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Biomarkers of oxidative damage to predict ischaemia-reperfusion injury in an isolated organ perfusion model of the transplanted kidney

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Abstract

Ischaemia-reperfusion (IR) injury is known to be a risk factor influencing both short and long-term graft function following transplantation. The pathophysiology of IR injury is suggested to involve elevated reactive oxygen species production resulting in oxidative damaged cellular macromolecules.

The objective of this study was to evaluate oxidative damage following IR using an isolated organ perfusion model of the transplanted kidney, in order to determine a simple, preferably non-invasive biomarker for IR injury.

Porcine kidneys were retrieved with 10 or 40 min warm ischaemic (WI) time and haemoperfused for 6 h on an isolated organ perfusion machine. ELISA was used to detect carbonyls, 8-isporostane and 8-hydroxy-2′-deoxyguanosine, representing protein, lipid and DNA damage respectively in pre and post reperfusion samples of plasma, urine and biopsy material.

Plasma carbonyl and 8-isporostane and were significantly increased in the 40 min group compared to pre-perfusion (0.96 ± 0.10 vs. 0.62 ± 0.06, $P < 0.001$ and 1.57(1.28–4.9) vs. 0.36(0.09–0.59), $P < 0.05$). The levels also correlated with creatinine clearance used to determine renal function ($r = -0.6150$, $P < 0.01$ and $r = -0.7727$, $P < 0.01$).

The results of this study suggest both plasma carbonyl and 8-isporostane to be reliable biomarkers to predict the level IR injury.

Keywords: *Ischaemia-reperfusion, oxidative damage, biomarker, carbonyl, 8-isporostane*

Introduction

Reperfusion is generally a favourable process, but the process of ischaemia-reperfusion (IR) itself may also cause damage due to the elevation in levels of reactive oxygen species (ROS) [1–3]. During IR elevated levels of superoxide ($O_2^{\cdot -}$) may be generated through several mechanisms. Univalent reduction of oxygen (O_2) to $O_2^{\cdot -}$ may result on reperfusion as a result of electron leakage from the electron transport chain because of impaired aerobic metabolism. Elevated $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2) are also produced from the reaction of hypoxanthine with xanthine oxidase due to break down of ATP [4,5]. Inflammatory cells are recruited on reperfusion and they produce $O_2^{\cdot -}$ from the reduction of oxygen during phagocytic respiratory burst [6]. During ischaemia, accumulation of $O_2^{\cdot -}$ and H_2O_2 may also occur as a result of reduced manganese superoxide dismutase (MnSOD) activity (which converts $O_2^{\cdot -}$ to H_2O_2) and the depletion of glutathione (a substrate for the reaction reducing H_2O_2 to water) [7,8].

During IR, $O_2^{\cdot -}$ and H_2O_2 may react to form more highly reactive species. For example reduction of proteins with iron sulphur centers during ischaemia leads to the release of iron (Fe^{2+}) and this may react with H_2O_2 to form the highly reactive hydroxyl radical ($OH^{\cdot}$) [9]. Also elevated peroxynitrite ($ONOO^-$) may be generated, where $O_2^{\cdot -}$ is able to react with nitric oxide (NO) a product of NO synthase which has been shown to be increased during ischaemia. Further $OH^{\cdot}$

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may be generated as a product of the decomposition of $ONOO^-$ [10]. As previously described, $O_2^{\cdot-}$ is produced by activated neutrophils on reperfusion and H_2O_2 production via the dismutation of $O_2^{\cdot-}$ is also used by neutrophil myloperoxidase to make hypochlorous acid (HOCl), another damaging ROS; HOCl is also able to react with $O_2^{\cdot-}$ to again produce the highly reactive $OH^{\cdot}$ [11].

If the pro-oxidant activity of ROS exceeds the antioxidant defence capacity, oxidative damage to cellular macromolecules may result and therefore determination of reliable biomarkers are potentially useful to predict the level of IR injury. In the clinical situation one may use this information to investigate if antioxidant supplementation may ameliorate the underlying risk to graft function mediated by ROS.

Recent studies have determined oxidant injury to cellular molecules including protein, lipid and DNA in small animal models of renal IR. Studies of protein oxidation have shown increased in carbonyl content, a common biomarker of protein oxidation, in tissue samples determined spectrophotometrically from rat kidney after IR injury [12]. A similar model demonstrated increased urinary excretion of 8-isoprostane (8-isoPGF$_2\alpha$) a well-known marker lipid peroxidation, generated by free radical catalyzed attack on arachidonic acid [13]. Accumulation of the more frequent and most studied oxidative DNA lesion 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) has also been demonstrated in tissue from rat kidney after IR [14]. Despite these findings no simple reliable non-invasive biomarker to predict IR have been recommended, and therefore, the aim of this study was to evaluate using an isolated porcine kidney perfusion model of the transplanted kidney if any of the above biomarkers, determined by simple reliable methodology, could be used for this purpose.

Materials and methods

Isolated organ perfusion

Kidneys were retrieved from large white pigs (schedule 1) sacrificed by electrocution and exsanguination and flushed with Soltran (Baxter, Norfolk, UK); after 10 or 40 min warm ischaemic time they were stored for 2 h on ice. Blood was collected (1 L) into a container containing 25,000 U of sodium heparin. The renal artery, vein and ureter were cannulated and the kidneys flushed with Gelofusine (B Braun Medical Ltd, Sheffield, UK) before reperfusion on an on an isolated organ perfusion machine according to previously described methodology [15]. In brief, the system based on cardiopulmonary bypass technology (Medtronic, Watford, UK) incorporated a centrifugal pump, flow and pressure transducers, heat exchanger, venous reservoir, membranous oxygenator and a temperature probe.

The system was first primed with 0.5 L Ringer's solution containing 25 mM Sodium Bicarbonate, 0.1 M Mannitol, 3.5 mM Cefuroxime and 50 μM Dexamethasone prior to addition of 0.5 L of autologous blood. The perfusate was infused with Nutrifex B (B Braun) containing 25 mM Sodium Bicarbonate and 100 units of insulin. Creatinine was added to the perfusate at an initial concentration of 1000 μM and during the first hour of reperfusion 0.1 mM Sodium Nitroprusside was administered after which 5% glucose solution was infused.

Experimental protocol

Kidneys were randomly retrieved with 10 or 40 min warm ischaemic time into two groups ($n = 6$). During reperfusion renal blood flow was continuously monitored and urine output was recorded. Samples were taken for serum and urinary creatinine every hour. The samples were analysed by the hospital pathology laboratory, and from the data creatinine clearance was calculated as an indication of renal function (urine creatinine × urine volume/plasma creatinine).

Urine samples at 1 and 6 h reperfusion were stored for the measurement of *N*-Acetyl-β-D-glucosaminidase (a marker of tubular damage) using a colorimetric assay (Bio-Quant, San Diego, USA) and also for 8-oxo-dG and 8-isoPGF$_2\alpha$ assays. Needle core biopsies ($n = 4$) were taken immediately prior to reperfusion, at 30 min and at 6 h, rapidly frozen using liquid nitrogen and stored at −80°C. Protein carbonyl detection was performed using this tissue. Two additional needle core biopsies were embedded in tissue-tek and used for the Apo Glow™ adenylate nucleotide ratio assay. Blood (4 ml) was sampled immediately prior to reperfusion, at 3 and 6 h. Plasma was separated at 1120*g* for 20 min at 4°C and 500 μl aliquots were stored at −80°C and used for the determination of carbonyl and 8-isoPGF$_2\alpha$. To minimise inter-assay variation all samples were compared in one plate, less time points were determined in assays requiring triplicate determinations.

Apo glow™ adenylate nucleotide ratio assay

Tissue viability was assessed using the adenylate nucleotide ratio assay kit (Cambrex Bio Science, Berkshire, UK). Frozen sections of 10 μM thickness were cut in triplicate and placed in an Eppendorf pre-cooled to −20°C. Nucleotide releasing reagent, 80 μl, was added to the sections and the sample was vortexed and brought to RT. Twenty microlitre of nucleotide monitoring reagent was added to the aliquot and the sample was placed in a 1250 Bio Orbit luminometer. An immediate voltage reading (A) on a 2210 LKB Bromma chart recorder was noted. A second reading (B) was taken after 10 min. The sample was removed,

20 μl of ADP converting reagent added and the sample placed back in the luminometer. A final reading (C) was taken after 5 min. A mean ratio was calculated by subtracting reading B from C and dividing by reading A for duplicate samples.

Measurement of protein carbonyl

Protein carbonyls were measured in plasma and tissue using an enzyme immunoassay (ELISA) kit (Zenith Technology Corp Ltd, Dunedin, NZ). Needle biopsy (20 μg) homogenates were prepared using an eppendorf homogeniser in only 25 μl of ultra-pure water to maximise protein concentration. Plasma and homogenates were centrifuged at 10,000*g* for 2 min and the supernatant taken for analysis. Standards and samples were derivatised with an excess of dinitrophenylhydrazine (DNPH) for 45 min at RT. For tissue samples twice the recommended volume was taken at the derivatisation stage to further increase protein concentration. Derivatised samples were applied to an ELISA plate in duplicate and incubated O/N at 4°C. The plate was further developed according to assay instructions; washed and blocked for 30 min at RT, washed and incubated with anti-dinitrophenyl (DNP)-biotin antibody for 1 h at 37°C, washed and incubated with streptavidin-horseradish peroxidase (HRP) for 1 h at RT. After a final wash the chromatin reagent was applied. The reaction was stopped after 7 min and the absorbance was determined at 450 nm.

Determination of 8-isoPGF$_2\alpha$

The levels of plasma and urine 8-isoPGF$_2\alpha$ were also determined by ELISA (Cayman Chemical Co, MI, USA). Plasma and urine samples were centrifuged at 10,000*g* for 2 min and the supernatant taken for analysis. Plasma and urine were diluted 10 fold and 5 fold respectively prior to analysis. The sample and standards in triplicate were added to the ELISA plate together with an 8-isoPGF$_2\alpha$-acetylcholinesterase (AchE) conjugate and incubated for 18 h. During incubation the 8-isoPGF$_2\alpha$ present in the sample competed with the 8-isoPGF$_2\alpha$-AChE conjugate for 8-isoPGF$_2\alpha$-rabbit antiserum binding sites on the precoated plate. The plate was washed and then developed by addition of the substrate to AchE. The plate was read at 405 nm after colour development for 60 min.

Detection of 8-oxo-dG

Urinary 8-oxo-dG was detected using the Japan Institute for the Control of Aging new 8-oxo-dG check enzyme immunoassay kit (Gentaur, Legerlaan, Br). Urine samples were centrifuged at 10,000*g* for 2 min and were applied undiluted. Standards and samples were added to the ELISA plate in triplicate together with the 8-oxo-dG monoclonal antibody and incubated for 1 h at 37°C. This antibody reacted competitively with 8-oxo-dG in the samples and 8-oxo-dG bound on the plate. The plate was washed and then incubated with the secondary antibody for 1 h at 37°C. After a further wash the plate was developed by addition of enzyme substrate. The plate was read at 450 nm after colour development for 15 min.

Statistical analysis

Levels of continuous variables such as serum creatinine were plotted against time and the area under the curve (AUC) for individual perfusion experiments was calculated using Microsoft Excel software. Mean values and standard deviations or median values and ranges were used as descriptive statistics. Analysis of data was carried out using GraphPad Instat Version 3 (GraphPad Software Inc., San Diego, CA USA). Difference between the two groups was compared by Student t-test or Mann–Whitney test. Differences between data collected at multiple time-points within the groups were compared by either one-way ANOVA with Turkey-Kramer post test or Kruskal-Wallis test with Dunn's post test. Pearson and Spearman *r* values were used to describe correlations. *p* values of <0.05 were considered statistically significant.

Results

Functional parameters and biochemical analysis of reperfusion

Three main parameters of function, namely creatinine clearance, urine output and renal blood flow of kidneys reperfused by isolated organ perfusion for 6 h (after 10 or 40 min warm ischaemic time followed by 2 h cold ischemic time) are shown in Table I represented as AUC. Each of the parameters was significantly lower in kidneys that had 40 min warm

Table I. Physiological parameters of reperfusion obtained from six kidneys subject to 10 or 40 min WI time and 2 h cold ischaemic time. For each parameter mean ± SDEV or median (and inter-quartile range) AUC are presented, calculated from plots of each parameter against reperfusion time. Creatinine clearance was calculated from results of urinary creatinine, urine volume and plasma creatinine. Statistical analysis was performed using student *t*-test or Mann–Whitney test.

Parameter (AUC)	10 min WI time	40 min WI time	*p* value
Creatinine clearance	9.0 (4.4–36.2)	1.9 (0.04–7.2)	0.04
Urine output	432 ± 175	154 ± 127	0.011
Renal blood flow	266 ± 54.7	137 ± 69.4	0.034

ischaemia compared to those that had 10 min warm ischaemia.

Figure 1 shows the ADP: ATP ratio (an indicator of tissue viability) pre and post reperfusion. A trend for reduced ratio after 6 h reperfusion was noted. Urinary *N*-Acetyl-β-D-glucosaminidase, a measure of tubular damage, normalised against creatinine was not significantly different in the 10 min group after 6 h reperfusion (17.5(16.1–90.75), $n = 6$) compared to 1 h (47.4(8.7–152.7), $n = 6$) or the 40 min group (22.1(6.2–123.1), $n = 5$) vs. (66(48.7–93.1), $n = 4$).

Protein oxidation in plasma

Plasma carbonyl was determined pre and post reperfusion after 10 or 40 min warm ischemic time, followed by 2 h cold ischaemic time (Figure 2A). The level of carbonyls after 3 h reperfusion were significantly higher in kidneys that had 40 min warm ischaemia compared to pre-perfusion. The level of significance increased following 6 h perfusion and at 6 h the levels of carbonyls were significantly higher compared to kidneys that had 10 min ischaemia.

Correlations between plasma protein carbonyls and parameters of renal function are shown in Table II. A significant negative correlation was shown between creatinine clearance (AUC) and plasma carbonyls after 6 h reperfusion (Figure 2B) and was also seen with creatinine clearance (6 h) after 6 h reperfusion. Plasma carbonyls after 3 h reperfusion also correlated creatinine clearance (AUC) and RBF (AUC). Correlations with urine output (AUC) were not quite significant.

Lipid peroxidation index in plasma

The levels of 8-isoPGF$_{2\alpha}$ determined pre and post reperfusion after 10 or 40 min warm ischemic time, followed by 2 h cold ischaemic time were also significantly higher in kidneys that had 40 minutes ischaemia when compared to pre-perfusion (Figure 3A). They too correlated negatively post

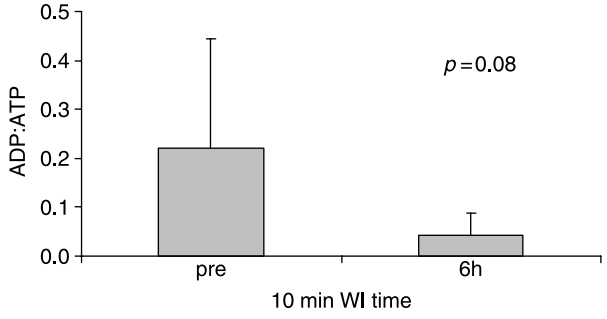

Figure 1. ADP: ATP ratio of kidney biopsies taken pre and 6 h reperfusion after 10 or 40 min WI time and 2 h cold storage. The ratio was determined using an adenylate nucleotide ratio assay kit. Data are mean and standard deviation of six kidneys. Statistical analysis was performed using student *t*-test.

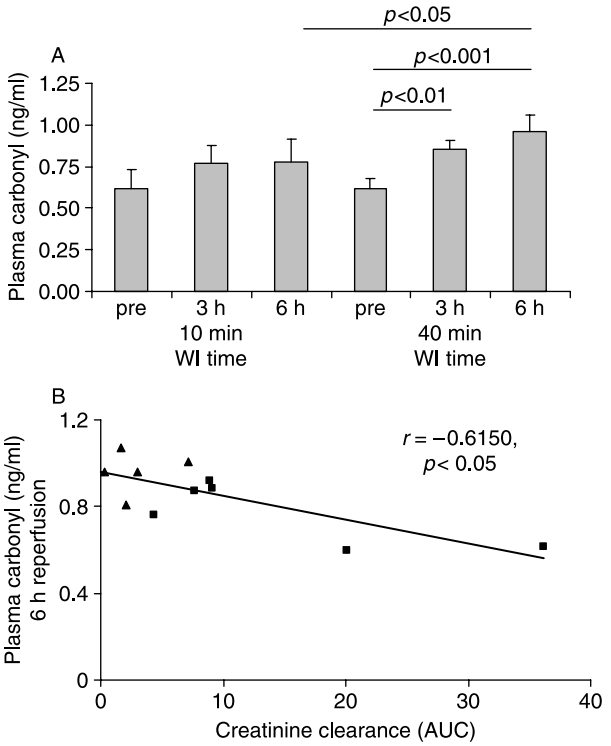

Figure 2. Plasma carbonyl pre and post reperfusion (after 10 or 40 min WI time and 2 h cold storage) determined by enzyme immunoassay. Data are mean and standard deviation ($n = 6$ for 10 min WI time and $n = 5$ for 40 min WI time). Statistical analysis was by ANOVA with Tukey-Kramer post-test (A). Spearman rank correlation between plasma carbonyl post reperfusion and creatinine clearance. ■ = 10 min WIT and ▲ = 40 min WIT (B).

reperfusion with creatinine clearance (AUC) (Figure 3B and Table II).

Protein oxidation in tissue

The results of carbonyl determination in biopsy tissue pre and post reperfusion after 10 or 40 min warm ischaemic time (followed by 2 h cold ischemic time) are shown in Figure 4. In the 10 min group, 4 of 6 kidneys had numerically greater carbonyl values at 30 min reperfusion compared to before reperfusion and all individual values were lower after 6 h reperfusion compared to 30 min in this group. There were no overall correlations between tissue carbonyl levels to any of the functional parameters, however, there was a positive correlation between renal blood flow (AUC) carbonyl levels after 30 min reperfusion (Figure 4B) in the 10 min group.

DNA oxidation products in urine

Urinary 8-oxo-dG normalised to creatinine measured at 1 and 6 h reperfusion following 10 or 40 min WI time (plus 2 h cold storage). The results show no significant difference in the levels of 8-oxo-dG in the 10 min group after 6 h reperfusion (5.1 (3.9–20.9), $n = 4$) compared to 1 h (5.9(3.5–10.2), $n = 4$) or the

Table II. Correlations between parameters of renal function and markers of oxidative damage.

Functional parameter		Plasma carbonyl 6 h	Plasma carbonyl 3 h	Plasma 8-iso-$PGF_2\alpha$ 6 h	Urine 8-iso-$PGF_2\alpha$ 6 h	Tissue carbonyl (10 min WI) 30 min
Creatinine clearance	AUC	−0.62 (0.05)	−0.62 (0.03)	−0.77 (0.01)	0.58 (0.09)	
	6 h	−0.61 (0.05)			0.71 (0.05)	
Urine output	AUC	−0.54 (0.08)	−0.56 (0.06)		0.71 (0.05)	
Renal blood flow	AUC		−0.73 (0.01)			0.93 (0.01)

Values are Pearson or Spearman correlation coefficient (and p values). p values of <0.05 considered statistically significant.

40 min group (4.8(3.2–10.9), $n = 4$) vs. (9.6(7.4–9.7), $n = 4$). There was no correlation between levels of 8-oxo-dG with renal function.

Lipid peroxidation products in urine

8-isoPGF$_2\alpha$ normalised to creatinine was also detected (Figure 5A) and individual data showed numerically greater levels of 8-isoPGF$_2\alpha$ at 6 h reperfusion compared to 1 h reperfusion in both groups. There was no difference between the 10 min group compared to the 40 min group after 6 h reperfusion. There was a significant positive correlation with creatinine clearance measured at 6 h and with urine output (AUC) (Figure 5B). The correlation between creatinine clearance (AUC) and 8-isoPGF$_2\alpha$ after 6 h reperfusion was not quite significant (Table II).

Discussion

The process of IR may cause underlying damage due to the elevation of ROS and highly specific and sensitive assays of oxidative damage are required to determine this injury as generally reperfusion is thought to be a beneficial process. Indeed in our system preliminary assessment of tissue viability (using the ADP: ATP assay) and urinary NAG, a well-known marker of tubular damage showed no significant reduction in tissue viability or increase tubular damage following reperfusion, if in fact there was a possible trend towards a decreased ADP: ATP ratio (greater viability) and this has previously been demonstrated in the literature with hypothermic preservation [16].

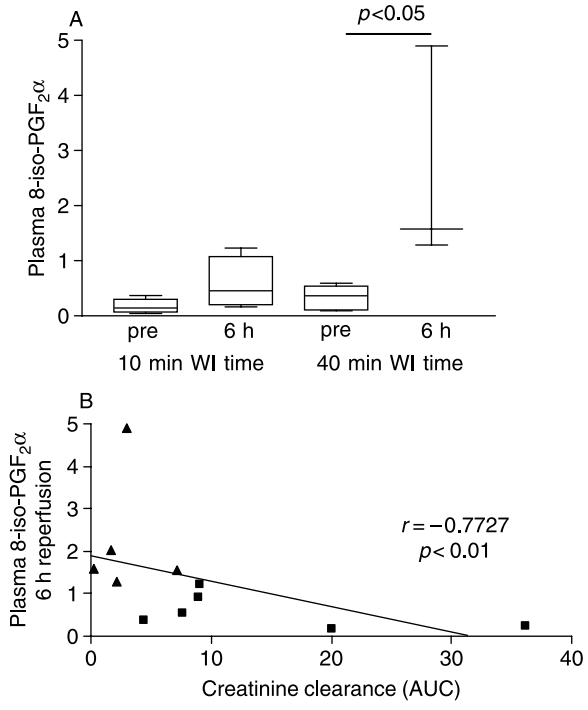

Figure 3. Plasma 8-iso-$PGF_2\alpha$ pre and post reperfusion (after 10 or 40 min WI time and 2 h cold storage) determined by enzyme immuno-assay. Data are median and range ($n = 6$ for 10 min WI time and $n = 5$ for 40 min WI time). Statistical analysis was by Kruskal-Wallis Test (nonparametric ANOVA) (A). Spearman rank correlation between plasma 8-iso-$PGF_2\alpha$ post reperfusion and creatinine clearance; ■ = 10 min WI time and ▲ = 40 min WI time (B).

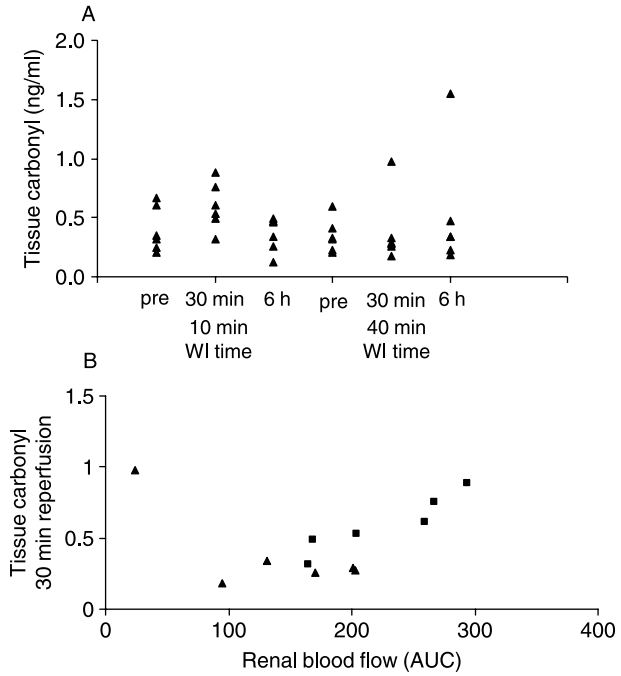

Figure 4. Levels of carbonyls detected in kidney biopsy taken pre, 30 min and 6 h reperfusion (after 10 or 40 min warm ischaemic (WI time) and 2 h cold storage) determined by enzyme immunoassay. Data are individual values of six kidneys (A). Scatter plot showing relationship between carbonyl levels in tissue at 30 min reperfusion and renal blood flow (AUC); ■ = 10 min WI time and ▲ = 40 min WI time (B).

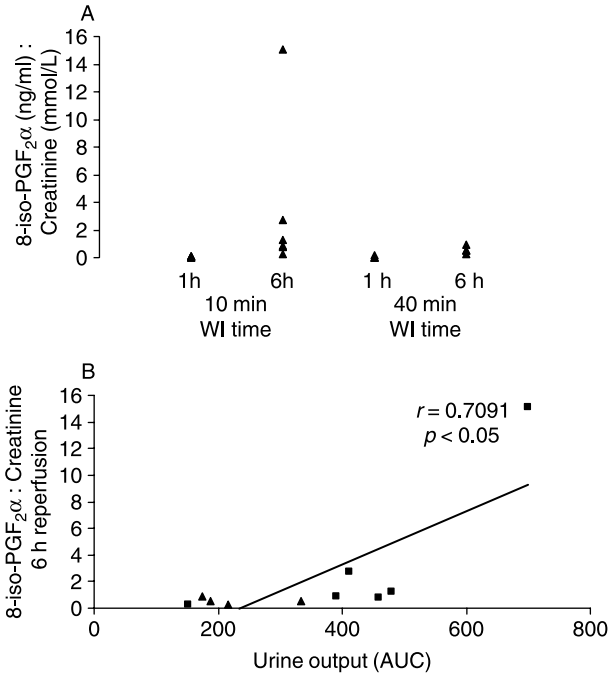

Figure 5. Urinary 8-iso-$PGF_2\alpha$ levels (normalised against creatinine) after 1 and 6 h reperfusion following 10 or 40 min warm ischaemic (WI time) plus 2 h cold storage of six kidneys; determined by enzyme immuno-assay (A). Data are individual values for 10 min WI time ($n = 6$) and 40 min WI time ($n = 4$). Spearman rank correlation between urinary 8-iso-$PGF_2\alpha$ after 6 h reperfusion and urine output (AUC); ■ = 10 min WI time and ▲ = 40 min WI time (B).

In order to detect ROS mediated IR injury, kidneys that had 40 min WI time were compared to those that had 10 min WI time where we predicted a higher level of damage with prolonged WI. In this model both groups of kidneys functioned based on creatinine clearance, renal blood flow, and urine output but better function was demonstrated in kidneys with a shorter WI time. Since the isolated organ perfusion system was designed to mimic what happens clinically, the addition of Sodium Nitroprusside and Mannitol were included in the organ perfusate, despite the fact they may influence the balance of ROS within the system.

Detection of oxidative damage is subject to many difficulties; specificity, stability, artefacts of storage and extraction, and the limits of detection are problems that frequently arise. Therefore immunochemical detection ensured high specificity and sensitivity of stable biomarkers in this study. Plasma and urine were assayed with relative ease, whilst more complex tissue determination was to provide additional information more localized to the initiation of oxidative damage.

Plasma protein carbonyls were shown to be significantly higher in kidneys that had 40 min WI compared to those that had 10 min after reperfusion. We suggest that prolonged warm ischemic time may have primed these kidneys towards oxidative damage on reperfusion. It has been suggested that the conversion of xanthine dehydrogenase to oxidase in the ischaemic kidney occurs over a period of about 30 min and thus more of this enzyme would be available in the kidneys with the longer warm ischaemic time for the generation of ROS [17]. Two of the three of core functional parameters (creatinine clearance and renal blood) flow were negatively correlated with plasma carbonyl levels suggesting this is a sensitive marker able to link oxidative damage to function. The correlation with function also suggests *in vitro* oxidation during reperfusion to be negligible.

Plasma 8-iso-PGF2α was also significantly increased in the kidneys that had 40 min WI after 6 h reperfusion compared to pre perfusion. Again, high levels of 8-iso-PGF2α were also associated with low creatinine clearance possibly due to elevated lipid peroxidation in those with poor function. A potential problem with 8-iso-PGF2α as a marker of lipid peroxidation is that as previous studies (in rat kidney) demonstrated cyclooxygenase dependent production of 8-iso-$PGF_2\alpha$ was observed during the ischaemic phase [18]. Our data suggests 8-iso-PGF2α increased following reperfusion and we suggest this is as a result of ROS produced following reoxygenation.

Carbonyls only were determined in tissue because of the simple and rapid extraction procedure. Carbonyl formation on hepatic proteins in rats exposed to high oxygen concentrations has been demonstrated previously [19]. In this study 4 of the 6 kidneys in the 10 min group, had numerically greater levels after 30 min reperfusion, which correlated with renal blood flow. The renal blood flow was greater in this group and would result in more oxygen reaching the tissue. Also in this group the level of carbonyls was numerically lower at 6 h reperfusion compared to 30 min reperfusion and this suggest damage may be irreversible. It is well known that moderately oxidized proteins are selectively and rapidly degraded by the proteosome and oxidative damage to cellular macromolecules has been shown to have effects on both apoptosis and phagocytosis. However it is still speculative as to whether oxidatively damaged targets could specifically induce the latter two processes as a protective mechanism [20–22].

8-oxo-dG was detected in urine where it is thought to represent a DNA repair product and an indirect marker of DNA damage, and is a simpler measurement than that from tissue which is more likely to induce artefactual damage [23]. Although there was no difference in the levels of 8-oxo-dG detected in this study, the sample size was limited, as some kidneys in the 40 min group did not produce urine. However, it was demonstrated that the assay could be used on porcine samples and that the levels of 8-oxo-dG were within the detectable range of the assay.

The levels of 8-iso-$PGF_2\alpha$ were also detected in the urine and individual kidneys showed numerically greater levels after 6 h reperfusion compared to 1 h reperfusion in both groups. As with the plasma 8-iso-$PGF_2\alpha$ data these results indicate, that 8-iso-$PGF_2\alpha$ was produced during the reperfusion phase. There was a positive correlation between function measured by urine output and the level of urinary 8-iso-$PGF_2\alpha$ and this was opposite to what was observed in plasma. It could be suggested that the kidneys that functioned better were able to excrete more 8-iso-$PGF_2\alpha$ and therefore avoided the build up of 8-iso-$PGF_2\alpha$ in plasma.

There appears to be some contradiction between tissue and plasma results and this suggests complex mechanisms. However, we cannot make an absolute comparison due to some differences in time points between the two types of sample. What happens in the tissue may relate more directly to what is happening in the kidney and therefore interpretation of plasma levels should take into consideration that they might be indirectly related to the cause of oxidative damage and also other conditions might contribute to the overall oxidative status. Further work is warranted in this area. However, one previous study of oxidative stress in a rat model suggested lipid peroxides (another marker of lipid peroxidation) in serum are useful to predict oxidative stress in tissue [24].

In summary, other investigations of oxidative damage in IR injury have been done in tissue samples from small animal models and to our knowledge this study is the first to address the use of non-invasive biomarkers to evaluate oxidative damage in a porcine isolated organ perfusion system. The anatomy and physiology of the porcine kidney are known to be very similar to human kidney and therefore this is a very representative model of the human transplanted kidney. From the evaluation we recommend protein carbonyls in plasma to be the most reliable biomarker to predict IR injury; demonstrated significant increase on reperfusion and correlated more consistently with parameters of function. Assays of plasma are the least complex and this assay was shown to be robust with an inter-assay coefficient of variation of $< 10\%$. We were able to use non-invasive biomarkers to demonstrate a link between the effect of IR on renal function and the involvement of ROS as previously implicated and these may be useful in the future to monitor oxidative damage in investigations of antioxidant intervention.

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