic islet transplantation in mice. The manuscript is well-written. The protocol is clearly described. However, minor revision is needed.

Minor Concerns: 1. What is the size of the insulin syringe needle used for portal vein injection (in line 113, step 2.5)? In the discussion (line 241-243), the authors stated that the needle size has to be at least 27G because the islets can be as large as 400um in size, but the listed insulin syringe in the supplemental table is with 31-gauge needle. Please clarify.
Answer: We apologize for the mistake. An insulin syringe with a 27G needle was used for the experiments. We updated this information in the discussion and supplemental table.

2. What is the volume of the blood drawn into the syringe (line 139, step 3.7.1)?
Answer: The volume of blood drawn into the syringe is approximately 25-50µl, which is enough to mix islets before infusion.

3. What is the approximate size of the Gelfoam that used to stop bleeding (line 145, step 3.7.5)?
Answer: The size of the Gelfoam used to stop bleeding is approximately 0.5cm x 0.5cm. We have added this information to the revised manuscript.

4. The headline for step 3.9 is missing (line 152).
Answer: This error has been corrected. Please see the updated manuscript for the correction.

5. What is the volume and temperature of the normal saline added to the peritoneal cavity before closing (line 163, step 3.11)?

Answer: We generally leave 0.5 mL of sterile warm saline (36-37 °C) in the abdominal cavity before closure. This information was added to the protocol.

6. Please provide the analgesia regimens and timing given to the recipient mice post-surgery.
Answer: The analgesia regimen used is Buprenorphine (0.5- 1 mg/kg, i.p), and this regimen was administrated every 12 hours for 48 hours after surgery. This information was added to the manuscript.

7. How long does it take to complete the procedures?
Answer: The whole infusion procedure takes between 15-20 mins to complete. This information was added to the manuscript.

8. In lines 241-243, a needle size of at least 27G is needed to "avoid" or to "ensure" the islet products' smooth flow?
Answer: This has been corrected.

9. In Figure 1 A&C, the fonts are not very reader friendly. Please use the formal font (Arial or Calibri) rather than the handwritten font.
Answer: We have revised the fonts as suggested.

10. Insulin staining histology should be provided.
Answer: An insulin staining histology was included in Fig 4.

Reviewer #3:
The efforts to redefine the technique of intraportal islet transplantation in rodents and to provide visualization of the methods might help others utilize this more clinically relevant animal model of islet transplantation, so the article has a relevant goal. However, there are major and minor concerns that should be addressed before the article could be published, described below:

1. The authors are claiming that by proposing the modifications in the procedure for islet intrahepatic transplantation in mice, they could reduce early mouse mortality from 10-30% to <5% (lines 236-237). However, the authors are not presenting the results confirming the improvement. The protocol for intrahepatic islet transplantation in mice was already published in JoVE Journal 1. The authors of submitted article are claiming that their methods are improving the mice mortality in comparison to the method published earlier (lines 65-67), but are not providing the results supporting that their methods are improving the mortality. It would be beneficial if the authors could show the percentage of mice with surgical complications after the intraportal islet transplantation and compare it for the 3 groups: without modification, after modifications: described as Method A (bleeding stopped with the gel form) and described as Method B (bleeding stopped with fat pad) with assessing also the procedure outcomes (glycemic control).

Answer: This is a great point. We want to clarify that our goal was to present alternative methods that can better ensure mouse survival after transplantation. If the bleeding was not appropriately stopped, 10-30% of mice would die. When bleeding occurred, 13 in 14 mice died within a couple of days post-surgery. In contrast, methods A and B prevent bleeding and reduce mice mortality. In study reported in (2), the authors lock portal vein bleeding by pressing the portal vein for 6 mins after transplantation, which would cause warm ischemia in the liver. Normal glycemic control can be achieved if enough islets were transplanted.

2. The language of article is not always clear and several sentences should be corrected:

- Page 3. „Abstract" Lines 33-34 "In this study, two improved procedures that target the major technical difficulty, post-infusion portal vein bleeding, are demonstrated."
Answer: This has been corrected in the revised manuscript.

Line 40-43 "In addition, this study shows the distribution of islet grafts in whole live section to offer guidance on how to locate islet graft post transplantation for analysis. Evidence of islet thrombosis post-transplantation, a typical feature for intrahepatic islet transplantation, is presented."
Answer: We have revised this.

- Page 4 "Introduction" Line 64: "Khatri and colleagues showed how to perform intraportal transplantation recently. - Step 1.3. STZ Injection
Please re-write the sentence" 1.3.1. Load the STZ solution into a 1 ml syringe accordingly to body weight.
Answer: We have corrected these sentences.

5. The figure 1 is lacking a good resolution to be readable.
Answer: We have changed the resolution of figure 1.

6. The figure 2D is not relevant for the article and could be skipped.
Answer: We have deleted Figure 2D as suggested.

7. Suggestion for minor correction to improve the protocol: Section 1.2 Prepare fresh STZ to make the protocol easier to follow, I suggest moving the part with the STZ doses for the different mouse strains with the example, directly under the step 1.2.1.
Answer: This has been revised.

Under the section 1.2.3 the table with the volume of STZ solution that should be given to the mouse accordingly to the body weight, should be moved.
Answer: This has been removed.

8. "2.1. Gently scrape islets attached to the cell culture dish" - islets are non-adherent and should not being attached to the culture dish, scraping shouldn't be needed; usually before hand-picking islets are just mixed in the culture medium. Please correct this step.
Answer: Islets attach to a normal cell culture dish loosely after culturing for a couple of days. Therefore, we have to gently scrape islets from the cell culture dish. If islets are cultured in a non-adherent dish, they don’t need to be scraped off.

References:
1. Jo J, Choi MY, Koh DS. Size distribution of mouse Langerhans islets. Biophys J 2007;93(8):2655-2666.
2. Khatri R, Hussmann B, Rawat D, Gurol AO, Linn T. Intraportal Transplantation of Pancreatic Islets in Mouse Model. J Vis Exp 2018(135).