Polysulfide Nanoparticles Ameliorate Ischaemia Reperfusion Injury in Renal Transplantation and Improve Kidney Function Post-Transplantation

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Keywords – Normothermic machine perfusion, kidney transplantation, ischaemia-reperfusion injury, nanoparticles, ROS scavenging

Running Title: Improving donor kidney health via ROS-scavenging with polysulfide nanoparticles

Abstract word count: 267 **Text word count:** 4584

Abbreviations

DGF – Delayed graft function

GTN - Glyceryl trinitrate

IRI – Ischaemia-reperfusion injury

IRR – Intra-renal resistance

NMP – Normothermic machine perfusion

NP – Nanoparticles

PPS-NP – Poly(propylene sulfide) (polysulfide) nanoparticles

RBF – Renal blood flow

ROS - Reactive oxygen species

SCS - Static cold storage

Abstract

Introduction Ischaemia-reperfusion injury (IRI) results in the pathophysiological generation of reactive oxygen species (ROS) with concurrent activation of the complement cascade. This manifests as delayed graft function, which has a significant impact on the longevity of the graft. We aimed to evaluate if ROS scavenging nanoparticles can be used to mitigate IRI related injury during normothermic machine perfusion (NMP).

Methods A randomised, two-stage, preclinical trial was used to assess the impact of poly(propylene sulfide) (polysulfide) nanoparticles (PPS-NPs) on parameters associated with IRI in a renal NMP system (experiment 1, n=6 vs 6). Paired porcine kidneys were randomised to receive either an NP-preservation flush followed by 6 hours of NMP with NP-perfusate, or control preservation flush and standard NMP. Following this, an allogeneic transplant-reperfusion model was used to evaluate if treatment with PPS-NPs improved renal haemodynamics post-transplantation (experiment 2, n=6 vs 6). Kidneys were perfused for 3 hours with or without NP, before being reperfused on a circuit primed with matched blood from genetically different donor pigs for 6 hours, without immunosuppression.

Results In experiment 1, all kidneys perfused well for 6 hours with physiological renal haemodynamics and biochemistry. Kidneys perfused with PPS-NPs had improved regional tissue perfusion on infra-red imaging. In experiment 2, renal haemodynamics were significantly improved during allogeneic reperfusion (post-transplant) after treatment with NP. Complement activation remained significantly lower in treated kidneys with a diminished TNF-α response. This translated into an improvement in tissue integrity.

Conclusion IRI was ameliorated following treatment with NPs during preservation and NMP. This was evidenced by an improvement in renal haemodynamics and diminished inflammatory markers upon reperfusion with allogeneic blood.

Introduction

Kidney transplantation remains the mainstay treatment option for patients with end stage renal disease, yet one of the limitations to its success is the inevitability of ischaemia reperfusion injury (IRI). This involves a cascade of events that begins at the point of organ retrieval and is further exacerbated at each stage of the transplant pathway. The current gold standard preservation technique of the donor kidney remains static cold storage (SCS), during which time there is no oxygen or nutritional support. As a result, metabolism becomes anaerobic, ATP is depleted, and there is a concurrent increase in intracellular calcium concentration and ion pump dysfunction [1]. At the same time, pH decreases, resulting in tissue acidosis. Upon reperfusion, reactive oxygen species (ROS) such as superoxide or hydrogen peroxide massively increase, because the machinery for the conversion of molecular oxygen into ROS had been upregulated under hypoxic conditions. High ROS levels then typically activate multiple immune pathways, including complement [1]; of note, ROS-mediated signalling may also contribute to maintaining active (through REDOX-sensitive transcription factors) inflammatory pathways with various origins [2]. Complement activation in the donor, and during storage, contributes to tissue injury and is associated with delayed graft function (DGF) post-transplantation [3]. Strategies to inhibit complement activation have been trialled, with some success [4, 5]. However, given that the activation of complement and the upregulation of ROS production machinery initiate within the graft prior to transplant, inhibition at this stage may be advantageous. This is now feasible via treatment during normothermic machine perfusion (NMP), a technique that enables organs to be preserved at normothermia with continuous oxygen and nutritional support. Treatment during NMP has additional advantages as it avoids potential off-target side-effects that can occur with systemic delivery of a therapy to the recipient post-transplant; in this way, NPs targeted to vascular endothelia can accumulate in the organ alone, and there e.g. act as slow-release drug depots in perfused kidneys or specifically inhibit specific inflammatory signalling [6-9]. It is worth noting that in this approach, when anti-CD31 antibodies fused with catalase (i.e. catalase targeted to inflamed endothelia) were perfused into lungs prior to transplantation, the resulting targeted ROS removal not only diminished ischemia-reperfusion injury but also prolonged the cold ischemia period and enhanced lung function [10]. Nanomaterials based on high-sulfur content organic polymers such as poly(propylene sulfide) (PPS) can scavenge ROS by sacrificially oxidizing their sulfur(II) atoms; since in PPS this action preferentially removes hydrogen peroxide[11, 12], the effect is similar to that of catalase, albeit with a different mechanism and not suffering of issues of denaturation or proteolytic degradation. As a result, PPS-based microparticles[13], nanoparticles (NPs)[14], micelles[15] and other self-assembled colloidal species[16] including gel-forming ones[17] have been shown to exert very significant anti-inflammatory effects both in *in vitro* and *in vivo* models of e.g. osteoporosis[15], wound healing[17], ischemic stroke[13, 14], or traumatic brain injury[13]. In this study, we investigated if the inclusion of catalase-mimetic PPS nanoparticles (PPS-NPs) during SCS and NMP could improve post-transplant outcomes via ROS scavenging. Herein we further confirm their validity in a kidney transplantation model.

Materials and Methods

Study Design:

This was a randomised, two-stage, preclinical trial to evaluate if polysulphide nanoparticle treatment can:

(Experiment 1) Mitigate ischaemia reperfusion during renal NMP. (Supplementary Figure 1a).

(Experiment 2) Improve renal haemodynamics in the immediate post-transplant period. (Supplementary Figure 1b).

Experiment 1: The Impact of Nanoparticles on NMP

Donor Organ Procurement:

Paired kidneys were obtained from n=6 Landrace pigs with a mean dry weight of 80 kg as previously described [18]. All pigs were veterinary inspected and culled in accordance with the European Union Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing. In brief, pigs were rendered unconscious via electrical stunning and sacrificed via exsanguination. Autologous blood was collected into a sterile receptacle containing 100 mL of normal saline supplemented with 40,000iU-unfractionated heparin (Fannin, UK). The abdomen was accessed using a midline laparotomy incision and the kidneys procured en-bloc and immediately placed on ice. The renal artery and ureter were isolated, cannulated and flushed with 20 mL of glyceryl trinitrate (GTN; Hameln, Germany – distributed by Gloucester, UK). Kidneys were randomised to receive a preservation flush with either 1 L 4°C hyperosmolar citrate solution (Soltran; Baxter Healthcare, Thetford, UK) or 1 L 4°C hyperosmolar citrate solution supplemented with PPS-NPs to a final concentration 0.25 mg/mL. Both preservation flushes contained 5,000iU-unfractionated heparin and were flushed at a hydrostatic pressure of 100 cm. Kidneys were then submerged in either 500 mL hyperosmolar citrate solution or 500 mL hyperosmolar citrate solution supplemented with 0.25mg/mL PPS-NPs according to their previous randomisation. All kidneys were placed on ice for a standardised static cold storage (SCS) time of 3 hours.

NMP:

Following 3 hours SCS, kidneys were perfused on separate NMP circuits for 6 hours as previously described [18]. In brief, the NMP circuit contained an organ chamber with a venous reservoir, a centrifugal pump (Medtronics, UK), a heat exchanger and an oxygenator (Chalice, UK). Prior to connecting the kidneys, the system was primed with approximately 1.4 L perfusate to ensure no air was in the circuit. This consisted of 500mL Ringer's solution supplemented with bovine serum albumin, 800 mL of autologous, cell-saved, leukocyte depleted packed red blood cells (prepared with a Cell Saver 5^+, Haemonetics, UK) to achieve a target haematocrit of 25-30%. The perfusate was further supplemented with broad spectrum antibiotics (Meropenem), dexamethasone, sodium bicarbonate, glucose, mannitol, and heparin. One circuit was also supplemented with (catalase-mimetic) PPS-NPs (to a final concentration of 0.25mg/mL of NPs). GTN and nutrition were infused into the circuit at a flow rate of 10 mL/hr. A gas mixture of 95% oxygen and 5% carbon dioxide was supplied to the membrane oxygenator. The circulating perfusate temperature was set to 38°C ± 1°C to rapidly re-warm the kidney to a physiological temperature.

Once the circuits were primed, kidneys were removed from ice and flushed with 200 mL 4°C Ringer's solution to remove any residual preservation solution. A pre-perfusion sample was taken for reference and kidneys were connected to the circuit via the renal artery. The mean arterial pressure was gradually increased until a target of 75 mmHg was achieved. At this time infusions of GTN and nutrition were commenced. The organ chamber was covered to retain humidity and organ temperature. Serial observations and blood gas analysis were recorded and correctable changes to abnormal blood gas physiology were instituted. Ringer's solution was used to substitute the exact volumes of urine produced.

Sample Collection during NMP:

Perfusate: Serial perfusate samples were collected. All samples were centrifuged at 1000 g for 10 minutes at 4°C. The supernatant was decanted into 1mL aliquots and stored at -80°C.

Urine: Serial urine samples were collected and aliquoted into 1 mL cryovials and stored at -80°C.

Biopsies: Needle-core biopsies were taken from the kidneys prior to NMP for baseline reference. Further needle-core biopsies were taken at 60 and 180 minutes for storage in 10% neutral buffered formalin. At the end of perfusion, the weight of kidneys was recorded, and wedge biopsies were stored in 10% neutral buffered formalin (Merck, Germany).

Infra-Red Imaging: Infra-red images were taken of the kidneys to determine perfusion homogeneity/heterogeneity. Images were taken prior to the kidneys being connected to the circuit, followed by 5-minute intervals for the first 30 minutes. Images were then collected every 10 minutes until 60 minutes, after which images were taken hourly until the end of perfusion.

Synthesis and Fluorescent Labelling of Poly(propylene sulfide) Nanoparticles:

Briefly, under an argon flow, 12.8 g of Pluronic F-127 was dissolved into 200 mL of degassed MilliQ water within a 250 mL round-bottom flask attached to a Radley's Tornado Overhead Stirring manifold. To ensure complete dissolution/dispersion, the solution was stirred at 500 rpm for 10 minutes. To this solution was then added a mixture containing 175 mg of 2,2'-(ethylenedioxy)diethanethiol (0.96 mmol) and 6.307 g propylene sulfide (86.4 mmol, 45 equiv.s per thiol), and the stir speed was increased to 1,000 rpm and the solution stirred for a further 10 mins. To initiate the polymerization, 292 mg of DBU (1.92 mmols, 1 equiv. per thiol) was added to the emulsion and the polymerization was allowed 3.5 hours to react. The pH was then lowered to ~8 with the addition of PBS salts (50 mM final concentration of phosphate) and acetic acid before adding 25 mg of 5-(iodoacetamido)fluorescein (0.049 mmol, 0.025 equiv.s per thiol) in 200 μL of a 1:1 DMF/THF solution followed 10 minutes later by 68 mg of the tetrafunctional cross-linker pentaerythritol tetraacrylate (PA_4, 0.19 mmol, 0.2 equiv.s to thiol) dissolved in 100 μL of THF. After an additional 1 hour of reaction time, a further 34 mg of PA4 (0.1 mmol, 0.1 equiv.s per thiol) was added in 100 µL of THF; the solution was brought to pH 7.4 with acetic acid and allowed to stir overnight (~16 hours). The reaction mixture was first filtered through sterile 0.45 µm PES filters and was dialyzed via hollow-fibre dialysis on a Millipore Labscale Tangential Flow Filtration (TFF) System using a Pellicon XL50 Biomax® 100 kDa MWCO membrane until the filtrate had a conductivity equal to that of deionized MilliQ water. The solution was concentrated to approximately 25 mg/mL of PPS-NPs and sterile filtered.

Total urine protein content:

Urine samples (80 µL) were centrifuged at 800 g for 10 mins at 0°C and the supernatant diluted 1:20 with MilliQ water. Total protein content was quantified using Pierce^{TM} BCA protein assay kit (Thermo Scientific, USA).

Albumin urine content:

Urine samples (80 µL) were centrifuged at 800 g for 10 mins at 0°C and the supernatant diluted 1:25000 or 1:50000. Albumin was quantified using an Albumin pig ELISA kit (Abcam, Cambridge, UK) according to manufacturer's instructions and protein levels were calculated.

DNA damage in plasma:

Quantification of oxidised DNA was performed using a DNA damage competitive ELISA kit (Invitrogen, USA) according to manufacturer's instructions with plasma samples diluted 1:8 in assay buffer.

TNF-α expression levels in plasma:

Quantification of TNF-α was performed on undiluted samples using a porcine TNF-α ELISA kit (Invitrogen, USA) with 20 pg/mL sensitivity according to manufacturer's instructions. Samples that were determined to be below the detection limit where quantified using <0.3 pg/mL sensitivity porcine TNF-α ELISA kit (Invitrogen, USA).

Histological Analysis:

Haematoxylin and eosin - Kidney tissue biopsies that were fixed in 10% buffered formalin solution were dehydrated and paraffin-embedded. Sections were cut (5 μm), de-paraffinized and stained with hematoxylin and eosin using the protocol set by the Leica Autostainer XL (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Samples were graded using the Remuzzi scoring system by a clinical histopathologist who was blinded to the sample groups.

Immunostaining - Kidney sections were incubated in 30% sucrose solution in 0.005 M PBS for 72 hr prior to being sectioned into 40μm slices using a freezing cryostat. The sections were permeabilized with a 0.3% v/v Triton X-100 in PBS (PBS-T) for 30 mins at room temperature followed by a blocking solution (2% normal goat serum in PBS-T) for 1 hour at room temperature. Tissue sections were washed three times with PBS-T, incubated with VCAM-1 monoclonal antibody (1:100, 1.G11B1 Invitrogen) at 4°C overnight, followed by incubation with goat anti-mouse Alexa 546 (1:500; Invitrogen) for 2 hr at room temperature and counterstained with HOESCHT (1:1000; Sigma-Aldrich) for 10min prior to imaging. The stained tissues sections were mounted using ProLong®Antifade mounting reagent (Thermo-Fisher Scientific) and imaged using a Leica SP5 confocal microscope (Wetzlar, Germany) with 40X magnification.

Experiment 2: Randomised study comparing post-transplant outcomes with or without PPS-NPs

Bilateral kidneys were retrieved from an additional n=6 pigs, as per experiment 1. Additional blood was also collected per experiment and blood matched to the donor pig using DiaClon Anti-A (Bio-Rad, Germany).

Experimental Design:

NMP - Kidneys were randomised to receive a preservation flush with or without PPS-NP supplementation. Based on data generated in experiment 1, kidneys were exposed to a standardised 3 hours SCS, followed by NMP for 3 hours rather than 6 with or without NP supplementation. Kidneys were then removed from the NMP circuit and flushed with 500 mL 4°C Soltran before being submerged in 500 mL 4°C Soltran and placed on ice for 1 hour.

Allogeneic Reperfusion – Whilst kidneys remained on ice for 1 hour, two new NMP circuits were primed with 1.2 L whole blood from a blood matched, genetically different donor pig to mimic the transplant procedure upon reperfusion, without immunosupression. The only supplements were 250 mg Meropenem and 10mL 15% glucose. No PPS-NPs were included in this part of the study. A syringe driver infused nutritional support (10mL/hour). After 1-hour SCS, kidneys were removed from ice and flushed with 200 mL 4°C Ringer's solution before being re-weighed and attached to the NMP circuit. Both kidneys were perfused for 6 hours at a target mean arterial pressure of 80 mmHg. Serial perfusate and urine samples were collected and stored as per experiment 1. At the end of the experiment, kidneys were removed from the circuit, weighed, and wedge biopsies were collected and stored in 10% neutral buffered formalin.

Assays:

Assays to quantify total urine protein and albumin content, DNA damage and TNF-α expression levels in plasma were all performed as per experiment 1.

Complement Assay:

Soluble C5b-9 was quantified by MesoScale Discovery (MSD). MSD GOLD 96-well streptavidin SECTOR plate was coated with 2µg/mL biotinylated anti-C5b-9 antibody (Abcam) and blocked with PBS containing 2% BSA 0.05% Tween 20 (v/v). Kidney perfusate was diluted three-fold and added to the plate. Human sC5b-9 (Complement Technology) at concentration ranging from 5 µg/mL to 0.01 µg/mL was used to generate a standard curve. Kidney samples and sC5b-9 standard were diluted in PBS containing 2% BSA, 0.05% Tween 20 and 10 mM EDTA. Ruthenylated anti-C6 monoclonal antibody (2 µg/mL) diluted in MSD Diluent 100 was used for detection. Plate was read using an MSD Sector Imager 6000 and sC5b-9 concentration was quantified from interpretation of electrochemiluminescent (ECL) signals using MSD Discovery Workbench 4.0.12.1 software.

Statistical Analysis: Statistical analysis was performed using Graphpad Prism v8.3 and JMP (JMP Statistical Discovery LLC). Data normality was formally assessed using the Shapiro-Wilk test. Two-way ANOVA was used to assess changes between groups over-time including RBF, IRR, biochemistry, and urine output. Paired t tests were used to compare individual time-points between groups.

For the complement assays a mixed model was fitted to the log transformed sC5b-9 concentration for each time point. The model terms were:

response variable: $\log_{10}$ concentration

fixed effect model: kidney

random-effect model: animal/kidney/replicate.

The model term ‘kidney’ also represents the treatment effect, as the two treatments (with and without PPS-NP) were applied to two paired kidneys. A significance test was performed as part of the model-fitting process to determine whether the mean with/without PPS-NP ratio was different from 1 (or equivalently, mean $\log_{10}$ ratio concentration was different from 0).

Results

Experiment 1 – The Impact of Nanoparticles on NMP

Haemodynamics: All kidneys were successfully perfused for 6 hours, achieving average renal blood flows (RBF) of 84±38.92 mL/min/100g in control kidneys and 102±31.86 mL/min/100g in treated kidneys. Overall, no significant differences were observed in RBF or IRR, although variability was much greater in control kidneys. No differences were observed between pre- and post-perfusion kidney weight, with control kidneys increasing on average by 6±12.5% and treated kidneys by -2.5±7.9% (Figure 1 A-C).

Biochemistry: All kidneys maintained physiological pH throughout the 6-hour experiment, without the need for additional sodium bicarbonate to manage acidosis. Glucose and sodium bicarbonate concentrations continued to increase within the perfusate of both groups. Lactate concentrations decreased within the first hour of perfusion in both groups, followed by a slight increase. From 4 hours, the concentration of lactate was significantly higher in the control group compared to the treatment group ($p=0.049$ (4hr), $p=0.038$ (5hr) and $p=0.046$ (6hr)) (Figure 1 D-G).

Kidney Function: All kidneys produced urine immediately following connection to the NMP circuit and maintained urine output for the 6-hour experiment. There was no significant difference in urine output between the two groups (Figure 1H).

Infra-Red Analysis: The average temperature of the kidneys did not change over-time between both groups. However, the variability in perfusion across the kidneys differed between the groups, with NP-treated kidneys demonstrating more homogenous perfusion (Figure 2; p=0.002).

Urinalysis: Albumin was detected in all urine samples in increasing concentrations over time. The relative albumin content was always lower in PPS-NP-treated kidneys compared to controls, although this did not reach statistical significance (Figure 3A).

Oxidised DNA: After baseline, oxidised DNA was detected within perfusate at all time points analysed. There was an increasing trend in both groups, but this was significantly greater in control kidneys at 1 and 3 hours. No difference was observed between groups by 6 hours (Figure 3B).

TNF-α expression: The expression of TNF-α increased in both groups over time with no significant differences noted at any time point (Figure 3C).

Histology: Mild morphological changes were observed in all kidneys with no significant differences between groups.

Immunostaining: PPS-NPs infiltrated renal tissue and appear to be associated with areas where VCAM-1 was not expressed. More generally, the PPS-NP treated kidneys displayed less VCAM-1 expression (Supplementary Figure 2).

Experiment 2 – Transplantation model using allogeneic blood.

Haemodynamics:

NMP - Both groups were successfully perfused for 3hrs (in autologous blood-based perfusate), reaching an average RBF of 61.38±17.03 mL/min/100 g in control kidneys and 71.86±9.83 in PPS-NP-treated kidneys. No significant differences were observed in RBF or IRR between groups. Similarly, percentage weight change did not differ between groups with an average change of 2.3±9.6% in control kidneys and -0.5±3.01% in PPS-NP-treated kidneys (Figure 4A and B).

Allogeneic Reperfusion – All kidneys were successfully reperfused for a further 6 hours in allogeneic blood, with low RBF observed within the first 30mins, increasing to an average of 34.30±19.06 mL/min/100 g in control kidneys and 53.17±10.86 mL/min/100 g in NP-treated kidneys. From 3 hours, RBF was significantly higher in kidneys that had received NP treatment compared to control kidneys, and this remained the case for the duration of the reperfusion period (p=0.019 (3hr), p=0.009 (4hr), p=0.013 (5hr) and p=0.015 (6hr)). Interestingly, IRR was only significantly different at 6 hours, although variability in NP-treated kidneys was lower throughout. Compared with pre-reperfusion, the greatest percentage weight change was observed in control kidneys (-18.00±7.35) compared to treated kidneys (-14.50±4.04) (Figure 4A and B).

Biochemistry:

NMP – The biochemical profile of all NMP circuits followed that observed during perfusion in experiment 1. Sodium, calcium, sodium bicarbonate and glucose concentrations increased during the experiments at an equal rate in both groups, with decreasing lactate concentrations also observed. The concentration of potassium did not change over time and remained within physiological parameters in both groups (averaging 4.018±0.95 mmol/L in control kidneys and 3.697±0.58 mmol/L in PPS-NP-treated kidneys) (Figure 5 A-F).

Allogeneic Reperfusion – All kidneys exhibited an acid-base imbalance within the first 30 minutes of reperfusion and required sodium bicarbonate supplementation to correct base excess. Following this, sodium bicarbonate, base and pH all increased at equal rates in both groups until the end of reperfusion. The concentration of potassium also increased in both groups. Conversely, the concentration of calcium, glucose and sodium remained stable in both groups. There was a marginal decline in lactate concentrations over-time from an average of 5.5±3.0 to 4.7±2.6 mmol/L in control kidneys and 5.4±2.9 to 3.5±1.2 mmol/L in PPS-NP-treated kidneys (Figure 5 A-F).

Kidney Function:

NMP – All kidneys began producing urine immediately following connection to the NMP circuit and this was maintained for the duration of the experiment. (Figure 6A)

Allogeneic Reperfusion – Kidneys in both groups produced urine within the first 30 minutes of reperfusion, to a greater quantity in NP-treated kidneys (10.42±11.72 in control kidneys and 15.92±14.91 in PPS-NP-treated kidneys). Following this, urine output rapidly declined in both groups with a cessation of production in some cases by the end of reperfusion (Figure 6B).

Complement Analysis:

NMP - The concentration of sC5-9b was significantly different between the groups at all time points assessed during perfusion, with lower concentrations in treated kidneys (Figure 7A).

Allogeneic Reperfusion - Upon reperfusion, there was no significant difference in the concentration of sC5-9b at baseline (p=0.8033). However, at all other time-points assessed (1, 3 and 6 hour) the concentration of sC5-9b was significantly less in NP-treated kidneys compared to control kidneys (Figure 7B; p=0.0091, p=0.0041 and p=0.0017 respectively).

Urinalysis:

NMP: The concentration of albumin and the total protein concentration was lower overall in NP-treated kidneys compared to controls. This became a statistically significant difference in albumin content at 30 minutes (Supplementary Figure 3A and B).

Oxidised DNA:

NMP – Oxidised DNA increased during NMP in both groups and was significantly higher in control kidneys compared to PPS-NP-treated kidneys at 3 hours (Supplementary Figure 3C)..

Allogeneic Reperfusion – There was an increase in oxidised DNA in both groups over time. Within the first hour of reperfusion this was numerically greater in control kidneys compared to treated kidneys, although this did not reach statistical significance (Supplementary Figure 3D).

TNF-α expression:

NMP: The expression of TNF-α increased at the same magnitude in both groups over time (Figure 8A).

Allogeneic Reperfusion: TNF-α expression increased in both groups within the first hour of reperfusion, decreasing thereafter. The expression was significantly higher in control kidneys compared to PPS-NP-treated kidneys from 3 hours (Figure 8B).

Histology: Histologic morphological changes were observed in all kidneys at the end of the 6-hour reperfusion, although these were observed to be only acute features. Overall, there was better preservation of tissue architecture in PPS-NP-treated kidneys, with less overall acute tubular necrosis (Supplementary Table 1).

Immunostaining: PPS-NPs were embedded within renal tissue following 6 hours of allogeneic reperfusion. As with Experiment 1, areas where PPS-NPs remained were negatively associated with VCAM-1 expression (Supplementary Figure 2).

Discussion

DGF represents a major clinical challenge following transplantation and is associated with poor long-term graft function and patient survival (estimated to be a 40% decrease in long-term survival) [19, 20]. IRI significantly contributes to DGF, with prolonged ischaemia time leading to an exacerbation of acute kidney injury and an increased risk of primary non-function [21]. There have been attempts to reduce IRI which include improvements to donor and recipient management, as well as therapeutic interventions (for example, calcium-channel blockers, L-arginine and N-acetylcysteine), but with limited clinical success [22]. Indeed, there are still no specific therapies available in the clinic to reduce IRI following transplantation. A primary challenge in treating IRI is that the process occurs in the graft during preservation. Therefore, treating the donor kidney prior to transplantation may mitigate intra-operative and post-transplant induced injury. With the advent of NMP, there is an opportunity to directly treat the donor kidney prior to transplant, specifically targeting pathways associated with IRI, with reduced off-target or systemic risks to the recipient. In this study, we have therefore evaluated if direct treatment of a donor kidney with a novel catalase-mimetic PPS-NP therapy could be used to improve perfusion parameters during preservation, and if this ameliorates post-transplantation IRI.

In experiment 1, the addition of the PPS-NP therapy during SCS and NMP resulted in homogenous perfusion with superior blood distribution. Equally, PPS-NP treatment was associated with significantly lower perfusate lactate compared to controls. Lactate is a surrogate marker for anaerobic metabolism and tissue perfusion, supporting the case for NP dependent optimisation of vascular integrity.

For this study, catalase-mimetic PPS-NPs were selected, based on their defined mode of action of converting hydrogen peroxide (H_2O_2) to water and O_2. H_2O_2 is highly destructive, contributing to the formation of superoxides, hypochlorite and hydroxyl. H_2O_2 oxidises proteins and DNA, and contributes to lipid peroxidation, all of which initiates inflammation, including complement activation [23]. We therefore speculated that treatment with catalase-mimetic PPS-NPs may have been exerting beneficial effects via ROS neutralisation and indirect complement inhibition, which are both intrinsically involved in IRI. Injury to the graft during ischaemia, and particularly at the point of reperfusion, is mediated by several factors which include the inappropriate production of ROS, comprising of H_2O_2, superoxide, hypochlorite and hydroxyl. Whilst an important biological process, the excess formation of ROS coupled with a reduction in antioxidant capacity ultimately leads to cell membrane injury, apoptosis, necrosis and graft dysfunction. The resulting tissue damage leads to the release of damage-associated molecular patterns (DAMPs) that activate the innate immune system via interactions with cell surface pattern recognition receptors. Complement activation ensues, with the formation of the terminal cytolytic membrane attack complex (C5-9b, also referred to as MAC), inducing direct renal injury via apoptosis of epithelial tubular cells [24]. There was a significant reduction in MAC (soluble C5-9b) in the perfusate of kidneys treated with the PPS-NP therapy, compared to untreated controls. PPS-NPs treated kidneys also displayed significantly reduced levels of oxidized DNA in the plasma perfusate indicating both reduced cell/tissue damage and lower levels of oxidative stress.

Following this, in Experiment 2 we assessed whether PPS-NP therapy during perfusion improved haemodynamics and/or altered terminal complement components in an ex vivo transplantation model using allogeneic (immunocompetent) blood reperfusion. Interestingly, kidneys that were exposed to PPS-NP therapy during (autologous) perfusion and subsequent allogeneic reperfusion (mimicking the immediate period following transplant) exhibited similar reduction in terminal cytolytic MAC. This was only significant from 1 hr after the start of allogenic blood reperfusion, demonstrating that the differences observed were a result of the NP treatment as the baseline perfusate was the same. There has been increasing momentum to develop treatments that block or attenuate complement following transplantation, but success in animal models and clinical trials has been variable. It is now recognised that targeted therapies mitigating inflammation and fibrosis post-transplantation are a priority to maximise the donor pool and improve patient outcomes. One such therapy in clinical utilisation is the C5 antibody Eculizumab, which has been used for the treatment of antibody-mediated rejection [25]. However, upstream activation of C3 and C4 can still occur with this treatment.

Following the reperfusion phase after exposure to PPS-NPs there was a significant improvement in renal haemodynamics, with a continual improved renal blood flow in treated kidneys. Furthermore, kidneys exposed to the treatment had a lower and more stable IRR, suggesting improved vascular tone and endothelial integrity. PPS-NP treated kidneys presented with better tissue integrity, reduced acute tubular necrosis, and a reduction in urine albumin concentration. This may be in response to reduced oxidative stress, cell/tissue damage, complement activation and MAC formation during reperfusion. The release of ROS and subsequent complement activation contributes to endothelial cell injury and death and causes the upregulation of leukocyte adhesion molecules. Neutrophil rolling on the vascular endothelium ensues, along with vascular wall remodelling, ultimately leading to narrowing of the lumen and reduced passage of blood flow [26]. Interestingly, we observed a reduction in VCAM-1 in the presence of NPs, suggesting ancillary roles of NP therapy in adhesion molecule downregulation. A potential explanation may be that MAC augments TNF-α production, which intrinsically upregulates several adhesion molecules, including VCAM-1. To evaluate this relationship, we quantified TNF-α and report diminished TNF-α perfusate levels in NP treated kidneys. TNF-α has been a therapeutic target in the transplant setting for decades, due to its pleiotropic roles in rejection, but to date, there have been no successful reports of TNF-α targeted treatments.

Summary:

PPS-NP therapy appears safe during static cold storage, NMP, and during allogeneic reperfusion. PPS-NP treatment was associated with improved tissue perfusion and haemodynamics, potentially via suppression of complement pathways and reducing oxidative stress and inflammatory pathways. This was evidenced by a reduction in the soluble form of the terminal cytolytic MAC, C5-9b, and a reduction in circulating TNF-α. Given that DGF is common post-transplant, further work evaluating NP therapies as a treatment strategy for IRI is warranted.

Data Availability:

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Figure Legends

Figure 1 - Renal haemodynamic, biochemical, and functional parameters during 6 hours of perfusion.

Renal haemodynamics remain stable during perfusion. Renal blood flow (A), pressure (B) and intra-renal resistance (C) remained within acceptable ranges during perfusion in both groups. Biochemical parameters remained similar between groups, with the exception of lactate which was significantly lower in treated kidneys from 4 hours (D-G). All kidneys regained function immediately and continued producing urine throughout (H). * = p≤0.05

Figure 2 – Temperature changes across the kidney during perfusion.

Average kidney temperature did not change over-time between both groups (A). However, there was less variability in perfusion across the kidneys in the treated group (B).

Figure 3 – Urine and perfusate analysis during perfusion.

Albumin was detected in all urine samples in increasing concentrations with no statistical significance between groups (A). Oxidised DNA was also detected within perfusate after baseline and was significantly greater in control kidneys at 1 and 3 hours. No difference was observed between groups by 6 hours (B). The expression of TNF-α increased in both groups over-time with no significant differences noted at any time point (C). * = p≤0.05

Figure 4 - Renal haemodynamics during perfusion and reperfusion.

Haemodynamics remained stable in both groups during perfusion (A). Upon reperfusion, renal blood flow was significantly higher after 3 hours in treated kidneys (A) with a more stable resistance (B). * = p≤0.05, ** = p≤0.01

Figure 5 – Biochemical parameters during perfusion and reperfusion.

Biochemical parameters remained stable between the groups throughout the perfusion and reperfusion phases. No differences were observed between groups (A-F).

Figure 6 – Kidney function during perfusion and reperfusion.

Urine output began in all kidneys as soon as they were placed onto the perfusion circuits with no differences noted (A). Urine output continued during reperfusion but diminished over time in both groups (B).

Figure 7 – Complement activation during perfusion and reperfusion.

The concentration of complement (soluble C5-9b) was significantly lower in treated kidneys at all time points assessed during perfusion (A). Upon reperfusion, there was no significant difference in the concentration of sC5-9b at baseline. However, at all other time-points assessed the concentration of sC5-9b was significantly less in NP-treated kidneys compared to control kidneys (B). * = $p \leq 0.05$, ** = $p \leq 0.01$

Figure 8 - TNF-α concentration in the perfusate.

The expression of TNF-α increased during perfusion at the same magnitude in both groups over time (A). During reperfusion**,** TNF-α expression increased in both groups within the first hour of reperfusion, decreasing thereafter. Expression was significantly higher in control kidneys compared to NP-treated kidneys from 3 hours (B). * = $p \leq 0.05$

Supplementary Figure 1a – Schematic overview of experiment 1.

Paired porcine kidneys were retrieved (n=6) and flushed cold with preservation solution as per clinical protocols. One kidney was randomised to receive a standard preservation flush, which the other received a preservation flush supplemented with nanoparticles. Kidneys were then stored on ice for 3 hours in preservation solution (with or without nanoparticles according to their flush). Kidneys were then perfused on a normothermic machine perfusion circuit for 6 hours with or without nanoparticle supplementation with real time haemodynamic, biochemical and urine output data recording and serial perfusate and urine collection. Biopsies were collected at the end of the experiment for histological analysis.

Supplementary Figure 1b – Schematic overview of experiment 2.

Paired porcine kidneys were retrieved (n=6) and treated as per experiment 1, except they were only perfused for 3 hours. Following this time, kidneys were flushed cold again with a standard preservation solution (no supplementations) and stored on ice for 1 hour while two new perfusion circuits were prepared. Each circuit was primed with whole blood that had been blood matched to the donor kidney to mimic a transplant setting. Kidneys were placed onto these circuits and perfused for 6 hours (with no supplementation of nanoparticles). Real time haemodynamic, biochemical and urine output data was recorded and serial perfusate and urine samples collected with a biopsy at the end of perfusion.

Supplementary Figure 2 – Kidney immunostaining of biopsies at the end of perfusion.

Representative images of kidney biopsy sections stained for nuclei (blue), VCAM-1 (red) and nanoparticles (green). Nanoparticles are still visible in treated kidneys and appear to be associated to areas where VCAM-1 is not expressed.

Supplementary Figure 3 – Protein and oxidised DNA concentrations in experiment 2.

Total protein and albumin within urine samples collected during perfusion showed minimal changes over 3 hours, although albumin was significantly higher in control kidneys at 30 minutes (A and B). As per experiment 1, oxidised DNA was detected during perfusion following baseline and was significantly higher in control kidneys compared to treated kidneys at 3 hours (C). No differences were observed in oxidised DNA between the groups during reperfusion (D).

Funding

This work was funded by a Prize Post-Doctoral Fellowship in collaboration with GlaxoSmithKline (GSK).

Disclosure: The authors declare no conflicts of interest

Author Contributions

JPS – Conceptualisation, methodology, formal analysis, investigation, writing (original draft), and visualisation.

RD – Methodology, formal analysis, investigation, resources and writing (review and editing).

AG – Methodology and Investigation

KA – Methodology and Investigation

AMF – Investigation and Resources

DD – Resources and data curation

MG – Resources

NG – Investigation, resources, and data curation

GC – Data curation

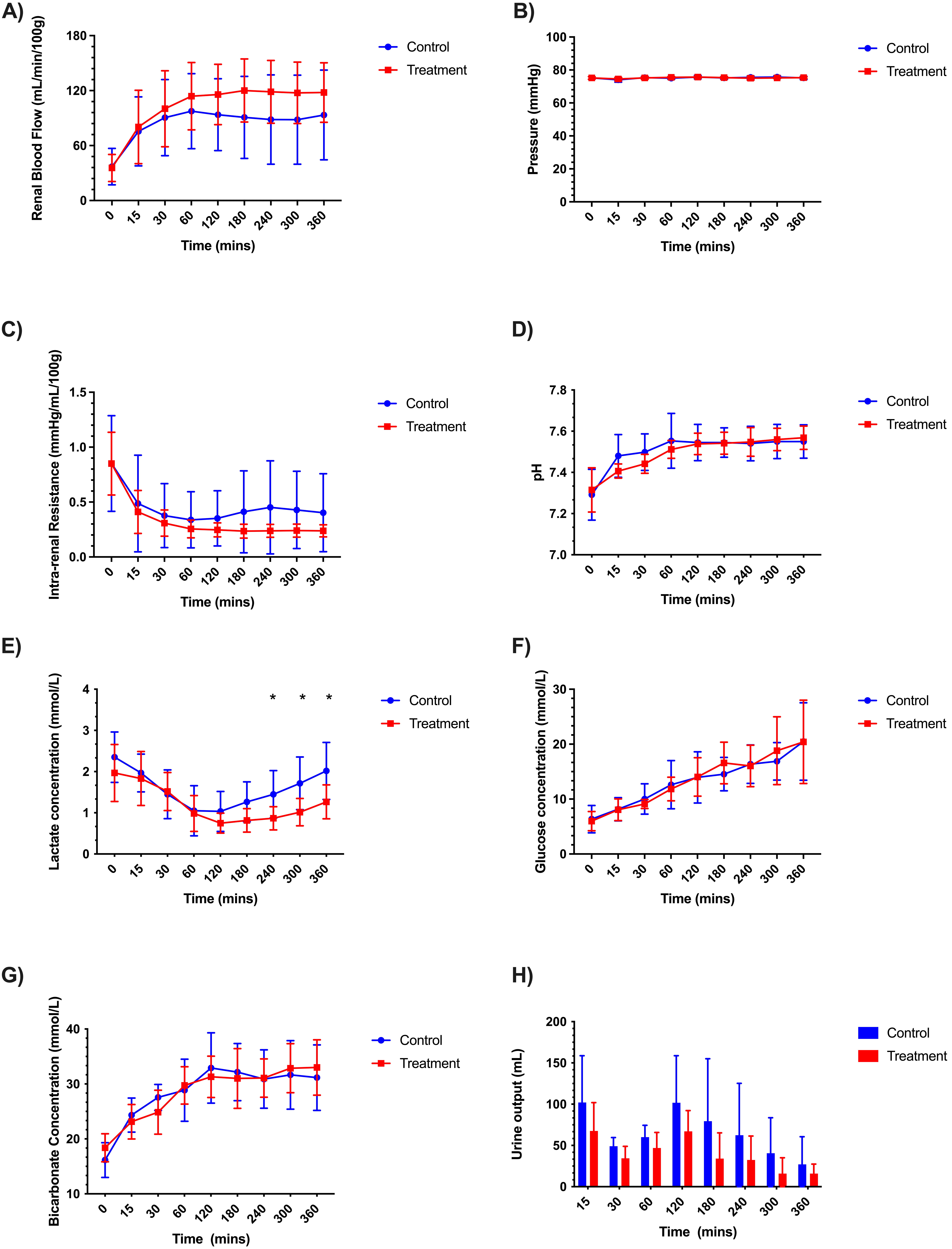
JB – Investigation

NG – Data curation

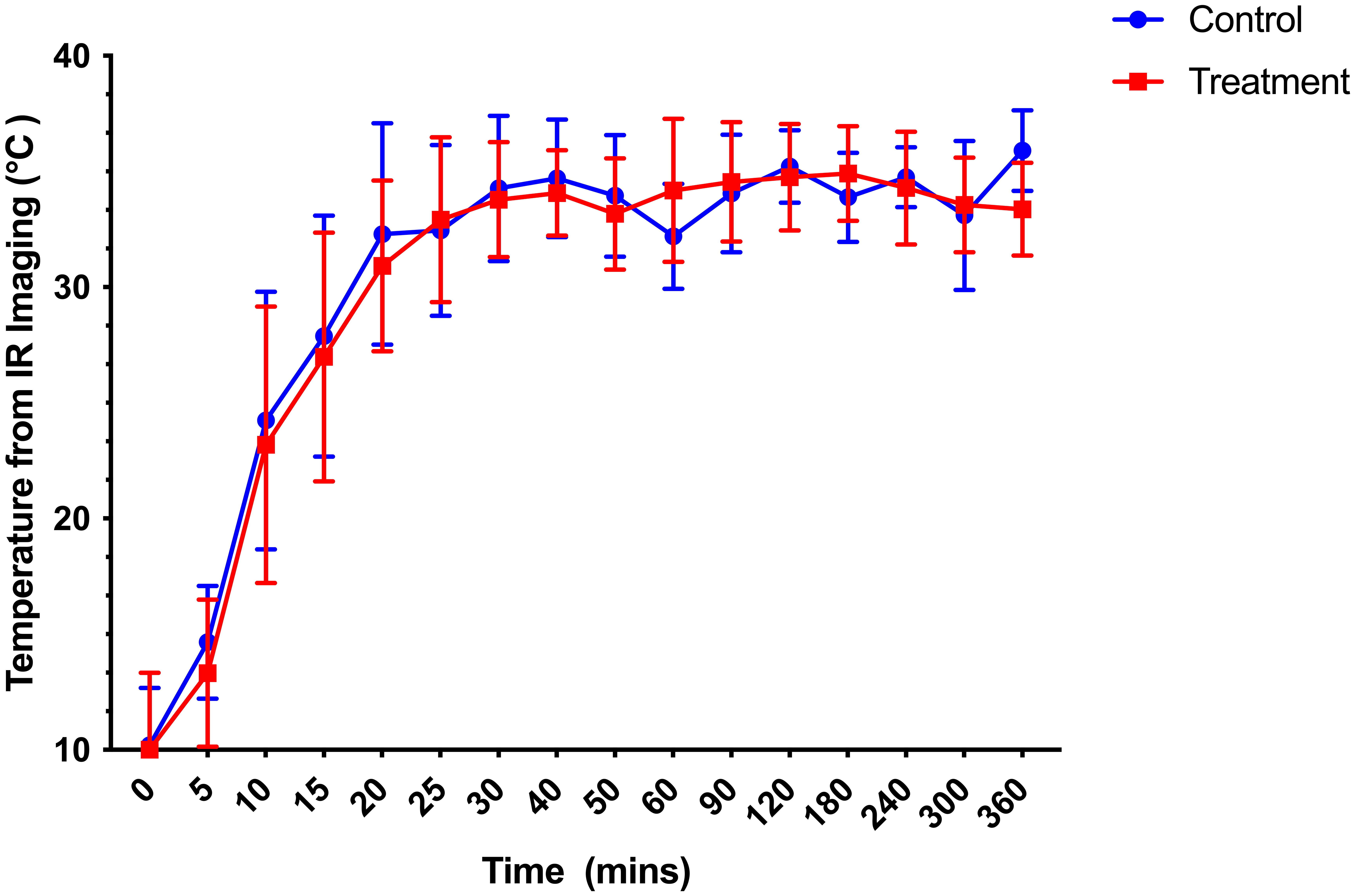
MR – Investigation, supervision, and funding acquisition

NT – Investigation, resources, data curation and writing (review and editing)

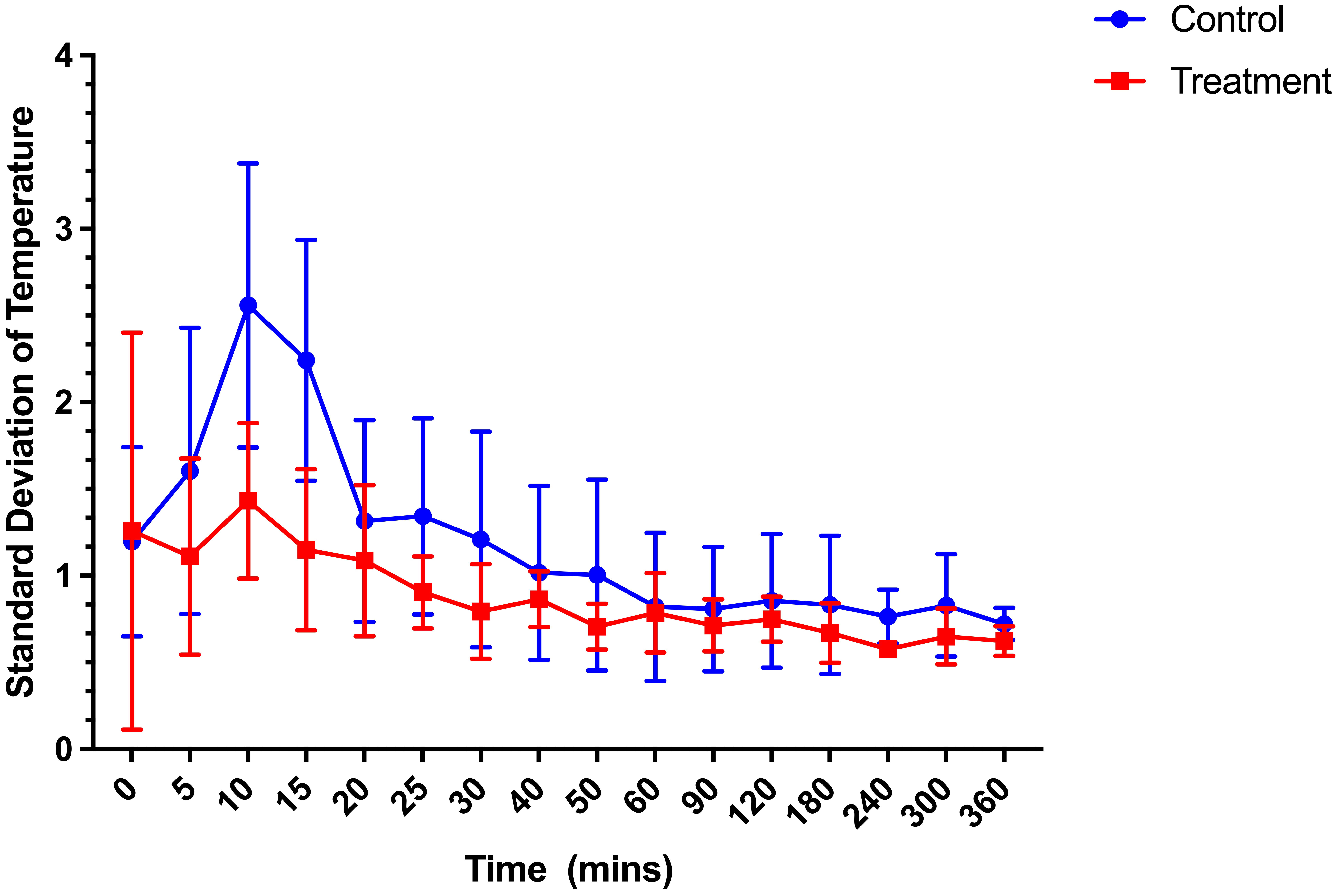
JEF – Conceptualisation, methodology, formal analysis, investigation, writing (original draft), supervision and funding acquisition.


A)
Renal Blood Flow (mL/min/100g)
0
60
120
180
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
B)
Pressure (mmHg)
0
20
40
60
80
100
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
C)
Intra-renal Resistance (mmHg/mL/100g)
0.0
0.5
1.0
1.5
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
D)
pH
7.0
7.2
7.4
7.6
7.8
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
E)
Lactate concentration (mmol/L)
0
1
2
3
4
*
*
*
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
F)
Glucose concentration (mmol/L)
0
10
20
30
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
G)
Bicarbonate Concentration (mmol/L)
10
20
30
40
0
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment
H)
Urine output (mL)
0
50
100
150
200
15
30
60
120
180
240
300
360
Time (mins)
Control
Treatment

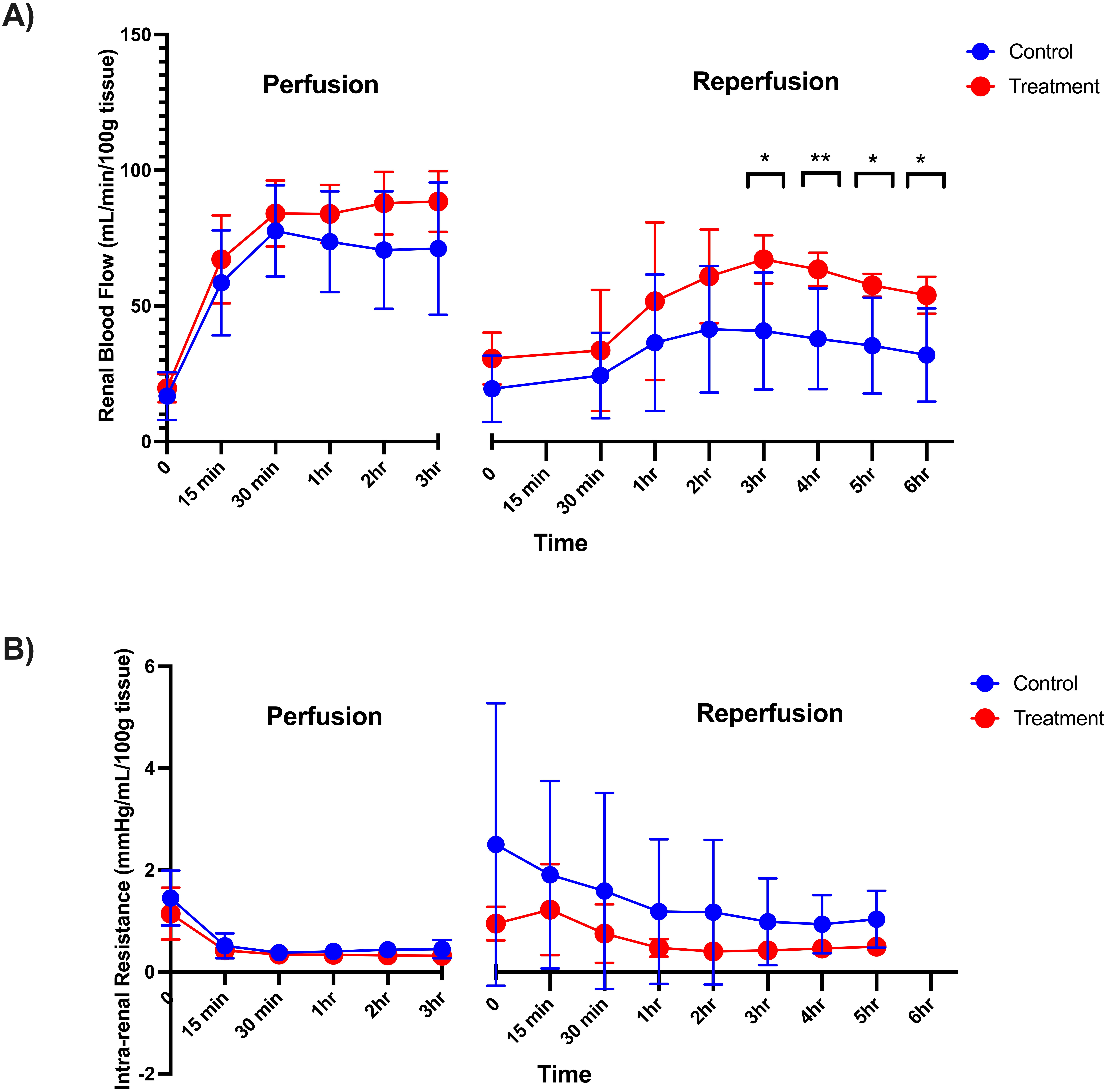
A)

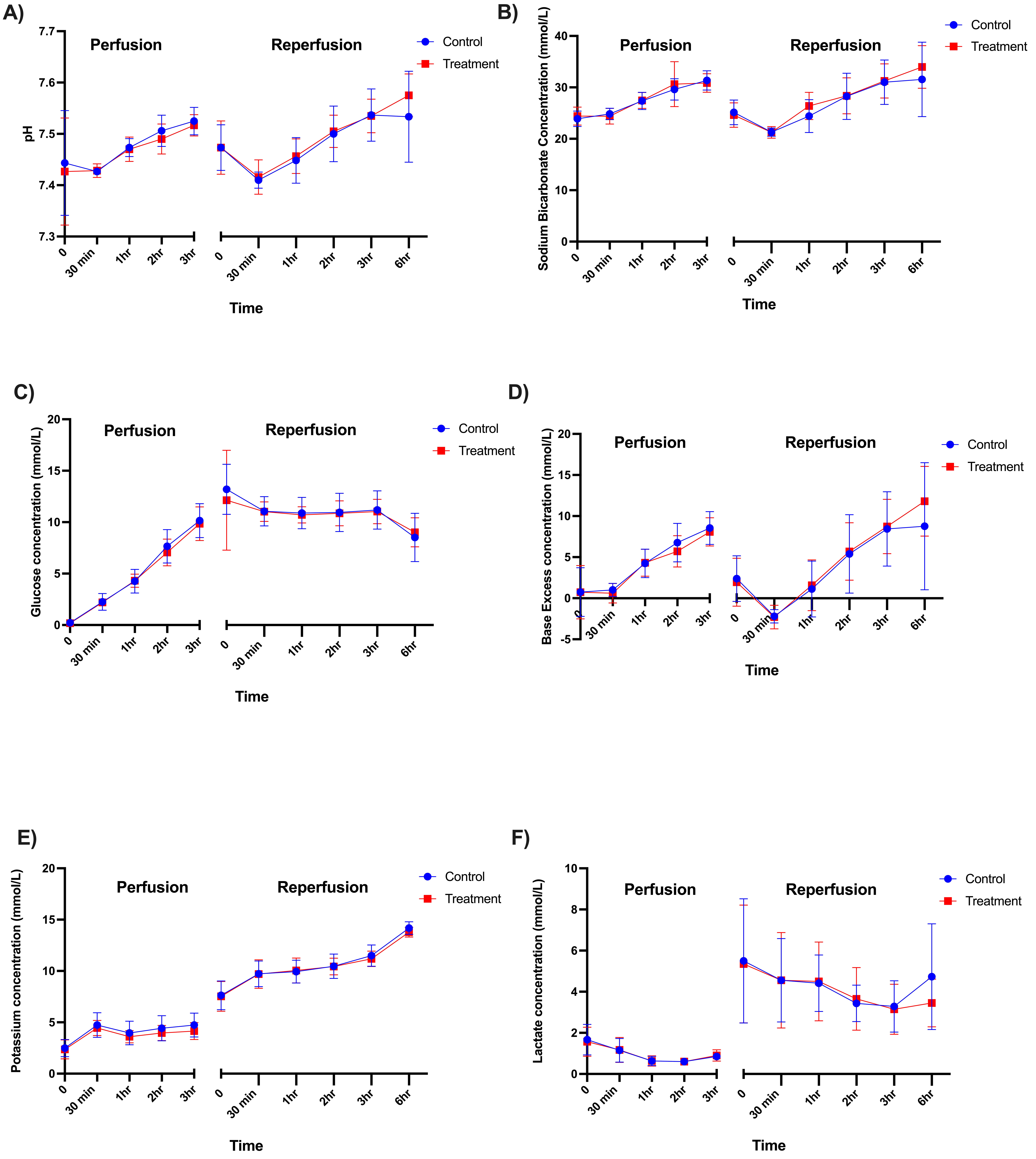

Control
Treatment
40
30
20
10
Temperature from IR Imaging (°C)
0
5
10
15
20
25
30
40
50
60
90
120
180
240
300
360
Time (mins)

B)


Control
Treatment
4
3
2
1
0
Standard Deviation of Temperature
0
5
10
15
20
25
30
40
50
60
90
120
180
240
300
360
Time (mins)

A)
Albumin Content in Urine (mg/mL)
4
3
2
1
0
15
30
60
180
360
Time (mins)
Control
Treatment
B)
*
*
Oxidised DNA Concentration in Perfusate (pg/mL)
20
15
10
5
0
0
60
180
360
Time (mins)
Control
Treatment
C)
TNF-alpha Expression In Perfusate (ng/mL)
5
4
3
2
1
0
0
60
180
360
Time (mins)
Control
Treatment


A)
Renal Blood Flow (mL/min/100g tissue)
150
100
50
0
Perfusion
Reperfusion
Control
Treatment
*
**
*
*
0
15 min
30 min
1hr
2hr
3hr
0
15 min
30 min
1hr
2hr
3hr
4hr
5hr
6hr
Time
B)
Intra-renal Resistance (mmHg/mL/mL/100g tissue)
6
4
2
0
-2
Perfusion
Reperfusion
Control
Treatment
0
15 min
30 min
1hr
2hr
3hr
0
15 min
30 min
1hr
2hr
3hr
4hr
5hr
6hr
Time


A)
pH
7.7
7.6
7.5
7.4
7.3
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time
B)
Sodium Bicarbonate Concentration (mmol/L)
40
30
20
10
0
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time
C)
Glucose concentration (mmol/L)
20
15
10
5
0
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time
D)
Base Excess concentration (mmol/L)
20
15
10
5
0
-5
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time
E)
Potassium concentration (mmol/L)
20
15
10
5
0
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time
F)
Lactate concentration (mmol/L)
10
8
6
4
2
0
Perfusion
Reperfusion
Control
Treatment
0
30 min
1hr
2hr
3hr
6hr
Time

A)
Perfusion
Urine output (mL)
250
200
150
100
50
0
15
30
60
90
120
180
Time (mins)
Control
Treatment
B)
Reperfusion
Urine output (mL)
40
30
20
10
0
30 mins
60 mins
2hr
3hr
4hr
5hr
6hr
Time
Control
Treatment

A)
B)
sC5b-9 concentration (ng/mL)
400
350
300
250
200
150
100
50
0
*
*
**
0
1
3
Time (hours)
● Control
■ Treatment
sC5b-9 concentration (ng/mL)
300
200
100
0
**
**
**
0
1
3
6
Time (hours)
● Control
■ Treatment

A)
Perfusion
TNF-alpha Expression (ng/mL)
6
4
2
0
0
60
180
Time (mins)
● Control
■ Treatment
B)
Reperfusion
TNF-alpha Expression (ng/mL)
0.3
0.2
0.1
0.0
*
*
0
60
180
360
Time (mins)
● Control
■ Treatment