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OPEN

A Pilot Study of Postoperative Animal Welfare as a Guidance Tool in the Development of a Kidney Autotransplantation Model With Extended Warm Ischemia

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Background. This pilot study aimed to maintain acceptable animal welfare in the development of a porcine autotransplantation model with severe and incremental renal ischemic injury, a model for usage in future intervention studies. Secondary aims were to develop and test methods to collect blood and urine without the need to restrain or use sedative and avoid transportation to optimize welfare of the pig. **Methods.** Kidneys from 7 female pigs were subjected to incremental durations of warm ischemia (WI) 30, 45, or 75 minutes by left renal artery and vein clamping. After static cold storage, contralateral nephrectomy was performed, and the injured graft was autotransplanted and animals observed for 14 days. Animal welfare was assessed and recorded using a structured scoring sheet before and 4 days after the kidney autotransplantation. Furthermore, blood samples were drawn daily the first week and every second day the following week using a semi-central venous catheter. An ostomy bag around the genitals was tested for urine collection. Measured glomerular filtration rate was calculated using renal clearance of chromium-51-labeled ethylenediamine tetraacetic acid on day 14. **Results.** None of the 7 animals died during the follow-up. The animal welfare was moderately affected when applying 75 minutes of WI (n = 2), and for that reason WI was not further increased. Pigs with lower WI had no observed welfare issues. With 75 minutes of WI peak, plasma creatinine was 1486 and 1317 $\mu\text{mol/L}$, reached on day 4. Lowest glomerular filtration rate levels were observed in the pigs with 75 minutes of WI. **Conclusions.** WI up to 75 minutes caused the intended severely impaired renal function without significantly compromising animal welfare. Blood and urine was collected postoperatively without sedation of the pigs or use of a metabolic cage.

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The gap between supply and demand of organs for kidney transplantation has been sought to be met by using “higher risk” donor organs from extended criteria donors or donation after circulatory death, but such kidneys are prone to a higher risk of delayed graft function.¹⁻⁷ The number of kidneys retrieved after circulatory death has significantly

increased in the past decade, contributing to reduced waiting lists, and they now constitute 28% of kidney transplantations in the United Kingdom,⁸ 18% in the United States,⁹ and even 58% in the Netherlands.¹⁰ The introduction of new strategies to improve graft function after the greater ischemic injury in

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A.K.K., U.M., M.E., B.K.M., B.J., J.H., C.M., R.J.P., and C.C.B. performed the critical revision.

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“higher risk” donor kidneys is of the utmost importance to first ensure organ quality but also to minimize the need for dialysis and improve patient well-being posttransplant. The porcine transplantation model has been proven to be an effective way to investigate innovative methods,^{2,11-17} as pig kidneys resemble human organs in both size and physiology^{2,14,18} as well as in metabolism, and therefore pig models have been widely used in recent years.¹⁹⁻²¹

An essential component in standardizing an experimental autotransplantation study is the animal itself, and attention to its well-being is crucial. In preclinical animal models, stress can lead to an activation of multiple systemic inflammatory responses,²² which in turn may have an impact on postoperative recovery and transplantation outcome.

Refinement (the principles of the 3Rs²³) refers to methods that can contribute to improved animal welfare. Perioperative approaches can, eg, be to keep invasive procedures and duration of general anesthesia to a minimum, sufficient surveillance of vital signs and corrections in consequence, and also to secure adequate fluid, and electrolyte balance while keeping the animal free from infections and pain during the observation time is quite important. Other welfare initiatives may include physical optimization, such as avoiding restraint, minimizing or avoiding transportation, and providing spacious facilities that allow mobility. Refinement also includes stimulation of animal activity by appropriate enrichment techniques as well as practice and rewarding desired tasks and/or behavior. The animal will be familiar with the procedures, and undesirable incidents are avoided.²⁴⁻²⁶ The 3Rs reduction of experimental animals is sought to be met by creating the highest possible amount of injury while balancing animal welfare. Proper amount of renal injury will gain meaningful results

and demonstrate difference between experimental groups, which in turn will reduce the number of animals needed in the experiment.

The autotransplantation pig model precludes interference of allogeneic confounders such as host-versus-graft reaction and the side effects of immunosuppressive medication.²⁷ An autotransplantation survival model including contralateral nephrectomy, to make continuous observation of the graft possible, may result in anuria, electrolyte imbalance, nausea, and fatigue, whereas a 2-kidney model may benefit from the healthy kidney, but the model has no clinical equivalent in a transplant setting. A model including contralateral nephrectomy will allow the use of blood and urine markers as read-outs of kidney function during the entire follow-up period, thus increasing the model's applicability.

The main goal of this pilot study was to evaluate changes in animal welfare after autotransplantation of kidneys that had suffered increasing renal injury to mimic the clinical setting of donation after circulatory death and additionally establish an upper limit of warm ischemia (WI) duration based on a pre-defined acceptable level of affected animal welfare. Secondary aims were to test usability of a semi-central venous catheter (CVC) for postoperative blood collections, as well as testing a noninvasive urine collection method.

MATERIALS AND METHODS

Animals and Welfare Assessment

Female 50-kg laboratory pigs (Danish Landrace and Yorkshire crossbreed) were used. Animals arrived at a specific pathogen-free housing facility 2 weeks before surgery for acclimatization and procedure training. The pigs were with at

TABLE 1.

Animal welfare score sheet used at the animal facilities at Department of Clinical Medicine, Aarhus University

	Points	Description
General appearance	Score normal animal 0, 1, 2, 3 for increase in severity	First impression of appearance in home pigpen
Respiration		Frequency/min.
Movement	0	Normal
	1	Limping gait and changed body posture
	2	Avoid supporting or crouched
	3	Laying down/sitting
Food intake	0	Normal food intake
	1	Eats up but slowly
	2	Does not eat up within 1 h
	3	No sign of food intake
Water intake		Describe if consumes water, only drinks when offered or no sign of water intake
Behavior	Score normal animal 0, 1, 2, 3 for increase in severity	Observe behavior in pigpen in relation to eating, movements, routines, curiosity, etc
Skin and fur/hair	Score normal animal 0, 1, 2, 3 for increase in severity	Color, hair loss, piloerection, skin changes
Defecation	0	Normal
	1	Minor changes in color, form and texture
	2	Moderate changes in color, form and texture
	3	Obstipation, diarrhea, blood
Urination		Describe if observed urination, urine in pen, no urine in pen
Surgical wound	0	Normal appearance for the given time after procedure
	1	Minor changes in color or observed swelling, warmth, local pain
	2	Moderate changes in color or observed swelling, warmth, local pain
	3	Lack of healing, infection, changes in color, swelling, warmth, local pain
Body temperature		Temperature in Celsius

It constitutes 11 items, which the animal keepers examine in their evaluation of each pig's welfare status. The scale range is between 0 and 21. If a pig scored ≥ 5 points, the affiliated veterinarian was to be contacted for possible initiatives to improve the welfare. A veterinarian contact was also done if a pig had abnormal respiration, no water intake or urination the last 24 h, or a fever. These items were therefore without assigned points.

least 1 neighbor but kept separately in the stable throughout the entire experimental period. The housing environment had a 12-hour light-dark cycle; animals were fed twice a day and provided water ad libitum and enrichment materials. Animal house staff, who were blinded to the WI duration, scored the pig's well-being on the day before first surgery and during the first 4 postoperative days. The scale consisted of the following parameters: general changes, respiration, movement, food and water intake, behavior, skin and fur/hair, defecation, urination, wound, and body temperature. The scale's range was between 0 and 21 (see Table 1). A score of 5 points or more required that the research veterinarian was contacted and the affected welfare should be improved (below 5 points) within 24 hours, or otherwise the animal should be terminated. Housing and surgical rooms were on the same site with no need for transportation of animals.

Ethics

Experiments complied with Animal Research: Reporting of In Vivo Experiments guidelines.²⁸ Furthermore, all animal care and procedures followed guidelines from the European Union (directive 2010/63/EU) and local regulations; the Animal Experiments Inspectorate approved the study (reference-number 2016-15-0201-01145). All personnel involved in the experiments had Federation of European Laboratory Animal Science Associations licenses in accordance with EU directive.

Experimental Design

Animal welfare of the 7 experimental pigs were evaluated after autotransplantations of donor kidneys exposed to incremental lengths of WI time to induce adequate graft injury. Pigs were allocated to WI of 30 ($n = 2$), 45 ($n = 3$), or 75 ($n = 2$) minutes, followed by static cold storage (SCS) before the autotransplantation. An overview of the experimental development and optimization is shown in Figure 1.

Pigs with 30 minutes of WI were studied first, followed by the pigs with 45 minutes of WI and lastly the pigs with 75 minutes of WI (see Figure 1). This order was chosen with incremental WI to monitor the degree of renal failure and its possible concomitant effect on animal welfare in each

pig before progressing to the next step and thus optimizing animal welfare. The experiments were conducted every other week, allowing acquired knowledge to be included into each subsequent experiment.

The surgical procedure duration may also influence animal welfare. In pigs 1–4, the 2 surgical procedures (kidney retrieval and kidney autotransplantation, described in detail below) were performed during a single anesthetic, and donor kidneys had SCS of an hour and a half. To expand the duration of SCS to a clinically relevant length of 16 hours, the procedures were performed on 2 consecutive days in the final 3 pigs. After contralateral nephrectomy, the ischemically injured and preserved grafts were autotransplanted by experienced surgeons. Animals were observed for 14 days.

Anesthetics and Analgesics

To allow vein cannulation, the fasting pigs were sedated with intramuscular injection of Midazolam (0.5 mg/kg). After induction of anesthesia with an intravenous administration of Ketamine (6 mg/kg) and Midazolam (0.5 mg/kg), intubation and ventilation followed using a mixture of atmospheric air and oxygen (2:1) to maintain expiratory P_{aCO_2} between 4.5 and 5.5 kPa. Anesthesia was maintained by Sevoflurane (gas, maintaining minimum alveolar concentration between 1.1 and 1.3) and intravenously administered Remifentanyl (0.03 and 0.06 mg/kg/h) for analgesia. Monitoring included heart rate, respiratory rate, oxygen saturation, and rectal temperature, whereas continuous blood pressure monitoring (noninvasive blood pressure) was done by placing a child's cuff (DURA-CUF 2751, GE Healthcare, Bronby, Denmark) on the right foreleg. Hourly venous blood gases (VBG) monitored levels of lactate, glucose, electrolytes, and pH. One liter of Ringer's acetate IV was administered within the first hour and afterwards with an infusion rate of 500 mL/h to keep normal hydration. Pigs 5–7 also received 500 mL of Ringer's acetate intraperitoneally before midline closure after transplantation, as pig 1–4's VBG on postoperative day 1 indicated a minor fluid substitution deficit. Five hundred milliliters of Ringer's acetate was given intravenously twice a day the first 2 postoperative days to all pigs. Antibiotics were administered twice per surgical day: 1500 mg Cefuroxime after intubation

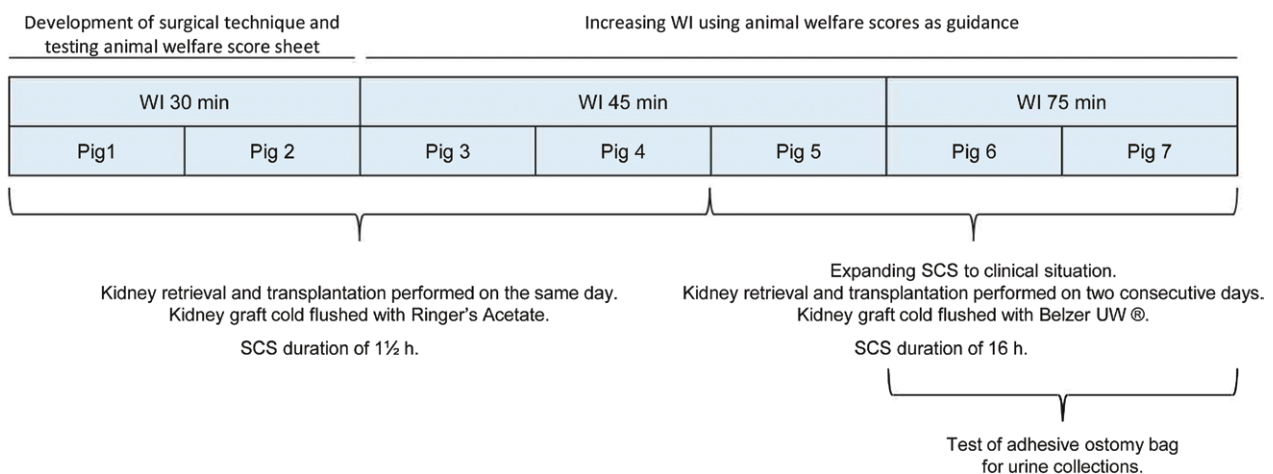


FIGURE 1. Experimental development. The first 2 pigs were used to establish the surgical technique while the WI was kept to a duration of 30 min. Furthermore, the animal caretakers' observation of the pigs was quantified on the animal score sheet. Pigs 3–7 had increasing renal WI to establish proper renal injury, whereas animal welfare scores were used as a guidance tool. SCS, static cold storage; WI, warm ischemia.

and 750 mg Cefuroxime 30 minutes before extubation and on postoperative day 1, 750 mg Cefuroxime IV was also given. Twenty milliliters of Ropivacain (7.5 mg/mL) was administered as local analgesic 30 minutes before extubation. Postoperative buprenorphine was injected intramuscularly 3 times a day: 0.02 mg/kg on the first and second day and 0.01 mg/kg on third and fourth postoperative days. Fourteen days after the transplantation, induction and maintenance of anesthesia followed the same methods as mentioned above. The euthanasia method was an overdose of pentobarbital during general anesthesia.

Surgical Procedure

For flow diagram, see Figure 2.

Kidney Retrieval

Under sterile conditions, a permanent semi-CVC (5 Fr, 20 cm, Careflow, BD, NJ) was placed in an auricular vein by the Seldinger technique and advanced into the jugular vein. The left kidney and vessels were exposed retroperitoneally and were freed with no-touch technique. The renal artery and, a few seconds later, the renal vein were clamped without administration of heparin. Following WI, the graft was flushed with 20 mL cold saline containing 5000 IU heparin and afterwards perfused with cold fluid, Ringer's acetate (Fresenius Kabi, Copenhagen, Denmark) in pigs 1–4 and Belzer UW (Bridge to Life, London, United Kingdom) in pigs 5–7, and put on cold storage at 4°C–6°C. Pigs 5–7 returned to the housing facility after extubation before the following autotransplantation.

Kidney Autotransplantation

A right nephrectomy was performed, and the left kidney graft was autotransplanted end-to-end to the right renal artery and vein. Before reperfusion, 3.33 mL/kg of mannitol (150 mg/mL) IV was given. The native ureter was anastomosed end-to-end to the graft pelvis, and a JJ-catheter (Urosoft 7/16, Angiomed GmbH, Karlsruhe, Germany) was inserted to overcome ureter edema. Following midline closure, the pigs returned to the stables for 14 days of observation and blood and urine collection.

Measurement of Renal Function at Day 14

The pigs were anesthetized again using the mentioned procedure. Intra-abdominal access to the right ureter was performed. After removing the JJ-catheter, a feeding tube was inserted instead and cannulated to the surface for urine

collection. At the end, a transplant nephrectomy was performed and the pig was terminated.

Blood Samples and Renal Function

Blood samples were collected from the CVC daily during the first week and every second day thereafter. While fed, the pig would stand still, and no sedation or restraint was needed. VBGs were analyzed on ABL90 FLEX (Radiometer, Denmark). For creatinine measurements, usage of the i-STAT (Abbott Diagnostics, East Windsor, NJ) analyzer and assays were used. Five milliliters of saline containing 100 IU heparin was injected into the CVC to prevent clotting. Measured glomerular filtration rate (GFR) was calculated as the renal clearance of chromium-51-labeled ethylenediamine tetraacetic acid (⁵¹Cr-EDTA) on day 14.

$$\text{GFR} = \frac{U_{(\text{cpm/mL})} \times \text{Uvol}_{(\text{mL/min})}}{\frac{(\text{PI1} + \text{PI2})_{(\text{cpm/mL})}}{2}}$$

where U is urine, P is plasma, and cpm is counts per minute. A priming dose of 2.25 MBq was administered intravenously followed by a constant ⁵¹Cr-EDTA infusion of 1.13 MBq/h. One hour of equilibration was completed. Hereafter, serial urine and blood samples were collected every 30 minutes for the first 2 hours and hourly for the last 2. The radioactivity measurements were, in 2-mL plasma samples and 1-mL urine samples, performed on the 2480 Wizard Gamma counter (PerkinElmer, Waltham, MA).

Urine Samples

An ostomy bag attached to the skin around the external genitals was used for urine collection (Figure 3). During feeding, the pigs stood still, enabling washing of the perianal area and genitals. After drying, the adhesive ostomy bag was placed so it would not interfere with defecation (further adhesive tape was required). If the bag fell off before urination, the procedure was repeated. At no point was it necessary to restrain the pigs, not even at detachment. No pigs tried to remove the ostomy bag, and several pigs defecated carrying the bag without contamination of the urine.

RESULTS

Seven renal autotransplantation procedures with injured grafts were conducted after introducing renal damage with

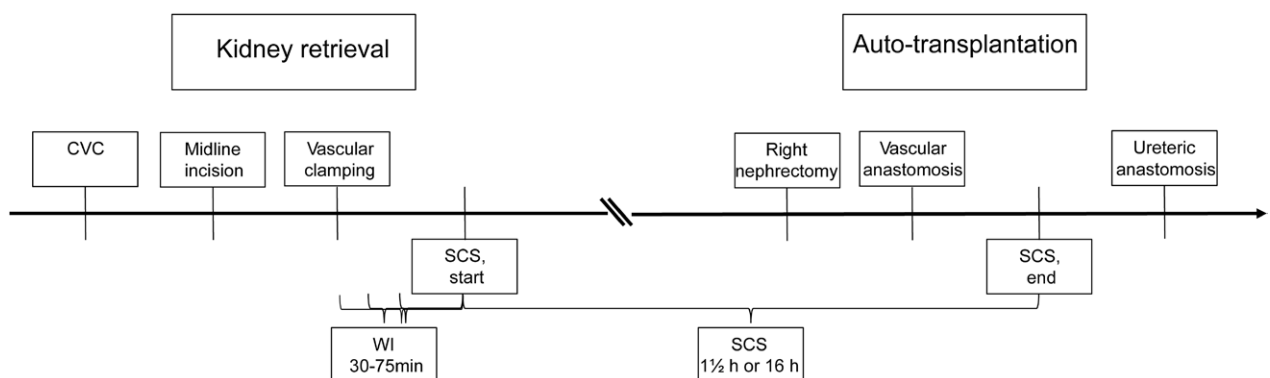


FIGURE 2. Flow diagram. Flow diagram of the surgical procedures. A CVC, used for perioperative and postoperative blood samples, was inserted before kidney retrieval. Graft kidneys were cold flushed and stored until right nephrectomy and the following transplantation. CVC, central venous catheter; SCS, static cold storage; WI, warm ischemia.

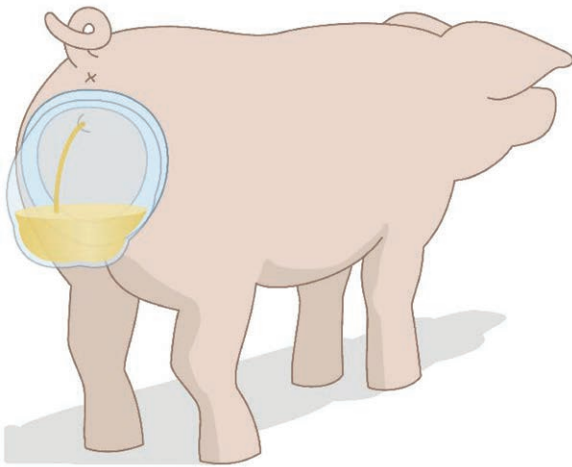


FIGURE 3. Urine collection. Avoiding bladder catheter or metabolic cage led to testing of adhesive ostomy bags. Their applicability and stability for the repetitive urine collections were established in the last phase of this pilot study.

30, 45, or 75 minutes of WI. Afterwards, a 14-day period enabled observation of the possible effects on animal welfare. No experimental animals died during the operations or the follow-up time, but the well-being of the pigs with 75 minutes of renal WI was moderately affected. Whereas maximal animal distress scores were 0 for the autotransplanted animals exposed to 30 and 45 minutes of WI, elevated distress scores for the pigs with 75 minutes of renal WI were observed on the first to third day posttransplant. One pig had a peak score of 5 (general changes, movement, eating, defecation), and 1 pig had a peak score of 10 (general changes, movement, behavior, defecation). Both pigs returned to acceptable behavior (points <5) within a day. Both pigs started to defecate, had improved food intake, and became more active and curious, but they appeared lethargic for the remaining follow-up period. A further increment of the renal WI was not initiated, as the pigs exposed to 75 minutes of WI had elevated welfare scores (≥ 5 points) for 24 hours.

For animal weight characteristics, selected peroperative data, and plasma parameters, see Table 2. All pigs had normal plasma creatinine at baseline (normal range 88–177 $\mu\text{mol/L}$) and highest levels were seen in pigs with 75 minutes of WI, who in addition had anuria until the second postoperative day, whereas peak plasma creatinine of 1486 (pig 6) and 1317 $\mu\text{mol/L}$ (pig 7) was reached on day 4 (Figure 4). Highest levels of plasma potassium were reached on the second day at 5.6 and 6.8 mmol/L (normal range for pigs 3.1–6.2 mmol/L ²⁹). The latter was successfully treated with 15 g of Calcium Resonium. With only 1 kidney present, a minor plasma creatinine increase from 61 to 115 $\mu\text{mol/L}$ was observed by the end of the follow-up period (Table 2), whereas electrolytes were in normal range (data not shown).

Table 2 also presents the results of GFR measured on postoperative day 14. Pigs with 75 minutes of WI had the lowest GFR values of 38 (pig 6) and 40 mL/min (pig 7).

Blood samples were drawn without distressing the animal, and there was no need for restraint or sedation. One pig extracted its CVC in the second week but with insignificant bleeding. As a result, we altered our suture technique to prevent future incidents.

To test whether urine collection was possible with an ostomy bag, it was placed on 2 animals at days 1, 2, 3, and 5. All urine collections were successfully completed, and it enabled confirmation of the anuria present in the pigs with 75 minutes of WI. However, use of the ostomy bag could be quite time consuming. Application would take less than half an hour, but withdrawal of the bag could be up to 4–6 hours later before the pig had urinated. During this urine collection procedure, no signs of animal distress were observed.

DISCUSSION

This study describes how animal welfare can be prioritized and used as a guidance tool in the development of a porcine kidney autotransplantation model. The significant WI, simulating the donation after circulatory death condition, resulted in severe posttransplant renal failure without the loss of any animals and with minimal animal distress. No infections or

TABLE 2.

Animal weight characteristics, selected peroperative data, and plasma parameters as well as GFR measured at day 14 by urinary clearance of chromium-51 labeled ethylenediamine tetraacetic acid

	WI 30 min		WI 45 min			WI 75 min	
	Pig1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7
Body weight, baseline, kg	50	50	51	49	49	48.5	52
Body weight gain (peak – baseline), kg	4.0	9.0	4.0	7.0	9.5	9.5	12.0
Left kidney weight, g	–	105	117	118	127	97	134
Right kidney weight, g	–	117	180	141	170	130	146
WIT, min	30	30	45	45	45	75	75
SCS, h	1.5	1.5	1.5	1.5	16	16	16
Peroperative fluid therapy (total volume of Day 1 and Day 0), L	3.0	4.0	4.0	5.5	6.5	6.2	6.5
Body temperature during renal ischemia, °C	38.0	36.6	37.0	38.3	37.6	38.4	37.5
Plasma creatinine, baseline, $\mu\text{mol/L}$	132	132	141	125	162	183	157
Plasma creatinine, peak, $\mu\text{mol/L}$	1167	840	981	1228	1055	1486	1317
Plasma creatinine, difference (end – baseline), $\mu\text{mol/L}$	63	62	72	59	101	115	61
Potassium, baseline, mmol/L	3.7	3.5	3.6	3.3	3.6	3.5	3.6
Potassium, peak, mmol/L	6.0	5.3	4.8	5.8	5.4	5.6	6.8
GFR, mL/min , day 14	50	48	53	56	44	38	40

GFR, glomerular filtration rate; SCS, static cold storage; WI, warm ischemia; WIT, warm ischemia time.

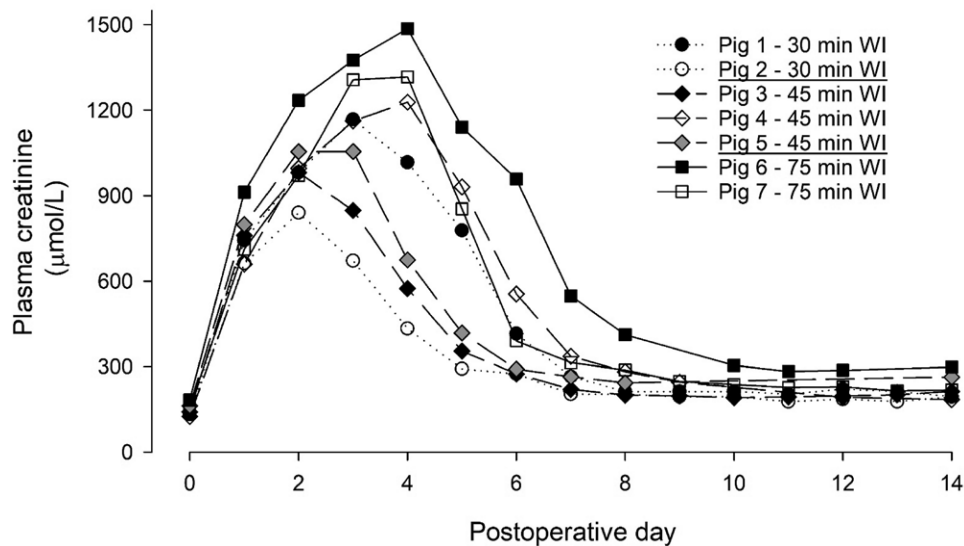


FIGURE 4. Postoperative plasma creatinine. Individual plasma creatinine ($\mu\text{mol/L}$) levels during the 14 days of follow-up. P-creatinine increased in all pigs reaching their peak levels between days 2 and 4, whereas Pigs 6 and 7 had the highest peaks. In each pig, a subsequent decline was observed and plasma creatinine reached a more stable plateau after 1.5 weeks. WI, warm ischemia.

serious complications were observed in the experimental period. In addition, it was possible to collect postoperative urine and blood samples continuously without compromising animal welfare.

Animal welfare is of great importance in regards to ethics and for optimal research results. In Denmark, animal death as an end point is not legal. A ceiling of 75 minutes of WI was chosen as a longer duration and would prolong the 48 hours of anuria, with consequently more severe uremia resulting in severely compromised welfare of the animal. Optimized welfare is not only important for ethical reasons but also because impaired welfare can have a negative impact on experimental results and introduce additional variation in important read-outs.^{22,30} Our methods to optimize welfare were introduced by several initiatives: (1) a 2-week acclimatization period before the first operation, (2) positive reinforcement training of the animals obviating restraint or sedation during sampling, (3) avoiding transportation as stables and surgical rooms were at the same site and we had no experimental animals that suffered from hypothermia or elevated lactate (a sign of stress) when arriving at the surgical room, (4) permanent intravenous access postoperatively, and (5) sampling of urine without use of catheterization. Learning from previously used methods,³¹⁻³⁵ an ostomy bag was successful for urine collection in our pilot study. Bladder catheterization was avoided, as it increases discomfort and risk of urinary tract infection.³⁶⁻⁴³ In addition, catheterization has been shown to relate to inflammatory cytokine production, immune cell infiltration, and mucosal lesions of both bladder and kidney.³⁷ The ostomy bag has the risk of falling off and is not advisable to be used for measurement of volume output, including 24-hour urine collection, which is a limitation to the study, but it does give the possibility to have spot urine for analysis without using a metabolic cage.

As a previous study concluded that 30 minutes of WI does not seem to cause substantial damage to the pig kidney⁴⁴ and another study detected irreversible renal damages after 180 minutes of WI,⁴⁵ we sought to conduct our pilot study with WI duration in-between. Not surprisingly, our transplant

model showed that applying 75 minutes of WI resulted in highest peak plasma creatinine. This pilot optimization study was not powered to get significant results, but the expected trend toward increasing plasma creatinine and decreased GFR was seen with prolonged WI duration. In some previous studies using ischemically injured grafts, the WI has been shorter, mainly between 20 and 60 minutes, resulting in a lower peak plasma creatinine,^{2,13,14,16,17} whereas other experimental transplantation studies have applied >75 minutes of WI, which on the other hand had mortality rates of 17%⁴⁶ and 50%⁴⁷ (90-minute WI), and 67%⁴⁶ (120-minute WI) in their untreated control groups. It is a delicate balance to ensure adequate injury while ensuring acceptable animal welfare and avoiding high mortality. Our decline of plasma creatinine after 9–14 days is a limitation of the porcine model in general and is consistent with previous studies.^{2,11,13-17} In addition, it is well known that removal of 1 kidney will stimulate an increase of renal perfusion and GFR with compensatory growth of the remaining kidney.⁴⁸ A 1-kidney GFR of 38 and 40 mL/min is below normal range for this size of pigs and represents a state of renal damage when compared with older studies. Frennby et al⁴⁹ measured GFR in 1-kidney pigs with reduced renal function and found a median GFR of 30 mL/min/50 kg, supporting this interpretation. Another study measured GFR in 19 healthy, 2-kidney pigs by administration of ⁵¹Cr-EDTA, and clearance range was 73–126 mL/min/50 kg,⁵⁰ which is in accordance with Hansen et al,⁵¹ who found a mean clearance of 97 mL/min in their 30- to 40-kg pigs. Other autotransplantation studies have mainly used creatinine clearance, which overestimates GFR because of tubular creatinine secretion, particularly when renal function is decreased. The lack of baseline GFR is another limitation of our model but was omitted to limit the surgical burden.

In conclusion, our animal model provides a unique opportunity to explore preclinical aspects of transplantation as well as methods to improve transplantation outcomes.¹⁸ With this pilot study, we were able to observe individual welfare of the experimental animals and develop our injured graft model for planned intervention studies. It was possible to study both

early and late postoperative outcomes, and the model gave easy access to measurement of various biomarkers in blood and urine, as well as GFR and tissue sampling. Combining these important characteristics and opportunities in 1 model makes it ideal for numerous future studies.

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