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TITLE:

A Simplified Model for Heterotopic Aortic Valve Transplantation in Rodents

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SUMMARY:

This protocol describes a simple and efficient method for the transplantation of aortic valve leaflets under the renal capsule to allow for the study of alloreactivity of heart valves.

ABSTRACT:

There is an urgent clinical need for heart valve replacements that can grow in children. Heart valve transplantation is proposed as a new type of transplant with the potential to deliver durable heart valves capable of somatic growth with no requirement for anticoagulation. However, the immunobiology of heart valve transplants remains unexplored, highlighting the need for animal models to study this new type of transplant. Previous rat models for heterotopic aortic valve transplantation into the abdominal aorta have been described, though they are technically challenging and costly. For addressing this challenge, a renal subcapsular transplant model was developed in rodents as a practical and more straightforward method for studying heart valve transplant immunobiology. In this model, a single aortic valve leaflet is harvested and inserted into the renal subcapsular space. The kidney is easily accessible, and the transplanted tissue is securely contained in a subcapsular space that is well vascularized and can accommodate a variety of tissue sizes. Furthermore, because a single rat can provide three donor aortic leaflets and a single kidney can provide multiple sites for transplanted tissue, fewer rats are required for a given study. Here, the transplantation technique is described, providing a significant step forward in studying the transplant immunology of heart valve transplantation.

INTRODUCTION:

Congenital heart defects are the most common congenital disability in humans, affecting 7 in 1,000 live-born children each year[1]. Unlike adult patients in which various mechanical and

bioprosthetic valves are routinely implanted, pediatric patients currently have no good options for valve replacement. These conventional implants do not have the potential to grow in recipient children. As a result, morbid re-operations are required to exchange the heart valve implants for successively larger versions as the children grow, with affected kids often requiring up to five or more open-heart surgeries in their lifetime[2,3]. Studies have shown that freedom from intervention or death is significantly poor for infants than older children, with 60% of infants with prosthetic heart valves facing re-operation or death within 3 years of their initial operation[4]. Therefore, there is an urgent need to deliver a heart valve that can grow and maintain function in pediatric patients.

For decades, attempts to deliver growing heart valve replacements have been centered on tissue engineering and stem cells. However, attempts to translate these valves to the clinic have been unsuccessful thus far[5–8]. For addressing this, a heart valve transplantation is proposed as a more creative operation for delivering growing heart valve replacements having the ability to self-repair and avoid thrombogenesis. Instead of transplanting the whole heart, only the heart valve is transplanted and will then grow with the recipient child, similar to conventional heart transplants or a Ross pulmonary autograph[9–11]. Post-operatively, recipient children will receive immunosuppression until the transplanted valve can be exchanged for an adult-sized mechanical prosthetic when the growth of the valve is no longer required. However, the transplant biology of heart valve transplant grafts remains unexplored. Therefore, animal models are needed to study this new type of transplant.

Several rat models have been previously described for heterotopic transplantation of the aortic valve into the abdominal aorta[12–18]. However, these models are prohibitively tricky, often requiring trained surgeons to operate successfully. Additionally, they are costly and time-consuming[19]. A novel rat model was developed to create a simpler animal model for studying the immunobiology of heart valve transplants. Single aortic valve leaflets are excised and inserted into the renal subcapsular space. The kidney is especially suited to study transplant rejection as it is highly vascularized with access to circulating immune cells[20,21]. While several others have utilized a renal subcapsular model to study the transplant biology of other allograft transplants such as pancreas, liver, kidney, and cornea[22–27], this is the first description of transplantation of cardiac tissue in this position. Here, the transplantation technique is described, providing a significant step forward in studying the transplant immunology of heart valve transplantation.

PROTOCOL:

The study was approved by the Committee of Animal Research following the National Institutes of Health Guide for Care and Use of Laboratory Animals.

1. Information on the animal model (Rats)

1.1 Use an operating microscope (see **Table of Materials**) with up to 20x magnification for all surgical procedures.

1.2 Use syngeneic (such as Lewis-Lewis) or allogeneic (such as Lewis-Brown Norway) strains

for the transplants as needed for the experiment.

1.3 Use rats of age between 5–7 weeks and bodyweight of 100–200 g that are appropriate for the experimental question.

2. Removal of fur, preparation of the skin, and anesthesia

2.1 Perform all operations under sterile conditions.

NOTE: The step is performed in a dedicated surgical space and under sterile conditions.

2.2 Place the rats into an anesthetic induction chamber and induce anesthesia with 5% isoflurane in oxygen. Maintain anesthesia with 3.5% isoflurane in oxygen throughout the procedure.

2.3 For the donor operation, remove the rat's fur from the umbilicus to the sternal notch using fur clippers. For the recipient operation, clip the hair over the surgical field at the posterior axillary line from the ribs to the pelvis. Next, prepare the skin with a surgical disinfectant.

2.4 Obtain a surgical plane of anesthesia before starting the procedure. Confirm adequate depth of anesthesia by firmly compressing the toes of the rat with forceps. If the rat withdraws to pain, titrate the anesthetic as needed.

2.5 Monitor the respiratory rate and the depth of anesthesia clinically throughout the procedure; the level of isoflurane is adjusted as needed to maintain a breathing rate of 55–65 breaths/min.

3. Donor operation

3.1 Prepare and anesthetize the rat as stated in step 2. Incise the skin from the xiphoid to the sternal notch using dissecting scissors. Perform a sternectomy by cutting the ribs on each side lateral to the sternum until optimal access to the heart is achieved.

3.2 Heparinize the rat with a 100 U/100 g of injection into the left atrium.

3.3 Sacrifice the donor *via* exsanguination.

3.4 Excise the thymus to improve the visualization of the great vessels. Then, remove the heart en bloc with the ascending aorta until the level of the innominate artery.

4. Preparation of aortic valve leaflets

4.1 Place the donor heart in a Petri dish immediately following the cardiectomy. Dissect the donor heart in an ice-cold cold storage buffer (see **Table of Materials**).

4.2 Using forceps and Vannas spring scissors, dissect the donor heart until only the aortic root remains with a 1 mm ventricular cuff proximal to the aortic valve.

4.3 Open the aortic valve by making a longitudinal cut to open the Sinus of Valsalva between the left and non-coronary sinuses to visualize all three leaflets.

NOTE: The cut should be the entire length of the Sinus of Valsalva. The actual dimensions depend on the size of the rat.

4.4 Excise each aortic valve leaflet individually. Specifically, use blunt forceps to grasp the edge of the leaflet and use Vannas spring scissors to excise the leaflet by cutting from one commissure down to the annulus, and then toward the next commissure.

NOTE: Take special care to only grasp the edge of the leaflet to minimize disruption of the valvular endothelial cells.

4.5 Store the samples following leaflet excision in ice-cold storage buffer solution until they are ready to be implanted in the recipient rat. Implant all the leaflets within 4 h of cold storage.

5. Recipient operation

5.1 Prepare and anesthetize the rat as stated in step 2. Use a heating pad maintained at 36–38 °C to perform the surgery.

5.2 Administer buprenorphine (0.03 mg/kg subcutaneously) to all recipient rats before surgery and every 6–12 h post-operatively as needed to alleviate the pain.

5.3 Place the rat in a right lateral recumbent position to access the left kidney.

NOTE: The left kidney is preferred due to its more caudal position relative to the right kidney.

5.4 Incise the skin over the flank longitudinally over 1-inch using scissors.

NOTE: The incision must remain smaller than the size of the kidney to provide enough tension to prevent the kidney from retracting back into the abdominal cavity during the procedure.

5.5 Similarly, incise the underlying abdominal wall.

5.6 Externalize the kidney

5.6.1 Using the thumb and forefinger, apply light pressure dorsally and ventrally while using curved forceps to lift the caudal pole of the kidney through the abdominal and skin incision. Externalize the cranial end of the kidney similarly.

5.6.2 Alternatively, the kidney may be externalized by grasping the perirenal fat and pulling upward with light tension.

NOTE: Take care not to grasp the kidney or the renal vessels directly.

5.6.3 Once the kidney is externalized, keep it moist with warm saline trickled onto the kidney.

5.7 Create a subcapsular pocket.

5.7.1 Lightly apply pressure to the renal capsule using one set of blunt forceps so that the renal capsule can be clearly distinguished from the underlying parenchyma. Simultaneously using another set of blunt forceps, carefully grasp the capsule and gently pull upward to create a hole in the capsule.

NOTE: Due to the delicate nature of the capsule, minimal force is required to establish this incision.

5.7.2 Continue using blunt forceps to extend the incision until a ~2mm space has been created to accommodate the aortic valve leaflet.

5.7.3 Develop a shallow subcapsular pocket that is slightly larger than the valve leaflet while lifting the edge of the incision with one set of forceps and advancing a blunt probe under the renal capsule.

5.8 Transplant the aortic valve into the subcapsular pocket.

5.8.1 Retrieve the aortic leaflet from cold storage and place it in the surgical field.

5.8.2 While lifting the edge fibrous capsule, advance the aortic leaflet into the subcapsular pocket with blunt forceps.

NOTE: Ensure the tissue is far enough away from the incision so that it is firmly secured under the capsule. Care should be taken to avoid damage to the underlying parenchyma or further ripping of the fibrous capsule.

5.8.3 The incision in the renal capsule can be left open.

5.9 Push the kidney gently back to its anatomical position using counter traction applied to the incision edges.

5.10 Close the abdominal incision with a running sterile surgical suture. Close the skin with staples.

5.11 Post-operative care

5.11.1 Following the operation, place the rat in a clean cage on a heating pad with access to food and water.

5.11.2 Monitor the animal daily to assess for routine wound healing and signs of pain or distress. Remove the staples after 7–10 days.

6. Collection of tissue for analysis

6.1 At selected endpoints after transplantation, euthanize the animal by exsanguination. Specifically, perform a median laparotomy and transect the abdominal aorta under 5% isoflurane in oxygen.

6.2 Mobilize the kidney and excise it by cutting the renal artery, vein, and ureter with scissors.

NOTE: Take care not to grasp the area containing the transplanted leaflet.

6.3 Place the kidney in formalin overnight, embed it in paraffin, and section it for the desired staining. Orient the specimen with the kidney capsule facing anteriorly and the kidney parenchyma facing posteriorly.

REPRESENTATIVE RESULTS:

A graphical depiction of the experimental design is provided for the rat model (**Figure 1**). Additionally, an aortic root dissected from the donor's heart and an individual aortic valve leaflet prepared for implantation is also shown in **Figure 2**. Next, a representative image of the position of the aortic valve leaflet under the renal capsule for implantation is shown in **Figure 3A** and after 3, 7, and 28 days within the recipient rat (**Figures 3B–D**), demonstrating the ease of locating and recovering the transplanted tissue.

The aortic valve leaflets retain their native architecture after heterotopic transplantation in syngeneic animals, demonstrating the utility of this model as a baseline to compare the immune response in allogeneic transplants. Specifically, histology with hematoxylin and eosin (H&E) staining revealed that valve leaflets in syngeneic transplants after 7 days were structurally intact with no signs of edematous swelling (**Figure 4A**). The structural integrity of the valve leaflet was further confirmed by immunohistochemistry for Alpha Smooth Muscle Actin (aSMA) and CD31 (**Figure 4B**).

FIGURE LEGENDS:

Figure 1: Experimental design of the heterotopic transplantation of the aortic valve under the renal capsule in rats. The heart is collected from the donor rat (**A**). The aortic valve leaflets are dissected and kept in cold storage (**B**) until the implantation process under the renal capsule in the recipient rat (**C**). The leaflets are then explanted at set time points and analyzed

microscopically (**D**).

Figure 2: Preparation of aortic valve leaflet for implantation. Example of an aortic root dissected from the donor heart (**A**) and further dissection of an aortic valve leaflet for implantation (**B**).

Figure 3: Visualization of aortic valve leaflet under the renal capsule. The aortic valve leaflet is visualized under the renal capsule at implantation (**A**), after 3 (**B**), 7 (**C**), and 28 days (**D**) in syngeneic animals and after 3 (**E**), 7 (**F**), and 28 days (**G**) in allogeneic animals.

Figure 4: Aortic valve leaflets remain structurally intact after transplantation under the renal capsule for 7 days in syngeneic animals. The top row shows H&E staining and immunostaining for DAPI, aSMA, and CD31 for control heart valves that were procured but not transplanted. The bottom row shows H&E staining and immunostaining for DAPI, aSMA, and CD31 in a syngeneic valve leaflet explanted after 7 days.

DISCUSSION:

Importance and potential applications

While mechanical and bioprosthetic heart valves are routinely used in adult patients requiring valve replacement, these valves lack the potential to grow and, therefore, are suboptimal for pediatric patients. Heart valve transplantation is an experimental operation designed to deliver growing heart valve replacements for neonates and infants with congenital heart disease. However, unlike the transplant immunobiology of conventional heart transplants, the transplant immunobiology of this new type of transplant remains poorly explored. Here, a unique rat model for subcapsular renal transplantation of aortic valve leaflets is described, providing a significant step forward in studying the transplant immunobiology of heart valve transplantation.

The renal subcapsular space provides an optimal environment to study transplant immunobiology of heart valves. The transplanted tissue is securely contained in a well-vascularized location with access to circulating immune cells[20]. Additionally, subcapsular models have previously been successfully utilized to test allograft rejection in many tissues such as the pancreas, liver, kidney, and the other cell types[22–27], indicating this model is justified in studying the immunogenicity of aortic valve leaflets.

This model has several protentional applications for studying the transplant immunology of aortic valves. First, the model may be used to determine the level of systemic immune suppression required for heart valve transplantation to prevent graft rejection, such as tacrolimus, mycophenolate, and steroids. Furthermore, several studies have indicated that valve tissue may be immunologically distinct from other heart tissue, as the valves are relatively spared during fulminant rejection of conventional heart transplants[28–30]. This model allows for exploration of this concept as the subcapsular space can accommodate various tissue types, such as valve leaflet and myocardium, to compare the immunogenicity of these tissues.

This model is advantageous because it is technically straightforward, quick, and has a high

survival rate with a low risk of complications. Because each donor can provide three aortic valve leaflets, one rat can serve as the donor for three different recipients. On average, the length of the donor operation was 27.2 min (n = 12), and the duration of the recipient operation was 29.7 min (n = 36). The survival rate of the recipient operation was 97.2% (n = 35/36), with one intraoperative death due to respiratory depression. Minimal bleeding due to trauma to the renal parenchyma while creating the subcapsular pocket was noted in 11.1% of recipient operations. However, the bleeding was easily controlled in all cases with compression from a cotton tip applicator. One sample was dislodged from the subcapsular space and not recovered upon explanation even after 7 days.

Previously, the valve leaflets were explanted by removing them from the subcapsular space and embedded, sectioned, and stained without any attached aortic tissue. However, this method is suboptimal as the leaflets themselves are extremely small, thin, and transparent, resulting in the loss of several samples in processing. Instead, it is recommended to remove the kidney en bloc and embed and section the tissue while still secured under the renal capsule to ensure no samples are lost. Additionally, this approach minimizes the trauma and manipulation of the leaflet.

Critical steps

The critical steps of the procedure are to establish a surgical plane of anesthesia, incising the abdominal wall over the kidneys, eviscerating the kidney, raising the subcapsular flap, insertion of the heterotopic transplant tissue, obtaining hemostasis, returning the kidney to the anatomical position, and closing the skin.

Modifications and troubleshooting

While this is the first description of transplantation of heart tissue under the renal capsule, several others have described transplantation of other tissue types in the renal subcapsular space[20,22–27]. In this protocol, minor adjustments were made to previous subcapsular models to optimize the technique and minimize complications. Specifically, while others have recommended using Vannas spring scissors to make the initial incision into the renal capsule[20,26], this method is more likely to cause trauma to the underlying parenchyma and result in subcapsular hematoma formation. Too much bleeding will result in distention of the capsule and compromise the security of the transplanted[26]. Therefore, blunt forceps should be used to open the capsule. Additionally, while some protocols advocate for the placement of commercial products with homostatic property over the capsular incision[26,31], this step is unnecessary as long as the tissue is advanced far enough into the subcapsular pocket.

In larger rats, the kidney may be covered in perirenal fat, and externalizing the kidney *via* lifting with curved forceps may not be feasible. In these cases, it is best to externalize the kidney by gently tugging the perirenal fat with forceps and pulling the kidney out of the abdominal cavity without causing damage or bleeding.

Comparison with existing heterotopic transplant models

While several other animal models for heterotopic aortic valve transplantation have been

previously described[12–18], the current protocol provides a straightforward and more practical alternative that improves previous models in several ways. First, due to the technically simple nature of the procedure, very little training is required to operate successfully. This is in stark contrast to previously described heterotopic aortic valve transplants into the abdominal aorta. Therefore, this model provides a more practical and cost-effective alternative to study aortic valve transplantation while minimizing the morbidity, pain, and mortality of the rats. Additionally, because only one aortic valve leaflet is needed for the recipient operation and each donor rat provides three leaflets, fewer donor rats are required for any given experiment. Furthermore, implanting tissue into the contralateral kidney or a separate subcapsular pocket may allow for internal control or comparison of immune responses to varying tissues within a single rat. In this case, the best approach is *via* a midline laparotomy incision.

In addition to the animal models describing heterotopic aortic valve transplantation into the abdominal aorta, other studies have utilized a subcutaneous model to study the immunogenicity of aortic valves[32]. While this approach is undoubtedly more straightforward than transplantation into the abdominal aorta, existing evidence suggests that subcutaneous implantation is a less effective method of antigen presentation[33,34]. The implanted specimen is also challenging to find and analyze. Therefore, the renal subcapsular space is proposed as a site of implantation that is both simplified yet optimal for studying aortic valve transplant biology.

In summary, the newly proposed model serves as an addition to scientists' armamentarium to study heart valve transplantation and supplements the previously described models.

Limitations

Although the transplantation of aortic valve leaflets under the renal capsule is an efficient method for studying alloimmunity *in vivo*, some limitations of this model exist. While the subcapsular space is well-vascularized, it does not offer the same hemodynamic environment as the sub-coronary position. This may affect the immune response to transplanted tissue. Some have hypothesized that the distinct immune properties observed in valve tissue may result from the high-pressure blood flow over the aortic valve in the sub-coronary position, nullifying the chemotactic response[28,35]. Furthermore, this model is insufficient to study the effect of alloreactivity on the valve function as the leaflets are not performing their physiologic function under the renal capsule. However, similar limitations exist for the heterotopic abdominal aorta transplant models as the success of these models relies on rendering the valve leaflets incompetent to avoid graft thrombosis [15,36].

Limitations to the protocol include the possibility of tissue becoming dislodged from the renal subcapsular space and un-recoverable (1 in 36 animals). Another limitation is the death of the animal during surgery (1 in 36 animals); however, the death was caused by the overdose of buprenorphine, and other methods for dosing of analgesia may be employed.

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DISCLOSURES:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1

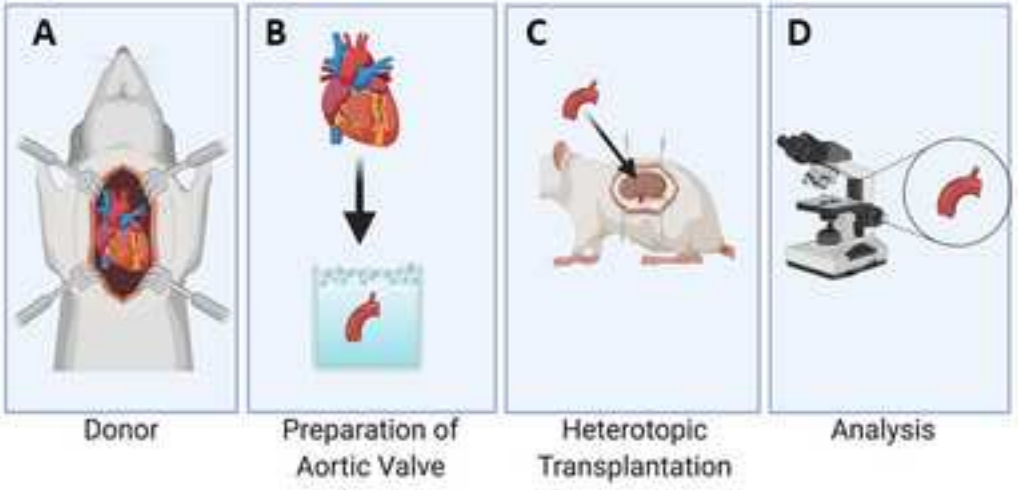

Figure 2

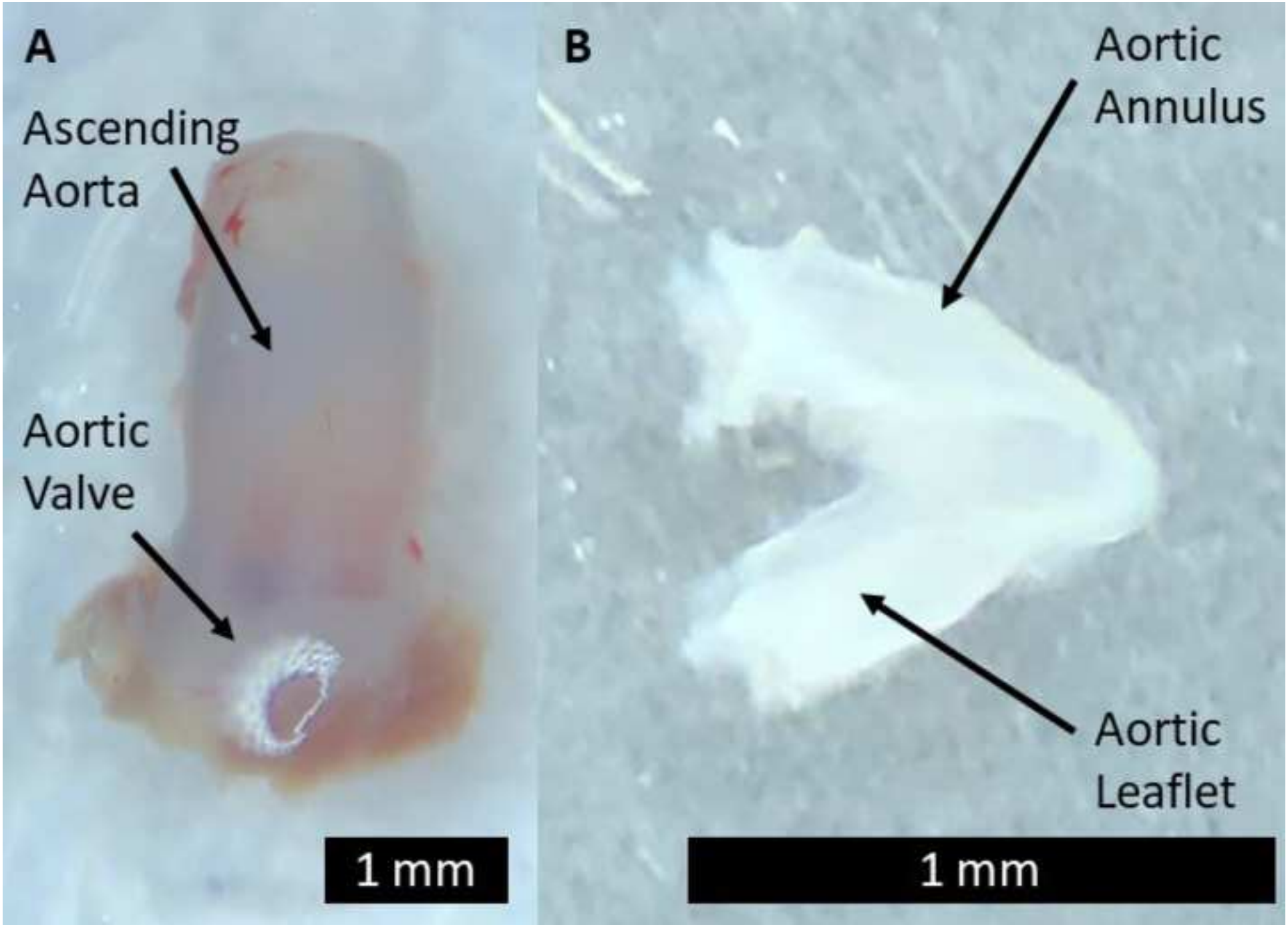

Figure 3

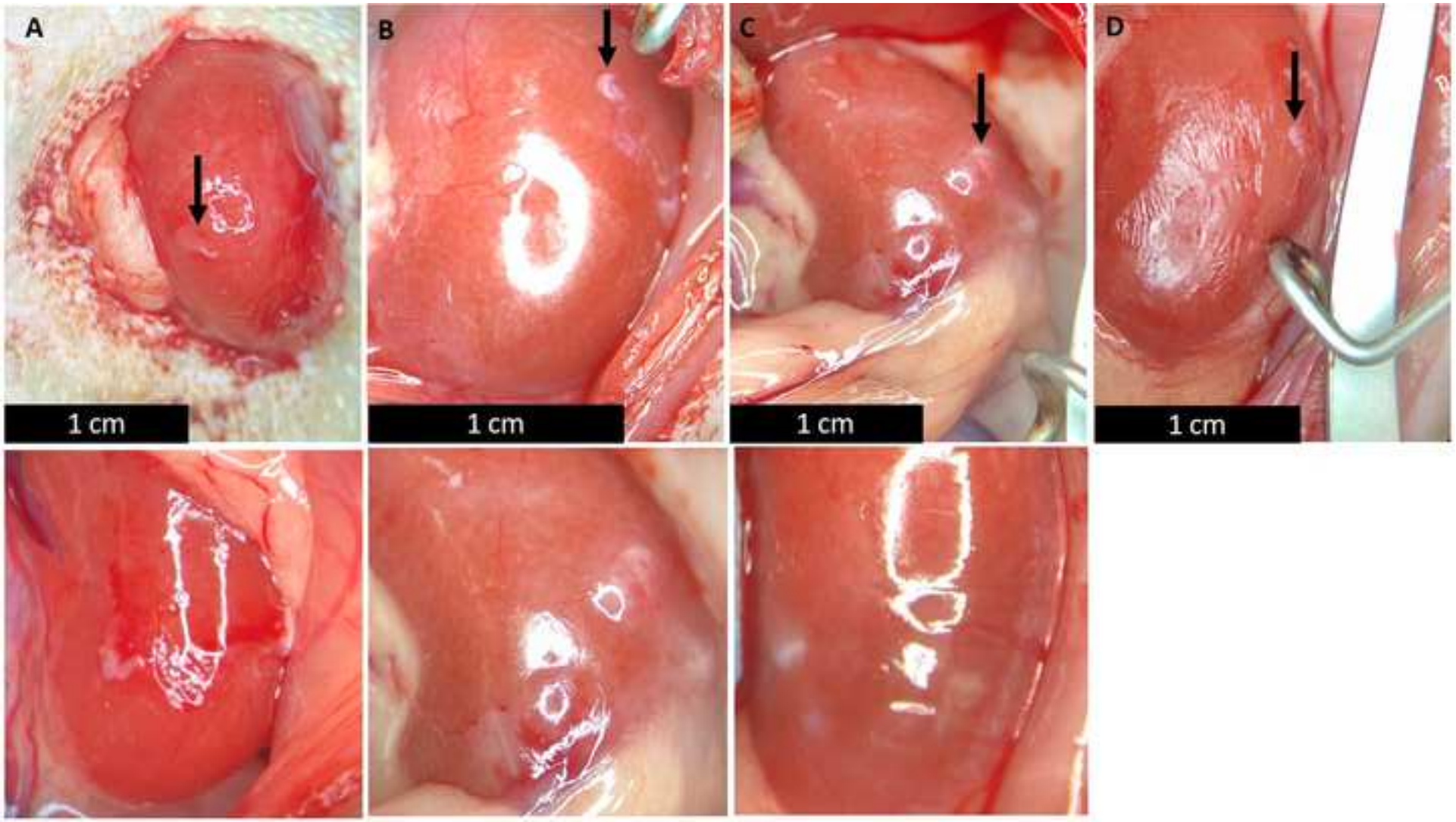

Figure 4

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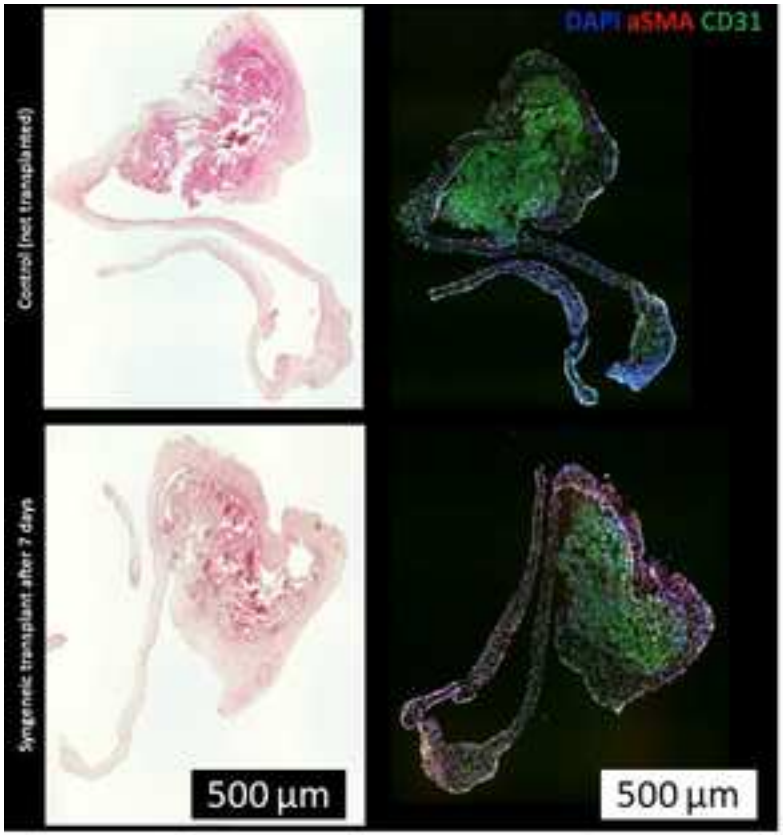

Revision Letter

Thank you for the opportunity to revise our manuscript. Please find the reviewer comments and detailed responses below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
RESPONSE: Thank you.

2. Please revise the following lines to avoid previously published work: 108-114, 153-154, 196-197.

RESPONSE: The changes were made.

3. Corresponding authors are different in the main manuscript (Dr. T. Konrad Rajab) and the Editorial software (Morgan Hill, where the authors give input while uploading the manuscript). Please clarify.
RESPONSE: Dr. Rajab is the corresponding author.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
RESPONSE: Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. Please sort the Materials Table alphabetically by the name of the material.

RESPONSE: There are no symbols and the table is already alphabetical. Therefore no further changes were made.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

RESPONSE: All steps contain less than 4 sentences and only 2-3 actions per step.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described

in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

RESPONSE: This was done.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

RESPONSE: Done.

9. Please add more details to your protocol steps:
Step 1.3: Please remove the name of the commercial place ,Charles River (Wilmington, MA) to the Table of Materials.

RESPONSE: Done.

Step 2.4: How much incision to be made?

RESPONSE: We clarified that this is done before an incision is made.

Step 2.5: Please specify how the respiratory rate and depth of anesthesia are monitored?

RESPONSE: We clarified that this is monitored clinically.

Step 3.1: Please mention how the sternectomy is performed. Else cite published References.
RESPONSE: We clarified that the sternectomy is performed by cutting the ribs.

Step 4.1: Please mention how the temperature is maintained.

RESPONSE: We clarified that the temperature is maintained by having the buffer on ice.

Step 5.7.1: Please specify the term “very little.” Can this be quantified?

RESPONSE: Yes. We clarified that the force should be as little as possible.

Step 5.10: Please remove the commercial term and use a generic term instead. Please add details in the Table of Materials.

RESPONSE: Polyglactin 910 was used instead of vicryl. The table was amended.

10. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and should be in line with the Title of the paper. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
RESPONSE: Done.

11. In the Discussion section, please include a paragraph on critical steps within the protocol
RESPONSE: Done.

12. Please add scale bars in the Figures.
RESPONSE: Thank you for this suggestion. Done.

13. Please spell out the journal titles in the References.
RESPONSE: This was done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a new and simple model of aortic valve leaflet implantation under the renal capsule in rats.

Major Concerns:

This nice experimental model of aortic avascular valve implant can be used by the scientists in their armamentarium, while exploring the complicated issue of valve replacement in newborn and pediatric cardiac surgery, but, for sure, cannot serve as a substitute to vascular models already described in the literature. These small laboratory animal models have intrinsically the bias of being heterotopic and it is indeed going too far, in my opinion, to not use at all the valves in their natural function. If we continue such reasoning and consider this model as an additional model and not an alternative one, the theory of using less animals cannot hold.

RESPONSE: Thank you for noting that this is a nice experimental model. We agree that it is useful in scientists' armamentarium. It is not intended to serve as a substitute the vascular models already described. Instead, it is intended to supplement them. This discussed in the section of the discussion entitled "*Comparison with existing heterotopic transplant models"*.

Minor Concerns:

Lines 61-68. I have some problems with this paragraph:

"partial transplantation"? Well, I do not understand why using this term if it means: "heart valve

transplantation" (I would say: implantation), as it is showed underneath.
RESPONSE: to clarify this point, we changed partial heart transplant to heart valve transplant.

"recipient children will receive immunosuppression…". Now, it seems immunosuppression is routinely used in valve implantation (or "transplantation", using authors terminology). To me, this is not true.
RESPONSE: This operation is different from artificial heart valve implantation. It is a heart valve transplantation and therefore it will require immunosuppression.

Lines 73-74: It is completely misleading to refer to Dr. Niimi paper as an example to show the difficulties of other models. Dr. Niimi's article does not describe a model of valve transplant, but of whole heart transplant. Furthermore, it uses another animal, 10 times smaller: the mouse.

RESPONSE: We used the Niimi paper as an example of neonatal rat heart valve transplant (neonatal rats are similar size to mice). However we agree that this may be misunderstood. Therefore we deleted this reference.

Lines 123-126: Please change place of 3.3 with 3.4. First exsanguinate (I do it by cutting the descending aorta); then remove the heart.

RESPONSE: Done

Recipient operation (from line 144 on):
It seems not clear to me if you put the leaflet in the anterior side or in the posterior side of the kidney. From the incision you describe, I can suppose it is put in the posterior side. The use of the anterior side is not described since, I suppose, you have to rotate the kidney and I am not sure if this is possible.
RESPONSE: Either side can be used depending on how the rat is positioned.

I take the opportunity here to notice that in Figure 1, the scheme of the recipient incision is completely different from the text (it is a xipho-pubic incision), so you need to change it.
RESPONSE: Thank you. Figure 1 was changed.

Line 178: "fibrous capsule". I do not understand what do you mean by "fibrous"?

RESPONSE: fibrous was deleted.

Lines 318-319: I mentioned above the article of Dr. Niimi: quite a different model in a different animal.
RESPONSE: agree, the reference to the Niimi article was deleted.

Lines 324-325. The use of contralateral kidney means another incision in the same animal, if we follow the text. I am not sure the Veterinary Service will be happy with it, or suggest anterior laparotomy: so,

another kind of surgery (supine position, not right and/or left lateral recumbent position; but only one, bigger incision, instead of two).

RESPONSE: We added the clarification that in this case, the best approach is via a midline laparotomy incision.

Figure 2B: I cannot see anything there.
RESPONSE: We added annotations to the figure to clarify.

Figure 3: The animal in this photo seems rather dehydrated. It needed some drops of saline over the tissues.
RESPONSE: This is how it looks during our experiments.

Page 18 of the .pdf file: I do not understand what is written here.
RESPONSE: Unfortunately, there is no page 18 in the submission PDF file.

Reviewer #2:
Morgan Hill et al. have created a novel model to investigate the immunological background of heterotopic valve transplantation. They have made a more practical, simpler and even cheaper surgical method compared to the already exsisting ones. The kidney is well suited to study transplant rejection as it is highly vascularized. The key point of this method is to implant the specimen carefully into the subcapsular pocket. The intervention is well planned both surgically and anaesthetically. Specimens are withdrawn after 7-10 days, then immunohistochemical methods are used to confirm the extent of rejection through certain tests.

RESPONSE: Thank you

Minor comments
1. It is a bit controversial that the immunological background of transplantation is being investigated on the valves, although it is described in the article that the valves are substantially spared during fulminant rejection.
RESPONSE: We agree, and point out that this is an area of active investigation.

2. I think it is important to describe what kind of immunosupressive treatment would be used. Most of the drugs used in adult heart transplantation have adverse effects on the kidneys (eg.: Cyclosporine, Tacrolimus, Daclizumab, Everolimus).

RESPONSE: We added the clarification that the immune suppressants would be tacrolimus, mycophenolate and steroids.

Questions:

1. Why these biomarkers were chosen (CD31, sSMA, DAPI)? Would you consider examining other biomarkers such as circulating DNAs and RNAs?

RESPONSE: These biomarkers were chosen because of their biological relevance and because our laboratory has experience ein this area. Other biomarkers such as DNAs and RNAs could be considered but we don't have experience with these.

2. Have you tested the tensile strength and the biomechanical characteristics of the valves removed after 7-10 days?

RESPONSE: We did not, but this is an interesting point that could be studied in future investigations. Unfortunately, we have no experience in this area either.

3. Has it been considered to isolate myofibrils from the removed valves and study their structure using different methods?

RESPONSE: We did not, but this is an interesting point that could be studied in future investigations. Unfortunately, we have no experience in this area either.

To summarize, I would suggest a major revision as it adds new and original information to the subject area. In addition it's findings could help the evolution of heterotopic transplantation, especially in cases of infants and young children. It could become a bridgeing method that could fit the gap between operations that implant infant-sized grafts and operations that settle adult-sized valves.
RESPONSE: Thank you

Reviewer #3:
Manuscript Summary:
This is a very interesting rat model of subcapsular valve implantation to study the transplant immunology but not the hemodynamics of the transplanted valve leaflets. The model is clearly described and explained and easy to perform after reading the whole procedure.
RESPONSE: Thank you

Major Concerns:
My major concern is the introduction and use of valves with growth potential in children that need immunosuppression. As a transplant pediatric cardiac surgeon I'm far from using immunosuppression, especially steroids, in non heart recipients because of their side effects. But I know that sensitization and immunological respond to allografts may accelerate degeneration and that is why this research is needed to confirm the need of immunosuppression.

RESPONSE: unfortunately, there are no good options for aortic valve replacement in neonates for example. In this case the side effects of steroids would be a small price to pay for a functional and growing heart valve.

Reviewer #4:
Manuscript Summary:
The protocol from Hill M et al. described a new heterotopic aortic valve transplantation model in rats, where aortic valve leaflets are implanted into the subcapsular space of recipient kidneys. The authors suggest that this is a simplified approach to study transplant immunology in heart valve transplantation, in particular, compared to heterotopic transplantation into another position.

Major Concerns:
1. A subcutaneous of heterotopic aortic valve transplantation in rodents has previously been reported (Khorramirouz R et al. Acta Histochem. 2018; 120: 282-291). Both of the heterotopic subcutaneous and renal capsule valve transplant models have similar limitations of not offering the same hemodynamic environment as would occur in a sub-coronary position. However, the subcutaneous model appears to have a much simpler technical procedure than transplantation of aortic valve leaflets under the renal capsule. Have the authors compared the histology, alloimmune responses, etc. between the two models? It would be important for the authors to highlight the advantages of their newly proposed model.

RESPONSE: Yes, we did subcutaneous implantations but found that the valves are difficult to find for analysis. Subcutaneous implantation is also a less-effective method of antigen presentation. This information was mentioned in the discussion under the section "comparison with exisiting heterotopic transplant models".

2. As a primary goal of this surgical procedure is to provide a model to study the allogeneic immune response in cardiac valve transplantation, it would be helpful to provide representative images of an allogeneic heart valve implanted in the subcapsular space of the kidney.

RESPONSE: Thank you, this is a good idea. We added the pictures in figure 3.

In the Protocol for "Rats", allogeneic (Lewis-Brown Norway) transplants appear to have been listed/performed. One wonders whether the subcapsular space would have comparable immunological access to other valve graft models which implant along the recipient aorta. As well, how well would the subcapsular space retain a valve leaflet without closure after acute rejection?

RESPONSE: This is an interesting question for future investigations.

3. The authors suggest that multiple leaflets could be implanted per kidney. What is the maximum number of leaflets that can be implanted and successfully retrieved? Does implantation of more than one leaflet per kidney increase the risk of complications or mortality?

RESPONSE: We implanted up to two leaflets, but it would easily be possible to implant four leaflets without difficulty. We noticed no increased risk of complications or increased mortality when multiple leaflets were implanted.

4. Is peri-transplant ischemic injury observed when implanting in the subcapsular space? Is there angiogenesis of renal blood vessels into the transplanted valve?
RESPONSE: Yes, we did observe angiogenesis of renal blood vessels. This is visualized grossly in Figure 2, particularly in panel C, D, and F

Minor Concerns:
1. The statement for an estimation of 78.8 animals needed to reach a 90% survival rate (Ref 19) refers to heterotopic cardiac transplantation in mice, and not aortic valve transplantation. It would be helpful for the authors to correct this reference and adjust their statement based on the practice necessary for mastery of the heterotopic valve transplant procedure in rats. Further, could the authors estimate the number of animals required to reach a 90% survival rate for their new protocol?

RESPONSE: Reference 19 was deleted. We only had one death in a series of 36 animals with this new protocol. Therefore 90% survival can be achieved immediately.

2. Could the authors state the total number of operations performed for this surgical procedure for the 97.2% survival rate.

RESPONSE: Of course. We added the information that the survival rate was n = 35 / 36 animals.

3. In "Donor Operation" section, "in bloc" should be "en bloc". This should also be corrected in the "Representative Results" section.

RESPONSE: Thank you. This was corrected.

4. Wisconsin Buffer should also be identified as University of Wisconsin (UW) solution, as it is more commonly known. Why was UW solution used for storage of the donor heart tissue?

RESPONSE: We identified Wisconsin Buffer as University of Wisconsin solution. This was used because it is the standard preservative in our lab.

5. In the "Recipient Operation", the authors suggest to "advance a blunt probe under the renal capsule to develop a shallow subcapsular pocket". Could the authors clarify the size/depth of the subcapsular pocket recommended. As well, why does the capsule incision not need to be closed? The authors suggest that the use of "GelFoam over the capsular incision… is unnecessary as long as the tissue was advanced far enough into the subcapsular pocket". It would be helpful to provide an estimation of the depth the valve leaflet should be implanted.

RESPONSE: In point 5.7.2 we clarified that a 2mm space should be created. We found capsule closure was unnecessary as the tissue was very secure under the capsule and easily recovered after 28 days without the use of GelFoam.

6. In Figure 1, the panel for the "Preparation of Valve" should show the aortic root and the dissected aortic valve leaflets for accuracy. Further, the "Transplantation under the renal capsule" panel shows an incision which much larger than described in the text. It would be more accurate to illustrate incisions that are smaller than the kidney, as recommended in the protocol. As well, both kidneys are exposed in the cartoon, but bilateral implantation is not suggested in this protocol.
RESPONSE: Thank you, we corrected the images.

7. The authors suggest that based on their previous experience, excising the aortic valve leaflet is not recommended, as this could result in sample damage/loss. If leaflets are left within the subcapsular space for embedding, are there recommendations for how to correctly orient the tissue for sectioning?

RESPONSE: We recommend orienting the capsule of the kidney anterior and parenchyma posterior. This was clarified in point 6.3.

The representative images in Figure 4 appear to show only the transplanted leaflets separate from the recipient kidney.

RESPONSE: This is correct.

8. The addition of representative higher magnification micrographs of the immunostaining for αSM actin and CD31 should be provided to get a better view of the tissue.
RESPONSE: We added higher magnification micrographs.

9. The formatting of the "Table of Materials" inadvertently moved some information to the second page in the PDF.
RESPONSE: This will be corrected in the final manuscript.

Reviewer #5:
Manuscript Summary:
The present manuscript describes a renal subcapsular transplant model in rodents as a practical and simpler method for studying heart valve transplant immunobiology.

Major Concerns:
limitations already recognised by authors. Indeed, the present model is insufficient to study the effect of alloreactivity on the function of the valve as the leaflets are not performing their physiologicfunction under the renal capsule

RESPOSNE: Thank you