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Preparation of Decellularized Kidney Scaffolds in Rats

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TITLE:

Preparation of Decellularized Kidney Scaffolds in Rats

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KEYWORDS:

Animal study, rat, kidney, decellularization, Triton X-100, sodium dodecyl sulfate

SUMMARY:

This protocol introduces a method to develop a scaffold using decellularized rat kidneys. The protocol includes decellularization and recellularization processes to confirm bioavailability. Decellularization is performed using Triton X-100 and sodium dodecyl sulfate.

ABSTRACT:

Tissue engineering is a cutting-edge discipline in biomedicine. Cell culture techniques can be applied for regeneration of functional tissues and organs to replace diseased or damaged organs. Scaffolds are needed to facilitate the generation of three-dimensional organs or tissues using differentiated stem cells in vivo. In this report, we describe a novel method for developing vascularized scaffolds using decellularized rat kidneys. Eight-week-old Sprague-Dawley rats were used in this study, and heparin was injected into the heart to facilitate flow into the renal vessels, allowing heparin to perfuse into the renal vessels. The abdominal cavity was opened, and the left kidney was collected. The collected kidneys were perfused for 9 h using detergents, such as Triton

X-100 and sodium dodecyl sulfate, to decellularize the tissue. Decellularized kidney scaffolds were then gently washed with 1% penicillin/streptomycin and heparin to remove cellular debris and chemical residues. Transplantation of stem cells with the decellularized vascular scaffolds is expected to facilitate the generation of new organs. Thus, the vascularized scaffolds may provide a foundation for tissue engineering of organ grafts in the future.

INTRODUCTION:

Cell culture techniques are applied for regeneration of functional tissues and organs to replace diseased or damaged organs. Allogenic organ transplantation is currently the most common treatment for irreversible organ damage; however, this approach requires the use of immunosuppression to prevent rejection of the transplanted organ. Moreover, despite advances in transplant immunology, 20% of transplant recipients may experience acute rejection within 5 years, and within 10 years after transplantation, 40% of recipients may lose their transplanted graft or die[1].

Advances in tissue engineering technologies have yielded in a new paradigm for transplantation of new organs without immune rejection using differentiated stem cells. After stem cell differentiation, a scaffold, called a synthetic extracellular matrix, is needed to facilitate the generation of three-dimensional organs and enable the new tissue to thrive within the recipient. Scaffolds from decellularized native organs have advantages, including a more effective environment for establishment of cells and enhancement of stem cell proliferation, although these mechanisms have not been fully elucidated[2]. In particular, the kidney is a suitable organ for scaffold generation because it has abundant circulation and a niche for stem cell establishment. Additionally, because of the complex structure of the kidney, it is difficult to artificially regenerate kidneys for organ transplantation.

In this report, we introduce a method of developing vascularized scaffolds using decellularized organs in a rat model to facilitate future animal studies for tissue engineering purposes.

PROTOCOL:

This study was approved by the administration of Pusan National University of Medicine and was conducted in accordance with ethical guidelines for the use and care of animals. (certificate no. 2017-119). Prior to any animal studies, institutional approval should be obtained.

NOTE: All surgical and anesthetic instruments/equipment and reagents recommended for successful surgical presentation and imaging of abdominal organs are detailed in **Table 1**.

1. Preparation procedures for harvesting of rat kidneys

1.1. In preparation for surgery, place 8-week-old Sprague-Dawley rats (weighing 200–250 g) on a warming pad. Place a rectal thermometer probe in the rectum to monitor core temperature.

1.2. Anesthetize the rat with a 5% mixture of isoflurane gas (induction: 5%, maintenance: 3%).

1.3. To start the operation, place the rat in a supine position after administration of anesthesia. Mount the four limbs of the rat on the operation table with tape.

1.4. Shave and clean the abdomen of the donor rat with germicidal soap. Apply 2% betadine for at least 1–2 min, and wipe with a 70% ethanol solution. Repeat this sequence three times.

1.5. Cover the operative field with a sterile fenestrated drape.

1.6. Make a vertical abdominal incision and expose the left kidney, ureter, abdominal aorta, and inferior vena cava.

1.7. Visualize and dissect the left kidney, ureter, abdominal aorta, and inferior vena cava just before cutting the pedicle.

2. Transcardial perfusion

2.1. Before surgery, prepare the perfusion solution.

2.1.1. Make 50 mL of perfusion solution per rat.

2.1.2. Mix 1x PBS with approximately 10 U/mL heparin (1 25 kU vial will make 2.5 L of PBS+Hep).

2.1.3. Mix equal volumes of 8% paraformaldehyde with 1x PBS to make the 4% PFA/1xPBS solution.

NOTE: 8% PFA made in water can be stored at 4 °C for up to 2 months. However, 4% PFA diluted in PBS is only stable for 1 week at 4 °C. Make the dilution fresh.

2.2. Extend the vertical abdominal incision cranially. Be sure to draw the scissors away from the organs when cutting to avoid damaging the internal organs.

2.3. Continue the incision through the rib cage, and then cut through the diaphragm by lifting the sternum.

2.4. Pin the loose flap of skin out of the way, and free the heart by tearing any connective tissue with the forceps.

CAUTION: **Do not use the scissors to free the heart** and this could result in unwanted bleeding.

2.5. Open the phosphate-buffered saline (PBS) line and ensure that the line is flowing before placing the needle into the left ventricle. Hold the heart gently with blunt forceps, and use a hemostat to control the needle. The needle should be inserted no more than 1/4 inch.

NOTE: Insertion greater than 1/4 inch may result in perforation to the other side of the tissue.

2.6. While supporting the heart with the needle and hemostat, locate the right atrium and snip through it with iridectomy scissors. Rest the hemostat on the rat's body, and make sure the needle is still positioned inside the heart.

NOTE: If the cut is sufficient, there should be blood in the body cavity as the pressure from the PBS flowing into the rat is relieved.

2.7. Carefully unpin the front feet and skin flap.

2.8. Continue perfusing PBS for 4 min or longer if there is still blood visible in the kidney and liver.

3. Kidney harvesting and decellularization

3.1. Harvest the left kidney with the abdominal aorta and inferior vena cava.

3.2. Ligate the ureter, thoracic aorta, superior vena cava, and branches of the abdominal aorta.

3.3. Keep the organ hydrated in Dulbecco's PBS (DPBS) in a 10 cm Petri dish.

3.4. Cannulate the abdominal aorta and inferior vena cava with a 23 Gauge catheter. To remove residual blood, connect the cannula with a peristaltic pump, and wash with DPBS (500 mL) and 16 U/mL heparin for 90 min at a rate of 5 rpm at 37 °C.

3.5. To decellularize the kidney, perfuse the kidney with 1% Triton X-100 (1 L) for 3 h and then with 0.75% sodium dodecyl sulfate (SDS) solution (2 L) for 6 h at a constant pressure of 40 mmHg.

NOTE: The kidney will become transparent after 8 h.

3.6. To remove residual SDS, perfuse the sample with 1% penicillin in distilled water (6 L) for 18 h (overnight) and then with sterile DPBS (500 mL) and 16 U/mL heparin for 90 min.

REPRESENTATIVE RESULTS:

The gross morphology of rat kidneys was dark red (**Figure 1A**). After decellularization, the kidney became pale and translucent (**Figure 1D**). Residual genomic DNA was assessed with a commercial kit according to the manufacturer's instructions, in decellularized kidney scaffolds and compared with that in native kidneys (control). Quantitative analysis confirmed that tissue genomic DNA was almost eliminated after decellularization. From 14 cases, the average DNA contents were 115.05 ng/μL for the control and 1.96 ng/μL for the decellularized scaffold. In total, 98.3% of DNA was removed (**Figure 2**), although the three-dimensional structure was maintained, and acellular gromeruli were preserved in the cortical parenchyma (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1. Rat kidneys subjected to renal arterial perfusion decellularization. (**A**) Immediately after the start of decellularization. (**B**) After Triton X-100 treatment. (**C**) After SDS buffer treatment. (**D**) After overnight scaffold washing.

Figure 2. DNA concentrations in control and decellularized rat kidneys, showing reduced DNA contents after decellularization.

Figure 3. Hematoxylin and eosin staining of control and decellularized kidney samples. (**A**) control cortex (**A`**) decellularized cortex (**B**) control medulla (**B`**) decellularized medulla (**C**) control vein (**C`**) decellularized vein. Scale bar, 100 μm.

DISCUSSION:

Various protocols have been used for decellularization of organs and other tissues. The optimal decellularization protocol should preserve the three-dimensional architecture of the extracellular matrix (ECM). In general, such protocols consist of lysing the cell membrane by physical processing or ionic solutions, dissociating the cytoplasm and nucleus from the ECM by enzymatic processing or detergents, and then removing cellular debris from the tissue[3]. Physical processes include scraping, solution agitation, pressure gradients, snap-freezing, nonthermal permanent electroporation, and supercritical fluids[2]. Cells on the external surface of a tissue or organ, such as the skin or small intestine, can be efficiently removed by mechanical processes combined with enzymes[4]. Ionic or nonionic detergents dissolve DNA/protein interactions, lipids, and lipoproteins, but can damage the ECM structure[5]. Enzymes remove the dissociated cytoplasm and nuclear material, but leave these materials in the ECM, which can cause an immune response[6]. The optimal agents for decellularization are determined by tissue thickness and density or the clinical use of the decellularized tissue.

For decellularization, we used a combination of nonionic and ionic detergents: Triton X-100 and SDS. Triton X-100, as a nonionic detergent, effectively disrupts lipid/lipid and lipid/protein interactions. However, Triton X-100 may also destroy the ECM ultrastructure owing to loss of glycosaminoglycan (GAG), laminin, and fibronectin contents. SDS, as an ionic detergent, effectively removes nuclear remnants and cytoplasmic proteins, but also disrupts the ECM ultrastructure by loss of GAG and collagen[3]. Although these agents destroy the microstructure of the ECM, SDS and Triton X-100 successfully remove all DNA contents[7,8]. This is essential because remaining DNA content within a scaffold can cause immune rejection. In tissue that has been properly decellularized, the DNA content should be less than 50 ng/mg[9,10].

In the method, the pressure of the decellularization perfusion was 40 mmHg. Pressure control is required for decellularization perfusion. The optimal perfusion pressure varies from organ to organ, and 60 mmHg is the optimal pressure for human and porcine kidney or heart decellularization[11]. In rats, 40 mmHg is considered sufficient for decellularization perfusion[12].

One promising treatment for replacing allograft transplantation is transplantation of stem cells using a vascularized scaffold. We hope that this protocol for organ decellularization may provide

a foundation for future tissue engineering studies.

ACKNOWLEDGMENTS:

This study was supported by a Biomedical Research Institute Grant from Pusan National University Hospital.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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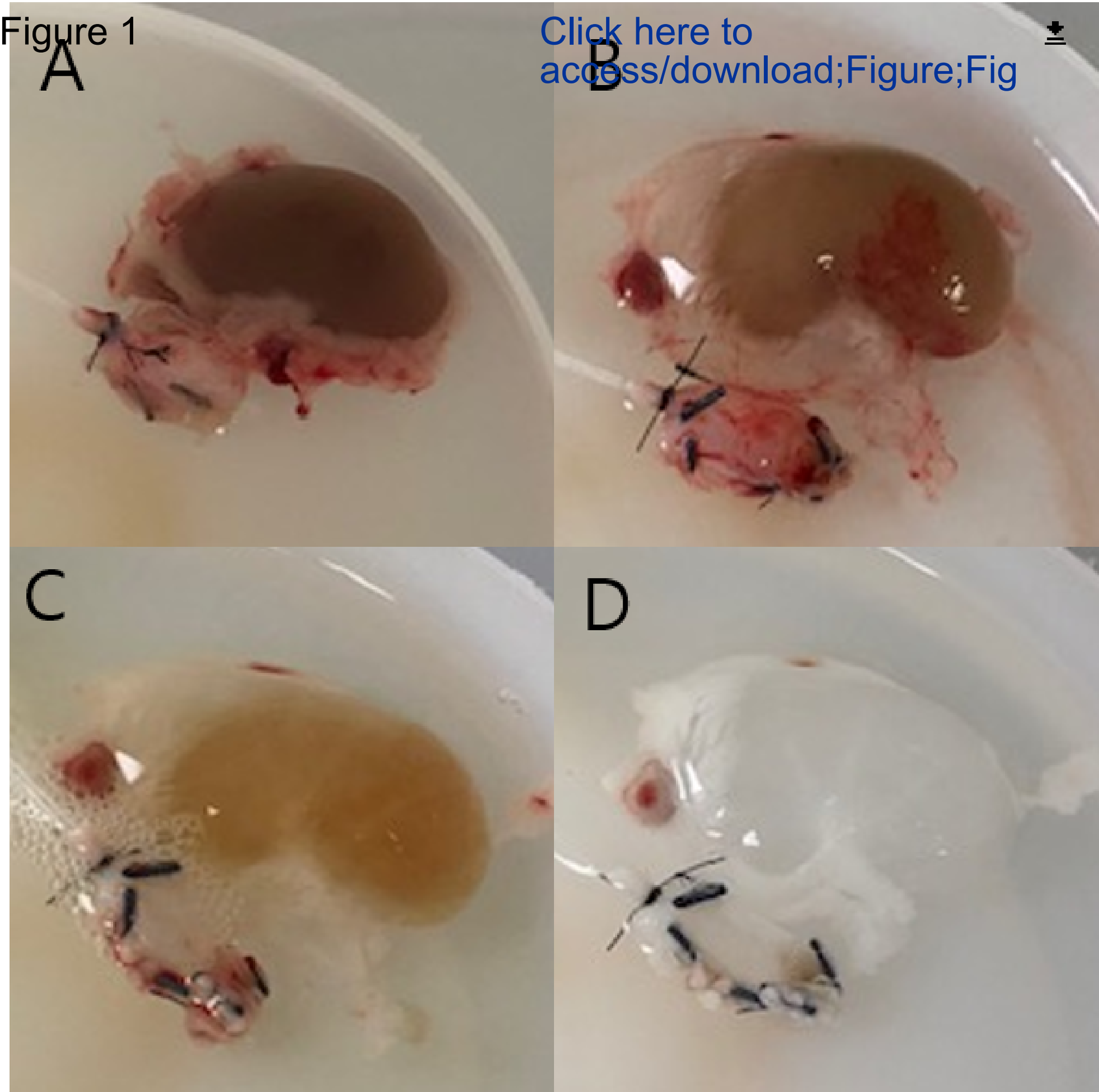

Figure 2

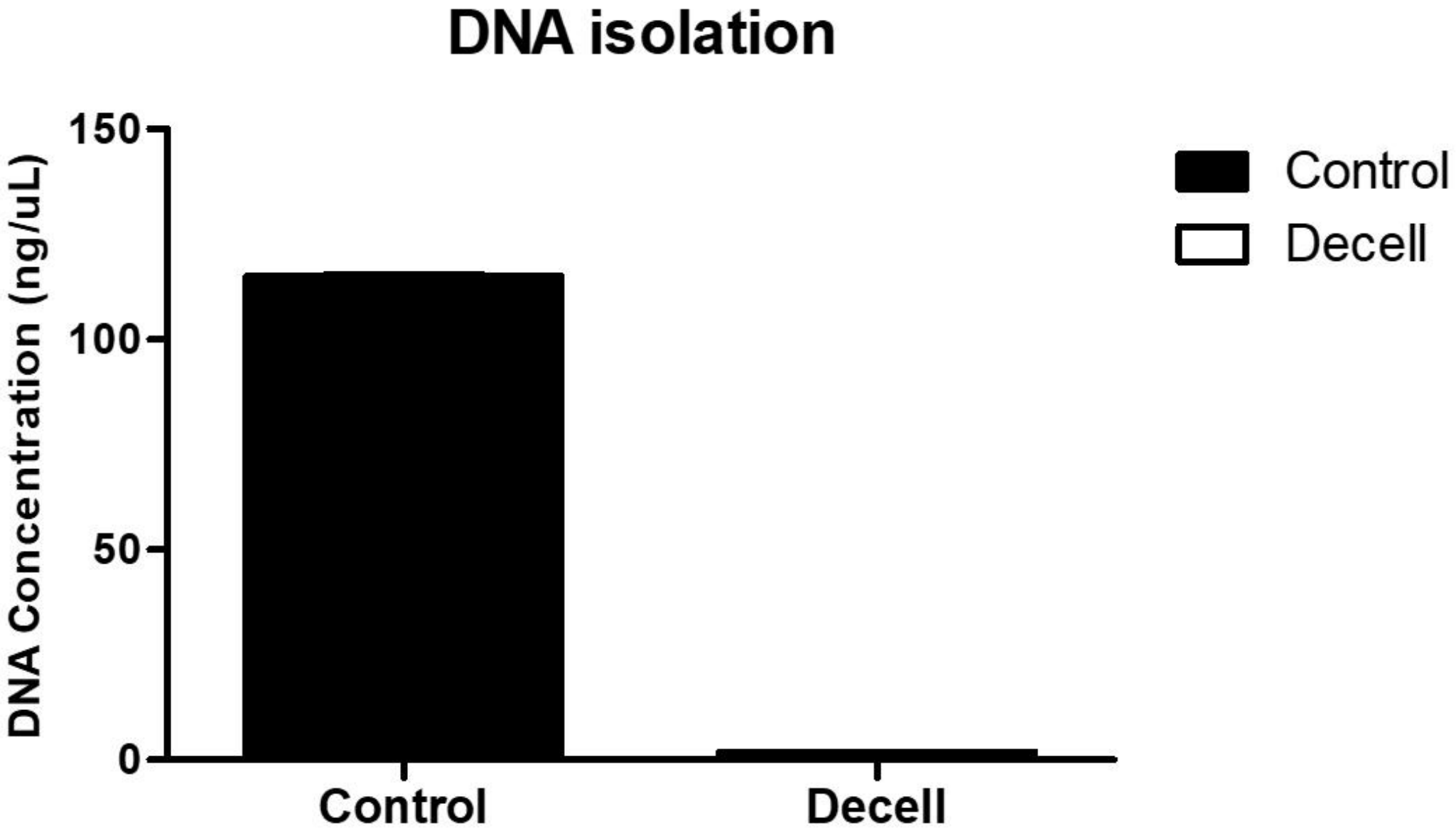

Figure 3

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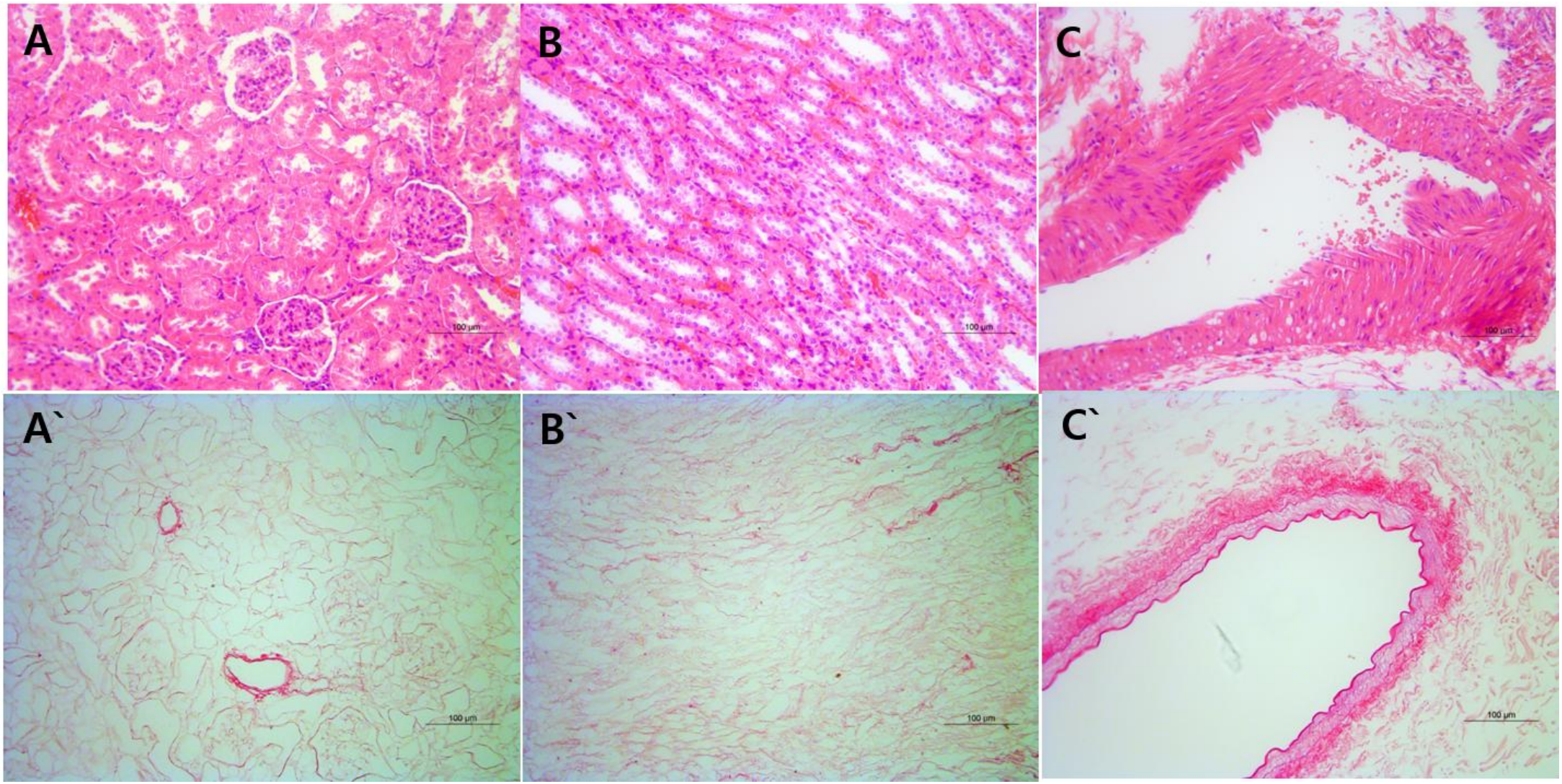

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 cc syringe (inject probes and vehicle solutic	Becton Dickins	305217	
10-0 ethilon for vessel anastomosis	Ethicon	9032G	
25 gauge inch guide needle(for vascular cath	Becton Dickins	305145	
3-0 PDS incision closure rat	Ethicon	Z316H	
3-0 Prolene incision closure rat	Ethicon	8832H	
3-0 silk spool vascular access/ligation in rat	Braintree Scier	SUT-S 110	
4-0 PDS incision closure mouse	Ethicon	Z773D	
4-0 Prolene incision closure mouse	Ethicon	8831H	
5-0 silk spool vascular access/ligation in mou	Braintree Scier	SUT-S 106	
Fine Scissors to cut fascia/connective tissue	Fine Science T	14058-09	
Halsey needle holder	Fine Science T	12001-13	
Kelly Hemostat for rats: muscle clamp to mini	Fine Science T	13018-14	
Polyethelyne 50 tubing, catheter tubing 100 ft	Braintree Scier	.023" × .038"	
Schwartz microserrefine vascular clamps	Fine Science T	18052-01 (straight) 18052-03 (curved)	
Surgical Scissors to cut skin	Fine Science T	14002-12	
Vannas-Tubingen Spring scissors for arteriot	Fine Science T	15003-08	

September 22, 2020

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[Designation]
Journal of Visualized Experiments

Dear Editor,

We are writing to thank you and the reviewers for the positive feedback regarding our manuscript (JoVE61856). We wish to express our appreciation for your in-depth comments, suggestions, and corrections, which have greatly improved the manuscript. We also thank the reviewers for constructive critique and their valuable comments that have helped us improve the quality of our manuscript. We have extensively revised our manuscript to address the concerns raised by the reviewers. Our point-by-point responses to the reviewers' comments are provided on the following pages.

We hope that the revised manuscript is now considered suitable for publication in *Journal of Visualized Experiments*. We look forward to hearing from you at your earliest convenience.

Sincerely,

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Responses to the comments of Reviewer 1

I thank the authors for addressing the comments.

The quality of the videos is not good. And the use of only adipose derived stem cells can convey the misconception of complete organ regeneration. Consider including in the discussion the article of Song et al., 2013 (DOI: 10.1038/nm.3154).

Response: We thank the reviewer for these comments. Thank you for your comments. First, regarding the quality of the video, we have submitted new video with higher quality in collaboration with the JoVE team. Second, we have excluded the text regarding the procedure for adipose-derived stem cell transplantation to avoid confusion and have instead focused on the decellularization procedure. Finally, we have carefully read and reviewed the article by Song et al. (2013), and we had actually referred to this article when we planned the study. Accordingly, we included a discussion of the article in the revised manuscript.

Major Concerns:

1) The videos are not of good quality. The footage was "shaky" and the focus was not always adequate. If possible, redo the footage using a tripod or camera mount.

Response: We appreciate this comment from the reviewer. We agree that the quality of the video could have been better. Accordingly, we filmed a new video in collaboration with the JoVE team.

2) Replace figure 3, as this appears to be a typical image of adipose tissue. Insert an image of the cortical tissue, preferably where the glomeruli appear. It is also without scale barr.

Response: We appreciate this comment from the reviewer. As suggested, we have replaced Figure 3 with an image of cortical tissue including glomeruli and have included a scale bar.

3) In the Protocol, 3.4-3.5: What was the flow used for the perfusion of the solutions (detergents, etc.), please include this information. Was the perfusion pressure controlled?

Response: We thank the reviewer for these questions. The perfusion pressure was controlled to 40 mmHg. We have included this information and additional information regarding the perfusion in the revised manuscript (page 2, lines 125-126).

4) Please insert the extraction protocol and measure the concentration of DNA (may be in supplementary material).

Response: We thank the reviewer for this comment. We used commercial kits for DNA extraction and followed the instructions provided with the kits. We would be happy to include this information, if the reviewer still thinks this detail is required.

Minor Concerns:

1) Please review the sentence (in Abstract Methods): "Decellularized kidney scaffolds were gently washed with 1% penicillin / streptomycin and heparin to remove all of the cellular debris and chemical residues."

For the "layman", the phrase can convey the idea that the 1% penicillin / streptomycin solution is to remove all of the cellular debris and chemical residues. What kind of solution? Why dextrose water? Perhaps the sentence can be deleted without prejudice to the abstract, or insert the sterilization and water type information.

Response: We thank the reviewer for this comment. We apologize for the error. Distilled water, not dextrose, was used. We have corrected this in the revised manuscript accordingly.

2) 2.9: "Open 4% paraformaldehyde (PFA) line and make sure it is flowing."

3) Why was PFA used? For histological analysis? If yes, please include this information. Or was the fixation used before step 3?

Response: We appreciate this comment form the reviewer. This information wad deleted because it was not meant to be included.

4) The question of the concentration of residual DNA is a little bit complex than it seems ... Each decellularization is one, and the protocol is not always repeated for each sample (Pereira et al., 2019. DOI https://doi.org/10.1038/s41598-019-48659-3). Please include the sample size in the figure 1.

Response: We appreciate this comment and agree with the reviewer. We have included the sample size (14 cases) as requested.

Responses to the comments of Reviewer 2

I thank the authors for addressing the comments.

Major Concerns:

General comments:

1. The manuscript needs editing for the use of English.

Response: We thank the reviewer for this comment. We have had the manuscript checked by at least two professional editors, both native speakers of English, to ensure appropriate language and grammar. The editing certificate has been included.

2. The manuscript needs to be checked carefully for correct spelling of English words (e.g. line 43 "three-dementional" should be "three-dimensional"; line 122 "decelluarization" should be "decellularization"; line 168 "form" should be "from").

Response: We thank the reviewer for this comment. We have carefully revised the manuscript to ensure that there are no spelling or grammatical errors throughout the text.

3. The title is awkward-why is it posed as a question?

Response: We thank the reviewer for this comment and agree with the reviewer's suggestion. Accordingly, we have corrected the title to improve impact and clarity.

4. The introduction is very short and should be expanded further to establish the significance

of research with decellularized organs, particularly the kidney.

Response: We appreciate this comment from the reviewer. Accordingly, we have expanded the Introduction as suggested.

5. The discussion is very short and should be expanded further.

Response: We appreciate this comment from the reviewer. Accordingly, we have expanded the Discussion as well.

6. The use of references is not appropriate. The reference list is very short, with only 6 cited papers and the references are ~10 years old. The reference list should be expanded significantly and current literature should be included.

Response: We appreciate this comment from the reviewer. As suggested, we have expanded the reference list and included more recent literature.

7. Throughout the protocol: add a period at the end of every step. This is not consistent throughout the manuscript.

Response: We thank the reviewer for this comment. We have checked that there is a period at the end of each step and have reviewed the manuscript for consistency.

Specific comments:

1. Introduction: please address why the rat is a good model for kidney studies.

Response: We appreciate this comment from the reviewer. We chose rats as a model animal to facilitate future animal studies of tissue engineering. This information has been added to the Introduction accordingly.

2. Line 100, Step 2.3: please explain incision more specifically, clarify "to just aside the lungs."

Response: We thank the reviewer for this comment. The description was not necessary and was therefore removed.

3. Line 136, Step 4.1: please describe the knife biopsy procedure.

Response: We thank the reviewer for this comment. The previous reviewer suggested that inclusion of information regarding recellularization with adipose-derived stem cells would confuse readers and convey the misconception that we performed complete organ regeneration. Accordingly, in the revised manuscript, we have excluded the procedure for adipose-derived stem cell transplantation and instead focused on the decellularization procedure. Therefore, this part was excluded.

4. Line 147, Step 4.9: please explain further. Are the stem cells in the supernatant, the pellet?

Response: We appreciate this comment from the reviewer. Because we have focused on the decellularization procedure, we have excluded this section.

5. Line 153, Step 5.2: please clarify the penicillin-streptomycin solution—what concentration and are these antibiotics in 1X PBS?

Response: We appreciate this comment from the reviewer. Because we have focused on the decellularization procedure, we have excluded this section.

6. Line 181, Step 6.13: please describe the post-operative analgesic for the rat. There should be alleviation of pain following the surgery and monitoring of the animal for pain and distress.

Response: We appreciate this comment from the reviewer. Because we have focused on the decellularization procedure, we have excluded this section.

7. Line 216: "Previously various protocol for decellularization were developed." Citations are necessary to support this statement. The authors should address how previous protocols are similar or different to the procedures that they are describing in the manuscript.

Response: We thank the reviewer for these comments. We have added more references to support the text as requested. Additionally, we have attempted to explain how previously published protocols are similar to or different from our procedure as suggested.