**Mitochondria-targeted anti-oxidant MitoQ ameliorates ischemia-reperfusion injury in pig and human models of kidney transplantation**

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**Running Head**

Efficacy of MitoQ in ameliorating kidney transplant IR injury

**Keywords:** Ischemia-reperfusion injury, kidney transplantation, oxidative damage, mitochondria-targeted antioxidant MitoQ, *ex vivo* normothermic perfusion, pig model of transplant Ischemia-reperfusion injury, human model of transplant Ischemia-reperfusion injury

**Abstract**

**Background:** Ischemia-reperfusion (IR) injury makes a major contribution to graft damage during kidney transplantation. Oxidative damage to mitochondria is an early event in IR injury. Therefore the uptake, safety and efficacy of the mitochondria-targeted antioxidant MitoQ were investigated in pig and human models of transplant IR injury.

**Methods:** MitoQ uptake by warm and cooled pairs of pig and declined human kidneys was measured when preserved in cold static storage or by hypothermic machine perfusion (HMP). Pairs of pigs kidneys were exposed to defined periods of warm and cold ischaemia, flushed and stored at 4°C ± MitoQ (50nm–250µM), followed by reperfusion with oxygenated autologous blood on an *ex vivo* normothermic perfusion (EVNP). Pairs of declined human kidneys were flushed and stored ± MitoQ (5µM–100µM) at 4°C for 6h and underwent EVNP with ABO group-matched blood.

**Results:** Stable and concentration-dependent uptake of MitoQ was demonstrated for up to 24h in pig and human kidneys. Total blood flow and urine output were significantly higher in the 50µM MitoQ-treated pig kidneys compared to the controls (p=0.006 and p=0.007 respectively). In proof-of-concept experiments, blood flow at 1h of EVNP was significantly higher in the 50µM MitoQ-treated human kidneys compared to controls (p=<0.0001). Total urine output was numerically higher in the 50µM MitoQ group compared to the control, but the differences did not reach statistical significance (p=0.054).

**Conclusion:** Mitochondria-targeted antioxidant MitoQ can be simply and effectively administered to ischemic pig and human kidneys during cold storage and may improve outcomes after transplantation.

**Introduction**

The relative shortage of organs over the last 10 years has necessitated the increasing use of less-than-ideal organs. Although primary non-function of kidney grafts is rare, it is associated with significant risks of recipient morbidity and mortality (1). Conversely, delayed graft function is common and can occur in up to 50% of kidney transplant recipients, necessitating a period of dialysis and longer in-patient stay (2). The injury endured by kidneys during transplantation also increases the risk of immunological rejection and contributes to long-term graft dysfunction (3). Consequently, two major challenges for increasing availability and improving outcomes of kidney transplantation are the development of interventions to increase the pool of transplantable organs by enabling utilization of organs that are currently rejected; and by improving the function and survival of currently transplanted organs.

One way to achieve these goals is to decrease the ischemia-reperfusion (IR) injury that is inevitably associated with organ transplantation. IR injury occurs when a tissue is exposed to a period of ischemia before reperfusion. Upon reperfusion there is a mitochondrial burst of reactive oxygen species (ROS) that initiates mitochondrial dysfunction, cellular injury and ultimately organ damage (4-6). The resulting mitochondrial dysfunction can also activate the innate immune system through release of damage associate molecular patterns (DAMPs) such as mitochondrial DNA (mtDNA) that augment the immune response that underlies organ rejection (7, 8).

Many marginal organs are currently not transplanted because factors such as age or background chronic pathology make them unlikely to survive the IR injury inherent in transplantation, or to retain function for years in the recipient (9, 10). Moreover, an increasing proportion of organs are now retrieved following deceased donation after circulatory death (DCD), when the organs are exposed to a period of warm ischemia before perfusion with cold preservation solution. This additional period of warm ischemia significantly increases the IR injury subsequently endured by the organs and doubles the likelihood of delayed graft function after kidney transplantation (11). This is in contrast with donation after brainstem death (DBD) when normoxemic organs can be perfused with cold preservation solution immediately after cessation of circulation. Therefore, preventing IR injury could extend the useful lifetime of transplanted organs in the recipient and also increase the pool of organs that can be used.

Conventional anti-oxidants have not previously been effective in preventing mitochondrial oxidative damage in clinical scenarios (12, 13), in large part because they are not rapidly taken up into mitochondria. Thus mitochondria-targeted antioxidants were developed, the most prominent of which is MitoQ (14, 15). MitoQ consists of a targeting lipophilic triphenyl phosphonium (TPP) cation linked to a ubiquinone moiety **(Fig. 1A).** Driven by the mitochondrial membrane potential, the TPP cation permits rapid uptake into cells and several-hundred fold accumulation within the mitochondrial matrix. The ubiquinone is then recycled by complex II to the active antioxidant ubiquinol that prevents oxidative damage (14, 15).

In previous mouse models it was shown that preloading an organ with MitoQ prior to reperfusion protects against mitochondrial oxidative damage and IR injury in the heart and kidney (16, 17). Here, these findings were translated to pig and human models of IR injury using an *ex vivo* normothermic perfusion (EVNP) circuit to reperfuse kidneys with oxygenated blood **(Fig. 1B),** thereby mimicking the IR damage inherent in transplantation. Pairs of pig **(Fig. 1C)** and declined human kidneys **(Fig. 1D)** were exposed to ischemia and stored at 4°C, with or without administration of MitoQ, followed by reperfusion**.** When MitoQ was administrated to ischemic kidneys during cold storage, MitoQ improves organ function and downregulates expression of inflammatory pathway genes. To our knowledge, this is the first study to demonstrate the efficacy of a mitochondria-targeted anti-oxidant in ameliorating IR injury in large animal and human models of kidney transplantation. This study provides large animal and proof-of-concept human model data that will prim the design of future in vivo studies and clinical trials for assessing the safety and efficacy of MitoQ in human kidney transplantation.

## Materials and Methods

### *MitoQ and control compound*

MitoQ ([10- (4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl) decyl] triphenylphosphonium methanesulfonate) was used complexed with β -cyclodextrin supplied by Antipodean Pharmaceuticals Inc and dissolved in organ preservation solution (Soltran®, Baxter Healthcare) for *ex vivo* use during organ preservation. MitoQ and control compound preparation is described in supplementary material.

***Preservation solutions***

The composition of Hyperosmolar citrate (Soltran®) solution, University of Wisconsin (Viaspan®) solution and KPS-1® solution are shown in supplementary material.

***Pig kidneys***

The surgical procedure for obtaining blood and kidneys from terminally-anaesthetized pigs is described in supplementary material.

***LifePort kidney transporter***

The use of the LifePort kidney transporter (Organ Recovery Systems) for hypothermic machine perfusion of kidneys is described in supplementary material.

***Human Kidneys***

Full ethical approval (research ethics committee reference number: 12/EE/0202) and informed consent was obtained for the use of human kidneys in the study. Human kidneys were selected for use in this study as described in supplementary material.

***Ex Vivo Normothermic Perfusion Circuit***

The *Ex vivo* normothermic perfusion (EVNP) apparatus is a modified pediatric cardiopulmonary bypass circuit that supplies a single pig or declined deceased human kidney with oxygenated and heated blood as shown in Figure 2.2 and in supplementary material.

***Uptake of MitoQ***

MitoQ concentration in pig and declined deceased human kidneys was measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) relative to a deuterated internal standard ($d_{15}$-MitoQ) (18) as described in supplementary material.

***Bulk RNA sequencing***

Bulk RNA sequencing was performed as described in supplementary material.

***Measurement of mtDNA release***

The release of mitochondrial and nuclear DNA into the circulation was assessed using the droplet

digital polymerase chain reaction (ddPCR) as described in supplementary material.

### *Histopathology*

Pig and human kidneys samples were fixed in 10%formalin, sectioned, stained with haematoxylin and eosin. Histopathological assessment of pig and human kidneys tissue biopsies was performed as described in supplementary material.

### *Statistical Analysis*

Kidneys were randomly assigned to different treatment groups, unless stated, and the data were analyzed blinded to the group identities. Data are presented as mean ± standard deviation (SD), unless otherwise stated. The difference between a pair of kidneys was analyzed using the two-tailed paired t-test. In cases where several conditions were compared, one-way analysis of variance (ANOVA) was used. In all subgroup analysis, p values were adjusted by multiple testing correction as specified in the figure legends. Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA). All tests were performed at the 5% significance level.

## Results

### *Model optimization for assessment of pig kidney IR injury using EVNP*

During a typical kidney transplant from a DCD donor there is a period of ~12 min warm ischemia during organ retrieval (19), followed by flushing of the organ with cold preservation solution and several hours of storage at ~4˚C. In many pig models of transplantation the animal is sacrificed by an approved method such as cerebral injury and exsanguination before the organ is removed. In our pilot experiments, this approach resulted in significant hemodynamic variability and inconsistent ischemic injury to the kidneys (data not shown). Therefore, pig kidneys were removed under terminal general anesthesia in order to establish a consistent and reproducible large animal model. Importantly, the aim of the study was not to accurately model DCD transplantation, but rather develop a model in which kidneys were exposed to consistent periods of warm and cold ischaemia prior to reperfusion. Furthermore, to minimize variation, two kidneys were compared from the same pig. Kidneys were exposed to periods of warm ischemia time (WIT), followed by flushing with cold preservation solution and storage at ~4˚C for periods of cold ischemia time (CIT). The two kidneys

from the same pig were then placed on two EVNP circuits and perfused simultaneously with autologous pig blood. This enabled one kidney from each pig to act as a control, thereby minimizing biological variation and enabling paired statistical analysis.

The function of left and right pig kidneys were compared on EVNP and opted to use right and left kidneys alternately in experimental groups to avoid any bias, as described in supplementary material **(Suppl. Fig 1)**. Then pig kidneys were exposed to different combinations of warm and cold ischaemia time. Based on the data shown in supplementary material **(Suppl. Fig 2)**, decision was taken to examine the effect of MitoQ on pig kidneys that had been exposed to 10 min WIT and 10h CIT, as this condition induced significant but not overwhelming organ damage.

***Administration and uptake of MitoQ by pig kidneys during organ preservation***

How effectively MitoQ was taken up by pig kidneys was examined first, as described in detail in supplementary material. When administered after the onset of ischemia, MitoQ was taken up in a concentration dependent manner and its tissue concentration remained stable over at least 24h of cold static storage **(Fig. 2A)**. MitoQ was also efficiently taken up by cold pig kidneys, but to approximately half the levels when administered to normothermic kidneys, and its tissue concentration remained stable over the following 12h **(Fig 2B)**. In some transplant centers, kidneys awaiting transplantation are transported and/or stored using a hypothermic machine perfusion (HMP) device, which allows the continuous perfusion of the kidney with cold preservation solution (20, 21). There was greater MitoQ uptake in kidneys that underwent HMP, most of which occurred within 6h of start of HMP and MitoQ tissue concentrations remained stable for at least 24h **(Fig. 2C)**. EVNP is yet another emerging method for preservation of kidneys while awaiting transplantation (20, 21). The tissue concentration of MitoQ in the kidney remained stable during 6h of EVNP **(Fig. 2D)**. It is noteworthy that EVNP is a closed, recirculating system, and that when administered to mice *in vivo*, the half-life for loss of MitoQ from the liver and kidneys is ~3h after a bolus i.v. injection (22). In summary, when administered immediately after the onset of ischemia, MitoQ is taken up by pig kidneys in a predictable and dose-dependent manner over a wide range of concentrations (5nM to 100μM; **Fig. 2E**). The tissue concentrations of MitoQ remain stable during subsequent cold storage or EVNP. MitoQ is also effectively taken up when administered to kidneys that are already in cold storage, although to approximately half the level achieved when administered to warm kidneys. Conversely, when administered during HMP, uptake of MitoQ is approximately twice that during CSS

(**Fig. 2F**). For clinical translation, therefore, the dose of MitoQ may need to be adjusted depending on whether MitoQ is administered at the time of retrieval at donor center (ie, to warm kidneys) or at the recipient center (ie, to cold kidneys), whether the kidneys are stored using CSS or HMP.

***Protection of pig kidney function by MitoQ***

The protective effect of MitoQ on pig kidneys at concentrations of 500nM, 5μM, 50μM, 100μM and 250μM was examined next. Pairs of pig kidneys from the same animal were exposed to 10 min WIT and randomized by alternating the order of right and left kidneys used in experimental groups to be flushed with cold Soltran® (500 ml) ± MitoQ. The paired kidneys were stored at 4°C for 10h and underwent 6h of EVNP simultaneously. Kidneys were perfused with autologous blood at set pressure of 80–90 mmHg (pump speed 1500 rpm). MitoQ did not improve kidney function at concentrations of 500nm or 5μM (**Suppl. Fig. 3A-E**).

Pig kidneys perfused with 50μM MitoQ had a normal appearance at the end of 6h of EVNP (**Fig. 3 A-B**). Compared to control kidneys treated with Soltran® alone, renal blood flow in the 50μM MitoQ group increased after 150 minutes of EVNP (**Fig. 3C).** To confirm that the effects of MitoQ were not due to the carrier molecule, the effects of the compound triphenyl methyl phosphonium (TPMP), which lacks the ubiquinone moiety were examined. 50μM TPMP had no effect on renal blood flow (**Fig. 3D**). 50μM MitoQ resulted in an increase in total renal blood flow (**Fig. 3E**) and total urine output (**Fig. 3F**). Creatinine clearance was almost doubled in the 50μM MitoQ group (31.6±11.4 vs. 16.1±4.1ml/min/100g AUC) but the difference did not reach statistical significance (p=0.065; paired t-test, **Fig. 3G**). 50μM MitoQ had no effect on fractional excretion of sodium (**Fig. 3H**). Compared to Soltran® alone, the control compound TPMP had no effect on total renal blood flow (**Fig. 3I**), total urine output (**Fig. 3J**), creatinine clearance (**Fig. 3K**) or fractional excretion of sodium (**Fig. 3L**).

MitoQ at 100μM caused changes in kidney function that were qualitatively similar to those at 50μM, with an increase in blood flow (**Suppl. Fig. 3A-B**), but no significant difference in urine output, creatinine clearance or fractional excretion of sodium (**Suppl. Fig. 3C-E**). When perfused with 250μM MitoQ, pig kidneys (n=2) developed progressively more abnormal appearance during 6h of EVNP with areas of poor perfusion and discoloration (**Suppl. Fig. 4A-E**). In summary, there was a dose-dependent effects of MitoQ on renal blood flow and urine production (**Suppl. Fig 5**).

As described in supplementary material, there were no significant differences between the control group (n=4) and the 50µM MitoQ group (n=4) in the total histopathology injury score (**Suppl. Table 1** and **Suppl. Fig. 6**) for the pig kidney biopsies at the end of CIT or 6h of EVNP. The histopathological manifestations of recovery from ATN depends on the kidney's ability to carry out cellular repair and regeneration to overcome the structural damage to the renal parenchyma and cellular death induced by IR injury. As the recovery from ATN may continue for several days or weeks, it is likely that 6h of EVNP is insufficient to allow any potential beneficial effects of MitoQ to manifest on histological assessment.

***Protection of human kidney function by MitoQ***

Next, the uptake of MitoQ by human kidneys that were retrieved for transplantation but subsequently declined was examined, as described in supplementary material. Declined deceased human kidneys, which were received after a variable length of CIT, were flushed with 500 ml Soltran® cold preservation solution supplemented with MitoQ at 5µM (n=3), 50µM (n=3) or 100µM (n=3). The donor demographics are shown in **Suppl. Table 2**. MitoQ was taken up efficiently by the cold deceased human kidneys in a dose-dependent manner and tissue MitoQ concentrations remained stable during the subsequent 6h of cold static storage (CSS) and 6h of EVNP (**Fig. 4A**).

Pairs of declined human kidneys from the same donor to test the safety and efficacy of MitoQ on kidney function were used, thus controlling for donor variables and cold ischemia times **(Suppl. Table 3**). One kidney from each pair was randomized to receive Soltran® supplemented with 50µM MitoQ and the contralateral control kidney received Soltran® only. The kidneys were subsequently stored at 4°C for 6h and then placed simultaneously on two EVNP circuits and perfused with ABO-compatible packed red cells for 3h (n=3 pairs) or 6h (n=4 pairs).

The renal blood flow remained stable during EVNP in the 50 µM MitoQ group and was significantly higher in the MitoQ group compared to the control group at the end of the first hour of EVNP (**Fig. 4B**), but not during the whole 3h of EVNP (**Fig. 4C**). The total urine output (**Fig 4D**) and creatinine clearance (**Fig. 4E**) were numerically higher in the MitoQ group compared to the control group but the difference did not reach statistical significance during 3h of EVNP. The fractional excretion of sodium was similar in the MitoQ and control groups (**Fig. 4F**). Although a trend towards protection was evident, MitoQ treatment did not have a statistically significant effect on the absolute levels of circulating mtDNA levels (**Fig. 4G**) or as a proportion of circulating nuclear DNA levels (**Fig. 4H**).

Increasing MitoQ concentration to 100µM did not have any additional protective effects on the endpoints examined (**Suppl. Fig 7**).

The effect of 50µM MitoQ perfusion on gene expression in 4 pairs of human kidneys that underwent EVNP was examined (**Suppl. Fig. 8**). Treatment with MitoQ significantly attenuated global changes in gene expression associated with EVNP, as evidenced by the fact that there were many genes significantly up or down regulated in the Soltran© group, and none in kidneys treated with MitoQ (**Fig. 5A**). Next, the change in expression of groups of genes within a common pathway rather than individual genes was assessed to identify important molecular pathways affected by the addition of MitoQ. This gene set enrichment analysis (GSEA) demonstrated that the addition of MitoQ had a substantial impact on a number of immune and inflammatory pathways (**Fig. 5B**). In particular, there was a marked reduction in genes involved in TNFα signaling via NF-κB, which included a number of pro-inflammatory molecules such as TNF and IL15RA (**Fig 5C**).

Finally, the degree of histopathological injury to human kidneys treated with Soltran®±MitoQ in a wedge biopsy taken at the end of CIT (baseline) and at the end of EVNP (3 or 6h) was examined (**Suppl. Table 4**). Similar to pig kidneys, there were no significant differences between the control group (n=7) and the MitoQ group (n=7) in the total histopathology injury score for the human kidney biopsies at the end of CIT or at the end of EVNP (**Suppl. Fig. 9**).

**Discussion and Conclusion**

Our data demonstrate that MitoQ is taken up by pig and human kidneys in a dose-dependent manner, and that tissue concentrations remain stable over several hours during cold-static storage, hypothermic machine perfusion or *ex vivo* normothermic perfusion. The eventual cold ischemia time prior to implantation can be difficult to predict at the time an organ is retrieved from the donor. The stability in the concentration of MitoQ after administration to the kidney is, therefore, clinically important; it would allow a therapeutic concentration to be achieved at the time of reperfusion irrespective of unforeseen changes in cold ischemia time. Compared to administration to cold kidneys, MitoQ tissue uptake is greater when it is administered to warm kidneys or to kidneys undergoing HMP. These results suggest that in clinical practice, MitoQ can be administered to the

kidney at the donor hospital, during transport or at the recipient hospital, although some adjustment in dosing may be required depending on the method of organ preservation.

Administration of MitoQ to the isolated kidney has the advantage that it avoids systemic absorption of MitoQ in the donor and or recipient, thus minimizing safety concerns. It is noteworthy, however, that MitoQ has been safely administered to human subjects in three phase II clinical trials (23-25) and is therefore a good candidate for clinical translation for the treatment of IR injury in kidney transplantation.

Administration of MitoQ to pig kidneys resulted in a dose-dependent improvement in renal function as measured by an increased renal blood flow, urine production and creatinine clearance. The protective effect was not observed when the carrier molecule TPP lacking the ubiquinone moiety was administered. The beneficial effects of MitoQ were consistent with our previous findings in mouse models of kidney and heart IR injury (16, 17).

It should be noted that our experimental design did not aim to model DCD transplantation precisely. Specifically, our model intentionally lacked the agonal phase after withdrawal of treatment and before circulatory arrest, which can take a variable time and can lead to exposure to inconsistent durations of warm ischaemia. Instead, the aim was to expose kidneys to consistent and reproducible periods of warm and cold ischaemia, which would be ideally suited for examining the uptake, safety and efficacy of MitoQ.

The human kidneys used in this study were, by necessity, declined for transplantation. The reasons for the decline of the kidneys included renal malignancy in kidneys with otherwise normal function, age, past medical history and poor perfusion of the kidney at the donor hospital by preservation solution. Some of the reasons for decline, such as presence of malignancy, would not be expected to affect the findings of the study. Moreover, using pairs of declined human kidneys, wherein each kidney in a pair was randomized to receive MitoQ or control, controlled for donor variability . Nonetheless, these limitations mean that the human kidneys used in these experiments were less than ideal and that 'better quality' transplanted kidneys may respond differently to MitoQ treatment. Despite these limitations, the effects of MitoQ on human kidneys were consistent with those demonstrated using pig kidneys and confirm the therapeutic potential of MitoQ in reducing IR injury in human kidney transplantation.

This improvement in human kidney function was associated with a downregulation of inflammatory pathways including TNFα signaling via NF-κB, TNF and IL15RA. These findings are consistent with the anti-inflammatory effects of MitoQ reported in previous studies. MitoQ has been shown to reduce cigarette-smoke extract-induced expression of the NLRP3 inflammasome, cleaved caspase-1 and IL-1β and activation of the cytosolic content of p-IκBα and the expression level of nuclear protein NF-κB p65 in endothelial cells (26). MitoQ reduced TNF-a-induced CXCL8 release from airway smooth muscle cells (27) and activation of the NLRP3 inflammasome and inflammatory cytokines IL-1 and IL-18 in models of colitis (28). MitoQ reduced NF-κB p65 and TNFα signaling in models of diabetes (29), reduced TNF-a and IL-6 levels following haemorrhage (30) and reduced NLRP3 activation and IL-1β secretion by mononuclear cells in response to ethanol (31). The present study confirms that the anti-inflammatory effects of MitoQ previously reported in animal models and in vitro can also be demonstrated at human whole organ level. Further studies are required to examine whether the short-term anti-inflammatory effects of MitoQ observed lead to long-term reduction in graft dysfunction.

A limitation of this study is that the duration of EVNP was restricted to 6h, which did not permit the investigation of long-term sequelae of IR injury such as histopathological recovery of kidneys from acute tubular necrosis and may miss any therapeutic efficacy or toxicity of MitoQ that could happen beyond this early reperfusion phase. Moreover, the kidneys did not endure the alloimmune response that is inherent in human kidney transplantation. The long-term safety and efficacy of MitoQ should therefore be assessed using an allogenic heterotopic pig kidney transplant model. Despite these caveats, this is the first study to identify MitoQ as a promising agent for amelioration of IR injury in pig and human models of kidney transplantation and successfully defines its mode of administration and therapeutic window, thus priming the design of future *in vivo* pig studies and human clinical trials.

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**Author Contributions**

M.H., M.P.M. and K.S-P. designed the experimental protocols. M.H. performed the experiments. K.C., D.F, C.F, N.G., A.J.D., J.M., A.B., S.A.H, M.L.N. and K.S-P. assisted with pig experiments and *ex vivo* perfusion experiments. A.L., A.V.G, T.E.B., A.M.J and A.M.G. assisted with mitochondrial assays. J.R.F. and M.R.C. performed and analysed RNA sequencing experiments. S.T. performed histopathological analysis of the samples. N.D. assisted with data analysis and presentation. M.H, M.P.M. and K.S-P. wrote the manuscript with assistance from all other authors.

**Fig. 1.**

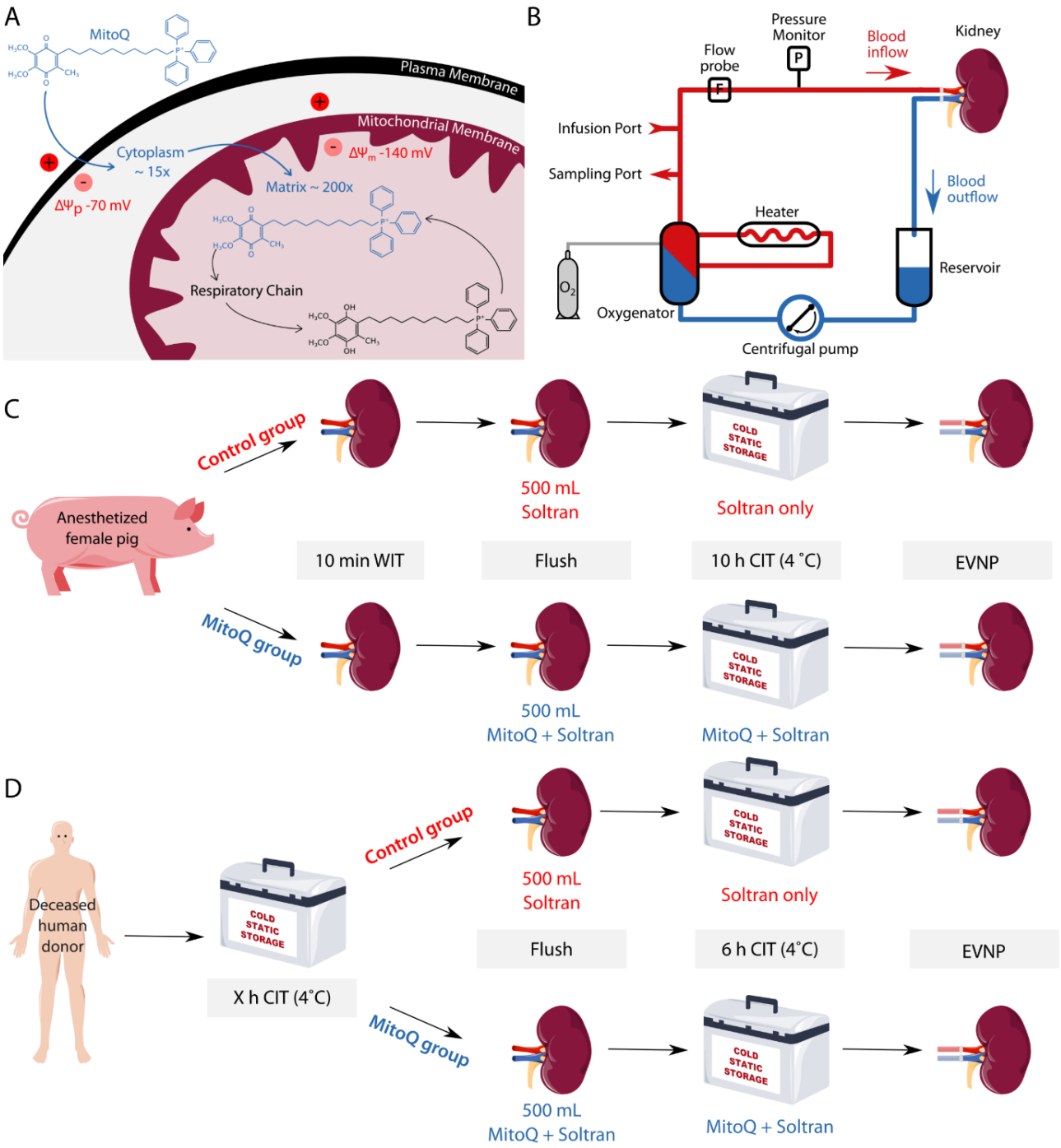



**Fig. 1. Experimental Design. (A)** The mitochondria-targeted antioxidant MitoQ is accumulated within mitochondria during organ storage, driven by the plasma membrane potential and the mitochondrial membrane potential. Within mitochondria, MitoQ is reduced to the antioxidant ubiquinol form that decreases oxidative damage to the mitochondria, thereby minimising IR

injury upon reperfusion. **(B)** The *ex vivo* normothermic perfusion circuit uses paediatric cardiopulmonary bypass technology to circulate blood using a centrifugal pump. The perfusate is warmed using a heat exchanger and oxygenated with a membrane oxygenator. Perfusion pressure is set at the onset of the experiment and flow rates monitored using an in-line flow probe. **(C)** Pairs of pig kidneys were removed under terminal general anaesthesia, exposed to 10 min of WIT at body temperature, then flushed and stored with cold preservation solution (Soltran®) with or without MitoQ at ~ 4˚C for 10h. The kidneys were then placed simultaneously on two EVNP circuits and perfused with normothermic oxygenated autologous blood for up to 6h. **(D)** Pairs of declined deceased human kidneys were received after having been exposed to a variable period of ischemia. The human kidneys were flushed and stored in Soltran® with or without MitoQ at ~4˚C for 6h. The kidneys were then placed simultaneously on two EVNP circuits and perfused with normothermic oxygenated ABO-compatible donated human blood (packed red cells).

**Fig. 2.**

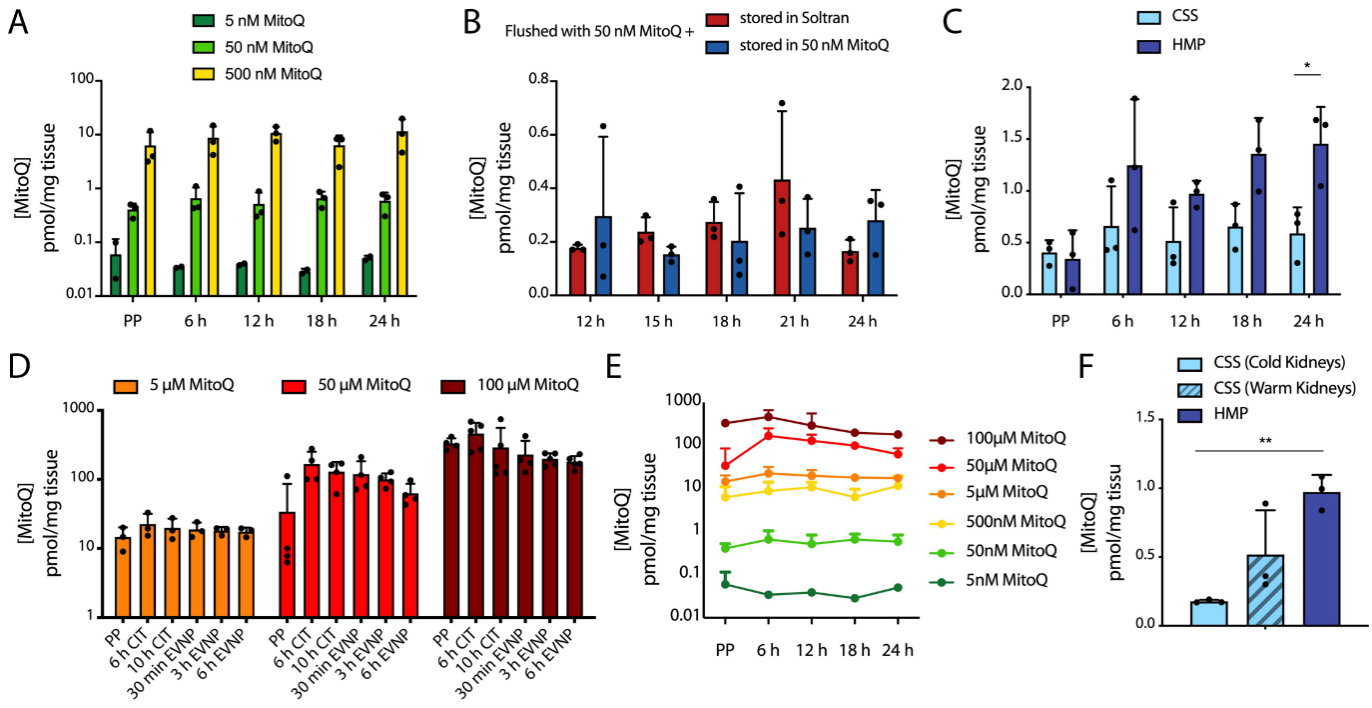



**Figure 2. Uptake of MitoQ by pig kidneys. (A)** Pig kidneys were removed under general anaesthesia, exposed to 10 min of warm ischemia and then flushed with 500 ml cold Soltran® supplemented with 5, 50 or 500nM of MitoQ and then stored in 500 ml cold Soltran® containing the same concentration of MitoQ (n=2 - 3 per group). MitoQ was taken up in dose-dependent manner and its tissue concentrations remained stable over at least 24h of CSS. PP: Post perfusion with MitoQ **(B)** MitoQ was effectively taken up when administered to cold kidneys that had already been in CSS for 12h. Thereafter, the tissue concentration of MitoQ remained stable up to at least 24h. After flushing with MitoQ, storage of kidneys in MitoQ or Soltran® alone did not have a significant effect on tissue MitoQ concentrations (n=3 per group). **(C)** There was a significantly higher uptake of 50nM MitoQ by kidneys at the end of 24h cold preservation when kidneys were preserved by HMP, which involved recirculation through the kidney of a solution containing MitoQ at 4°C (1.5±0.4 vs. 0.6±0.3 pmol/mg; p=0.024 repeated measures 2-way ANOVA followed by Sidak multiple comparisons test; n=3 per group). **(D)** MitoQ was taken up by warm kidneys in a dose-dependent manner at 5µM (n=4), 50µM (n=4) and 100µM (n=5; p<0.001; repeated measures two-way ANOVA followed by Tukey multiple comparisons test). MitoQ tissue concentrations were stable for up to 10h

CSS and during 6h of subsequent EVNP. **(E)** MitoQ was taken up by kidneys in a concentration-dependent manner when administered to warm kidneys after 10 min WIT over a range of 5nM to 100µM. Data on 5-500nM are from panel A. **(F)** At the end of 24h, compared to administration to warm kidneys followed by CSS, MitoQ tissue concentrations were higher when kidneys were stored in HMP and lower when MitoQ was administered to cold kidneys using 50nM MitoQ concentration (p=0.007, ANOVA; data are replotted from values in A-C). Data are mean±SD; * p≤0.05, ** p≤0.01.

**Fig. 3.**

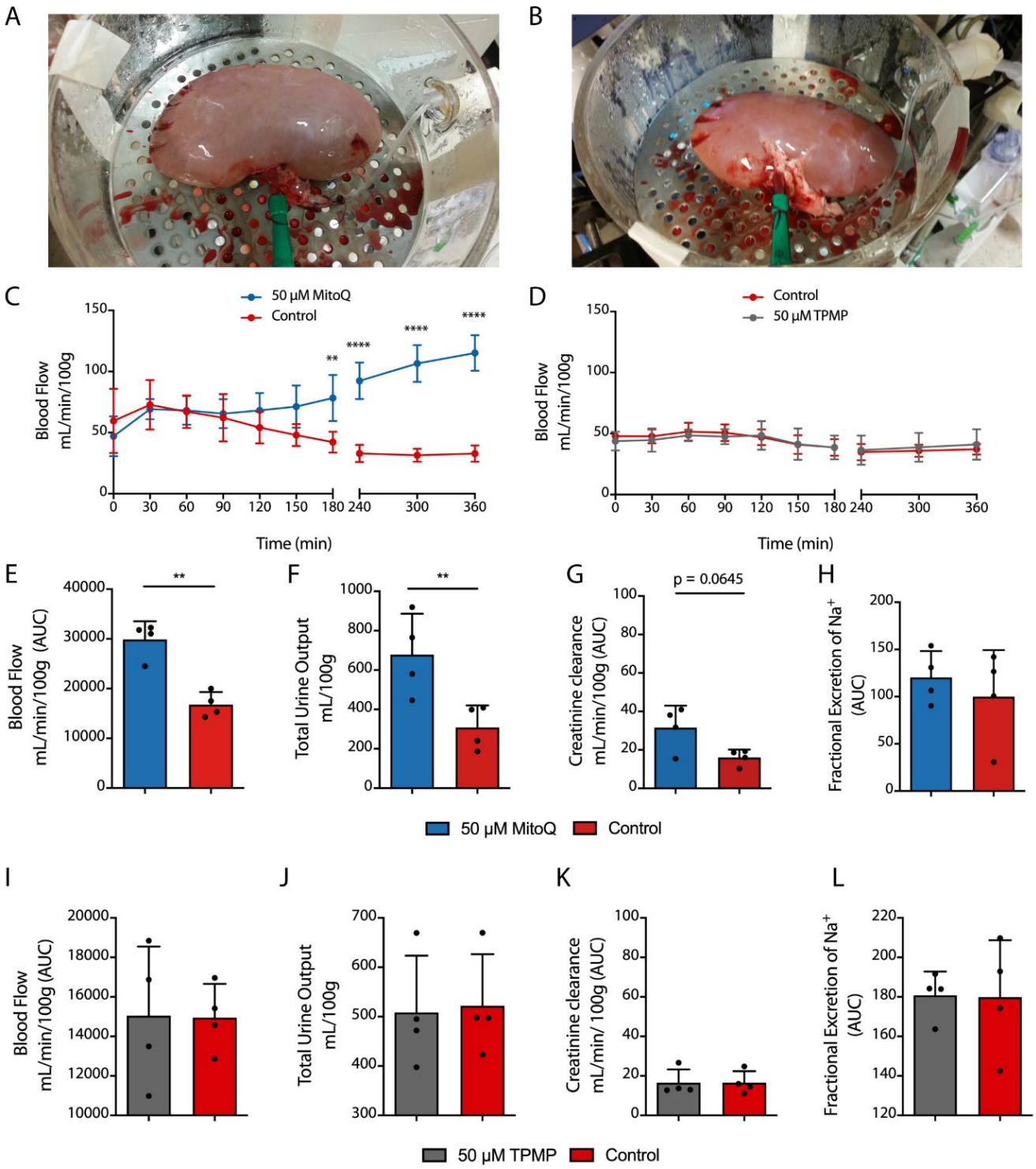



**Figure 3**. **Efficacy of 50µM of MitoQ on pig kidney function.** Pairs of pig kidneys were exposed to 10 min of warm ischemia, then flushed with and stored in 4°C Soltran® supplemented with or without 50µM MitoQ for 10h, followed by 6h of EVNP (n=4 per group). Kidneys treated with MitoQ **(A)** and Soltran only **(B)** had a normal appearance after 6h of EVNP

(representative images). Towards the end of EVNP, renal blood flow was significantly higher in kidneys treated with 50µM MitoQ **(C)** but not TPMP **(D)** (n=4). Total blood flow **(E)** (AUC, p=0.006; paired t-test) and urine output **(F)** (p=0.007; paired t-test) was significantly higher in the 50µM MitoQ group compared to the control group). **(G)** Creatinine clearance was numerically higher in the 50µM MitoQ group compared to the control during 6h of EVNP, but the difference did not reach statistical significance (31.6±11.4 vs. 16.1±4.1ml/min/100 g AUC; p=0.065; paired t-test). **(H)** There was no significant difference in fractional excretion of sodium between the 50µM MitoQ group and the control group. Compared to Soltran® alone, perfusion with the control compound TPMP did not affect total renal blood flow **(I)**, total urine output **(J)**, creatinine clearance **(K)** or fractional excretion of sodium **(L)**. Data are mean ± SD; ** p≤0.01, **** p≤0.0001.

**Fig 4.**

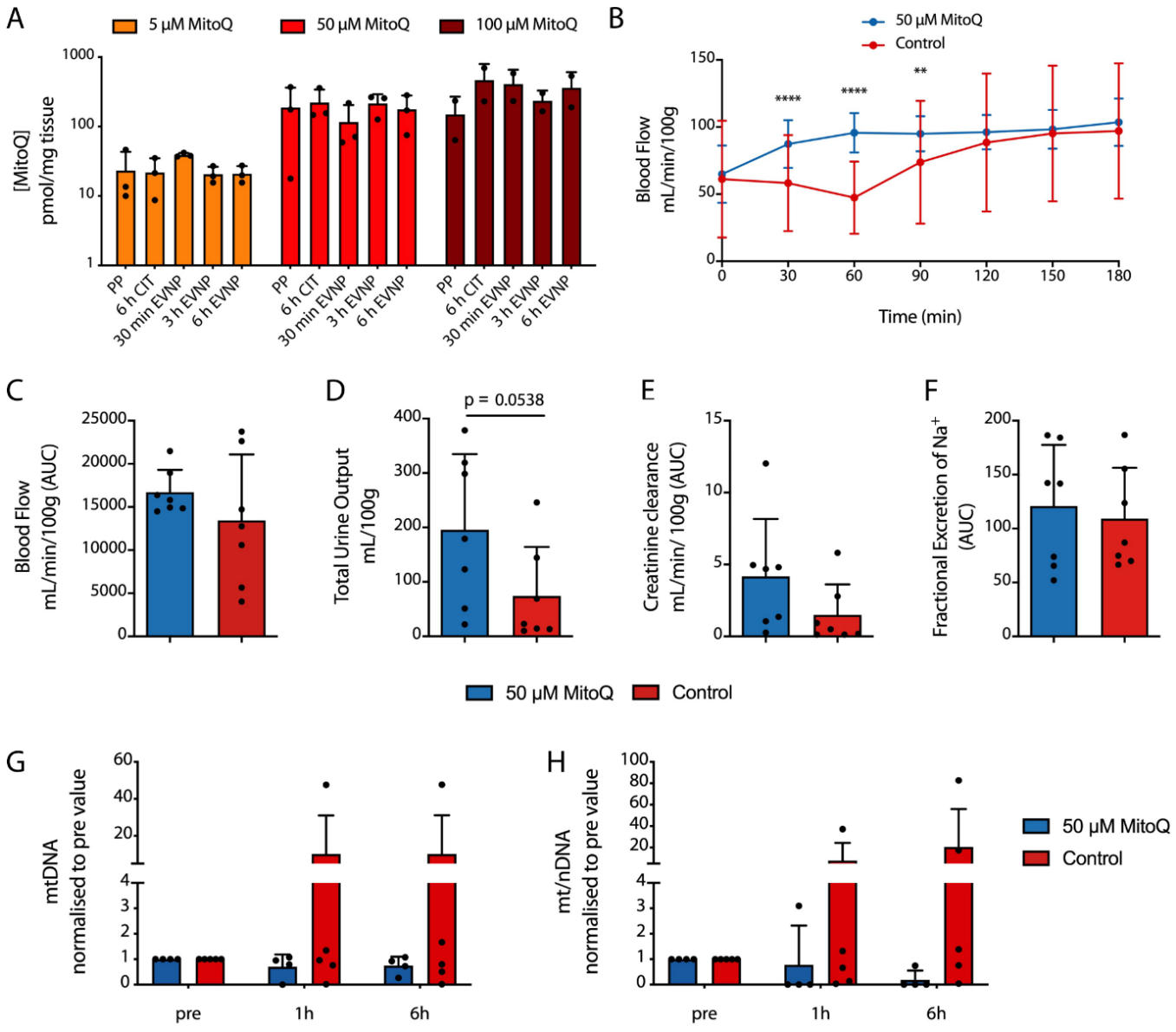



**Figure 4. Uptake and efficacy of MitoQ in ameliorating IR injury in human kidneys. (A)** Declined deceased human kidneys were flushed with 500 ml cold Soltran® supplemented with various concentrations of MitoQ as indicated, and then stored in 500 ml cold Soltran® containing the same concentration of MitoQ in CSS. There was a dose-dependent rise in MitoQ tissue concentrations (n=3 per group; $p<0.001$; repeated measures two-way ANOVA followed by Tukey multiple comparisons test) but MitoQ tissue concentrations subsequently remained stable over 6h of EVNP compared to the immediate post-perfusion (PP) levels. **(B)** An additional 7 pairs of declined deceased human kidneys were used to investigate the efficacy of MitoQ.

One kidney from each pair was flushed with Soltran® and the contralateral kidney with Soltran® + 50µM MitoQ. Both kidneys from each pair were then subjected to 6h CIT and subsequently underwent 3h of EVNP at the same time. There was a significant difference between the MitoQ group and the control group at the end of the first hour of EVNP (95.8±14.8 vs. 47.4±27.0 ml/min/100 g; p=<0.0001, repeated measures two-way ANOVA followed by Sidak multiple comparisons test), but not during the entire 3h period (p=0.216; paired t-test) **(C)**, total urine output **(D)** and creatinine clearance **(E)** were numerically higher in the 50µM MitoQ group compared to the control during 3 h of EVNP, but the differences did not reach statistical significance (p=0.054 and p=0.152 respectively; paired t-test) . **(F)** There was no significant difference between the MitoQ group and the control group during 3h of EVNP in fractional excretion of sodium. Normalised to pre-EVNP levels, MitoQ treatment did not have a significant effect on the absolute levels of circulating mtDNA levels (**G**) or as a proportion of circulating nuclear DNA levels (n=4-5; MitoQ at 50 or 100µM; **H**). Data are mean ± SD; ** p≤0.01, **** p≤0.0001.

**Fig. 5.**

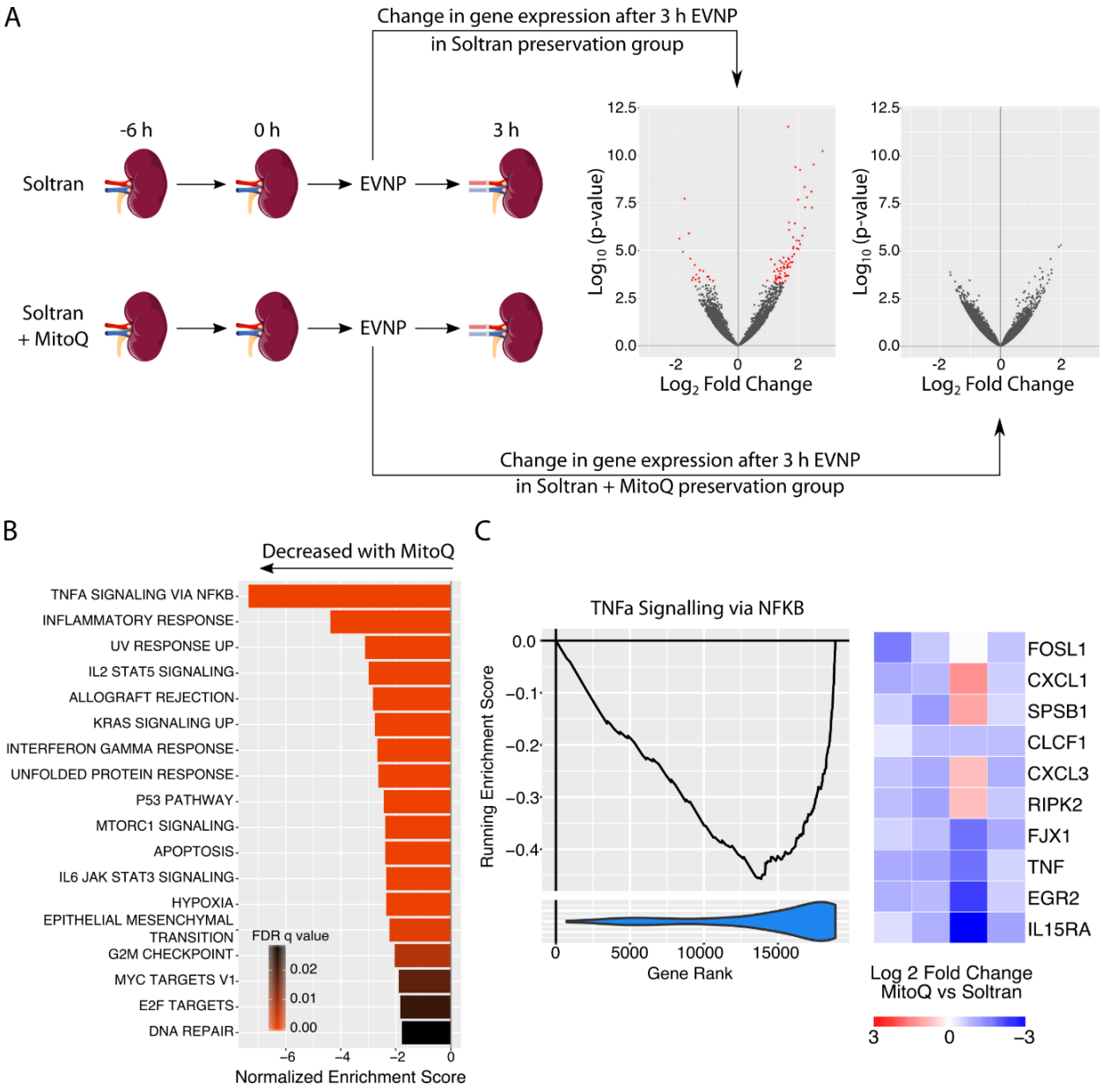



**Figure 5**. **Transcriptional changes in human kidneys following EVNP. (A)** Following treatment with Soltran® ± MitoQ, biopsies were taken from pairs of kidneys just prior to and after 3h of EVNP and the transcriptome assessed by RNASeq. Volcano plots show the differential expression of genes which occurred during EVNP. Genes in red are significantly differentially expressed (adjusted p value <0.05). The number in the plot is the number of genes in each direction which were significantly altered. **(B)** Gene Set Enrichment Analysis (GSEA) of changes in gene expression between the kidneys which were treated with MitoQ or control following

EVNP. GSEA was performed against the hallmarks database from MsigDB. **(C)** Left panel – Enrichment plot for the TNFα via NF-κB pathway in B. Line indicates running enrichment score and the violin plot represents the position of the genes in the gene set within the ranked list of genes. Right panel – top 10 genes from the leading edge of the enrichment plot. Heatmap indicates the log2-fold change in gene expression when comparing each kidney to its contralateral pair relative to UW following perfusion.

**List of Supplementary Material**

**Suppl. Fig. 1**. Comparison of the function of left and right pig kidneys upon reperfusion on the EVNP circuit.

**Suppl. Fig. 2.** Effect of WIT and CIT on pig kidney function.

**Suppl. Fig. 3.** Effect of 500nM, 5µM and 100µM MitoQ on pig kidney function.

**Suppl. Fig. 4.** Effect of 250µM MitoQ on pig kidney function.

**Suppl. Fig 5.** Dose-dependent effect of MitoQ on pig kidney function after ischemia-reperfusion.

**Suppl. Fig. 6.** Histopathological assessment of pig kidneys tissue biopsies.

**Suppl. Fig. 7.** Effect of MitoQ on human kidney transcriptome.

**Suppl. Fig. 8.** Effect of MitoQ on human kidney histopathological injury.

**Supplementary Table 1.** Histopathological scoring system for the degree of tubular injury in pig kidneys.

**Suppl. Table 2.** Deceased donor demographics whose kidneys were used to investigate the uptake of MitoQ.

**Suppl. Table 3.** Deceased donor demographics whose declined kidneys were used to investigate the effect of MitoQ on kidney function.

**Suppl. Table 4.** Histopathological scoring system for human kidneys.