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TITLE:

Orthotopic Kidney Auto-Transplantation in a Porcine Model Using 24 Hours Organ Preservation And Continuous Telemetry

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SUMMARY

Large animal models play an essential role in preclinical transplantation research. Due to its similarities to the clinical setup, the porcine model of orthotopic kidney auto-transplantation described in this article provides an excellent in vivo setting for the testing of organ preservation techniques and therapeutic interventions.

ABSTRACT

In the present era of organ transplantation with critical organ shortage, various strategies are employed to expand the pool of available allografts for kidney transplantation (KT). Even though, the use of allografts from extended criteria donors (ECD) could partially ease the shortage of organ donors, ECD organs carry a potentially higher risk for inferior outcomes and postoperative complications. Dynamic organ preservation techniques, modulation of ischemia-reperfusion and preservation injury, and allograft therapies are in the spotlight of scientific interest in an effort to improve allograft utilization and patient outcomes in KT.

Preclinical animal experiments are playing an essential role in translational research, especially in the medical device and drug development. The major advantage of the porcine orthotopic auto-transplantation model over ex vivo or small animal studies lies within the surgical-anatomical and physiological similarities to the clinical setting. This allows the investigation of new therapeutic methods and techniques and ensures a facilitated clinical translation of the findings. This protocol provides a comprehensive and problem-oriented description of the porcine orthotopic kidney auto-transplantation model, using a preservation time of 24 hours and telemetry monitoring. The combination of sophisticated surgical techniques with highly standardized and state-of-the-art methods of anesthesia, animal housing, perioperative follow up, and monitoring ensure the reproducibility and success of this model.

INTRODUCTION

Since the first successful human renal transplantation between identical twins, performed by the pioneering group of the Nobel prize laureate surgeon in 1954, Joseph Murray[1], kidney transplantation (KT) has evolved as the mainstay of treatment for patients with end-stage renal disease (ESRD)[2]. KT shows superior long-term clinical outcomes and quality of life compared to dialysis[2]. Short- and long-term survival rates after KT improved continuously, due to advances in surgical techniques, organ preservation, immunosuppressive therapy, and critical care, hence KT became widely available on a global scale[2-4].

Due to critical organ shortage, there is a continuously increasing gap between allograft supply and demand[3,5,6]. In 2018, approximately 12,031 patients were waiting for KT in Germany, however, only less than 20% (2,291 patients) could receive a donor kidney due to the extreme shortage in organs for transplantation[7]. Unfortunately, not only the absolute number of organ donors, but also the general quality of the allografts offered for transplantation have declined in the past decades[8,9]. An increasing tendency was observed in the numbers of predamaged or

"marginal" kidney allografts that had to be accepted for transplantation[10]. The use of ECD allografts may reduce waiting time and waiting list morbidity and mortality, it is, however, associated with an increased incidence of graft-related complications such as primary graft non-function (PNF) and/or delayed graft function (DGF)[8-10]. Further research is essential to optimize allograft utilization, expand the donor pool and protect and recondition marginal allografts which ultimately may improve patient outcomes[3,6].

Due to the resource-intensive and complex nature of large animal transplantation models, a large number of studies are performed using small animals or in ex vivo settings[11-15]. Although these models can deliver important scientific data, the translation of these findings to the clinical setting is often limited. The porcine model of orthotopic kidney auto-transplantation is a well-established and reproducible model that allows testing of new innovative treatment approaches in a clinically relevant in vivo setting, with potentially longer follow-up periods and abundant possibilities for repetitive sample collection[16,17]. Beyond the advantage of the comparable size, which allows relatively direct translation into the clinical setting (particularly for medical device development and drug dosage), the surgical-anatomical and physiological similarities in terms of ischemia-reperfusion injury (IRI) response and kidney damage, support the use of this model in translational research[17-19]. This model also provides an excellent training opportunity to prepare young transplant surgeons for the technical challenges of clinical organ transplantation[20].

There are also multiple differences compared to the human setting and various technical modifications of the model can be found in the literature[16,17,19-21]. This article comprehensively describes technical details, pitfalls, and recommendations which can aid to establish the model of porcine orthotopic kidney auto-transplantation. The described telemetry and video monitoring method as well as our specifically designed housing facility allows a close-up severity assessment and clinical observation of the animals. The use of a percutaneous urinary catheter and designated porcine jackets provide the possibility of a detailed assessment of kidney function without the use of metabolic cages. These technical modifications are described as potential solutions to comply with the modern challenges of the 3R principle (Replacement, Reduction and Refinement) and improve animal experiments using large animal models[22].

PROTOCOL

The present study was designed according to the principles of the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines[23]. Experiments were performed in accordance with the institutional guidelines and the German federal law regarding the protection of animals. The full ethical proposal was approved by the responsible authorities (Governmental Animal Care and Use Committee, LANUV NRW – "Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen", Recklinghausen, Germany, Protocol ID: 81-02.04.2018.A051). All animals in the present study received humane care according to the principles of the "Guide for the Care and Use of Laboratory Animals" (8th edition, NIH Publication, 2011, USA) and the Directive 2010/63/EU on the protection of animals used for

scientific purposes (Official Journal of the European Union, 2010). Female German landrace pigs were obtained from a hygienically optimized barrier breeding facility (Heinrichs GbR, Heinsberg, Nordrhein-Westfalen). **Figure 1** depicts the summary of the described experimental protocol.

1. Animals and housing

1.1. Use female German landrace pigs (or comparable) for this protocol. Deliver the animals to the research facility 14 days before the first surgery (telemetry implantation) for acclimatization and house them in a temperature- and humidity-controlled barrier environment with a 12 h light and dark cycle (**Figure 2**).

1.2. Mount two telemetry receivers on the ceiling of the room which allows the registered data to be transferred directly to a PC located in the observation room. Ensure that animals are observed visually during the regular visits by the veterinary officers and by the animal caretaker in charge (every 8 h and on-demand).

NOTE: Furthermore, in this experiment a real-time camera footage with integrated thermal imaging connected to the local network was used. Details of the housing facility used in this study are depicted in **Figure 2**.

2. Basic techniques and common procedures

2.1. Fast the animals overnight before surgery.

2.2. Premedicate by an initial intramuscular injection of azaperone (4 mg/kg) and atropine (0.1 mg/kg), followed by an injection of ketamine (15 mg/kg) 10 min later.

2.3. After premedication, weigh the animal and transfer it directly from the housing facility to the central OR facility anesthesia preparation room.

2.4. Cannulate one of the large ear veins using an 18 G peripheral venous catheter. Monitor the animal by a standard ECG and pulse oxymetry.

2.5. Initiate the anesthesia with propofol (3 mg/kg).

2.6. Expose the vocal cord with a laryngoscope and insert a 7.5 mm endotracheal tube. The cuff is blocked with air according to manufacturer´s recommendations.

2.7. Insert an oro-gastric drainage tube to remove fluid and air from the stomach.

2.8. Insert a urinary catheter via the urethra.

2.9. Subsequently, trim the skin in the area of the surgical incision.

2.10. Apply eye ointment to prevent drying of the cornea during surgery.

2.11. After orotracheal intubation, maintain anesthesia with isoflurane (final expiratory 1.45-2.0 Vol.%) and fentanyl (3 – 7.5 µg/kg/h).

2.12. Ensure active intraoperative temperature control of the animal by a heating pad and using warmed air. Insert a rectal probe to monitor body temperature (target temperature 36.5 °C - 37.5 °C).

2.13. Administer antibiotic prophylaxis using cefuroxime (35 mg/kg i.v.). Infuse Ringer solution at 4 mL/kg/h and increase to 8 mL/kg/h after skin incision. Administer a prophylactic dose of pantoprazole (40 mg i.v.) over the ear vein access.

2.14. Perform all surgical procedures under sterile conditions according to the general principles of surgical asepsis and antisepsis. Disinfect the surgical field with povidone-iodine solution and cover with surgical drapes.

3. Telemetry implantation

3.1. Prepare the animal for surgery following the steps described under section 2 of the protocol and confirm proper anesthesia by a decreasing heart rate and a lack of conscious movement of the animal.

3.2. Collect blood and urine samples to determine individual baseline lab values.

3.3. Mark the incision sites using a permanent marker.

3.4. To implant the arterial sensor of the telemetry transponder, perform a 3-4 cm incision in the groin. Expose and dissect the artery in a 360˚ fashion.

3.5. Using an Overholt clamp pull through two-vessel loops below the artery and secure them with mosquito clamps.

3.6. After making an arteriotomy using #11 blade scalpel, insert the arterial sensor. Close the arteriotomy using 5-0 polypropylene suture with single knot sutures and secure the arterial sensor using one of these sutures.

3.7. Make a 3-4 cm large incision on the left flank of the animal and create a subcutaneous pouch for the transponder by blunt dissection.

3.8. Tunnel the telemetry transponder to the flank and fix it to the muscle fascia (3-0 polypropylene, single knot). Tunnel the red and white ECG electrodes to the right and left side of the thorax. Make two 1 cm incisions and secure the electrodes in the muscle tissue to ensure a good ECG signal with single knot sutures (3-0 polyglactin).

3.9. Commence registration of telemetry data and check the various signals (e.g., body temperature registered by the transponder body itself, arterial blood pressure, and ECG signals).

3.10. Close the incisions in the groin, at the left flank and the two small thoracal incisions using muscle and subcutaneous sutures (3-0 polyglactin) and close the skin using a non-absorbable monofilament suture (e.g., 2-0 Prolene).

3.11. Use a spray film dressing to seal the incision sites.

3.12. At this time point make the animal wear a designated porcine jacket which the animal wears for the rest of the study period. Replace jackets with a clean jacket following every surgical intervention.

NOTE: To record stable baseline data, telemetry transponders are implanted 14 days before the index surgery (left nephrectomy, please also see **Discussion**).

4. Nephrectomy and kidney graft retrieval

4.1. Prepare the animal for graft retrieval surgery following the procedures described in section 2.

4.2. After the induction of anesthesia, cannulate the external jugular vein. Following the sterile disinfection of the surgical field, a 4 cm incision is made on the right side in the jugular groove.

4.3. Dissect subcutaneous tissue and muscle to expose the external jugular vein.

4.4. Expose and dissect the vein in a 360˚ fashion.

4.5. Using an Overholt clamp pull through two-vessel loops below the vein and secure them with mosquito clamps.

4.6. Tunnel the jugular catheter to the back of the animal. For this, position the pig on its left side. Use the Seldinger method to insert the jugular catheter.

4.7. Close the opening on the vein and secure the catheter using 5-0 polypropylene suture.

4.8. Close the incision in two layers (e.g., 3-0 polyglactin for the muscle and subcutaneous and 2-0 polypropylene for the skin).

4.9. Secure the catheter to the skin with multiple sutures (2-0 polypropylene).

4.10. Test the jugular vein catheter for free aspiration and injection. Subsequently, switch

the intravenous line from the ear vein cannula to the central venous line.

4.11. Following surgical disinfection and draping, perform a median laparotomy to open the abdomen (25-30 cm). Use a standard abdominal retractor to expose the surgical field.

4.12. Use wet and warm abdominal towels to cover the colon and the small bowel. Ask the second assistant to hold the bowel to the direction of right hemi-abdomen exposing the kidney and its vascular structures.

4.13. Open the peritoneal layer and dissect the left kidney and the ureter from any adherent tissue using the monopolar cautery, bipolar forceps, and fine scissors.

4.14. Ligate and divide the left ureter (3-0 polyglactin) leaving an at least 10 to 12 cm long segment.

4.15. Complete the dissection of the left renal vein(s) and artery to their origin from inferior vena cava and aorta, respectively.

NOTE: Avoid injury and opening of the large lymphatic vessels in this anatomical region. Also be aware of a potential injury to the azygo-lumbar vein joining to the renal vein near to its origin from the vena cava.

4.16. Dissect and ligate the azygo-lumbar vein between two ligatures (3-0 polyglactin).

4.17. Prepare for the back-table dissection using a bowl of ice and a sterile cover.

4.18. To retrieve the graft kidney, clamp the renal artery and the renal vein close to the aorta and the vena cava with vascular clamps. Remove the kidney graft by cutting the vessels with a scissor close to the clamps then hand the kidney over to the back-table team.

4.19. Close the stump of the renal artery using a 5-0 polypropylene suture. Close the renal vein using a two-layer continuous suture with 5-0 polypropylene. Remove the vascular clamps.

4.20. After checking the area for bleeding or lymphatic leakage, close the abdomen in 4 layers.

NOTE: Peritoneum: 3-0 polyglactin running suture; fascia: 0 polyglactin running suture; subcutaneous layer: 3-0 polyglactin running suture; skin: skin staplers after kidney retrieval surgery, to facilitate re-opening the abdomen the following day and 2-0 polypropylene single knot sutures after the transplantation procedure for definitive closure.

4.21. After applying sterile wound dressing, return the animal to the housing facility and allow to recover following endotracheal extubation. For postoperative analgesia, use buprenorphine (0.05 – 0.1 mg/kg) intramuscularly every 8 h until auto-transplantation.

5. Back-table and organ preservation

5.1. After graft retrieval, immediately cannulate the renal artery using a standard 14 G (orange) peripheral catheter and fix it using a tourniquet prepared from 3-0 polyglactin.

5.2. Rinse the kidney with cold organ preservation solution.

5.3. After flushing with 500 mL of organ preservation solution, remove the arterial cannula, wrap the kidney graft in sterile organ bags and store in organ preservation solution with a target cold ischemic time (CIT) of 24 h at 4 °C using a computer-controlled cooling circuit.

NOTE: A brief post-preservation flush is recommended using 500 mL of 4 °C normal saline solution.

6. Contralateral nephrectomy and orthotopic kidney auto-transplantation

6.1. During the recipient operation, adapt premedication and initial anesthesia to the restricted renal metabolism and avoid the use of ketamine. Induction is performed with propofol (3-5 mg/kg i.v.), midazolam (0,25 mg/kg i.v.), and atropine (0.1 mg/kg i.m.). Thereafter, the preoperative preparation is identical to the procedures described in section 2.

6.2. Maintain anesthesia with isoflurane (final expiratory 1.45-2.0 Vol.%) and fentanyl (3 - 7,5 µg/kg/h) and propofol (2 – 4 mg/kg/h).

6.3. Check and continuously monitor ECG, pulse oximetry, rectal temperature and the function of the telemetry transponder.

NOTE: Strict anesthesia and blood pressure control is of crucial importance during the implantation procedure.

6.4. In rare cases where the arterial blood pressure signal registered over the telemetry transponder is not satisfactory due to the supine position of the animal, place a further arterial catheter into the right femoral artery using percutaneous puncture and the Seldinger technique.

6.5. Following sterile draping, reopen the median laparotomy and expose the surgical field using the abdominal retractor. The colon and small bowel are placed to the left side of the abdomen to expose the intact right kidney.

6.6. Similar to the donor procedure, dissect the contra-lateral kidney and its vessels from the surrounding tissue. Dissect the right renal vein and renal artery in the direction of the kidney hilum to ensure sufficient vessel length for anastomosis.

6.7. 2 - 5 min before vascular clamping, inject natrium-heparin intravenously (100 I.U./ kg).

6.8. Clamp the right renal artery and the right renal vein using vascular clamps. The right kidney is removed. The vessels are checked for integrity before starting the anastomoses.

6.9. Place the preserved graft kidney into the abdomen and start the venous and arterial anastomoses.

6.10. From this point onwards, keep the mean arterial pressure over 80-90 mm Hg to ensure a good early perfusion of the kidney graft following reperfusion. Achieve this partially by adequate volume management and partially by the administration of norepinephrine (0.1 - 1.0 µg/kg/min as a continuous infusion using the mean arterial blood pressure and heart rate for monitoring the efficiency).

6.11. Perform end-to-end anastomosis of the renal vein:

6.11.1. After placing two corner stitches using 5-0 polypropylene, suture the back wall in a continuous fashion.

6.11.2. Tie the cranial corner stitch and tie it together with the thread used for the back wall.

6.11.3. After finishing the back wall, use the cranial corner stitch to suture the front wall in a cranio-caudal direction. Flush the vein with a heparinized saline solution (100 I.U./mL). Tie the caudal corner stitch.

NOTE: In case of a size mismatch between the donor and recipient sides, a small growth-factor can be used to ensure a wide and sufficient anastomosis. There are many possible variations of the porcine renal vein branches. In the case of complex venous anatomy, a modified anastomosis approach is necessary (see **Figure 3**).

6.12. Perform the end-to-end anastomosis of the renal artery:

6.12.1. Use a 6-0 polypropylene cranial corner stitch to perform the arterial anastomosis. Placing a further caudal, supporting corner stitch which is later removed, is optional.

6.12.2. Suture the back wall in a continuous fashion. After arriving at the caudal corner remove the second corner stitch (if applicable).

6.12.3. Suture the front wall with the other end of the double-armed 6-0 polypropylene suture. Flush the artery with a heparinized saline solution (100 I.U./mL). Tie the two threads at the caudal corner.

6.13. Record the times needed for performing both anastomoses with a target warm ischemia time of <40 min.

6.14. Reperfuse the kidney by opening the venous vascular clamp and subsequently the arterial clamp. Check for significant bleeding.

6.15. If no significant bleeding from the anastomoses is observed, unwrap the kidney graft and pour warm normal saline solution in the abdomen covering the reperfused graft.

6.16. Reposition the graft, if needed, to ensure homogeneous reperfusion and avoid congestion.

6.17. Administer papaverine topically to the outside of the renal artery and the arterial anastomosis (5 mL undiluted).

6.18. After reperfusion, infuse 250 mL of 20% glucose solution to induce osmotic dieresis followed by the administration of a single dose of 80 mg of furosemide.

NOTE: Following this, initial urine production may be observed.

6.19. To ensure urinary drainage, pass a 12 French pediatric urine catheter through the abdominal wall of the right flank of the animal, retroperitoneally.

6.20. Secure the catheter in the ureter using ligatures (2-0 polyglactin) and block the catheter with 2 mL saline. Further sutures are used the adapt and secure the ureter to the peritoneum of the abdominal wall (2-0 polypropylene). The catheter is also secured to the skin with at least two single knot sutures (2-0 polypropylene).

6.21. Close the peritoneal layer over the kidney to prevent dislocation of the kidney graft and kinking of the vascular anastomoses (3-0 polyglactin).

6.22. Close the abdomen in a similar 4-layer fashion as described earlier for the graft retrieval.

6.23. Following abdominal closure, maintain normothermia on the OR table.

NOTE: Mean arterial blood pressure should be maintained over 80 mm Hg until the animal is awake and is in a prone position.

6.24. Following abdominal closure, use color Doppler ultrasound to ensure adequate arterial and venous perfusion of the kidney graft (**Figure 4**). Monitor the animal closely until its fully awake and can walk and drink spontaneously. The animals are given 1 L of Ringer solution during the recovery phase.

6.25. Subsequently, return the animal to its box in the housing facility.

7. Follow up, sample and data collection

7.1. Provide the animals water ad libitum as soon as they can drink spontaneously. Provide solid food from postoperative day 1.

7.2. For postoperative analgesia, administer buprenorphine (0.05 – 0.1 mg/kg) intramuscularly every 8 h for 72 h, give pantozol (40mg i.v.) once a day for 72 h. Provide antibiotic treatment (cefuroxime 35 mg/kg i.v. 2x daily) and thrombosis prophylaxis (500 mg of acetylsalicylic acid from postoperative day 1) during the whole observation period until the end of the experiment.

NOTE: If bleeding complications occur, aspirin is discontinued.

7.3. Register continuous telemetry data throughout the observation period. Ensure that the animals are visited at least every 8 h by the veterinary officer and/or by an experienced veterinary technician and their clinical condition is evaluated using a score sheet which is used as a basis to prematurely terminate the experiment if required by the clinical condition of the animal.

NOTE: These so-called humane endpoint criteria are defined as described previously[24].

7.4. Perform daily sample collection using the central venous line and the percutaneous urinary catheter. Change urinary bags (2,000 mL) 2x daily.

7.5. Following sample collection or the administration of fluids or drugs, block the central venous catheter with heparinized saline solution (100 I.U./mL) between every use to avoid occlusion and cover it with a new sterile cap.

7.6. Following the corresponding observation period of 5 to 7 days, sacrifice the animals in deep anesthesia following relaparotomy, sample collection and explantation of the kidney graft. Sacrifice is performed using a single injection of pentobarbital (50 - 60 mg/kg i.v.).

NOTE: In compliance with the 3R principle, the remaining organs and tissues of the sacrificed animals may be used for various ex vivo research and educational purposes in in-house institutes.

REPRESENTATIVE RESULTS

Our group has several years of experience with solid organ transplantation models in small- and large animals and utilized the porcine orthotopic kidney auto-transplantation model, obtaining reproducible results in various experimental settings[16,25-27]. Depending on the experimental setup, we recommend performing 3 to 5 auto-transplantations as preliminary experiments which ensures a sufficient learning curve of the whole experimental team. In the present setting 5 transplantations were required to train a surgeon, with 8 years of previous experimental- and 5 years of clinical surgical experience in the field of transplantation surgery,

in performing these experiments. This can differ depending on the previous exposure of the surgeon to these techniques.

Within the frameworks of this protocol, the results of a set of 5 porcine orthotopic kidney auto-transplantation experiments are demonstrated. Transponder implantation was successful in each animal with sufficient telemetry signals throughout the observation period (except one animal with partial transponder dysfunction). Knife-to-skin interval for the transponder implantation was 85 min ± 5 min (**Table 1**). Following graft retrieval, all animals recovered well in the housing facility. Knife-to-skin interval for the retrieval surgery was 135 min ± 32 min (including approximately 30-45 min for the insertion, tunneling and securing of the jugular catheter). The left kidney was stored in a cold water-bath with a target cold ischemia time of 24 h (24 h ± 30 min). The following day, after anesthesia induction and relaparotomy, the contralateral (right) kidney was removed followed by the orthotopic auto-transplantation of the cold stored left kidney graft as described earlier. Knife-to-skin interval for the auto-transplantation surgery was 168 min ± 27 min (including the explantation of the right kidney). Warm ischemia time was 34 min ± 7 min. Each implanted kidney graft had a minimal but direct urine production following reperfusion. Following abdominal closure, color Doppler ultrasound showed satisfactory arterial and venous perfusion of the kidney in all cases (**Figure 4**). All animals recovered from the anesthesia and no significant complications were observed throughout the observation period. Daily blood and urine samples were collected. All pigs were in good clinical condition during the follow-up and were sacrificed after 5 days. Serum creatinine and potassium values peaked on POD3-4. The blood pH has remained within normal ranges (**Figure 5**). Urine output recovered to normal values over the first four postoperative days. White blood cell count was slightly increased at the end of the follow-up period (**Figure 5**). Body temperature, measured by continuous telemetry monitoring, showed slight fluctuations over the postoperative period.

FIGURE LEGENDS

Figure 1: Study flowchart and protocol. Abbreviations used: POD-postoperative day; ECG-electrocardiography.

Figure 2: Animal housing facility with real-time and continuous telemetry monitoring of up to 6 animals. (**A**) Schematic blueprint of our facility suitable for the housing and telemetry monitoring of up to 6 animals. The size of the single holding boxes was determined based on the guidelines of the EU Directive 2010/63 and ETS 123 Appendix A. Panels A-E show representative images of the organization of our facility. (**B**) Animal room for the housing of 6 animals. (**C**) Observation room with a PC used for the continuous registration of telemetry data. (**D**) Real-time video and thermal footage of the animals. (**E**) Individual holding ensuring acoustic and olfactory contact of the animals with their companions to avoid social isolation.

Figure 3: Orthotopic kidney auto-transplantation and anatomical variations and reconstruction possibilities. (**A**,**B**) The steps of the orthotopic kidney auto-transplantation model in case of a ”standard” vascular anatomy. (**C**) Variation 1: while one larger vein comes

with the donor kidney, there are two veins on the recipient side. Management: the smaller vein is closed by a ligature and the anastomosis is performed end to end between the renal veins. (**D**) Variation 2: while one larger vein comes with the donor kidney, there is no suitable recipient vessel on the contralateral side (e.g., size mismatch). Management: end to side anastomosis of the renal vein to the inferior vena cava. (**E**) Variation 3: two similar-sized veins on both sides. Management: reconstruction by two venous anastomoses. (**F**) Variation 4: while two similar-sized veins come with the donor kidney, there is no suitable recipient vessel on the contralateral side. Management: end to side anastomosis of the renal vein to the inferior vena cava in the case of two renal veins. (**G**) Variation 5: a donor kidney comes with a vein showing an early bifurcation, while there is one large vein on the contralateral side. Management: end to end anastomosis of the short common channel of the donor renal vein with one large vein on the recipient side. (**H**) Variation 6: while the donor kidney comes with a single renal vein with an early bifurcation, there is no suitable recipient vessel on the contralateral side. Management: end to side anastomosis of the short common channel of the donor renal vein to the inferior vena cava. This figure depicts a handful of the more frequent variations and is not statistically comprehensive in terms of all variations possible in German landrace pigs. Abbreviations used: KG-kidney graft; RK-right kidney; IVC-inferior vena cava; AO-aorta

Figure 4: Representative color Doppler ultrasound images, directly after orthotopic kidney auto-transplantation and abdominal closure. (**A**) Color Doppler ultrasound is performed directly following the implantation of the kidney and abdominal closure, to ensure good arterial and venous perfusion of the kidney graft and to screen for potential iatrogenic vascular kinking. Ultrasound was also used daily and on-demand, based on the clinical performance of the animal to screen for various problems. (**B-E**) Representative ultrasound images of a kidney graft following implantation. The image of the kidney graft with and without color Doppler (**B,C**) shows an excellent arterial (**D**) and venous perfusion (**E**). This figure show representative images from the same animal.

Figure 5: Representative laboratory findings and telemetry data of the orthotopic kidney auto-transplantation model with a cold ischemia time of 24 h. (**A**) Serum potassium values (**B**) Serum creatinine values (**C**) pH (**D**) White blood cell count (WBC) (**E**) Urine output. Abbreviations used: POD-postoperative day. (**F**) Mean body temperature registered by telemetric monitoring throughout the observation period in four consecutive kidney transplantation (no data presented from the 5^{th} animal due to partial transponder dysfunction).

Figure 6: Examples of possible peri-operative complications and pitfalls. (**A-C**) Postoperative congestion of the transplanted kidney graft on POD3 following orthotopic kindey auto-transplantation. (**D**) The reason for the congestion was identified as catheter kinking due to an overtightened suture on the level of the skin. After readjusting the suture the congestion resolved almost completely in 24 h. (**E**) Here an other kidney graft on POD2 following orthotopic kidney auto-transplantation is shown. Asterix (*) shows a fluid collection around the underpole of the graft (bloody collection vs. lymphocele). Due to our technique with closure of the peritoneum over the kidney these collections are usually self limiting due to the advantageous effects of local compression. Animals should be monitored closely in terms of the

local finding, signs of bleeding or infection. (**F**) Qualified color Doppler ultrasound performed daily (and on demand) in the housing facility has, besides its academic utilization (e.g., documentation, registration of arterial resistence indices), a crucial diagnostic role in recognizing potential complications in the early subclinical phase.

Table 1. Description of the required human resources and time-schedules for performing various experimental steps of the porcine kidney auto-transplantation model.

DISCUSSION

The porcine model of KT allows the investigation of novel therapeutic approaches and medical devices in a clinically relevant large animal setting[15,17,21]. The anatomical, pathophysiological and surgical-technical similarities between the porcine and human setting can facilitate the clinical interpretation of data and the rapid translation of the findings and techniques into clinical testing[15-19,21].

The model of orthotopic kidney auto-transplantation does not only comply with the 3R principle by reducing the numbers of required animals compared to allo-transplantation, e.g. no separate donor animal is required, but also provides a unique opportunity to investigate the effects of IRI and preservation injury without the confounding effects of the immunological response and immunosuppressive drugs[17,21].

Slight modifications of the protocol allow modeling a broad spectrum of clinical situations. To mimic KT using donation after circulatory death (DCD) kidneys, vascular structures are clamped for 30 to 60 min in situ before kidney retrieval, while prolonged cold ischemia times (24 hours and longer) can be applied to model extensive preservation injury[16,17,28,29].

Although, the porcine KT model is surgically less challenging than solid organ transplantation models in small animals (e.g. rats and mice)[26], there are multiple technical aspects and pitfalls which have to be kept in mind to improve outcomes and avoid specific complications[17].

Failing to avoid the large lymphatic vessels around the inferior vena cava and the aorta during graft retrieval or implantation due to technical mistake or anatomical variations, can lead to a high output lymphatic fistula and post-operative abdominal fluid collection, infection, and potentially technical failure. Lymphatic vessels should be completely avoided during surgery or closed with 5-0 or 6-0 polypropylene sutures. It is wise to also avoid the use of bipolar or any other coagulation device in case of lymphatic leaks. It usually leads to worsening of the situation. In case of a low output lymphatic leakage, our team has a good experience with the application of fibrin-based collagen patches (e.g., Tachosil)[30], however, their high cost limits their application in this setting.

In the present protocol we demonstrate a transperitoneal approach for kidney retrieval and auto-transplantation. This is a major technical difference compared to the clinical situation, where kidney grafts are usually implanted into the iliac fossa using an extraperitoneal approach. Although, most groups use a transperitoneal and an orthotopic approach in the porcine model,

heterotopic transplantation to the iliac fossa is also possible in pigs[31]. However, due to the relatively low diameter of the external iliac artery in 30-40 kg pigs and its tendency to vasospasm makes it sometimes difficult to perform the end-to-side anastomosis of the renal artery to the external iliac artery[31]. Concerning the fact that we retrieve the left kidney via a transperitoneal approach to perform a subsequent auto-transplantation, it is more feasible to perform the implantation by reopening the same incision and using a straigtforward orthotopic approach, especially that per-protocol it is also required to remove the native right kidney to ensure that the animal will recover with only one predamaged kindey. The comprehensive description of all possible technical variations of the model is beyond the scope of this protocol and has been summarized by others in comprehensive review articles[31].

Dislocation of the transplanted kidney graft and consequential kinking of the vascular anastomoses is a major source of failure in the porcine KT model, rapidly leading to vascular occlusion and complete failure of the experiment, due to a surgical complication. To avoid this, following auto-transplantation we close the peritoneal layer over the kidney with a running suture using 3-0 polyglactin. Furthermore, color Doppler ultrasound is performed directly following the implantation of the kidney and abdominal closure, to ensure good arterial and venous perfusion of the kidney graft. Ultrasound is also used daily and on-demand, based on the clinical performance of the animal, to screen for kidney perfusion, post-renal problems (e.g. obstruction or kinking of the urinary catheter), and fluid collection due to lymphatic fistula, bleeding or infection (**Figure 4** and **Figure 6**).

As 24 hours of cold ischemia often leads to functional impairment and delayed graft function, the animals may require on-demand medical therapy if it is considered necessary by the veterinary officer. This may include infusion therapy using 5% glucose and/or Ringer solution administered via the central venous line, furosemide bolus injections (in case of oliguria/anuria depending on the clinical state and laboratory results, 60-80 mg bolus injections up to 200 mg/day), and the oral administration of Sodium Polystyrene Sulfonate (Resonium A) in case of severe hyperkalemia[32]. To avoid experimental bias, the veterinary officer responsible for the post-transplant veterinary care of the animals must be blinded for the applied treatment and grouping.

Although, the anatomy of the renal artery is rather straightforward in German landrace pigs with usually one artery to reconstruct, there is a wide spectrum of anatomical variations of the renal vein branches which require certain surgical creativity during the venous reconstruction. Frequently two (or more) renal vein branches join on different levels between the kidney hilum and the inferior vena cava. The most frequently observed variations and the possible reconstruction options[17] are shown in **Figure 3**.

Following the first surgical intervention (day -15, telemetry implantation), all animals receive a porcine jacket which they wear throughout the whole period of the experiments. This provides excellent protection against accidental injuries and dislocation of the implanted catheters and provides room for the storage of the urine collection bags. The use of these jackets is also a feasible solution to eliminate the need for metabolic cages for the assessment of creatinine

clearance as a refinement method according to the 3R Principle.

Our housing facility integrates the use of telemetry and video-based peri-operative monitoring. Although, these methods cannot replace the regular visits by the veterinary officer and technicians, they facilitate rapid interventions and improve severity assessment to further refine our experimental settings for the future. There is a wide spectrum of indications for the use of an implantable telemetry device in large animal models[33]. Although, close monitoring of clinical paramters following major surgery such as ECG, blood pressure, temperature is considered to be standard in the human clinical setting of a surgical intensive- and intermediate care unit, in experimental surgery monitoring is mostly discontinued when the animal is waking up from anaesthesia[33-35]. Therefore, telemetry provides a feasible way for the continuous monitoring of these animals. 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, or sacrifice of the animal to avoid suffering). Besides these "real-time" monitoring aspect, our group is currently focusing on the severity assessment and refinement of animal experiments[36-38]. 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 (e.g., analgesia) in laboratory animals.

In terms of implantable telemetry, a period of at least 12 days after implantation of the measurement system is recommended to ensure stable and optimal measurement data (based on personal communication). After discussing this issue with various manufacturers providing telemetry solutions for large animals as well as with other research groups using these systems in various experimental settings, we decided to integrate a 14 day period between telemetry implantation and kidney transplantation. During the earlier days, deviations may still occur due to the movement of the animal as the scarring and healing processes are still uncomplete.

Despite its advantages, the above-described model has certain limitations. The complexity and required resources and infrastructure are the most important limitations of the model. The time-consuming experimental protocol, complex techniques, and intense peri-operative follow up necessitate the availability of a significant housing and OR capacity and require the involvement of a larger team, including doctoral fellows, surgeons, veterinary officers, and technicians (**Table 1**). Therefore, based on our empirical observations, it is usually unfeasible to perform more than two procedures a day. A further disadvantage of the porcine model compared to small animal models is the limited possibility of mechanistic and molecular-biological investigations. In the present protocol only 5-days of follow up was reported. This was suitable to demonstrate the most important experimental characteristics of the model, however, this relatively short follow up may not be sufficient to answer certain specific research question (e.g. long-term recovery of function vs. acute damage). Therefore, a project related extension of the follow up might be necessary. This manuscript describes our current "best-practice" in the experimental setting of porcine orthotopic 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. Description and justification of each and every methodical aspect would be beyond the scope of the present protocol and has been discussed elsewhere[31]. Finally, it is also difficult to replicate the exact clinical situation of ECD KT in the porcine model where elderly donors, allografts with acute kidney injury and donors with multiple co-morbidities and chronic diseases such as hypertension, diabetes mellitus or arteriosclerosis represent a major part of the marginal donor pool[8,9].

Notwithstanding the above-mentioned limitations as well as technical and logistical challenges, this well-established and reproducible large animal model of KT provides a unique opportunity to investigate novel therapies and techniques to improve organ preservation and clinical outcomes and represents an excellent platform for younger surgeons to master organ transplantation techniques in a large animal model.

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DISCLOSURES

The authors have no conflict of interest to disclose.

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Figure1

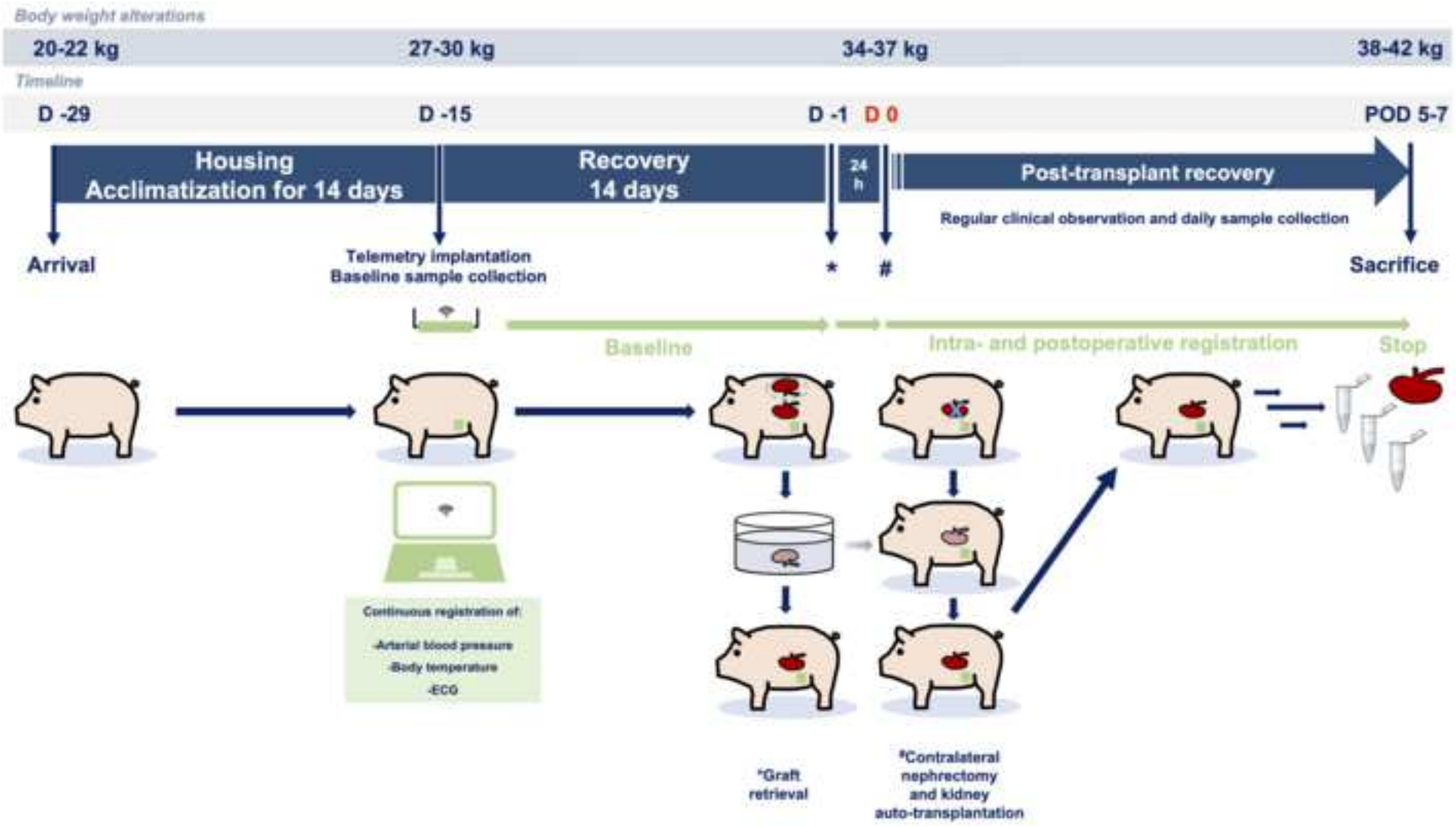

Figure2

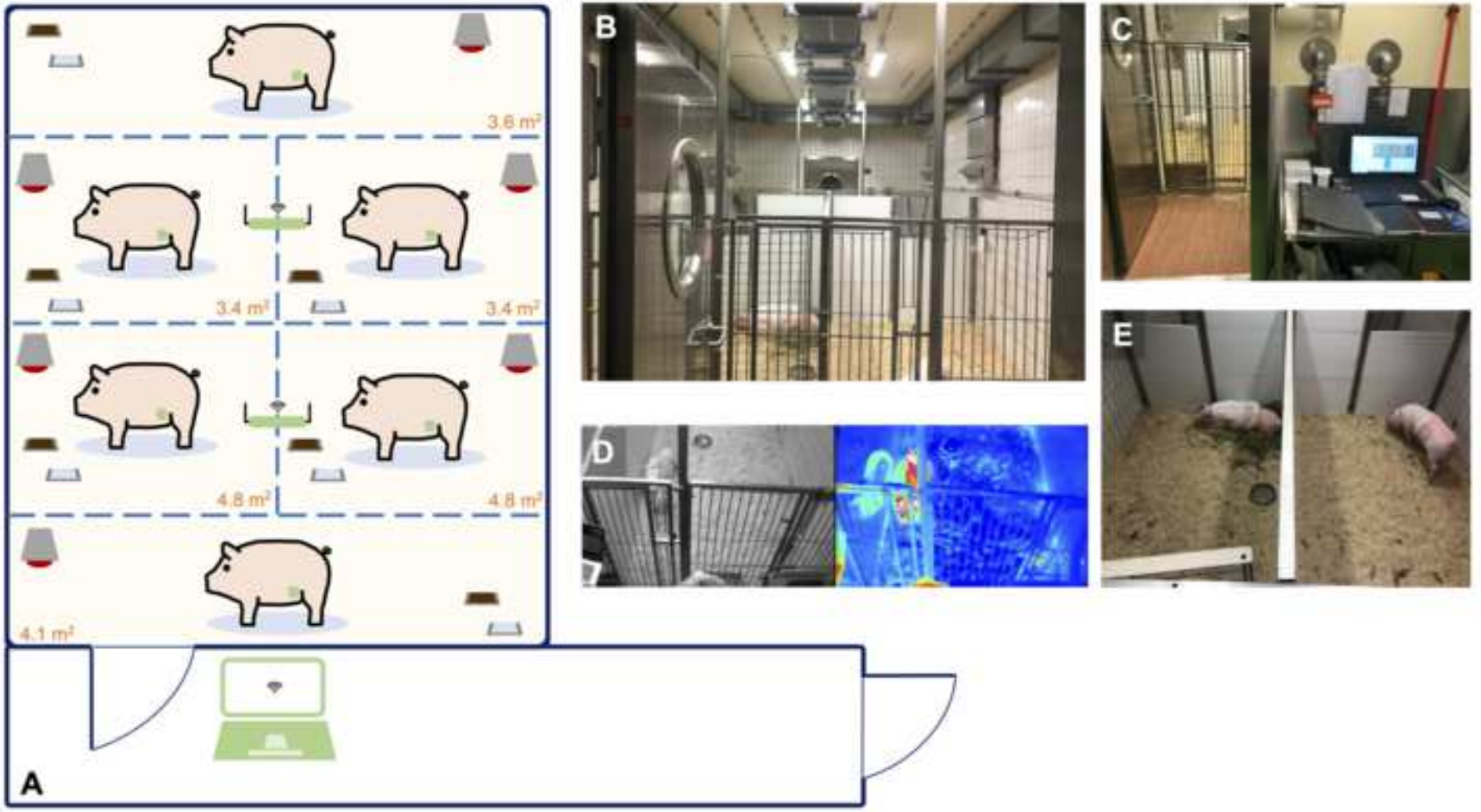

Figure3

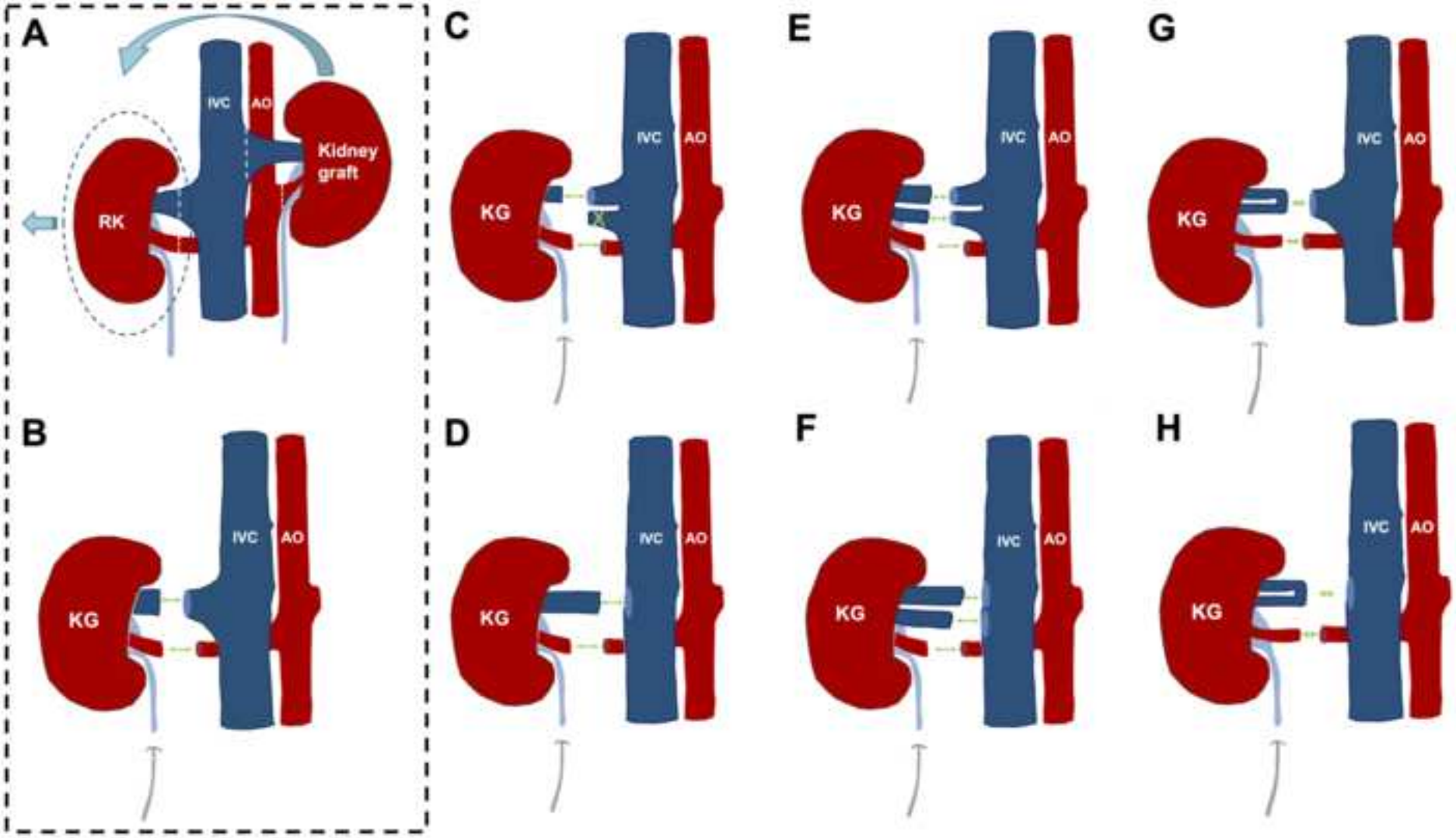

Figure4

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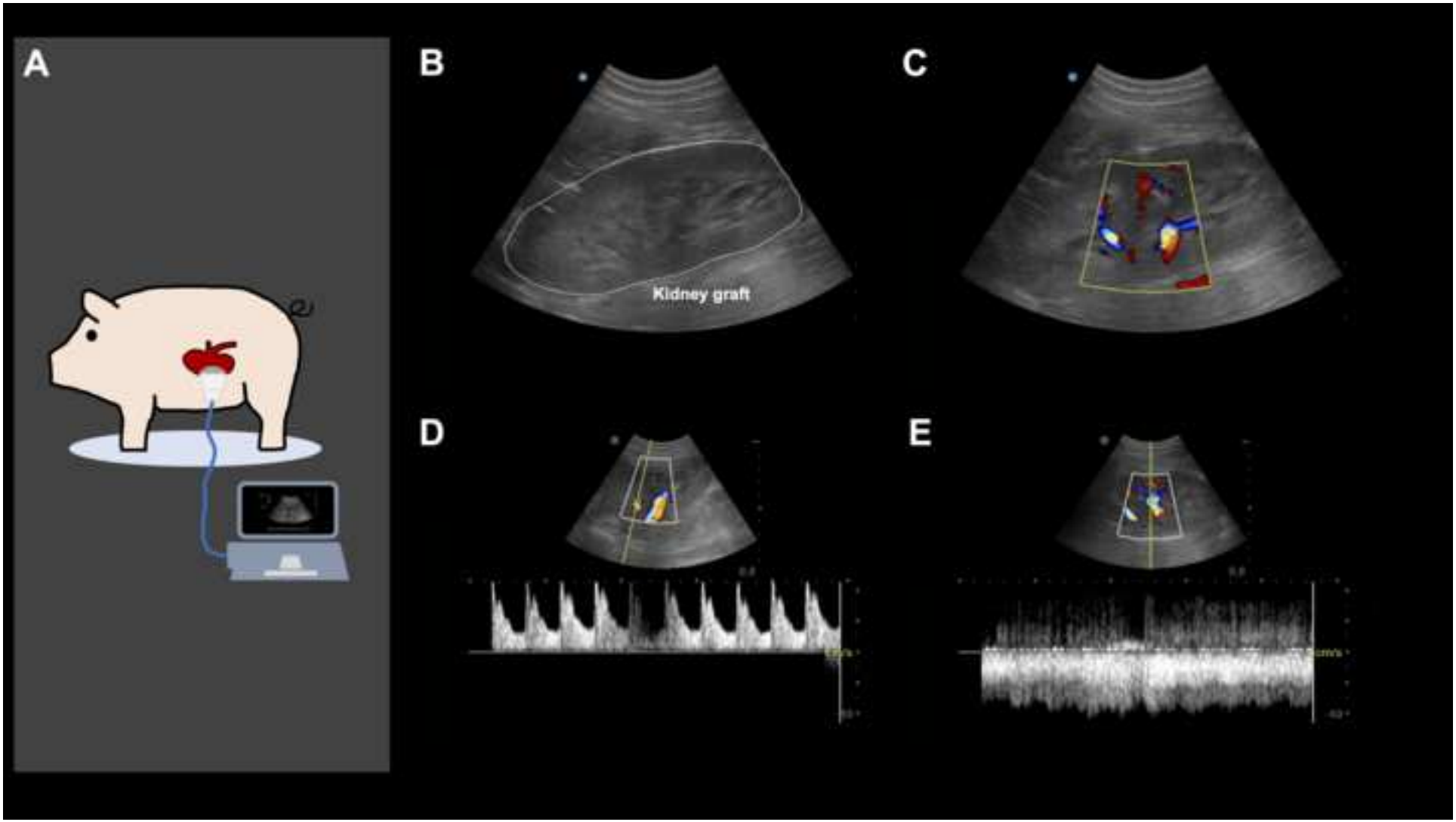

Figure5

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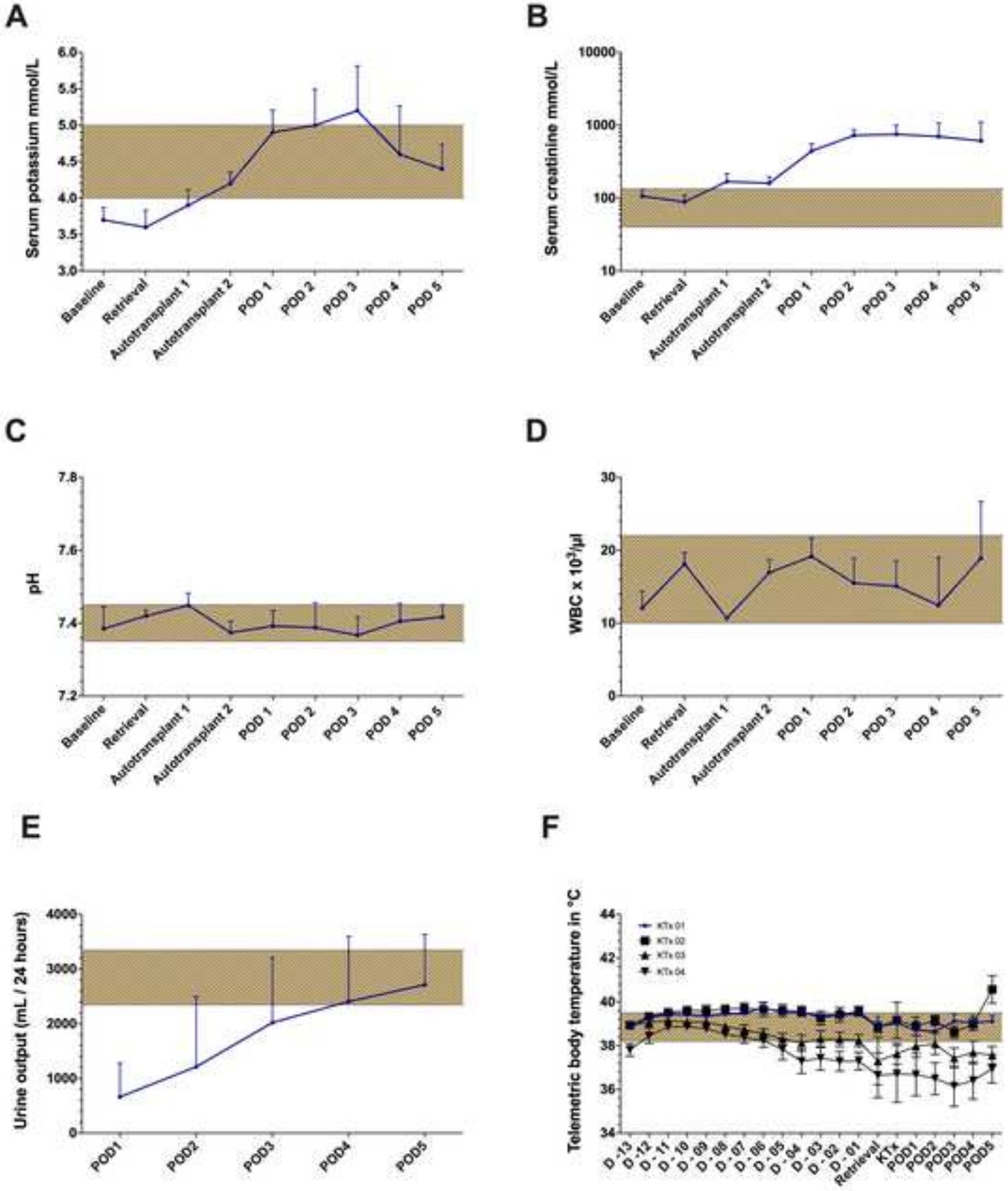

Figure6

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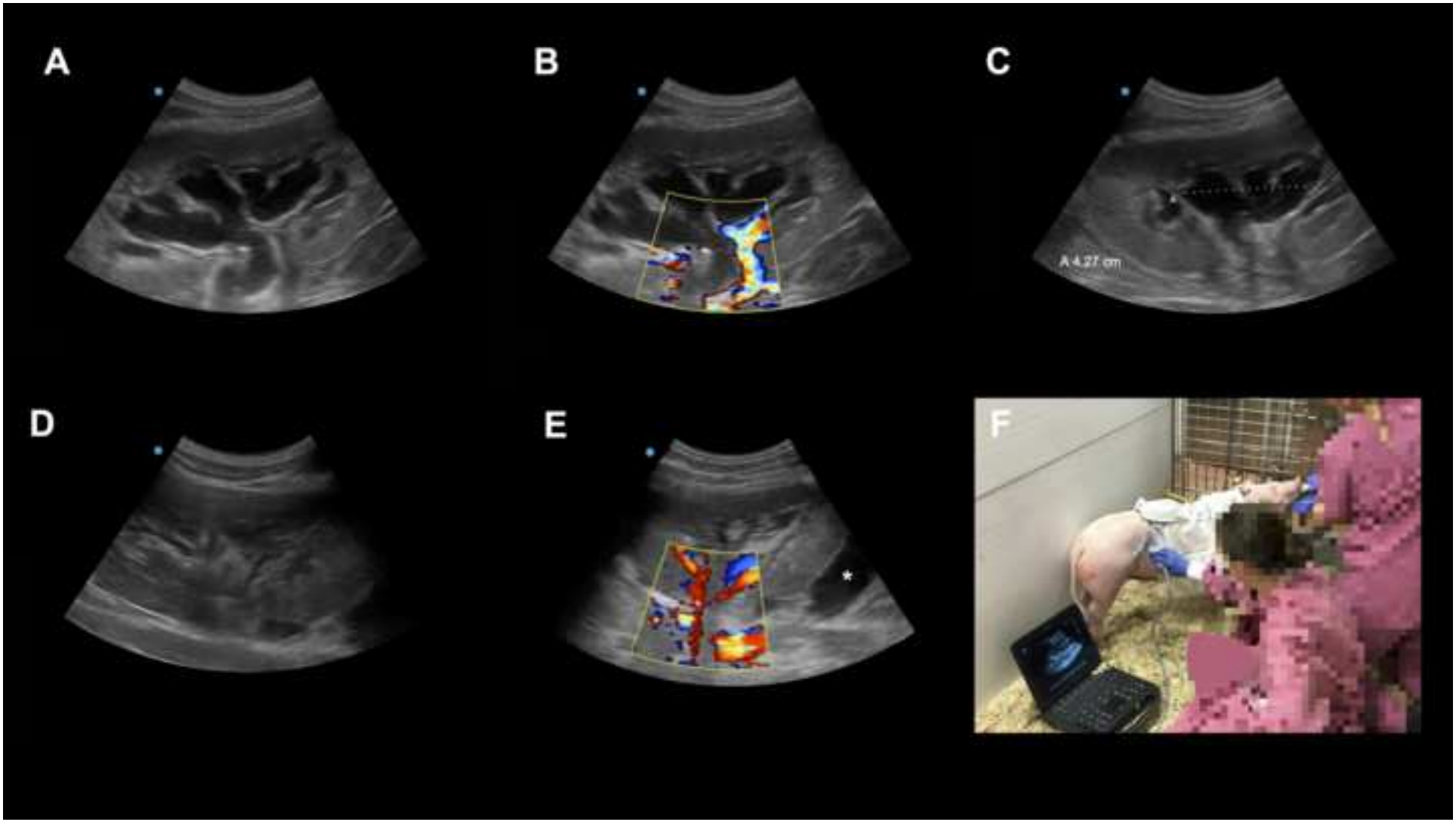

Experimental task/step	Days	Time (min)	Surgeon
Preopreative care	D-29 to D-15	n.a.	
Telemetry implantation surgery	D-15	85±5	1
Postoperative care following telemetry implantation	D-15 to D-1	n.a.	
Graft retrieval surgery	D-1	135±32	1
Kidney auto-transplantation surgery	D 0	168 ±27	1
Postoperative care following kidney auto-transplantation	D 0 to D5	n.a.	
Sacrifice	D 5	n.a.	

Veterinary officer	Veterinary technician	Laboratory technician	Doctoral student	Total Nr
1	1		1	3
1	1	1	1	5
1	1		1	3
2	1	2	2	8
2	1	2	2	8
2	1		2	5
2		1	1	4

Name of Material/Equipment	Company
Anesthesia materials, drugs and medications	
Aspirin 500mg i.v., powder for solution for injection	Bayer Vital AG, Leverkusen, Germany
Atropine sulfate solution for injection, 100mg	Dr. Franz Köhler Chemie GmbH, Bensheim, Germany
Bepanthen ointment for eyes and nose	Bayer Vital AG, Leverkusen, Germany
BD Discardit II syringes, 2ml, 5ml, 10ml,20ml	Becton Dickinson GmbH, Heidelberg, Germany
BD Micolance 3 (20G yellow) Cannula	Becton Dickinson GmbH, Heidelberg, Germany
BD Venflon Pro Safety (20G pink)	Becton Dickinson GmbH, Heidelberg, Germany
Buprenorphine (Buprenovet)	Bayer Vital AG, Leverkusen, Germany
Cefuroxime 750mg, powder for preparing injection solution	FRESENIUS KABI Deutschland GmbH, Bad Homburg, Germany
Covidien Hi-Contour, Endotracheal Tube 7,5 with Cuffed Murphy Eye	Covidien Deutschland GmbH,Neustadt/Donau, Germany
FENTANYL 0,5 mg Rotexmedica solution for injection	Rotexmedica GmbH Arzneimittelwerk, Trittau, Germany
Furosemide-ratiopharm 250 mg/25 ml solution for injection	Ratiopharm GmbH, Ulm, Germany
Glucose 5% solution for infusion (500ml, 250ml)	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Glucose 20% solution for infusion	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Heparin-Sodium 5000 I.E./ml	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Isoflurane-Piramal (Isoflurane)	Piramal Critical Care Deutschland GmbH, Hallbergmoos, Germany
Ketamine (Ketamine hydrochloride) 10%	Medistar Arzneimittelvertrieb GmbH, Ascheberg, Germany
MIDAZOLAM 15mg/3ml	Rotexmedica GmbH Arzneimittelwerk, Trittau, Germany
NaCl 0,9% solution for infusion (500ml,1000ml)	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Norepinephrine (Arterenol)	Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany
Organ preservation solution (e.g. HTK)	Dr. Franz Köhler Chemie GmbH, Bensheim, Germany
Pantoprazole 40mg/solution for injection	Laboratorios Normon,Madrid, Spain
Paveron N 25mg/ml solution for injection (Papaverine Hydrochloride)	LINDEN Arzneimittel-Vertrieb-GmbH, Heuchelheim, Germany

Pentobarbital (Narcoren)	Boehringer Ingelheim vetmedica GmbH, Ingelheim, Germany
Propofol 1% (10mg/ml) MCT Fresenius	FRESENIUS KABI Deutschland GmbH, Bad Homburg, Germany
Ringer solution	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Sterofundin ISO solution for infusion (1000ml)	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
Stresnil (Azaperone) 40mg/ml	Elanco
Urine catheter ruffle 12CH	Wirutec Rüsch Medical Vertriebs GmbH, Sulzbach, Germany
Surgical materials	
Appose ULC Skin Stapler	Covidien Deutschland GmbH,Neustadt/Donau, Germany
Cavafix Certo 375	B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany
EMKA Easytel +L-EPTA Transponder	emka TECHNOLOGIES S.A.S,Paris,France
EMKA Reciever and Data Analyzer System	emka TECHNOLOGIES S.A.S,Paris,France
Feather Disposable Scapel (11)(21)	Feather, Japan
Prolene 2-0, blue monofil VISI-BLACK, FS needle	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
Prolene 3-0,blue monofil,FS1 needle	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
Prolene 5-0 (simply angulated, C1 needle) blue monofil VISI-BLACK	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
Prolene 5-0 (double armed, C1 needle) 60cm	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
Prolene 6-0 (double armed, C1 needle) 60cm	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
Sempermed derma PF Surgical Gloves Seril Gr. 7, 7.5, 8	Semperit investment Asia Pte Ltd, Singapore
Sentinex® PRO Surgical Gowns Spunlace XL 150cm	Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany
Tachosil	Takeda Pharma Vertrieb GmbH & Co. KG, Berlin, Germany
Telasorp Belly wipes (green 45x45cm)	PAUL HARTMANN AG,Heidenheim, Germany
Pediatric urine catheter	Uromed Kurt Drews KG, Oststeinbeck, Germany

VICRYL- 0 MH Plus	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
VICRYL - 3-0, SH1 Plus needle, 75cm	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
VICRYL - 3-0, SH1 Plus needle, 4*45cm	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
VICRYL - ligatures Sutupak purple braided, 3-0	Johnson & Johnson Medical GmbH - Ethicon Deutschland, Norderstedt, Germany
3M™ Standard Surgical Mask 1810F	3M Deutschland GmbH, Neuss, Germany
Surgical instruments	
Anatomical forceps Standard	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Atraumatic tweezers steel, De Bakey Tip 1,5mm 8"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Bipolar forceps 16 cm straight, Branch 0,30 mm pointed, universal fit	Bühler Instrumente Medizintechnik GmbH,Tuttlingen, Germany
Bulldog clamp atraumatic,curved, De bakey 78 mm, 3"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
DE BAKEY-SATINSKY vascular clamp 215mm	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Dissecting scissors Mayo,250 mm, 10"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Dissecting scissors Metzenbaum-Fino, 260 mm, 101/4"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Draeger CATO Anesthetic machine with PM8050 Monitor	Dräger, Drägerwerk AG & Co. KGaA, Lübeck, Germany
Fine Tweezers, ADSON 180 mm	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Gosset abdomenal wall spreader	CHIRU-INSTRUMENTE, Kaierstuhl,Germany
HALSTEAD MOSQUITO,curved, surgical 125mm	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
HF surgical device ICC 300, Electrocautery	Erbe Elektromedizin Gmbh; Tübingen, Germany
MICRO HALSTED-MOSQUITO 100mm, curved	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Micro steel needle holder straight 0,5mm, with spring lock	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Microsurgical/watermaker tweezers LINZ 150mm 6" Ergo round handle	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
needle holder Mayo-hegar,190 mm, 71/2"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany

Overhold Slimline Fig. 0 8 1/2"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Sterile Gauze 10X10	Paul HaRTMANN AG,Heidenheim, Germany
Suction tip OP-Flex Handpiece Yankauer	Pfm Medical AG, Köln, Germany
surgical forceps Standard 5 3/4"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
surgical scissors standard pointed-blunt (thread/cloth scissors)175 mm, 7"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Titanit vascular scissors POTTS-SMITH,185 mm, 71/4"60°	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Tunneling instrument	Marina Medical Instruments Inc,Davies,US
Vessel loops	Medline International Germany GmbH,Kleve, Germany
Wound spreaders Weitlander, Stump,110 mm, 41/4"	ASANUS Medizintechnik GmbH, Tuttlingen, Germany
Further material	
Heating pad	Eickemeyer - Medizintechnik für Tierärzte KG, Tuttlingen, Germany
Laryngoscope, customized	Wittex GmbH, Simbach, Germany
Rectal temperature probe	Asmuth Medizintechnik, Minden, Germany
Spray wound film	Mepro-Dr. Jaeger und Bergmann GmbH, Vechta, Germany
Sterile organ bag	Raguse Gesellschaft für medizinische Produkte, Ascheberg, Germany
swine jacket small, adult Landrasse swine 30-50kg, customized for Emka Telemetry and urinary catheterization	Lomir Biomedical Inc., United Kingdom
Ultrasound device, Sonosite Edge-II	FUJIFILM SonoSite GmbH, Frankfurt, Germany
Urine bag 2000ml Volume	ASID BONZ GmbH, Herrenberg, Germany

Catalog Number	Comments/Description
4324188	antiplatelet agents
1821288	parasympatholytic agent, premedication
1578675	eye ointment
300928, 309050,309110, 300296	syringes
305888	venous catheter
4491101	venous catheter
794-996	analgesia
J01DC02	antibiotics
COV-107-75E	endotracheal Tube
4993593	opioide analgetic agent
1479542	loop diuretics
3705273,03705422	infusion fluid
4164483	osmotic diuresis
15782698	anticoagulant
9714675	volatile anaesthetic agent
0004230	general anaestetic agent
828093	hybnotica, sedative agent
864671.8779	infusion fluid
16180	increase in blood pressure
should be decided based on preference and experimental design	organ preservation
11068	proton pump inhibitor
2748990	spasmolytic agent for vasodilatation

1,204,924,565	used for euthanasia
654210	general anaesthetic agent
1471411	infusion fluid
1078961	Infusion fluid
797-548	sedative
RÜSCH-180605-12	transurethral urinecatheter
8886803712	skin stapler
4153758	central venous catheter
L-EEEETA 100	telemetry transponder
Reviever	telemetry receiver
8902305.395	scapel
EH7038H	skin
EH7694H	skin
EH7227H	vascular
KBB5661H	vascular
EH7228H	vascular
4200782,4200871,4200894	surgical gloves
19302	surgical gown
MAXI 9,5 x 4,8 cm	haemostasis
4542437	abdominal towel
PZN 03280856	used for the uretero-cutaneus stoma

V324	fascial closure
W9114	subcutaneous suture, peritoneal suture,
V780	subcutaneous suture, peritoneal suture,
V1215E	threats for ligature
3M-ID 7000039767	surgical mask
PZ0260	anatomical forceps
GF0840	anatmical atraumatic forceps
08/0016-A	biopolar forceps
GF0900	bulldog clamps
GF1661	vascular clamp
SC2232	Scissors for dissection
SC2290	Scissors for dissection
106782	Ventilation System
ADSONPZ0571	fine forceps
09-621512	abdominal retractor
KL2291	mosquite clamps
20132-043	cautery, biopolar
KL2187	mosquite clamps
MN1324D	microsurgical needle holder
MN0087	fine microsurgical forceps
NH1255	needle holder

KL4400	overholds
401725	sterile gauze
33032182	suction
PZ1260	surgical forceps
SC1522	surgical Scissors
SC8562	Pott scissors
MM-TUN06025	subcutaneous tunneling
VLMINB	hold and adjust the vessel
WH5210	wound care
648050 MHP-E1220	maintain body temperature during surgery
333222230	expose the vocal cord
ASD-RA4	measure body temperature
2830	keep sterile condition
800059	organ preservation
SS J1LAPMP	swine jackets to pretect implanted catheters and store urine bag
V21822	ultrasound and color Doppler
2062578	disposable urine bag connected to the uretero-cutaneous fistula catheter

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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.

RWTH AACHEN UNIVERSITY

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.

RWTH AACHEN UNIVERSITY

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