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Isolation and Decellularization of whole porcine pancreas

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Dear Sir/Madam,

This is an invited article by editor Nandita Singh.

We hereby submit our manuscript entitled "**Isolation and Decellularization of whole porcine pancreas**" by the authors Vijay K Kuna et al. to be considered for publication in your esteemed Journal of Visualized Experiments.

All authors have read and approved submission of the manuscript and the manuscript has not been published and is not being considered for publication elsewhere in whole or part in any language.

SSH holds shares in VeriGraft AB, a biotechnology company in the field of regenerative medicine. The other authors have no conflicts of interest.

We sincerely hope that the editors of Journal of Visualized Experiments find these results potentially interesting and important.

Hoping for a favorable response.

Sincerely,
Vijay K Kuna (on behalf of all authors)

TITLE:

Isolation and Decellularization of a Whole Porcine Pancreas

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KEYWORDS:

Pancreas, porcine pancreas dissection, tissue engineering, decellularization, regenerative medicine, extracellular matrix

SUMMARY:

Tissue engineering of the whole pancreas is a challenge because of its exocrine and endocrine functions. We show a method for the dissection of an intact porcine pancreas and the process of successful decellularization by perfusion of detergents Triton X-100, sodium deoxycholate, and deoxyribonuclease.

ABSTRACT:

Tissue engineering of the whole pancreas can improve current treatments for diabetes mellitus. The ultimate goal is to tissue engineer pancreas from an allogeneic or xenogeneic source with human cells. A demonstration of methods for the efficient dissection, decellularization, and recellularization of porcine pancreas might benefit the field. Akin to human pancreases, porcine pancreases have a special anatomical arrangement with three lobes (splenic, duodenal, and connection) rounded by the duodenum and small intestine. The duodenal lobe of the pancreas connects to the duodenum by several small blood vessels. Tissue engineering of the pancreas is complicated because of its exocrine and endocrine nature. In this paper, we show a detailed protocol to dissect the whole porcine pancreas and decellularize it with detergents while saving its structure and some extracellular matrix components. To achieve complete perfusion, the aorta is chosen as inlet and the portal vein as outlet. The other blood vessels (hepatic artery, splenic vein, splenic artery, and mesenteric artery) and bile duct are ligated. To prevent the formation of thrombus, the pig is heparinized and, immediately after dissection, the organ is

flushed with cold heparin. To inhibit the action of exocrine enzymes, the pancreas decellularization is set at 4 °C. The decellularization is performed by perfusion of Triton X-100, sodium deoxycholate, and deoxyribonuclease, with an intermittent and final extensive washing. With a successful decellularization, the pancreas appears white, and a histological evaluation with hematoxylin and eosin shows an absence of nuclei with a preserved extracellular matrix structure. Thus, the proposed method can be used to successfully dissect and decellularize whole porcine pancreas.

INTRODUCTION:

Diabetes mellitus is characterized by the presence of increased levels of glucose in blood. It is recognized as a major public health challenge in most countries[1]. High levels of blood glucose affect the blood vessels and nervous system, causing damage to the eyes, the heart, and the kidneys, and extremity ischemia. Traditional methods of treatment include injections of exogenous insulin, drugs, and lifestyle changes. Putting aside a cure for the disease, in some cases, available treatments fail to maintain the insulin at therapeutic levels, resulting in hyperglycemia. Although the transplantation of islets or whole pancreas eliminates the disease, it is not commonly done because of a shortage of suitable donor organs and because of the risks and difficulties involved from immunosuppression and encapsulation[2].

Current improvements in the field of tissue engineering and regenerative medicine possess the capacity for providing a solution for these issues. With the technique of decellularization, the cellular material from a human or animal donor can be removed while the important extracellular matrix (ECM) proteins, growth factors, and signaling molecules are preserved in the scaffold. Such scaffolds can potentially be transplanted without the need for immunosuppression, to restore the organ function after recellularization with the recipient's own non-immunogenic stem cells[3,4]. The tissue-engineered organs from allogeneic or xenogeneic sources can be used in clinical transplantation, as the major extracellular matrix proteins are conserved among species and might not be rejected after transplantation[5].

Decellularization is a well-explored method involving the optimal use of physical forces, chemical detergents, and enzymes in a physiological setting to remove cells and nuclear material from a tissue or organ. Recellularization is a procedure of seeding cells back into the acellular organ. It is an intellectually tough procedure, requiring a large number of cells, an optimum cell-seeding strategy, and a bioreactor system for the culture of the organ at physiologically acceptable conditions like temperature, pressure, and gases[6].

The pancreas can be considered a challenging tissue for tissue engineering because of its exocrine and endocrine capacities. The exocrine tissue secretes several digestive enzymes, while the endocrine part secretes hormones, including insulin. The decellularization of intact pancreases from mouse[7,8], human[9], and pig[10] has already been reported using enzymes (trypsin, deoxyribonuclease [DNase]) and non-ionic (Triton X-100) and ionic detergents (sodium deoxycholate [SDC] and sodium dodecyl sulfate [SDS]). However, following the published protocols, we struggled with a successful dissection and complete perfusion and decellularization while maintaining an ECM structure. We speculated that the applied detergents during the

decellularization cause lysis of the cells, thereby releasing digestive enzymes into the organ. The released enzymes will cause an irreversible damage to the ECM scaffold and make it inefficient for decellularization and recellularization. A design of the method that effectively decellularizes pancreas while inhibiting the action of digestive enzymes may solve the problem. We chose the strategy of Peloso *et al.*, of decellularization of the pancreas at a cold temperature, although they did not report on why cold temperature is used[9]. At the same time, we designed a dissection strategy with modifications from *Taylor et al.* by choosing the aorta as a perfusion inlet over the coeliac trunk (CT) and the superior mesenteric artery (SMA)[11].

In a recently published article[12], we demonstrate a method for the effective isolation and decellularization of porcine pancreas while preserving some ECM components. In this paper, we show a detailed description of how to dissect a whole porcine pancreas containing splenic, duodenal, and connection lobes, and present a stepwise protocol for successful decellularization.

PROTOCOL:

The dissection of a porcine pancreas and the decellularization procedure presented here follow the ethical guidelines of the University of Gothenburg.

1. Preparation of the Decellularization Set-up

1.1. Using 3 x 5 mm silicone tubes, connect in series the detergent container to the degasser and then to the pancreas in the organ chamber *via* the peristaltic pump (see **Figure 1**).

1.2. Using another 3 x 5 mm silicon tube, connect the organ chamber to the detergent collection container *via* the peristaltic pump to collect the perfused detergent.

1.3. Keep the whole set-up (except the detergent collection container) at 4 °C.

2. Preparation of Decellularization Solutions

2.1. Solution 1 (phosphate-buffered saline [PBS]): Add 8 g of (137 mM) sodium chloride, 0.2 g of (2.7 mM) potassium chloride, 1.44 g of (10 mM) sodium phosphate, and 0.24 g of (1.8 mM) potassium phosphate to 1 L of ultrapure water and stir until dissolved. Adjust the pH to 7.4 with hydrochloric acid (HCl). In total, 3.2 L of this solution is required.

2.2. Solution 2 (PBS + heparin): To 1 L of Solution 1, add 3.4 mL of heparin (17 international units [IU]/mL). Prepare this solution fresh and keep it on ice until it becomes cold. In total, 1.2 L of this solution is required.

2.3. Solution 3 (ultrapure water + sodium azide + disodium ethylenediaminetetraacetic acid [EDTA]): To 1 L of ultrapure water, add 1.86 g of (5 mM) EDTA and 200 mg of (0.02%) sodium azide. Stir until the salts are dissolved. Cool the solution to 4 °C before use. In total, 22 L of this solution is required.

2.4. Solution 4 (PBS + sodium azide + EDTA): To 1 L of Solution 1, add 200 mg of (0.02%) sodium azide and 1.86 g of (5 mM) EDTA. Stir until the salts are dissolved. Cool the solution to 4 °C before use. In total, 1 L of this solution is required.

2.5. Solution 5 (ultrapure water + sodium azide): To 1 L of ultrapure water, add 200 mg of (0.02%) sodium azide. Stir until the salts are dissolved. Cool the solution to 4 °C before use. In total, 260 L of this solution is required.

Note: EDTA forms a precipitate with SDC and was excluded from Solution 5 since it will be used for immediate washing before and after the SDC treatment.

2.6. Solution 6 (SDC + Triton X-100): To 940 mL of ultrapure water, add 40 g of (4%) SDC, 60 mL of (6%) Triton X-100, 200 mg of (0.02%) sodium azide, and 69.6 mg of (0.4 mM) phenylmethylsulfonyl fluoride (PMSF). Stir until dissolved. Cool the solution to 4 °C before use. Add PMSF before use. In total, 9.6 L of this solution is required.

2.7. Solution 7 (DNase): Add 10,000 Kunitz units of DNase-I in 250 mL of (40 Kunitz units/mL) Dulbecco's PBS containing $CaCl_2$ and $MgCl_2$. Prepare fresh and use immediately. Warm the solution to 37 °C before use.

2.8. Solution 8 (PBS + sodium azide): To 1 L of Solution 1, add 200 mg of (0.02%) sodium azide. Stir until the salts are dissolved. Cool the solution to 4 °C before use. In total, 1 L of this solution is required.

3. Dissection of the Porcine Pancreas

Note: In this study, porcine pancreases were dissected from euthanized, heparinized (400 IU/kg) female pigs weighing 45 kg from a farm.

3.1. Place the pig on the dissection table in the supine position.

3.2. Make a midline incision from the xiphoidal process to the pubic bone (approximately 40 cm) using a scalpel, exposing all abdominal organs.

3.3. Locate the splenic, duodenal, and connection lobes.

3.4. Locate the level of the major duodenal papilla and ligate the duodenum orally from that site using two separate sutures.

3.5. Ligate the distal esophagus with two separate sutures and cut between the ligatures with scissors to take the stomach out.

3.6. Separate the connective tissue from the colon to reach the small intestine.

3.7. Separate the connective tissue of the colon that attaches to the splenic lobe of the pancreas.

3.8. Remove the colon from the small intestine after ligating the arteries.

3.9. Find the mesenteric vein and the mesenteric artery and ligate with one suture where they appear caudally of the pancreas.

3.10. Ligate the splenic artery and vein together with one suture, close to the spleen, in the hilum, and cut distally with scissors to remove the spleen.

3.11. Follow the duodenum until the duodenal and connection lobes are cleared and ligate the duodenum at the end with two separate sutures.

3.12. Dissect the portal vein and ligate it with one suture to prevent any blood leakage from the liver. The portal vein serves as an outlet during the decellularization.

3.13. Dissect and ligate the bile duct and hepatic artery with two sutures.

3.14. Find the aorta under the renal vein and dissect it in the cranial direction from muscle and connective tissue until it reaches the pancreatic area.

3.15. Flip the pancreas gently over and dissect the aorta, keeping the SMA and the CT intact, and cut the aorta cranial to the latter with scissors.

3.16. Cut the remaining surrounding tissues with scissors and take the pancreas out.

3.17. Using a 50-mL syringe, flush the organ through the aorta with Solution 2 until it perfuses the whole organ or until the whole organ becomes cold.

4. Preparation of the Porcine Pancreas for Decellularization

4.1. Keep the pancreas at 4 °C or on ice throughout the process.

4.2. Cut the sutures of the duodenum using scissors, clean it of food by flushing 50 - 150 mL of ultrapure water using a 25-mL pipette, and ligate it again with sutures.

4.3. Ligate one end of the aorta and all branches besides the SMA and CT with sutures to prevent leakage.

4.4. Insert from the other end of the aorta a 4-mm arteriotomy cannula and ligate it with sutures.

4.5. Perfuse the pancreas with Solution 3 for 1 h at 20 mL/min using the decellularization set-up.

Note: Look for any leakages from all sides of the organ and ligate all open vascular branches with sutures, except the portal vein.

4.6. Freeze the pancreas at -20 °C in Solution 4 until the start of the decellularization.

5. Decellularization of the Porcine Pancreas

5.1. Thaw the pancreas at 4 °C.

5.2. Run the peristaltic pump in the decellularization set-up with Solution 3 at 20 mL/min until no air bubbles are seen in the detergent inlet tube.

5.3. Place the pancreas in the decellularization container and connect the detergent inlet tube to the aorta of the pancreas. Wash the organ by perfusion with Solution 3 overnight at 20 mL/min at 4 °C.

5.4. Pour out the solution left in the organ chamber.

5.5. Replace Solution 3 with Solution 5 and perfuse the pancreas for 30 min at 20 mL/min at 4 °C. Pour out the solution in the organ chamber.

5.6. Add Solution 6 and perfuse the pancreas for 8 h at 20 mL/min at 4 °C. Pour out the solution in the organ chamber.

5.7. Wash the organ by perfusion with Solution 5 for 96 h at 20 mL/min at 4 °C. Pour out the solution in the organ chamber.

5.8. Prepare the pancreas for DNase treatment by recirculating 500 mL of Dulbecco's PBS containing $CaCl_2$ and $MgCl_2$ for 30 min at 37 °C. Pour out the solution in the organ chamber.

5.9. Add 250 mL of Solution 7 and perfuse the pancreas for 4 h at 20 mL/min at 37 °C. Pour out the solution in the organ chamber.

5.10. Wash the organ by perfusion with Solution 5 for 120 h at 20 mL/min at 4 °C.

5.11. Store the organ in Solution 8 at 4 °C for short periods or at -20 °C for long periods.

6. Verification of Decellularization

6.1. Using scissors, cut 3- to 10-mm biopsies from all lobes of the pancreas and fix them in formaldehyde for 48 h at room temperature.

6.2. Wash the pieces in ultrapure water for 15 min, process them in a tissue processor following standard protocols, and embed them in paraffin.

6.3. Cut 5-µm sections using microtome and stain them by Meyer's hematoxylin and 0.2% alcoholic eosin (HE) following standard protocols.

6.4. View the slides under a light microscope to check for the loss of nuclei.

Note: A piece from a fresh tissue processed in the same way can be used as a control to check for the presence of nuclei.

REPRESENTATIVE RESULTS:

Representative porcine pancreas dissection pictures, which can help in locating and dissecting the mesenteric artery and vein, the portal vein, and the aorta under the renal vein, are shown in **Figures 2A**, **2B**, and **2C** (red arrows), respectively. **Figure 3A** shows the gross morphology of a normal pancreas, which appears light pink and contains splenic, connection, and duodenal lobes. After decellularization, the pink color is lost and the decellularized pancreas looks pale white in color. The gross morphology picture showing splenic, connection, and duodenal lobes of a decellularized pancreas is shown in **Figure 3B**. **Figure 3C** shows the presence of many blue nuclei in a normal pancreas by staining with HE. In a decellularized pancreas, the HE staining showed a loss of nuclei, as no blue nuclei are seen (**Figure 3D**).

FIGURE LEGENDS:

Figure 1: Preparation of the perfusion set-up. Using a 3 x 5 mm silicone tube, as shown in the set-up, connect in series the detergent container to the degasser, the pump, and the organ chamber. For the detergent outlet, use another 3 x 5 mm silicone tube and connect the organ chamber *via* the peristaltic pump to another container.

Figure 2: Pictures of a porcine pancreas dissection. (**A**) Location of the mesenteric artery and vein (red arrow). (**B**) Location and dissection of the portal vein (red arrow). (**C**) Dissection of the aorta under the renal vein (red arrow).

Figure 3: Gross morphology and HE staining of normal and decellularized pancreases. (**A**) Gross morphology of a normal pancreas. (**B**) Gross morphology of a decellularized pancreas. (**C**) HE staining shows the presence of blue nuclei in a normal pancreas. (**D**) HE staining shows the absence of blue nuclei in a decellularized pancreas.

DISCUSSION:

The proposed protocol, using perfusion of SDC and Triton X-100 at 4 °C, will decellularize whole porcine pancreas successfully. The challenge in this technique is the dissection of the intact pancreas containing all the three lobes without damaging the parenchyma and its supplying vessels, as well as the ligation of the other vascular branches of the specimen in order to perfuse the organ without leakage. The porcine pancreas has a different anatomy compared to the human pancreas. It consists of three lobes and stays in close contact with the small intestine by partly surrounding it. We dissected the duodenum together with the pancreas, as several small

blood vessels from pancreas connect the intestine. During the optimization studies, blunt cutting of these blood vessels has shown leakage and an incomplete perfusion of solutions.

Since the aorta connects to the pancreas through the coeliac and superior mesenteric arteries, we chose the aorta as an inlet to keep the perfusion simple by only using one cannula and, therefore, one inlet. In our experience, ligation of the aorta above the CT and below the SMA will decrease the time of dissection and reduces the risk of damaging any of the two vessels. In addition to a heparinized pig, we also noticed that a perfusion of cold heparin *via* the aorta immediately after the dissection helps in achieving perfusion of solutions throughout the organ. We speculate if this occurs by preventing the formation of blood clots in the blood vessels. The initial perfusion of a pancreas with ultrapure water after dissection will lyse red blood cells and remove the blood remnants in the organ, thereby preventing the formation of blood clots. This period can also be used to find any unligated small branches of veins and arteries, as blood flow can be easily noticed above the background.

We chose to keep the whole decellularization procedure at cold temperatures (4 °C), as this will hinder the action of exocrine enzymes that release from exocrine cells of the pancreas. The exocrine enzymes, when not inhibited, can cause a deleterious effect on cells and the ECM, as they can digest cell membranes and proteins[12]. As freezing and thawing can effectively burst the cells, we included a freeze/thaw step, initially even before the perfusion of detergents[4,13]. The initial wash after thawing will remove the remnants of cell bursts. The detergent treatment we used is a mix of SDC and Triton X-100 at unusually high concentrations and at a high perfusion speed. We chose this approach to achieve faster decellularization by removing the exocrine cells that damage the ECM. We speculate that a hard and fast protocol is beneficial for pancreas decellularization, as less time will be available for pancreatic enzymes to interact with the ECM, thus preserving good ECM components. To preserve the ECM components, we also added serine protease inhibitor (PMSF) to the detergent solutions, as that will inhibit the activation of enzymes released from exocrine cells[14]. Sodium azide is added to all decellularization solutions, as it acts as a bacteriostatic agent, thereby inhibiting the chance of bacterial contamination[15].

The pancreas decellularized following this protocol showed a preservation of ECM structures and the ECM proteins collagen and elastin. However, a significant loss of glycosaminoglycans was noticed in the decellularized pancreas. The pancreas decellularized in this fashion also showed promise for the attachment of human fetal pancreatic stem cells and the expression of exocrine and endocrine markers in pieces recellularized for 14 days[12]. However, to generate an intact and functional pancreas, further research is required in evaluating correct cell sources, cell types, cell seeding strategies, and bioreactor culture.

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DISCLOSURES:
S.S.H. holds shares in Verigraft, a company that has licensed the technology of blood vessel tissue engineering. The other authors have nothing to disclose.

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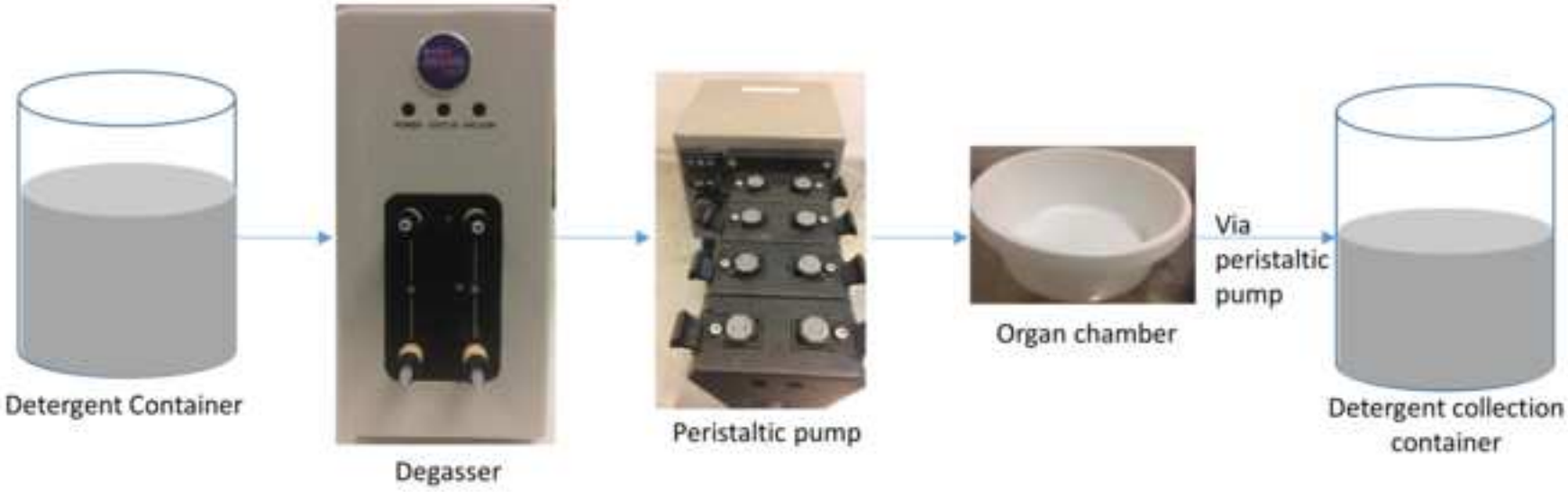
Figure



Figure

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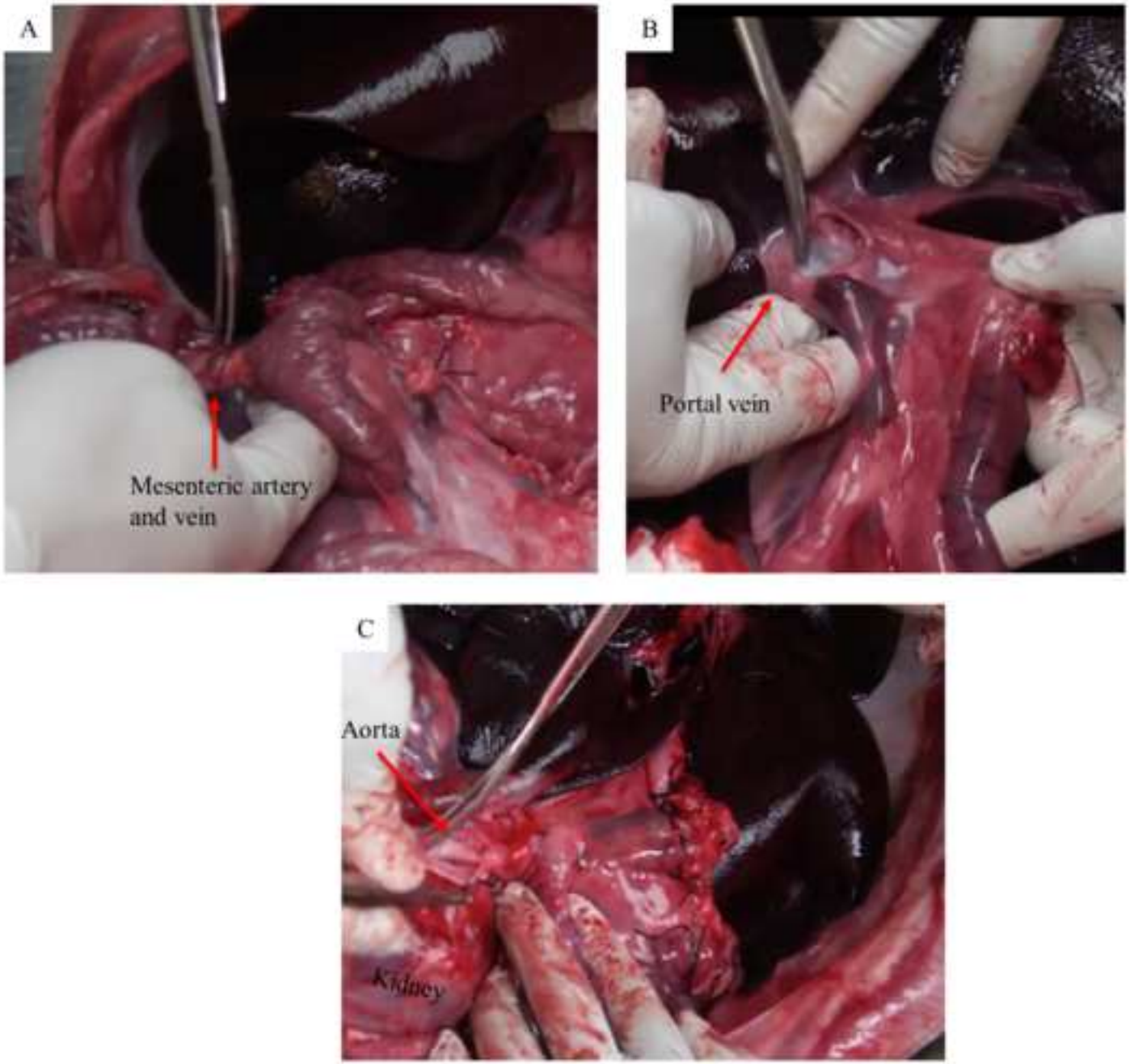

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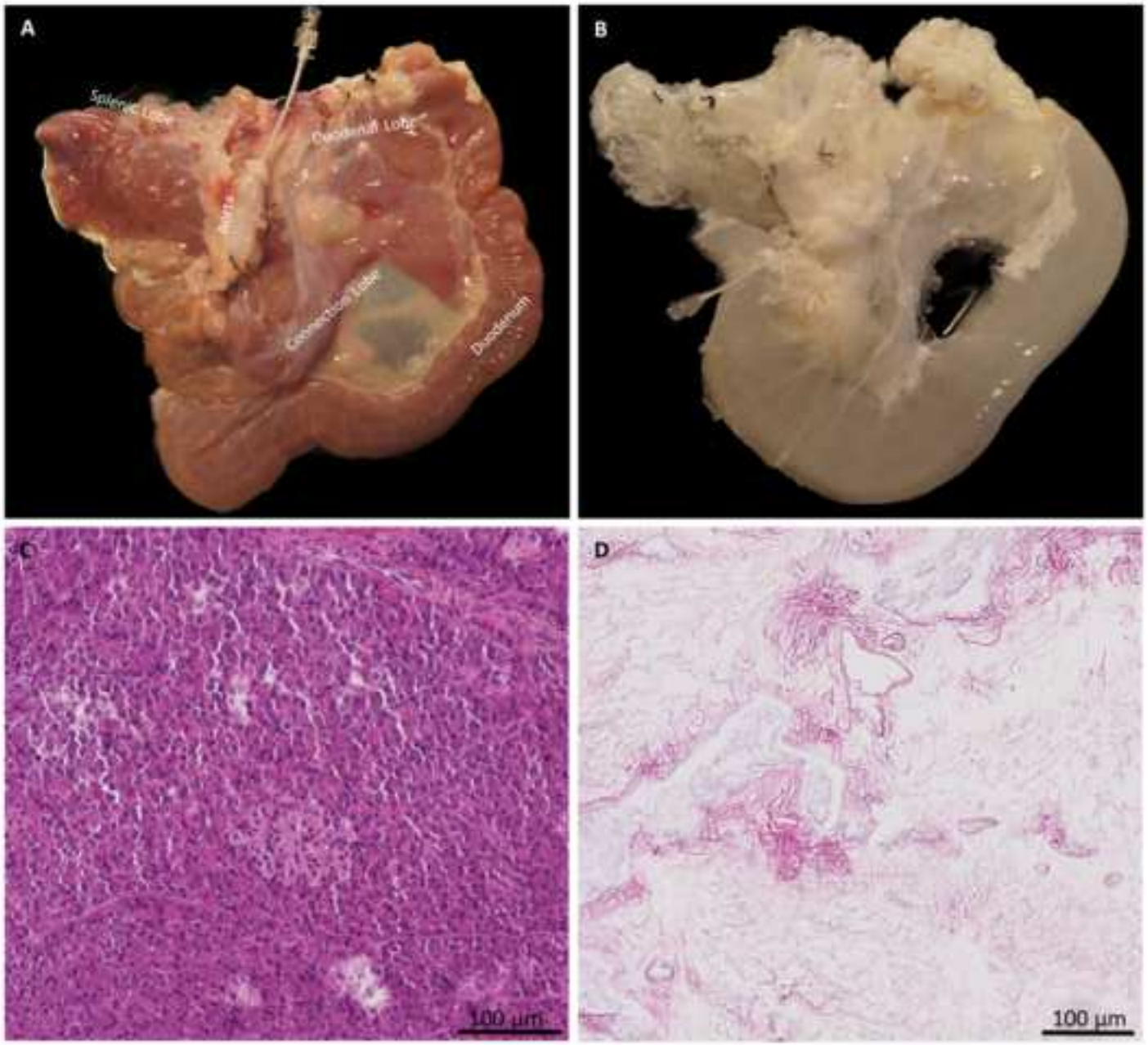

Table of Materials

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Name of Reagent/ Equipment	Company	Catalog Number
4 mm DLP arteriotomy cannula	Medtronic	31104
Degasser	Biotech AB	0001-6484
DNase-I	Worthington	LS0020007
Dulbecco's PBS with $CaCl_2$ and $MgCl_2$	Sigma Aldrich	D8662
EDTA disodium salt dihydrate	AlfaAesar	A15161.OB
Heparin	Leo	387107
Peristaltic pump	Oina	SP-1X4
PMSF	Roche	10837091001
Potassium chloride	Sigma Aldrich	P5405
Potassium hydrogen phosphate	Sigma Aldrich	P9791
SDC	Sigma Aldrich	30970
3 x 5 mm Silicon tube	VWR	2280706
Sodium Azide	Sigma Aldrich	71290
Sodium chloride	Sigma Aldrich	13423
Sodium hydrogen phosphate	Merck	71640-M
Suture	Vömel	14817
50 mL Syringe	Becton Dickinson	300137
Triton X-100	AlfaAesar	A16046.OF

Comments/Description
Unstable in aqueous solution. Should be added fresh before perfusion.

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Dear Sir,

We wish to humbly thank and express our appreciation to the editors of Journal of Visualized Experiments for allowing us to resubmit our paper. We also appreciate the constructive criticism from the reviewers that has indeed helped us to improve the contents of the manuscript.

Response to the queries regarding the manuscript:

JoVE58302

Title: Isolation and decellularization of whole porcine pancreas

We have answered in red all the questions posed by the editors and reviewers. Our changes are marked with track changes in the manuscript.

Editorial comments:

1. Please proofread; there are still some grammar and usage errors, some of which have been corrected in the attached manuscript.

We have proofread again to our best knowledge correcting grammar.

2. Introduction: Do you mean 'high degree of sequence similarity/identity'? Proteins either are or are not homologous; there is no degree involved.

We corrected the line 88 to "major extracellular matrix proteins are conserved among species and might not be rejected after transplantation".

3. Protocol section 2: How much of solutions 4 and 8 are needed?

The information is added in lines 154 and 173 respectively.

4. Section 3: For each step with sutures, could you clarify how many are needed?

The number of sutures are clarified in all steps.

5. Section 5: How exactly will you empty solutions from the organ chamber? Pour them out?

We pour out the solution. It is clarified in all steps of section 5.

6. 6.4: What kind of microscope?

The light microscope is clarified in 6.4.

7. Results: What do you mean '(please note that this figure will be replaced by a clear view of the dissection in final edited version of the manuscript)'? Do you mean to replace it by, e.g., stills from the video? It may be best to keep the original figures; note that we will use the original .tif files for the final manuscript.

On the day of video filming, with our photo camera, we take the pictures where the anatomy is clearly seen and will change the pictures present in Figure 2 now with newly taken ones.

8. Please remove 'Figure 1/Figure 2/etc.' from the Figures themselves.

We followed your suggestion and removed the Figure1/2 from figures.

9. You mention that Figures are reused in your questionnaire. Please obtain explicit copyright permission to reuse any figures. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The pictures we are using are from a published project but is different from the published one. For this reason, we are not referring in figures to the article. However, we have obtained permission to use data from a published project. The email conversation is uploaded as a word document.

From: Gabriella Anderson <Gabriella.Anderson@sagepub.co.uk>
Subject: RE: Permission request
Date: 23 January 2018 at 16:22:00 GMT+1
To: "erik.elebring@gu.se" <erik.elebring@gu.se>
Cc: "Knowles, Jonathan" <j.knowles@ucl.ac.uk>

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Let me know if you have any further queries.

Kind regards,

Gaby

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From: Erik Elebring <erik.elebring@gu.se>
Date: Wednesday, 10 January 2018 at 20:43
To: Jonathan Knowles <j.knowles@ucl.ac.uk>
Subject: Permission request

Dear Prof Jonathan Knowles and Prof Hae-Won Kim,

I contact you regarding our article "Cold-perfusion decellularization of whole-organ porcine pancreas supports human fetal pancreatic cell attachment and expression of endocrine and exocrine markers" recently published in your journal.

An editor of Journal of Visualized Experiments (JoVE) has recently contacted us after reading above mentioned article and is interested in publishing the method in their journal. If you accept this we will mainly focus on the method of isolation of the pig pancreas and the actual process of decellularisation.

Therefore, we kindly request your permission to use some of the pictures taken which are included in the study published in your journal but not used for publication.

We would be very grateful for a positive response.

Kind regards,

Erik Elebring

Laboratory for Transplantation and Regenerative Medicine

University of Gothenburg