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Renal ischemic postconditioning in a laparoscopic porcine model

Preliminary results

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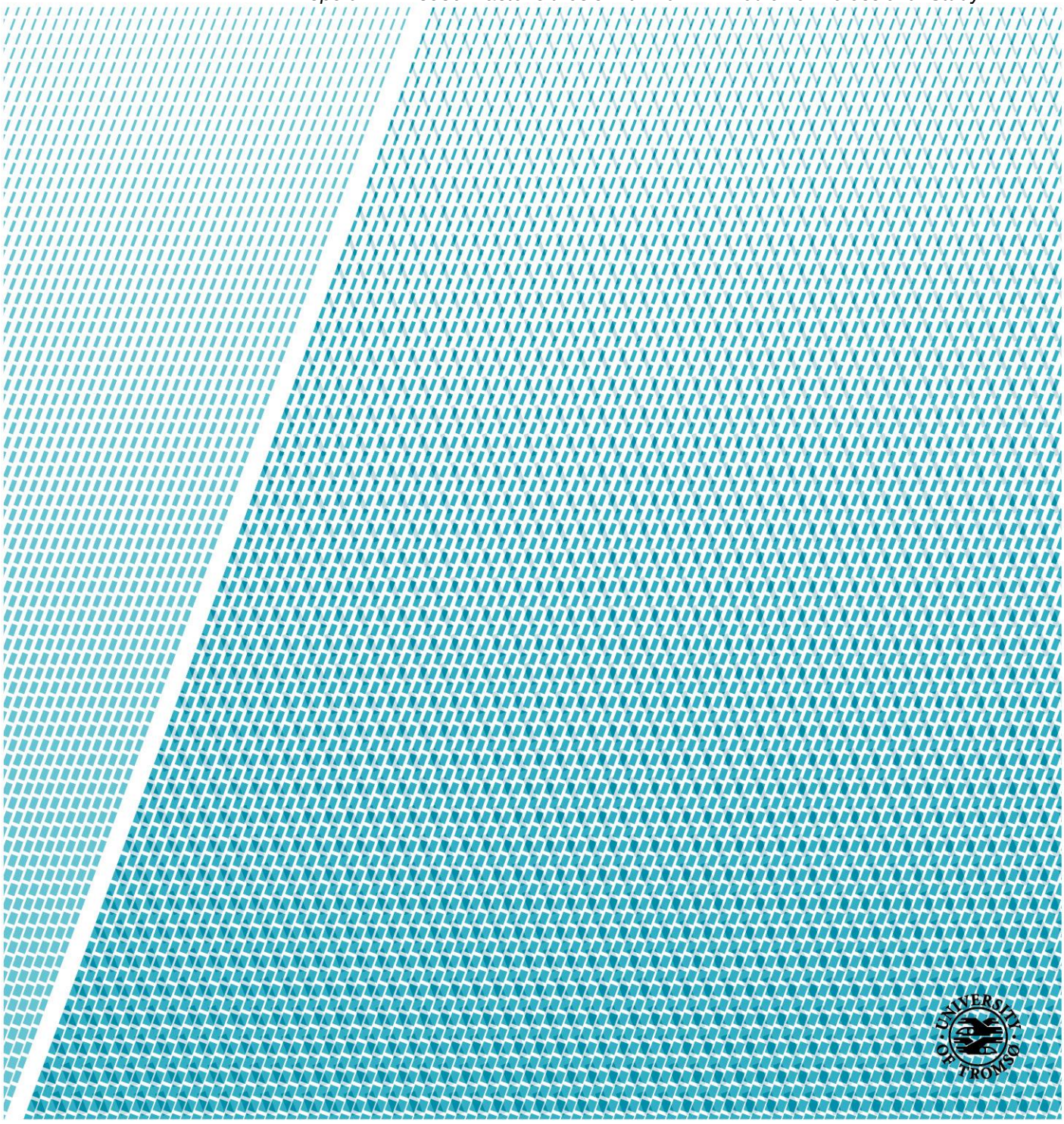


Forword

The aim of this report is to evaluate surgical experimental results obtained from an *in vivo* porcine model of ischemic/reperfusion injury.

The idea to conduct my own surgical experiment on pigs to explore the effect of postconditioning on kidney function began with an informal meeting with my colleagues Dr. Erling Aarsæther and Dr. Marius Roaldsen during lunch at the hospital cafeteria at University Hospital of Northern Norway (UNN). I had already been involved with the PhD project of Dr. Roaldsen for over two years as an assistant medical student. During those previous two years, I had assisted Dr. Roaldsen with many of his 29 pig experiments exploring anesthesia in ischemi/reperfusion injury, ischemic preconditioning as well as the development of a novel two kidney model. That work has become the foundation for Dr. Roaldsen's PhD dissertation.

When confronted with the idea of choosing a Master's thesis project, I balked at the idea of using already established data from either a database or from Dr. Roaldsen's pigs. This gave me slippery knowledge about data and results where I had not had my hands in on the whole process from start to finish. Further, after having performed a preconditioning experiment in a single kidney model, we became increasingly curious to the idea of postconditioning. When the news came down that the large animal surgical laboratory at The University of Tromsø would be closing for repairs and upgrades I jumped at the opportunity to have my own project before the lab closed doors for over a year. Immediately after I finished my practical year of rotations during my fifth year, I performed all the experiments on 16 pigs over the course of 4 long, intense weeks with mandatory visits back to the facility to check on my pigs every night.

When working with a pig surgical experimental model, it is, in the least, resource and time consuming, and as I found, constantly developing and improving. This project would not have been possible without the groundwork that Dr. Marius Roaldsen had done in developing the surgical model and experimental protocols. This laid the foundation for my project. Acknowledgement is given to him for many informal meetings and email exchanges, as well as assistance at the beginning of my surgical experiments and proofreading and advice during the data analysis and writing stage.

A project of this magnitude involving many large pigs that must be kept alive for weeks, cared for and fed as well as receiving assistance in the surgical, cellular and histological

laboratories would not be possible without the help of others. I would like to acknowledge the expertise and work of those involved with this project at The University of Tromsø's Large Animal Surgical Laboratory, Department for Comparative Medicine and The Institute for Cardiovascular Research. Those who have assisted and receive my gratitude include: Victoria Steinsrud, Hege Hagerup, Remi Osnes, Ragnhild Osnes, Harry Jensen and Siri Knudsen.

Histopathological expertise and assistance in the form of slide preparation and evaluation include Dr. Samer Al-Saad, Dr. Elin Richardsen and technical staff at the Department for Clinical Pathology at UNN.

Trine Kalstad is due thanks for her days spent pipetting and incubating ELISA analyses for this project. Her motivation and initiative to "get it done" has been a motivating factor for me to strike while the iron is hot.

I would like to acknowledge the assistance during the laparoscopic experiments to Dr. Didrik Kjønås. His cool demeanor, professionalism and humor were appreciated by all especially during more stressful times at the start of the surgical phase of the experiments.

The backbone of this entire project has been Dr. Erling Aarsæther whose work founded and secured funding for the project at the Department of Urology at UNN. Amidst a very hectic work day with multiple surgeries and patients to attend to, he has always found time to meet with me and discuss this project. His genuine enthusiasm and interest for research has been a source of inspiration.

Finally, I wish to extend my greatest gratitude to my family who has supported me through this entire process to the point of planning writing vacations so that I could sit in a wilderness cabin for days on a husky farm without the distractions of the city and home obligations.

Table of Contents

Introduction …… 1

Materials and Methods ……... 5

Results …… 8

Discussion …… 11

Conclusion ……14

References ……. 15

Tables and Figures……. 21

Appendix I: GRADE Evaluations ……. 26

Appendix II: Master's Project Contract …….....32

Abstract

Background: During procedures such as nephron sparing surgery, kidney transplantation and thorocoabdominal aorta aneurysm surgery the kidney is subjected to ischemia and reperfusion injury which may lead to loss of renal function. It has been proposed that an intermittent and brief repeated sequence of ischemia and reperfusion immediately after the main ischemic insult, known as ischemic postconditioning (IPoC) may protect the kidney from ischemic/reperfusion injury. The aim of the study was to evaluate an IPoC protocol in an *in vivo* porcine model of warm ischemic injury with 75 minutes occlusion of the left renal hilum followed by 48 hours reperfusion.

Method: 16 hybrid pigs were randomised to either warm renal ischemia only (control) or warm renal ischemia followed by 6 x 15 seconds IPoC prior to laparoscopic surgery. Following anesthesia, the left kidney hilum was clamped for 75 minutes and the intervention group then received 6 x 15 seconds postconditioning. Serum creatinine and urea was measured following 24 hours and 48 hours respectively. Blood was analysed for neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and tumor necrosis factor alpha (TNFα). Just before 48 hours of reperfusion, urine was collected from each kidney separately for 60 minutes and sampled for analysis of NGAL. Urinary biomarker analysis of uNGAL and uKIM-1 was conducted between three groups: sham right kidney, IR left kidney, and IPoC left kidney.

Results: Having only IR or IPoC significantly decreased corrected creatinine clearance (corCrCl) compared to not receiving an intervention ($p<0.001$). The IPoC group (n=4) had a higher corCrCl after 48 hours of reperfusion compared to the IR only group (n=3) ($p= 0.04$). There was a significant increase in uNGAL in IR group from PreOp to POD 2 ($p=0.02$). There was a significant effect of IR and IPoC on levels of uNGAL compared to sham right kidney ($p=0.001$). Having only IR or IPoC significantly increased uNGAL compared to receiving no IR ($p=0.003$).

Conclusion: Preliminary results suggest that ischemic postconditioning attenuates warm ischemia/ reperfusion injury in a laparoscopic double kidney porcine model of warm ischemia.

Introduction

Because the surgeon wishes to work in a bloodless field, it is a common surgical practice to occlude the renal arteries and/or veins preceding interventions that involve the kidneys or aorta. Common surgical procedures that involve occlusion of the renal hilum include a partial nephrectomy in renal cancer or the aorta in the proximity of the kidney hilum, known as a thoracoabdominal aorta aneurysm. Another type of surgical intervention that involves restricting the blood supply to the kidneys is during kidney transplantation. In these interventions, the kidney cells are deprived of the oxygen rich blood flow as well as the venous exchange of carbon dioxide and metabolic molecules until the oxygenated blood supply and venous return are resumed. Unfortunately this sequence of restricting and resuming blood flow to tissue causes a two-pronged injury known as ischemia and reperfusion injury (IRI).

Although remarkably capable of adapting and responding to reduced blood flow, the kidney is very sensitive to ischemia and reperfusion (I/R). The pathogenesis of IRI to kidney cells is a complex cascade of events leading to injury and dysfunction to epithelial and endothelial cells all linked by a strong inflammatory and oxidative stress response (1, 2). During periods of ischemia, ATP depletion in the epithelial cells causes cell injury or death by necrosis or apoptosis, especially in the high energy demanding proximal tubular cells (1). These cells then slough off and accumulate in the lumen followed by backleak of filtrate. This ultimately leads to impaired glomerular filtration and a dramatic reduction in glomerular filtration rate (GFR). Endothelial cells of the kidney are important for vascular tone and permeability, blood flow regulation to tissue and modulation of coagulation and inflammation (1). Damage to these cells cause vasoconstriction and increased permeability and has profound effects on oxygen and metabolite delivery to tissue beds as inflammation (1) as well as release of cytokines, such as TNF-α and IL-6, and chemokines leading to activation of inflammatory processes and ROS causing oxidative stress and lipid peroxidation (2).

Inflammation and oxidative stress play a major role in all phases of IRI and is therefore an important target for therapeutic strategies. Proinflammatory cytokines such as TNFα, IL-6 and IL-1β (3) and chemokines produced by the injured epithelial and endothelial cells recruit leukocytes and macrophages to the site of ischemic injury. This aggravates injury, causes swelling of the endothelial cell, impedes blood flow and increases the inflammatory response

and subsequent injury (1, 4, 5). Reactive oxygen species (ROS) are also released by the damaged tissue which causes oxidative stress characterized by impairment of mitochondrial oxidative phosphorylation, ATP depletion, increased intracellular calcium and activation of membrane phospholipid proteases (2). When blood flow is returned to the ischemic tissue, this rush of oxygen rich blood and metabolites to previously deprived tissue produces oxygen free radicals that cause lipid peroxidation and oxidative damage of proteins and DNA that lead to further damage, apoptosis and/or necrosis (6).

Depending upon the extent and duration of ischemia, acute kidney injury (AKI) is a common consequence of many types of surgical interventions and disease states that cause hypo-perfusion of the kidneys. AKI due to IRI is common among sepsis patients, thoracoabdominal aortic surgical patients, after partial nephrectomy and cardiac surgical patients. It has been reported that AKI is reported in up to 7% of hospitalized patients (7) and 25% of ICU patients will develop AKI (3). Though renal replacement therapy is the gold standard in treating AKI, mortality among ICU patients who experience AKI is reported up to 70% (8). Therefore, AKI is associated with significantly increased mortality, length of stay and hospital costs (7) and much attention is being given to this increasingly common condition.

AKI has most recently been defined according to either the RIFLE (Risk, Injury and Failure; and Loss in End Stage Kidney Disease) or AKIN (Acute Kidney Injury Network) criteria. Though both criteria differ in several ways, both are defined by clinical outcomes such as sudden reduction in kidney function measured by an acute increase in serum creatinine (sCr) and blood urea nitrogen (BUN) and duration of events, either 48 hours afterward or 7 days respectively. In 2012, the Kidney Disease: Improving Global Outcomes (KDIGO) group of AKIN published the practical clinical guidelines and have further focused on urinary output (UO) along with sCr as key indicators of AKI (9) . Currently sCr, BUN and UO are the most commonly used biomarkers of AKI in the hospital, however; it is often argued that sCr is not a reliable indicator of AKI due to its variation from patient to patient in regards to muscle mass, gender, ethnicity, age and hydration status (10). On the other hand, UO has been reported recently to perhaps be a better and under- utilized predictor of AKI (8) .

Several more recent biomarkers of AKI have received a lot of attention and have showed promising results pre-clinically and clinically. Among these that have received the most attention are neutrophil gelatinase-asociated lipocalin (NGAL) and kidney injury molecule-1

(KIM-1) (3). Both small proteins are released from injured epithelial proximal tubular cells. Though sCr and BUN continue to be used primarily in the clinic, it has been shown clinically that using a combination of functional biomarkers such as uNGAL and uKIM-1 together rather than alone improve the predictive value of AKI (11).

The commonly accepted warm ischemia time during surgical interventions to the kidney is 25-30 minutes without risking irreversible kidney injury (12). Though this arbitrary time limit has been debated often in the literature, it remains a guideline for surgeons clamping the kidney hilum (13). During procedures such as partial nephrectomy and repair of thoracoabdominal aneurysms, this 30-minute limit may be challenging even to the experienced surgeon. There is a need to find strategies to reduce IRI to the kidney. One such strategy is called ischemic conditioning (IC). The idea is that short repeated periods of ischemia directly or distant (remote) to the organ either before (pre-), during (peri-) or after (post-) the main ischemic insult cause a protective response in the organ. This phenomenon was first described by Murry and colleagues in a landmark article in 1986 when he reported the protection of heart myocardium in dogs after 4 repeated 5-minute occlusions of the circumflex coronary artery before 40 minutes of occlusion (14) . This has subsequently become known as ischemic preconditioning (IPC) and it is used in the clinic to protect cardiac surgery patients. However, clinical use of IPC to protect the kidneys has been stunted by contradicting pre-clinical studies among other obstacles (15).

Ischemic Preconditioning and Postconditioning

The concept that IPC can also be applied to attenuate kidney injury has been explored by numerous experiments mostly using small animal models; however, only a handful of experiments have utilized the pig model. Preclinical results have mostly been disappointing (16-19) but have contributed to a greater knowledge about standard preconditioning algorithms and experimental models. Most pig experiments have used the single kidney model in either a laparoscopic or open surgical technique with sCr, and BUN as kidney functional outcomes and histology for morphological outcome. None of the porcine preclinical IPC trials have used a bilateral kidney model; however, a bilateral kidney model has been used in a few warm ischemia porcine experiments (20-23).

The proposed protective mechanism of IPC in the kidney is associated with the release of several autocoids (adenosine, opioids and bradykinin) that trigger a signalling cascade that ultimately protects the cells from mitochondrial dysfunction, oxidative stress, inflammation and apoptosis/necrosis (24). Of increasing interest is the role of renal innervation in the mechanism of ischemic conditioning (24, 25). Clinically translation from the lab to the operating room has been lacking while more standardized methods, models and promising results are being reported.

Whereas IPC is performed before the onset of the ischemic insult, application of a similar intermittent repeated sequence of clamping and releasing the blood supply to an organ after the ischemic insult is known as ischemic postconditioning (IPoC). This concept was first reported by Zhao and colleagues in 2003 (26) in a dog experimental model examining the protective effect of clamping and unclamping the left anterior descending coronary artery 3 x 30 seconds in the reperfusion phase during acute myocardial infarct. IPoC has shown promising experimental results in attenuating IRI in a variety of animal organs either directly or remotely (27). A meta-analysis of IPoC by Jonker *et al* published in 2016 (28) reported 39 publications whereas only one was a dog and none were pigs. Jiang et al reported attenuation of renal IRI by IPoC with an postconditioning algorithm of 6 x 15 seconds of on/off clamping of the kidney hilum in a single kidney dog model (29). Most recently, Hunter and colleagues (2015) reported reduction of renal IRI by IPoC and a 6 x 15 second postconditioning algorithm similar to Jiang et al in the first porcine experiment (30). Both experiments utilized a single kidney model with an open surgical procedure. In addition to reporting kidney functional and histological outcomes as other IPC experiments, they reported inflammation and oxidative stress biomarkers that are focused on the proposed protective molecular mechanisms of IPoC such as TNFα, malondialdehyde (MDA) and superoxide dismutase (SOD).

The reported protective molecular mechanisms of IPoC are centered on oxidative stress, apoptosis and inflammation (24, 31) . Upon reperfusion, the sudden rush of oxygenated blood to ischemic tissues creates reactive oxygen species (ROS) such as O_2-, H_2O_2 and hydroxyl radicals that damage the epithelial and endothelial cells. Biomarkers of oxidative stress in plasma include superoxide dismutase (SOD) that scavenges ROS, antioxidant GSH and malondialdehyde (MDA) which is a by-product of lipid peroxidation. It is proposed that IPoC is a mediator in protecting the cells against oxidative stress by supporting anti-oxidant activity and maintaining mitochondria membrane integrity as to avoid release of cytochrome c to the

cytosol thus activating apoptotic pathways (31-34). It has also been demonstrated that IPoC protects against IRI induced inflammation by downregulation of COX-2 and thereby stimulating pro-inflammatory and oxidative stress mediators (33). Biomarkers of inflammation that has been shown to be reduced in IPoC are TNFα, IL-6 and lower levels of myeloperoxidase (MPO) which is a biomarker for neutrophil infiltration (24, 31, 35, 36). An in-depth review of molecular mechanisms is beyond the scope of this report and is reviewed by Kierulf-Lassen (24).

Surgical technique and experimental model

The practice of minimally invasive surgery such as laparoscopic and robot assisted surgery has become an acceptable and widely used method for surgical procedures such as partial nephrectomy in cancer patients and kidney transplantation. The ideology is such that less invasive procedures which involve only a few small incisions limit trauma, inflammation, infection and recovery time. Laparoscopy has been shown to decrease blood loss, decrease need for analgesics and limit hospital stay and speed-up recovery (37). This experiment utilized laparoscopic surgical techniques to approximate the increasingly common clinical scenario. It is the only laparoscopic porcine IPoC experiment to the best of the author's knowledge.

The pig as a research animal has become more commonly used in laparoscopic and open surgical models in many specialties of surgical research. In contrast to small animals, the pig has a multilobular and multipapillary urinary system and renal physiology that more closely resembles that of humans (38). Most laparoscopic experiments have developed a single kidney model to explore IRI and ischemic conditioning, and only a few have utilized a double kidney model. A double kidney model was used in this experiment to emulate the clinical scenario where most often the surgical procedure is being performed on a patient with two intact kidneys.

The aim of this experiment was to evaluate an IPoC protocol in an *in vivo* laparoscopic porcine model of renal ischemic/reperfusion injury.

Materials and methods

The experimental protocol was approved by the local steering committee of the Norwegian Animal Experiments Authority. Animal care was done in accordance with the

guidelines for the care and use of laboratory animals published by the U.S National institutes of Health (NIH Publication No. 85-23, revised 1996).

16 domestic hybrid pigs (Noroc: Norwegian landrace and Yorkshire hybrids) from a local supplier were acclimated in the animal research facility 10-14 days before the experiment. The pigs were a mix of castrated males and females with weight 37-50 kg, average 43 kg. They were fasted overnight with free access to water and block randomized into two different groups, ischemia and reperfusion (IR control) or ischemia/reperfusion and postconditioning (IPoC). Since this is a double kidney model, the right kidney to each animal that did not receive any intervention is used as a negative control (sham) in comparison of urinary results. The author was the primary surgeon for all portions of the experiments. An overview of the experimental design is depicted in Figure 1.

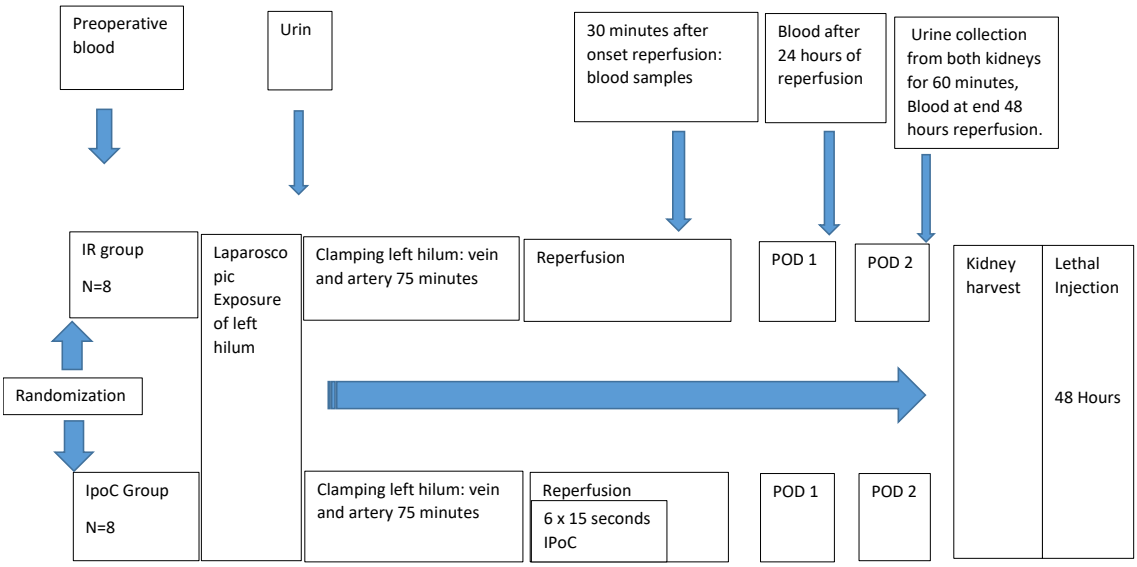
The animals were premedicated with 15 mg/kg ketamine, 2 mg/kg atropine and 1 mg/kg midazolam. General anesthesia was induced with fentanyl in an ear vein in addition to 4% isoflurane on a ventilation mask. The induction was followed by continuous isoflurane 2 %, fentanyl 0,02 mg/kg/h and midazolam 0,3 mg/kg/h. General anesthesia was maintained with 2 % isoflurane on a ventilation mask. The animals were intubated and mechanically ventilated using a volume controlled ventilator adjusted according to capnography measurements and arterial blood gases. The circulating volume was maintained by 10 mg/kg/h saline infusion. All animals received Penovet ® 1.5 ml/10 kg i.m. (Boehringer Ingelheim, Denmark) before the onset of surgery. When the animals were fully sedated and intubated, a central venous catheter was introduced into the jugular vein and an arterial cannula was inserted in the superficial femoral artery for hemodynamic measurements. The core temperature was maintained between 37-39 ^{o}C using a thermal mattress.

In full anesthesia, access to the abdominal cavity was gained through open placement of laparoscopic trocars in the midline. After verifying that the first port was inside the abdominal cavity, pneumoperitoneum was accomplished with a pressure of 12 mmHg. One 12 mm trocar and two 5 mm trocars were inserted under visual guidance. The left kidney was identified and the hilum was mobilised and cleared. 2500 IU heparin was administered intravenously 3 min before clamping. A Satinzky clamp was then placed over the hilum, artery and vein, and then clamped for 75 minutes. In the control group, after 75 minutes, the Satinzky clamp was opened and reperfusion of the kidney was observed by visualizing the change in color. In the postconditioning group, after 75 minutes, the Satinzky clamp was removed for 15

seconds and then clamped again for 15 seconds a total of six times (6 x 15 seconds off and 15 seconds on). Buprenorfine (Temgesic) 30 µg/kg i.m. and a fentanyl patch 50 µg/hour were administered for postoperative analgesia. All the instruments were then removed and the abdomen was closed. The pigs were then extubated and kept alive for 48 hours with free access to food and water. Blood was sampled before surgery, after 30 minutes of reperfusion, and then after 24 and 48 hours of reperfusion directly from the central venous catheter. Blood and urine was then centrifuged for 15 minutes and analysed immediately and samples stored at -80 ° C.

On postoperative day 2 (POD 2), the pigs were premedicated and intubated as previously described. If mean arterial pressure was under 45 mmHg such that there was no urinary output, pentobarbital and corresponding concentrations were substituted for isoflurane. In those cases, induction with fentanyl 0.2 x (kg) and pentobarbital 0.1 x (kg) was given with continuous infusion afterward of fentanyl plus midazolam 0.69 x (kg) and pentobarbital 0.06 x (kg). The pig was then placed in a supine position and incised with a midline laparotomy. Both ureters were exposed and urine was collected separately from the left and right kidney for 60 minutes via a ureterostomy using a 12 ch Foley catheter. At 48 hours of reperfusion the left and right kidneys were harvested and placed in formalin 10% for histologic examination. The pigs were then immediately euthanized with intravenous injection of 20 mg/kg sodium pentobarbital and potassium chloride.

Figure 1. Experimental Design Flow Chart



Urine and Blood Sample Measures

Neutrophil gelatinase-associated lipocalin (NGAL) was analysed in urine and blood samples. The urine and blood samples were stored at -80°C. Concentration levels of NGAL were obtained using a pig specific NGAL ELISA kit (BioPorto Diagnostics, Denmark). Intra-assay CV% was < 12%.

Kidney injury molecule-1 (KIM-1) was analysed in urine stored at -80°C. Concentration levels of uKIM-1 were obtained using a pig specific KIM-1 ELISA kit (Abcam). Intra-assay CV% was 12.7%.

Tumor necrosis factor α (TNFα) was analysed in blood samples stored at -80°C. Concentration levels were obtained using a pig specific TNFα ELISA kit (AbCam). Intra-assay CV% was <12%.

All ELISA had duplicate samples that were added to pre-coated wells and performed following manufacturer`s instructions. Analysis and calibration of all ELISA concentrations were performed using Perkin Elmer Wallac 1420 Multilabel Counter.

Statistics and Graphing

All statistical analysis was performed with computerized software (IBM SPSS statistics 24, Chicago, IL). The Kolmogorov-Smirnov test was applied to test for a normal distribution. P-values for blood serum measurements between two groups were calculated by either the two-way t-test for independent samples for normal distributed data or Mann-Whitney U test for non-normal distributed data where noted. Urinary measurement p-values between three groups were calculated by one-way ANOVA. Data are presented as mean ± standard deviation. Values of $p < 0.05$ were considered statistically significant. All graphs were produced with Prism ® 7 software (GraphPad Software, San Diego, California, USA).

Results

Two animals, one from each group, did not survive to POD 2. Both died due to respiratory complications during anesthesia either shortly after or during the first operation. All other animals survived to POD 2 without complications. One animal has been excluded from the study due to failure of urine collection from the right kidney (sham) during POD 2. In addition, that animal was the only one with a larger right kidney compared to left kidney by

mass (right 113 grams, left 96.5 grams). The animal was therefore excluded from the study due to a right pelvi-ureteric junction stenosis. For blood analysis the final results are therefore presented as IR group (n=6) and IPoC (n=7). Six animals failed to produce urine from the left ischemic kidney during POD 2. Histologic analysis is forthcoming; however, preliminary urinary analysis results are therefore presented only for those animals that produced urine from both the left and right kidney during POD 2. For urinary analysis the final results are presented as IR group (n=3), IPoC (n=4) and right kidney negative (sham) control (n=7).

Baseline Characteristics

Baseline characteristics are summarized in table 1 and statistical results were obtained with independent t-test. There was no significant difference between weight ($p= 0.5$), mean arterial pressure (MAP) during PreOp and POD 2 operations ($p= 0.2$ and $p= 0.6$ respectively), Hb on PreOp and POD 2 operations ($p=0.7$ and $p=0.2$), BUN PreOp ($p=0.7$) and sCr PreOp ($p= 0.5$) between the IR or IPoC groups. Weights between left and right kidneys at harvest on POD 2 had no significant difference (left $p=0.6$ and right $p=0.6$).

Traditional renal function biomarkers

Serum Creatinine (sCr)

There was no significant difference in serum creatinine between the groups at either sampling period ($p \geq 0.4$) (Fig. 2).

Blood urea nitrogen (BUN)

There was no significant difference in blood urea nitrogen levels between the groups at either sampling period ($p\geq 0.5$).

Corrected Creatinine Clearance (corCrCl)

CorCrCl was calculated by utilizing the following formula for finding body surface area (BSA) of laboratory miniature pigs proposed by Itoh *et al.* (39):

$$100 \times BSA(m^2) = 7.98 \times \text{body weight } (kg)^{2/3}$$

There was a significant effect of either IR or IPoC on levels of corCrCl ($p<0.001$). Planned contrasts revealed that having only IR or IPoC significantly decreased corCrCl compared to not receiving any treatment ($p<0.001$). Further, receiving IPoC significantly increased corCrCl compared to receiving only IR without IPoC ($p=0.04$) (Fig. 3).

Urinary Output (UO)

Though numerically lower, there was no significant effect of either IR or IPoC on UO between the groups ($F(2,11)=1.94$, $p=0.190$, $\omega=0.345$). Planned contrasts revealed that having any treatment of only IR or IPoC did not significantly reduce UO compared to right kidney sham group ($t(11)=-1.48$, $p=0.166$, $r=0.41$). Further, IPoC did not significantly increase UO compared to IR group ($t(11)=-1.44$, $p=0.177$, $r=0.40$) (Fig. 4).

Tubular injury biomarkers

Serum neutrophil gelatinase-lipocalin (sNGAL)

PreOp sNGAL levels deviated significantly from normal distribution ($D(13)=0.219$, $p=0.028$). However, POD 2 sNGAL levels did not deviate significantly from normal distribution ($D(13)=0.135$, $p=0.298$).

There was a significant increase in sNGAL in the IR group from PreOp to POD2 ($p=0.023$). There was not a significant increase in sNGAL in the IPoC group from PreOp to POD 2 ($p=0.209$). There was no significant difference between PreOp sNGAL levels between the IR and IPoC groups ($p=0.775$, Mann-Whitney U test). There was no significant difference between POD 2 sNGAL levels between IR and IPoC groups ($p=0.238$, unpaired independent t-test) (Fig. 5).

Urinary neutrophil gelatinase-lipocalin (uNGAL)

There was not a significant difference in right kidney uNGAL levels between IR and IPoC groups ($p=0.114$, unpaired ind. t-test). There was a significant effect of IR and IPoC treatment on levels of uNGAL ($F(2,11)=14.17$, $p=0.001$, $\omega=0.81$). Planned contrast revealed that having IR or IPoC significantly increased uNGAL compared to receiving no IR ($t(5)=5.36$, $p=0.003$, $r=0.85$). However, receiving IPoC after IR did not significantly decrease uNGAL in the left kidney compared to IR only ($t(5)=-0.559$, $p=0.600$, $r=0.24$) (Fig. 6).

Urinary kidney injury molecule-1 (KIM-1)

There was not a significant effect of IPoC treatment compared to IR group and right kidney control; however, a large effect score was determined ($F(2,11)=2.715$, $p=0.189$, $\omega=0.64$). Planned contrast revealed that having IPoC treatment compared to IR only did not reduce uKIM-1 levels although a large effect score was determined ($t(2.3)=1.40$, $p=0.289$, $r=0.67$) (Fig. 7).

Inflammation Biomarker

Tumor necrosis factor alpha (TNFα)

There was no significant difference in plasma levels of TNFα between IR and IPoC groups at any sampling period (Fig. 6). Numerically, mean TNFα levels 30 minutes after reperfusion in IPoC kidneys decreased from PreOp levels compared to IR kidneys whose mean levels increased slightly (IR= 1689.6 ± 774 pg/ml and IPoC=1065.9 ±563 pg/ml) (Fig. 8).

Discussion

This is perhaps the first study to investigate ischemic postconditioning in a laparoscopic double kidney porcine model in warm ischemia. The preliminary results from these experiments suggests that postconditioning preserves and may protect renal function during warm ischemia and reperfusion.

Postconditioning was first described by Zhao *et al* in an *in vivo* dog model of cardiac protection (26). Since, there has been few experiments investigating renal postconditioning in large-animals. It is a relatively simple procedure that can possibly preserve and protect the kidney from post-ischemic injury. The mechanism of injury during reperfusion and the molecular mechanisms of IPoC protection has been the subject of several investigations and reviews. Reperfusion leads to oxygen free radical injury, oxidative stress and lipid peroxidation that promotes inflammation in the kidney (2). IPoC studies in rats have been reported to support anti-oxidant activity by showing lower levels of H_2O_2, increased levels of anti-oxidant GSH, increased SOD and lower levels of the lipid peroxidation biomarker malondialdehyde (MDA) (24, 33, 40). Jiang and colleagues' dog model reported significant lower levels of MDA compared to control group as well as higher SOD meaning that less SOD is consumed due to

higher levels of ROS presumably formed during the IRI (31, 33, 34). Hunter et al reported a significant increase in TNFα in control animals during POD 1 but not in IPoC animals as well as lower uNGAL concentrations (30).

It is commonly known that when one kidney is injured in the presence of a functional second kidney, the uninjured kidney will hypertrophy and undergo compensatory changes to maintain renal plasma flow and GFR (41-43). The most common large-animal experimental model of renal warm ischemic conditioning is a surgically created single kidney model (17-19, 30, 44-48). It has been previously proposed and shown that a single kidney large-animal model is more tolerant to injury from IR than a two-kidney model (10, 49). Therefore, many of the results from solitary kidney models may be underestimating the damage incurred by the ischemic kidney in the presence of a normal functioning second kidney. In addition, many of the published large-animal studies use an open surgical approach whereas it is now more increasingly common in the surgical theatre with laparoscopic or robot-assisted laparoscopic surgery when conducting nephron sparing surgery (23). Therefore, this study wished to investigate the most common clinical scenario of two kidneys during laparoscopic surgery.

When evaluating ischemic kidney function in the paired kidney model, serum functional biomarkers such as Cr and BUN are not reliable. The second normal functioning kidney will compensate for the injured ischemic kidney. Therefore, traditional renal functional measures that define AKI are not very useful to gauge the function of the injured kidney. To overcome this limitation and to lateralize functional data to a specific kidney, this model utilized a ureterostomy on POD 2 to collect urine directly from each kidney for 60 minutes immediately preceding 48 hours of reperfusion. In this way, the second non-ischemic kidney in each animal was used as a negative control (sham). A similar model has been used before to investigate kidney function during renal ischemia in a double kidney laparoscopic porcine model (23).

Unfortunately, urine was not collected from 6 of the left kidneys and thus reduced the number of sampling groups in urinary analysis (n=3,4 and 7). At the end of 60 minutes of urine collection, failure to aspirate any urine from the kidney pelvis or catheter end prior to removal of the catheter from the ureter verified that no urine was produced. Therefore, those animals were excluded from this urinary results analysis. However, as this is a preliminary report, histological analysis is forthcoming and, though speculative, may correlate level of kidney morphological injury to those kidneys that failed to produce urine.

Two previous studies of postconditioning in single kidney large-animal studies have utilized 60 minutes of ischemia (29, 30). It has been shown that ischemia times greater than 75 minutes in a double kidney laparoscopic porcine model produced deleterious effects on kidney function (20). 75 minutes of warm ischemia was used to produce measurable outcomes biochemically and histologically as previous studies have shown limited injury from ischemia times less than 75 minutes (22, 30, 44). Though some may argue that 75 minutes of warm ischemia is not clinically relevant, it is necessary in research to produce measurable results. Further, as there is not yet definitive evidence to prove otherwise, the commonly accepted maximum allowable warm ischemic time interval of 25-30 minutes is used as a *de facto* "rule of thumb" measurement. In fact, large-animal porcine studies have suggested that the solitary kidney can withstand and recover from warm ischemic times of up to 90-minutes (44, 46).

The reason why 48 hours of reperfusion was chosen is based on previous work of Jablonski et al (50) who proposed that at 48 hours one finds the most extensive morphological changes on histological examination. Beyond 48 hours, the kidney begins to repair and regenerate. Moreover, other studies have shown that peak sCr and uNGAL levels peak before or near POD 2 (23, 30, 44). As it would be desirable to continue measuring the animals to full-recovery, it is resource and time consuming.

It is commonly argued that sCr is not a reliable biomarker of kidney function because its release varies with age, sex, diet, muscle mass, drugs, activity level and hydration status (51, 52). The presence of sCr in serum is used along with correction factors (Cockcroft-Gault Equation) to estimate GFR; however, it is not reliable to use measurements based on sCr to detect acute deterioration of kidney function. The reserve capacity of healthy nephrons compensate for GFR in the presence of injury thus preventing a significant increase in sCr until 50% of nephrons are lost (51). An increase in sCr-based GFR measurements will occur only after there is considerable loss of nephrons, thus underestimating the initial, acute injury. Therefore, other more reliable functional biomarkers have been studied recently to detect acute functional decline such as uNGAL and uKIM-1, perhaps the two most promising especially when used in combination (53).

This study showed that corCrCl in the IPoC kidney group was significantly higher in POD 2 after 75 minutes of warm ischemia followed by 6 x 15 seconds of postconditioning compared to IR only left kidneys and control right kidneys. CorCrCl is used as a urinary functional measurement for GFR when sCr cannot be used. With this bilateral kidney model, corCrCl is

a functional measurement of glomerular function of each kidney. Further, though not significant, there were numerically lower uNGAL, uKIM-1 and UO levels in IPoC animals on POD 2 compared to IR animals. In addition, though not statistically significant, large effect size scores were determined for the effect of IPoC groups compared to IR and right kidney groups in regard to lower levels of uKIM-1 and UO. It was observed that mean TNFα levels in IPoC animals decreased after postconditioning treatment 30 minutes after reperfusion compared to IR animals whose mean levels increased. Though speculative due to absence of significance and low study numbers, the trend is clear and these results suggest protection from IRI by IPoC and the need for further studies.

The limitations of this study include the absence of comorbidities and low sample numbers. Using young animals that come from the same genetic stock produces a homogenous study group that lacks the human clinical scenarios of advanced age, smokers, existing comorbidities and medication use. This has been a defining argument for why clinical translation of ischemic conditioning strategies are still not routine clinical practice (54). Moreover, porcine studies are known to be underpowered and have low sample numbers mainly due to the high cost and resource demanding nature of the studies. Jonker *et al* suggest that future studies should prioritize using both sexes, large animals with relevant comorbidities, more transplant models and better reporting through use of ARRIVE guidelines (28). This study used both sexes, large animals and procedures that have been used with reported success in two earlier large-animal IPoC studies (29, 30). Further, this study has attempted to expand the knowledge of IPoC with regards to investigating a clinically relevant scenario of two kidneys during laparoscopic operation. More research into this protective mechanism of IPoC is warranted.

Conclusion

Despite low sample numbers, these preliminary results suggest that a IPoC treatment preserves kidney function during warm ischemia and reperfusion. Coupled with evidence gained from two previous large-animal IPoC studies (29, 30) that find similar protective results, it can be argued that IPoC has the potential for limiting injury from renal ischemic/reperfusion in the clinical setting.

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Tables and Figures

Table 1: Baseline Characteristics (Mean ± S.D.)

Baseline characteristics	IR Group PreOp	IPoC PreOp	P-Value	IR Group POD 2	IPoC POD 2	P-value
Weight kg	42.5 ± 3.8	44.0 ± 3.6	p=0.5	-	-	-
Sex (M/F)	5/3	4/4	-	-	-	-
MAP mmHg	78.5 ± 16.3	69.0 ± 8.8	p=0.2	67.4 ± 17.0	72.5 ± 14.7	p=0.6
Hb mg/dl	9.9 ± 0.8	10.1 ± 1.5	p=0.7	9.8 ± 0.5	9.4 ± 0.6	p=0.2
sCr mg/dl	0.95 ± 0.1	0.99 ± 0.1	p=0.5	1.45 ± 0.3	1.33 ± 0.23	p=0.5
BUN mmol/L	1.0 ± 0.2	1.1 ± 0.4	p=0.7	2.1 ± 0.7	1.7 ± 0.5	p=0.7

Figure 2: Serum Creatinine

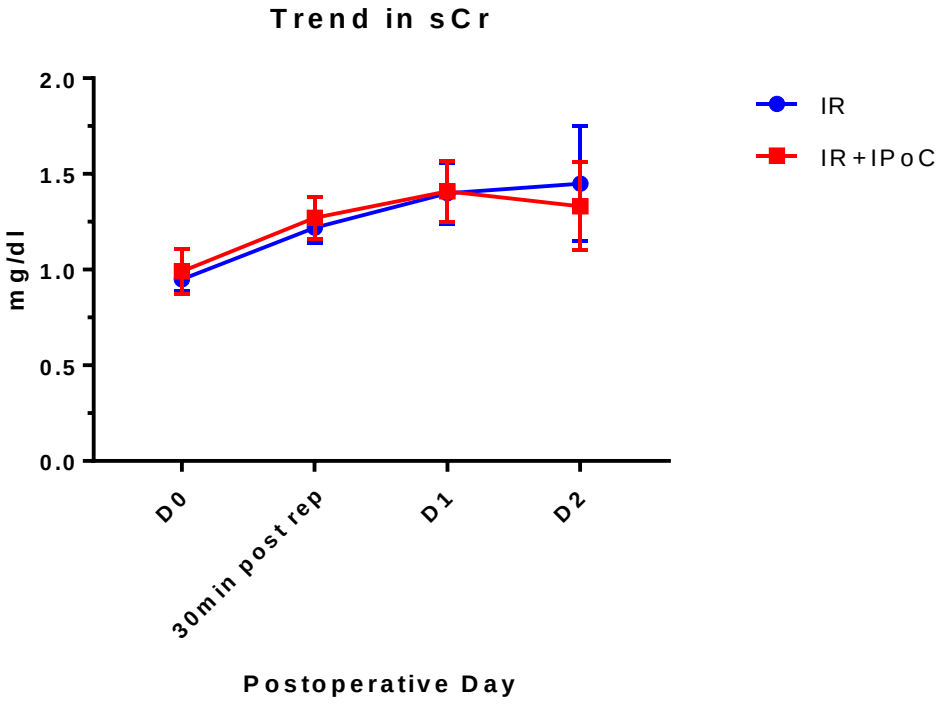


Figure 3: Corrected Creatinine Clearance

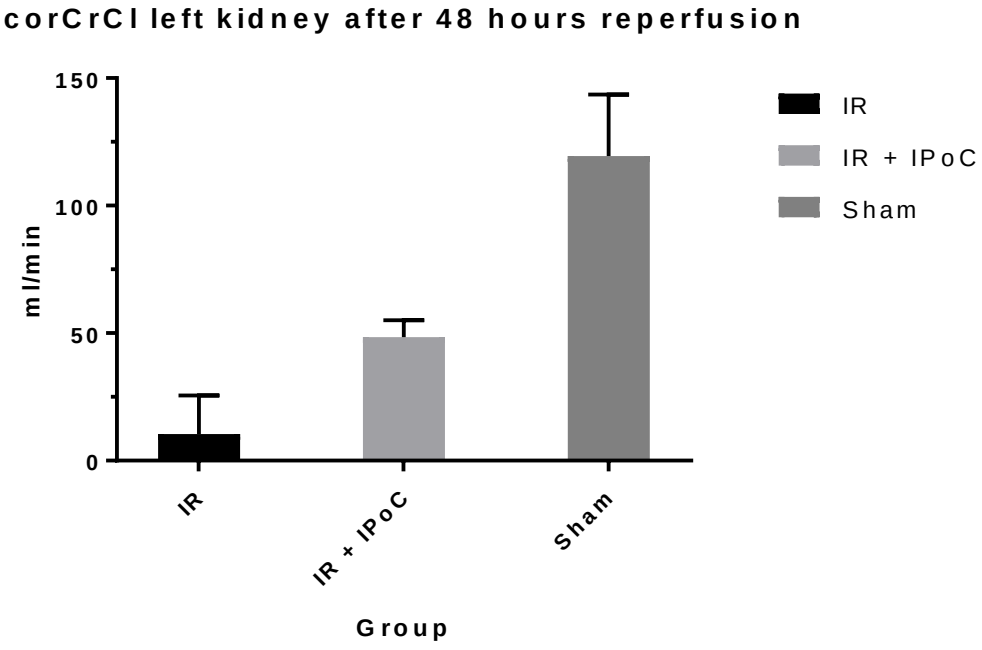


Figure 4: Urinary Output

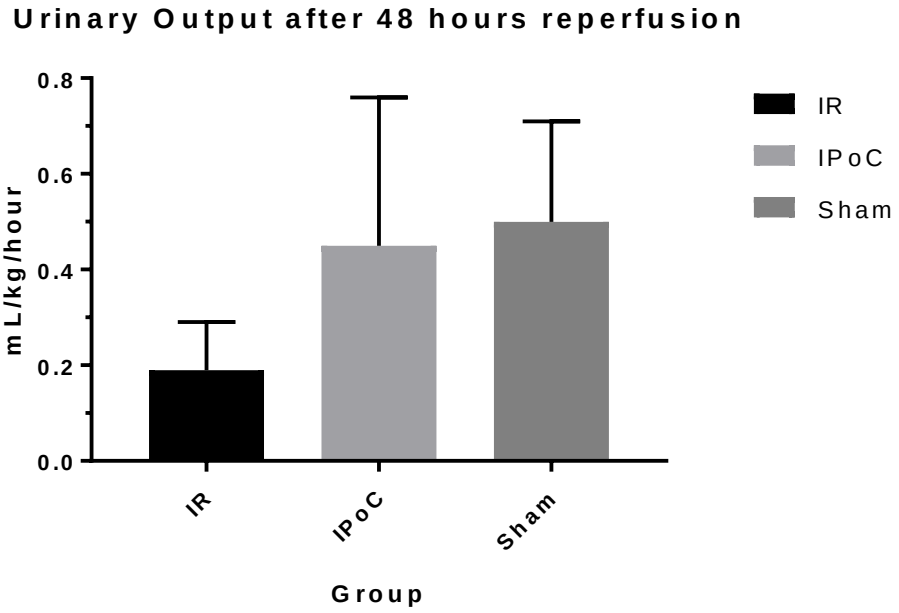


Figure 5: Serum NGAL

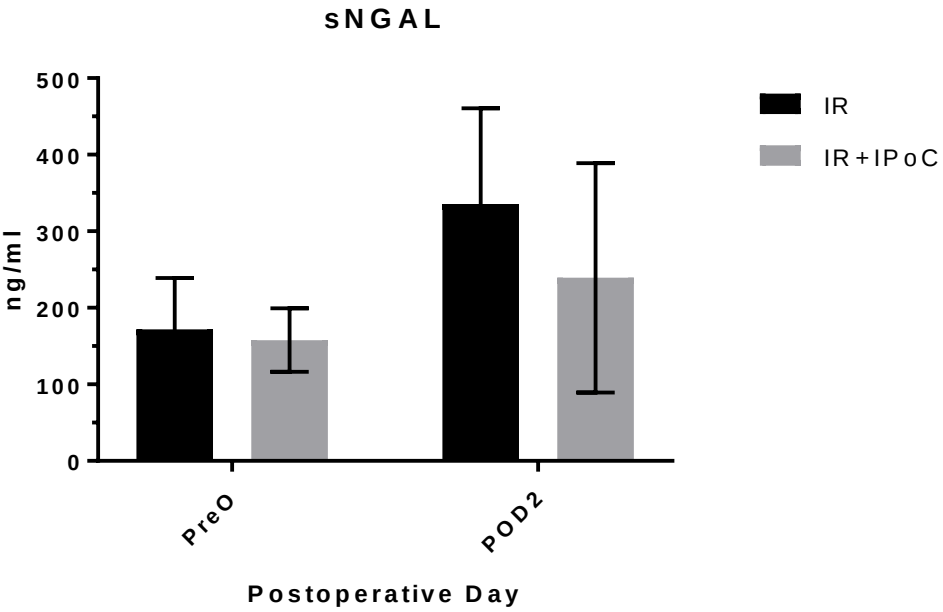


Figure 6: Urinary NGAL

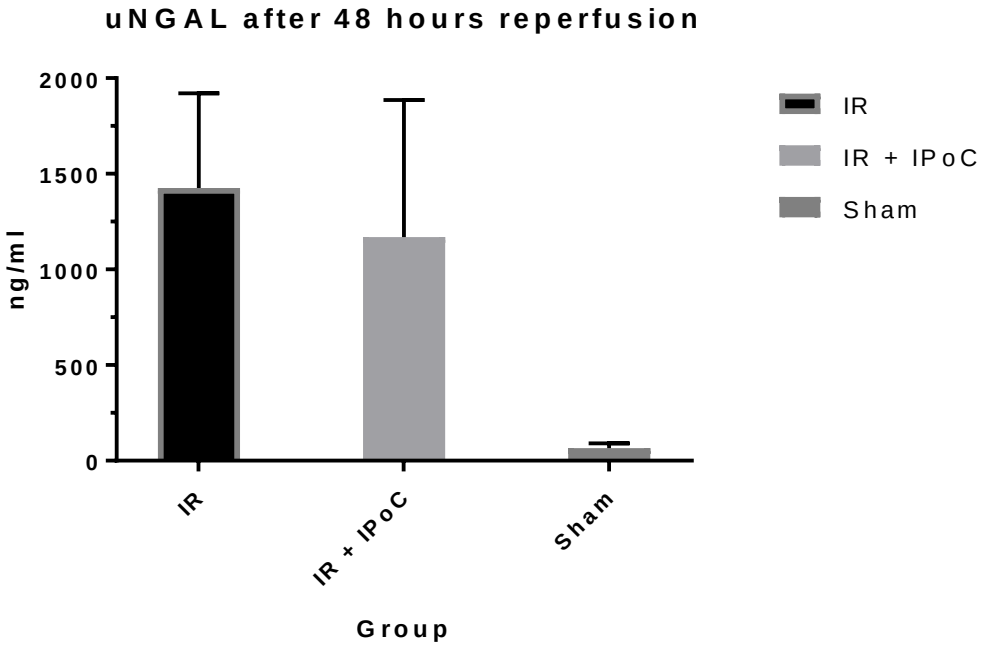


Figure 7: Urinary KIM-1

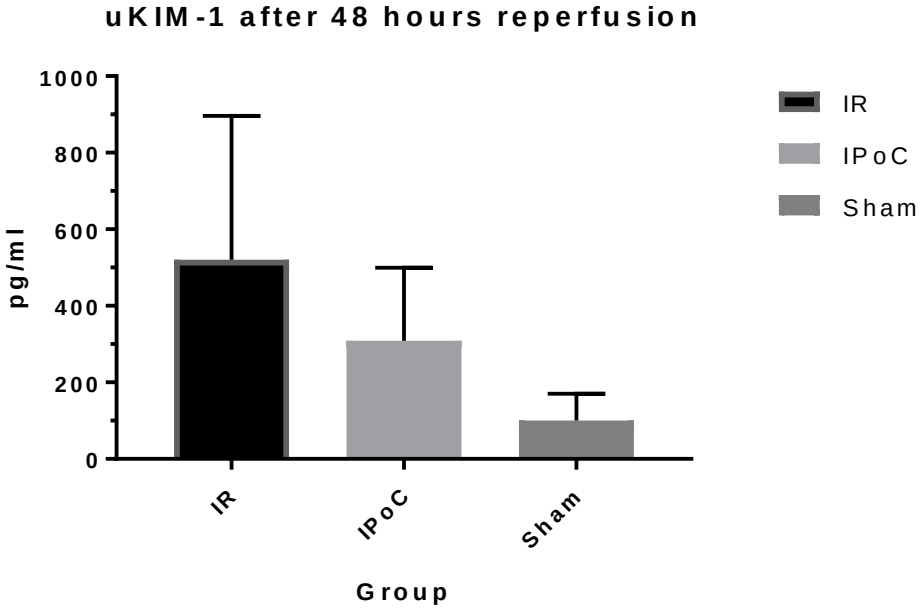
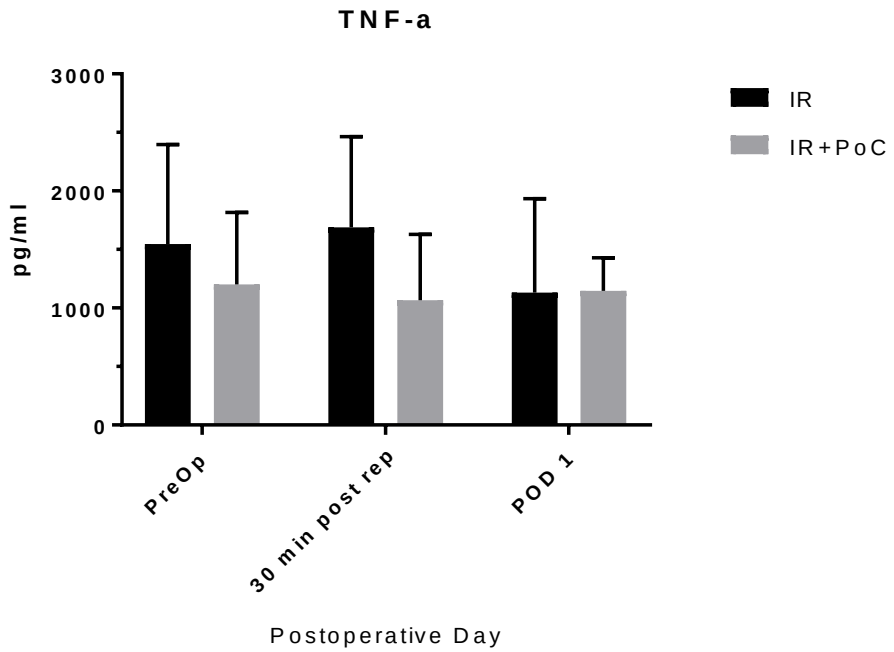


Figure 8: Serum TNF-α



Reference: Jonker SJ, Menting TP, Warle MC, Ritskes-Hoitinga M, Wever KE. Preclinical Evidence for the Efficacy of Ischemic Postconditioning against Renal Ischemia-Reperfusion Injury, a Systematic Review and Meta-Analysis. PloS one. 2016;11(3):e0150863.	**GRADE High**	
	Dokumentation level	Ib
	Recommendation	Stron for

Purpose	**Material and method**	**Results**	**Discussion/Comments:**
Ischemic postconditioning (IPoC) is a promising treatment strategy for renal IRI, but early clinical trials have not yet replicated the promising results found in animal studies.	Present a systematic review, quality assessment and meta-analysis of the preclinical evidence for renal IPoC, and identify factors which modify its efficacy. Statistics: MD or SMD used tin meta-analysis. I^2 and R^2 were used to assess heterogeneity. Publication bias assessed with funnel plots, Duval and Tweedies trim and fill analysis and by performing Eggers test for small study effects. Histology using Jablonski scores. .	We identified 39 publications studying >250 control animals undergoing renal IRI only and >290 animals undergoing renal IRI and IPoC. Healthy, male rats undergoing warm ischemia were used in the vast majority of studies. Four studies applied remote IPoC, all others used local IPoC. **Meta-analysis showed that both local and remote IPoC ameliorated renal damage after IRI for the outcome measures serum creatinine, blood urea nitrogen and renal histology.** Subgroup analysis indicated that IPoC efficacy increased with the duration of index ischemia. Measures to reduce bias were insufficiently reported	1. Risk of Bias: Low. 2. Indirectness: High. All lesser animals. No primates. 3. Inconsistency: Low. 4. Imprecision: Low. 5. Publication bias: Low. **Strengths:** Meta-analysis. **Weaknesses:** Data obtained in a period just before Hunter et al published. No pig studies included. Only one dog, all the rest are mice or rats.
Conclusion			
High efficacy of IPoC is observed in animal models, but factors pertaining to the internal and external validity of these studies may hamper the translation of IPoC to the clinical setting. The external validity of future animal studies should be increased by including females, comorbid animals, and transplantation models, in order to better inform clinical trial design. The severity of renal damage should be taken into account in the design and analysis of future clinical trials.			
Country			
Netherlands			
Year data collection			
2007-2015			

Reference: Hunter JP, Hosgood SA, Barlow AD, Nicholson ML. Ischaemic conditioning reduces kidney injury in an experimental large-animal model of warm renal ischaemia. The British journal of surgery. 2015;102(12):1517-25.	**GRADE High**	
	Dokumentation level	Ib
	Recommendation	Strong for

Purpose	**Material and method**	**Results**	**Discussion/Comments:**
The aim of the study was to assess the effects of direct and remote ischaemic conditioning in a porcine model of renal warm ischaemia-reperfusion injury.	Pigs (50 kg) underwent laparotomy and 60-min occlusion of the left renal pedicle followed by right nephrectomy. Animals were divided into three groups: untreated controls (n = 8); direct postconditioning involving six 15-s cycles of clamping then releasing of the left renal artery (n = 7); or remote periconditioning involving four 5-min cycles of clamping then releasing of the left common iliac artery (n = 8). After 7 days kidney tissue was harvested, and blood and urine samples were collected on postoperative days 1, 3 and 7. Statistical analysis: Mean (s.e.m.) or mean (s.d.). ANOVA. Unpaired t-test with Bonferroni correction. $P < 0.025$ significant. Mann-Whitney U also used for not normally distributed. Categorical data $\chi 2$.	The direct postconditioning group had a lower area under the serum creatinine curve (mean(s.d.) 1378(157) versus 2001(1022) μmol/l · day respectively; P = 0.036) and peak creatinine level (316(46) versus 501(253) μmol/l respectively; P = 0.033) compared with values in control animals. There was a significant increase in serum levels of tumor necrosis factor α on day 1 in control animals but not in the conditioning groups (P = 0.013). Urinary levels of neutrophil gelatinase-associated lipocalin increased over the study period in both the control and remote groups (P = 0.001 for both), but not in the direct group (P = 0.176)	1. Risk of Bias: Low. Randomization done before penning of animals and each day of surgery. 2. Indirectness: High. Pig used instead of nonhuman primate 3. Inconsistency: Low. No presumed heterogeneity in results. 4. Imprecision: Low. OIS and ARRIVE stated, however: no calculation for stat. power. 5. Publication bias: Low. There were not many significant finds. **Strengths:** OIS (though no calculation) and ARRIVE**.** Second time that 60 minutes of WI and 6 x 15 seconds IPoC used in a large-animal study that showed effectiveness (See Jiang et al.). Thorough statistical analysis. **Limitations:** Few significant finds. Pig study. Not applicable to transplant research because of confounding factors of immunosuppression and cold ischemia. Intra-group comparisons of uNGAL absent. No mention of ELISA CV%. No CI reported.
Conclusion Postconditioning applied directly to the renal artery was shown to reduce renal injury. Furthermore, new evidence is provided that shorter cycles of ischaemic postconditioning than previously described can protect against renal injury			
Country **UK**			
Year data collection 2015			

Reference: Jiang B, Liu X, Chen H, Liu D, Kuang Y, Xing B, et al. Ischemic postconditioning attenuates renal ischemic/reperfusion injury in mongrel dogs. Urology. 2010;76(6):1519.e1-7.	**GRADE Moderate (high)**	
	Dokumentation level	Ib
	Recommendation	Strong for

Purpose	**Material and method**	**Results**	**Discussion/Comments:**
To investigate the effect of Postcond on renal ischemia-reperfusion (I/R) injury in a canine model.	40 adult male mongrel dogs were randomly divided into five groups of **eight** dogs each. Animals underwent **60 minutes** of renal pedicle occlusion followed by reperfusion for 72 hours. Postcond was performed by 15-second, 30-second, or 1-minute I/R for six or three cycles. Blood and urine were collected at different reperfusion time points (24, 48, and 72 hours), and blood urea nitrogen (BUN), creatinine (Cr) levels, urine N-acetyl-β-D-glucosaminidase (NAG), and Cr levels were assayed. Kidney samples were harvested after I/R, and renal superoxide dismutase (SOD), malondialdehyde (MDA), and myeloperoxidase (MPO) concentrations were measured, respectively. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay in the tissue samples. Statistical analysis: All data expressed as mean +/- s.d. Kolmogorov-Smirnov test applied. ANOVA student's-Newman-Kuels test. Intragroup differences analyzed with a repeated measures of variance.	Compared with the sham group, I/R resulted in renal dysfunction, decreased SOD levels, increased MDA and MPO levels, and increased apoptosis indexes. However, Postcond attenuated the aforementioned effects, the protection of which in the Postcond of 15-second reperfusion/ischemia for six cycles was the most notable.	1. Risk of Bias: High. No mention of randomization and blinding. 2. Indirectness: Low-Moderate. Pig model. Histology and biochemical outcomes reported. 3. Inconsistency: Low heterogeneity presumed. 4. Imprecision: OIS and CI not reported. 5. Publication bias: Moderate. Many significant results reported, only dog study on IPoC published. **Strengths:** Thorough statistical analysis. Tested several IPoC algorithms with several groups. Histology, ELISA and TUNEL staining with functional outcomes. **Weaknesses**: No OIS or ARRIVE**.** No mention of ELISA CV%. No CI reported.
Conclusion Postcond exerts protective effects on renal (I/R) injury.			
Country **China**			
Year data collection 2010			

Reference: Silberstein JL, Sprenkle PC, Su D, Power NE, Tarin TV, Ezell P, et al. Neutrophil gelatinase-associated lipocalin (NGAL) levels in response to unilateral renal ischaemia in a novel pilot two-kidney porcine model. BJU international. 2013;112(4):517-25.	**GRADE Moderate (high)**	
	Dokumentation level	Ib
	Recommendation	Strong for

Purpose	**Material and method**	**Results**	**Discussion/Comments:**
Test a porcine two-kidney model for evaluating the effect of controlled acute kidney injury (AKI) related to induced unilateral ischemia on both renal units (RUs) To use neutrophil gelatinase-associated lipocalin (NGAL) and physiological serum and urinary markers to assess AKI and renal function **Conclusion** AKI as measured by increases in NGAL and decreased renal function as measured by decreases in CrCl, are specific to the RU exposed to ischaemia. **Country** **USA** **Year data collection** 2013	Twelve female Yorkshire pigs had bilateral cutaneous ureterostomies placed laparoscopically with identical duration of pneumoperitoneum for all cases. An experimental group (n = 9) underwent induced unilateral renal ischaemia with left hilar clamping of timed duration (15, 30, 60 min) and a control group (n = 3) had no induced renal ischaemia. Urine was collected and analysed from each RU to assess creatinine and NGAL concentration preoperatively and at multiple postoperative time points. Serum was collected and analysed daily for creatinine and NGAL levels. Statistical comparisons were made using the rank-sum and sign-rank tests.	Three pigs were excluded because of intra-operative and postoperative complications. In the RUs that experienced renal ischaemia (n = 7),the median urine volume was lower (P = 0.04) at 6, 12, 24 and 48 h and the median NGAL concentration was higher (P = 0.04) at 12 and 48 h compared with the RUs of control pigs that experienced no renal ischaemia (n = 2). When comparing the ischaemic (left) RU of the pigs in the experimental group with their contralateral non-ischaemic (right) RU, ischaemic RUs had a lower median cumulative urine volume at 6, 12, 24 and 48 h (P = 0.05) and a higher median NGAL concentration at 12, 24 and 48 h (P < 0.05). At 48 h, no significant increase was found in serum NGAL in pigs in the experimental group compared with controls (P = 0.2). Creatinine clearance (CC) was lower in ischemic RUs compared with non-ischaemic RUs 1 day after surgery (P = 0.04) with decreasing CC as the duration of ischaemia increased**.** Urine NGAL increased in ischemic kidney with little change in urine NGAL in contralateral kidney. Ischemic kidneys (n=7) had lower (P=0.04) median urine volume at 6, 12, 24 and 48 hours and median NGAL higher (P=0.04) at 12 and 48 hours compared with kidneys of control pigs (n=2).	1. Risk of Bias: Low 2. Indirectness: Moderate. Pigs urinary system close to humans. No histology reported with biochemical outcomes. 3. Inconsistency: Low. No heterogeneity in results. 4. Imprecision: Moderate risk. OIS not given. 5. Publication bias: Low. There were not many significant finds. Strengths: Novel urine collection system under normal physiological conditions. (Mixed sexes. Histology and biochemical outcomes. Limitations: Fluid intake not measured during urine collection period. Very low sample numbers. No individual pig weight measurements. Pigs kept at ambient temperature during surgery (temp. times?). ARRIVE guidelines not mentioned. 3 of 12 pigs in final analysis. No statistical power calculation (OIS). No effect scores given in statistics.

Reference: Behrends M, Walz MK, Kribben A, Neumann T, Helmchen U, Philipp T, et al. No protection of the porcine kidney by ischaemic preconditioning. Experimental physiology. 2000;85(6):819-27.	**GRADE Moderate (high)**	
	Documention level	Ib
	Recommendation	Strong

Purpose	Material and method	Results	Discussion/Comments:
Investigate the influence og ischemic preconditioning (IPC) on postischemic function and morphology in porcine kidneys.	Preclinical RCT: Two kidney, open surgical pig model, 19 mixed sex total, 40-60 kg. Enflurane anesthesia. Three groups. Group 1 (n=8): 60 min ischemia Rt kidney, 8 hrs reperfusion, terminated Group 2 (n=8): IP group ,3x 10 minutes Ischemia followed by 10 minutes reperfusion and 60 minutes WI, 8 hours reperfusion and terminated. Group 3 (n=3): 3x10 minutes IP protocol w/o 60 minutes WI afterward. 8 hrs reperfusion, terminated. IPC algorithm: 10 minutes Rt artery/vein clamping followed by 10 minutes reperfusion. Blood and sCr, inulin GFR, urea, haemodynamics and histology (PAS). Statistical methods: Two-way ANOVA, unpaired t-test for histology and mean and SEM given. No effect scores given, only p values.	Haemodynamics: no difference Inulin, creatinine and urea: no sig. difference between IPC group and control. Histology: more severe damage in IPC group compared to control. The reperfused kidneys did not excrete inulin, creatinine or urea in both groups, although renal blood flow during reperfusion was similar to baseline. Morphological damage ranged in both groups from single cell necrosis to disseminated patchy necrosis; the number of pyknotic cells tended to be higher in the IP group than in the placebo group (27.0 +/- 7.1 vs. 15.6 +/- 5.6%, n.s.). In anaesthetized pigs, IP did not therefore attenuate renal dysfunction and morphological damage resulting from 60 min of renal normothermic ischaemia followed by 8 h of reperfusion.	1. Risk of Bias: Low 2. Indirectness: Low (Pigs urinary system close to humans). Histology reported with biochemical outcomes. 3. Inconsistency: Low. No heterogeneity in results. 4. Imprecision: Moderate risk. OIS not given. 5. Publication bias: Low. It is a negative study article. **Strengths:** No effect article. Many time period recordings. Mixed sexes. Histology and biochemical outcomes. **Limitations:** Low sample numbers. Unclear if same main operator for every pig. ARRIVE guidelines not mentioned. No statistical power calculation (OIS). No effect scores given in statistics.
Conclusion No protection of IPC to porcine kidney			
Land Germany			
Year data collection 2000			

Note about GRADE evaluations and checklists: Since all GRADE evaluations were pre-clinical animal studies, I used methodology from the following articles for evaluation:

1. Goldet G, Howick J. Understanding GRADE: an introduction. Journal of evidence-based medicine. 2013;6(1):50-4.

2. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW. SYRCLE's risk of bias tool for animal studies. BMC medical research methodology. 2014;14:43.

3. Wei D, Tang K, Wang Q, Estill J, Yao L, Wang X, et al. The use of GRADE approach in systematic reviews of animal studies. Journal of evidence-based medicine. 2016.

4. Animal research: reporting in vivo experiments: the ARRIVE guidelines. The Journal of physiology. 2010;588(Pt 14):2519-21.