Postconditioning effects of argon or xenon on early graft function in a porcine model of kidney autotransplantation

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Authorship contribution:

JDD: acquisition and analysis of the data, critical revision of the work; SR: conception and design of the work, acquisition and interpretation of the data, critical revision of the work; EL: analysis and interpretation of the data, critical revision of the work; WM: analysis and interpretation of the data, critical revision of the work; DM: interpretation of the data, critical revision of the work; JP: interpretation of the data, critical revision of the work; IJ: conception and design of the work, acquisition, analysis, and interpretation of the data, drafting of the work, critical revision of the work. All authors gave final approval of the version to be published and all agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Abbreviations:

AST, aspartate aminotransferase

CIT, cold ischaemia time

CVP, central venous pressure

H-FABP, heart-type fatty acid binding protein

IFCC, International Federation of Clinical Chemistry

IQR, interquartile range

IRI, ischaemia-reperfusion injury

LOD, limit of detection

MAP, mean arterial pressure

N_2, nitrogen

O_2, oxygen

POD, postoperative day

TUNEL, terminal deoxynucleotidyl transferase end labelling

WIT, warm ischaemia time

Summary

What is already known:

Ischaemia reperfusion injury is inevitable during renal transplantation and can lead to delayed graft function and primary non-function. Based on mainly small animal experiments, noble gases (i.e. argon and xenon) have been proposed to minimise this ischaemia-reperfusion injury and improve outcomes after transplantation.

What is new:

The hypothesis that postconditioning with argon or xenon inhalation would improve graft function was tested in a porcine kidney autotransplant model. Peak plasma creatinine was similar in the control, argon and xenon groups. No other secondary outcome parameters, including animal survival, were affected by the intervention. Xenon was associated with an increase in autophagy and pro-apoptotic markers.

What is the potential impact on future practice:

Despite promising results in small animal models, postconditioning with argon or xenon in a translational model of kidney autotransplantation was not beneficial. Clinical trials would require better results.

Abstract

Background: Ischaemia reperfusion injury is inevitable during renal transplantation and can lead to delayed graft function and primary non-function. Pre-, re-, and postconditioning with argon and xenon protects against renal ischaemia reperfusion injury in rodent models. The hypothesis that postconditioning with argon or xenon inhalation would improve graft function in a porcine renal autotransplant model was tested.

Methods: Pigs (predetermined n=6/group) underwent left nephrectomy after 60 min of warm ischaemia (renal artery and vein clamping). The procured kidney was autotransplanted in a separate procedure after 18 hours of cold storage, immediately following a right nephrectomy. Upon reperfusion, pigs were randomised to inhalation of control gas (N_2 70%+O_2 30%), argon 70%+O_2 30%, or xenon 70%+O_2 30% for 2h. Primary outcome parameter was peak plasma creatinine, secondary outcome parameters included further markers of graft function (creatinine course, urine output), graft injury (aspartate aminotransferase, heart-fatty acid binding protein, histology), apoptosis and autophagy (western blot, TUNEL-staining), inflammatory mediators and markers of cell survival/growth (mRNA and tissue protein quantification), and animal survival. Results are presented as median (interquartile range). ANOVA or Kruskal-Wallis were used where indicated.

Results: Peak plasma creatinine was similar between the groups: control 20.8 mg/dL (16.4-23.1); argon 21.4 mg/dL (17.1-24.9); xenon 19.4 mg/dL (17.5-21.0); p =0.607. Xenon was associated with an increase in autophagy and pro-apoptotic markers. Creatinine course, urine output, injury markers, histology, survival, and inflammatory mediators were not affected by the intervention.

Conclusions: Postconditioning with argon or xenon did not improve kidney graft function in this experimental model.

Introduction

Despite enormous advances in the field of renal transplantation, ischaemia reperfusion injury (IRI) remains a problem that complicates patient care. IRI plays a major role in primary non-function, delayed graft function, long-term graft failure, morbidity, and mortality.[1] The increasing use of kidneys procured from expanded criteria donors or donated after circulatory death makes the need for treatment of IRI even more relevant as these grafts are particularly vulnerable to the harmful effects of ischaemia and reperfusion.[2, 3] Noble gases, via anti-inflammatory and pro-survival mechanisms, are believed to convey organ protective effects during IRI in a wide variety of medical conditions, including acute kidney injury and renal transplantation.[4-6] A meta-analysis confirmed that xenon inhalation attenuates IRI and prolongs survival in rodent models of renal IRI and renal transplantation.[7] Saturation of organ preservation solutions with argon had renoprotective effects in transplant models.[7-10] However, the results in large animal models are disappointing, stressing the importance of performing well-designed large animal studies before considering clinical trials.[7] The pig is physiologically and anatomically close to the human and provides an excellent model for renal IRI studies.[11] The hypothesis that postconditioning with argon or xenon inhalation would improve renal graft function was tested in a porcine model of renal autotransplantation.

Methods

Animals

Female, prepubescent pigs (3-4 months old) (TOPIG 20, Toon Janssen, Nijmegen) were housed with an artificial 12h light-dark cycle and became accustomed to their surroundings for at least 2 days before surgery. Pigs were kept in a conventional, closed housing system. After surgery they were kept in single pens allowing visual, olfactory, and auditory contact with each other. Pigs were fasted 12h before the experiment with access to tap water. Food (MPig-H, ssniff, Soest, Germany) was

introduced after the transplantation. The Animal Care Committee (KU Leuven, P115-2013) approved the study and all experiments were performed according to European guidelines.[12]

Anaesthesia and surgery

The procedure has been described previously.[13] In brief, pigs were premedicated with intramuscular xylazine (2mg/kg, VMD, Arendonk, Belgium) and tiletamine (8mg/kg, Virbac, Barneveld, The Netherlands). After orotracheal intubation with a cuffed tube, anaesthesia was induced and maintained with a continuous infusion of propofol (6-10mg/kg/h, AstraZeneca, Ukkel, Belgium) and fentanyl (8µg/kg/h, Janssen-Cilag, Beerse, Belgium). Cefazolin 2g (Mylan, Hoeilaart, Belgium) was given at induction. Catheters were placed in the left carotid artery and external jugular vein. Following a median laparotomy, a suprapubic bladder catheter was placed. The left renal artery and vein were clamped for 60 min to initiate warm ischaemia, mimicking donation after circulatory death. The kidney was procured, flushed with 500 mL of cold (4°C) histidine-tryptophan-ketoglutarate solution (100 cm H_2O) and cold-stored for 18h. After weaning from the ventilator, the pig was returned to the pen and received a standard regimen of analgesia and crystalloids as previously described.[14]

The following day, the same pig was anaesthetised in a similar fashion and underwent a right nephrectomy followed by autotransplantation of the left kidney. Mean arterial and central venous pressure were monitored during transplantation. Post-transplant, animals received standardised fluid replacement[14] and analgesia by transdermal buprenorphine (70µg/h for 3 days, Grünenthal, Sint-Stevens-Woluwe, Belgium). Twenty-four hours after reperfusion, a percutaneous ultrasound guided biopsy was taken under sedation [xylazine (2mg/kg, VMD, Arendonk, Belgium) and tiletamine (8mg/kg, Virbac, Barneveld, The Netherlands)] and local anaesthesia (lidocaine 1%, B Braun, Melsungen, Germany). Animals were assessed daily by trained and experienced researchers supported by a veterinary advisor. Ten days post-transplant, the pig was anaesthetised again. After re-laparotomy and graft inspection, biopsies were taken and animals were euthanised by intravenous potassium chloride injection. In any case where clinical assessment showed clear evidence of a

deteriorating condition that would most likely lead to death, it was predetermined that the animal was to be euthanised as described above.

Study groups

Animals were electronically randomised (MS Excel 2013®) to receive ventilation with control (70% N_2 + 30% O_2), argon (70% argon + 30% O_2), or xenon (70% xenon + 30% O_2) gas mixture (Fig. S1). Allocation to the treatment group was revealed at the start of the transplantation as the necessary gases needed to be prepared. Inhalation treatment started 5 min before graft reperfusion and continued until 2h post-reperfusion. Argon was administered from prefilled gas mixture cylinders (AirLiquide Belgium, Brussels, Belgium). Xenon (AirLiquide Belgium) was administered and monitored using a closed circuit respirator (Felix Dual™, AirLiquide Medical Systems, Paris, France) in the automatic mode. The team performing primary outcome analyses was blinded to the treatment arm.

Sample collection

Central venous blood samples were obtained via the venous catheter at baseline, 15 min, 60 min, 3h, 6h postreperfusion and daily thereafter. Six-hour urine collections were performed on postoperative day (POD) 1, 3, 6, 8, and 10. Urine and blood samples were centrifuged at 1000G for 10 min, aliquoted in 1 mL microtubes and stored at -20°C until analysis. Needle biopsies (14G), from deep cortex–outer medulla were taken at baseline, 24h post-reperfusion, and POD10. Tissue was stored in formalin (6%), paraffin embedded and processed for periodic acid-Schiff staining and terminal deoxynucleotidyl transferase end labelling (TUNEL), or snap-frozen for western blot or qPCR.

Analyses

The primary outcome parameter was peak plasma creatinine, secondary outcome parameters included further markers of kidney graft function (urine output, creatinine course), kidney graft injury (aspartate aminotransferase (AST), heart-fatty acid binding protein (H-FABP), histology), apoptosis and autophagy (western blot, TUNEL-staining), inflammatory mediators and markers of cell survival/growth (mRNA, tissue protein quantification), and animal survival.

Biomarkers

Plasma levels of creatinine and AST were assessed by the kinetic Jaffé method (Hitachi/Roche Modular P) and the IFCC method (limit of detection (LOD) 4IU/L, Hitachi/Roche Modular P), respectively. Plasma levels of H-FABP (HK403, LOD 391pg/mL, Hycult Biotech, Uden, the Netherlands) were measured using an enzyme-linked immunosorbent assay (ELISA).

Protein quantification

Western blot was performed with primary antibodies for Anti-LC3, anti-S6, anti-phospho-S6, anti-cleaved caspase 3, anti-Beclin 1, anti-Bax, anti-SQSTM1/P62, and anti-GAPDH as an internal control, and secondary antibodies anti-mouse IgG HRP-linked and anti-rabbit IgG HRP-linked (Suppl. Methods).

Erythropoietin and vascular endothelial growth factor (VEGF) concentrations in kidney tissue homogenates (20µl PBS/mg tissue) were determined by ELISA according to the manufacturer's instructions (Suppl. Methods).

qPCR

After sample preparation (Suppl. Methods), real-time PCR reaction was performed on a LightCycler 96W system (Roche Diagnostics, Vilvoorde, Belgium) with Taqman Fast Universal PCR Master Mix and Taqman Gene Expression Assays for Hypoxia-Inducible Factor one alpha (HIF 1α, Ss03390447¬_m1) (ThermoFisher Scientific, Aalst, Belgium). For Tumour Necrosis Factor alpha (TNFα), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Toll-like Receptor 4 (TLR-4) and β-actin (housekeeping) primers and probes were designed with Primer3 version 0.4.0 (Kaneka EuroGentec, Seraing, Belgium) (sequences in Table S1). A two-step amplification program was used: 95°C for 1 min followed by 45 cycles of amplification (95°C for 5 sec, 60°C for 30 sec). Target mRNA expression was quantified as fold change relative to the housekeeping gene and baseline tissue sample.

TUNEL

Oligonucleosomal DNA cleavage via TUNEL was detected in 5 µm tissue sections that were deparaffinised in 100% toluene (200 mL, 2x5 min), rehydrated in distilled water (5 min) and pre-treated with 3% citric acid (200 mL, 60 min) to remove tissue calcification. Endogenous peroxidase was quenched by incubating sections for 15 min in 3% hydrogen peroxide (200 mL). TUNEL was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Merck Chemicals, Overijse, Belgium).

Histopathology

Periodic acid-Schiff stains were assessed for six morphological features by a blinded, experienced pathologist as previously described[14]: brush border integrity, tubular cell aspect, tubular nuclei changes, intratubular detachment, interstitial oedema, and interstitial inflammatory infiltrate. These features were semiquantitatively scored: (0) none, (1) mild, (2) moderate, and (3) severe morphological changes.

Statistics

A minimum of 6 animals per group were needed to detect a change of 25% in peak creatinine (2-way α 0.05, β 0.10) between control, argon, and xenon for repeated measures ANOVA testing, assuming a creatinine peak of 19.7 mg/dL and a standard deviation of 2.14 mg/dL (based on previous data[14]) (G*Power Version 3.1.5.1). Data are presented as median (interquartile range, IQR). After testing for normal distribution (Shapiro Wilk test), continuous variables were compared between groups (ANOVA or Kruskal-Wallis analysis of variance with a post-hoc Dunn's test). Within group changes were assessed by repeated measures ANOVA using a mixed model. Survival at POD10 was compared by Fishers-Freeman-Halton test. Analyses were performed with SPSS20 and GraphPad Prism 5.03 for Windows. P values <0.050 were considered significant. Creatinine was corrected for a body weight of 40kg; AST and, H-FABP were corrected to a kidney weight of 100g.

Results

To obtain 6 animals in each group for final analysis, 21 pigs were operated (6 controls, 7 argon, 8 xenon). One pig from the argon group was excluded at POD6 because of a severe skin infection unrelated to surgery. Because the infection might have been subclinically present at POD1, this could have affected the primary endpoint. Two animals from the xenon group were excluded (POD7, POD10) because of a massive renal haematoma, most likely related to percutaneous biopsies on POD1. This tissue injury might have compromised renal function, influencing the primary outcome. These animals were excluded from the analysis and replaced.

Baseline and operative characteristics

Baseline and operative characteristics were similar between the 3 groups (Table 1). Peri-operative haemodynamic variables were comparable between groups, except for central venous pressure at 2h post-reperfusion being slightly higher in the xenon group compared to control despite similar fluid volumes administered.

Kidney graft function

Peak creatinine concentrations were similar (Table 1). Creatinine peaked at median on POD5 in controls, POD5 in the argon group and POD4 in the xenon group (p=0.586). All grafts experienced an initial period of anuria and picked up urine production at POD3 (IQR 3-4 in each group, p=0.980). Daily urine output and plasma creatinine were similar between the groups (Fig. 1).

Kidney graft injury

Peak AST and peak H-FABP concentrations, markers of cellular injury,[14] were similar in all groups (Table 1). On histology, acute tubular necrosis was present 24h post-reperfusion but no differences were seen between groups (Table 2). At POD10, there was a similar recovery to minimal injury in all groups, consistent with the functional recovery of the kidney graft and clinical course of the animal.

Survival

In the control group, 4/6 animals (67%) survived until POD10. One animal suffered from a severe rectal prolapse on POD6 and was euthanised, the other pig was found dead in its pen on POD7 having shown no signs of clinical distress hours before. Autopsy revealed unspecific mild pulmonary oedema in this animal. Both animals had urine output and peak creatinine had been reached. Survival was 6/6 (100%) in the argon and xenon groups. The numeric survival difference was not significant (p=0.290).

Inflammatory mediators and effectors of cell survival/growth

There were no differences in the expression of TLR-4, ICAM-1, TNFα, IL-6, and HIF-1α nor in the protein levels of HIF-1 α downstream effectors erythropoietin and VEGF (Fig. 2) between the groups. Within group analyses showed significant reductions in the expression of TLR-4, ICAM-1, erythropoietin, and VEGF at 24h compared to 45min post-reperfusion (Table S2).

Autophagy and apoptosis

Western blot analysis did not show significant differences between argon and control groups with respect to apoptosis (Bax, cleaved Caspase 3) and autophagy (LC3I, LC3II, Beclin) pathways (Fig. 3A). In the xenon group, a significant decrease in the autophagy marker phosphorylated S6 (pS6) and an increase in the pro-apoptotic markers cleaved Caspase-3 and Bax was observed (Fig. 3B).

Despite this increase in pro-apoptotic markers, TUNEL analysis did not show any obvious differences in apoptosis between the groups (Fig. S2).

Discussion

In a porcine model of renal autotransplantation, 2h of postconditioning with 70% argon or xenon was unable to improve kidney graft function, graft injury, or animal survival. An anti-inflammatory effect of argon or xenon was not detectable. A pro-apoptotic effect of xenon in tissue biopsies 24h post-reperfusion was seen but the elevated levels of Bax and cleaved caspase 3 had no apparent clinical

repercussion. These findings contradict the outcomes of previous experiments in animal models of acute kidney injury and renal transplantation in which the administration of argon or xenon improved renal function and prolonged animal survival.[7] In rodents, xenon was found to be particularly effective, offering protection independently from the time point of administration. Xenon *pre*conditioning has repeatedly demonstrated to improve renal function and histological injury in various murine and rat models.[4-6, 15, 16] Both *pre*- and *post*conditioning with xenon increased graft function and survival and reduced graft fibrosis in rat transplant models.[4-6] *Re*conditioning with xenon, cold-storing rat kidneys in a xenon-saturated preservation solution, resulted in improved graft function and histology,[9, 17] although it was detrimental in a pig model of renal transplantation.[10] Likewise, *re*conditioning kidney grafts with argon saturated perfusion solutions improved graft function in rats and pigs.[9, 10]

The exact mechanisms underlying noble gas-induced organ protection remain largely obscure but most likely involve anti-inflammatory and pro-survival mechanisms.[7] As an example, xenon has been demonstrated to activate HIF-1α and its downstream effectors including erythropoietin and VEGF in rodents.[15] However, evidence for HIF1α-activation by nobles gases in humans is scarce and controversial. [18, 19] It was recently shown that xenon anaesthesia, in patients undergoing off-pump cardiac surgery, was neither associated with a reduction of acute kidney injury nor with an increase of erythropoietin plasma levels.[20] In the present study neither HIF-1α expression nor protein levels of erythropoietin and VEGF, the downstream effectors of HIF-1α, were affected by argon or xenon.

Xenon was found to reduce apoptosis in various small animal IRI models in brain, heart and kidneys.[4,17] However, an increase of apoptosis after xenon treatment has been described in porcine cardiac IRI where xenon demonstrated cardioprotective effects.[21] These differences could suggest that noble gases might have different molecular effects in small and large animals which might have contributed to the lack of clinical effects of argon and xenon in this model. Indeed, only a few studies have examined the effects of these noble gases in large animal models and found the organ

protective effects of noble gases to be less obvious in large animals compared to small animals.[7] Moreover, reported results in pigs are contradictory. While some investigators have shown decreased infarct volumes and improved cardiac and neural function with xenon after cardiac and cerebral IRI in pigs,[22-24] others were unable to confirm these protective qualities.[25] Furthermore, porcine kidneys have recently been observed to show an improvement with argon but not xenon reconditioning.[10]

Several other factors might have contributed to the discrepancy between the results presented here and those previously obtained in other, mainly small animal models. Argon and xenon were administered in a pure postconditioning setting at a fixed concentration of 70% for 2h. Nearly the maximum possible argon or xenon concentrations were used as evidence suggests a stronger organ protective effect with higher concentrations.[26-28] Considerably higher concentrations cannot be administered in clinical routine as a further increase of inhaled noble gas concentrations under normobaric conditions would result in hypoxic gas mixtures. The choice for postconditioning during 2h was based on evidence that noble gases exert similar organ protective effects in either conditioning setting, including postconditioning.[7] Postconditioning was a pragmatic choice as this strategy could be easily implemented in current clinical practice. Nevertheless, postconditioning inherently means starting treatment of IRI when the injury cascade has already been set in motion and no single agent administered after reperfusion has shown effectiveness in the clinical setting. Argon-reconditioning of porcine kidneys during cold storage has been shown to improve graft function,[10] suggesting that noble gas administration earlier in the IRI cascade is possible.

These findings open the door for organ treatment during preservation, not only by adding gas to preservation solutions but potentially also adding it to dynamic preservation and resuscitation techniques.[29] In fact, Hosgood et al. have administered argon during 1h of normothermic machine perfusion of pig kidneys, showing an improved renal function compared to controls.[30] Furthermore, if ethical and legal challenges of donor (pre)treatment could be overcome,[31] transplantation lends itself

to *pre*-, *per*- and *post*conditioning. Although 2h of postconditioning is a commonly used timeframe in studies addressing noble-gas organ protection the possibility that the duration of inhalation was too short cannot be excluded. Longer periods of noble gas inhalation would need to be investigated to address the relationship between cumulative effects and whether they alter allogeneic immunity.

Both warm and cold ischaemia are important contributors to IRI.[1] Kidney injury induced in this model might have been too excessive to detect a noble gas organ protective effect. In contrast, one could also argue that the injury was not excessive enough, making it difficult to show any organ protective effects. Nevertheless, the injury elicited was severe enough to result in kidney dysfunction with a median anuric period of 3 days after which function gradually recovered. As such it represents a reproducible model for delayed graft function in the pig, and similar models are used by other groups.[11] The study was powered to detect a 25% change in peak creatinine so a smaller effect on graft function might have been missed. However, both peak creatinine values and creatinine course were identical in the 3 groups, making it unlikely that a smaller but clinically relevant effect of the noble gases was missed.

In conclusion, postconditioning with argon or xenon did not improve kidney graft function in this experimental model. Better results would be needed to consider clinical trials.

Declaration of interest

SR has received an unrestricted research grant from Air Liquide Belgium.

DM, JP and IJ are holders of a named chair in abdominal transplant surgery at the KU Leuven of the Centrale Afdeling voor Fractionering.

JP holds a named chair in abdominal transplant surgery at the KU Leuven of the Institut George Lopez group.

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The data of this study can be made available to other researchers upon their request by contacting the corresponding author.

This study was not preregistered in an independent, institutional registry.

References

1. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 2002; **346**: 580-590.

2. Cho YW, Terasaki PI, Cecka JM, Gjertson DW. Transplantation of kidneys from donors whose hearts have stopped beating. *N Engl J Med* 1998; **338**: 221-225.

3. Lim WH, McDonald SP, Russ GR, Chapman JR, Ma MK, Pleass H*, et al.* Association Between Delayed Graft Function and Graft Loss in Donation After Cardiac Death Kidney Transplants-A Paired Kidney Registry Analysis. *Transplantation* 2017; **101**: 1139-1143.

4. Zhao H, Luo X, Zhou Z, Liu J, Tralau-Stewart C, George AJT*, et al.* Early treatment with xenon protects against the cold ischemia associated with chronic allograft nephropathy in rats. *Kidney International* 2014; **85**: 112-123.

5. Zhao H, Watts HR, Chong M, Huang H, Tralau-Stewart C, Maxwell PH*, et al.* Xenon treatment protects against cold ischemia associated delayed graft function and prolongs graft survival in rats. *Am J Transplant* 2013; **13**: 2006-2018.

6. Zhao H, Yoshida A, Xiao W, Ologunde R, O'Dea KP, Takata M*, et al.* Xenon treatment attenuates early renal allograft injury associated with prolonged hypothermic storage in rats. *FASEB Journal* 2013; **27**: 4076-4088.

7. De Deken J, Rex S, Monbaliu D, Pirenne J, Jochmans I. The Efficacy of Noble Gases in the Attenuation of Ischemia Reperfusion Injury: A Systematic Review and Meta-Analyses. *Crit Care Med* 2016; **44**: e886-896.

8. Irani Y, Daniel L, Gaudart J, Magalon G, Hardwigsen J. Kidney storage conditions are dramatically improved by an argon satured cold storage solution. *Am J Transplant* 2010; **10**: 494.

9. Irani Y, Pype JL, Martin AR, Chong CF, Daniel L, Gaudart J*, et al.* Noble gas (argon and xenon)-saturated cold storage solutions reduce ischemia-reperfusion injury in a rat model of renal transplantation. *Nephron extra* 2011; **1**: 272-282.

10. Faure A, Bruzzese L, Steinberg JG, Jammes Y, Torrents J, Berdah SV*, et al.* Effectiveness of pure argon for renal transplant preservation in a preclinical pig model of heterotopic autotransplantation. *J Transl Med* 2016; **14**: 40.

11. Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, Hauet T. Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model. *J Biomed Biotechnol* 2011; **2011**: 532127.

12. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063.

13. Jochmans I, Lerut E, Heedfeld V, Wylin T, Pirenne J, Monbaliu D. Reproducible model for kidney autotransplantation in pigs. *Transplant Proc* 2009; **41**: 3417-3421.

14. Jochmans I, Lerut E, van Pelt J, Monbaliu D, Pirenne J. Circulating AST, H-FABP, and NGAL are early and accurate biomarkers of graft injury and dysfunction in a preclinical model of kidney transplantation. *Ann Surg* 2011; **254**: 784-791; discussion 791-782.

15. Ma D, Lim T, Xu J, Tang H, Wan Y, Zhao H*, et al.* Xenon preconditioning protects against renal ischemic-reperfusion injury via HIF-1alpha activation. *J Am Soc Nephrol* 2009; **20**: 713-720.

16. Jia P, Teng J, Zou J, Fang Y, Zhang X, Bosnjak ZJ*, et al.* miR-21 contributes to xenon-conferred amelioration of renal ischemia-reperfusion injury in mice. *Anesthesiology* 2013; **119**: 621-630.

17. Zhao H, Ning J, Savage S, Kang H, Lu K, Zheng X*, et al.* A novel strategy for preserving renal grafts in an ex vivo setting: Potential for enhancing the marginal donor pool. *FASEB Journal* 2013; **27**: 4822-4833.

18. Stoppe C, Ney J, Brenke M, Goetzenich A, Emontzpohl C, Schalte G*, et al.* Sub-anesthetic Xenon Increases Erythropoietin Levels in Humans: A Randomized Controlled Trial. *Sports medicine (Auckland, NZ)* 2016; **46**: 1753-1766.

19. Balachandran A, Streiner DL, Signorile JF. Comment on "Sub-anesthetic Xenon Increases Erythropoietin Levels in Humans: A Randomized Controlled Trial". *Sports medicine (Auckland, NZ)* 2017; **47**: 379.

20. Al Tmimi L, Van Hemelrijck J, Van de Velde M, Sergeant P, Meyns B, Missant C*, et al.* Xenon anaesthesia for patients undergoing off-pump coronary artery bypass graft surgery: a prospective randomized controlled pilot trialdagger. *Br J Anaesth* 2015; **115**: 550-559.

21. Sopka S, Mertens C, Roehl AB, Schiffl K, Rossaint R, Classen-Linke I. Effects of xenon and isoflurane on apoptosis and inflammation in a porcine myocardial infarction model. *Annals of Anatomy* 2013; **195**: 166-174.

22. Chakkarapani E, Dingley J, Liu X, Hoque N, Aquilina K, Porter H*, et al.* Xenon enhances hypothermic neuroprotection in asphyxiated newborn pigs. *Annals of neurology* 2010; **68**: 330-341.

23. Fries M, Nolte KW, Coburn M, Rex S, Timper A, Kottmann K*, et al.* Xenon reduces neurohistopathological damage and improves the early neurological deficit after cardiac arrest in pigs. *Crit Care Med* 2008; **36**: 2420-2426.

24. Hein M, Roehl AB, Baumert JH, Bantes B, Bleilevens C, Bernstein N*, et al.* Establishment of a porcine right ventricular infarction model for cardioprotective actions of xenon and isoflurane. *Acta Anaesthesiol Scand* 2008; **52**: 1194-1203.

25. Fries M, Coburn M, Nolte KW, Timper A, Kottmann K, Kuru TH*, et al.* Early administration of xenon or isoflurane may not improve functional outcome and cerebral alterations in a porcine model of cardiac arrest. *Resuscitation* 2009; **80**: 584-590.

26. Brucken A, Kurnaz P, Bleilevens C, Derwall M, Weis J, Nolte K*, et al.* Dose dependent neuroprotection of the noble gas argon after cardiac arrest in rats is not mediated by K(ATP)-channel opening. *Resuscitation* 2014; **85**: 826-832.

27. Homi HM, Yokoo N, Ma D, Warner DS, Franks NP, Maze M*, et al.* The neuroprotective effect of xenon administration during transient middle cerebral artery occlusion in mice. *Anesthesiology* 2003; **99**: 876-881.

28. Ma D, Hossain M, Chow A, Arshad M, Battson RM, Sanders RD*, et al.* Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. *Annals of Neurology* 2005; **58**: 182-193.

29. Jochmans I, Akhtar MZ, Nasralla D, Kocabayoglu P, Boffa C, Kaisar M*, et al.* Past, present and future of dynamic kidney and liver preservation and resuscitation. *Am J Transplant* 2016; **16**: 2545-2555.

30. Hosgood S, Hamed M, Adams T, Nicholson M. Ex-vivo normothermic perfusion with the noble gas argon in an experimental model of kidney transplantation. *Transplant International* 2016; **29**: 7-7.

31. Feng S. Donor intervention and organ preservation: where is the science and what are the obstacles? *Am J Transplant* 2010; **10**: 1155-1162.

Figure Legends

Fig. 1: Boxplots of plasma creatinine (Panel A) and urine output (Panel B) for Control, Argon, and Xenon. Differences between the three groups were not significant.

Fig. 2: Boxplots of fold changes in expression of TLR-4 (Panel A), ICAM-1 (Panel B), IL-6 (Panel C), TNFα (Panel D), HIF-1α (Panel E) mRNA expression and erythropoietin (Panel F) and VEGF (Panel G) protein levels at 24h post-reperfusion. p-values are given in the table, none reached significance, * marks protein levels instead of mRNA levels. At 45 min post-reperfusion: n=5 in all groups; at 24h post-reperfusion: n=4 in Control, n=4 in Argon, n=5 in Xenon; at 10 days post-reperfusion: n=3 in Control; n=5 in Argon, n=4 in Xenon.

Fig. 3: Boxplots of the band intensity quantification of western blot in tissue biopsies 24h post-reperfusion (5 biopsies were available per group for western blot): Argon compared to Control (Panel A), Xenon compared to Control (Panel B). All results were corrected for the housekeeping protein GAPDH. Band intensity of pS6/S6 was lower for Xenon versus Control (p=0.022) and higher for cleaved Casp3 (p=0.034) and Bax (p=0.043).

Supplementary appendix

Fig. S1 Experimental design

Fig. S2 Representative images of TUNEL in Control (Panel A), Argon (Panel B), and Xenon (Panel C). All images are taken at 20 times magnification.

Table S1 Sequence of Primers and Probes

Table S2 Within group comparison of changes in expression of TLR-4, ICAM-1, IL-6, TNFα, HIF-1α mRNA expression, and erythropoietin and VEGF protein levels. (Boxplots in Fig. 2 of the main text).

Table 1. Baseline characteristics, kidney function, and plasma kidney injury markers of pigs undergoing kidney autotransplantation after 60 min of warm ischaemia and 18h of cold storage and receiving postconditioning with control gas, argon, and xenon.

Parameter	Control (n=6)	Argon (n=6)	Xenon (n=6)	p-value
Baseline characteristics				
Weight Pig (kg)	38.2 (36.7-39.3)	35.7(32.7-42.1)	43.5 (37.8-47.1)	0.054
Weight Kidney (g)	135 (115-163)	121 (102-128)	154 (138-161)	0.065
WIT (min)	60 (60-60)	60 (59-60)	60 (59-60)	0.532
CIT (h:min)	18,0 (18,0-18,1)	18,0 (18,0-18,0)	18,0 (18,0-18,2)	0.820
Time for anastomosis (min)	30 (28-31)	29 (26-30)	28 (27-31)	0.614
MAP reperfusion (mm Hg)	95 (90-136)	95 (84-99)	118 (108-123)	0.128
CVP reperfusion (mm Hg)	12.0 (11.0-14.5)	12.5 (11.3-14.0)	14.5 (12.8-15.5)	0.348
MAP 1h (mm Hg)	98.0 (85.0-129.5)	107 (92-117)	106 (96-123)	0.994
CVP 1h (mm Hg)	11.0 (10.5-15.0)	12.0 (10.0-12.3)	14.0 (12.8-15.3)	0.098
MAP 2h (mm Hg)	107 (101-147)	116 (100-133)	119 (104-122)	0.875
CVP 2h (mm Hg)	9.0 (9.0-12.5)*	12.0 (10.0-16.0)	14.0 (13.5-14.8)*	0.027*
Kidney function				
Peak Creatinine (mg/dL)	20.8 (16.4-23.1)	21.4 (17.1-24.9)	19.4 (17.5-21.0)	0.607
Kidney injury markers				
Peak AST (IU/L)	228 (156-253)	184 (147-216)	189 (149-206)	0.205
Peak H-FABP (ng/mL)	278 (221-359)	216 (183-328)	223 (206-250)	0.236

CIT, cold ischaemia time; CVP, central venous pressure; H-FABP, heat-fatty acid binding protein; MAP, mean arterial pressure; WIT, warm ischaemia time.

Creatinine is corrected for body weight (per 40 kg), AST and H-FABP are corrected for kidney weight (per 100 g). Medians (interquartile range) is given.

* post-hoc Dunns shows a significant difference between control and xenon.

Table 2: Histological scoring of ischaemia-reperfusion injury in a pig kidney autotransplantation model with 60 min of warm ischaemia and 18 h of cold storage and receiving postconditioning with control gas, argon, and xenon.

Variable	**Control**	**Argon**	**Xenon**	**p-value**
Baseline	0 (0-0), (n=6)	0 (0-0), (n=6)	0 (0-0), (n=6)	>0.999
24h after transplantation (*)	5 (4-5), (n=5)	6 (5-10), (n=4)	7 (4.5-11), (n=5)	0.145
End of follow-up ($)	2.5 (1-4), (n=2)	4 (1-5), (n=4)	2.5 (2-4), (n=4)	0.748

Values are presented as median (inter quartile range).

Six morphological features (brush border integrity, aspect of the tubular cells, changes to the tubular nuclei, intratubular detachment, interstitial oedema, and interstitial inflammatory infiltrate) were assessed for morphological changes on periodic acid-Schiff stained sections according to a semiquantitative score: (0) no abnormality, (1) mild, (2) moderate, and (3) severe morphological changes.[9]

(*) in 2 cases the biopsy was of insufficient quality for PAS scoring, in 2 cases a safe ultrasound guided biopsy proved difficult and either none or only one biopsy (for tissue processing and not pathology) was taken.

($) in 2 cases (control group) the experiment was stopped before the 10 day follow-up period, in 4 cases the biopsy was of insufficient quality to be scored reliably, in 2 cases the biopsy was not taken.

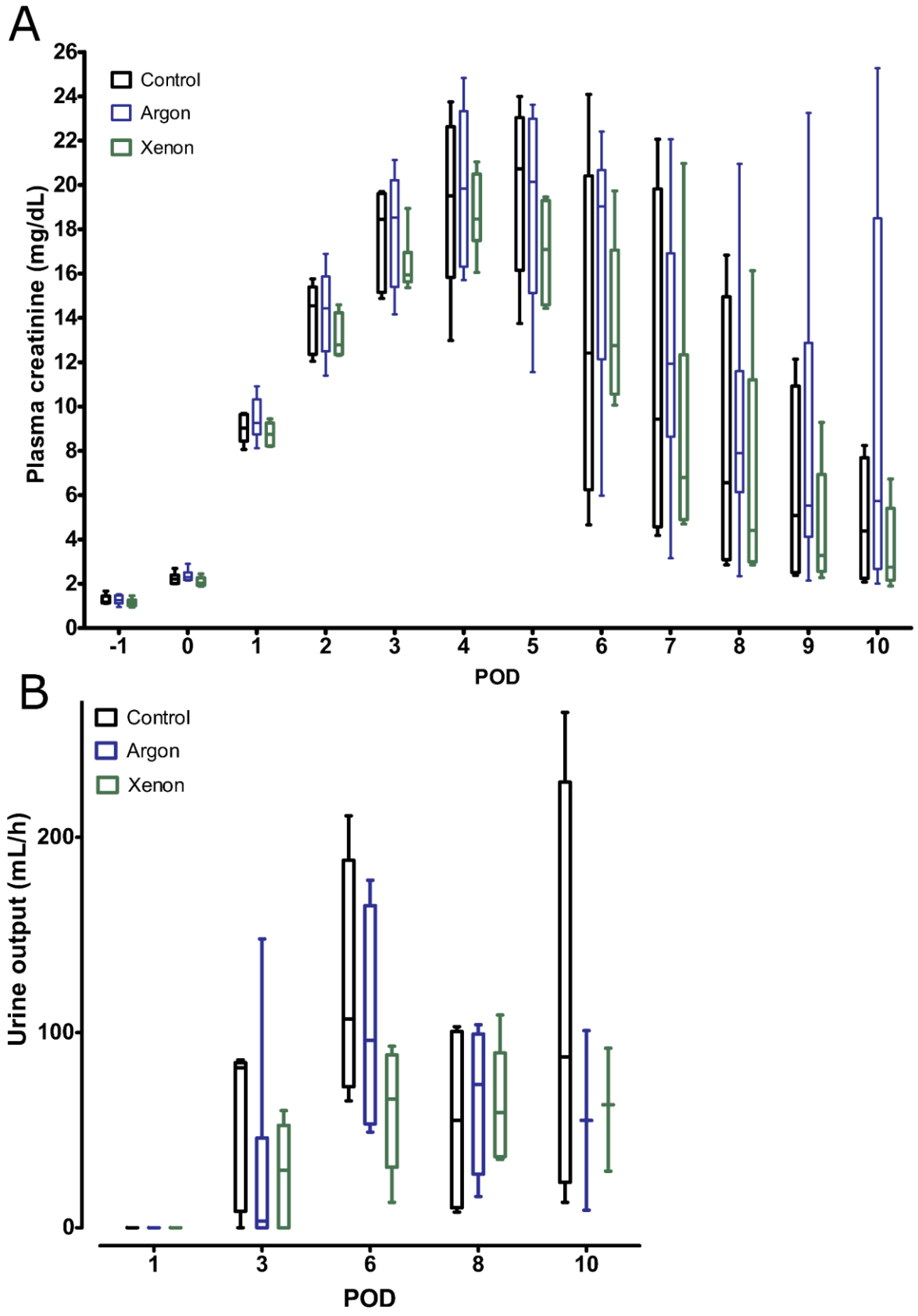
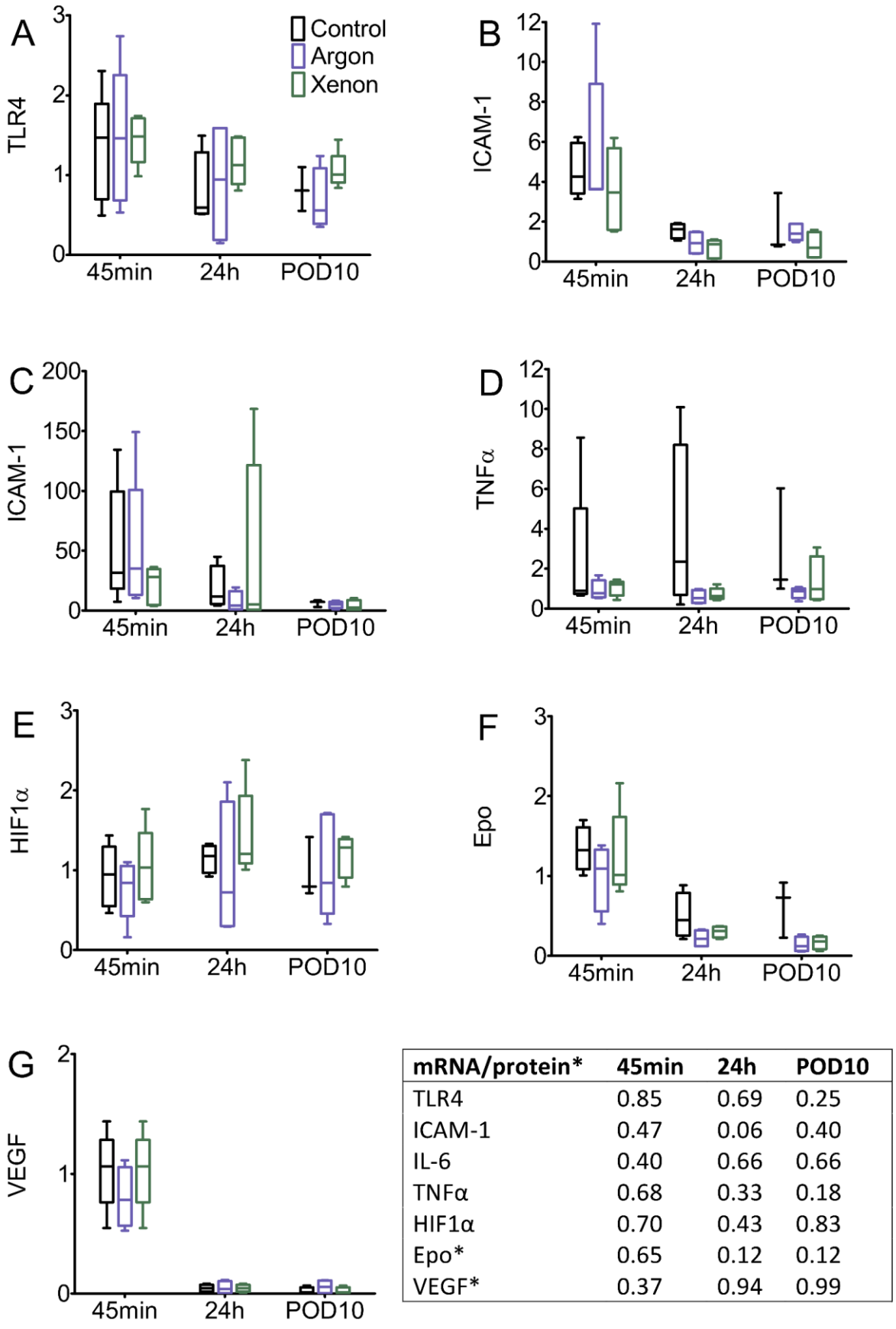

A
Control
Argon
Xenon
Plasma creatinine (mg/dL)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
-1
0
1
2
3
4
5
6
7
8
9
10
POD
B
Control
Argon
Xenon
Urine output (mL/h)
0
100
200
1
3
6
8
10
POD

Fig 1



mRNA/protein*	45min	24h	POD10
TLR4	0.85	0.69	0.25
ICAM-1	0.47	0.06	0.40
IL-6	0.40	0.66	0.66
TNFα	0.68	0.33	0.18
HIF1α	0.70	0.43	0.83
Epo*	0.65	0.12	0.12
VEGF*	0.37	0.94	0.99

Fig. 2

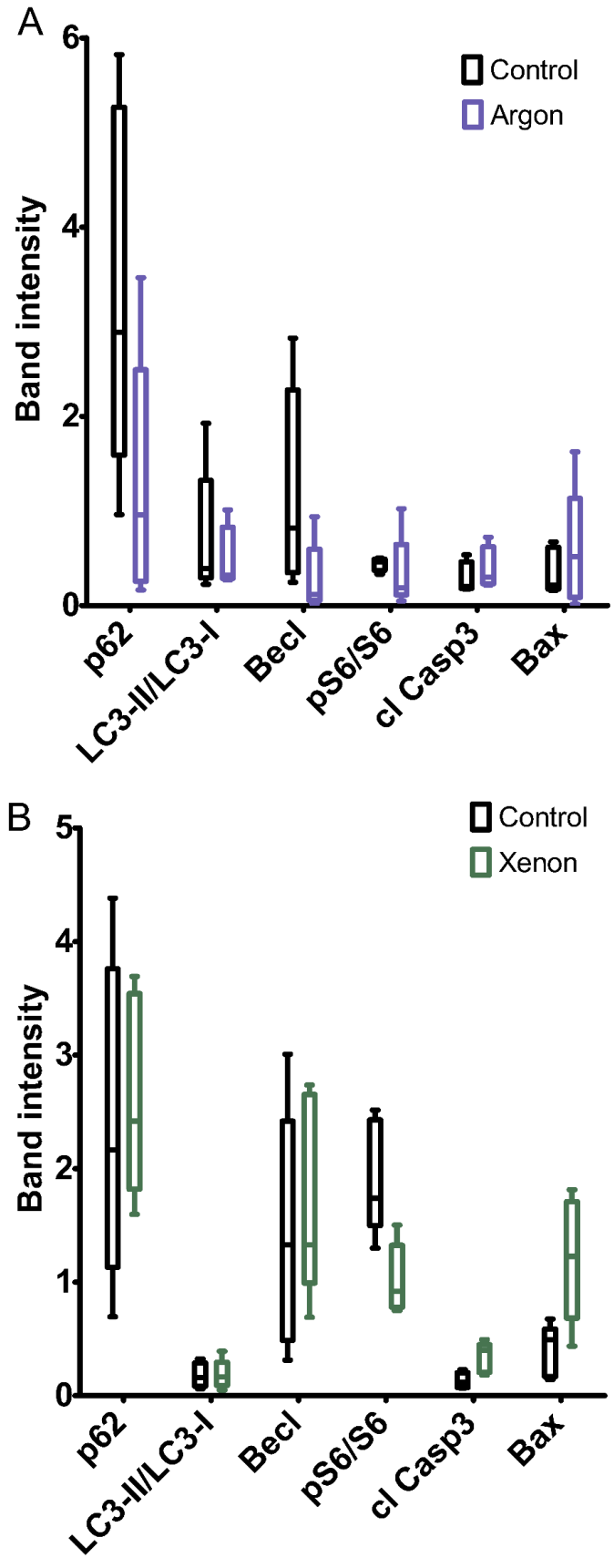

A
Band intensity
6
4
2
0
Control
Argon
p62
LC3-II/LC3-I
Becl
pS6/S6
cl Casp3
Bax
B
Band intensity
5
4
3
2
1
0
Control
Xenon
p62
LC3-II/LC3-I
Becl
pS6/S6
cl Casp3
Bax

Fig. 3

Supplementary appendix to

Postconditioning effects of argon or xenon on early graft function in a porcine renal autotransplant model

Supplementary Methods

Protein quantification

After tissue homogenisation, Bradford protein assay (Sigma-Aldrich, Diegem, Belgium) determined protein concentrations. Samples for Western Blot underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis after priming with a Laemmli buffer containing β-mercatoethanol (Mini-PROTEAN Tetra cell module with AnyKD Mini-PROTEAN TGX gels from Bio-Rad, Temse, Belgium). Utilising the semi-dry Trans-Blot Turbo Transfer system (Bio-Rad), proteins were blotted on polyvinylidene difluoride membranes. Membranes were blocked, incubated with the primary antibody overnight, washed, incubated with the secondary horseradish peroxidase coupled antibody and washed again. Immunoreactive bands were visualised through enhanced chemoluminescence (Pierce ECL Western Blotting Substrate; Perbio Science, Erembodegem, Belgium). Band intensity was quantified by Chemidoc MP System and Imagelab software (Bio-Rad). Primary antibodies: Anti-LC3 (5F10, Nanotools, Teningen, Germany), anti-S6, anti-phospho-S6, anti-cleaved caspase 3 (2217, 4858, 9664, Cell Signaling Technologies; Bioké, Leiden, the Netherlands), anti-Beclin 1 (LS-B3203, LifeSpan Biosciences; Bio-Connect, TE Huissen, the Netherlands), anti-Bax (sc-493, Santa Cruz Biotechnology, Bio-Connect), anti-SQSTM1/P62, and anti-GAPDH (P0067, G8715, Sigma-Aldrich NV, Diegem, Belgium) as an internal control. Secondary antibodies: anti-mouse IgG HRP-linked and anti-rabbit IgG HRP-linked (7076, 7074S, respectively, Cell Signaling Technologies, Bioké).

qPCR

After tissue homogenisation in TRIzol Reagent (ThermoFisher Scientific, Aalst, Belgium), total RNA was extracted (RNeasy isolation kit, Qiagen, Antwerp, Belgium) according to the manufacturer's instructions. cDNA was synthesised from 200ng RNA (Moloney Murine Leukemia Virus Reverse Transcripase, ThermoFisher Scientific, Aalst, Belgium). Real-time PCR reaction was performed on a LightCycler 96W system (Roche Diagnostics, Vilvoorde, Belgium) with Taqman Fast Universal PCR Master Mix and Taqman Gene Expression Assays for Hypoxia-Inducible Factor (HIF)1α (Ss03390447¬_m1) (ThermoFisher Scientific, Aalst, Belgium). For Tumour Necrosis Factor (TNF)α, Intercellular Adhesion Molecule (ICAM)-1, Interleukin (IL)-6, Toll-like Receptor (TLR)-4 and β-actin (housekeeping) primers and probes were designed (Kaneka EuroGentec, Seraing, Belgium, Table S1). A two-step amplification program was used: 95°C for 1 min followed by 45 cycles of amplification (95°C for 5 sec, 60°C for 30 sec). Target mRNA expression was quantified as fold change relative to the housekeeping gene and baseline tissue sample.

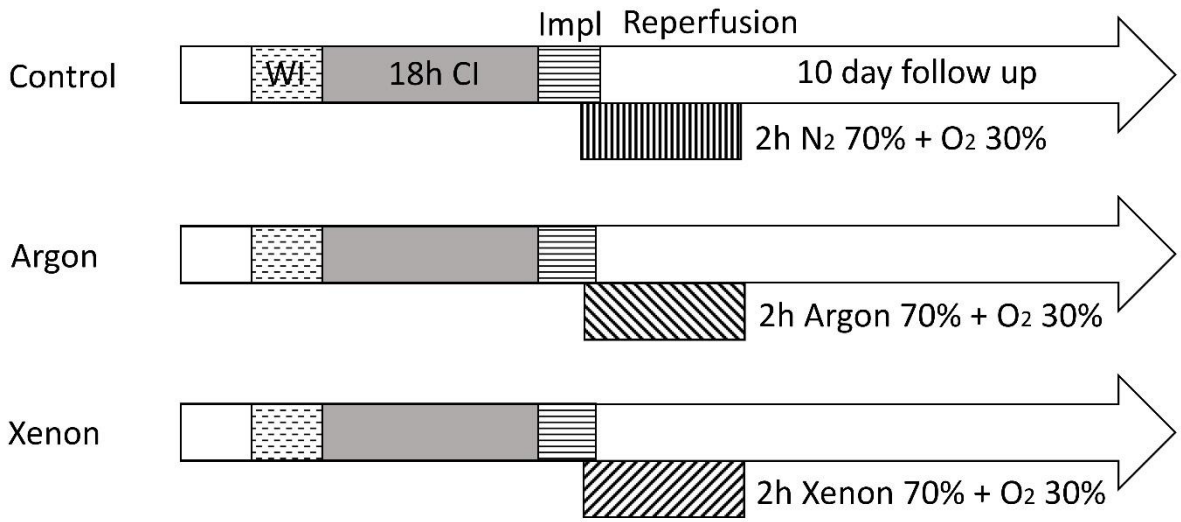


Fig. S1 Experimental design. After being subjected to 60 min of warm ischaemia, followed by 18h of cold ischaemia, the left kidney is transplanted into the donor animal after a right nephrectomy is performed. Five minutes before reperfusion treatment is started and is continued for 2h. Animals are followed up for 10 days.

CI, cold ischaemia; Impl, implantation; WI, warm ischaemia, N_2, nitrogen; O_2, oxygen

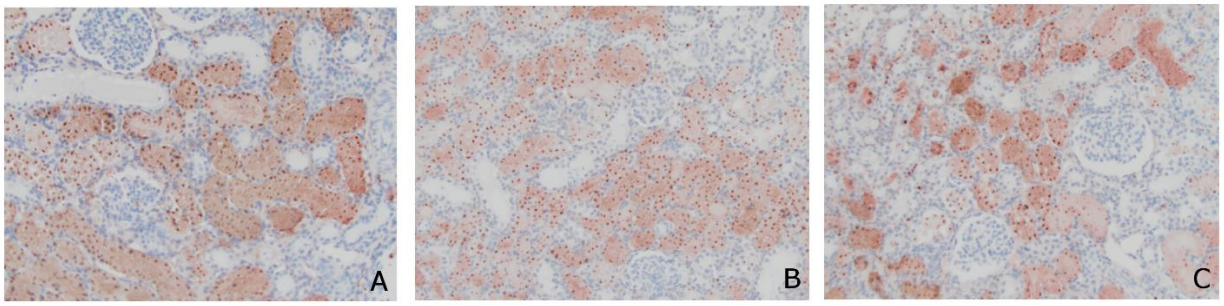


Fig. S2: Representative images of TUNEL in Control (Panel A), Argon (Panel B), and Xenon (Panel C). All images are taken at 20 times magnification.

Table S1 Sequence of Primers and Probes

	Sequentie probe	Seq. FW primer	Seq. REV primer	Product size (bp)	Reference seq.
Beta-actine	FAM-CGGCCCCTCCATCGTCCA-TAMRA	5'-AGAGCGCAAGTACTCCGTGT-3'	5'-AAAGCCATGCCAATCTCATC-3'	210	AY550069
	200 nM	300 nM	300 nM		
	70,10°C	60,08°C	60,04°C		
ICAM	FAM-ACCCCACCCCCAGGCCTC-TAMRA	5'-GGATGCCAGAGGACAGGATA-3'	5'-TTCCCAGTTGTGTGTTTCCA-3'	235	NM_213816.1
	200 nM	300 nM	300 nM		
	69,03°C	60,03°C	59,98°C		
IL6	FAM-TTCAGTCCAGTCGCCTTCTCCCTGG-TAMRA	5'-AGCTATGAACTCCCTCTCCACAA-3'	5'-GGCAGTAGCCATCACCAGAAG-3'	79	M80258.1
	300 nM	200 nM	200 nM		
	72,44°C	61,49°C	62,11°C		
TLR4	FAM-CCTGCACGCAAGGGTCCCAGCTC-TAMRA	5'-GCCTTTCTCTCCTGCCTGAG-3'	5'-AGCTCCATGCATTGGTAACTAATG-3'	83	NM_001113039.1
	300 nM	300 nM	300 nM		
	74,95°C	61,57°C	61,54°C		
TNFalpha	FAM-TGGCCTGGACAACCAGGCA-TAMRA	5'-CCCCTGTCCATCCCTTTATT-3'	5'-AAGCCCCAGTTCCAATTCTT-3'	200	NM_213969.1
	300 nM	300 nM	300 nM		
	68,51°C	60,01°C	59,94°C		

Table S2 Within group comparison of changes in expression of TLR-4, ICAM-1, IL-6, TNFα, HIF-1α mRNA expression and erythropoietin and VEGF protein levels. (Boxplots in Fig. 2 of the main text).

Group	Marker	Change between	estimate	p-value	95% CI	
Control	TLR-4	45 min - 24h	0,59	0,063	-0,05	1,22
		24h - POD 10	-0,01	0,974	-0,66	0,64
Argon	**TLR-4**	**45 min - 24h**	**0,77**	**0,012**	**0,22**	**1,31**
		24h - POD 10	0,19	0,469	-0,40	0,78
Xenon	**TLR-4**	**45 min - 24h**	**0,39**	**0,033**	**0,04**	**0,74**
		24h - POD 10	0,11	0,484	-0,24	0,46
Control	**ICAM-1**	**45 min - 24h**	**2,78**	**0,005**	**1,17**	**4,39**
		24h - POD 10	-0,30	0,676	-1,98	1,38
Argon	**ICAM-1**	**45 min - 24h**	**4,28**	**0,015**	**1,12**	**7,43**
		24h - POD 10	-0,56	0,706	-3,88	2,76
Xenon	**ICAM-1**	**45 min - 24h**	**0,77**	**0,023**	**0,14**	**1,39**
		24h - POD 10	-0,19	0,454	-0,77	0,39
Control	IL-6	45 min - 24h	42,94	0,160	-22,69	108,58
		24h - POD 10	9,40	0,751	-61,69	80,48
Argon	**IL-6**	**45 min - 24h**	**47,94**	**0,047**	**0,89**	**94,99**
		24h - POD 10	2,36	0,915	-47,36	52,07
Xenon	IL-6	45 min - 24h	15,96	0,578	-47,85	79,76
		24h - POD 10	44,53	0,145	-19,27	108,33
Control	TNFα	45 min - 24h	1,76	0,411	-2,96	6,49
		24h - POD 10	3,07	0,189	-1,85	7,99
Argon	TNFα	45 min - 24h	0,16	0,414	-0,27	0,59
		24h - POD 10	-0,27	0,213	-0,73	0,19
Xenon	TNFα	45 min - 24h	0,25	0,247	-0,21	0,71
		24h - POD 10	-0,07	0,718	-0,54	0,39
Control	HIF-1α	45 min - 24h	-0,06	0,832	-0,65	0,54
		24h - POD 10	0,18	0,529	-0,46	0,81
Argon	HIF-1α	45 min - 24h	-0,27	0,269	-0,80	0,26
		24h - POD 10	-0,06	0,827	-0,63	0,52
Xenon	HIF-1α	45 min - 24h	-0,18	0,560	-0,87	0,51
		24h - POD 10	0,22	0,465	-0,47	0,91
Control	**Epo**	**45 min - 24h**	**0,72**	**0,016**	**0,20**	**1,25**
		24h - POD 10	-0,12	0,588	-0,65	0,40
Argon	Epo	45 min - 24h	0,44	0,089	-0,10	0,98
		24h - POD 10	-0,06	0,802	-0,59	0,48
Xenon	**Epo**	**45 min - 24h**	**1,10**	**0,005**	**0,52**	**1,68**
		24h - POD 10	0,17	0,504	-0,42	0,76
Control	**VEGF**	**45 min - 24h**	**1,01**	**<0,001**	**0,68**	**1,33**
		24h - POD 10	0,02	0,891	-0,31	0,36
Argon	**VEGF**	**45 min - 24h**	**0,74**	**0,001**	**0,44**	**1,05**
		24h - POD 10	-0,01	0,946	-0,31	0,29
Xenon	**VEGF**	**45 min - 24h**	**1,01**	**<0,001**	**0,68**	**1,34**
		24h - POD 10	0,02	0,873	-0,33	0,38