Tolerance of the Human Kidney to Isolated Controlled Ischemia

Dipen J. Parekh,* Joel M. Weinberg,† Barbara Ercole,* Kathleen C. Torkko,‡ William Hilton,* Michael Bennett,§ Prasad Devarajan,§ and Manjeri A. Venkatachalam||

*Department of Urology and | Department of Pathology, University of Texas Health Science Center San Antonio, San Antonio, Texas; †Division of Nephrology, Department of Internal Medicine, University of Michigan and Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, Michigan; ‡Department of Pathology, University of Colorado, Aurora, Colorado; and §Center for Acute Care Nephrology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio

ABSTRACT

Tolerance of the human kidney to ischemia is controversial. Here, we prospectively studied the renal response to clamp ischemia and reperfusion in humans, including changes in putative biomarkers of AKI. We performed renal biopsies before, during, and after surgically induced renal clamp ischemia in 40 patients undergoing partial nephrectomy. Ischemia duration was >30 minutes in 82.5% of patients. There was a mild, transient increase in serum creatinine, but serum cystatin C remained stable. Renal functional changes did not correlate with ischemia duration. Renal structural changes were much less severe than observed in animal models that used similar durations of ischemia. Other biomarkers were only mildly elevated and did not correlate with renal function or ischemia duration. In summary, these data suggest that human kidneys can safely tolerate 30–60 minutes of controlled clamp ischemia with only mild structural changes and no acute functional loss.

Ischemia to the human kidney has been implicated as a common contributor to acute and chronic kidney injury from diverse medical and surgical causes.1–3 However, to our knowledge the direct effect of controlled ischemia on human renal structure and function has never been evaluated prospectively. Current understanding of renal ischemia is derived from animal studies, the renal transplant setting, and retrospective human studies that report conflicting data regarding the response and tolerance of the human kidney to ischemia.1–6 These studies suggest harmful effects from renal ischemia, resulting in the present dogma of limiting renal ischemia time during surgical procedures to within 20–30 minutes.6 Clinical trials in the field of AKI based on promising animal data have disappointed, suggesting that the human and small-animal kidney responses to ischemia may be qualitatively and quantitatively different.7,8 They have also generated interest in better understanding of the human kidney ischemia response to allow optimal design of strategies to treat AKI, store kidneys for transplantation, and more safely use renal clamp ischemia during abdominal surgery.

New biomarkers of renal injury are receiving increasing attention in an effort to achieve earlier diagnosis than provided by serum creatinine, better assess the extent and severity of parenchymal insults from different causes, and serve as markers during clinical trials.9–14 However, their relationship to underlying structural changes during AKI in humans is unknown.

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D.J.P. and J.M.W. contributed equally to this work.
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Correspondence: Dr. Dipen J. Parekh, Department of Urology, University of Miami Miller School of Medicine, Dominion Tower, 5th Floor, 1400 NW 10th Avenue, Miami, FL 33136. Email: parekhd@med.miami.edu

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Small renal masses make up 48%–66% of all renal tumors that are diagnosed and 38% of all renal tumors that are excised. There are approximately 65,000 new cases of renal cancer just in the United States, of which about 45,000 are amenable to nephron-sparing surgery. Despite the potential for a better outcome, only 10% of patients eligible for nephron-sparing actually undergo a partial nephrectomy because of renal ischemia concerns; most have radical nephrectomy, which is technically easier. Several technically challenging nonclamping approaches have been recently proposed to avoid renal ischemia, with significantly higher complication rates. Using open partial nephrectomy for excision of a renal mass involving direct clamp occlusion of the renal blood vessels, we have conducted a prospective study that evaluates for the first time the structural response of the human kidney to clamp ischemia and initial reperfusion in association with assessment of short-term renal functional outcomes and novel biomarkers. Our results document substantial resistance of the human kidney to clamp ischemia and have implications for both the basic understanding of ischemic injury in human kidneys and the care of patients requiring isolated controlled ischemia for partial nephrectomy and other procedures.

RESULTS

Patient demographic characteristics, intraoperative measures, and characteristics of renal ischemia are summarized in Table 1. No protocol-related complications occurred. Duration of ischemia in 82.5% of patients was >30 minutes. The mean duration of warm ischemia was 32.3 minutes (range, 15–53 minutes; n=27) and duration of cold ischemia was 48 minutes (range, 30–61 minutes; n=13). Data were analyzed to determine differences by warm and cold ischemia, and no statistically significant differences were discovered. Cold and warm data were pooled for further analyses.

Renal Clearance Function

Serum creatinine was significantly increased by a mean ± SEM of 23.6%±5.0% relative to baseline at 24 hours, then returned toward its baseline; it was not significantly different from baseline at 72 hours (Figure 1, A and C). Serum cystatin C was not significantly changed at 2 or 24 hours (Figure 1, B and D). There was no correlation between the change in creatinine and the change in cystatin C at 24 hours (Figure 1E). No significant correlations were seen between changes in serum creatinine and cystatin C and ischemia time (Figure 1, F and G). Simlarly, no correlation was seen between tumor size and duration of ischemia (Supplemental Figure 1A), creatinine increase at 24 hours (Supplemental Figure 1B), or cystatin C increase at 24 hours (Supplemental Figure 1C).

Renal Biopsy Results

The availability of high-quality biopsy material is an unprecedented aspect of this study. The samples were obtained from needle biopsies directed perpendicular to the capsule under direct visualization. They were of good size and were generally 10–12 mm in length. The samples usually contained both cortex and inner and outer stripes of the outer medulla, with excellent sampling of S3 segments that were easily identifiable on the basis of their structure and the nearby tubules.

Structural features were well preserved in the biopsy specimens (Figures 2–4, 9 and Supplemental Figures 2, 3, and 4 for large high resolution files). By electron microscopy, end-clamp samples showed ischemic mitochondrial changes with prominent swelling but little brush-border damage (Figures 2B and 3B). Thick sections with greater area for examination provided a wider range of scores than did the ultrastructure (Figure 2A, C, D), but the correlation between the blinded, quantitative scoring for the thick sections and the ultrastructure was good (Supplemental Figure 5). Overall severity of structural injury at the end of ischemia (Figure 2G) and post-clamp (Supplemental Figure 5) did not correlate with peak creatinine or ischemia duration. The only significant relationship was the r value for peak serum creatinine ratio versus end-clamp thick section score (Figure 2G). The corresponding β value was not significant. The end-ischemia changes improved during the 5 minutes of reflow before the post-clamp biopsies in most cells (Figure 2, A–F, and Figure 3C), although mitochondrial condensation developed rarely (Supplemental Figure 3). Tubule injury produced by ischemia or hypoxia is sensitively reflected by alterations of actin and integrins. Cellular ATP depletion decreases protein tyrosine phosphorylation, and intercellular adhesion molecule-1 (ICAM-1) is an important early mediator of the inflammatory component of ischemic AKI. Therefore, we used these indices to further assess injury produced by ischemia, as well as to confirm

Table 1. Patient demographic characteristics, intraoperative measures, and characteristics of renal ischemia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants (n)</td>
<td>40</td>
</tr>
<tr>
<td>Mean age (range) (yr)</td>
<td>55 (28–84)</td>
</tr>
<tr>
<td>Women (%)</td>
<td>26 (65)</td>
</tr>
<tr>
<td>Race/ethnicity, n (%)</td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>8 (20)</td>
</tr>
<tr>
<td>Hispanic grouped</td>
<td>32 (80)</td>
</tr>
<tr>
<td>Preoperative eGFR (MDRD), n (%)</td>
<td></td>
</tr>
<tr>
<td>&gt;90 ml/min per 1.73 m²</td>
<td>24 (60)</td>
</tr>
<tr>
<td>60–89 ml/min per 1.73 m²</td>
<td>12 (30)</td>
</tr>
<tr>
<td>30–59 ml/min per 1.73 m²</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Estimated blood loss (ml)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>249.4</td>
</tr>
<tr>
<td>Median (range)</td>
<td>200 (50–1000)</td>
</tr>
<tr>
<td>Median size of tumor (range) (cm)</td>
<td>4.1 (2.0–8.0)</td>
</tr>
<tr>
<td>Ischemia time (min)</td>
<td></td>
</tr>
<tr>
<td>Warm (mean)</td>
<td>32.3</td>
</tr>
<tr>
<td>Cold (mean)</td>
<td>48</td>
</tr>
<tr>
<td>Mean (combined)</td>
<td>37.4</td>
</tr>
<tr>
<td>Median (combined) (range)</td>
<td>32 (15–61)</td>
</tr>
</tbody>
</table>

eGFR, estimated GFR; MDRD, Modification of Diet in Renal Disease.
the observations made by strictly morphologic criteria. Rhodamine phalloidin staining of periodate-lysine-paraformaldehyde–fixed frozen sections for polymerized actin (Figures 4 and 5 and Supplemental Figure 4) confirmed the brush-border preservation seen in the thick sections and ultrastructure. β1 integrin was also almost unaffected (Figures 4 and 5). Phosphotyrosine and ICAM-1 were affected in the end-clamp and post-clamp samples, but not in all patients (Figures 5). The frequency with which they changed was similar to that seen for actin (Figure 5).

**Injury Biomarkers**

Small but significant increases of serum neutrophil gelatinase–associated lipocalin (NGAL) were seen at 2 and 24 hours after surgery (Figure 6A); it also significantly increased with increasing ischemia time (Figure 6B). However, serum NGAL was not related to peak serum creatinine (Figure 6C) or end-ischemia morphology score (Figure 6D). Urine outputs in all patients were high, reflecting the postoperative hydration provided and the presence of an uninjured contralateral kidney. They averaged 200±26 ml per hour for the first 2 hours after surgery and 114±9 ml per hour subsequently. Urine creatinine concentrations tracked appropriately with the dilution expected from these high urine outputs (Supplemental Figure 6).

Creatinine-factored urine biomarkers are summarized in Figure 7, and unfactored values are given in Supplemental Figure 7. All urine biomarkers had significant changes. There were early increases of liver fatty acid–binding protein (LFABP) and N-acetylβ-D-glucosaminidase (NAG). Urine NGAL increased moderately at all times. Kidney injury marker-1 (KIM-1) was maximally increased at 24 hours. IL-18 was increased at all time points, along with urine albumin. However, the median increases of the novel urine biomarkers were only modest in

![Figure 1](image-url)
In addition, urine biomarker levels did not correlate with duration of ischemia (Figure 8), peak serum creatinine (Supplemental Figure 8), or end-ischemia thick section score (Supplemental Figure 9). No significant differences were seen between warm and cold ischemia in the urine biomarkers or serum NGAL (Supplemental Figure 10). No ischemia-induced podocyte alterations were associated with the increased albuminuria (Figure 9).

**DISCUSSION**

Ischemic damage to human kidneys is implicated in approximately 50% of cases of AKI from diverse causes, including hypovolemic states, sepsis, cardiopulmonary bypass, and aortic and renal surgeries.1–3 Our study addresses three important issues: the tolerance of the human kidney to isolated controlled clamp ischemia, the nature of structural injury to the kidney that develops during and immediately after hilar clamping, and the behavior of biomarkers in this clinical scenario. To our knowledge, the effect of ischemia on renal structure and function has not been prospectively assessed in a clinical setting. Lack of clarity on this subject has prevented widespread adoption of partial nephrectomy for small renal masses because of technical difficulties induced by limiting renal ischemia times to <30 minutes; retrospective human and small-animal studies have suggested deleterious effect of ischemia times >20–30 minutes.6,15

Clinically, the most common denominator underlying AKI in patients is shock, which is almost always complicated by...
other concurrently operative processes that also injure the kidney, such as tissue damage, sepsis, and nephrotoxins. The relative contributions of these factors differ between patients; moreover, other variables obscure the analysis of shock-induced renal ischemia *per se* in the clinical context. As such, an understanding of ischemic effects on the human kidney and a determination of the thresholds of ischemic severity required to produce injury have remained elusive. It is mainly for this reason that clamp ischemia-reperfusion injury in small mammals has emerged as a popular paradigm to derive insights into the pathobiology of ischemic AKI. The model lends itself to standardization and reproducibility in a range that permits experimentation to test treatment modalities. However, translation of results from the small animal models to the human context has failed to live up to expectations, underscoring a pressing need to study renal ischemia in humans under clinical circumstances. Understandably, this is difficult to do in a standardized manner. In this regard, despite constraints imposed by unique patient-specific differences in the study population and the need to vary ischemic intervals according to the context of individual surgeries, the overall design of the clamp ischemia-reperfusion analysis in the present cohort of patients has yielded useful information. It is internally controlled to a degree by variations of ischemic intervals and, furthermore, by inclusion through clinical necessity of both cold and warm ischemia in the same study population.

Despite the longer durations of ischemia used in the present study, >30 minutes in >80% of the patients, only a mild transient increase of creatinine, averaging 23.6%, was seen; cystatin C, an alternate, possibly more sensitive marker of GFR, did not change. Normal contralateral renal function may have masked injury observed in our study. However, if the clamped kidney had sustained severe injury and decreased clearance, we would expect functional changes similar to those seen in donor nephrectomy studies in which serum cystatin C increased 37% and creatinine increased 19% at 24 hours, and both increased 52%–62% at 72 hours. Increased creatinine without a concomitant decrease in clearance could indicate a transient defect of tubule creatinine secretion in our cohort.

Figure 3. Examples of the range of ultrastructural changes seen at each sampling time showing reversal of ischemia-induced pathology with brief reflow. (A) Pre-clamp. (B) End-clamp. (C) Post-clamp. Size marker, 10 microns.
In prior reports, the extent of structural damage during human AKI of diverse causes has appeared to be less than that seen in animal models during severe reduction of renal function.\(^4,30\)--\(^32\) However, the previous studies were generally retrospective, had relatively small numbers of patients with heterogeneous insults, and the biopsies were usually performed late (i.e., days after the insult). We believe this study represents the first prospective comprehensive analysis of human kidneys subjected to sustained clamp ischemia over time frames typically used in the experimental models that have contributed to our understanding of the cellular response to renal ischemia.\(^33,34\) Consistent with the limited apical structural damage, basal \(\beta_1\) integrin, which is highly sensitive to ischemia in animal models and to hypoxia of isolated tubules,\(^21,22\) was almost unaffected. In maximally hypoxic isolated tubules, immunodetectable phosphotyrosine is entirely lost and then recovers during reoxygenation as a function of ATP availability.\(^22\)

In vivo, phosphotyrosine is similarly lost during ischemia but can then rebound with hyperphosphorylation of focal adhesion proteins during reflow.\(^23\) Both end-clamp and post-clamp samples had areas of decreased and increased phosphotyrosine signal relative to the pre-clamp baseline; however, this did not occur in every patient, as might have been expected from the insult durations used. Secondary inflammation plays a major role during ischemic AKI,\(^2\) and ICAM-1 expression is known to increase and to contribute to progressive tissue damage.\(^24,37\) Changes of ICAM-1 were detected in many of the cases despite the short time frame of the study. Cell necrosis and apoptosis were not expected because they develop only after a delay.\(^2,32\)

Several possibilities may account for the resistance to renal injury in this study. Two factors could have preserved tissue ATP and contributed to the mild changes of actin, \(\beta_1\) integrin, and phosphotyrosine relative to animal and isolated tubule models. One is cooling of the kidney\(^38\) during the open surgical procedure; this may have occurred, even during the warm ischemia conditions, to a greater degree than in animal models when the clamped kidney is returned to a closed abdominal cavity for the ischemic period. Another is the presence of collaterals in the renal capsule.\(^39\) However, our failure to detect differences of function and structure between the hypothermic and normothermic groups of patients with renal ischemia during nephrectomy strongly argues against the contribution of cooling effects to the protection. The mitochondrial alterations are also consistent with a strong ischemic insult to equivalent degrees in both groups. Although the contribution of a collateral blood supply from capsular vessels

**Figure 4.** Representative immunofluorescence results from a single patient illustrating perturbations of actin, phosphotyrosine and ICAM-1 distribution but not of \(\beta_1\) integrin in end-clamp and post-clamp biopsies. Actin was detected using rhodamine phalloidin. The other images show staining with primary antibodies for the indicated proteins and CF\(^\text{TM}\)488A green-fluorescent secondary antibody. Size marker, 10 microns. pTyr, phosphotyrosine.
is difficult to assess, renal perfusion by such vessels is likely to be confined to the most superficial cortex. In this regard, the biopsy specimens that we obtained were of an adequate length and were cut perpendicular to the surface under direct vision, so that deeper cortical and medullary tissue was invariably present. We failed to detect significant differences between the more superficial areas of the biopsy specimens and the deeper zones. Thus, the protection that we observed is unlikely to have been due to such collaterals. Other explanations for the absence of progressive functional changes are the use of mannitol or protective factors present in the human kidney that are absent in rodents, such as tubule cell production of LFABP.40 Previously reported information on monkey kidneys suggests similar or even greater resistance with lack of consistent functional impairment at clamp durations of <90 minutes.41 Also relevant is a human study in which minimal functional changes developed after aortic cross-clamping averaging 65 minutes.42

Novel biomarkers have been reported to be more sensitive in predicting clinical AKI and its outcomes compared with serum creatinine. Although several of these biomarkers have been explored in settings of AKI from diverse causes, their role in clinical practice is not yet fully defined.9–14 There is controversy in the literature regarding whether urinary biomarkers should be factored for creatinine in the setting of AKI because of decreased creatinine resulting from lower clearance.43 We believe that creatinine-factored biomarkers are more appropriate for the present study because of the maintained creatinine clearance from the contralateral uninjured kidney, but we have presented the data in both ways. All biomarkers in our study showed significant changes consistent with their underlying biology. However, the changes were relatively small and did not correlate with extent of structural alteration during ischemia, duration of ischemia, or the transient increases of creatinine.

The lesser discriminative power of the biomarkers in this setting compared with other AKI settings10,12,25 may be explained by the lack of more severe and sustained injury and is consistent with the mild

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**Figure 5.** Quantitation of ischemia-induced changes of actin, phosphotyrosine, ICAM-1 and β1 integrin. (A) End-clamp and post-clamp sections were compared with the corresponding pre-clamp section and were assigned one of three categories: (1) neither end-clamp nor post-clamp sample was different from the corresponding pre-clamp sample, (2) end-clamp was different from pre-clamp, or (3) post-clamp reflow was different from pre-clamp. (B) Apical brush-border rhodamine phalloidin staining for polymerized actin was graded on a scale of 1–5 as described in Complete Methods in Supplemental Data. *P<0.05 versus pre-clamp (Pre). pTyr, phosphotyrosine.

**Figure 6.** Serum NGAL increases after ischemia as a function of ischemia duration, but does not correlate with changes of serum creatinine or end-clamp thick section morphology score. (A) Preoperative and 2- and 24-hour postoperative values for each patient. Bars indicate medians. (B) Relationship between ischemia duration and ratio of peak serum NGAL to preoperative values. The 95% confidence intervals were −0.01 to 0.60 for r and 0.01–0.08 for β. (C) Ratio of peak value to the preoperative value versus thick section score.
Figure 7. Increases of multiple urine biomarkers. Bars indicate medians. Significant differences are as indicated.

Figure 8. Urine biomarker changes do not correlate with ischemia duration. Biomarker values are ratios to the preoperative values measured at the time they peaked for all variables except albumin, which are 24-hour values to allow clearing of early postoperative hematuria. None of the biomarkers was significantly correlated with duration of ischemia.
structural injury induced by the surgical procedure. This study is the first to correlate biomarkers with mild structural changes in humans. Although urinary biomarkers were not useful for predicting early functional AKI in this setting, our findings do not preclude their use in other clinical contexts where more severe structural injury develops.

The RIFLE (Risk, Injury, Failure, Loss, ESRD) and AKIN (Acute Kidney Injury Network) criteria for categorizing AKI provide a valuable tool for standardizing assessment of the process in clinical trials and practice.44 We have not primarily used them in reporting the present data for several reasons: (1) Because all patients had high urine outputs due to the presence of the contralateral kidney and the intentional hydration, classification according to the criteria is entirely predicted by the creatinine ratios. Using those ratios directly as we have done presents more thorough data than lumping them into categories would. (2) As discussed previously, because of the discrepancies between behavior of creatinine and cystatin C, it is not clear that the changes of creatinine in fact represent the decreases of clearance implicit in applying the AKI staging. (3) Also, the standard criteria for the AKIN and RIFLE categorizations do not apply in this clinical setting because the insult was unilateral.

However, because of the importance and wide application of these categorizations, we have analyzed the data using them with these caveats in mind (Supplemental Table 1 and Supplemental Figure 11). As indicated in Supplemental Table 1, we have modified the AKIN criteria to account for the fact that the contralateral kidney is unaffected so that the expected increments of creatinine due to loss of function of the partial-nephrectomy kidney would be no more than half the magnitude of those used for the standard criteria. Even after our stringent modification, none of the patients in our study experienced clinically significant stage 3 AKIN at 48 hours. This analysis showed the only measures that significantly differed between patients classified as having AKI at 24 hours or ≥48 hours were serum creatinine (as expected because that was the basis for the AKI categorization) and end-ischemia thick

Figure 9. Albuminuria is not accompanied by ischemia-induced alterations of podocytes. Glomeruli were examined in detail in sections from six of the patients with the highest levels of urinary albumin at 2 and 24 hours. Images shown are end-clamp (A–C) and post-clamp (D–F) from a patient whose albumin-to-creatinine ratios were 2366 μg/mg pre-clamp, 11,500 μg/mg at 2 hours of recovery, and 2930 μg/mg at 24 hours of recovery. Although focal podocyte effacement (arrow in F) was seen in some end-clamp and post-clamp samples, it was also seen in pre-clamp samples to the same extent (not shown); the ultrastructure was most notable for large areas of completely normal podocytes (arrow in B) in all samples despite the albuminuria. Focal blebbing of endothelial cells (arrow in C) was also seen in some samples but could be found at all time points, including pre-clamp (not shown). Size marker is 5 microns.
section score. In the latter case, the effect across all groups was significant but the individual differences between them were not.

The relatively small sample size may serve as a limitation, with the possibility of a broader range of outcomes in a multi-institutional setting with more patients and greater power for statistical analysis. Structural studies were unavoidably limited to the ischemia period and 5 minutes of reperfusion, which does not cover the subsequent 12- to 24-hour period during which injury further progresses in small animal models from the more extensive initial injury they develop. It would not have been practical or ethical to perform biopsies at later points in this clinical context. Although we cannot obtain the structural correlate at those later points, there were certainly no later functional changes, such as those that develop in the small-animal models, to suggest later worsening. On the basis of the results, clamp ischemia times up to 60 minutes may be safe in patients with two functional kidneys undergoing open partial nephrectomy, which will expand indications for partial nephrectomy in management of small renal masses.

In summary, this study provides the first detailed analysis of structural and functional responses of the human kidney to controlled clamp ischemia. It documents a greater than expected resistance to injury with little or no acute renal functional loss. Overall, variations of ischemic durations from 15 to 61 minutes had no effect on the injury produced, assessed functionally by creatinine and cystatin C, structurally by light and electron microscopic and immunofluorescence analysis, and by biomarkers excreted in the urine. Urinary biomarkers did increase, but to variable degrees, and did not correlate with ischemia durations or with structural changes. Notably, other than mitochondrial swelling, the structural changes were uniformly mild, of a degree that would be considered reversible by criteria guided by the results of small-animal studies. Increases of serum NGAL were indeed higher with increasing ischemia durations. Nevertheless, these increases bore no relationship to the structural changes documented during and after ischemia. Although the results in no way diminish the value of small-animal models for understanding pathophysiology and studying interventions, the structural data, taken together with the evidence for functional preservation in this study and the limited prior work, imply unexpectedly high tolerance of the human kidney to clamping during isolated controlled ischemia that needs to be considered in translational studies.

### CONCISE METHODS

#### Study Design

Between January 2009 and October 2010, 40 patients were enrolled in this prospective clinical study after the University of Texas Health Science Center San Antonio Institutional Review Board approved the study and participants provided informed consent. A single surgeon conducted all procedures. A data safety and monitoring committee periodically monitored the study progress. The surgeon was blinded to the study results until study completion.

#### Study Participants

Any patient with a renal mass who was undergoing an open partial nephrectomy and was at least 18 years of age was eligible for inclusion. Excluded were patients scheduled for a laparoscopic partial nephrectomy, pregnant patients, and patients with a solitary kidney or ESRD.

#### Surgical Procedure and Renal Biopsies

Study participants underwent an open partial nephrectomy for renal mass excision. The kidney was mobilized and the vascular pedicle was isolated in the standard fashion. After induction of anesthesia and before clamping of the hilar vessels, all study participants received 25 g intravenous mannitol. For all participants, needle biopsies were performed in the kidney with an 18-gauge needle biopsy gun at a site remote from the tumor at specified time points: one biopsy just before renal pedicle clamping, one biopsy every 10 minutes during ischemia up to unclamping of renal vessels, and one biopsy at 5 minutes after reperfusion. Biopsy protocol was modified to focus more on endpoint biopsies after review of the first 18 patients indicated sufficient data on ischemia time course. The subsequent 22 participants underwent biopsies at the following time points: two biopsies just before renal pedicle clamping, two biopsies at end of maximum duration of ischemia, and two biopsies after 5 minutes of reperfusion. One core was processed for ultrastructure and the other for immunofluorescence, as described in the Supplemental Data. All biopsies were done under direct visualization from non–tumor-bearing areas of the kidney.

A Satinsky vascular clamp was used to control the renal hilum en bloc. The decision to use warm or cold ischemia was based on the size and complexity of the renal mass, with cold ischemia reserved for the most complex lesions. Surface hypothermia for cold ischemia was induced by using ice slush for 10–15 minutes after clamping of the hilum. Renal mass excision with parenchymal reconstruction was performed in a standard fashion. All patients received intravenous normal saline at 125–150 ml per hour in the postoperative 24 hours. All complications were recorded and graded according to the Clavien classification system.

#### Serum and Urine Biomarker Collection and Analysis

Serum and urine were collected at the preoperative visit; at 2, 6 (urine only), and 24 hours after surgery; and then daily until discharge (serum only). Full assays were done on all available samples from the first 24 hours. Serum was analyzed for creatinine, cystatin C, and NGAL. Urine was analyzed for creatinine, NGAL, N-acetyl-β-D-glucosaminidase, IL-18, kidney injury marker-1, LFABP, and albumin. Serum creatinine was measured in the hospital clinical laboratory. The other samples were stored at −80°C until assay by the procedures specified in the Complete Methods in Supplemental Data.

#### Analysis of Biopsy Specimens

Biopsy specimens processed for light microscopy of toluidine blue–stained thick sections and ultrastructure were analyzed by two of the authors (M.A.V. and J.M.W.), who were blinded to the clinical information from the patients during the evaluation, using the
methods and grading procedures described in the Complete Methods in Supplemental Data.

Statistical Analyses
For the statistical analyses, underlying data distributions were determined using the Shapiro-Wilk test. Parametric tests (t test, paired t test, ANOVA) were used for normally distributed data, nonparametric tests (Wilcoxon rank-sum, Wilcoxon signed-rank, Kruskal-Wallis tests) were used for data with skewed distributions, and a chi-squared test was used for categorical data.

To study the relationship of biomarkers to recovery time, vertical scatter plots were used to display the data at various time points after surgery. Median values were plotted and differences at each time point from preoperative values were determined. To correlate urine and serum biomarkers with functional changes and ischemia time, the ratios of biomarker peak values to preoperative values were calculated. These data were plotted with the creatinine ratio (24 hours to preoperative values) or ischemia time on XY graphs. Functional biomarkers (creatinine, serum cystatin C, and GFR) ratios (24 hour to preoperative) were also plotted against ischemia time. Peak ratios of structural biomarkers were also plotted against peak creatinine ratio. Linear regression analyses were used to fit lines to the data, and an F statistic was calculated to determine whether the slope of the regression line was significantly different from zero. Power analysis indicated that with a sample size of 40, we have 80% power to detect a moderate correlation of ±0.43 or greater compared with the null hypothesis of no correlation ($r=0.0$; two-sided test, $\alpha=0.05$) and to detect a slope of ±0.41 or greater compared with the null hypothesis of slope=0 (two-sided test, $\alpha=0.05$, assume SD for both $x$ and $y$=1).

To determine differences in morphology scores between the three sampling times, a paired analysis was done comparing pre- to end-clamp, pre- to post-clamp, and end- to post-clamp. A paired t test was used to test for differences and a Bonferroni correction was used to adjust the $P$ values for multiple testing. Statistical analyses were performed using SAS software, version 9.2 (SAS Institute, Inc., Cary, NC) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). All tests were two sided with significance set at $P<0.05$.

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DISCLOSURES
None.

REFERENCES

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Supplementary Material
for
Tolerance of the Human Kidney to Isolated Controlled Ischemia

Dipen J. Parekh¹*, Joel M. Weinberg²*, Barbara Ercole¹, Kathleen C. Torkko³, William Hilton¹, Michael Bennett⁴, Prasad Devarajan⁴ and Manjeri A. Venkatachalam⁵.

¹Department of Urology, University of Texas Health Science Center San Antonio, San Antonio, Texas, ²Division of Nephrology, Department of Internal Medicine, University of Michigan and Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, Michigan, ³Department of Pathology, University of Colorado, Aurora, CO, ⁴Center for Acute Care Nephrology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, and ⁵Department of Pathology, University of Texas Health Science Center San Antonio, San Antonio, Texas
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Any patient with a renal mass undergoing an open partial nephrectomy and at least 18 years old was eligible for inclusion. Exclusion criteria included patients scheduled for a laparoscopic partial nephrectomy, pregnant patients, and patients with a solitary kidney or end stage renal disease.

Surgical Procedure and Renal Biopsies
Study participants underwent an open partial nephrectomy for renal mass excision. The kidney was mobilized and the vascular pedicle was isolated in the standard fashion. After induction of anesthesia and prior to clamping of the hilar vessels all study participants received 25 g of intravenous mannitol. Needle biopsies with an 18 gauge needle biopsy gun (Bard Monopty) were obtained from the kidney at a site remote from the tumor on all study participants at specified time points: one biopsy just prior to renal pedicle clamping, one biopsy every 10 minutes during ischemia up to unclamping of renal vessels and one biopsy at five minutes after reperfusion. The biopsy protocol was modified to focus more on endpoint biopsies after review of the first 18 patients indicated sufficient ischemia time course data. The subsequent 22 participants underwent biopsies at the following time points: two biopsies just prior to renal pedicle clamping, two biopsies at end of maximum duration of ischemia and two biopsies after five minutes of reperfusion. One core was processed for ultrastructure and the other for immunofluorescence. All biopsies were done under direct visualization from non-tumor bearing areas of the kidney.

A Satinsky vascular clamp was used to control the renal hilum en bloc. The decision to use warm or cold ischemia was based on the size and complexity of the renal mass with cold ischemia reserved for the most complex lesions. Surface hypothermia for cold ischemia was induced by using ice slush for 10-15 minutes after clamping of the hilum. Renal mass excision with parenchymal reconstruction was performed in a standard fashion. All patients received intravenous normal saline at 125-150 ml/hr in the post-operative 24 hours. All complications were recorded and graded according to the Clavien classification system.

Serum and Urine Biomarker Collection and Analysis
Serum and urine were collected at the preoperative visit and at 2, 6 (urine only), and 24 hours postoperatively and then daily until discharge (serum only). Full assays were done on all available samples from the first 24 hours. Serum was analyzed for creatinine, cystatin C, and neutrophil gelatinase-associated lipocalin (NGAL). Urine was analyzed for creatine, NGAL, N-acetyl-beta-D-glucosaminidase (NAG), interleukin-18 (IL-18), kidney injury marker-1 (KIM-1), liver fatty acid binding protein (LFABP) and albumin. Serum creatinine was measured in the hospital clinical laboratory. The other samples were stored at -80°C until assay. The laboratory investigators were blinded to the sample sources and clinical outcomes. The urine NGAL ELISA was performed using a commercially available assay (NGAL ELISA Kit 036; Biporto, Grusbakken, Denmark) that specifically detects human NGAL. The intra-assay coefficients of variation were 2.1% (range: 1.3-4.094) and inter-assay variation was 9.1% (range: 6.8-18.1%). Urine IL-18 and L-FABP were measured using commercially available ELISA kits (Medical &Biological Laboratories Co., Nagoya,
Japan and CMIC Co., Tokyo, Japan, respectively) per manufacturer's instructions (12). The urine KIM-1 ELISA was constructed using commercially available reagents (Duoset DY1750, R & D Systems, Inc., Minneapolis, MN) as described previously. Urine microalbumin was measured by immunoturbidimetry using MALB flex reagent on a Siemens Dimension Xpand Plus with HM clinical analyzer (Siemens Healthcare USA, New York, NY). Intra- and inter-assay CV values for the assay are 2.3% and 5.9%, respectively. Creatinine values were obtained using a colorimetric creatinine kit based on the Jaffé reaction (Enzo Life Sciences, Plymouth Meeting, PA). Intra- and inter-assay CV's for the creatinine assay are 2.4% and 3.15% respectively. Cystatin C was measured by N-latex nephelometry on a Siemens BNII clinical nephelometer (Siemens Healthcare USA, New York, NY). The inter- and intra-assay coefficient variations were 5% for batched samples analyzed on the same day and <10% for samples measured 6 months apart. The plasma NGAL ELISA was performed using an established and validated assay as previously described. Briefly, microtiter plates precoated with a mouse monoclonal antibody raised against human NGAL (#HYB211-05; AntibodyShop, Gentofte, Denmark) were blocked with buffer containing 1% bovine serum albumin, coated with 100 µl of samples (plasma) or standards (NGAL concentrations ranging from 1 to 1,000 ng/ml), and incubated with a biotinylated monoclonal antibody against human NGAL (#HYB211-01B; AntibodyShop) followed by avidin-conjugated horseradish peroxidase (Dako, Carpinteria, CA). TMB substrate (BD Biosciences, San Jose, CA) was added for color development, which was read after 30 minutes at 450 nm with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA).

Renal biopsy specimen processing and analysis

Thick sections for toluidine blue and thin sections for electron microscopy: Needle biopsy cores obtained using 18 gauge Bard Monopty biopsy instruments were immediately ejected into 4% formaldehyde and 1% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.2. After subdivision into multiple pieces, the tissue was washed in cold 0.1M Na cacodylate buffer pH 7.4, fixed over ice in the dark in 1% osmium tetroxide in water for 1 hour, washed in cold water and incubated overnight in the dark in cold, saturated uranyl acetate in water. After thorough washing in cold distilled water, the tissue was dehydrated in ascending concentrations of cold acetone in water. After incubation in absolute acetone with 2 changes, the tissue was transferred to propylene oxide with 3 changes to remove acetone, and embedded in epoxy resin by standard techniques. One micron thick sections of epoxy embedded tissue were cut with diamond knives and stained with toluidine blue. After light microscopic verification of the tissue architecture, the block was trimmed further for microtomy using diamond knives to obtain 600-800 Å thick sections. The sections were mounted on copper grids, stained with uranyl acetate and lead citrate and prepared for electron microscopy. Sections were viewed using a JEOL 1230 electron microscope and images recorded digitally.

Immunostaining and rhodamine phalloidin staining on frozen sections: Biopsies were ejected into periodic acid–lysine–paraformaldehyde (PLP), transported to the laboratory, and stored in PLP overnight (18-24 hours) at 4 degrees C. They were then washed x3 in PBS (phosphate buffered saline) and stored at 4 degrees C in PBS-0.02% NaN3. For sectioning, they were cryoprotected in 10% sucrose–PBS for 1 hour, then 30% sucrose–PBS, prior to embedding in OCT compound, freezing in isopentane, and storage at -80°C. Cryosections of 6 µm thickness were cut on a Reichert-Jung Frigidocut-N 2800 cryostat and placed on glass slides pre-coated with 1% poly-L-lysine, then stored at -80°C until stained. For staining, slides were rinsed in phosphate buffered saline (PBS), then permeabilized with 0.3% Triton X-100 in PBS for 4 min. at room temperature. They were then dip washed for 2 min. in PBS prior to 10 min. room temperature blocking with 10% Donkey Serum (S30, Millipore, Billerica, MA). Following another PBS wash, primary antibodies diluted in 10% donkey serum in PBS were applied for 60 min. at
room temperature in a humidified chamber. Primary antibodies were mouse monoclonal antibodies to phosphotyrosine (4G10, Millipore, 1:50), β1 integrin (MAB1981, Millipore, 1:100), and ICAM-1 (sc-107, Santa Cruz Biotechnology, Santa Cruz, CA, 1:50). After antibody exposure, slides were washed and CF™488A–conjugated donkey anti-mouse IgG (20014, Biotium, Hayward, CA) was applied at a 1:100 dilution in PBS as the secondary. After another 60 min. slides were rinsed with PBS and then treated with a 1:40 dilution of rhodamine phalloidin (00027, Biotium) followed by postfixing in 1% paraformaldehyde in PBS. Slides were then dried and sections were overlaid with Prolong Gold with DAPI (Invitrogen, Carlsbad, CA), cured overnight at room temperature, sealed with nail polish and stored at -20 C until viewing. Viewing was done on a Leica DM IRB fluorescence microscope (Leica, Bensheim, Germany) using a 40x HCX PL Fluotar lens and L5, N2.1, and A filter sets for CF488, rhodamine phalloidin, and DAPI respectively. Images were captured using an Olympus DP70 camera and DP controller software (Olympus, Center Valley, PA).

**Grading criteria for quantitation of thick section and ultrastructure morphology, immunofluorescence, and rhodamine phalloidin staining for actin**

Biopsy specimens processed for light microscopy of toluidine blue-stained thick sections and ultrastructure were analyzed by two of the authors (MAV and JMW) who were blinded to the clinical information from the patients during the evaluation.

**Thick section evaluation:** Single pre-clamp, end-clamp, and post-clamp slides for each case.

0 – Normal
1 – mild focal tubule epithelial swelling, occasional tubules with blebs.
2 – moderate to severe tubule epithelial swelling affecting a minority of tubules. Swollen cells exhibit rounded prominent and discrete organelles. Tubules with blebs, focal. Focally frayed brush borders.
3 – Severe tubule epithelial cell swelling affecting a majority of tubules. More extensive blebbing affecting a larger population of tubules than in (2). Frayed brush borders.
4 – More extensive and severe tubule epithelial cell swelling. Necrosis of epithelium affecting a minority of tubules. Severe blebbing and fraying of brush borders.
5 – Necrosis of a majority of tubules.

**Ultrastructure analysis:** 10-15 sets of EM photographs for each biopsy totaling >6000 images on 178 biopsies were reviewed.

**Parameters assessed:**

a) Reversible mitochondrial swelling proximal tubule (PT) vs. distal tubule (DT).
b) Mitochondrial condensation.
c) Mitochondrial permeability transition lethal swelling.
d) Brush border membrane (BBM) clubbing, fragmentation, thinning, discontinuities.
e) Apical membrane blebbing PT vs. DT.
f) Presence of free blebs in lumens.
g) Expansion of intercellular spaces – ± biopsy artifact since seen in pre-clamp biopsies.
h) Pale cytosol with otherwise intact structure – ± biopsy artifact since seen in pre-clamp biopsies.

**Composite Scale:**

0 - absolutely pristine.
1 - Minimal BBM discontinuity, apical membrane blebbing without shedding , mild mitochondrial swelling limited to DTs. Mild occasional intercellular expansion. Occasional pale cells.
2 - Moderate mitochondrial swelling in PTs, moderate to severe swelling DTs. Mitochondrial condensation. BBM fragmentation, thinning or discontinuities. Occasional luminal blebs.
3 - BBM thinning, fragmentation. Lumenal bleb casts. Uniform higher amplitude mitochondrial swelling in PTs and DTs, but with preservation of cristae and overall architecture. Changes present in any tubules, but not present in all.
4 - Stage 3 changes seen in every tubule.
5 - Presence of necrotic cells with large amplitude permeability transition-type mitochondrial swelling, plasma membrane disruption.

Quantitation of alterations of apical membrane integrity by rhodamine phalloidin staining for polymerized actin:
Parameters assessed were thinning, irregularity and absence of the brush border and apical membrane blebbing.
0 – No abnormalities in any tubules on the section
1 – <10% of tubules with any abnormalities. These were invariably mild
2 – 10-30% of tubules with abnormalities.
3 – 30-50% of tubules with abnormalities
4 – 50-70% of tubules with abnormalities
5 – >70% with abnormalities and/or substantial numbers of tubules with complete BBM loss, bleb casts

Semiquantitative analysis of immunofluorescence results for phosphotyrosine, β1 integrin, and ICAM-1 and of rhodamine phalloidin staining for actin:
End-Clamp and Post-Clamp sections were compared to the corresponding Pre-Clamp section and were assigned one of three categories: 1) Neither End-clamp nor Post-Clamp different from the corresponding Pre-Clamp, 2) End-Clamp different, 3) Post-Clamp different. Signal locations for each protein in normal tubules (Supplemental Figure 2) and types of changes seen in the End-Clamp and Post-Clamp samples for each parameter assessed as part of this analysis were:
1) Phosphotyrosine – This signal was basally located in most tubules, but was also seen in apical membranes of S3 segments of the proximal tubule. It became more heterogeneous in affected injury samples with loss of signal in some tubules, but intensification in others.
2) β1 integrin – Fluorescence was seen in the glomerular capillaries and basally in all tubule segments in pre-clamp samples. The abnormality seen was decreased signal intensity and was seen in only one of the Post-Clamp samples.
3) ICAM-1 – Fluorescence was seen in the glomeruli and peritubular capillaries in pre-clamp samples. The change in the End-Clamp and Post-Clamp samples was an increase in extent and intensity.
4) Actin – Fluorescence was intense in the apical brush border membrane of proximal tubules and was also present basally in proximal tubules and both basally and apically in distal segments of pre-clamp samples. The main changes in the end-clamp and post-clamp were in the proximal tubule brush border membrane as described above.

Statistical Analyses
For the statistical analyses, underlying data distributions were determined using the Shapiro-Wilk test. Parametric tests (t-test, paired t-test, ANOVA) were used for normally distributed data, non-parametric tests (Wilcoxon Rank Sum, Wilcoxon Signed Rank, Kruskal-Wallis) were used for data with skewed distributions, and categorical data were analyzed using a chi-square test.
To study the relationship of biomarkers to recovery time, vertical scatter plots were used to display the data at various time points post-surgery. Median values were plotted and differences at each time point from preoperative values were determined. Functional biomarkers (creatinine and serum cystatin C) ratios (24 hour to pre-operative) were plotted against ischemia time. To correlate urine and serum
biomarkers with functional changes and ischemia time, the ratios of biomarker peak values to preoperative values were calculated. These data were plotted with either the creatinine ratio (peak to preoperative values) or ischemia time on XY graphs. Peak ratios of structural biomarkers were also plotted against peak creatinine ratio. Linear regression analyses were used to fit lines to the data and an F statistic was calculated to determine if the slope of the regression line was significantly different from zero. Power analysis indicated that with a sample size of 40, we have 80% power to detect a moderate correlation of ± 0.43 or greater as compared to the null hypothesis of no correlation ($r=0.0$; two-sided test, alpha=0.05) and to detect a slope of ± 0.41 or greater as compared to the null hypothesis of slope=0 (two-sided test, alpha=0.05, assume standard deviation for both x and y=1).

To determine differences in morphology scores between the three sampling times, a paired analysis was done comparing pre- to end-clamp, pre- to post-clamp, and end- to post-clamp. A paired t-test was used to test for differences and a Bonferroni correction was used to adjust the p-values for multiple testing. Statistical analyses were performed using SAS ver. 9.2 (SAS Institute, Cary, NC) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). All tests were two-sided with significance set at $p<0.05$.

Reference List


### Supplemental Table 1. “One Kidney” AKIN Categorization

<table>
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<tr>
<th>Stages</th>
<th>Total Number of Evaluable Patients</th>
<th>24 hrs</th>
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<td>8</td>
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<tr>
<td>Stage 2 (&gt;1.5x)</td>
<td>4</td>
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<td></td>
</tr>
<tr>
<td>Stage 3 (&gt;2.0x)</td>
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<tr>
<td><strong>Total</strong></td>
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<td>9/38 = 24%</td>
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<th>Warm Ischemia</th>
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<td>7/26 = 27%</td>
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<tr>
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<td>Stage 3</td>
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<table>
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<th>Cold Ischemia</th>
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<tr>
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<td>0/2</td>
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*a. Creatinine criteria for each stage (fold increase vs preoperative or increment) are as indicated in first column. 2 of the 40 patients did not have sufficient serum creatinine data for this analysis.*
Supplemental Figure 1. Relationships between tumor size, ischemia time and clearance markers. A) tumor size vs. ischemia time, B) tumor size vs. ratio of creatinine at 24 hours to preoperative, C) tumor size vs. 24 hour cystatin C to preoperative.
Supplemental Figure 2. Appearance in pre-clamp samples of each of the parameters assessed by immunofluorescence. Left panels show the green fluorescent signals from the primary antibody and the CF$_{TM}$488A-conjugated secondary, middle panels are the red rhodamine phalloidin signals for polymerized actin, right panels are an overlay that also shows the DAPI-stained nuclei. Size marker 10 microns.
Supplemental Figure 3. Ultrastructure showing severe mitochondrial condensation during reflow. The changes shown here were found in small areas of 2 cases. The images are three magnifications from the same region. Size markers are: A) 10 microns, B) 2 microns, C) 0.5 microns.
Supplemental Figure 4. Additional examples of rhodamine phalloidin staining. Sets of three images from the pre-clamp, end-clamp, and post-clamp samples from one case. Size marker is 10 microns.
Supplemental Figure 5. Relationships between thick section scores, EM scores, ischemia time, and creatinine changes. A) Correlation between thick section and ultrastructure (EM) scoring including all points sampled. Overlapping points are not separated. 95% confidence intervals were 0.29 to 0.61 for $r$ and 0.47 to 0.97 for $\beta$. B) Relationships between ischemia time and ratio of peak serum creatinine to preoperative versus the pre-clamp and post-clamp thick section scores. Circles are warm ischemia. Triangles are cold ischemia.
Supplemental Figure 6. Urine creatinine concentrations preoperatively and at 2, 6, and 24 hours of recovery. Values are means±SEM. *P < 0.05 vs. preoperative (Pre) value.
Supplemental Figure 7. Urine biomarkers as absolute concentrations not factored for creatinine. Bars indicate medians. Pre, preoperative.
Supplemental Figure 8. Relationships between changes of serum creatinine and biomarkers. Serum creatinines and biomarker are ratios between the peak and preoperative (Pre-op) values.
Supplemental Figure 9. Relationships between creatinine-factored urine biomarkers and end-clamp thick section scores. Biomarker values are the ratios of the peak to preoperative values.
Supplemental Figure 10. Serum NGAL and urine biomarkers categorized according to whether patients were subjected to warm or cold ischemia during the clamping period. Values are means±SEM. None of the cold vs. warm differences were statistically significant.
Supplemental Figure 11. Behavior of biomarkers and structural parameters relative to ‘modified AKIN’ categorization. The ‘modified AKIN’ categorization is detailed in Appendix Table 1. Values are means±SEM for each parameter sorted according to whether patients had no AKI or met the ‘modified AKIN’ criteria at 24 hours or at ≥96 hours. S – serum, U – urine. Other abbreviations are as in the text. Scores for thick section morphology and ultrastructure are as defined in Methods. The other values are all ratios of the peak value measured to preoperative (Pre-op). --#-- indicates overall significant differences among the conditions (P<0.001 for creatinine and P=0.013 for thick section morphology). * indicates significantly different (P<0.05) from the corresponding “No AKI” group.
High Resolution Main Paper Figures
for
Tolerance of the Human Kidney to Isolated Controlled Ischemia
Figure 2
Pre-Clamp

Figure 3A
Figure 3B

End-Clamp
Post-Clamp

Figure 3C
Figure 4
Figure 9