BOLD-MRI assessment of Intrarenal Oxygenation and Oxidative Stress in Patients with Chronic Kidney Allograft Dysfunction

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Abstract

Blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) uses deoxyhemoglobin as an endogenous contrast agent for the noninvasive assessment of tissue oxygen bioavailability. We hypothesized that intrarenal oxygenation was impaired in patients with chronic allograft nephropathy (CAN). Ten kidney-transplant recipients with CAN and 9 healthy volunteers underwent BOLD-MRI. Medullary R2* (MR2*) and cortical R2* (CR2*) levels (measures directly proportional to tissue deoxyhemoglobin levels) were determined alongside with urine and serum markers of oxidative stress (OS): hydrogen peroxide (H₂O₂), F₂-isoprostanes, total nitric oxide (NO), heat shock protein 27 (HSP27) and total antioxidant property (TAOP). Mean MR2* and CR2* levels were significantly decreased in CAN (increased local oxyhemoglobin concentration) compared to healthy volunteers (20.7/s±1.6 vs. 23.1/s±1.8, p=0.03 and 15.9±1.9 vs. 13.6/s±2.3, p=0.05, respectively). There was a significant increase in serum and urine levels of H₂O₂ and serum HSP27 levels in patients with CAN. Conversely, urine NO levels and TAOP were significantly increased in healthy volunteers. Multiple linear regression analyses showed a significant association between MR2* and CR2* levels and serum/urine biomarkers of OS.

BOLD-MRI demonstrated significant changes in medullary and cortical oxygen bioavailability in allografts with CAN. These correlated with serum/urine biomarkers of OS, suggesting an association between intrarenal oxygenation and OS.
Introduction

Kidney transplantation has become the treatment of choice for patients with end-stage kidney disease (ESRD) (36; 66; 75). Yet, long-term transplant outcomes have not significantly improved (37; 45; 46; 66). This is, in part, due to chronic allograft nephropathy (CAN), the leading cause of kidney transplant failure (13; 17; 51; 54). CAN is characterized by progressive kidney dysfunction, interstitial fibrosis, tubular atrophy, glomerulosclerosis and vascular occlusive changes that include dropout of peritubular capillaries (13; 17; 33; 51; 54). A better understanding of CAN’s pathogenesis may lead to the development of strategies to prevent/delay its development/progression. There is evidence that both immune and non-immune insults, including oxidative stress (OS) contribute to CAN (5; 18; 19; 43; 54; 64). OS may lead to kidney tissue injury through inflammatory, apoptotic and fibrotic processes (3; 5; 18; 19; 29; 41; 43; 72; 73). Evidence shows that the burden of OS is increased in experimental and human CAN (5; 10; 18; 19; 68). However, it remains unknown whether intrarenal oxygenation affects OS in CAN. Insufficient oxygen supply, increased oxygen requirements and/or inappropriate oxygen extraction could all affect OS. To date there has been no safe, noninvasive technique available to assess intrarenal oxygenation.

Blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) is a noninvasive method to assess tissue oxygen bioavailability, using deoxyhemoglobin as an endogenous contrast agent (20; 58-60). Oxyhemoglobin is a diamagnetic molecule that creates no magnetic moment as oxygen molecules are bound to iron, while deoxyhemoglobin is a paramagnetic molecule that generates magnetic moments by its unpaired iron electrons (20; 40; 56; 71). Higher levels of deoxyhemoglobin result in increased magnetic spin dephasing of blood water protons and decreased signal intensity on T2* (apparent spin-spin relaxation time)-
weighted MR imaging sequences (40; 56; 58-60; 71). The apparent relaxation rate or R2* (=1/T2*) may be calculated as the slope of loge (intensity) versus echo time and is directly proportional to the tissue content of deoxyhemoglobin (58-60). Increased R2* levels imply increased deoxyhemoglobin (decreased oxyhemoglobin) and decreased partial pressures of oxygen (PaO2) in tissues (58-60). BOLD-MRI can therefore be used to determine intrarenal oxygen bioavailability (40). This technique has been used to investigate human and experimental models of kidney disease including aging (23; 60), diabetes (24), acute kidney transplant dysfunction (20; 65), acute ischemic kidney injury and unilateral ureteral obstruction (6; 35; 57).

We recently demonstrated changes in medullary R2* (MR2*) levels in association with acute transplant rejection (20; 65). This led us to assess changes in intrarenal oxygenation in chronic transplant injury. We sought to determine if there was a relationship between impaired intrarenal oxygenation and OS in CAN. We hypothesized that CAN, as a chronic inflammatory condition, was associated with abnormal intrarenal oxygen bioavailability and increased OS. To validate these hypotheses and to assess the effect of a possible intervention, we prospectively studied 19 subjects (10 kidney transplant recipients with CAN and 9 healthy volunteers) in a pilot analysis of BOLD-MRI, OS biomarkers, and acute angiotensin receptor blockade (ARB).
Materials and Methods

Patients

Subjects in this study included a group of healthy volunteers (n=9) and a group of transplant recipients with CAN (n=10) recruited between late 2003 and mid-2005. Healthy volunteers were selected from the nephrology section and through collegial relationships at the university. Healthy volunteers had to be free from chronic illness and not taking any medications with the exception of allergy medications, birth-control, anti-depressants and anti-anxiety medications. Kidney transplant recipients were selected from the transplant clinic at the UW Madison Hospital and Clinics. They were at least 12 months post-transplant and needed to be in stage 3 chronic kidney disease (CKD) (estimated glomerular filtration rate (eGFR) between 30 and 59 ml/min (42)) based on the National Kidney Foundation classification of CKD (1). The diagnosis of CAN was clinical and defined by the presence of proteinuria (>200mg/g creatinine), a 0.3 mg/dl rise in serum creatinine compared to baseline, and hypertension. However, in most cases, the clinical diagnosis was confirmed by pathology. To participate in the study, patients also had to be off angiotensin receptor blockers (ARBs), angiotensin converting enzyme inhibitors (ACE (-)), statins or diuretics BOLD-MRI. All participants had to be off vitamin supplements for 24 hours prior to the BOLD-MRI and free of any acute illness at the time of the study visit.

Exclusion criteria: Potential participants with hazardous metallic implants and cardiac pacemakers, hypersensitivity to losartan or any component of the formulation, hypersensitivity to other ARBs, known transplant artery stenosis, orthostatic hypotension or who were pregnant were excluded.

Procedure: After human subjects committee approval was obtained potential participants were approached by the principal investigator or study coordinator during their regular clinic visit.
Individuals were given a synopsis of the study including risks and benefits of participation. After informed consent was obtained participants presented to the General Clinical Research Center (GCRC) for one-half day. Each participant was asked to verify that they had not been recently acutely ill and then were asked to provide a brief medical history including co-morbid conditions and current medications. Baseline vital signs were then recorded and a blood and urine sample was collected before the first BOLD-MRI. Blood and urine samples were assayed for standard blood chemistries, BUN, creatinine, albumin, C-reactive protein, and urine protein, creatinine, and sodium. A urine pregnancy test was performed on all female participants of child-bearing age. In addition, aliquots of serum and urine samples were assayed for biomarkers of OS as described below. Following the first BOLD-MRI, patients returned to the GCRC and received a 50mg oral dose of losartan. They then rested for about two hours while having their vital signs monitored and had another set of blood and urine samples collected just prior to the second BOLD MRI study (Figure 1).

**BOLD-MRI Technique**

MR imaging was performed with a 1.5-Tesla system (Sigma; GE Healthcare, Waukesha, WI) and a four-element torso phased-array surface coil. BOLD-MRI was performed using a multi-gradient-recalled-echo sequence with 16 echoes at a gap of 1 mm and a 5-mm section thickness that was prescribed in the coronal plane as described previously (20; 65). The scanning parameters were: repetition/echo time, 87ms/8-41.8ms; flip angle, 40°; bandwidth, ± 62.5 kHz; field of view, 32-34 cm; matrix, 256 x 128; and number of signals acquired, one. Each set of 16 T2*-weighted images was acquired during an 11-second breath hold. Three sections were obtained in the coronal plane for each transplanted kidney as the coronal planes have shown to present less variability (6; 65). Mean R2* values were recorded in units of 1/sec. Color R2*
maps were generated and regions of interest (ROIs) were placed in the medulla and cortex using Functool on the Advantage workstation (GE Healthcare, Waukesha, WI). The color R2* map was windowed to provide a visual range of R2* values from low to high, with blue representing the lowest R2* value (area of lowest deoxyhemoglobin concentration) and red representing the highest R2* value (area of highest deoxyhemoglobin concentration). Six to ten ROIs were placed in the medulla, and six to ten ROIs were placed in the cortex, resulting in 12-20 ROIs per kidney per subject.

**Biomarkers of oxidative stress**

To avoid significant bias from assay variability, each sample was measured in triplicate and the experiment was repeated three times for each of the biomarkers assessed.

**Heat shock protein 27 (HSP27)**

Serum and urine HSP27 levels were measured using an ELISA Kit from Stressgen Biotechnologies (Victoria, BC Canada, cat # EKS-500) according to manufacturer’s recommendations. Briefly, recombinant HSP 27 was serially diluted from 25 – 0.39ng/mL with the sample diluent to generate a standard curve. The anti HSP 27 rabbit polyclonal antibody (pAb), the HRP- anti rabbit IgG conjugated antibody (cAb) and wash buffer were diluted based on kit specifications. 100µl of each dilution of standard was added in duplicate to the HSP27 specific mouse monoclonal antibody coated plate. 100µl of sample diluent was added to serve as the blank. 50µl of sample diluent was added to all empty wells followed by the addition of 50µl of samples in duplicate (1:2). Plates were covered and incubated for 1 Hr at Room Temperature (RT). After incubation, plates were washed 6 times with 1x wash buffer. 100µl of the diluted pAb was added to all wells except the blank. The plate was incubated for 1 Hr at RT and then washed as previously described. 100µl of the diluted cAb was added to all wells except the
blank. The plate was incubated for 30 mins at RT and washed again. The plate was developed for 15 mins at RT in the dark with 100µl/well of TMB solution and terminated with 100µl/well of Acid stop solution. The plate was read at 450 nm using the Synergy™ HT Multi-Detection Microplate Reader and analyzed using the KC4 software (Bio-Tek, Winooski, VT). HSP27 levels were reported in pg/ml.

Nitric Oxide (total)

Serum and urine total NO levels (Nitrate/Nitrite) were measured using the NO (total) Detection Kit from Stressgen Biotechnologies (Victoria, BC Canada, cat # EKS-310) according to manufacturer’s recommendations. Briefly, reaction Buffer, NADH Reagent and Nitrate Reductase Enzyme were diluted based on kit specifications. The Nitrate Standard Solution was serially diluted from 100-3.125 µM with the reaction buffer to generate a standard curve. Serum and urine samples were diluted 1:10 and 1:50, respectively, in reaction buffer prior to addition to the plate. 50µl of each diluted sample and standard was added in duplicate to the plate including 50µl of reaction buffer to serve as the 0 standard. 200µl of reaction buffer was added in duplicate to serve as the blank. 25µl of NADH and 25µl of Nitrate Reductase Enzyme were subsequently added to all wells except the blanks. The plate was covered, mixed gently and incubated at 37°C for 30 mins. 50µl of Griess Reagent I and 50µl of Griess Reagent II were subsequently added to all wells except the blanks. The plate was covered, mixed gently and incubated at room temperature for 10 mins. The plate was read at 550 nm using the Synergy™ HT Multi-Detection Microplate Reader and analyzed using the KC4 software (Bio-Tek, Winooski, VT). Total NO concentrations were reported in nanomoles (nM).
Hydrogen Peroxide (H$_2$O$_2$)

Serum and urine H$_2$O$_2$ levels were measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit from Molecular Probes, Invitrogen Corporation (Carlsbad, CA, # A-22188) according to the manufacturer’s recommendations. Briefly, serial 50 µl dilutions of H$_2$O$_2$ (standard curve), and urine supernatants were placed into individual wells of a 96 well microplate. A 50 µl volume of the Amplex Red reagent/HRP solution was then added to each microplate well containing the standards and samples. The plate was incubated at room temperature for 30 minutes, protected from light. The plate was read at 590 nm using the Synergy™ HT Multi-Detection Microplate Reader and analyzed using the KC4 software (Bio-Tek, Winooski, VT). H$_2$O$_2$ concentrations were reported in micromoles (µM).

8-iso Prostaglandin F$_{2\alpha}$ (F$_2$ isoprostanes)

Serum and urine 8-iso Prostaglandin F$_{2\alpha}$ levels were measured using the ELISA Kit from Stressgen Biotechnologies (Victoria, BC Canada, cat # EKS-200) according to manufacturer’s recommendations. Briefly, assay Buffer and Wash Buffer were diluted based on kit specifications. The 8-iso Prostaglandin F$_{2\alpha}$ (PGF) standard solution was serially diluted from 100,000-6.1 pg/mL with assay buffer to generate a standard curve. 100 µl of each dilution of standard was added in duplicate to the goat anti-rabbit IgG polyclonal antibody coated plate. 50 µl of assay buffer was added to all empty wells followed by the addition of 50 µl of samples (serum and urine) in duplicate (1:4). 50 µl of alkaline phosphate conjugated PGF and 50 µl of rabbit polyclonal PGF were added to all wells except the blanks. The plate was covered and shaken for 2 Hrs at RT. The plate was then washed 3 times with wash buffer. 200 µl of p-Npp Substrate solution was added to each well and incubated for 45 mins at RT. The reaction was terminated with 50 µl/well of Stop solution. The plate was read at 405 nm using the Synergy™ HT
Multi-Detection Microplate Reader and analyzed using the KC4 software (Bio-Tek, Winooski, VT). F$_2$ isoprostane concentrations were reported in picograms per ml (pg/ml).

Total Antioxidant Property

Serum and urine total antioxidant property (TAOP) was measured using the Cayman Chemical antioxidant detection kit (Ann Arbor, MI, cat # 709001) according to manufacturer’s recommendations. Briefly, serum (1:25) and urine (1:15) were diluted in Assay Buffer. Trolox was also diluted in the Assay Buffer to yield the standard curve from 0 – 0.330 milli molar (mM): 0, 0.044, 0.088, 0.135, 0.18, 0.225, 0.330. 10ul of diluted standard or samples were added to each well in duplicate. 10ul of metmyoglobin and 150ul of chromogen was also added to each well. 40ul of hydrogen peroxide solution (441µM) was added to each well to initiate the reaction. Plate was incubated on a shaker for 5 minutes at room temperature and read at 750nm using the Synergy™ HT Multi-Detection Microplate Reader and analyzed using the KC4 software (Bio-Tek, Winooski, VT). TAOP concentrations were reported in millimoles (mM).

Statistical Analyses

This was a pilot study to determine whether intrarenal oxygenation measured by BOLD-MRI was different between healthy volunteers and renal transplant recipients with CAN, whether there was a correlation between intrarenal oxygenation and biomarkers of OS in patients with CAN and whether a single dose of Losartan could alter intrarenal oxygenation. Numerical data between the groups were compared using the student’s $t$ test or the Wilcoxon rank sum test for parametric and nonparametric data, respectively. Paired tests were used to compare the effect of Losartan. All numerical data, including R2* (1/s) values were expressed as means ± standard deviation (SD). Chi-square analyses of contingency tables or Fisher’s Exact test were performed to evaluate the frequency of nominal data between groups. Multiple linear regression and
Spearman rank order correlation analyses were performed to determine the strength of association between serum and urine biomarkers of OS and medullary, cortical and Medullary:Cortical R2* values. These analyses were performed using SigmaStat (SPSS Inc., Chicago, IL) and MedCalc Statistical Softwares (Mariakerke, Belgium). A $p$ value $\leq 0.05$ was considered as significant. Data are reported as mean ± standard deviation unless otherwise indicated.
Results

Subject characteristics

We studied 10 patients with CAN and 9 control subjects. Baseline characteristics are depicted in Table 1. The average time between the transplant and BOLD-MRI imaging was 3.9 ± 1.1 years for the patients with CAN. All patients had clinical CAN and 6/10 had biopsy proven CAN based on clinically indicated biopsies prior to the study (62; 63). Only one patient had a previous history of acute rejection with return of the serum creatinine back to baseline following steroid treatment. Five patients had a history of end-stage renal disease (ESRD) secondary to diabetes, four had a history of hypertension and one had a history of chronic glomerulonephritis. This patient and the patient with a history of acute rejection were among those with biopsy confirmed CAN. None of the patients had an acute rise in serum creatinine prior to study enrollment and imaging. Five patients received living donor transplants and 5 patients had undergone deceased donor transplantation. Average donor age was 38.5 years ± 3.3 (p=0.3 compared to healthy volunteers). Patients were all first-time transplant recipients except for one patient (second transplant). Induction immunosuppression included basiliximab in 5 patients, alemtuzumab in 3 patients and OKT3 in 2 patients. Maintenance immunosuppression was a combination of a calcineurin inhibitor or CNI (cyclosporine A/tacrolimus in 5/5 patients), mycophenolate mofetil and prednisone.

The CAN group was significantly older (49.3±3.9 vs. 32.6±4.3 years, p=0.01) and had significantly increased serum creatinine (1.7±0.1 vs. 0.9±0.1 mg/dL, p<0.0001), proteinuria (237.35 vs. 96±12 mg/g creatinine, p=0.005) and fractional excretion of sodium (FeNa) levels (1.4%±0.3 vs. 0.7%±0.1, p=0.02) compared to the control population. The CAN group also had lower levels of serum albumin (4.2±0.1 vs. 4.5±0.1 g/dL), urine pH (6±0.2 vs. 6.9±0.3, p=0.03)
and urine specific gravity (1.010±0.002 vs. 1.018±0.002, p=0.002) compared to healthy controls. Blood tacrolimus (TAC) and cyclosporine A (CsA) levels were 4±1 and 86.8±26.4 ng/ml, respectively. There were no patients or healthy controls on vitamins, over the counter antioxidants or antihistaminics. Two healthy volunteers was on venlafaxine (75 mg daily) and one was on a birth control pill (ethinyl estradiol/levonorgestrel 20mcg/0.1 mg). There were no significant differences in gender, ethnicity, mean arterial blood pressure (MAP), hematocrit and C-reactive protein (CRP) levels between the two groups.

BOLD-MRI Analyses

- Coronal color R2* maps. Representative color maps of R2* values from a healthy volunteer and a kidney allograft with CAN in the coronal planes are presented in Figure 2, Panels (a) to (d). The blue color represents the lowest R2* value (low deoxyhemoglobin concentration), and green, yellow and red show increasing values of R2* (increased deoxyhemoglobin concentration). Usually, there are more blue areas in the cortex, and more green, yellow and red spots in the medulla, corresponding to the corticomedullary oxygen gradient. There was a loss of corticomedullary oxygen differentiation in CAN. Increasing blue areas (decreased deoxyhemoglobin) appeared in both the medulla and the cortex of kidney allografts with CAN.

- Medullary R2* (MR2*), Cortical R2* (CR2*) and Medullary:Cortical R2* (MCR2*) values. Bar graphs of medullary, cortical and medullary:cortical R2* ratios are presented in Figure 2 (f). Mean MR2* levels were significantly (approximately 10%) decreased in CAN (increased local oxyhemoglobin concentration) compared to healthy volunteers (20.7/s±1.6 vs. 23.1/s±1.8, p=0.03). Similarly, mean CR2* levels were decreased (by 14%) in CAN (15.9/s±1.9 vs. 13.6/s±2.3, p=0.05). Intrinsic regulatory mechanisms
maintain intrarenal oxygen bioavailability and perfusion gradients between the medullary and cortical regions of the kidney (9; 21; 25; 26). We have previously defined the MCR2\(^*\) ratio as a surrogate marker of normalized intrarenal oxygen bioavailability (20). The MCR2\(^*\) ratios were nearly unchanged between the two groups (1.5±0.2 and 1.53±0.2, p=0.67).

**Biomarkers of OS**

We simultaneously assayed serum and urine levels of hydrogen peroxide (H\(_2\)O\(_2\)), total nitric oxide (NO), F\(_2\) isoprostanes, heat shock protein 27 (HSP27) and total antioxidant properties (TAOP). Each of these molecules may represent some component of OS from both pro- and anti-oxidant states, thus providing a panel of kidney-related ROS.

Surrogate markers of OS were measured in the serum and urine of all subjects at baseline and following losartan. Hydrogen peroxide levels were significantly higher in the serum (24.8±9.3 vs. 5.9±1.6 \(\mu\)M, p=0.04) and urine (8.3±1.6 vs. 6±2.3 \(\mu\)M, p=0.05) of patients with CAN (Figure 3 (a)). Similarly, serum HSP27 levels were increased in patients with CAN (8.6±2.9 vs. 2.3±1.1 pg/ml, p=0.02, Figure 3 (c)). Conversely, urine NO levels and TAOP were significantly increased in healthy volunteers (945±149 vs. 443±134 nM, p=0.009 and 67.6±12.8 vs. 29.4±5.5 mM, p=0.03, Figures 3 (b) and (e) respectively). Serum and urine F\(_2\) isoprostane levels were not statistically different between the two groups (Figure 3 (d)). Inter and intra-assay variability (coefficient of variation) was < 15% for all assays. Yet, when considered as a whole, these experiments demonstrated a pro-oxidant profile in patients with CAN.

**Correlation between intrarenal oxygenation and OS**

To determine whether there was a correlation between intrarenal oxygenation and OS in CAN, we performed multiple linear regression and Spearman rank order correlation analyses
using MR2*, CR2* and MCR2* as dependent variables and serum and urine biomarkers of OS as independent variables (Tables 2 (a) and (b)). Multiple linear regression analyses showed that MR2* levels could be predicted by a combination of CR2*, urine F2-isoprostane and NO levels ($R^2=0.97$, $p<0.001$, Table 2(a)), whereas CR2* levels could be predicted by a combination of urine NO and H2O2 levels ($R^2=0.71$, $p<0.03$, Table 2(a)). Conversely, urine F2 isoprostanes, NO, HSP27 and H2O2 levels could be predicted by various combinations involving MR2* and CR2* levels. Interestingly, Mean arterial Pressure (MAP) could be accurately predicted by a linear regression formula involving MCR2*, hematocrit, urine F2 isoprostanes and H2O2 levels as variables ($R^2=0.92$, $p<0.03$).

Spearman rank order correlation analyses demonstrated a significant correlation between MR2* levels and age, CR2*, serum HSP27 and urine NO (Table 2 (b)) as well as between CR2* and MCR2* levels and age, urine NO and urine F2 isoprostanes. MAP correlated with eGFR, hemoglobin, serum F2 isoprostanes and total NO levels (Table 2 (b)). There was no correlation between calcineurin-inhibitor dose/blood levels and intrarenal oxygenation or OS biomarkers (data not shown). Overall, these analyses showed a significant correlation between intrarenal oxygenation measured by BOLD-MRI and serum/urine biomarkers of OS.

**The impact of Losartan on intrarenal oxygenation.**

ARBs, including losartan may improve/delay disease progression in CAN (7; 11; 44). Losartan has also antioxidant properties (12; 16) that could lead to decreased cortical oxygen consumption through increased local NO availability (4). We hypothesized that losartan might improve BOLD-MRI-measured intrarenal oxygenation. In this pilot study we decided to first examine the short-term effects of the drug. Because the terminal half-life of the drug, taken
orally, is 2 hours (physicians desk reference, PDR-2006), BOLD-MRI as well as blood and urine sample analyses were repeated 1.5 to 2.5 hours after ingesting losartan (50mg).

Losartan was associated with a modest, yet significant decrease in CR2* levels in healthy volunteers (15.5/s±1.9 pre-losartan vs. 14.5/s±1.3 post-losartan, p=0.05, Figure 3 (a2)). This corresponded to increased MCR2* levels in these subjects (1.5±0.2 vs. 1.6±0.2, p=0.04, Figure 3 (a3)). MR2*, CR2* and MCR2* levels did not change significantly in patients with CAN. Similarly, losartan had no significant impact on serum/urine levels of OS biomarkers (data not shown). Finally, there was no significant change in urine sodium excretion rates (Figure 3 (b)).
Discussion

The current study shows that oxyhemoglobin bioavailability measured by BOLD-MRI is increased in the medulla and cortex of allografts with CAN. Impaired intrarenal oxygenation was associated with impaired OS balance as there was a significant relationship between medullary, cortical and medullary:cortical R2* levels and urinary and serum biomarkers of OS. Finally, a single oral dose of losartan increased cortical oxygenation in healthy volunteers, but had minimal effect in kidneys with CAN.

BOLD-MRI has recently been utilized to examine intrarenal oxygenation in human and experimental models of kidney disease (Table 3) (6; 20; 23; 35; 57; 60; 65; 69). These studies have shown impaired regional oxygenation patterns in unilateral ureteral obstruction (57), acute ischemic kidney injury (6; 35), experimental hypertension (57) acute kidney transplant rejection (20; 65) and aging (69). While ischemic models of kidney injury (acute ischemic renal failure and aging) have understandably resulted in increased MR2* and CR2* levels (decreased regional oxyhemoglobin concentrations) (6; 35; 69), inflammatory and immune-mediated kidney injury has been associated with decreased MR2* (increased oxyhemoglobin) values, probably as a result of decreased tissue oxygen extraction (20; 57; 65) (Table 3). In addition, studies inhibiting sodium (Na⁺) reabsorption (by furosemide, water diuresis and tempol) have shown increased medullary oxygenation through reduced nephron work load (23; 57; 60). Yet, there have been no studies assessing regional oxygenation patterns in CAN.

The current study shows decreased MR2* and CR2* levels in CAN, suggesting that oxygen bioavailability (oxyhemoglobin levels) is increased in both the medulla and cortex of kidney allografts. Enhanced medullary and cortical oxyhemoglobin concentrations may result from increased oxygen delivery, decreased oxygen extraction/consumption, or a combination of
these two factors. It is unlikely that allografts with CAN have greater oxygen delivery through increased perfusion (34; 67). We have recently demonstrated that both medullary and cortical blood flow rates are decreased in kidney allografts undergoing acute rejection (unpublished data). Although the current study has not examined regional blood flow rates, it is unlikely that increased intrarenal O2 bioavailability in CAN is secondary to greater renal blood flows. We believe that the mechanisms of decreased O2 uptake in CAN may include: salt wasting and tubular damage, inflammation and OS, decreased GFR or a combination of these factors.

Tubular dysfunction and/or drop-out is a histopathological hallmark of CAN (70). Renal O2 consumption is largely dedicated to Na+ reclamation (9; 22; 53), of which the majority occurs in the cortex with adjustment of the final urine Na+ content in the medulla (9; 22; 53). Urine concentrating ability is impaired in CKD resulting in isosthenuria and increased Na+ excretion, a finding consistent with our observations. It is therefore likely that salt wasting (increased FeNa and decreased urine specific gravity) in CAN contributed to increased oxygen bioavailability. Furthermore, it has been recently shown that increases in luminal Na+ concentration and/or flow rate can increase the generation of superoxide in mTAL and reduce nitric oxide bioavailability, thereby increasing local OS (2). It is therefore possible that salt wasting results in OS with further limitation of O2 uptake. Congruent with this hypothesis, BOLD-MRI studies have shown that decreased nephron workload through inhibition of Na+ reabsorption may increase medullary oxygen bioavailability experimentally (23; 24; 60).

CAN is a state of chronic inflammation/OS. This pro-inflammatory/oxidant milieu results from immune and non-immune insults including graft infiltrating cells, pro-inflammatory cytokines, glomerular hyperfiltration and hypertension. These insults result in tubulo-interstitial injury and atrophy (3; 5; 18; 19; 29; 41; 43; 72; 73) and the progressive reduction in nephron
mass could lead in turn to salt wasting, OS and decreased oxygen uptake and consumption by tubules, creating a “vicious circle” (Figure 5). Finally, a decrement in GFR may be a protective mechanism to reduce oxygen consumption in the medulla (9). It is thus likely that multiple factors (decreased GFR, OS, inflammation and salt wasting) contribute to increased intrarenal oxygen availability in CAN.

Other transplant-related factors may affect intrarenal oxygen bioavailability. For example, CNIs may affect intrarenal perfusion and decrease urinary NO levels (8). Likewise, sympathetic denervation in transplanted kidneys results in a significant decrease in local norepinephrine concentrations (28) with a potential effect on intrarenal oxygen bioavailability. Yet, experimental studies suggest that the medullary blood flow may be insensitive to sympathetic nerve activity (25) and that changes in renal vascular resistance are not explained by denervation (27). As far as CNI use and oxygen bioavailability are concerned, we have been unable to show a correlation between the dose and/or the blood level of CNIs and medullary or cortical R2* values in both acute (20) and chronic allograft dysfunction (current study, data not shown). A group of kidney transplant recipients with similar GFRs (stage 3 CKD) and no CNI-therapy would have been an ideal control group but this was out of the scope of this study.

OS is increased in CAN (5; 10; 18; 19; 68), yet the relationship between intrarenal oxygenation and OS remains obscure. We assayed a number of different biomarkers to complete an OS profile for this pilot study. Urinary H$_2$O$_2$ levels may be a valuable biomarker of OS in certain conditions (30; 76). NO is a free radical synthesized by endothelial and inducible NO synthase enzymes (eNOS and iNOS) (39). Deleterious effects of NO are observed when it reacts with superoxide anion to form peroxynitrite (ONOO$^-$), a potent pro-oxidant molecule (49). In the kidney however, NO counters the effects of superoxide anion and H$_2$O$_2$ (26), balancing out
potential OS injury. Isoprostanes are biologically active products of arachidonic acid metabolism formed by non-enzymatic lipid peroxidation (49). Urinary F2 isoprostanes provide a non-invasive index of lipid peroxidation in patients with obesity, hypertension and cardiovascular disease (15; 26; 48; 55). Finally, HSP27 is a constitutive and inducible stress protein with anti-oxidant properties, including inhibition of H2O2-mediated cell death (18; 32; 61). Normally, high concentrations of HSP27 are present in medulla tissue (18; 38; 50). Urinary and serum H2O2, and serum HSP27 levels were increased in CAN, whereas urinary TAOP and total NO levels were decreased, confirming an increased OS burden (Figure 3). We believe that greater serum HSP27 levels in CAN were a result of an upregulated response to OS. There was a close correlation between biomarkers of OS and intrarenal oxygenation. Multiple linear regression and spearman rank order correlation analyses demonstrated that MR2*, CR2* and MCR2* could accurately be predicted by various combinations of OS markers (Tables 2a and 2b). It is therefore possible that intrarenal oxygenation is involved in the generation of reactive oxygen/nitrogen species (ROS/RNS) and that conversely, increased ROS/RNS result in tubular injury and decreased oxygen extraction/consumption in CAN. Various stimuli, e.g. calcineurin inhibitors (8; 14), inflammation (31; 52; 74), hypoalbuminemia (31) and acidic urine (47) could have contributed to the generation of ROS/RNS (5; 10; 18; 19; 68) (Figure 5).

Losartan has antioxidant properties and may improve or delay progression in CAN (7; 11; 12; 16; 44). We hypothesized that losartan would improve intrarenal oxygenation by decreasing oxygen consumption through increased local NO availability (4). However, losartan did not improve intrarenal oxygenation in patients with CAN and produced only modest, yet significant decreases in CR2* levels in healthy volunteers. This may be secondary to the large burden of OS/inflammation in kidney allografts with CAN. A greater dose, multiple doses,
intravenous drug delivery or prolonged treatment may be necessary to observe difference in intrarenal oxygenation in CAN. There is also the possibility that the solitary injured kidney had changes in perfusion in response to the ARB distributed equally throughout cortex and medulla. Unfortunately, the nature of these studies prohibited formal assessment of perfusion by BOLD-MR techniques.

Our study was thus limited by the lack of perfusion analyses, the absence of longitudinal data on these patients and the inability to demonstrate a causal relationship between increased OS and greater intrarenal oxyhemoglobin levels in CAN. However, despite these limitations, this pilot study is provocative in suggesting that oxygenation patterns are different in CAN and moreover, are strongly associated with OS. Our therapeutics to date have not used oxygen delivery as an outcome of therapy but it may well be the case that optimal tissue oxygenation, not hypoxia nor hyperoxia, is a target of therapy. The association in CAN between aberrant kidney oxygenation and OS is important and may provide leads as to how to slow loss of transplant function.

Acknowledgements

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Reference List


45. **Meier-Kriesche HU, Schold JD and Kaplan B.** Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? *Am J Transplant* 4: 1289-1295, 2004.


216. 4-1-0936.

Ref Type: Generic


### Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Chronic Allograft Nephropathy (CAN)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>9</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>4/5</td>
<td>7/3</td>
<td>0.37</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>9/9</td>
<td>7/10</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>32.6 ± 4.3</td>
<td>49.3 ± 3.9</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27 ± 1.7</td>
<td>28.3 ± 1.2</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>87.7 ± 2.5</td>
<td>95.5 ± 3.5</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Serum creatinine (mg/dL)</strong></td>
<td>0.9 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Estimated GFR (MDRD) ml/min</strong></td>
<td>94.8 ± 4.7</td>
<td>46.5 ± 3.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Hematocrit (%)</strong></td>
<td>42.3 ± 1.5</td>
<td>39.2 ± 0.9</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Albumin (g/dL)</strong></td>
<td>4.5 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Calcineurin inhibitor levels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>TAC (n=5)</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>CsA (n=5)</td>
<td>86.8 ± 26.4</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CRP mg/dL</strong></td>
<td>0.9 ± 0.8</td>
<td>0.2 ± 0.1</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Urine Protein:Creatinine (mg/g)</strong></td>
<td>96 ± 12.1</td>
<td>237 ± 35.3</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Urine pH</strong></td>
<td>6.9 ± 0.3</td>
<td>6 ± 0.2</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Urine Specific Gravity</strong></td>
<td>1.018 ± 0.002</td>
<td>1.010 ± 0.002</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Urine Na (mEq/L)</strong></td>
<td>109.5 ± 17.9</td>
<td>83.9 ± 15.7</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>FeNa (%)</strong></td>
<td>0.7 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviations. BMI: Body Mass Index, CRP: C-reactive protein, CsA: cyclosporine A, FeNa: Fraction of excretion of Na, MAP: Mean Arterial Pressure, MDRD: Modification of Diet in Renal Disease formula, TAC: tacrolimus.
Figure 1. Intrarenal Oxygenation and Oxidant Stress in patients with CAN

- Subjects
- General Clinical Research Center (GCRC)
- Vital Signs
  Blood and Urine Collections
- BOLD-MRI
- Losartan 50 mg
- Vital Signs
  Blood and Urine Collections
- BOLD-MRI
Figure 2. Baseline BOLD-MR Color R2* map of coronal sections

(a) Normal (grey scale)  (b) Normal (color)  (c) CAN (grey scale)  (d) CAN (color)

(f) Baseline R2* values in Healthy Volunteers ( ) and patients with chronic allograft nephropathy (CAN, )

Figures a→d: Grey and Color R2* maps in the coronal planes, of normal kidney allograft and transplants with chronic allograft nephropathy (CAN). Blue represents the lowest R2* value (lowest deoxyhemoglobin concentration), and green, yellow and red show increasing R2* values. Color map scale is similar in all figures. Figures f: bar graphs representing R2* values (mean and SD) of medullary, cortical and medullary:cortical ratios in healthy volunteers compared to patients with CAN.
Figure 3. Serum and Urine markers of oxidative stress

Data represented as the arithmetic mean and the standard error of the mean

* p values represent significant differences between healthy volunteers and patients with chronic allograft nephropathy (CAN).

H2O2: hydrogen peroxide, NO: total nitric oxide (nitrates/nitrites), HSP27: heat shock protein 27
### Table 2. Intrarenal oxygenation and oxidative stress in chronic allograft nephropathy are correlated

#### (a) Multiple Linear Regression Analyses

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Multiple Linear Regression Analyses</th>
<th>R²</th>
<th>*p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOLD</td>
<td>Medullary R₂* (MR₂*)</td>
<td>6.923 + (0.945 x CR₂*) + (0.0000815 x UF₂*) - (0.00146 x UNO)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Cortical R₂* (CR₂*)</td>
<td>19.472 - (0.00518 x UNO*) - (0.564 x UH₂O₂*)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>M:C R₂*</td>
<td>1.313 + (0.00000851 x UF₂*) + (0.0257 x UH₂O₂*) - (0.0143 x SH₂O₂)</td>
<td>0.84</td>
</tr>
<tr>
<td>OS</td>
<td>Urine F₂ isoprostanes (F₂)</td>
<td>-37191.367 + (10174.393 x MR₂*) - (11132.035 x CR₂*)</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Urine Nitric Oxide (UNO)</td>
<td>2975.627 - (85.026 x MR₂*) - (97.771 x UH₂O₂*) - (101.880 x UHSP27*)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Urine HSP27 (UHSP27)</td>
<td>-0.446 + (1.466 x CR₂*) - (1.406 x MR₂*) - (1.946 x FeNa*) + (3.271 x albumin)</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Urine H₂O₂ (UH₂O₂)</td>
<td>19.112 - (0.723 x CR₂*) - (0.00572 x UNO*) - (0.694 x UHSP27*)</td>
<td>0.76</td>
</tr>
<tr>
<td>MAP</td>
<td>MAP (mmHg)</td>
<td>217.902 - (37.299 x MCR₂*) - (2.325 x Ht*) + (0.00482 x SF₂*) + (2.490 x UH₂O₂*)</td>
<td>0.92</td>
</tr>
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</table>

#### (b) Spearman Rank Order Correlation

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent variable</th>
<th>Correlation Coefficient</th>
<th>*p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medullary R₂*</td>
<td>Age</td>
<td>0.80</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Cortical R₂*</td>
<td>0.78</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Serum HSP27</td>
<td>0.73</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Urine Nitric Oxide</td>
<td>-0.70</td>
<td>0.02</td>
</tr>
<tr>
<td>Cortical R₂*</td>
<td>Age</td>
<td>0.65</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Urine Nitric Oxide</td>
<td>-0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>M:C R₂*</td>
<td>Urine F₂ isoprostanes</td>
<td>0.88</td>
<td>0.0001</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>eGFR</td>
<td>-0.66</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Hgb</td>
<td>-0.67</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Serum F₂ isoprostanes</td>
<td>0.67</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Serum Nitric Oxide</td>
<td>-0.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>

FeNa: Fraction of Excretion of Na, Ht: hematocrit, MAP: Mean Arterial Pressure, OS: Oxidative Stress
Figure 4. The effect of Losartan (50mg) on intrarenal oxygenation and urine Na⁺

(a) R₂* values in Healthy Volunteers (---) and patients with CAN (-----) before and after Losartan

(a.1) Medullary R₂*

(a.2) Cortical R₂*

(a.3) Medullary:Cortical R₂* ratio

(b) Urine Na⁺ in Healthy Volunteers (■) and patients with chronic allograft nephropathy (CAN, □)

(b.1) Spot urine Na⁺ (mEq/L)

(b.2) Fraction of Excretion of Na⁺
### Table 3. BOLD-MRI, MR2* and CR2* levels in experimental and human studies

<table>
<thead>
<tr>
<th>Condition</th>
<th>Potential Mechanism</th>
<th>Species</th>
<th>MR2*</th>
<th>CR2*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral Ureteral Obstruction</td>
<td>Inflammation</td>
<td>Pig</td>
<td>↓</td>
<td>↑</td>
<td>Pederson et al [57]</td>
</tr>
<tr>
<td>Acute Ischemic Renal Failure</td>
<td>Ischemia</td>
<td>Swine</td>
<td>↑</td>
<td>↑</td>
<td>Alford et al [6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pig</td>
<td>↑</td>
<td>↑</td>
<td>Juillard et al [35]</td>
</tr>
<tr>
<td>Tempol in hypertension</td>
<td>Free radical scavenger</td>
<td>Rat</td>
<td>↓</td>
<td>No change</td>
<td>Pederson et al [57]</td>
</tr>
<tr>
<td>Furosemide</td>
<td>↓ Na(^+) reabsorption</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>Epstein et al [23]</td>
</tr>
<tr>
<td>Water diuresis</td>
<td>↓ Na(^+) reabsorption</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>Prasad et al [60]</td>
</tr>
<tr>
<td>Age</td>
<td>Ischemia</td>
<td>Human</td>
<td>↑</td>
<td>↑</td>
<td>Simon-Zoula et al [69]</td>
</tr>
<tr>
<td>Acute transplant rejection</td>
<td>Immune/Inflammation</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>Sadowski et al [65]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Djamali et al [20]</td>
</tr>
<tr>
<td>CAN</td>
<td>Immune/Inflammation</td>
<td>Human</td>
<td>↓</td>
<td>↓</td>
<td>Djamali et al</td>
</tr>
</tbody>
</table>

ATN: acute tubular necrosis, CAN: Chronic Allograft Nephropathy
Figure 5. Potential interactions between intrarenal oxygenation and OS in CAN

CNI: calcineurin inhibitors, GFR: glomerular filtration rate, FeNa: fraction of excretion of Na, OS: oxidative stress