Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences
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The Pig as an Animal Model for Kidney Transplantation

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Uppsala
2016

Degree Project 30 credits within the Veterinary Medicine Programme

ISSN 1652-8697
Examensarbete 2016:27

The Pig as an Animal Model for Kidney Transplantation

Grisen som modelldjur för njurtransplantation

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Degree Project in Veterinary Medicine

Credits*: 30 hec*
Level*: Second cycle, A2E*
Course code*: EX0736*

Place of publication*: Uppsala*
Year of publication*: 2016*
Number of part of series*: Examensarbete 2016:27*
ISSN*: 1652-8697*
Online publication*: http://stud.epsilon.slu.se*

Key words*: Pig, Kidney, Transplantation, Rejection, Ischemia/reperfusion*
Nyckelord*: Gris, Njure, Transplanation, Avstötning, Ischemi/reperfusion*

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SUMMARY

A significant amount of the world's population suffers from chronic kidney disease. Renal failure arises when less than 25 % of the kidneys function properly. The treatments available for humans that suffer from renal failure are dialysis or renal transplantation, of which transplantation is considered to be the treatment of choice. Transplantations increase quality of life, lengthen the patients' lifespans and are cost effective. However, many transplanted kidneys are subjected to substantial ischemia/reperfusion damage, which could lead to graft loss.

Animal models for kidney transplantation are crucial to gain more information about ischemia/reperfusion injury (IRI) and rejection mechanisms. The pig provides a good animal model for kidney transplantation studies. The porcine urological system is very similar to that of humans, both in aspects of physiology and anatomy. Moreover, pigs are relatively cheap, easy to breed and raise less ethical concerns than non-human primates.

This master degree project includes a literature review and summary of a renal transplantation study, which is a collaboration between Uppsala University and the Department of Clinical Sciences, SLU, Uppsala. The study is included in the DIREKT program financed by EU-FP7. The aim of the renal transplantation study was to investigate whether polyethylene glycol conjugated phospholipid (PEG-lipid) could reduce IRI and thus help to prevent rejection of a transplanted kidney. The pigs were given a two-week acclimatization period during which they received social training for 15 minutes per pig and day. Thereafter, they were easy to handle under stress-free conditions. Kidneys were successfully transplanted under general anesthesia and the pigs recovered well after surgery. All pigs started to produce urine intraoperatively, and urine was found in the bladder of all pigs within three days after surgery by ultrasound examination. The pigs were euthanized on day 4 and 5 post surgery. Thus, the pigs lived long enough to collect relevant data regarding thromboinflammation. The present study confirmed that the pig constitutes an excellent animal model for kidney transplantation studies.

SAMMANFATTNING

Kronisk njursjukdom drabbar många människor världen över. Njursvikt uppkommer då njurens funktion understiger 25%. Behandlingsalternativen som erbjuds människor med njursvikt är dialys eller njurtransplantation där den senare är det bästa alternativet. Transplantation ökar livskvalitet, förlänger patientens livslängd och är kostnadseffektiv. Dock utsätts många transplanterade njurar för betydande ischemi/reperfusionsskador som kan leda till avstötning av organet.

Djurmodeller för njurtransplantation är viktiga för att öka förståelsen om ischemi/reperfusionsskador och avstötningsmekanismer. Grisen är en utmärkt djurmodell för njurtransplantationsstudier. Grisens urologiska system är mycket likt människans, både vad gäller fysiologi och anatomi. Dessutom är grisar relativt billiga och enkla att föda upp.

Denna masteruppsats innehåller en litteraturstudie samt en sammanfattning av en njurtransplantationsstudie som är ett sammarbete mellan Uppsala Universitet och Kliniska Vetenskaper, SLU, Uppsala. Studien är inkluderad i DIREKT-programmet och är finansierat av EU-FP7. Målet med denna transplantationsstudie var att undersöka om polyetylenglykol konjugerad fosfolipid (PEG-lipid) kan minska ischemi/reperfusionsskador och därmed hindra avstötning av en transplanterad njure. Grisarna i denna studie gavs två veckors acklimatiseringsperiod, då de blev socialt tränade i 15 minuter per gris per dag. Efter detta var samtliga grisar enkla att hantera. Njurar var framgångsrikt transplanterade under allmän narkos och mottagargrisarna återhämtade sig bra efter operationen. Alla transplanterade njurar producerade urin intraoperativt och vid ultraljudsundersökning av urinblåsan efter operation hittades urin i samtliga blåsor inom tre dagar. Mottagarna avlivades dag 4 och 5 efter operation. Därmed levde de tillräckligt länge för att relevanta data gällande tromboinflammation kunde insamlas. Denna studie bekräftar att grisen är en utmärkt djurmodell för njurtransplantationsstudier.

CONTENTS

INTRODUCTION

Kidney transplantation is the treatment of choice among human patients with end-stage renal disease. However, the waiting time to receive an organ is long and the risk of graft failure within the first year among kidneys from diseased donors is as high as 4.7 % (USRDS Annual Data Report 2014). Five year graft survival has been reported to be 78-87 % (Montalti et al. 2005, Soler et al. 2005, Karatzas et al. 2011). Graft survival needs to be improved and for this animal models are crucial. The pig's urological system is very similar to that of humans and has been well studied (Sampaio et al. 1998, Grosse Siestrup et al. 2002, Nath et al. 2014), suggesting it provides a suitable animal model for renal research and procedures.

This master degree essay is part of a larger study, which aims to investigate whether polyethylene glycol conjugated phospholipid (PEG-lipid) could reduce damage in renal transplants associated with ischemia/reperfusion injury (IRI). This essay will focus on the usefulness of the pig as an animal model in kidney transplantation studies.

LITERATURE REVIEW

Anatomy and physiology of the human kidneys

The human kidneys are a paired organ localized behind the peritoneum on each side of the vertebral column. They stretch from the 12th thoracic vertebra to the third lumbar vertebra. Although the two kidneys together weigh less than 0.5 % of the total body weight of a human, the kidneys receive around 20 % of the cardiac output. This allows a high blood pressure in the glomerular capillary network leading to an ultrafiltration of plasma. Each human kidney is made up of 800 000 to 1 200 000 nephrons, which in turn are composed of a glomerulus and a tubule. The glomerulus is situated in the outer part of the kidney, the cortex, whilst the tubule stretches from the cortex to the inner part of the kidney, the medulla (as reviewed by Giebisch and Windhager 2012).

The kidneys have many vital functions. One is their ability to excrete waste products, such as metabolites and toxins, through the urine. Another is their regulation of electrolyte, fluid and acid-base balance. Also, the kidneys produce hormones that are essential in the erythropoiesis, the calcium metabolism and the regulation of blood pressure (as reviewed by Giebisch and Windhager 2012).

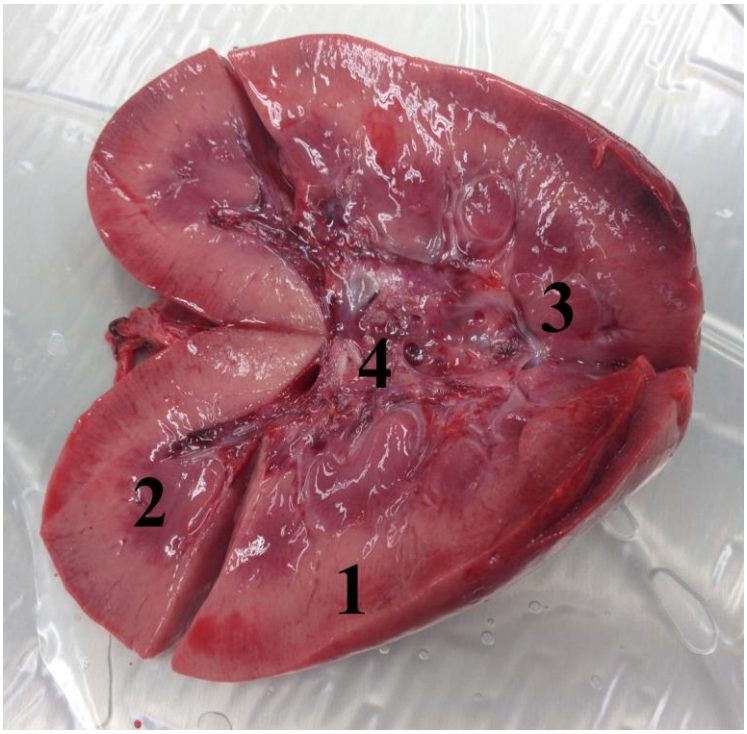


Figure 1. *Anatomy of the porcine kidney. (1) cortex; (2) medulla; (3) papilla; (4) pelvis.*

Renal failure

The kidneys can be damaged in many ways, for example by toxins, drugs, infections, ischemia or diseases, such as diabetes mellitus. The damage can be reversible or permanent. Severe renal disease emerges when 66-75 % of the nephrons are no longer working, leading to the kidneys not being able to concentrate urine which in turn results in polyuria and polydipsia. Renal failure arises when more than 75 % of the nephrons in both kidneys are non-functional. Renal failure means that excretory function is impaired and substances such as urea and creatinine begin to accumulate in the blood. This accumulation is termed azotemia. When azotemia becomes severe, clinical symptoms such as anorexia, vomiting, diarrhea, anemia, gastric ulceration, lethargy and depression arise. This state is called uremia (as reviewed by Squires 2006).

Renal failure can be acute or chronic. Acute renal failure (ARF) develops rapidly while chronic renal failure (CRF) typically takes several months, or even years, to develop. ARF is often reversible if correct treatment is applied, whereas CRF usually is permanent (as reviewed by Squires 2006).

To help the diagnosis of renal disease and renal failure, measurements of plasma or serum creatinine and urea are very useful. Urea is synthesized in the liver from carbon dioxide and ammonia and is almost exclusively excreted in the urine. Urea is a small molecule of 60 daltons that is freely filtered through the glomerular basement membrane. Creatinine is mainly produced in the skeletal muscles through breakdown of creatine, thus the muscle mass of a person or an animal will affect the level of blood creatinine. Like urea, creatinine is a small molecule (113 daltons) that is freely filtered in the glomerulus (as reviewed by Squires 2006).

According to the USRDS Annual Data Report (2014) around 14 % of the American population have chronic kidney disease (CKD). CKD is divided into five stages based on estimated glomerular filtration rate (eGFR) and urine albumin/creatinine ratio (ACR). eGFR decreases and albuminuria increases as kidney function declines. People with eGFR less than 60 mL/min/1.73 m² or ACR above 30 mg/g are considered to have reduced kidney function. Renal failure is classified as having eGFR below 15 mL/min/1.73 m² (USRDS Annual Data Report, 2014).

Risk factors

Several factors have been associated with a higher risk for CKD and renal failure, of which diabetes mellitus (DM) is the leading one. Around 40 % of all patients with DM also have CKD. Furthermore, older age, hypertension, cardiovascular disease and obesity all increase the risk of CKD and renal failure (USRDS Annual Data Report 2014).

Cats, dogs and pigs

Kidney disease is a common cause of death in older cats and around 16 % of all cats above the age of 15 are estimated to have chronic kidney disease (as review by Kit Sturgess 2013). In a study by Pelander et al. (2015) 1.6 % of insured dogs in Sweden were diagnosed with kidney disease. However, it is likely that the true prevalence of dogs with kidney disease are underestimated, partly due to the fact that dogs (and cats) are not routinely screened for renal disease.

Moreover, juvenile nephropaties, such as polycystic kidney disease, have been recognized in cats, dogs and pigs. Polycystic kidney disease is characterized by multiple cysts of various sizes in both kidneys. They form early in life and increase in size and numbers as the animal ages, leading to chronic kidney disease and eventually renal failure. The disease is hereditary in Persian cats, of which approximately 38 % are affected (Lees 2007).

Transplantation

When CKD stage five, i.e. renal failure, is reached, there are three treatment choices; hemodialysis, peritoneal dialysis and transplantation. Hemodialysis means that the patient's blood is filtered through a dialysis machine with a special filter, which mimics the normal work of the kidneys. This usually needs to be performed three times per week for four hours each time. In peritoneal dialysis a catheter is placed within the patient's abdomen and the peritoneum functions as a filter for waste products. Hemodialysis is by far the most common dialysis technique (National Kidney Foundation, 2015).

Kidney transplantation is the best choice for patients with end-stage renal disease as it increases quality of life (Shrestha et al. 2010, de Mendonça et al. 2014), reduces the intensity of chronic pain (Nourbala et al. 2007), is cost effective (OPTN 2012) and lengthens the lifespan of the patient (Wolfe et al. 1999).

Kidneys are donated from either living donors or deceased donors. The risk of graft failure is substantially lower among living donor transplant recipients, with a probability of graft failure within the first year of 1.8 % versus 4.7 % among grafts donated from deceased donors (USRDS Annual Data Report 2014). Deceased donors are divided into standard criteria donors (SCD) and extended criteria donors (ECD). ECD are defined by having an increased risk of graft failure compared to SCD. Included in ECD are donors above the age of 60 years or between 50-59 years with two of the following criteria: cerebrovascular accident as the cause of death, hypertension or serum creatinine greater than 0.015 g/L at the time of death (UNOS Policy 3.5, 2002). However, although ECD kidneys by definition have a higher risk of graft failure, Zádori et al. (2015) found that graft survival was comparable between SCD and ECD. Moreover, in a study by Lionaki et al. (2014) graft survival of ECD kidneys were found to be highly satisfactory, suggesting that ECD grafts can be transplanted safely.

Shortness of organs and an increased demand of donated kidneys are large problems in the treatment of CKD. In 1998, the median waiting time to receive a renal transplant in the USA was 2.7 years. This was increased to 4.2 years in 2008 (OPTN 2012).

A brief history of transplantation

The first successful human kidney transplantation was performed in 1947 at the Peter Bent Brigham Hospital in Boston. The kidney, which was derived from a newly deceased donor, was connected to the big artery and vein in the patient's elbow. It was functional until the patient's own kidneys had regained their function. When an organ is transplanted between two individuals of the same species it is called allotransplantation. Autotransplantation is when an organ is transplanted from one place to another on the same body. Several attempts to transplant kidneys were made over the following years, but most patients seemed to be more susceptible to infections and soon died in renal failure. In 1954, there was a breakthrough in transplantation history when a kidney was transplanted between two identical twins. The recipient lived for nine years before dying of a heart attack (as reviewed by Petechuk 2006).

Between 1954 and 1964, 600 kidneys in the U.S. were transplanted from living donors with a two year survival rate of 50 %. Many of the recipients died of opportunistic infections or developed cancer due to the immunosuppressive medication they were given. Another breakthrough came in 1979 when cyclosporin A was introduced as immunosuppressive therapy. This drug could in many cases be used as the only immunosuppressant and thus reducing the many side effects of immunosuppressive therapy. The development of the drugs tacrolimus and mycophenolic mofetil further enhanced the effectiveness of transplantation (as reviewed by Petechuk 2006).

During the last decades there has been a marked increase in renal allograft survival. Grafts from deceased donors had a median half-life of 6.6 years in 1989, which increased to 8 years in 1995 and 8.8 years by 2005 (Lamb et al. 2011).

Xenotransplantation is defined as transplantation across species and is a potential solution to the problem of organ shortages. The pig is the donor species of choice because of the similarity between pig and human anatomy and physiology and because they raise less ethical considerations than non-human primates. Experiments with non-human primates as animal models for human recipients have been carried out during the last decades (Lexer et al. 1986, Loss et al. 2001, Hisashi et al. 2008).

Ischemia/reperfusion injury

Ischemia/reperfusion injury (IRI) is a complex process that constitutes a huge challenge in transplantation surgery as it can cause delayed graft function (DGF) and graft loss. DGF is defined by the need for dialysis within 7 days post transplantation (OPTN 2012, Cavaillé-Coll et al. 2013). DGF leads to 41 % increased risk of early graft loss within 3.2 years and is associated with a higher risk of acute rejection (Yarlagadda et al. 2009) and worse long term survival, with 23 % survival after 5 years as compared to 68 % in grafts that functioned properly after transplantation (Karatzas et al. 2011). DGF is also associated with longer hospital stay and an increase in expenses of about 5,000 USD per graft (Englesbe et al. 2008). Delayed graft function has been reported to occur in 31-34 % of transplantations (Faenza et al. 2001). Ischemia, with subsequent hypoxia, emerges when circulatory death of the donor occurs (Chen et al. 2015). However, allografts from living donors are also subjected to warm ischemia, *id est* ischemia under normothermic conditions, starting with arterial clamping. Organ injury continues during retrieval and storage, where the grafts are subjected to cold ischemia, *id est* ischemia under hypothermic conditions (as reviewed by Gulec 2011). With reperfusion, the cells are exposed to oxygen and the free radicals generated, which lead to further damage, called the "reflow paradox" (Menger et al. 1992).

When oxygen is reintroduced to the cells, a massive reactive oxygen species (ROS) production is commenced (Menger et al. 1992, Massberg & Messmer 1998). ROS are highly unstable oxygen molecules with unpaired electrons in their outer shell that make it possible for them to interact and oxidize proteins, lipids and DNA. For example, they cause lipid peroxidation in the cell membranes, leading to further vasoconstriction and a decreased microvascular perfusion (Gulec 2011).

The mechanisms of ischemic injury are intricate. As no oxygen reaches the cells, ATP stores are depleted and aerobic metabolism is stopped. Anaerobic metabolism leads to an accumulation of byproducts, such as lactate, that cause intracellular acidosis and hyperosmolarity. The osmotic imbalance subsequently leads to an influx of water, resulting in cellular edema. Eventually, ischemia leads to cell death by several different pathways (as reviewed by Chen et al. 2015). Prolonged cold ischemia of grafts from deceased donors is strongly associated with long-term graft loss (Salahudeen et al. 2004, Gwinner et al. 2008), which might be associated to a longer period of leukocyte-endothelium interactions (Massberg & Messmer 1998).

Damage to the endothelial glycocalyx is clearly linked to IRI, as damaged kidneys due to ischemia have reduced microvascular perfusion and loss of glycocalyx integrity (Snoeijs et al. 2010). The glycocalyx consists of membrane-bound proteoglycans and associated glycosaminoglycans that cover the endothelial surface and aid to maintain the endothelial function (Yang et al. 2013). Thus, glycocalyx injury leads to increased vascular permeability, interstitial edema and endothelial cell swelling (Henry & Duling 1999, van den Berg et al. 2003, Vink & Duling 2000). It is suggested that ways to improve capillary blood flow and decrease endothelial glycocalyx damage could improve early graft function (Snoeijs et al. 2010).

During reperfusion the complement system is activated and cytokine and chemokine production are commenced. The release of cytokines and chemokines lead to a recruitment of leukocytes, in particular neutrophils, during the first hour post reperfusion (Miura et al. 2001). Neutrophils produce NADPH oxidase, which causes oxidative stress, secrete cytotoxic elastases and further enhance the recruitment of inflammatory cells, such as monocytes and T-cells (as reviewed by Tizard 2009). Inhibition of neutrophil infiltration leads to decreased blood levels of creatinine and urea and results in better graft survival (Miura et al. 2001). Thus, infiltration of neutrophils is associated with an increased risk of graft dysfunction.

The complement system is a vital part of the innate immune response and is, for example, involved in bacteria elimination and the clearance of debris after inflammatory injury. There are three different pathways by which the complement system can be activated: the classical pathway, the alternative pathway and the lectin pathway. Activation of all three pathways result in C3 formation, making C3 a central complement component (as reviewed by Tizard 2009). The complement system has a significant impact on graft survival, since renal production of C3 is associated with an increased risk of acute renal transplant rejection (Pratt et al. 2002, Damman et al. 2011). Another common result of the three pathways is C5b-9, alias membrane attack complex (MAC), which causes IRI in mouse models (Zhou et al. 2000). The complement system is in this case probably activated by the alternative pathway since absence of C4, which is an important part of the classical pathway, does not help to prevent graft failure (Zhou et al. 2000, Lin et al. 2006). Thurman et al. (2005) observed an increase in alternative pathway activation in kidneys suffering from acute tubular necrosis and it has also been shown that inhibition of C5a, which is an essential component of the alternative pathway, decrease reperfusion injury (Amsterdam et al. 1995). This is a strong indication that the alternative pathway activation is a key mediator of reperfusion injury and the inhibition of this pathway would thus help protect the kidney from acute renal failure.

When an allo- or xenograft is transplanted into a non-immunosuppressed recipient it is rapidly damaged. This damage is due to instant blood-mediated inflammatory reaction (IBMIR), which is initiated by platelet adhesion (Moberg et al. 2005). Rood et al. (2007) showed that by inhibiting complement activity with cobra venom and adding dextrane sulfate as anticoagulation, damage caused

by IBMIR was reduced. This demonstrates that coagulation and complement systems are part of IBMIR. Moreover, there is evidence that polymorphonuclear granulocytes constitute a substantial part of IBMIR (Moberg et al. 2002, Gustafson et al. 2011).

Kidneys from deceased donors are subjected to a longer period of warm ischemia before being harvested as compared to kidneys from living donors. Kidneys donated after cardiac death (DCD) thus suffer substantially more ischemic injury than living donor kidneys. Moreover, DCD kidneys have a smaller blood vessel diameter, resulting in 42 % lower capillary perfusion in the early reperfusion period (Snoeijs et al. 2010). Significantly more recipients of DCD compared to living donor kidneys have to continue dialysis for some time after transplantation due to DGF (OPTN 2012).

Organ preservation

After being harvested from the donor, organs must be preserved in a suitable solution until they can be transplanted into a recipient. The first static cold storage solution was introduced in 1969 (Collins et al. 1969). Continuous research led to the development of University of Wisconsin (UW) solution in 1988. UW solution has since then been widely used, although it is now replaced by Histidine-tryptophan-ketoglutarate (HTK) solution in many transplantation centers. Components of the UW solution include glutathione, which helps the cell regenerate ATP and maintain membrane integrity, and adenosine that provides the cell with substrates for regeneration of ATP (as reviewed by Southard et al. 1990). HTK contains NaCl, KCl, MgCl, CaCl, histidin, tryptophan, mannitol and ketoglutarate. The electrolyte composition protects the graft from ischemic damage by minimizing its energy requirements and histidine acts as a buffer that delays the fall in pH (Custodiol [HTK] prescribing information 2004). Which solution that is superior remains unclear, with some evidence of equal function (Lynch et al. 2007, Klaus et al. 2007) and some of reduced long-term graft survival in HTK (Stewart et al. 2009).

Static cold storage versus hypothermic machine perfusion

There are two ways to store kidney grafts after retrieval from a donor; static cold storage (CS) and hypothermic machine perfusion (HMP). CS has long been the preservation of choice. In recent years, however, the interest for HMP has grown. In CS, the graft is first flushed, then cooled with a preservation solution and transported on ice. In HMP, the blood is flushed out and thereafter the graft is connected to a machine that continuously pumps a fluid through the vessels of the kidneys at a temperature of 1 to 10 ℃ (Moers et al. 2009).

HMP is superior to CS, as it reduces the risk of delayed graft function and has a higher rate of graft survival during the first year after transplantation (Moers et al. 2009). Three years after transplantation, HMP has survival rates of 86 % in expanded criteria donors as compared to CS with survival rates of 76 % (Moers et al. 2013). Also, patients receiving kidneys from HMP storage have lower serum creatinine levels at discharge from the hospital (Sedigh et al. 2013). There seems to be no surgical disadvantages with using HMP instead of CS (Moers et al. 2009, Sedigh et al. 2013).

In a study with autotransplantation of kidneys in beagle dogs, the two machines mostly used for HMP, ORS LifePort and Waters RM3, were compared. It was concluded that both machines worked satisfactory, but the cycle must remain pulsatile. Keeping the machine on a nonpulsatile flow was associated with greater graft injury (Lindell et al. 2013).

Figure 2. *ORS LifePort Kidney Transporter (LKT 101P) is one of the most frequently used machines for HMP in transplantation centers.*

Studies in hearts also show encouraging results in favor to HMP. Hearts conserved with HMP have a lower lactate level and a better contractility level than hearts stored in SC solution (Caenegem et al. 2014). Likewise, HMP provides better protection of function and metabolism in liver grafts than CS solution (Li et al. 2015, Jia et al. 2015).

Polyethylene glycol

Polyethylene glycol (PEG), with the chemical formula $C_{2n}H_{4n+2}O_{n+1}$, is a colloid with high molecular weight. PEG has a low toxicity and if attached to protein medications it has the ability to slow the clearance of the protein and thus prolong the effect of the medicine. It can also be used in preservation solutions. PEG can protect the graft from initial inflammation and thus reduce injuries caused by ischemia and cold storage (Dutheil et al. 2006). The PEG molecules also have the ability to bind to cell membranes, forming a coat that will hinder the approach of other cells and molecules. In this way, PEG can ”hide” the graft from the recipient’s immune system and thus help reduce inflammation (Thuillier et al. 2011).

Different weights and concentrations of PEG are currently being studied. Dutheil et al. (2006) showed that with PEG 20 kDa, you will need a concentration of 60 g/L in order to get the same results as with PEG 35 kDa at a concentration of only 2 g/L. These concentrations, however, are too low to effectively protect the graft from cell injury during a cold preservation time of 24 hours. PEG 35 kDa did, nevertheless, manage to successfully reduce the cell damage at a concentration of 30 g/L (Dutheil et al. 2006). In this study however, 2 g/L was compared to 30 g/L, so it is possible that a concentration between these values would provide the same results as 30 g/L.

When comparing Institute Georges Lopez (IGL) solution with PEG 35 kDa at a concentration of 1 g/L to “solution de conservation des organes et des tissus” (SCOT) containing 30 g/L PEG 20 kDa Thuillier et al. (2011) found that kidneys treated with SCOT had less fibrosis and secreted only a low degree of proteins in the urine as compared to IGL. The authors theorize that PEG 20 kDa at 30 g/L provides better immunocamouflague than PEG 35 kDa at 1g/L and thus demonstrate the importance of the PEG solution being in a high enough concentration.

As mentioned previously, ROS production with subsequent lipid peroxidation is one of many challenges in IRI. PEG 20 kDa does not seem to be able to limit the damage achieved by ROS even at a concentration of 60 g/L, whereas PEG 35 kDa managed to diminish ROS production even at 1 g/L concentration, indicating that size matters (Dutheil et al. 2006).

PEG 20 kDa at a concentration of 30 g/L has been shown to improve lung graft function when added to a solution with low kalium content. The use of less kalium helped to reduce bronchospasm and vasospasm and the use of PEG led to an even greater reduction in cell swelling and vasoconstriction (Jayle et al. 2002). Moreover, PEG 20 kDa added to a solution with low kalium content leads to better creatinine clearance and less proteinuria in kidney grafts 16 weeks after transplantation compared to

grafts treated with either a PEG solution with high kalium content or the regular UW solution (Faure et al. 2004).

Surface modification of pancreatic islets provided by PEG-lipids has been shown to effectively protect against IBMIR, mainly by preventing platelet adhesion (Lee 2011, Hwang et al. 2011, Teramura & Iwata 2011).

Immunosuppressive therapy

Immunosuppressive management is one of the keys to successful organ transplantation. An induction therapy is given for a short period of time followed by a maintenance therapy, usually given for the rest of the patient's life. Most patients are treated with a combination of tacrolimus and mycophenolate. The use of steroids has decreased during the last decade (OPTN 2012).

Tacrolimus, or FK-506, is a second generation calcineurin inhibitor that suppresses the activation of interleukin-2-dependent T-cells. It has a narrow therapeutic window and many factors, such as gastrointestinal motility, other medications and liver function, can affect its pharmacokinetic abilities. If combined with products that are potentially nephro- or neurotoxic, these effects might be enhanced by tacrolimus (FASS 2015). Moreover, tacrolimus has been shown to disturb the glucose metabolism (Vincenti et al. 2007).

Mycophenolic acid (MPA) is the active metabolite of mycophenolate mofetil. It suppresses the immune system by inhibiting inosine monophosphate dehydrogenase, which is an enzyme essential for the T and B lymphocyte proliferation (Sintchak et al. 1996). The absorption of MPA from the intestines increases after the first week, probably due to the enterohepatic circulation (Regazzi et al. 1997).

Immunosuppressive therapy is associated with a higher risk of developing cancer as well as an increased susceptibility to infections. Graft recipients who receive immunosuppressive therapy run an increased risk of getting thyroid cancer (Kim et al. 2014), lymphomas, lip cancers, renal carcinomas (Penn 1999), Kaposi´s sarcoma as well as lung and bronchial cancers (Sampaio et al. 2012). Common cancers in the general population, such as breast and prostate carcinomas, were not increased among graft recipients (Penn 1999). Because of the serious adverse effects of immunosuppressive therapy it is important to keep it at its lowest functional level.

Transplantation complications

As previously mentioned there is a significant increased risk of infections and developing tumors after transplantation. Development of hepatitis C, stroke, severe arthritis, spinal stenosis and ischemic heart disease have also been reported after renal transplantations (Shrestha et al. 2010). Moreover, the presence of diabetes or hypertension increases the risk of graft loss within five years after transplantation (OPTN 2012).

Animal models for kidney transplantation

Animal models have played important roles in the understanding of pathophysiology of different diseases and in the search for novel treatments. In the research on renal IRI, animal models are essential. Most experiments use small animal models, such as rats or mice. However, before the results can be applicable to humans, research with large animal models is needed. Advantages with large animal models compared to small are the possibility to perform thorough clinical and diagnostic

examinations, collect repeated blood samples or biopsies and the organs being more similar to human organs. The three large animal species most extensively used in transplantation experiments are pigs, dogs and nonhuman primates (as reviewed by Giraud et al. 2011).

Rodents

Kidney transplantations with the rat as an animal model have been done since the beginning of the 1960s. The rat has since then helped to provide valuable information regarding organ preservation and transplantation, graft rejection and immunosuppression. There are several advantages of utilizing rodents in kidney transplantation studies, including low cost to breed and house the animals and the fact that they are easy to maintain (as reviewed by Schumacher et al. 2003). The diameter of the kidney vessels and the ureter are large enough to successfully perform anastomoses, although this requires a skilled microsurgeon. The availability of genetically manipulated mice is a huge asset in the work to map out the precise mechanisms of graft rejection and ischemia/reperfusion injury (Tian et al. 2010).

The first successful renal transplantation in the mouse was reported in 1973 (Skoskiewics et al. 1973, see Tian et al. 2010 p.e91). Since then great improvements concerning the transplantation technique in rodents have been made. In the late 1990s a new technique with a 82.5 % survival rate and a total operation time of an average of 70 minutes (35 minutes less than previous methods) in mice was presented (Han et al. 1999). For mouse kidney transplantations there is a long learning curve and even for skilled microsurgeons it takes at least 50 procedures before the surgeon feels confident with the procedure. In the same study, the intraoperative failures were as high as 56 %, mostly due to arterial thrombosis, and only 8 % of the animals survived long-term (Martins 2006). In another study, 14 day survival rates of 70-80 % in transplanted mice kidney grafts were reported. Causes of graft failure included thrombosis of the renal artery, a narrow outflow of the renal vein and fistulation of the ureter (Tian et al. 2010).

Another disadvantage with rodents is that even a short period of renal artery occlusion leads to extensive necrosis of the proximal tubules (Kennedy & Erlich 2008). This reaction is much more severe in rodents than in humans (as reviewed by Rosen & Heyman 2001), underscoring the importance of studying large animal models as well.

Dogs

In the beginning of transplantation research, the dog was the most commonly used large animal model. The first renal transplantation in the dog was conducted already in 1902 by Emerich Ullman, when a dog's kidney was autotransplanted to its neck. The kidney actually produced some urine, resulting in this experiment being viewed as successful (Ullman 1902, see: Shrestha et al. 2015 p.65). Since then, canine models have, for example, been used in intrasplenic hepatocyte transplantations (Dunn et al. 2000) and in evaluations of new immunosuppressants (Furukawa et al. 2000, An et al. 2015).

Canine kidneys have been shown to function properly after 5-6 days of hypothermic organ preservation, indicating that dog kidneys are not as sensitive as human kidneys (McAnulty et al. 1989, Rijkmans et al. 1984). Moreover, there are other animal models, such as pigs and rodents that are cheaper and easier to breed (as reviewed by Snoeijs et al. 2011). In Sweden, dogs are no longer purpose-bred and thus have to be imported at a high cost (personal communication Marianne Jensen Waern, Department of Clinical Sciences, SLU). Consequently, there has been a decrease in the use of dogs in transplantation studies in Sweden over the last decades.

Pigs

Pigs are frequently used in several areas of biomedical research, mainly in studies on cardiovascular, integumentary, digestive and urological systems (as reviewed by Laber et al. 2002). There are several advantages with the porcine model. Pigs are in many aspects more similar to humans than dogs and rodents and their anatomy and physiology are well studied, which serves as an excellent basis for further research. Moreover, pigs have relatively low costs and are, in contrast to dogs, not considered to be pets. In Sweden, sows produce a mean of 13.3 piglets per litter, of which 11 survive until weaning. Sows produce more than 24 piglets per year, indicating that they are easily and fast bred (Pig Win 2014).

Both domestic farm breeds (mainly Yorkshire, Landrace, Hampshire and Duroc) and miniature breeds (such as Yucatan, Hanford, Sinclair and Göttingen) are used in biomedical research. The quick growth of domestic breeds results in physiological and anatomical changes that make them unsuitable for chronic studies reaching longer than 3 weeks, since larger pigs can be more difficult and dangerous to handle. In such experiments miniature breeds are preferred (as reviewed by Laber et al. 2002).

When using pigs in research, there are several aspects that need to be taken into account. It is, for example, important to consider how to best transport and handle them, since they are easily stressed. The stress experienced can trigger subclinical disease and also make the pigs more susceptible to new infections. An acclimatization period of two weeks is important to cover the incubation time of most infectious diseases in Sweden. Furthermore, two weeks is enough to adapt the intestinal microflora to the new environment (Melin et al. 1997).

If individually housed, the pigs should be within sight and sound of each other to avoid social deprivation. The pens, including the feeders and waterers, should be built in a way to make it impossible for the pigs to tear them down. It is encouraged to use straw and wood shavings since these provide an excellent enrichment in addition to promoting normal rooting behavior and contributing to insulation. Pigs are intelligent and social animals that easily adjust to new routines. It is important to let them get acclimatized to their new environment and then begin social training with gentle handling techniques (as reviewed by Smith and Swindle 2006). It is recommended that the pigs are delivered at least two weeks before the experiment begins (Kaiser et al. 2005).

The anatomy and physiology of the urinary system of the pig is more similar to that of humans than most other animal species. The kidneys of pigs and humans are multipapillary (as reviewed by Giebisch and Windhager 2012, Dyce et al. 2010), while those of rodents and dogs are unipapillary (as reviewed by Dyce et al. 2010). Moreover, the pig kidney collecting system is very similar to that of humans and the mean number of calices per collecting system is 8.6 in pig kidneys compared to 8.2 in human kidneys (Sampaio et al. 1998). In adult humans the weight of each kidney ranges from 115 to 170 g (as reviewed by Giebisch & Windhager 2012), whereas the kidneys of a 20-30 kg pig each weigh 150-180 g (Zonta et. al. 2005). Furthermore, pigs weighing 30 kg are still easy to handle and they are large enough to conduct kidney transplantation without limitations due to their size (Golriz et al. 2012). Thus 30 kg pigs are ideal in kidney transplantation studies.

The intrarenal arteries are similar but not identical between pigs and humans. Pigs have almost always one artery per kidney (Perreira-Sampaio et al. 2004) compared to humans where 27-30 % have multiple renal arteries (Sampaio & Passos 1992, Satyapal et al. 2001). Also, in over 90 % of pig kidneys the renal artery divides into a caudal and cranial branch (Perreira-Sampaio et al. 2004), while the human renal artery primarily divides into an anterior and a posterior branch (Sampaio & Aragao 1990). Moreover, the renal metabolism is very similar between human and pig kidneys (Nath et al. 2014).

During renal transplantation surgery in pigs it is important to keep in mind that the porcine ureter has a narrow lumen and a fragile mucosa that is very susceptible to edema (Zonta et al. 2005). The middle and distal segment of the ureter receive blood from the common iliac artery and necrosis due to

ischemia is a substantial risk if long segments of ureter are being used (Golby and White, 1971, see Golriz et al. 2012 p.127). Thus, if possible, it might be better to use only the proximal part of the porcine ureter.

Renal biopsies are a standard procedure in renal transplantation clinic and research. In porcine kidneys, biopsies should be taken as far peripheral as possible, preferably through the calyceal fornix. Punctures in the cranial infundibulum or the renal pelvis are discouraged due to high risk of arterial damage (Pereira-Sampaio et al. 2004).

Anesthesia and analgesia

During kidney transplantation in pigs it is essential to provide excellent anesthesia and analgesia to prevent intra- and postoperative complications and avoid pain in the animals.

Anesthesia is preferably induced in a stress-free manner in the pig's home pen. Intramuscular (i. m.) injections are painful because of the density of the muscle mass, thus subcutaneous (s. c.) injection is recommended. All common anesthetic agents that can be given i. m. can also be injected s. c. with the same induction time (Swindle and Smith 2013). All pigs undergoing general anesthesia should be intubated since pigs are prone to laryngospasm and fluid often accumulates in the pharyngeal region during anesthesia. If anesthesia is maintained by the use of a volatile anesthetic drug, isoflurane is preferred since it provides the least cardiodepressant effect while maintaining proper anesthesia.
To prevent hypovolemia, infusion of Ringer Acetate, or a similar solution, at a dose of 1-4 mL/kg/h is recommended (Pehböck et al. 2015). Due to a high metabolism and a sparse body hair coat, pigs are very susceptible to hypothermia. Accordingly, heat should be added during anesthesia if needed.

The use of proper analgesia during surgical procedures in pigs is very important. Buprenorphine is an analgesic that maintains proper analgesia for 8-12 hours in pigs (Swindle and Smith 2013). Buprenorphine given intramuscular reduce isoflurane minimum alveolar concentration by 50 % (Malavasi et al. 2008). Morphine is another substance extensively used. When administered intravenously in pigs it results in analgesia with respiratory depression (Steffey et al. 1994). Common side effects of morphine in humans include obstipation, nausea and vomiting (FASS 2015). The use of morphine extradurally, however, results in analgesia without the previously mentioned side effects. Extradural morphine has an onset time of approximately 30 minutes and makes it possible to reduce isoflurane concentration during general anesthesia (Malavasi et al. 2006).

If surgical wounds are present post op the pigs should be kept individually to avoid cannibalism.

Swine Leukocyte Antigen

The swine leukocyte antigen (SLA) complex, which is part of the porcine major histocompatibility complex (MHC), is readily mapped out (Ho et al. 2010, Gao et al. 2014). It plays a significant role in the porcine immune response and the importance of SLA matching in transplantation studies is well known (Roussi et al. 1996, Haller et al. 1999). Roussi et al. (1996) found that crossed bone marrow transplantations between SLA-identical pigs provides a good model for studying the roles of von Willebrand factor in hemostasis and thrombosis. In the study by Haller et al. (1999) thymic tissue were transplanted across SLA mismatched pigs, which led to a loss of thymic tissue in many recipients, despite the use of immunosuppression.

The human correspondence to SLA is called HLA. It has long been known that a positive crossmatch, *id est* the recipient has pre-formed cytotoxic antibodies against the graft, is associated with a higher risk of immediate failure after transplantation (Patel & Terasaki 1969, O'Rourke et al. 1999, O'Malley et al. 1999). These pre-formed antibodies are more common among multiparous females, patients with

a history of blood transfusion and patients receiving a second or third transplant (Patel & Terasaki 1969).

Transplants from related donors have a higher survival rate than those from unrelated donors (Patel & Terasaki 1969). Transplantations between HLA-identical siblings have the highest survival rates, underscoring the importance of HLA matching (Terasaki et al. 1995). Several studies have shown that a positive crossmatch leads to worse long-term survival (Bryan et al. 1998, O'Malley et al. 1999). However, according to OPTN (2012) the five year survival is similar between living related and unrelated donors and Terasaki et al. (1995) found that grafts from living donors with positive HLA crossmatch still have a higher survival rate than grafts from deceased donors.

Nonhuman primates

Nonhuman primates (NHP), especially macaques, have been essential in transplant immunology studies. Their immune system is more similar to that of humans than rodents or dogs and experimental results can often be directly translated to humans (as reviewed by Kean et al. 2012). Furthermore, NHP have large bodies and long lifespans that enable elongated studies with multiple procedures, such as repeated biopsies or blood samples. Despite their usefulness, NHP are not as extensively used as pigs or rodents, much due to ethical concerns. They are viewed as intelligent animals with a high level of self-awareness. Compared to pigs and rodents, NHP reproduce slower, as they only produce 1-2 offspring per year. Furthermore, they are more difficult to care for and are more expensive. Another disadvantage is the possible transmission of infectious diseases from NHP to humans (as reviewed by Estep et al. 2010).

The European Union legislation, Directive 2010/63/EU acts to replace, reduce and refine the use of animal models in scientific research. It stipulates minimum standards for housing and care and stresses that the procedure should cause as little pain and suffering as possible. The directive states that NHP should only be used to help avoid, prevent, diagnose or treat debilitating or life-threatening conditions in humans where the procedure cannot be achieved by the use of other animal models (Directive 2010/63/EU of the European Parliament and of the Council).

TRANSPLANTATION STUDY WITH PIGS, SLU 2015

The present experiment is a collaboration between experts (physicians, veterinarians and nurses) in innate immunity, transplantation, comparative medicine, imaging and pathology from Uppsala University and the Department of Clinical Sciences at SLU. The project is a part of DIREKT financed by EU-FP7. The aim of the study is to investigate whether PEG-lipid could reduce damage in renal transplants associated with ischemia/reperfusion injury. Moreover, the usefulness of the pig as an animal model for kidney transplantation experiments will be closely studied.

MATERIAL AND METHODS

Animals and Housing

Eleven specific pathogen free pigs (Yorkshire x Hampshire) were obtained from the University herd (Lövsta, SLU, Sweden). Three female pigs from the same litter were used as donors. Six pigs from another litter, unrelated to the donors, of both sexes were used as recipients. Two pigs of both sexes from the same litter as the recipients were used as controls. The pigs arrived on the 27th of Mars and

were housed at the Department of Clinical Sciences, SLU, Uppsala, Sweden. Upon arrival the donors were six and the recipients seven weeks old. The recipients were kept in individual pens measuring approximately 2.5 m² within sight and sound of each other. The donors were kept together in one pen measuring 4.3 m². Straw and wood shavings were used as bedding. A 14:10 h light/dark schedule was used and an infrared lamp (24 h) was placed in the corner of each pen. Pigs were fed commercial pig feed (SOLO 330, Lantmännen) twice daily, the amount according to SLU regimen for growing pigs (Göransson and Lindberg 2011). Water was provided *ad libitum*. The pens were cleaned twice daily. After a 14 days acclimatization period the recipients weighed 32.4 ± 2.2 kg, the controls 30.7 ± 2.5 kg and the donors 27.0 ± 3.1 kg. At the time for surgery, the donors were eight and the recipients nine weeks old.

Acclimatization and social training

The recipients were given three days to adjust to their new environment in their own pen, thereafter social training was initiated. Each pig was given 15 minutes of social training for a total of eleven days. During the acclimatization period, the pigs were also trained to tolerate ultrasound evaluations of their urinary bladder and trained to step onto an electronic scale. As rewards, the pigs were offered pieces of apples, pears or bananas.

Experimental design

All procedures were approved by the Ethical Committee for Animal Experimentation, Uppsala, Sweden. The experiment ran for a total of three weeks.

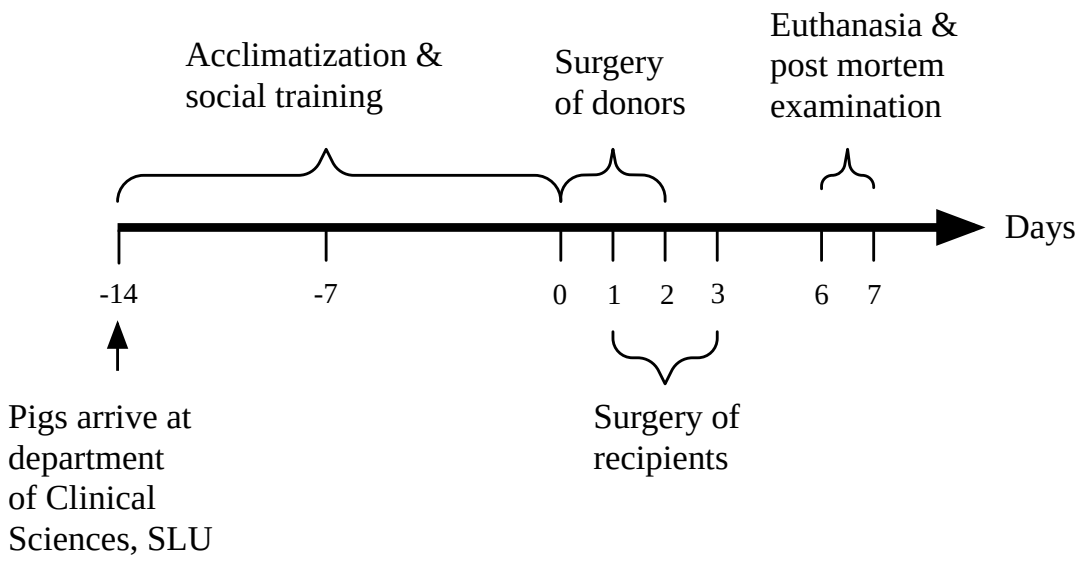


Figure 3. *Time line of the experimental design of the present study. The pigs were acclimatized and socially trained. The donors underwent double nephrectomy and the recipients received either a PEG or a control kidney.*

All pigs were typed for Swine Leukocyte Antigen (SLA) at the Faculty of Veterinary Medicine in Vienna before the commencement of the experiment. Three pairs as similar to each other as possible in respect to these tests were chosen within the recipient litter (A1 female, A2 male, B1 female, B2

male, C1 female and C2 female). These pigs were mismatched against the three donors. The recipient pigs were randomly assigned to receive either PEG (A1, B2 and C2) or control kidney (A2, B1 and C1) from their donor. Two pigs from the same litter as the recipients were used as controls (D1 female and D2 male).

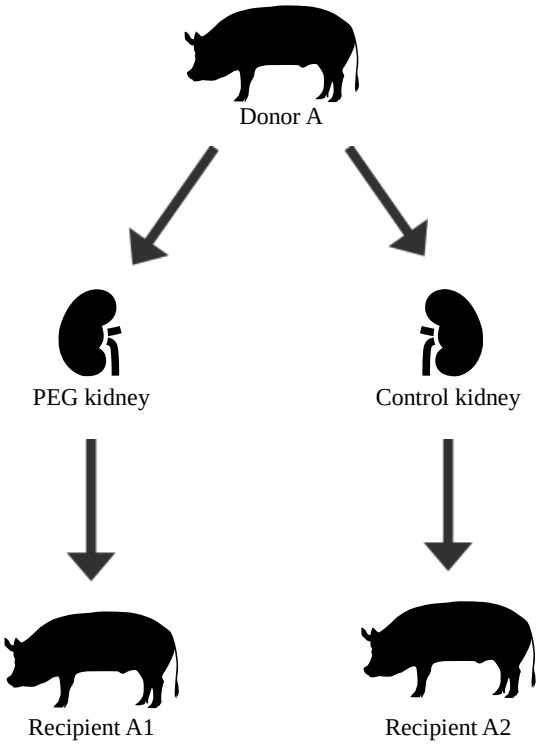


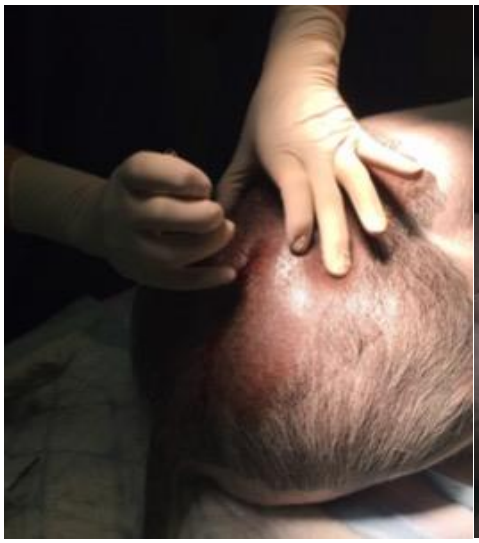
Figure 4. *The two recipients (who were double nephrectomized) within a pair received either PEG-treated or an un-treated kidney from the same donor.*

Surgery and Anesthesia

Anesthesia

Anesthesia was induced in the pigs' home pens with a combination of xylazin (Rompun® vet. 20 mg/mL, Bayer) at a dose of 2.2 mg/kg b.w. i.m. and tiletamin and zolazepam (Zoletil® 100 mg/mL, Virbac) at a dose of 5 mg/kg b.w. i.m. Buprenorphine (Vetergesic® 0.3 mg/mL, Orion Pharma) was given at a dose of 0.01 mg/kg b.w. i.m for further analgesia. Bensylpenicillinprokain (Penovet® vet. 300 mg/mL, Boehringer Ingelheim) at a dose of 21 mg/kg b.w. i.m. was given prior to surgery. After induction, pigs were moved to the preparation room, where they were endotracheally intubated. Anesthesia was maintained with isoflurane mixed with oxygen. The concentration of isoflurane was adjusted between 0.6 and 1.5 % to reach adequate depth of anesthesia. In addition 0.5-1.0 mL of Zoletil® was given i.v. when the anesthesia became too shallow. In the recipients, a venous catheter was placed in *vena jugularis* through *vena auricularis* by Seldinger technique (Zeltner 2013). This catheter was used for blood sampling throughout the experimental period and was filled with heparinized saline (Heparin LEO, 5000 IU/mL, Leo Pharma) at a concentration of 100 IE/mL, when not used. In one of the recipients (C1) a catheter could not be placed. All pigs were also given an epidural injection with morphine (Morfin Epidural Meda®, 2 mg/mL) before surgery. The morphine was diluted in saline and 1 mL of the solution was given for the first 40 cm of vertebral length, adding an extra 1.5 mL for every 10 cm of vertebral length. Ringer Acetate were given in an amount of 240-300 mL each hour of the surgery. Dobutamine at a dose of 0.4-2.0 mL/kg/h was infused when the mean arterial blood pressure sank below 60, which it foremost did at reperfusion, removal of the

native kidneys and when the intestines were held outside of the body. Gelatine and sodium chloride (Gelofusine® 40 mg/mL and 7 mg/mL, Braun) were given i.v. to maintain blood pressure.



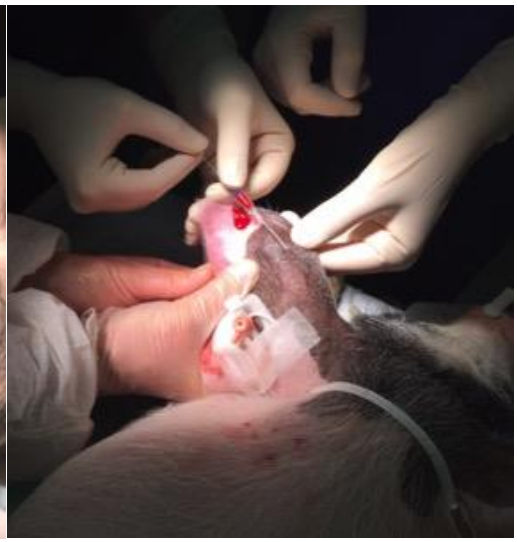


Figure 5 (left). *An epidural with morphine was given to the recipients in the preparation room.*

Figure 6 (right). *In the recipients, a venous catheter was placed in vena jugularis through vena auricularis by Seldinger technique.*

During anesthesia the pigs were continuously monitored regarding blood pressure (mmHg), heart rate (beats/min), respiratory rate (breaths/min), ECG, end-tidal carbon dioxide (mmHg/kPa), oxygen saturation (%), end-tidal isoflurane gas (%), inspiratory/expiratory oxygen (L/min), minute ventilation (L/min), inspiratory maximum pressure (cmH_2O) and body temperature (°C).

Surgery

In the preparation room, the pigs were placed in dorsal recumbency and socks were placed on their claws to prevent heat loss. The surgical area was shaved and cleaned thoroughly with soap and chlorhexidine solution.

Subsequently, the pigs were moved to the surgical theater where surgery was initiated. In the donors the incision was made in *linea alba*. Both kidneys and ureters were carefully dissected loose. The supra- and infrarenal aorta and the inferior *vena cava* on each side were cross-clamped to prevent blood flow. Thereafter the kidneys were promptly removed *en bloc* and flushed with cold HTK (Custodiol®, Köhler Chemie GmbH, Germany). The surgery was completed within 75 minutes. The donors were euthanized under general anesthesia by an intravenous overdose of potassiumchloride (Addex® Kaliumklorid 2 mmol/mL, Fresenius Kabi AB).

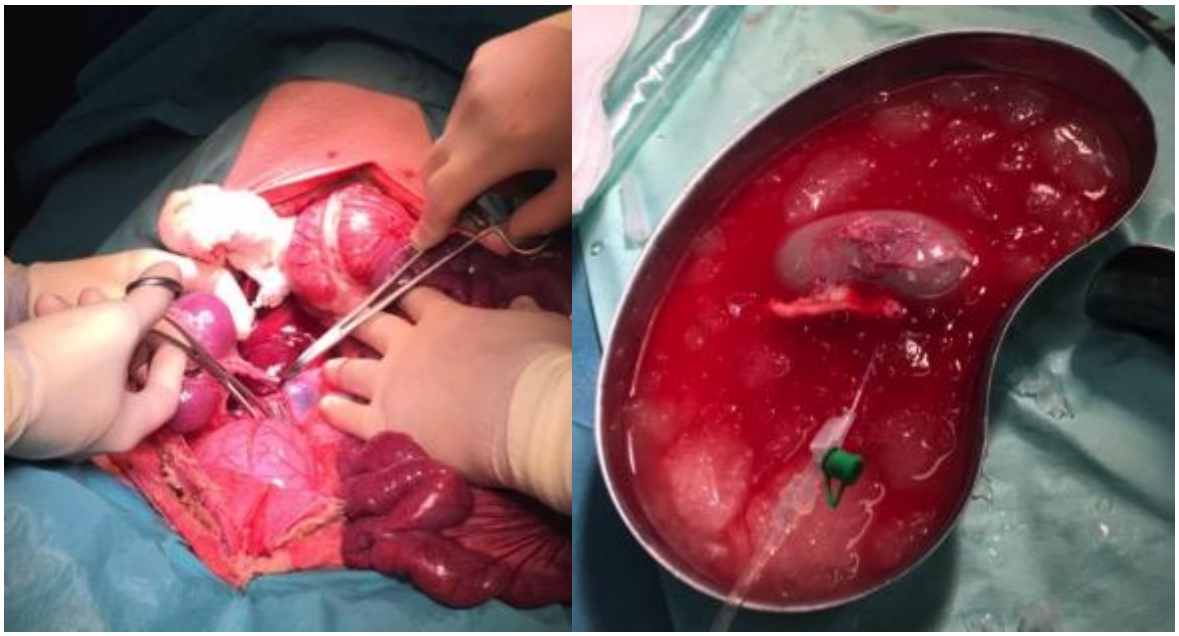


Figure 7 (left). *Donor nephrectomy.*

Figure 8 (right). *Donated kidney placed in ice water and flushed with cold HTK solution.*

The donated kidneys were stored in a refrigerator at 4 °C (cold static preservation) for at least 24 hours. 45 minutes prior to surgery one kidney, randomly chosen, were perfused manually with polyethylene glycol lipid 2 mg/mL and the other with 20 mL of histidine-tryptophan-ketoglutarate solution. Afterwards, both kidneys were stored in a perfusion solution at 4 °C for at least 40 minutes.

In the recipients, a subumbilical midline incision was made, keeping the surgical trauma to a minimum. The kidney was heterotopically transplanted to dorsally of the right inguinal canal with closeness to the inferior *vena cava* (IVC), due to the short venous segment of the right kidney. The right common iliac artery and the caudal IVC were carefully mobilized, after which the blood vessels of the graft were clamped. Both artery and vein of the transplant were anastomosed by an end-to-side procedure with running 6-0 prolene sutures. When completed, reperfusion was initiated. The middle and distal segment of the ureter were then dissected loose, leaving only the proximal segment, which was implanted by ureteroneocystostomy to the top of the bladder using 6‑ 0 polydioxanone.

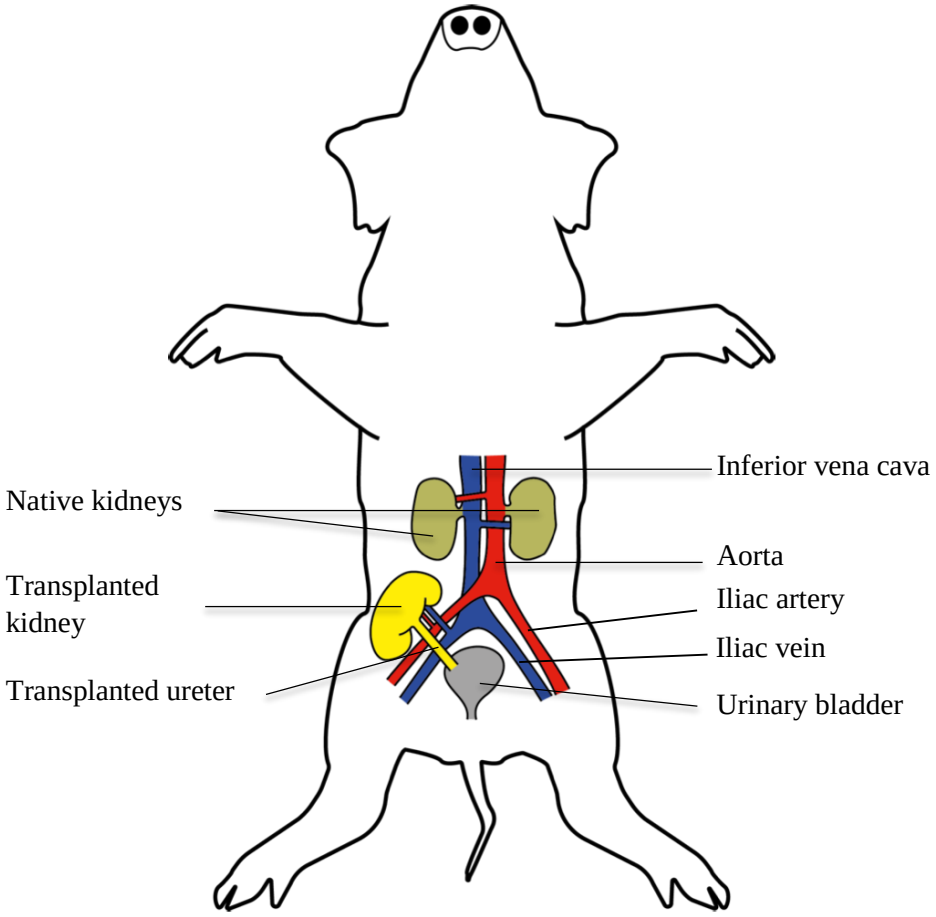


Figure 9. *The kidney graft was transplanted to dorsally of the right inguinal canal of the recipient. Thereafter the pig's native kidneys were removed.*

The native kidneys of the recipient were removed at the end of the surgery to prevent intraoperative morbidity due to fluid/electrolyte imbalance and hemodynamic depression. Both native kidneys were reached from the subumbilical midline incision and removed after closing the renal vessels and the ureters with a LigaSure Impact™ vessel-sealing device. The incision in the abdominal wall was closed using an absorbable 2-0 vicryle suture and the skin was closed using staples. The surgery of the recipients lasted between 1.75 and 3.25 hours.

Two wedge biopsies, at a size of approximately 5x5x5 mm, were taken with a scalpel from the cortex of transplanted kidney before and 15 minutes after reperfusion. One biopsy was snap frozen in liquid nitrogen and the other was placed in 4 % Paraformaldehyde (PFA).

The surgery was performed by two experienced transplantation surgeons from Uppsala Academic Hospital.

Postoperative care

The recipients were closely monitored postoperatively and extubated when regaining swallowing reflex. The pigs were constantly monitored by the research staff for at least five hours after surgery.

One pig (B2) stopped breathing shortly after extubation and was intubated again and ventilated manually until spontaneous breathing returned. This pig could later be successfully extubated.

After the surgery was completed all recipients were given 0.01 mg/kg b.w. buprenorphine (Vetergesic® 0.3 mg/mL, Orion Pharma) i.v. or i.m. when expressing signs of pain. Enrofloxacin (Baytril® vet. 100 mg/mL, Bayer) was given at a dose of 2.6 mg/kg b.w. i.v. combined with either bensylpenicillinprokain (Penovet® vet. 300 mg/mL, Boehringer Ingelheim) at a dose of 21 mg/kg b.w. i.m. or bensylpenicillinsodium (Bensylpenicillin® 1g, Meda) at a dose of 0.06 mL/kg b.w. i.v. Baytril® was given once daily for three days. The pigs also received Penovet® once daily or Bensylpenicillin® twice daily for two days.

During the consecutive days clinical appearance was assessed several times each day. The pigs that had trouble standing received massage and were helped to stand up. When the pigs were able to walk they were, one at a time, let out of their pen and allowed to move freely in the stable. They were also cuddled with and offered fruits several times each day. Weight was recorded at least three times per week.

Blood analyses

EDTA-preserved blood was analyzed for total and differential white blood cell counts, hematology (EPK, Hb, EVF, MCV, MCHC, reticulocytes), and thrombocytes by an electronic cell counter validated for porcine blood (Advia 2120, Siemens, Erlangen, Germany). Serum samples were analyzed for enzyme activities; aspartate amino transferase (ASAT), alanine aminotransferase (ALAT), γ-glutamyltransferase (GT), glutamate dehydrogenase (GLDH) and creatinine (crea) with automated equipment (Architect C4000, Abott Diagnostics, North Ryde, Australia). These parameters were measured at the Section of Clinical Chemistry, SLU, Uppsala.

Urine sampling

After surgery, the pigs were carefully monitored and free flow urine samples were collected when possible. The urine was evaluated macroscopically and specific gravity was measured with a refractometer. pH, blood and protein were measured by urine test strips.

Ultrasound

After surgery, the urinary bladders were examined twice daily at 9 a.m. and 6 p.m. by ultrasound (Imago 1401MG05, ECM, France). The purpose of the ultrasound examinations was to investigate whether the transplanted kidney produced urine or not.

Each kidney was at one point examined by ultrasound (Logiq e R6, GE Healthcare, Wauwatosa, U.S.A.) using linear (10 MHz) and curvilinear (4 MHz) probes. The examination took place on day 2 (A1 and A2), 3 (C1 and C2) and 4 (B1 and B2) post surgery. The length and echogenicity of the kidney as well as the corticomedullary definition were estimated. Furthermore, it was assessed whether the renal pelvic region was dilated. The blood flow in the kidney was evaluated by color doppler.

These examinations were performed by an experienced veterinary radiologist.

Euthanasia and Necropsy

At the end of the experiment, the pigs were euthanized in their home pens by an intravenous overdose (12 mL) of pentobarbital sodium (Euthasol vet. 400 mg/mL, Virbac). Since one pig did not have a venous catheter, this pig received 2.5 mL of tiletamin and zolazepam (Zoletil® 100 mg/mL, Virbac) and 3 mL of xylazin (Rompun® vet. 20mg/mL, Bayer) i.m. followed by 5 mL of pentobarbital sodium (Euthasol vet. 400 mg/mL, Virbac) i.c.

Immediately after confirmed death, two biopsies including the cortex, medulla and renal pelvis, at a size of approximately 10x5x5 mm, were taken with a scalpel from the transplanted kidney for further analyses, such as complement activities. One sample was snap frozen in liquid nitrogen and the other was placed in 4 % PFA.

Figure 10. *After confirmed death of the recipient, biopsies were taken from the transplanted kidney.*

All pigs underwent post-mortem examinations at the Department of Biomedical Sciences and Veterinary Public Health, Section of Pathology, SLU, Uppsala.

Statistical analyses

Data is expressed as mean ± SD. Groups were compared with nonparametric Mann-Whitney *U* test and p values $\leq$ 0.05 were considered statistically significant.

RESULTS

Acclimatization and social training

At first the pigs expressed signs of fear and stress when the trainer entered their pen, shown by them running around or pressing themselves into a corner. At the end of the first session, four out of six pigs became curious and came to sniff the trainer. Three pigs allowed the trainer to brush them the first day. On the fourth day of social training the trainer were allowed to brush and touch the ears of all pigs but one. After the eleven days of social training all pigs tolerated handling well and none got stressed when research staff entered their pen.

Figure 11. *After the 14 days acclimatization period the pigs were very calm around the staff.*

General appearance after surgery

The pigs showed signs of pain and weariness the first 12-24 hours after surgery, whereafter general appearances were improved. All pigs started to eat within 24 hours after surgery. B2 (PEG) and C1 (non-PEG) had difficulties with putting weight on their left hind leg the first day after surgery. After having received massage and standing training a couple of times they were able to walk. There was no difference in general appearance between PEG-treated and non-PEG-treated pigs.

Daily weight gain is presented in chart 1. Before surgery, the daily weight gain was 1.1 ± 0.1 kg in the controls, 1.0 ± 0.1 kg and 1.0 ± 0.0 kg in the pigs that were to receive a PEG and a non-PEG kidney, respectively. The daily weight gain after surgery was less for all recipients compared to the controls. The two controls had a daily weight gain of 1.0 ± 0.0 kg, whereas the pigs with a PEG kidney lost 0.2 ± 0.4 kg per day and the pigs with a non-PEG kidney lost 0.2 ± 0.3 kg per day. There were no difference in daily weight gain between PEG and non-PEG treated transplanted pigs (p=1).

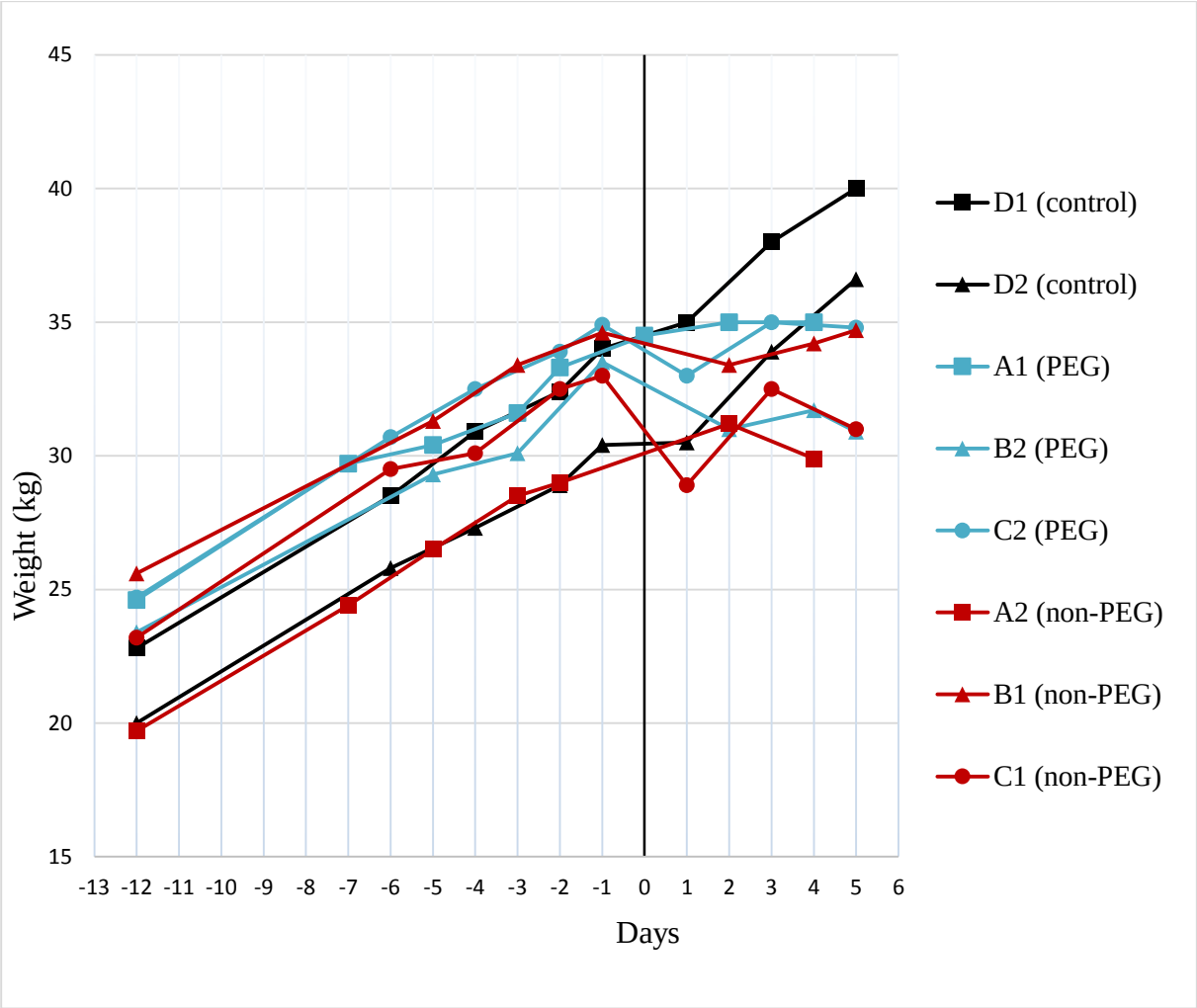


Chart 1. *Weight gain before and after surgery (day 0).*

B1 and B2 were euthanized on day five post surgery, since B1 (PEG) was found to be in poor condition. On day four after surgery, C1 (non-PEG) showed signs of pain and had diarrhea. She received analgesia, but the next day she was still in pain and her abdomen was distended. A2 (non-PEG) started vomiting day four post op and had lost weight. Therefore, C1, C2, A1 and A2 were euthanized the very same day, *id est* five and four days after surgery, respectively.

Blood analyses

Hematology and biochemical analyses before surgery were within reference range for all pigs.

Urine analyses

After the transplantation was completed, urine was collected directly from the bladder by cystocentesis, showing that all of the transplanted kidneys had started to produce urine soon after reperfusion.

Urine specific gravity, blood and protein are presented in chart 2-4. Urine pH was 5 in all samplings but one. Pig C1 (non-PEG) had pH 7 on day three after surgery, but measured 5 the next day. The color of all samplings, but one, was yellow. Pig C1 (non-PEG) had orange-red colored urine on day three after surgery, but on day 4 this pig produced yellow urine. There were no significant difference

between PEG and non-PEG in any of the measurings; urine pH (p=1), urine specific gravity (p=0.81), hematuria (p=0.52) and proteinuria (p=0.73).

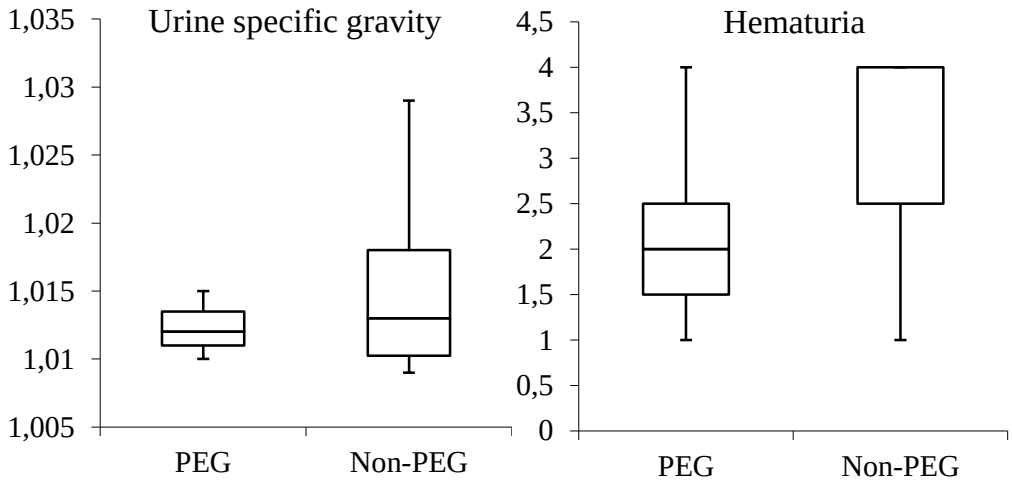


Chart 2 (left). *Box and whisker chart of urine specific gravity.*

Chart 3 (right). *Box and whisker chart of hematuria.*

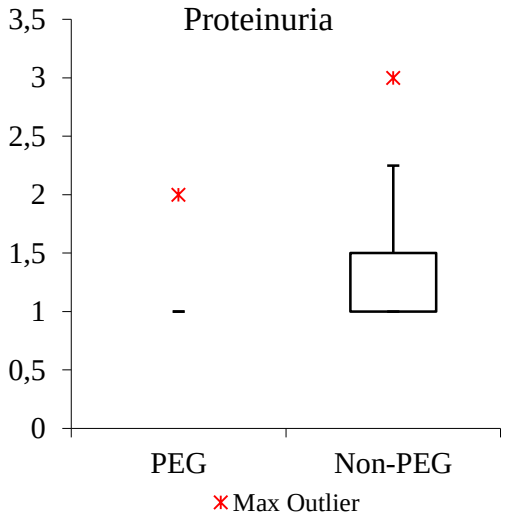


Chart 4. *Box and whisker chart of proteinuria.*

Ultrasound evaluation

Bladder ultrasound

Urine was found in the bladder on day one in four out of six pigs. Within three days urine was found in the bladder of all pigs.

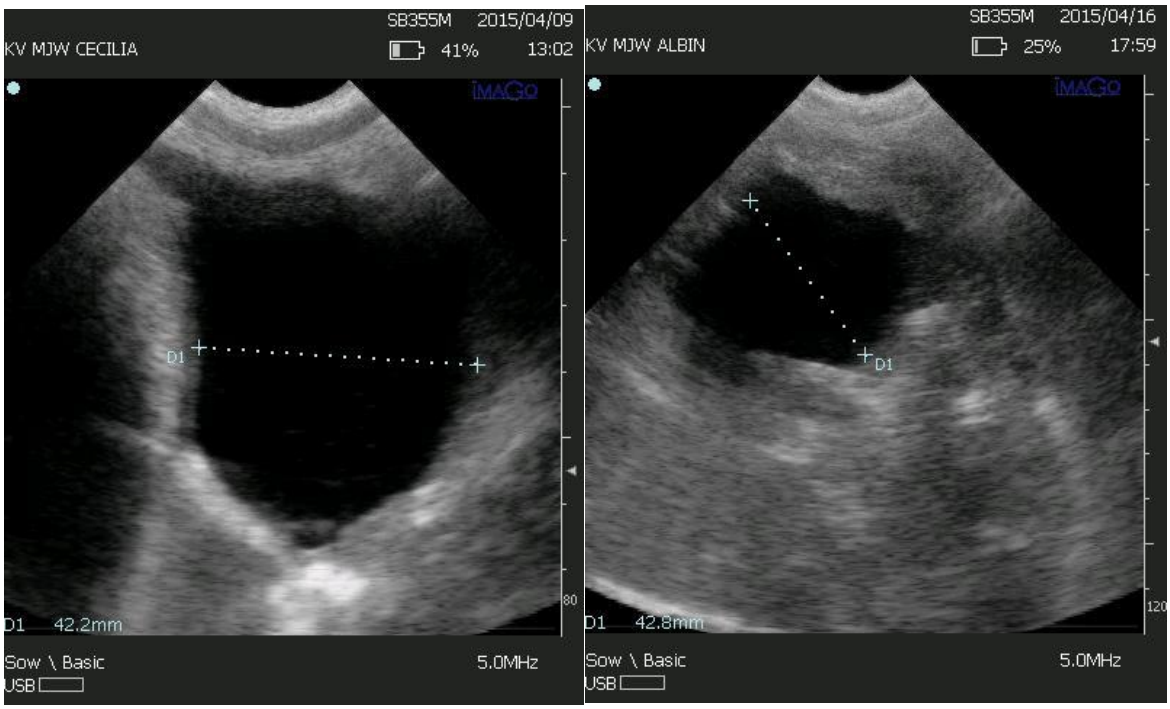


Figure 12. *Ultrasounds of the bladders of C1 (to the left) and A2 (to the right).*

Kidney ultrasound

The blood flow in all kidneys was assessed as very good. The renal pelvis was judged as moderately dilated from day three after surgery The mucosal thickening at the transition from ureter to renal pelvis is presented in table 1.

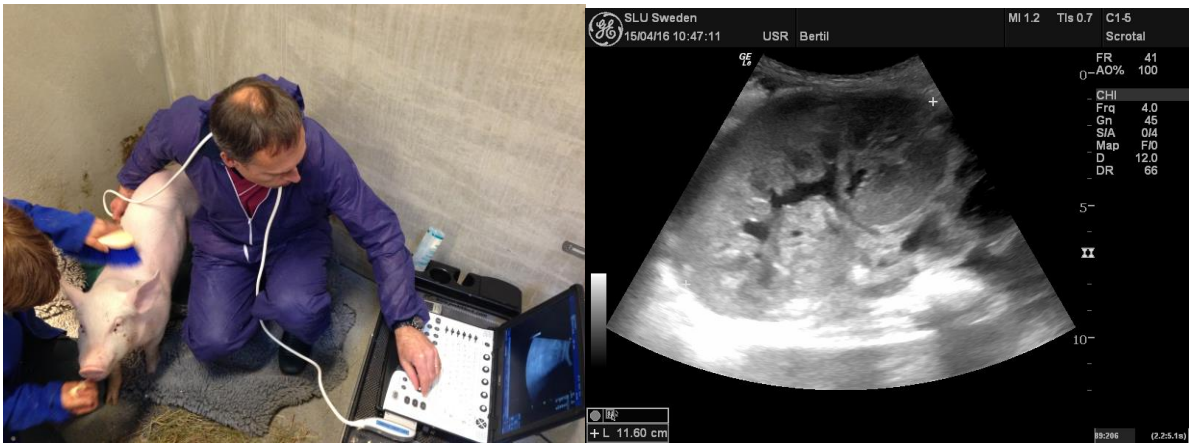


Figure 13. *Kidney ultrasound.*

Pig	A1 (PEG)	A2 (Non-PEG)	B1 (Non-PEG)	B2 (PEG)	C1 (Non-PEG)	C2 (PEG)
Days after transplantation	2	2	4	4	3	3
Mucosal thickening	None	None	3 mm	None	4 mm	6 mm

Table 1. *Mucosal thickening at the transition from ureter to renal pelvis.*

Post mortem examination

In two out of the three donor pigs, the visceral pleura stretched from the caudal lung lobes to the diaphragm. These were assessed as incidental findings. All other findings were directly related to the anesthesia and nephrectomy.

In all recipients, diffuse lymphocytic nephritis, acute tubular necrosis and renal bleedings were found. In two out of the six recipients, gauze pads were found in the abdomen with fibrinous adhesions to colon spiralis (C1) and the kidney (A2). These two pigs also had gastric ulceration. There were no other abnormal findings.

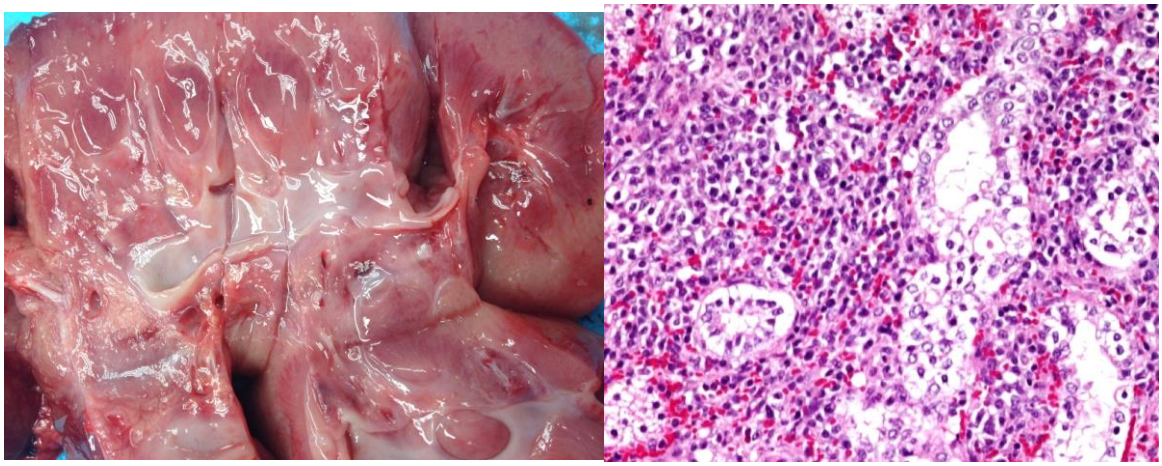


Figure 14. *Kidney of A1 (PEG) post mortem macroscopically (to the left) and histologically (to the right). Pictures taken by the Department of Biomedical Sciences and Veterinary Public Health, Section of Pathology, SLU.*

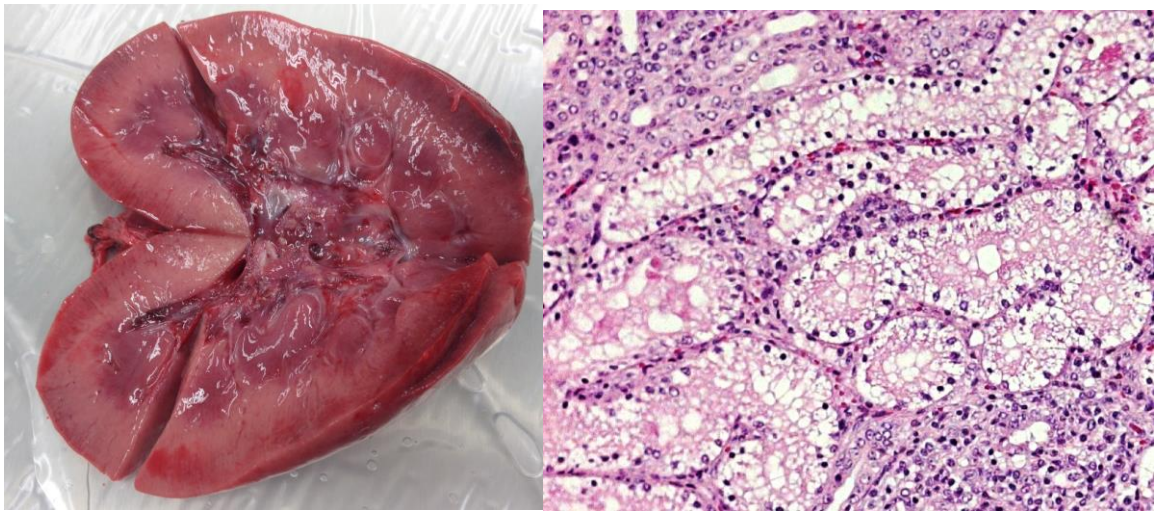


Figure 15. *Kidney of A2 (non-PEG) post mortem macroscopically (to the left) and histologically (to the right). Pictures taken by the Department of Biomedical Sciences and Veterinary Public Health, Section of Pathology, SLU.*

DISCUSSION

The present study confirmed that pigs provide an excellent animal model for renal transplantation studies. There are several advantages with pigs as urological models, including that the urological system of pigs is more similar to humans than those of most other animal models, both in aspects of physiology and anatomy.

The acclimatization period was successful, demonstrating the importance of including such a period in most procedures with pigs. In the literature, it is recommended that the pigs are delivered at least two weeks prior to the beginning of the experiment (Kaiser et al. 2005). In this period the pigs will have time to get accustomed to the environment and the food. Furthermore, the incubation time of most infectious diseases in Sweden will be covered. After the two weeks acclimatization period in the present experiment the pigs were very tame and easy to handle. They were not stressed when a member of the staff entered their pen, which is important for the animal welfare. On clinical examination before the surgeries all pigs were found healthy. Also, the intestinal microflora have been shown to adapt to a new environment within this acclimatization period (Melin et al. 1997), which should support the recovery after surgery.

In the present study, the pigs weighed around 30 kg at the time of surgery. At this size, they were large enough to undergo kidney transplantations and small enough to be easily handled. We included both female and male pigs in the experiment, although female kidneys have been shown to be more resistant to ischemia/reperfusion injury than male kidneys in a study with mice. It is, however, concluded that this difference between the sexes is attributable to testosterone (Park et al. 2014). In the present study, the pigs that had not yet reached sexual maturity and thus there should be minimal difference between female and male kidneys.

In the present experiment, the controls gained 1.0 kg per day. All pigs were easily handled during the three week experimental period. However, their fast growth makes large pigs unsuitable for chronic studies reaching longer than this period. Thus, in chronic studies miniature pigs are preferred. The Hanford minipig, for example, is social and easily trained and often used for urological studies (as reviewed by Giraud et al. 2011).

The anesthetic protocol was appropriate. Before the onset of surgery the pigs were given systemic buprenorphine and epidural morphine. The proper use of analgesics before and during surgery enabled less use of isoflurane and provided better pain relief during and after surgery. During surgery, there were some critical points where the blood pressure of the pigs sank. These include reperfusion of the transplanted kidney, nephrectomy of the native kidneys and when the intestines where held outside of the body. Dobutamine was given when the mean arterial pressure (MAP) sank below 60, which made MAP rise to approvable levels. Muscle relaxants, such as pancuronium or vecuronium, can be administered during surgery (as reviewed by Swindle 2008). We chose not to use muscle relaxants since it can mask pain during the procedure.

All pigs survived the surgery and none was sacrificed before four days post transplantation. However, one of the pigs suffered intraoperative complications at reperfusion, because the vein of the transplant was accidentally obstructed. Measures to correct this were immediately taken. Although this pig lost some blood and was anesthetized for a longer period of time than the other pigs, the pig recovered well and could continue in the study. As demonstrated in other experiments, intraoperative complications are not uncommon. He et al. (2013) performed renal transplantations in ten pigs with an anastomosis success rate of 70 %. In one pig the renal anastomosis failed due to a complex renal anatomy and in two pigs the arterial anastomoses were unsuccessful. In a study by Han et al. (2013) one out of eight pigs died after surgery, probably due to anesthetic overdose. Other intraoperative complications reported include thermal bowel injury and splenic injury resulting in blood loss and splenectomy (Hunter et al. 2015). In conclusion, kidney transplantation in pigs can be performed but requires a skilled surgeon.

Postoperative care is of outermost importance. In the present study, one of the pigs stopped breathing when extubated. Spontaneous breathing returned after he was intubated and ventilated manually. Death due to laryngospasm after extubation has previously been reported (He et al. 2013), making extubation a critical step in pig surgical procedures. Thus, it is very important to be prepared for these situations and be able to quickly intubate the pig again.

It is discouraged to keep newly operated pigs in groups since surgical wounds can be attacked by other pigs. Moreover, the pigs would rip out each other's venous catheters if kept in a group. Thus, the pigs in the present study were kept in individual pens with visual and auditory contact with the other pigs to stimulate their social needs.

During the present experiment the staff spent a lot of time in the stables post surgery to monitor the pigs. In order to facilitate for the staff, video cameras, which could be accessed from another room, could have been used. With video cameras, it would also be possible to better monitor when the pigs start urinating post surgery.

Two pigs had trouble with putting weight on their left hind leg the day after surgery. One theory to why this happened is that they might have been lying on their left side for too long, leading to muscle and/or nerve damages. After surgery the pigs were very tired and did not move much, thus they would lay in the same position for several hours unless the staff moved them. In future experiments it would be preferable that the staff made sure that the pigs switched positions at regular intervals the first 24 hours after surgery. The pigs in the present experiment received massage and standing training, which probably helped them to be able to walk again.

In five out of six pigs a vein catheter was successfully placed in *vena jugularis* through *vena auricularis* by Seldinger technique. Compared to a surgical technique where the catheter is placed directly into *vena jugularis*, this technique is quicker and less invasive. It is also associated with a lower risk of infections, bleedings and placement complications (McBride et al. 1997, Ahmed & Mohyuddin 1998). However, placing the catheter in *vena jugularis* through *vena auricularis* can be difficult even for an experienced surgeon. In the present experiment, the catheter could not be placed in one of the pigs. This could be due to the use of xylazin (Rompun® vet. 20 mg/mL, Bayer), which initially cause hypertension, followed by vasodilation and hypotension in some patients. Another pig in the study did not like that the staff touched her ear. This pig moved her head during blood samples and intravenous injections, which made it difficult to keep the procedures sterile and stress free. For this particular pig it might have been better to use a surgical technique with subcutaneous tunneling to the scapula. In one of the pigs, the catheter stopped working the first day after surgery. In future experiments it would therefore be a good idea to place two vein catheters, one through each ear.

Post surgery, the research staff drew blood from the venous catheters without stressing the animals. This is important both for the animal welfare and to be able to take the samples as sterile as possible. No pig was found to have thrombophlebitis on post mortem examination, indicating that catheters have been well maintained.

In order to facilitate the collection of urine, a urinary catheter can be placed in female pigs. However, the placement of a urinary catheter is highly discouraged in male pigs as they have many folds in their urethra (Golriz et al. 2012). A urinary catheter would also increase the risk of infection in the urinary bladder. Thus we chose not to catheterize any of the pigs. Instead, free flow urine was collected whenever possible and we managed to successfully retrieve urine from all the recipients. There were no differences between PEG and non-PEG in any of the urine analyses. It is, however, important to remember that the results are based on a small sample with three pigs in each group. With a larger study group there might have been statistically significant results. In a later experiment performed by our group, we collected urine samples from nine healthy pigs of similar age and breed as the ones in the present experiment. The urine specific gravity was similar between the two groups. It was thus considered normal for the pigs in the present experiment and showed that the pigs were able to

concentrate their urine. The color of the urine samples was yellow in all but one sample from day three post surgery. Nontheless, blood was present in all samples from operated pigs, indicating microhematuria. In a study by McDonald et al. (2004) persistent hematuria in humans was defined as having at least 1+ of blood on the urine stick in more than 75.0 % of the clinic visits over a period of at least 4 weeks. The prevalence of persistent hematuria was found to be 13.3 % of 640 renal transplant recipients. Hematuria was associated with higher median serum creatinine and earlier graft failure (McDonald et al. 2004). The hematuria found in the operated pigs might be a sign of glomerular damage. Protein was also found in the urine of all operated pigs. Kang et al. (2009) found that five year graft survival was 83.0 % in patients with proteinuria of 0.2-0.5 g/day at one year post-transplant, 70.0 % in patients with proteinuria of >0.5 g/day and 97.1 % in patients with no proteinuria.
They conclude that even minimal proteinuria after transplantation increases the risk of early graft loss. Other studies have had similar results with worse long term survival if proteinuria was found after 3 or 12 months (Roodnat et al. 2001, Borrego et al. 2013). Proteinuria might indicate commencement of graft rejection in the pigs.

At the kidney ultrasound the renal pelvis and mucosa, at the transition from the ureter to the renal pelvis, were normal in the pigs operated two days earlier. In the pigs operated three and four days earlier the renal pelvis were moderately dilated and the mucosa was thicker than normal in three out of four pigs. This suggests that these kidneys were subjected to acute rejection (Rigler et al. 2013).

Under stressful conditions, pigs are prone to develop gastric ulcers. Pigs C1 and A2 were found to have gastric ulceration on post mortem examination. This was probably due to the fact that gauze pads had unintentionally been left in their abdomens after surgery. To prevent this we should, of course, have made sure to count all gauze pads before closure of the abdomen and we also could have used histamine blockers (as reviewed by Golriz et al. 2012) after surgery.

Jensen-Waern et al. (2012) showed that it is possible to use tacrolimus and mycophenolic acid as immunosuppressive management in pigs. In the present study, however, the pigs were not given immunosuppressive treatment since we did not want the interpretation of the results to be complicated by immunosuppression. By not treating the pigs, which had received mismatched grafts, rejection would be inevitable. Rejection can be seen histologically on the third day after surgery and chemically on the fourth day (Pennington 1992, see Golriz et al. 2012 p.128). Thus, in the present study, the pigs lived for 4-5 days after transplantation. Death from renal failure, if native kidneys have been removed, happens around day 10 (Pennington 1992, see Golriz et al. 2012 p.128). In this study, the pigs were euthanized before dying of renal failure.

The present experiment showed very promising results and was repeated some months later to confirm the results.

CONCLUSIONS

The pig is an excellent animal model for kidney transplantation studies. Pigs are relatively cheap, reproduce quickly and young animals with the right training are easy to handle. Moreover, their anatomy and physiology are well studied and have been found to be very similar to that of humans.
In the present study we kept pigs anesthetized for a longer period of time and showed that it is possible to perform kidney transplantations in a similar manner to the procedure performed in humans.
The animals recovered after surgery and their general appearances were acceptable at least until day 4 after surgery. It was possible to perform clinical examinations, bladder ultrasounds and receive blood samples from the pigs every day under non-stressful situations. Moreover, this period was sufficient to collect relevant data, such as blood samples and renal biopsies, regarding thromboinflammation systemically and in the grafts.

The pig will most likely continue to be the animal model of choice in future renal transplantation studies.

ACKNOWLEDGEMENTS

The research leading to these results has receivied funding from the European Community's Seventh Framework Programme under grant agreement number 602699 (DIREKT). This study has been performed as a collaboration between various professions at Uppsala University and the Swedish University of Agricultural Sciences. I want to thank everyone that participated in this study.
Special thanks to my supervisor Marianne Jensen Waern and assistant supervisor Elin Manell for your support. I also want to thank Anneli Rydén for teaching me about anesthesia in pigs and
Alireza Biglarnia for showing me how to perform kidney transpantations.

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