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Early Urinary Biomarkers of Warm and Cold Ischemic Injury in an Experimental Kidney Model

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Background. **Early urinary biomarkers may be useful in determining the severity of ischemic injury in donation after circulatory death (DCD) kidneys. The aim of this study was to evaluate the efficacy of a collective series of urinary biomarkers in relation to the warm and cold ischemic intervals.**

Methods. **Porcine kidneys were retrieved after 0, 10, and 25 min of warm ischemia (WI), then preserved by static cold storage (CS) for period of 2 and 18 h. After preservation, kidneys were reperfused on an isolated organ perfusion system to assess renal function and injury. Levels of IL-6, TNFα, endothelin-1 (ET-1), and neutrophil gelatinase-associated lipocalin (NGAL) were measured in urine samples after 3 h of reperfusion.**

Results. **There was no significant difference in renal functional parameters or urinary biomarkers between the WI times when kidneys were stored for 2 h ($P > 0.05$). After 18 h CS, kidneys with 10 and 25 min of WI demonstrated a significant decline in renal function compared with kidneys without WI ($P < 0.05$). Levels of ET-1 and NGAL were significantly higher in kidneys with 25 min WI (25 m ET-1, 30.1 ± 21.2, *versus* 0 m 2.25 ± 1.5 pg/mL; $P = 0.002$: NGAL, 25 m 77 ± 51 *versus* 0 m 10 ± 0.1 pg/mL; $P = 0.005$). Levels of IL-6 and TNFα were significantly higher in kidneys with 10 and 25 min of WI ($P = 0.001$, 0.001).**

Conclusion. **Early urinary biomarkers are a useful means to determine graft injury. ET-1 and NGAL are more accurate in predicting the severity of ischemic injury compared with inflammatory markers.**

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INTRODUCTION

Delayed graft function (DGF) is a significant problem in kidneys from donation after circulatory death (DCD) donors [1, 2]. The cumulative effects of the warm ischemic interval before organ retrieval, the hypothermic conditions during preservation and reperfusion after transplantation are the main contributors of early graft dysfunction [3]. Although DGF appears to have little impact on long term graft survival [4, 5], it is associated with complications and poorer outcome in the early transplant phase [6].

Previously, we conducted a series of *ex vivo* experiments to determine the relative effects of warm and cold ischemic injury on the renal function and metabolism in a large animal *ex vivo* kidney model of the heart beating and DCD donor [7]. The results showed a progressive decline in renal function and metabolism in kidneys with prolonged static cold storage after 10 and 25 min of warm ischemic injury. No significant differences were found between the warm ischemic intervals when kidneys were stored for just 2 h. The study suggested that limiting the cold ischemic interval was of paramount importance when transplanting kidneys from DCD donors.

The noninvasive measurement of early urinary biomarkers can be valuable indictors of kidney injury [8] and may be useful in predicting the severity of ischemic injury in kidneys from DCD donors. Furthermore, they may be used to predict graft function and allow patients to be treated accordingly. Many potential biomarkers have been identified but none have been used to

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determine the degree of injury in relation to the warm and cold ischemic interval [9]. The aim of this study was to evaluate the efficacy of a collective group of urinary biomarkers measuring inflammation, interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), endothelial cell injury, endothelin-1 (ET-1), and acute kidney injury, neutrophil gelatinase-associated lipocalin (NGAL) in the early phase of reperfusion using an *ex vivo* porcine kidney model of the heart beating and DCD donor.

METHODS

Organ Retrieval

HB donor (0 Min Warm Ischemic Time)

Under the Home Office (Scientific Procedures Act 1986), large white pigs weighing 40–50 kg underwent a general anesthetic, insertion of a central line for the withdrawal of blood, and midline laparotomy as previously described [7]. Both kidneys were immobilized and a double-balloon catheter (Porges, Sarlat, France) inserted into the aorta *via* the iliac artery and inflated. An isolated segment of aorta was then perfused by gravitational hydrostatic pressure of approximately 100 cm H_2O with hyperosmolar citrate solution (Soltran; Baxter Healthcare, Thetford, UK) at 4°C, allowing rapid perfusion of the kidneys. A large vessel cannula was inserted into the vena cava to allow venous drainage. The animal was then exsanguinated *via* the caval cannula and the kidneys removed *en bloc*. Kidneys were then stored on ice for a period of either 2 or 18 h ($n = 5$ per group).

Controlled / Uncontrolled DCD Model (10 and 25 min WI Time)

Under Home Office Schedule 1 regulations, large white pigs were sacrificed by electrocution followed by exsanguination. Approximately 2 L of blood was collected into a sterile receptacle containing 25,000 units of heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK). The blood was then transferred into CPDA-1 blood bags (Baxter Healthcare) for storage. The kidneys were retrieved after 10 or 25 min of *in situ* warm ischemia (WI) and flushed with hyperosmolar citrate at 4°C (Soltran; Baxter Healthcare,) and a hydrostatic infusion pressure of 100 cm H_2O. Again, these kidneys were preserved using static cold storage for periods of 2 or 18 h ($n = 6$ per group) [7].

Reperfusion

After cannulation of the renal artery, vein and ureter kidneys were flushed with Gelofusine at 4°C (B. Braun, Sheffield, UK) and the kidneys placed immediately on an isolated organ preservation system (IOPS) for 3 h at a temperature of 38–39°C and mean arterial pressure set at 85 mmHg. Priming solutions and nutrient supplements were added to the IOPS and kidneys reperfused at normal body temperature with autologous blood for 3 h. Creatinine (Sigma-Aldrich, Steinheim, Germany) was added to the perfusate to achieve an initial circulating concentration of 1000 μmol/L. Renal blood flow (RBF) and mean arterial pressure (MAP) were recorded continuously and intrarenal resistance (IRR) calculated (MAP/RBF). Urine output was also measured during reperfusion. Biochemical analysis of serum and urine samples were carried out at hourly intervals [7]. A sample of urine was collected into a sterile container after 3 h of reperfusion and stored at –80°C until analyzed [7].

IL-6 and TNFα

Urine levels of IL-6 and TNFα were determined by the quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, MN). The samples and standards were added in duplicate to the precoated ELISA plate and incubated for 2 h (IL-6; polyclonal antibody specific for porcine IL-6 or TNFα). After washing an enzyme-linked polyclonal antibody specific to IL-6 or TNFα was added. After a further incubation period of 2 h, the plate was washed and developed with the addition of a substrate solution. A stop solution was then added and the plate read at 450 nm.

Endothelin- 1

Urine levels of endothelin 1 (ET-1) were determined using an enzyme immunometric assay (EIA) kit (Assay Designs, Ann Arbor, MI). The samples and standards were added in duplicate to the precoated plate and incubated for 1 h at room temperature (monoclonal antibody-specific for ET-1). After washing, the HRP labeled monoclonal antibody to ET-1 was added and incubated for 30 min at room temperature. A substrate solution was added after washing then incubated for a further 30 min at room temperature. A stop solution was added to stop the substrate reaction and the optical density read at 450 nM using a spectrophotometer.

NGAL (Neutrophil Gelatinase-Associated Lipocalin)

Urine levels of NGAL were determined using a pig NGAL sandwich ELISA kit (BioPorto Diagnostics, Gentofte, Denmark). The samples were diluted 1/5000 then added in duplicate to the precoated wells. A blank and series of concentrations of calibrator standards were also added to the plate in duplicate. The samples and calibrators were incubated for 1 h at room temperature. After washing, Biotinylated Pig-NGAL antibody was added. The plate was then incubated for 1 h at room temperature. HRP-Streptavidin was added after washing and again the plate incubated for 1 h under the same conditions. After washing, TMB substrate was added and the plate incubated for 10 min at room temperature in the dark. A stop solution was then added and the optical density read at 450 nM using a spectrophotometer.

Statistical Analysis

Values are presented as means ± SD. Levels of continuous variables such as RBF were plotted against time and the area under the curve (AUC) for individual perfusion experiments were calculated using Excel software (Microsoft, Reading, UK). Mean AUC and 3 h values were compared using Kruskal-Wallis test with Dunn's post-test (Graphpad Software, San Diego, CA, USA).

RESULTS

Renal Function (Table 1)

There was no significant difference in the renal blood flow (RBF) or intrarenal resistance (IRR) between the WI times when kidneys underwent 2 h of CS. After 18 h of static cold storage, the RBF was significantly reduced and IRR higher in kidneys with 10 and 25 min of WI ($P = 0.0001$, 0.0015; Table 1).

Although levels of Serum creatinine (Cr) and creatinine clearance (CrCl) were numerically higher in kidneys with 25 min WI and 2 h of CS, there was no significant difference compared with the 0 and 10 min WI groups ($P > 0.05$; Table 1). Cr levels were significantly higher and CrCl lower in kidneys with 10 and 25 min WI after 18 h of CS compared with kidneys without warm ischemic injury ($P = 0.0002$, 0.0001; Table 1).