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Vineeta Bajaj, Ph.D.
Review Editor
Journal of Visualized Experiments

Aachen, 25th June 2020

Re-submission of *"A detailed surgical protocol for a porcine model of kidney auto-transplantation using 24-hours organ preservation and continuous telemetry" to Journal Of Visualized Experiments*

Dear Editors,

thank you very much for the favourable evaluation of our manuscript and the constructive comments from the referees. Following corrections to our article, we kindly ask you to reconsider the enclosed manuscript entitled *"A detailed surgical protocol for a porcine model of kidney auto-transplantation using 24-hours organ preservation and continuous telemetry"* to be published in JoVE.

We would like to thank you for giving us the opportunity to re-submit a revised version of our manuscript. We are grateful to the reviewers for their extensive and insightful comments and input and were able to address all remarks in detail and provided further data to improve the quality of our research.

We thank you in advance for expediting the review process of our manuscript and hope the revised version is now suitable for publication in JoVE.

Please find below our point to point responses to the reviewers' comments.

Yours sincerely,

Zoltan Czigany, M.D., Ph.D.

RWTH AACHEN UNIVERSITY

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Revised accordingly.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Thank you for the editorial reminder, we have revised the manuscript accordingly. Line spacing is set as “single” for the whole manuscript.

3. We only note equal first author contribution, please remove the equal last author contribution.

Revised accordingly.

4. Please provide an email address for each author.

Co-Author correspondence has been provided on the title page on the revised manuscript.

5. Please remove the line header from all the pages.

The header has been removed as suggested.

6. Please reword lines: 37-40, 67-70 as it matches with previously published literature.

We apologize for this mistake and have rewritten the corresponding parts in our revised manuscript.

7. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Revised accordingly.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Revised accordingly.

9. 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.

Revised accordingly.

10. The Protocol should contain only action items that direct the reader to do something.

Revised accordingly.

11. 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.

Revised accordingly.

12. Please ensure you answer the "how" question, i.e., how is the step performed?

Revised accordingly.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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.

Revised accordingly. Highlighted text has been reduced and the protocol has been revised.

14. Please ensure that the live animal will be available for filming.

Thank you for the editorial reminder, we will make sure that live animals and all required material all available for filming.

15. Please ensure that the results are described with respect to your experiment performed.

All representative results refer to the described protocol and experiments. ,

16. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Figure legends are now presented together at the end of the representative results as requested.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. 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]."

All figures are either property of our research group (such as color photos and ultrasound images) and/or generated by our team. No copyright issues are raised.

18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Our discussion has been revised to put more focus on the above-mentioned aspects. Furthermore, due to the extensive explanations requested by the four referees we had to extend our discussion.

19. Please do not abbreviate the journal titles in the references section.

Although, we have used the JOVE EndNote Styling downloaded from the JOVE website which does seemingly include abbreviations of the journal titles, we have revised this in the current version of our manuscript.

20. Please sort the materials table in alphabetical order.

Revised accordingly.

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Reviewer #1:

A detailed protocol for a pig kidney transplantation experiment is described. This should be a good guide when writing a paper on a pig kidney transplant experiment.

Major Concerns:
1) A detailed protocol for a pig kidney transplantation experiment is described. This should be a good guide when writing a paper on a pig kidney transplant experiment. However, normal transplants are performed between different individuals and when looking at long-term transplant performance, immunosuppressive agents are used. Therefore, it is an inadequate preclinical model.

We would like to express our gratitude for the valuable expert comments of reviewer. We principally agree with the referee in terms of the clear difference between allo- and autotransplantation model but would like to address this comment further. Animal models of auto-transplantation are broadly used in the field of organ preservation and in every subfield of transplantation research which is not dealing with immunological responses of allograft rejection. The auto-transplantation model allows an isogenic transplantation (similarly to the situation when we transplant organs between identical twins, as it was the case in the setting of the first human kidney transplantation performed by Murray in 1953). This is indeed different compared to the setting where allografts are transplanted to genetically not-related recipients. Nevertheless, the lack immune rejection in this setting and the avoidance of complex confounding factors such as immunosuppressive medication can be considered as an advantage of this model which allows us to focus on the effects of preservation damage and ischemia-reperfusion injury, representing the main research directions of our working group [1-15]. Moreover, the model of auto-transplantation also complies better with the 3R principle by reducing the numbers of required animals compared to allotransplantation by 50%, as no separate donor animal is required. We also would like to add that even if we have described a model of autotransplantation exactly the same surgical and anaesthesia techniques are applicable for an allotransplantation setting (except the lack of a detailed description of an immunosuppression protocol described by others previously). Based on its excellent reproducibility, feasibility to selectively test the effects of preservation damage and IRI as well as its good compliance with the 3R principle, we cannot agree with the referee that this would be an inadequate preclinical model.

2) This research team has extensive experience with kidney transplantation using pigs. In the case of the kidney after circulatory arrest, even with autologous transplantation, I would like you to describe your experience with each time of circulatory arrest (e.g., 30 minutes, 60 minutes, etc.) to what extent it interferes with the recovery of renal function.

We thank the reviewer for this constructive comment. Donation after circulatory death is a major risk for delayed graft function and primary non-function/graft loss. Unfortunately, within the frameworks of the present project we did not perform any comparative studies on the effects of 30 and 60 minutes warm ischemia. (Although, these studies are planned

and currently ongoing, we are not able to provide a suitable amount of data on this). According to our previous experience with this model, however, 60 minutes of warm ischemia combined with 24 hours of cold storage in HTK represents an almost lethal damage to the kidney with little chance of a recovery of renal function, therefore we can only recommend this model in the setting of acute experiments without animal survival.

3) The biggest drawback of the pig experiment compared to the mouse experiment is the limited means of elucidating the mechanism. Little molecular biological or immunological analysis is possible. Comments should be made on this shortcoming.

We cordially thank the referee for raising this important aspect, with which we fully concur. On the one hand, it is a well-known drawback of a large animal model that only limited investigation of subcellular mechanisms is possible (e.g. lack of knock out stains, reduced availability of molecular kits and assays etc). On the other hand, in contrast to mouse experiments, using a large animal model allows us to test various “clinic ready” treatment concepts. A very good example for this is the use of various organ preservation techniques such as machine perfusion. Organ preservation experiments can be performed in pigs in clinically relevant setting [16]. Very little modification or “downsizing” is required compared to rodent experiments. As our group has many years of experience with both small and large animal models of solid organ transplantation [1, 4, 10, 13, 15, 17-22], we strongly believe that the use of both small and large animals models has their own specific advantages, limitations and indications. To further stress this, we have revised the limitations section of our manuscript as suggested by the referee.

4) By the way, you even explain how to put in telemetry, but what is the significance of putting in telemetry? Is the only purpose to remotely check that your blood pressure is stable?

The authors thank the reviewer for his/her comment and apologize for presenting the details of the telemetry protocol too briefly. The use of a telemetry device can be multi-purpose. Firstly, it reflects the postoperative state of the animal by monitoring parameters such as ECG, arterial blood pressure, temperature continuously. We believe that all these data contribute to the early detection of possible postoperative complication accurately and timely (e.g. haemorrhagic shock, or sepsis detected by increasing temperature, hypotonia and tachycardia). This may facilitate timely intervention (e.g. introduction of therapeutic antibiotic therapy, fluid substitution, discontinuation of anticoagulation). Besides these “real-time” monitoring aspect, our group is currently focusing on the severity assessment and refinement of animal experiments. Retrospective analysis of a large amount of collected telemetry data in these experiments may allow us to better stratify the severity of these kind of surgical interventions and optimize perioperative care in laboratory animals. To further address this we have modified the discussion of our manuscript accordingly.

Reviewer #2:

Manuscript Summary:
This article describes a model of renal auto-transplantation using a large animal model and telemetry monitoring.

Major Concerns:
1. It is an interesting piece of work but the innovative aspect is not obvious. This model of renal autotransplantation in pigs has been known since the 80s and has allowed many publications.

We would like to express our gratitude for the valuable expert comments of reviewer. We fully agree with the reviewer that the model of kidney auto-transplantation in pigs has been widely carried out since the 80s. Thanks to various reproducible and feasible small and large animal models of organ transplantation, great progress have been made in the field of molecular biology research, immune rejection and immunosuppression, dynamic organ preservation techniques, modulation of ischemia-reperfusion and preservation injury. Nevertheless, comprehensive publications and state-of-the-art protocols (especially with a well-documented video protocol) about the exact techniques and pitfalls are still hard to find. Most teams dealing with these models are using in-house protocols and sometimes struggling with various technical problems which can only be solved by personal communication with other teams. A previous JOVE publication of our group by Nagai et al. on the rat liver transplantation model has been viewed over 25 000 times by researchers from various institutions worldwide according to the usage statistics of the JOVE website (URL: https://www.jove.com/video/4376/surgical-procedures-for-rat-model-partial-orthotopic-liver) [18]. Therefore, we believe that such comprehensive protocols are of interest for the transplant community and for the broad readership of JOVE.
Therefore, even if some basic techniques of the auto-transplantation model itself were described before, multiple aspects of our protocol are novel including the state-of-the-art housing facility with telemetry and video monitoring, the utilization of porcine jackets to avoid the use of confined metabolic cages. All these refinement procedures improve severity assessment of laboratory animals and comply with the modern interpretation of the 3R principle.

Minor Concerns:
2. Title is concise and the summary is relatively clear. The 24 hours cold ischemia time in the pig model is a classical duration, it is closer to clinical reality (18 hours). The actual extended duration would therefore be 36-48 hours.

Although, the length of cold preservation is largely heterogeneous between studies using various porcine kidney transplantation models, the 24 h preservation time is indeed frequently used. The wording “prolonged” may not be exact enough in the present setting. Therefore, we decided to remove the word “prolonged” from the title and from our manuscript and simply replaced it with “24-hours” organ preservation. We thank the reviewer for drawing attention to this important aspect which helped us to improve the clarity of our manuscript.

3.Telemetry requires more justification. In the background, several references are missing. Transplantation surgeon formation may not be of great argument when dealing with end to end anastomosis in orthotopic kidney transplantation as it is not the usual technique in human.

We appreciate the constructive remark of the reviewer and apologize for not being clear enough on the telemetry. Please also see our answer Reviewer #1 Answer 4. According to the best of our knowledge, this is the first study integrating implantable telemetry monitoring in a large animal model of kidney auto-transplantation. We have revised the current version of the manuscript accordingly and included the benefits of using a telemetry and also updated the reference list accordingly (please see Line 570-586).

We principally agree with the referee in terms of the differences in surgical techniques between human and porcine kidney transplantation and its significance for but would like to specify our considerations further. Indeed, end-to-end anastomosis is not the typical technique in the human kidney transplantation setting. However, in certain settings it is required to use the internal iliac artery for an end-to-end anastomosis with the renal artery (e.g. massive atherosclerosis of the external iliac artery, dissection of the external iliac artery, two renal arteries which are non-feasible to reconstruct using alternative techniques) [23]. Therefore, mastering these anastomosis techniques is essential for every junior transplant surgeon and also helps to achieve solid skills and more confidence when performing “standard” cases. These basic techniques are also essential for the anastomosis of portal vein and hepatic artery in the setting of liver transplantation on the further way of the carrier path of a transplant surgeon.
It should be noted, however, that there is no defined standard for the vascular anastomoses in porcine kidney transplantation. Our working group had tried various techniques in the past and we realized that the end-to-end anastomosis approach is a feasible was to perform the kidney auto-transplantation procedure. As there are basically no arterial variations of the main renal artery (always one main artery in German landrace pigs) it is a straightforward anastomosis. In case of an early division of the vein or with double veins, it may be necessary to modify the surgical approach and perform an end-to-side anastomosis of the vena cava. With the use of a color Doppler ultrasound directly after fascial closure and postoperatively to avoid vascular kinking, the topical use of papaverine and the postoperative administration of aspirin ensures that we almost never experience significant vascular problems.

Advantages of the end-to-end anastomosis are:
1, No complete clamping of the aorta/vena cava is required ->hemodynamic stability
2, No need to completely dissect the aorta and vena cava which significantly reduces the risk of a major high-output lymphatic leakage.

The best value of this article is telemetry which provide continuous monitoring and the use of jackets. Objectives need to be clarified. Information on ethical statement are provided. The study design is accurately described but some points may require discussion and clarification:

4. Why 5-7 days follow up? As telemetry is an expensive procedure which may increase experiment burden, complexity and pain, it may be worthwhile - if possible - to extend follow-up to really get complete recovery of renal function?

We are grateful to the reviewer for her/his encouraging comments. In this protocol-oriented manuscript we have attempted to describe our current experimental setting. The follow up can be extended to a period which is suitable to answer the project specific research questions (e.g. long-term recovery of function vs. acute damage). To further underline this we have included the possibility to extend follow up to our revised manuscript.

5. Why 14 days prior to transplantation procedure? is there any variation between first and second week of recovery justifying this duration?

We thank the reviewer for drawing attention to this important aspect. The period of 14 days between telemetry implantation and kidney transplantation is based on thorough internal discussions and considerations. After discussing this issue internally and then with various telemetry manufacturers (EMKA; DSI; TSE) as well as with other research groups and experts using these systems in various experimental settings, we decided to leave a 14-day period between telemetry implantation and kidney transplantation. A period of at least 12 days after implantation of the measurement system is recommended to ensure stable and optimal measurement data. During the earlier days, deviations may still occur due to the movement of the animal as the scaring and healing processes are still vulnerable and not final. Concerning the fact that in the present experimental setting, the animals are sacrificed 5 days after kidney transplantation, these potential disturbances and undesired variances of the measurement would fall exactly to our post-transplant period. In order to avoid effects of the telemetry implantation on the study results and to ensure that the animals fully recover from the initial surgery, we decided to establish a 14-day re-convalescence period between surgeries. To be more clear on our considerations, we have revised our manuscript accordingly (see Line 587-593).

Experimental procedure is partially described. Some add can be of interest: 6.-Is bladder catheter usefull only to provide urinary example at each anesthesia? Or to monitor during surgery? (but it should be of small interest in such quick procedures).

As it is the case for every “how I do it” type experimental protocol, we believe that certain minor aspects including the use of a bladder catheter are facultative. Here we attempted to describe our current “best-practice” experimental protocol. We insert a bladder catheter due to experimental considerations. This easy to perform and low-risk procedure (which is performed in anesthesia, therefore also completely painless for the animals) is the easiest way to collect baseline urinary samples e.g. at the time-point of organ retrieval. The alternative would be the direct puncture of the bladder which we considered as high

risk. If no baseline urine samples are required per protocol, the transurethral bladder catheter may be avoided at the investigators' discretion. To improve clarity on this we have included the following parts to our discussion: "*This manuscript describes our current "best-practice" in the experimental setting of porcine kidney auto-transplantation. While certain steps are mandatory to successfully establish this model, minor aspects (e.g. the intraoperative use of a bladder catheter, arterial catheter placement to the femoral vs. carotid artery) are facultative and may be avoided/altered at the investigators` discretion." (Line 608-614).*

7. Why choosing femoral artery instead of carotid artery? Any infection or arterial catheter dysfunction due to leg movements or animal lying position?

We thank the reviewer for drawing attention to this important aspect. Although, we use both techniques in various experimental settings with good results we selected to use the femoral artery in this model. The catheter is placed under sterile conditions and then fixed on the skin using multiple sutures and sterile tape, therefore dislocation does not represent a major issue. Infection we have not observed as the percutaneous catheter is only placed for approximately 2 hours during the recipient procedure and removed afterwards. We also avoid the use of a percutaneous catheter if the telemetry device delivers satisfactory data on the arterial pressure despite the supine position of the animal. It should be mentioned, however, that the use of the carotid artery is certainly not "wrong" and also possible at the investigators` discretion (please see also our answer 6).

8. Why choosing Propofol and Isoflurane? Any protocol to choose which one to increase or decrease?

By increasing the propofol dosage, a significant and prompt deepening of the anaesthesia can be achieved, whereas a pure TIVA = total intravenous anaesthesia usually leads to long recovery phases and an increased risk of hypotension. However, a stable mean blood pressure is absolutely necessary to maintain the initial perfusion of the kidney. Maintaining anaesthesia via isoflurane alone would increase the required fentanyl dosage over the whole anaesthesia period (due to the lacking analgesic potential) which also leads to a prolonged post-anaesthesia recovery phase and possibly the risk of respiratory depression during recovery.
Combination anaesthesia using isoflurane, propofol and fentanyl is used to improve the depth of anaesthesia while minimizing the post-sleep phase. This protocol has proven to be very effective, reproducible and safe in our hands: Isoflurane 1.5 vol. % Fentanyl 7.5 µg/kg/h Propofol (2) - 4 mg/kg/h.

9. How to deal with movement during this curare-free anesthesia?

We would like to thank the referee for this expert comment. In deep surgical anesthesia we basically never observe any significant movements of the animal which would interfere with the surgical procedure. Therefore, this is not a clinically relevant issue even without the use of muscle relaxants. The rationale behind avoiding the use of muscle relaxants is to facilitate rapid post-operative recovery with spontaneous breathing and early endo-tracheal extubation of the animal.

10. How is arterial cannulation for organ flushing realized (type, ligature). Do you keep canula during preservation to realize end preservation flushing?

The reviewer raises a question of outstanding relevance. We would like to apologize for not describing our approach in detail. For the arterial cannulation a standard 14 G (orange) peripheral venous catheter is used which is fixed using a tourniquet prepared from 3-0 Vicryl. After initial flush, the catheter is removed to avoid any damage of the artery over the 24 hours of cold preservation, however, a brief post-preservation cold-flush is also applied. We have revised our manuscript and included these details to our protocol (see Page 6, Bac table and organ preservation).

11. Why aspirin is provided as it can make bleeding and therefore be discontinued which can alter results? Any thrombosis event or bleeding event data records from the team experience?

We are grateful to the reviewer for this valuable expert comment. The use of anti-coagulants and thrombocyte aggregation inhibitors in major surgical experiments is indeed always a question of balancing the risk of anastomosis complications and bleeding. In our current experimental setting no significant bleeding complications have been observed with the used dosage of aspirin (e.g. no hemorrhagic shock or major hemodynamically relevant bleeding). If minor bleeding from the abdominal wound is observed with the building of hematoma, we recommend to stop aspirin administration.
Other research groups even recommend the preoperative administration of aspirin for 3 days before implantation to prevent any vascular complications [24]. As with the implementation of the present aspirin administration protocol (combined with the post-implantation color Doppler ultrasound, intraoperative heparin administration and the right anastomosis techniques) arterial complications are extremely rare, we restricted the use of aspirin for the postoperative period to reduce the risk of intraoperative bleeding.

12. Near-complete peritoneal closure is a good point, and can also be justified as it can help with lymphatic leakage (lymphocele marsupialization)

We would like to thank the reviewer for her/his encouraging comments.

13.-How is infusion managed? The authors state that infusions are performed in case of delayed resumption of function, how are they performed with animals outside the metabolic cage? Are infusion line kept during all 5 days follow-up?

The central venous line is tunnelled and sutured to the back of the animal (this is also required for the administration of medication such as antibiotics, pantroprazol etc), therefore it can be used for on demand infusion of intravenous fluids in the housing facility. We continuously monitor urine output and oral intake and if the latter is in imbalance compared to the urine output and the animals clinical state justifies, on demand fluid infusion is performed by the veterinary officer. Veterinary officers involved in the daily care of the animals are not informed about the experimental grouping of the animals to avoid experimental bias. Following fluid administration, the infusion line is disconnected and the central venous line is blocked with heparinized solution and covered with a sterile

cap again. We have revised our protocol to include the most important information on the handling of the central venous line (see point 7.5).

14. Information on experimental animals are relatively well described and the rational for pig choice also Housing is also well described. Some informations on experimental outcomes could be useful. Anastomosis and procedure duration, adverse events (catheter dysfunction, infection - if available), can be of interest.

We thank the reviewer for drawing attention to this important aspect. To provide more data on operative outcomes now we have included the data on the time required for organ retrieval, total implantation time, warm ischemia time (see Representative results and Table 1). Furthermore, to include some potential complications we have prepared a further figure showing representative postoperative ultrasound images of potential complications, such as catheter dysfunction and lymphocele (see Figure 6). We believe that this suggestion of the reviewer helped us to improve our manuscript.

15.Experimental groups are small groups! n=3. For instance, it seems difficult to explain 6 anastomosis variations with this subset. Maybe procedures from other protocol can help in capturing these situations and should therefore be reported.

This issue raised by the reviewer is certainly highly important which we would like to address further. As we have described in our figure legends of Figure 3, our figure depicts a handful of the more frequent variations and is not statistically comprehensive in terms of all variations possible in German landrace pigs. This figure is also not part of our representative results part, it is just a visual guide for future investigators and experimental surgeons who are planning to establish this model. To describe the whole spectrum of anastomosis variations on a populational level with a corresponding incidence would probably require a very large sample size. Considering the sample size: as JOVE is a methods journal with more focus on the experimental protocol itself than on experimental findings, we decided to only use a small data subset of our very recent experiments to demonstrate the performance of this model. However, to satisfactory reflect to the comment of the reviewer we have included the data of two more recent experiments. We think this relatively small subset of data is satisfactory to show the main aspects and performance of the model and n=5 is also comparable with previous protocols reported in JOVE using porcine as an experimental model [25-27].

16.Results presentation are well done but may be enhanced with telemetry results follow up after surgery (arterial pressure for example).

We thank the reviewer for pointing out this detail, which we have specified in the revised version of our manuscript accordingly. A comprehensive analysis of the telemetry data would have been beyond the scope of the present manuscript and it requires a longer period of time than the relatively short time frame which was available for the revision of our paper. Nevertheless, to satisfactory address the suggestion of the referee, we have included exemplary telemetry data of mean body temperature changes over the study period (see Figure 5).

17.Anatomical description is an interesting point to help surgical procedure. Did author experienced variations also with azygo-lumbar vein? Its presence can modify and hamper the complete dissection of the left renal vein and can therefore give a false impression of a double vein that complicates and makes anastomosis less reproducible.

We would like to express our gratitude to the reviewer for sharing her/his valuable experience. The azygo-lumbar vein may indeed complicate surgical dissection of the renal vein in certain situations, especially on the "donor side" where the renal vein is usually divided near the vena cava. Here we pay great attention to avoid accidental damage to the vein. If the lumbar vein joins the renal vein very near to the vena cava it can usually be preserved without any issues. Otherwise we usually just dissect and divide the vein between two 3-0 ligatures. Nevertheless, to raise attention of the reader for the presence of this anatomical aspect we have revised our manuscript accordingly (see Point 4.16 and 4.17).

18.Number of animal is very small and need some further justification (was only a developing/learning curve protocol ?)

Please see our answer to question 15.

19.The choice of orthotopic (not even mentioned in the manuscript but it is the correct description of the model) is not usually performed in clinical practice.

The authors thank the reviewer for his comment and would like to specify their considerations. For this please also see our answer to question 3. Indeed, orthotopic kidney transplantation is not a typical technique in human setting excepts some special situations [28]. Nevertheless, there is no defined standard for porcine kidney transplantation. Although, most groups use a transperitoneal and orthotopic approach, heterotopic transplantation to the iliac fossa is also possible [24]. However, due to the relatively low diameter of the external iliac artery in 30-40 kg pigs and its tendency for vasospasm makes it sometimes more difficult to perform the end-to-side anastomosis of the renal artery to EIA. As for the auto-transplant model we retrieve the left kidney via a transperitoneal approach, it is more feasible to perform the implantation by reopening the same incision, especially that we are also required to remove the native right kidney to allow the animal the recover with only one predamaged kidney.
However, to further emphasize these differences between the experimental and clinical setting we have revised the limitations sections of our manuscript accordingly. We have also included the word "othotopic" throughout the manuscript to better describe the model as suggested by the referee.

20.Was it a necessity to be able to collect urine with transparietal ureterostomy?

The transparietal ureterostomy allows us an easy and reproducible way to collect urine over the follow up period without the use of a metabolic cage. The catheter is sutured to the skin and hidden below the porcine jacket, therefore does not significantly disturb the animal. However, as it was mentioned for other surgical aspects of the procedure, there is certainly now established standard or ultimately correct way to perform the ureteral

reconstruction. For experimental protocols with long follow up periods of multiple weeks, the more anatomical approach of ureteral reconstruction with uretero-cystostomy or uretero-ureterostomy may be more suitable and should be considered.
Nevertheless, the ureter of the porcine has a narrow caliber and fragile mucosa which is susceptible to edema during surgical management. The middle and distal segments of the ureter receive blood from the common iliac artery and its branches. Ischemic necrosis is a risk if long segments of donor ureters are used for the anastomosis [24].

Reviewer #3:

Manuscript Summary:

This is an interesting protocol from an established unit in the field of experimental organ preservation and transplantation. Several questions are raised and should be addressed. The protocol is of interest to fellow researchers in the field.

Major Concerns:
1) Orthoptic location (ie. anastomosis to renal artery and vein vs iliacs or distal aorta / IVC - reasons for this choice and potentially options for and against should be mentioned. If this is to be a clinically comparative protocol this is not a kin to routine clinical practice.

First of all, the authors would like to thank the reviewer for her/his encouraging comments and would like to specify their considerations. We agree with the reviewer and believe that this protocol will be a comprehensive guide for our peers who are dealing with large animal models of kidney transplantation.

As the question concerning our approach on the anastomoses and orthotopic location of the kidney have been raised by reviewer #2, please also see our previous answers to question 3 and 19 from reviewer #2. Although, there is no ultimately right way or general consensus to perform these techniques, we believe that a transperitoneal approach in our case is probably the most feasible way due to multiple reasons:

-The use of the autotransplantation model required the retrieval of the left kidney then the explantation of the right kidney the next day and the subsequent implantation of the "damaged" left kidney after 24 hours of preservation. For this a retroperitoneal approach would be more complicated, takes longer and may cause larger trauma, due to a bilateral intervention. (retroperitoneal retrieval on the left side, retroperitoneal nephrectomy on the right side and subsequent implantation to the iliac axis using a 3rd retroperitoneal access).

-Due to the relatively low diameter of the external iliac artery in 30-40 kg pigs and its tendency for vasospasm makes it sometimes more difficult to perform the end-to-side anastomosis of the renal artery to the EIA

To further address this issue and mention the technical differences between the orthotopic and heterothopic approach, we have revised our manuscript accordingly (see see revised Discussion).

2) Background to the development of this protocol - historically and significant changes to the protocol from initial start and reasoning behind these would be useful for readers and units wishing to implement such a research program.

The authors would like to thank the reviewer for this constructive suggestion. Our group is using the model of porcine kidney auto-transplantation for well over 10 years now in various settings [21, 29]. Although, some modifications have been made, the basic techniques of the model remained the same. Over the years we have reached a "best-

practice" presented here in this protocol. We are afraid that comprehensively describing every modification and model upgrade would be beyond the scope of this manuscript and would require to be presented within the frameworks of a special article. Nevertheless, we have attempted to describe all the possible pitfalls we have observed in the past in our discussion (e.g. vascular kinking after abdominal closure, lymphatic fistulas).

3) Retrieval of the donor organ could be through a lateral incision / retroperitoneal (especially left sided) vs the midline laparotomy approache chosen - and then need for re-laparotom of retrieval of a carrel patch with the donor kidney for subsequent use during implantation?. Can the authors discuss the reasoning for this choice?
4) Can the authors comment on the technique In there experience ? - this is normally easier to perform and more reliable than end to end anastomosis to renal arteries especially if complex reconstructions are required as described by the authors.

We thank the reviewer for these expert comments and considerations. The choice of the exact technique remains dependent on the surgeon's preference and experience. Although, in this protocol, we have attempted to show our "best-practice", there are other techniques which are comparably successful. The retroperitoneal approach for retrieval has been described before and may be a good alternative to avoid opening the abdomen for the retrieval surgery [24]. In the settings of auto-transplantation we see the use of a Carrel patch a bit problematic. Retrieval of the kidney graft with an aortic patch would generate a defect in the aorta which has to be reconstructed by suture or by using a vascular patch (e.g. autologous or allogenic material vs. e.g. bovine pericardium). This approach is more time consuming and technically complex, with high risk of major bleeding from the aorta. As the arterial anastomosis is usually very straightforward in an end-to-end approach (suturing usually two 6-8 mm arteries with good quality arterial wall and without atherosclerosis), therefore we think that the risk and complexity of the retrieval with an aortic patch and subsequent aortic reconstruction would overweigh the benefits.

Although, we attempted to most comprehensively describe our approach and its limitations, we are afraid that including all these potential technical aspects would be beyond the scope of such a protocol and would rather belong into a comprehensive technical review article on the topic [24]. To emphasize this fact, we have revised our discussion accordingly.

5) Timings - for each step and suggestions for a timetable - days etc. How long for each part of the protocol for example and if this changed upon refinement etc. These details again would enable a unit seeking to start this work to understand the resource implications and learning curve.

We thank the reviewer for drawing attention to this important aspect. We have now revised our manuscript and included the times required for the various experimental steps. Further, as the procedure is time consuming and binds a large amount of resources and OR-capacity it is usually unfeasible to perform more than 2 procedures a day. This we have also included to our discussion section of the revised manuscript to guide future researchers in establishing this protocol (see Table 1 and Representative Results).

6) Management of the ureter - what is the rational to ureteric canulation vs a ureter-ureteric (due to the implant location chosen) or ureteric-bladder. If the rationale is only for telemetry purposes could not urethral cathterisation be a less invasive, simpler process? The authors rational for this should be discussed.

As the same question has been asked by reviewer 2, please see also answer 20 reviewer #2. The authors thank the referee for this valuable comment.

7) Cost suggestions for implementation of such a program and personel required would be both useful for an interested unit. Do the authors have such information avalaible to include in the article?

We thank the reviewer for raising this issue of outstanding relevance. An exact cost-assessment is difficult, as it is largely depends on the country where the experiment are performed (OR costs, materials, animals etc). We believe our comprehensive material list may help other groups the rapidly assess the approximate costs of these experiments according to the local situation. However, it may be more interesting to know more information on human resources. To describe our approach on this we have generated a table for our revised manuscript, suggesting the number of team members and their level of training required for each experimental phase (see Table 1).

Minor Concerns:
8) Description of the anatomical issues (anatatomical variants described) and management of each of the lettered diagrams in Figure 3.

To further improve the description and management strategies of the venous anatomical variants, we have revised our figure legend to Figure 3 accordingly. Thank you for this great suggestion.

9) Figure 1 - more detailed descriptions of contents of A-G please.

We thank the reviewer for drawing attention to this aspect. We have improved the figure legends for Figure 1 (now Figure 2).

10) Figure 4 - define descriptive stats, how many animals used to generate this data? Definition of auto-transplant 1 and auto-transplant 2 is needed? No mention in text of 2nd auto-transplant.

Figure 4 just intend to demonstrate our practice with the use of color Doppler following kidney transplantation. These pictures are images from the same animal. In certain cases the compression of the kidney graft following fascia closure may lead to vascular kinking with fatal complications including arterial and venous thrombosis. If perfusion of the graft is not satisfactory, the abdominal incision must be opened and the kidney hast to be repositioned in a way to avoid kinking. With this technique (combined with the use of intraoperative heparin administration, postoperative aspirin therapy and the right surgical techniques) we were able to almost completely eliminate vascular complications during

follow up. To further clarify that the pictures are from the same animal we have revised our figure legends accordingly.

Reviewer #4:

Manuscript Summary:
I enjoyed reading this interesting manuscript very much. In my view, it is well written and sufficiently comprehensive. The manuscript describes a protocol for porcine renal autotransplantation. Special features that the authors have incorporated are continuous telemetry of the pig, isolated graft urine collection through percutaneous ureterostomy and a vest that each pig wears to protect catheters and lines, as well as urine collection bags. Limitations of the protocol are also well discussed, which is an asset of the current paper.

We would like to thank the referee for her/his encouraging words and constructive suggestions.

Major Concerns:
1. I was surprised to read that these authors gain access to the kidneys by means of a laparotomy. Please explain this choice and discuss in the manuscript why an inraperitoneal approach was chosen, instead of extraperitoneal dissection (which can also easily be done via a midline incision in pigs). Bowels may be more disturbed when a transperitoneal approach is utilized. Please comment on the occurrence of (sub)ileus postoperatively.

This is indeed an important, yet challenging comment. Please see also reviewer #3 question 3 and reviewer #2 question 19 which are referring to the same topic. As both procedures are relatively short, we do not usually observe clinically significant paralytic ileus. Animals receive water directly after surgery when they are fully awake and food is provided from the first postoperative day ad libitum. Intraoperatively we continuously cover the bowel with wet and warm towels and pay a great attention to its sufficient circulation which is very critical in pigs. To further address the possible benefits and disadvantages of using an extraperitoneal vs. transperitoneal approach we have revised our manuscript accordingly (see revised discussion).

2. The percutaneous urine collection methodology is not new, but still very elegant. Nevertheless, performing this correctly can be challenging for researchers who do not have experience with the method. I would have liked to see a more detailed description of percutaneous catheter placement and also how urine collection bags are secured, how often they are changed, which type they are, etcetera.

We are grateful for the reviewer's interest in our technique and would like to clarify it further. Therefore, we have paid more attention to these details in our revised protocol and of course included all materials required into our material list (see Point 6.19, 6.20 and 6.21, 7.4).

Minor Concerns:
3. Figure 1A appeared a bit simplistic to me.

Figure 1A is schematic presentation of the blueprint of our housing facility. This drawing may serve as a guide for other researchers when planning similar housing facilities with video surveillance and telemetry monitoring. To further improve our manuscript, we have revised the figure legends of Figure 1 accordingly.

4. All figures and especially photographs should be presented in a much higher resolution than I found in the reviewer pdf.

Thank you for this remark. All original figures are in high resolution complying with the JOVE guidelines on image formatting which however may appear in a reduced quality in the reviewer pdf.

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Reference

1. Czigany Z, Bleilevens C, Beckers C, Stoppe C, Mohring M, Fulop A, et al. Limb remote ischemic conditioning of the recipient protects the liver in a rat model of arterialized orthotopic liver transplantation. PloS one. 2018;13(4):e0195507. Epub 2018/04/05. doi: 10.1371/journal.pone.0195507. PubMed PMID: 29617450.
2. Czigany Z, Bleilevens C, Möhring M, Beckers C, Fulop A, Neumann U, et al. Effects of various remote ischemic conditioning protocols in a rat model of arterialized orthotopic liver transplantation. European surgical research Europaische chirurgische Forschung Recherches chirurgicales europeennes. 2016;57(Suppl 1):3.
3. Czigany Z, Hata K, Lai W, Schwandt T, Yamamoto Y, Uemoto S, et al. A Dual Protective Effect of Intestinal Remote Ischemic Conditioning in a Rat Model of Total Hepatic Ischemia. Journal of clinical medicine. 2019;8(10). Epub 2019/09/29. doi: 10.3390/jcm8101546. PubMed PMID: 31561505; PubMed Central PMCID: PMCPMC6832347.
4. Czigany Z, Iwasaki J, Yagi S, Nagai K, Szijarto A, Uemoto S, et al. Improving Research Practice in Rat Orthotopic and Partial Orthotopic Liver Transplantation: A Review, Recommendation, and Publication Guide. European surgical research Europaische chirurgische Forschung Recherches chirurgicales europeennes. 2015;55(1-2):119-38. Epub 2015/08/01. doi: 10.1159/000437095. PubMed PMID: 26228574.
5. Czigany Z, Lurje G. Hypothermic Oxygenated Machine Perfusion (HOPE) for Liver Transplantation of Human Liver Allografts From Extended Criteria Donors (ECD) in Donation After Brain Death (DBD); a Prospective Multicenter Randomized Controlled Trial (HOPE ECD-DBD). BMJ Open. 2017. Epub Under Review.
6. Czigany Z, Lurje I, Schmelzle M, Schoning W, Ollinger R, Raschzok N, et al. Ischemia-Reperfusion Injury in Marginal Liver Grafts and the Role of Hypothermic Machine Perfusion: Molecular Mechanisms and Clinical Implications. Journal of clinical medicine. 2020;9(3). Epub 2020/04/05. doi: 10.3390/jcm9030846. PubMed PMID: 32244972.
7. Czigany Z, Lurje I, Tolba R, Neumann UP, Tacke F, Lurje G. Machine perfusion for liver transplantation in the era of marginal organs - new kids on the block. Liver international : official journal of the International Association for the Study of the Liver. 2018. Epub 2018/08/22. doi: 10.1111/liv.13946. PubMed PMID: 30129192.
8. Czigany Z, Turoczi Z, Kleiner D, Lotz G, Homeyer A, Harsanyi L, et al. Neural elements behind the hepatoprotection of remote perconditioning. The Journal of surgical research. 2015;193(2):642-51. Epub 2014/10/01. doi: 10.1016/j.jss.2014.08.046. PubMed PMID: 25266602.

9. Czigany Z, Turoczi Z, Onody P, Harsanyi L, Lotz G, Hegedus V, et al. Remote ischemic perconditioning protects the liver from ischemia-reperfusion injury. The Journal of surgical research. 2013;185(2):605-13. Epub 2013/08/21. doi: 10.1016/j.jss.2013.07.018. PubMed PMID: 23953788.

10. Emontzpohl C, Stoppe C, Theissen A, Beckers C, Neumann UP, Lurje G, et al. The Role of Macrophage Migration Inhibitory Factor in Remote Ischemic Conditioning Induced Hepatoprotection in A Rodent Model of Liver Transplantation. Shock. 2018. Epub 2019/01/03. doi: 10.1097/shk.0000000000001307. PubMed PMID: 30601408.

11. Meister FA, Czigany Z, Bednarsch J, Bocker J, Amygdalos I, Morales Santana DA, et al. Hypothermic Oxygenated Machine Perfusion of Extended Criteria Kidney Allografts from Brain Dead Donors: Protocol for a Prospective Pilot Study. JMIR research protocols. 2019;8(10):e14622. Epub 2019/10/16. doi: 10.2196/14622. PubMed PMID: 31613224.

12. Meister FA, Czigany Z, Bednarsch J, Boecker J, Wiltberger G, Rohlfs W, et al. Hypothermic oxygenated machine perfusion-Preliminary experience with end-ischemic reconditioning of marginal kidney allografts. Clinical transplantation. 2019;33(10):e13673. Epub 2019/07/25. doi: 10.1111/ctr.13673. PubMed PMID: 31332838.

13. Schreinemachers MC, Doorschodt BM, Florquin S, van den Bergh Weerman MA, Reitsma JB, Lai W, et al. Improved preservation and microcirculation with POLYSOL after transplantation in a porcine kidney autotransplantation model. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2009;24(3):816-24. Epub 2008/10/14. doi: 10.1093/ndt/gfn559. PubMed PMID: 18849394.

14. Srinivasan PK, Yagi S, Doorschodt B, Nagai K, Afify M, Uemoto S, et al. Impact of venous systemic oxygen persufflation supplemented with nitric oxide gas on cold-stored, warm ischemia-damaged experimental liver grafts. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2012;18(2):219-25. Epub 2011/10/12. doi: 10.1002/lt.22442. PubMed PMID: 21987402.

15. Yagi S, Nagai K, Kadaba P, Afify M, Teramukai S, Uemoto S, et al. A novel organ preservation for small partial liver transplantations in rats: venous systemic oxygen persuf fl ation with nitric oxide gas. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(1):222-8. Epub 2012/11/07. doi: 10.1111/j.1600-6143.2012.04310.x. PubMed PMID: 23126657.

16. Schlegel A, de Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. Journal of hepatology. 2013;58(2):278-86. Epub 2012/10/16. doi: 10.1016/j.jhep.2012.10.004. PubMed PMID: 23063573.

17. Nagai K, Yagi S, Afify M, Bleilevens C, Uemoto S, Tolba RH. Impact of venous-systemic oxygen persufflation with nitric oxide gas on steatotic grafts after partial orthotopic liver transplantation in rats. Transplantation. 2013;95(1):78-84. Epub 2012/12/25. doi: 10.1097/TP.0b013e318277e2d1. PubMed PMID: 23263502.

18. Nagai K, Yagi S, Uemoto S, Tolba RH. Surgical procedures for a rat model of partial orthotopic liver transplantation with hepatic arterial reconstruction. Journal of visualized experiments : JoVE. 2013;(73):e4376. Epub 2013/03/26. doi: 10.3791/4376. PubMed PMID: 23524839.

19. Bessems M, Doorschodt BM, Albers PS, Meijer AJ, van Gulik TM. Wash-out of the non-heart-beating donor liver: a matter of flush solution and temperature? Liver international : official journal of the International Association for the Study of the Liver. 2006;26(7):880-8. Epub 2006/08/17. doi: 10.1111/j.1478-3231.2006.01295.x. PubMed PMID: 16911472.

20. Bessems M, t Hart NA, Tolba R, Doorschodt BM, Leuvenink HG, Ploeg RJ, et al. The isolated perfused rat liver: standardization of a time-honoured model. Laboratory animals. 2006;40(3):236-46. Epub 2006/06/29. doi: 10.1258/002367706777611460. PubMed PMID: 16803641.

21. Doorschodt BM, Schreinemachers MC, Behbahani M, Florquin S, Weis J, Staat M, et al. Hypothermic machine perfusion of kidney grafts: which pressure is preferred? Annals of biomedical engineering. 2011;39(3):1051-9. Epub 2010/12/17. doi: 10.1007/s10439-010-0228-7. PubMed PMID: 21161683.

22. Fabry G, Doorschodt BM, Grzanna T, Boor P, Elliott A, Stollenwerk A, et al. Cold Preflush of Porcine Kidney Grafts Prior to Normothermic Machine Perfusion Aggravates Ischemia Reperfusion Injury. Scientific reports. 2019;9(1):13897. Epub 2019/09/27. doi: 10.1038/s41598-019-50101-7. PubMed PMID: 31554887; PubMed Central PMCID: PMCPMC6761287.

23. Matheus WE, Reis LO, Ferreira U, Mazzali M, Denardi F, Leitao VA, et al. Kidney transplant anastomosis: internal or external iliac artery? Urol J. 2009;6(4):260-6. Epub 2009/12/23. PubMed PMID: 20027554.

24. Golriz M, Fonouni H, Nickkholgh A, Hafezi M, Garoussi C, Mehrabi A. Pig kidney transplantation: an up-to-date guideline. European surgical research Europaische chirurgische Forschung Recherches chirurgicales europeennes. 2012;49(3-4):121-9. Epub 2012/11/23. doi: 10.1159/000343132. PubMed PMID: 23172014.

25. Spetzler VN, Goldaracena N, Knaak JM, Louis KS, Selzner N, Selzner M. Technique of porcine liver procurement and orthotopic transplantation using an active porto-caval shunt. Journal of visualized experiments : JoVE. 2015;(99):e52055. Epub 2015/05/21. doi: 10.3791/52055. PubMed PMID: 25992583; PubMed Central PMCID: PMCPMC4542501.

26. Ribeiro RVP, Alvarez JS, Yu F, Adamson MB, Fukunaga N, Serrick C, et al. A Pre-Clinical Porcine Model of Orthotopic Heart Transplantation. Journal of visualized experiments : JoVE. 2019;(146). Epub 2019/05/14. doi: 10.3791/59197. PubMed PMID: 31081813.

27. Knaak JM, Spetzler VN, Goldaracena N, Louis KS, Selzner N, Selzner M. Technique of subnormothermic ex vivo liver perfusion for the storage, assessment, and repair of marginal liver grafts. Journal of visualized experiments : JoVE. 2014;(90):e51419. Epub 2014/08/26. doi: 10.3791/51419. PubMed PMID: 25145990; PubMed Central PMCID: PMCPMC4672992.

28. Musquera M, Peri LL, Alvarez-Vijande R, Oppenheimer F, Gil-Vernet JM, Alcaraz A. Orthotopic kidney transplantation: an alternative surgical technique in selected patients. Eur Urol. 2010;58(6):927-33. Epub 2010/10/05. doi: 10.1016/j.eururo.2010.09.023. PubMed PMID: 20888120.

29. Doorschodt BM, Schreinemachers MC, Florquin S, Lai W, Sitzia M, Zernecke A, et al. Evaluation of a novel system for hypothermic oxygenated pulsatile perfusion preservation. The International journal of artificial organs. 2009;32(10):728-38. Epub 2009/11/28. PubMed PMID: 19943234.