Kidney International, Vol. 67 (2005), pp. 1142–1151

^{1}H-NMR–based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants

Natalie Serkova, T. Florian Fuller, Jost Klawitter, Chris E. Freise, and Claus U. Niemann

Department of Anesthesiology, Biomedical MRI/MRS, University of Colorado Health Sciences Center, Denver, Colorado; Department of Urology, CCM Charité Universitaetsmedizin Berlin, Germany; and Division of Transplantation, Department of Surgery, and Department of Anesthesia and Perioperative Care, University of California, San Francisco, California

^{1}H-NMR–based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants.

Background. Severe ischemia/reperfusion (IR) injury is a risk factor for delayed graft function. Delayed graft function remains difficult to predict, and it currently relies primarily on serum creatinine (SCr), urine output, and occasionally on graft biopsy. ^{1}H-NMR (nuclear magnetic resonance spectroscopy) based metabolomics was used to establish IR-specific metabolic markers in both blood and kidney tissue. These markers were compared to SCr and graft histology.

Methods. Male Lewis rats were used for kidney transplantation. Two cold ischemia (CI) groups (24- and 42-hour) and two transplantation groups [after 24 (TX24) and after 42 hours (TX42) of CI] were compared to a control group. Whole blood and kidney tissue were collected for further analysis.

Results. SCr levels taken 24 hours after transplantation were 1.6 ± 0.12 mg/dL (TX24) and 2.1 ± 0.5 mg/dL (TX42), ($P =$ n.s.). Histology samples revealed mild injury in the TX24 group and severe injury in the TX42 group. A significantly decreased level of polyunsaturated fatty acids (PUFA) and elevated levels of allantoin, a marker of oxidative stress, was found in the renal tissue. In the blood, both trimethylamine-N-oxide (TMAO), a marker of renal medullary injury, and allantoin were significantly increased. Allantoin levels were low in both the control and CI groups. Levels were significantly increased after reperfusion (control 0.02 ± 0.03 μmol/mL, TX24 1.13 ± 0.22, and TX42 1.89 ± 0.38, $P < 0.001$), and correlated with cold ischemia time ($r = 0.96$) and TMAO ($r = 0.94$).

Conclusion. The ^{1}H-NMR metabolic profiles of both the mild and severe IR groups revealed significant changes consistent with graft histology, while the SCr did not.

Renal transplantation has several major post-transplant complications, which include delayed graft function (DGF), toxicity of calcineurin inhibitors, acute and chronic graft rejection. These can contribute to immediate postoperative compromise in graft function and even graft loss. A prolonged cold ischemia time has been shown to contribute to DGF. Delayed graft function is known to be an independent risk factor for acute rejection and is considered along with acute rejection and old donor age to be a predictor of late graft failure [1–3].

Unfortunately, DGF appears to amplify the detrimental effects of old donor age on long-term graft outcome [4]. Currently, urine output and serial serum creatinine (SCr), both nonspecific indicators of kidney function, are most commonly used to detect DGF. The distinction between DGF and acute rejection within the first 24 hours of transplantation remains difficult and frequently requires renal biopsy. An accurate, early, noninvasive method that can detect the severity of ischemia/reperfusion (IR) injury would not only facilitate management, but could also help to distinguish poor renal function that is secondary to acute rejection.

Several isolated metabolites in the blood and urine have been found to be indicators of kidney injury following transplantation. These metabolites are intermediates or end products of cellular processes and, therefore, reflect the global integrated response of an organ or entire biological system to pathophysiologic stimuli [5]. Nuclear magnetic resonance spectroscopy (NMR) has been shown in different studies to predict long-term outcome of renal grafts based on energy state in vivo [6], and may be able to document medullary injury based on metabolic markers ex vivo [7, 8]. However, significant information may be missed if obtained metabolic profiles cannot be integrated into the clinical context. One may be able to demonstrate renal injury by NMR, but will not necessarily be able to distinguish the etiology. Different well-established metabolic end points that can identify a particular pathophysiologic stage will be required for the accurate diagnosis of specific disease processes. The determination of these profiles, primarily using blood, is termed metabolomics, and this may allow insight into complex clinical scenarios. Indeed, ^{1}H-NMR–based metabolomics is a promising approach

Key words: oxidative stress, allantoin, ischemia reperfusion injury.

Received for publication April 15, 2004
and in revised form July 15, 2004
Accepted for publication September 28, 2004

used to investigate and analyze complex multiparametric metabolic data [9, 10]. It has been recently used to distinguish serum metabolic markers that predict coronary artery disease and hypertension [10]. In the area of kidney transplantation, various NMR-based publications have reported trimethylamine-N-oxide (TMAO) to be a urine marker for nonspecific medullar injury [7, 8, 11, 12].

Using ^{1}H-NMR–based quantitative metabolomics, it was our goal in the present study (*1*) to distinguish metabolic markers for IR injury in a rat kidney transplant model; (*2*) to establish concentration ranges for distinguished markers that correlate with the degree of IR injury; and most importantly (*3*) to compare our findings with clinically commonly used end points such as SCr and graft histology.

METHODS

Definition of mild and severe ischemic injury

We previously determined the relationship between duration of cold storage in UW and rat kidney graft survival. The survival rate of grafts with 24-hour cold storage (mild ischemic injury) was 100%. The survival rate of 42-hour cold-stored grafts (severe ischemic injury) was only 40% [13]. In the severe ischemia reperfusion group, recipients of renal grafts typically died of renal failure between postoperative day 2 and 3.

Animal surgery

All animal experiments were carried out at the University of California at San Francisco with approval by the UCSF Committee on Animal Research. Animal care was in agreement with the National Institute of Health guidelines for ethical research (NIH publication no. 80–123, revised 1985). Inbred male Lewis rats (200–250 g body weight) were used as both donors and recipients for heterotopic kidney transplantation. Donor animals were anesthetized with isoflurane, and the left kidney was exposed through a midline incision. Following mobilization of the left kidney, an arterial flush catheter was then placed in the abdominal aorta. Both kidneys were flushed with University of Wisconsin solution (UW solution, DuPont, Wilmington, DE, USA) after the clamping of the suprarenal aorta and incision of the vena cava. Both kidneys were removed and placed in cold UW solution. After the end of cold ischemia time, the right kidney was snap frozen in liquid nitrogen for ^{1}H-NMR analyses of "cold ischemia" kidney extracts. The left kidney was used for transplantation. The recipient animal was anesthetized with isoflurane. Before transplantation, blood samples ("native") were collected from the recipient. Through a midline incision, the native kidneys were exposed, removed, and snap frozen for ^{1}H-NMR analyses of "native" kidney extracts. The recipient's abdominal aorta and vena cava were then exposed, and an end-to-side anastomosis between the donor renal vessels (using an aortic patch) and the recipient aorta and vena cava was performed. An end-to-end anastomosis between the donor and the recipient ureter was performed. All recipients were sacrificed 24 hours after transplantation. Blood and kidney samples were snap frozen in liquid nitrogen for further ^{1}H-NMR analysis.

Study groups

Renal tissue was examined in 5 different groups: native (NAT, $N = 8$); 24 hours of cold ischemia only (CI24, $N = 6$), 42 hours of cold ischemia only (CI42, $N = 6$), and cold ischemia (24-hour, 42-hour) followed by transplantation and 24 hours of reperfusion, (TX24, $N = 8$) and (TX42, $N = 6$). Blood was analyzed in native rats (NAT) and in recipient rats after transplantation and reperfusion (TX24 and TX42).

Graft function and histology

In the native group, blood samples for SCr measurement were taken from the recipient before transplantation, while in the 24- and 42-hour group the samples were from the time of sacrifice. Kidney cross-sections were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for histologic evaluation under a light microscope.

Animal recovery after surgery

Recipient recovery was closely monitored. Animal behavior and appearance were assessed 1 hour, 3 hours, 12 hours, and 24 hours after surgery. The clinical signs of poor initial graft function and ill-appearing animals included a lack of urine production, lethargy, ruffled fur, and guarding upon abdominal palpation. Upon sacrifice, all animals underwent necropsy. Animals with intra-abdominal pathology such as hemorrhage, bowel obstruction, and urine leak were excluded from the study.

Kidney acid extraction for NMR

Kidney tissues were snap frozen in liquid nitrogen and extracted using 12% perchloric acid (PCA) according to a protocol developed in our laboratory [14]. Briefly, 0.2 to 0.8 g of frozen kidney tissue was used per a PCA extract. Tissues were weighed and powdered in a mortar in the presence of liquid nitrogen before 4 mL of ice-cold PCA was added. The samples were vortexed, homogenized using an electrical homogenizer, and centrifuged for 20 minutes at 1300*g* at 4°C. The supernatants were collected and the pellets were redissolved with 2 mL PCA before being vortexed and centrifuged. The supernatants were combined, neutralized to pH 7 using potassium hydroxide (KOH), and centrifuged again to remove potassium perchlorate. The supernatants that

contained water-soluble endogenous metabolites were then lyophilized overnight to remove water for NMR experiments. The water-soluble extracts were then dissolved in 0.45 mL of deuterium oxide (D_2O) prior to ^{1}H-NMR. The pH of each extract inside of the 5-mm NMR tube was checked using a thin pH electrode. If necessary, deuterated chloride (DCl) and sodium hydrogen (NaOD) were added to adjust pH = 7.

After the second centrifugation, the pellets were redissolved in 4 mL ice-cold water and pH = 7 was adjusted using KOH. The pellet suspensions that contained lipid fraction were then lyophilized overnight to remove water for NMR experiments. The lipid extracts were dissolved in 1 mL of deuterated chloroform/methanol mixture (2:1, vol/vol) prior to ^{1}H-NMR.

Blood methanol/chloroform extraction for NMR

All blood samples were processed using dual chloroform/methanol extraction [15]. One mL of heparin-preserved whole blood was carefully vortexed and processed with 2 mL of cold chloroform/methanol (1:1, vol/vol). After centrifugation, the supernatants were collected and the pellets were resuspended with 1 mL of chloroform/methanol, centrifuged, and the supernatants were collected. The supernatant was washed with 1 mL ice-cold water. The water phase was removed and added to the pellet. Two mL of water was added, and the pellet was centrifuged, and the supernatant was lyophilized overnight (water-soluble extracts). Afterwards, the water-soluble part was dissolved in 0.45 mL D_2O and analyzed by proton NMR. The lipids in the organic phase were evaporated to dryness under a stream of nitrogen at 50°C. The lipid extracts were dissolved in 0.6 mL of deuterated chloroform/methanol (2:1, vol/vol) for NMR analysis.

Proton quantitative NMR on blood and kidney extracts

All water-soluble and lipid extracts from kidney and blood tissue were analyzed using a 500 MHz high-resolution Bruker DRX system (Bruker Biospin, Inc., Fremont, CA, USA). An inverse TXI 5-mm probehead was used for all experiments. In order to suppress water residue in extracts, a standard Bruker water presaturation sequence was used ("zgpr") (operating frequency for proton channel: 500.24 MHz; power level pl1 = 3 dB; power level for water suppression pl9 = 55 dB; power angle p1 = 7.5 μsec (90° pulse); power angle for water suppression p12 = 60 μs; water suppression at O1 = 4.76 ppm; relaxation delay d1 = 12.85 sec (5×T1); delay for power switching d12 = 20 μs; short delay d13 = 3 μs; spectral width sw = 12 ppm; total number of scans ns = 40). An external standard substance, trimethylsilyl propionic-2,2,3,3,-d_4 acid (TMSP, 20 and 50 mmol/L in D_2O) was added into a thin glass capillary. The final TMSP concentration (0.5 mmol/L and 1.2 mmol/L) in the capillary was calculated prior to NMR experiments on study extracts using a standard amino acid solution. The TMSP capillary was placed into the NMR tube during the experiment (0.5 mmol/L for water-soluble extracts and 1.2 mmol/L for lipid extracts), and served as an external standard which allowed for absolute metabolite quantification in each study extract. ^{1}H chemical shifts were referred to TMSP signal at 0 ppm. After performing Fourier transformation and making phase and baseline corrections, each ^{1}H peak was integrated using 1D WINNMR program (Bruker Biospin, Inc., Fremont, CA, USA). The absolute concentrations of single metabolites were then referred to the TMSP integral and calculated according to equation 1:

$$Cx = \frac{Ix : Nx \times C}{I : 9} \times V : M \quad (1)$$

where Cx = metabolite concentration, Ix = integral of metabolite ^{1}H peak, Nx = number of protons in metabolite ^{1}H peak (from CH, CH_2, CH_3), C = TMSP concentration, I = integral of TMSP ^{1}H peak at 0 ppm (:9 since TMSP has 9 protons), V = volume of the extract, M = weight of kidney tissue or volume of blood sample.

Statistical analysis

All data are present as mean ± standard deviations. Stepwise logistic regression modeling was performed in order to distinguish a specific marker in outcome based on values of a set of predictor variables as widely described in the literature [16]. In addition, a correlation analysis between the absolute concentrations of distinguished markers and cold ischemia time ($t_1 = 24$ and $t_2 = 42$) was performed using SPSS 11.5. One-way analysis of variance (ANOVA) test (SPSS 11.5; SPSS, Inc., Chicago, IL, USA) was performed in order to evaluate statistically significant changes in metabolites between the study groups. The P value <0.05 was recognized as being statistically significant. Postoperative SCr were compared to native groups using ANOVA with Dunnett's correction.

RESULTS

Animal recovery after surgery

All recipients with 24-hour cold stored kidneys (N = 6) fully recovered 1 hour after surgery as assessed by ambulation, grooming, drinking, and feeding. All animals in this group showed good urine production on postoperative day 1. Two recipients in the 42-hour cold ischemia group also demonstrated a quick recovery from surgery that was comparable to animals in the 24-hour group. Four animals in the 42-hour cold ischemia group showed slow recovery. A lack of urine production, lethargy, ruffled fur, and guarding upon abdominal palpation was present in all 4 animals. However, none of the animals

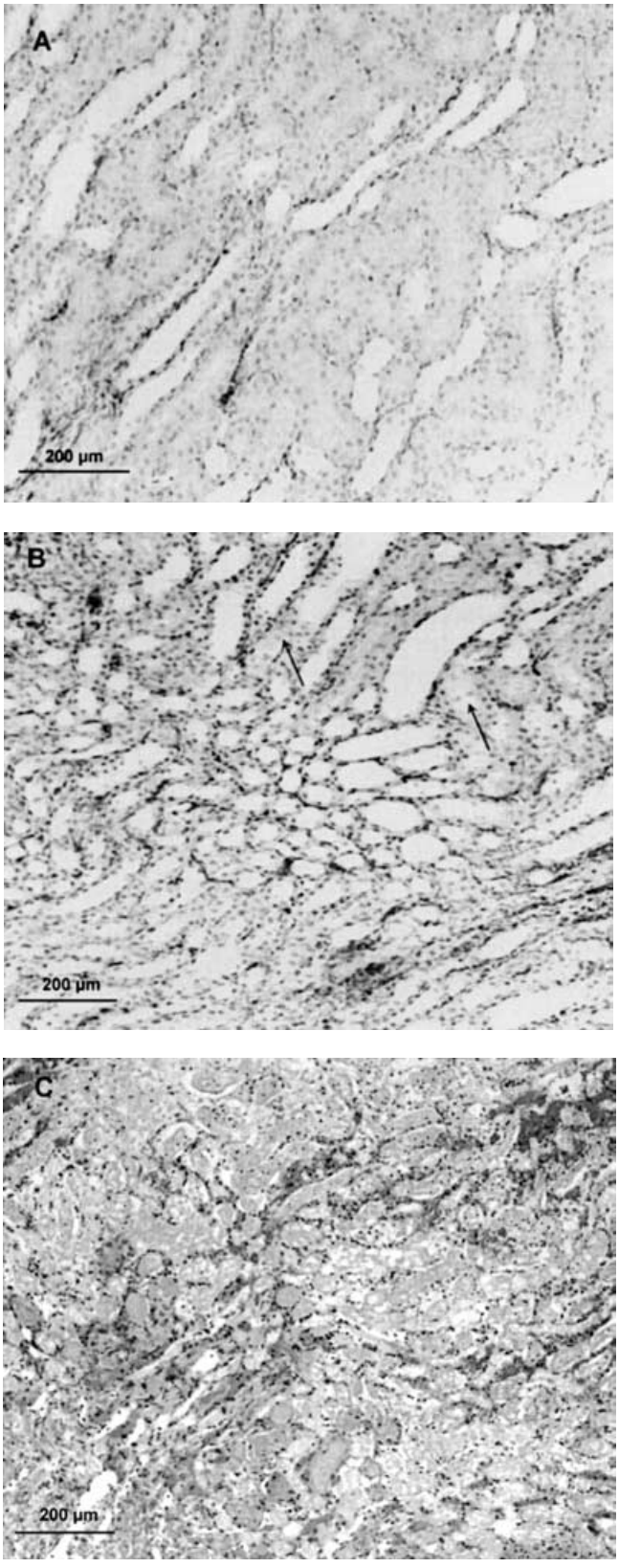

Fig. 1. (*A*) Representative light micrographs of native kidneys with no ischemia reperfusion injury. (B) Representative light micrographs of 24 hour cold-stored rat kidney grafts 24 hours after transplantation. Mild to moderate IR injury resulted in proximal tubular dilatation with few epithelial casts (arrows). (B) also shows a widely intact tubular architecture in the outer medulla. (*C*) Representative light micrographs of 42-hour cold-stored rat kidney grafts 24 hours after transplantation. Severe I/R injury caused widespread necrosis of proximal tubular cells. (C) also shows the outer medulla with bandlike areas of frank tubular necrosis and congested capillaries.

died before the end of the observation period. There was no difference in body weight between animals after 24 hours of transplantation.

Serum creatinine levels after 24 hours of reperfusion

Serum creatinine in sham operated rats was 0.47 ± 0.1 mg/dL. Twenty-four hours after transplantation SCr was not different for grafts after 24 or 42 hours of cold ischemia. Mean SCr was 2.1 ± 0.48 mg/dL in the TX42 group and 1.6 ± 0.12 mg/dL in the TX24 group (P = n.s.). Both were significantly higher than in control animals ($P < 0.01$). The two rats in the 42-hour cold ischemia/24-hour reperfusion group that appeared to be in better clinical condition had lower SCr levels.

Graft histology

Grafts subjected to either 24 hours or 42 hours of cold storage presented with different patterns of histologic damage after 24 hours of transplantation. The proximal tubules, which are predominantly located in the outer medulla, are known to be most susceptible to ischemia reperfusion injury, while the renal cortex remains largely unaffected. Grafts subjected to 24 hours of cold storage prior to transplantation (TX24) showed mild to moderate histologic damage, presenting as proximal tubular dilatation and epithelial casts formation (Fig. 1B). In contrast, 42-hour cold stored grafts (TX42) showed irreversible damage in most cases. Typically, the outer medulla showed large areas of frank tubular necrosis surrounded by viable but severely damaged proximal tubules (Fig. 1C).

Metabolic changes in kidney tissues

Over 30 metabolites were identified and quantified from kidney extracts by ^{1}H-NMR. The absolute concentrations of major endogenous metabolites from native kidney extracts are presented in Table 1. The determination of the baseline metabolic profiles in normal kidney tissue is crucial in order to allow for the comparison to injured kidneys. Renal tissue contained carbohydrates (glycogen and glucose) and triglycerides as their nutritional source. There was no hydroxybutyrate (ketone bodies). High concentrations of cellular osmolytes such as inositol, betaine, TMAO, and taurine were detected in the native kidney, as well as intermediate amino acids from the citrate cycle (glutamate, glutamine, citrate, aspartate). End products of glycolysis (lactate

Table 1. Absolute concentrations of major endogenous metabolites quantified from native blood and kidney extract by ^{1}H-NMR

Metabolites	Chemical shift *ppm*	Kidney *μmol/g*	Blood *μmol/mL*
Acetate	1.95	0.09 ± 0.10	0.17 ± 0.02
Alanine	1.48	1.05 ± 0.20	0.65 ± 0.03
Allantoin	5.39	0.34 ± 0.16	0.02 ± 0.03
Asparagine	2.96, 4.00	n.d.	0.42 ± 0.26
Aspartate	2.82+2.68	0.56 ± 0.09	n.d.
Betaine/CH_3	3.26	1.16 ± 0.21(+TMAO)	n.d.
3-OH-butyrate	1.20	n.d.	0.08 ± 0.03
Citrate	2.80 + 2.67	0.91 ± 1.15	0.42 ± 0.09
Citrulline	1.58, 3.15	n.d.	0.72 ± 0.07
Cholesterol	0.69	6.91 ± 1.00	1.90 ± 0.10
Cholines	3.21	14.23 ± 1.98	1.35 ± 0.15
Creatinine+creatine	3.02 − 3.04	0.79 ± 0.22	0.23 ± 0.08
Dimethylamide	2.72	0.06 ± 0.06	n.d.
Glucose	5.23 + 4.64	2.87 ± 0.42	4.16 ± 1.35
Glutamate	2.36	3.01 ± 0.57	0.33 ± 0.10
Glutamine	2.46	1.05 ± 0.19	0.29 ± 0.08
Glutathione (GSH)	2.53	0.84 ± 0.22	0.31 ± 0.11
Glycerol-6-phosphate	3.99	12.57 ± 2.17	1.06 ± 0.24
Glycerophosphocholine	3.23	0.99 ± 0.11	n.d.
Glycogen	5.41	0.34 ± 0.04	n.d.
Inositol	4.06	2.72 ± 1.08	n.d.
Lactate	1.32	1.75 ± 0.28	1.31 ± 0.39
Monounsaturated fatty acids (MUFA)	5.42 (with PUFA)	10.30 ± 2.72	1.11 ± 0.07
Phosphatidylcholine	3.57	3.13 ± 0.42	0.28 ± 0.06
Phosphatidylethanolamine	3.10	1.15 ± 0.22	n.d.
Polyunsaturated fatty acids (PUFA)	2.84	78.52 ± 13.02	8.12 ± 0.72
Pyruvate	2.38	1.40 ± 0.57	0.36 ± 0.05
Taurine	3.43	4.91 ± 1.20	n.d.
Total triglycerides (TG)	0.88	119.87 ± 30.22	13.22 ± 2.88
Triacylglycerol (TAG)	4.16	10.17 ± 4.17	1.02 ± 0.34
Trimethylamide	2.88	n.d.	0.01 ± 0.02
Trimethylamine-N-oxide (TMAO)/CH_3	3.27	1.16 ± 0.21 (+betaine)	0.24 ± 0.04
Valine+leucine+isoleucine	0.99 − 1.04	3.17 ± 1.50	1.18 ± 0.19

n.d., nondetectable. TMAO and betaine concentrations are based on three CH_3 groups.

Table 2. Changes in distinguished metabolic markers of I/R in rat kidney tissue in ischemic (CI24, CI42) and transplant groups (TX24, TX42)

Kidney metabolites	NAT	CI24	CI42	TX24	TX42
Allantoin	0.34 ± 0.16	0.02 ± 0.05[a]	0.02 ± 0.06[a]	1.89 ± 0.49[a,b]	2.62 ± 0.19[a,b,c]
PUFA	78.52 ± 13.02	64.91 ± 12.00	54.27 ± 13.46[a]	51.04 ± 3.51[a,b]	38.91 ± 8.60[a,c]

PUFA, polyunsaturated fatty acids. All data are given as mean ± SD. The concentrations are given as μmol/g.
[a] $P < 0.01$ to the control group.
[b] $P < 0.05$ to the corresponding cold ischemia groups.
[c] $P < 0.02$ between TX24 and TX42.

and alanine) were present in the kidney. Kidney tissues also contained high concentrations of lipid metabolites (triglycerides, cholesterol, poly- and monounsaturated fatty acids).

Cold storage in UW solution significantly changed the metabolic composition of the kidney. A significant increase in glycogen and other carbohydrates ($P < 0.0001$ vs. native kidney) was observed at 24 and 42 hours of cold ischemia. As expected, the lactate concentration was greatly increased, up to 380% during cold ischemia. In the lipid extracts, a decrease of polyunsaturated fatty acids (PUFA) was seen in cold ischemia groups versus native kidney (spectra not shown, Table 2). There were no significant differences in metabolic composition at the end of 24 and 42 hours of cold ischemia time (groups CI24 and CI42), indicating that after 24 hours of cold storage, kidneys had already switched their metabolism to adapt for ischemic conditions.

Transplanted kidneys exposed to reperfusion for 24 hours (TX24 and TX42) demonstrated characteristic changes in their metabolic profiles. The most pronounced difference between the TX and CI groups was the dramatic increase of an initially not identified peak at 5.39 ppm (Fig. 2). Peak identification was performed using two-dimensional 2D-H,C-HSQC NMR, which allows for the exact structure analysis (chemical shifts for allantoin: $\sigma_{1H} = 5.39$ ppm; $d_{13C} = 66$ ppm). It was identified as a (CH) group of allantoin (Fig. 4), the end product of xanthine metabolism and uric acid, and a marker of oxidative stress. In order to confirm the identified structure we also ran HSQC on a standard solution of 10 mmol/L allantoin (Sigma Aldrich, St. Louis, MO, USA).

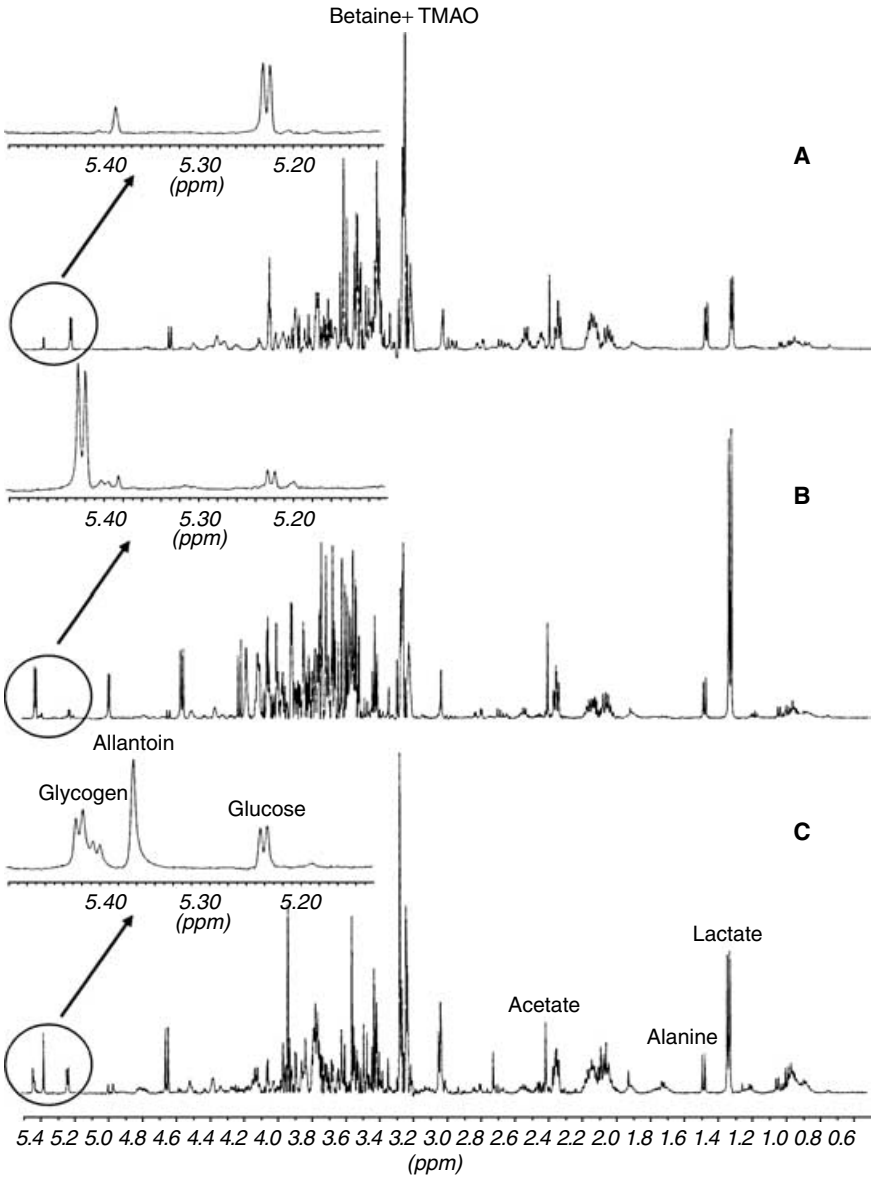

Fig. 2. Representative ^{1}H-NMR of kidney extracts. (*A*) Native kidney (NAT). (*B*) Twenty-four hours of cold ischemia (CI24). (*C*) Twenty-four hours after transplantation followed after 24 hours of cold ischemia (TX24). TMAO, trimethylamine-N-oxide.

Allantoin concentrations were low in the native kidney (0.34 ± 0.16 μmol/g tissue) and at the end of cold ischemia (range 0.02 to 0.12 μmol/g for 24 to 42 hours). Allantoin levels increased significantly after 24 hours of reperfusion (1.89 ± 0.49 μmol/g in TX24 and 2.62 ± 0.19 μmol/g in TX42, $P < 0.01$ between TX24 and TX42; Table 2). The stepwise logistic regression analysis revealed that from 30 metabolites quantified from kidney extracts, only two—allantoin and PUFA—were different among study groups (Table 2 and Fig. 3A). Even though significant allantoin concentrations appeared only after reperfusion, its concentrations correlated well with cold ischemia times ($r = 0.95$). A three-dimensional group distribution based on allantoin, PUFA concentrations, and cold ischemia time allowed for visual cluster analysis of the 5 study groups (Fig. 3A).

Metabolic changes in rat blood

From blood extracts, over 50 metabolites were identified and quantified. Out of 8 animals in the native group (Table 1), allantoin concentrations were below the limit of quantification for NMR in the blood of 6 animals. Allantoin peaks appeared in the blood in both TX24 and TX42 group following reperfusion (Fig. 2). Allantoin concentrations were significantly higher in transplanted rats with 42-hour cold storage when compared with 24-hour cold storage (Table 3, $P < 0.002$). The correlation coefficient for allantoin concentrations was excellent for cold ischemia time ($r = 0.96$).

The allantoin concentrations in 2 TX42 rats with good initial graft function as determined by clinical appearance, SCr, and urine output were lower in comparison with the concentrations in 4 rats from the same group

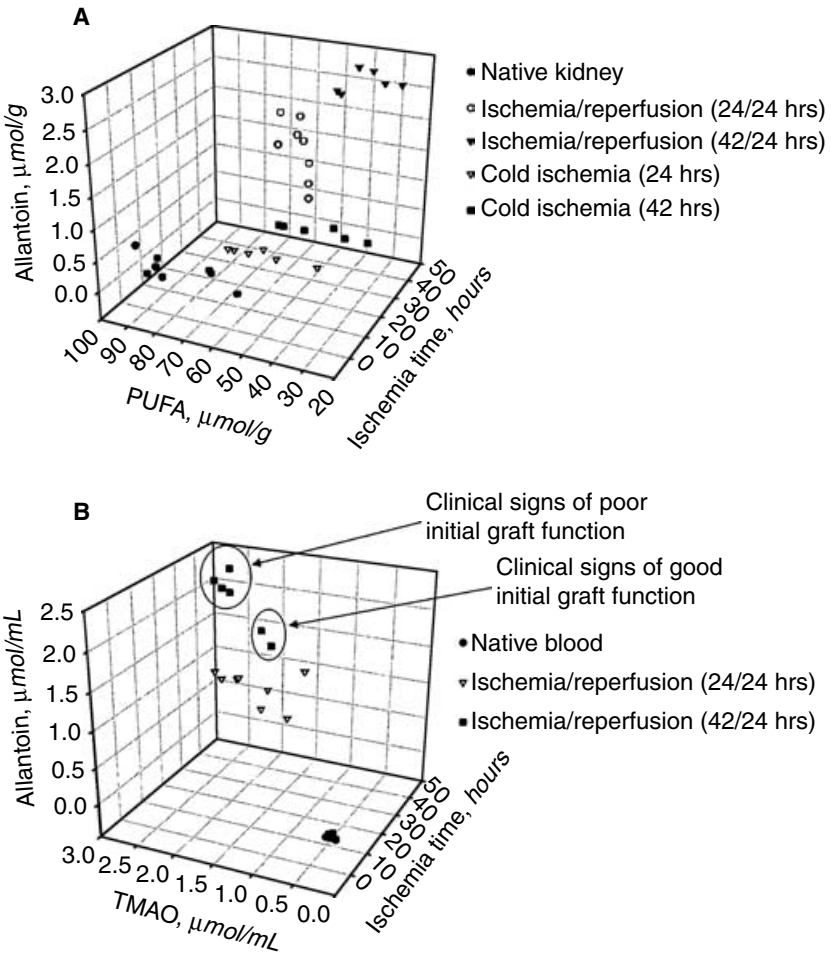

Fig. 3. (*A*) Distribution of study groups based on endogenous concentrations of allantoin and PUFA in rat kidney as function of cold ischemia time. Correlation with ischemia time: $r = 0.95$ (allantoin), $r = -0.91$ (PUFA); correlation between allantoin and PUFA: $r = -0.87$. (*B*) Distribution of study groups based on endogenous concentrations of allantoin and TMAO in rat blood as function of cold ischemia time. Correlation with ischemia time: $r = 0.96$ (allantoin), $r = 0.94$ (TMAO); correlation between allantoin and PUFA: $r = 0.94$. Abbreviations: PUFA, polyunsaturated fatty acids; TMAO, trimetylamidne-N-oxide.

who had signs of poor initial graft function (Fig. 3B). The allantoin concentrations in these 2 rats were 1.52 and 1.33 µmol/mL compared to 1.99 to 2.32 µmol/mL in the 4 rats with poor function.

A second metabolic blood marker, trimethylamidoxide (TMAO), which was identified by logistic regression correlated well with cold ischemia time ($r = 0.94$). TMAO is a nonspecific marker of medullary injury, and its concentration correlated well with elevated allantoin levels ($r = 0.95$).

Because allantoin is a direct product of uric acid, we determined the concentrations of uric acid in rat serum from NAT, TX24, and TX42 groups. We used an enzymatic assay since uric acid has no CH-bounded groups and, as such, produces no NMR signal. However, uric acid concentrations were below the linear range for the assay and, therefore, of no significance.

Table 3. Changes in distinguished metabolic markers in rat blood in transplant groups (TX24, TX42)

Blood metabolites	NAT	TX24	TX42
Allantoin	0.02 ± 0.03	1.13 ± 0.22^{a}	$1.89 \pm 0.38^{a,b}$
TMAO	0.24 ± 0.04	1.79 ± 0.42^{a}	$2.45 \pm 0.32^{a,b}$

TMAO, trimetylamine-N-oxide. All data are given as mean ± SD. The concentrations are given as µmol/mL.
[a] $P < 0.00001$ to the control group.
[b] $P < 0.005$ between TX24 and TX42.

DISCUSSION

The attempt to distinguish the etiologies of poor initial function of renal grafts has led to an increasing number of studies. Detection of DGF currently relies mainly on serial SCr measurements and urine output, but this approach cannot distinguish between DGF, acute rejection, and drug nephrotoxicity. Knowledge about the etiology of poor postoperative graft function is necessary for clinical decision-making, including the choice of immunosuppressive therapy. At times, renal biopsy is required to determine the etiology of impaired graft function. Using specific metabolic changes in peripheral blood to identify DGF may have important clinical implications, largely because this method is relatively noninvasive. It is unlikely that diagnostic blood tests will be able to entirely replace renal biopsy, but they may prove to be adequate in the majority of cases.

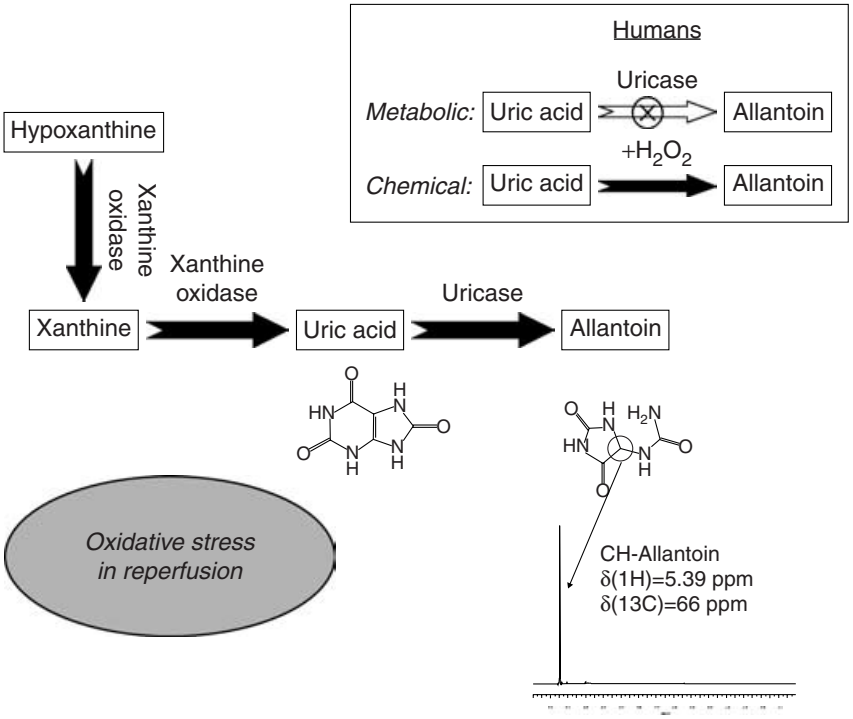

Fig. 4. Metabolic and chemical pathways of allantoin formation from uric acid (hypoxanthine/xanthine pathway). Xanthines are highly sensitive to oxidative stress during reperfusion. Increased activity of xanthine oxidase leads to increased degradation of xanthines. Accumulation of end products allantoin (in mammals) can be used as a sensitive marker for reperfusion injury in rat kidney transplantation. In humans, allantoin is produced by the chemical reaction between uric acid and H_2O_2 since uricase is not present. Allantoin (molecular weight: 158.12) is detectable a singlet at 5.39 ppm in ^{1}H-NMR (66 ppm in ^{13}C-NMR).

Proton-NMR spectroscopic analysis allows for the simultaneous detection, identification, and quantification of hundreds of low-molecular-weight (max. 20 kD) metabolites within a biological matrix [15]. The use of NMR-based metabolomics on tissue biopsies and body fluids allows for the assessment of dynamic changes in global metabolism and, specifically, for noninvasive blood markers, which represents a novel and robust method of assessing organ response to pathophysiologic stimuli [5, 10, 17]. Several publications have demonstrated the feasibility of NMR for evaluating different clinical scenarios in transplantation [8, 12]. Most publications have reported the relative ratios of one metabolite to another, which, due to the complicated dynamic changes in metabolism, can often overlook significant changes.

Previous studies have reported the production of reactive oxygen species (ROS), lipid peroxidation, and antioxidant capacity in renal transplantation [18–22]. However, the interpretation and comparison (e.g., lipid peroxidation between groups) of a single metabolic end point can be confounded by variations in either experimental or physiologic status. For example, oxidative stress is not only seen with IR during kidney transplantation, but also with diabetes [23] and hypertension [24]. The interpretation is further complicated by the fact that both hypertension and diabetes are often simultaneously present in kidney recipients.

Reactive oxygen radicals are produced by different pathways. These pathways include various enzymes such as lipoxygenases, peroxidases, NADPH oxidase, and xanthine oxidase. Xanthine oxidase is a molybdenum-requiring enzyme that uses molecular oxygen and produces H_2O_2, which in turn may be used for further reactions in the xanthine pathway in humans (Fig. 4). Up-regulation of the xanthine pathway has been demonstrated to be an important contributor to oxidative stress in transplantation, burn traumas, pulmonary endothelial cells exposed to tobacco smoke, and hypertensive and global ischemia models [24].

As expected, we did not detect any metabolic differences between the cold storage periods without reperfusion that were due to oxidative stress. Only after the reperfusion of the graft did the different levels of oxidative stress become apparent. Twenty-four hours after cold storage and transplantation, we were able to distinguish allantoin, a marker of oxidative stress after ischemia/reperfusion, not only in renal tissue but also in blood. In mammals, allantoin is a metabolic end product of uric acid and, therefore, of xanthine metabolism (Fig. 4). The levels of allantoin after reperfusion correlated strongly with the duration of cold ischemia. The increased concentrations of allantoin after the reperfusion of the ischemic kidney indicate up-regulation of xanthine oxidase with increased degradation of xanthines. Uricase (the enzyme which catalyzes the reaction from uric acid to allantoin) is highly active in mammals and, therefore, uric acid was not detected in rat plasma in our study. In humans, by contrast, uricase activity has not been reported, but allantoin has been documented. The detection of allantoin in humans is likely to be secondary to a nonenzymatic reaction, as shown in Figure 4. Several studies involving diabetic human subjects and volunteers

under extreme exercise have demonstrated elevated allantoin concentrations that can be attributed to oxidative stress [23, 25]. Furthermore, the preliminary results from a clinical study performed at our institution indicate that the recipients of cadaveric renal allografts that have had prolonged cold ischemia may experience elevated allantoin as well as uric acid concentrations 24 hours after transplantation (data not shown). In the present study, the high concentrations of allantoin in rat renal grafts might have 2 possible explanations: the increased degradation in xanthine pathways through uricase (not present in humans, but elevated uric acid concentrations can be expected); and an increased H_2O_2 production through a chemical pathway (can be expected in human recipients).

Similarly, decreased PUFA levels were observed in renal grafts, which were inversely correlated with allantoin levels. Tissue concentrations of PUFA have been shown to be a sensitive marker of lipid peroxidation [26, 27]. PUFA are the fatty acids that undergo peroxidation under oxidative stress conditions. Therefore, their decreased concentrations are a marker for increased lipid peroxidation. During ischemia/reperfusion injury, lipid peroxidation is increased in the tissue, while PUFA concentrations are greatly decreased [27]. Markedly decreased PUFA levels were seen in the TX42 group (38.91 ± 8.60 vs. 78.52 ± 13.02 μmol/g when compared with native kidney, $P = 0.0001$). In contrast, the TX24 group had significantly higher PUFA levels (51.04 ± 3.51 vs. 38.91 ± 8.60 when compared to TX42 group, $P = 0.0163$), which indicates a lower degree of lipid peroxidation. After 42 hours of cold ischemia and 24 hours of reperfusion, the kidney still experienced significantly elevated levels of oxidative stress. However, these findings in renal tissue are unlikely to offer any advantage over the classical histologic analysis of kidney biopsies.

Blood allantoin levels were highly correlated with the degree of renal medullary injury as determined by TMAO. The osmolyte, TMAO, is synthesized in the renal medulla and is an established marker of medullary renal injury. Hauet et al demonstrated in several studies that TMAO correlated with the degree of renal injury inflicted by different mechanisms. Most relevant to the present study was their finding that metabolic markers of injury significantly increased with the duration of cold ischemia and different preservation solutions [7, 8, 12]. While to a certain extent the degree of medullary injury can be estimated by TMAO, the etiology of this injury remains undetectable. For instance, significantly elevated blood TMAO levels were demonstrated in rats treated with clinically relevant cyclosporine doses when compared to controls [15]. Of note is that observed TMAO blood levels in this study are 3 to 5 times (24-hour and 42-hour, respectively) higher than levels reported by our group for cyclosporine toxicity. In contrast, using the same NMR protocol as in the present study, allantoin was not detected after cyclosporine treatment, indicating a completely different magnitude of oxidative stress [15]. In addition, our previous report [18] demonstrated that allantoin levels were less elevated (in both kidney as well as blood tissues) when donors were pretreated with N-acetyl-cysteine, a putative free radical scavenger that is known to ameliorate oxidative stress. It is possible that further evaluation of metabolic blood profiles may allow for the distinction not only between IR and drug toxicity but also rejection. There are no ^{1}H-NMR–based studies of blood that reliably distinguish rejection from other pathologies.

In view of our promising data, it would be worthwhile to perform a prospective clinical study using quantitative metabolomics. In this study, kidney transplant patients with elevated SCr levels would undergo blood sampling for assessment of their metabolic profiles. These metabolic "fingerprints" could then be correlated with the histologic findings previously obtained by renal biopsy. One could then determine early metabolic profiles to predict and to differentiate between IR injury, rejection, and drug toxicity.

CONCLUSION

In the present study, we were able to demonstrate the correlation of metabolic markers that independently serve as surrogates for oxidative stress and renal medullary injury. Unlike SCr values, which were not different between transplant groups, the distribution of allantoin and TMAO in blood or allantoin and PUFA in kidney tissue (Fig. 3) was significantly different between groups and demonstrated no overlap between 24 and 42 hours of cold ischemia. Metabolic patterns were also consistent in terms of the severity of histologic damage. Nuclear magnetic resonance spectroscopy is well suited to identify and, more importantly, to quantify metabolic profiles under different conditions. A metabolic blood profile that was based on the correlation of independent markers of oxidative stress (allantoin) and medullary injury (TMAO) instead of nonspecific SCr was able to quantify the severity of IR injury in a rat kidney transplant model. An important future clinical application of quantitative metabolomics may be the distinction between IR injury, early rejection, chronic rejection, and drug nephrotoxicity.

ACKNOWLEDGMENT

Supported in part by NIH R01 DK065094-01 (N.S., J.K., and C.U.N.).

Correspondence to Claus U. Niemann, M.D., University of California at San Francisco, Department of Anesthesia and Perioperative Care, 521 Parnassus Avenue, C 450, San Francisco, CA 94143–0648.
E-mail: niemannc@anesthesia.ucsf.edu
No reprints available.

REFERENCES

1. Boom H, Mallat MJ, de Fijter JW, *et al*: Delayed graft function influences renal function, but not survival. *Kidney Int* 58:859–866, 2000
2. Moreso F, Seron D, Gil-Vernet S, *et al*: Donor age and delayed graft function as predictors of renal allograft survival in rejection-free patients. *Nephrol Dial Transplant* 14:930–935, 1999
3. Dragun D, Hoff U, Park JK, *et al*: Prolonged cold preservation augments vascular injury independent of renal transplant immunogenicity and function. *Kidney Int* 60:1173–1181, 2001
4. Hetzel GR, Klein B, Brause M, *et al*: Risk factors for delayed graft function after renal transplantation and their significance for long-term clinical outcome. *Transpl Int* 15:10–16, 2002
5. Nicholson J, Lindon JC, Holmes E: "Metabonomics": Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate analysis of biologocal NMR spectroscopic data. *Xenobiotica* 29:1181–1189, 1999
6. Seto K, Ikehira H, Obata T, *et al*: Long-term assessment of post-transplant renal prognosis with 31 P magnetic resonance spectroscopy. *Transplantation* 72:627–630, 2001
7. Hauet T, Gibelin H, Richer JP, *et al*: Influence of retrieval conditions on renal medulla injury: Evaluation by proton NMR spectroscopy in an isolated perfused pig kidney model. *J Surg Res* 93:1–8, 2000
8. Richer J, Baumer TH, Gibelin H, *et al*: Evaluation of renal medulla injury after cold preservation and transplantation: noninvasive determination of medullar damage by proton nuclear magnetic resonance spectroscopy of urine and plasma. *Transplant Proc* 32:47–48, 2000
9. Nicholson J, Wilson ID: Opinion: Understanding 'global' systems biology: Metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* 2:668–676, 2003
10. Brinde J, Antti H, Holmes E, *et al*: Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nature Med* 8:1439–1444, 2002
11. Foxall PJ, Mellotte GJ, Bending MR, *et al*: NMR spectroscopy as a novel approach to the monitoring of renal transplant function. *Kidney Int* 43:234–245, 1993
12. Hauet T, Gibelin H, Godart C, *et al*: Kidney retrieval conditions influence damage to renal medulla: Evaluation by proton nuclear magnetic resonance (NMR) pectroscopy. *Clin Chem Lab Med* 38:1085–1092, 2000
13. Fuller TF, Freise CE, Serkova N, *et al*: Sirolimus delays recovery of rat kidney transplants after ischemia/reperfusion injury. *Transplantation* 76:1594–1599, 2003
14. Serkova N, Jacobsen W, Niemann CU, *et al*: Sirolimus, but not the structurally related RAD (everolimus), enhances the negative effects of cyclosporine on mitochondrial metabolism in the rat brain. *Br J Pharmacol* 133:875–885, 2001
15. Serkova N, Klawitter J, Niemann CU: Organ-specific response to inhibition of mitochondrial metabolism by cyclosporine in the rat. *Transpl Int* 16:748–755, 2003
16. Porter C, O'Donnell C, Crawford ED, *et al*: Predicting the outcome of prostate biopsy in a racially diverse population: A prospective study. *Urology* 60:831–835, 2002
17. Beckwith-Hall B, Holmes E, Lindon JC, *et al*: NMR-based metabonomic studies on the biochemical effects of commonly used drug carrier vehicles in the rat. *Chem Res Toxicol* 15:1136–1141, 2002
18. Fuller TF, Serkova N, Niemann CU, *et al*: Influence of donor pretreatment with N-acetylcysteine on ischemia/reperfusion injury in rat kidney grafts. *J Urol* 171:1296–1300, 2004
19. Kosieradzki M, Kuczynska J, Piwowarska J, *et al*: Prognostic significance of free radicals: Mediated injury occurring in the kidney donor. *Transplantation* 75:1221–1227, 2003
20. Oehlschlager S, Albrecht S, Hakenberg OW, *et al*: Measurement of free radicals and NO by chemiluminescence to identify the reperfusion injury in renal transplantation. *Luminescence* 17:130–132, 2002
21. Perez Fernandez R, Martin Mateo MC, De Vega L, *et al*: Antioxidant enzyme determination and a study of lipid peroxidation in renal transplantation. *Ren Fail* 24:353–359, 2002
22. Shoskes DA, Shahed AR, Kim S, *et al*: Oxidant stress and antioxidant capacity in urine of renal transplant recipients predict early graft function. *Transplant Proc* 33:984, 2001
23. Benzie I, Chung W, Tomlinson B: Simultaneous measurement of allantoin and urate in plasma: Analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem* 45:901–904, 1999
24. Suematsu M, Suzuki H, Delano FA, *et al*: The inflammatory aspect of the microcirculation in hypertension: Oxidative stress, leukocytes/endothelial interaction, apoptosis. *Microcirculation* 9:259–276, 2002
25. Mikami T, Kita K, Tomita S, *et al*: Is allantoin in serum and urine a useful indicator of exercise-induced oxidative stress in humans? *Free Radic Res* 32:235–244, 2000
26. Deiana M, Aruoma OI, Rosa A, *et al*: The effect of ferric-nitrilotriacetic acid on the profile of polyunsaturated fatty acids in the kidney and liver of rats. *Toxicol Lett* 123:125–133, 2001
27. Niemann C, Saeed M, Akbari H, *et al*: Close association between the reduction in myocardial energy metabolism and infarct size: Dose-response assessment of cyclosporine. *J Pharmacol Exp Ther* 302, 1123–1128, 2002