Noninvasive evaluation of renal pH homeostasis after ischemia reperfusion injury by CEST-MRI

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Title

Noninvasive evaluation of renal pH homeostasis after ischemia-reperfusion injury by CEST-MRI

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Running Title

MRI-CEST pH mapping of acute kidney injury

Keywords

acute kidney injury, ischemia reperfusion injury, MRI, CEST, pH homeostasis

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ABBREVIATION USED

AKI   acute kidney injury
ATN   acute tubular necrosis
KIRI  kidney ischemia-reperfusion injury
sCr   serum creatinine
BUN   blood urea nitrogen
CEST  chemical exchange saturation transfer
ROI   Region of interest
H&E   hematoxylin and eosin
Abstract

Acute kidney injury (AKI) in mice caused by sustained ischemia followed by reperfusion is associated with acute tubular necrosis and renal dysfunctional blood flow. Despite the principal role of the kidney is the maintenance of acid-base balance, current imaging approaches are unable to assess this important parameter and clinical biomarkers are not robust enough in evaluating the severity of kidney damage. Therefore, novel noninvasive imaging approaches are needed to assess in vivo the acid-base homeostasis. This study investigates the usefulness of magnetic resonance imaging (MRI) - chemical exchange saturation transfer (CEST) pH imaging in characterizing moderate and severe AKI in mice following unilateral ischemia-reperfusion injury. Moderate ischemia (20 min) and severe ischemia (40 min) were induced in Balb/C mice that were imaged at 4 time points thereafter (days 0, 1, 2, 7). A significant increase of renal pH values was observed as early as one day after the ischemia-reperfusion damage for both moderate and severe ischemia. MRI-CEST pH imaging distinguished the evolution of moderate from severe AKI. A recovery of normal renal pH values was observed for moderate AKI, whereas a persisting renal pH increase was observed for severe AKI at day 7. Renal filtration fraction was significantly lower for clamped kidneys following impairment of glomerular filtration. Notably, renal pH values were significantly correlated with the histopathological score. In conclusion, MRI-CEST pH mapping is a valid tool for the noninvasive evaluation of both acid-base balance and renal filtration in patients with ischemia-reperfusion injury.
INTRODUCTION

Ischemic renal injury is a severe clinical problem in nephrology and the major cause of acute kidney injury (AKI).\textsuperscript{1} Hospitalized patients commonly experience AKI that is strongly associated with poor prognosis and high mortality.\textsuperscript{2} Moreover, patients may develop a progressive renal dysfunction even with initial recovery of renal function.\textsuperscript{3} Despite advances in clinical care and therapeutic interventions, morbidity and mortality remain high, largely due to the incomplete understanding of AKI pathophysiology and to the limitation of currently techniques for diagnosis. In fact, conventional biomarkers such as blood urea nitrogen (BUN) and serum creatinine (sCr) are unable to fulfil diagnostic criteria.\textsuperscript{4} BUN production is inconstant and can be altered by non renal factors, whereas sCr values become pathological if more than half of the glomerular filtration rate is lost. In addition, increases in blood concentrations become detectable only 24 hours after the occurrence of the pathological event.\textsuperscript{5} Therefore, the noninvasive evaluation and monitoring of renal function may allow a timely and definitive diagnosis of AKI.

Functional magnetic resonance imaging (MRI) provides several approaches, such as diffusion-weighted imaging,\textsuperscript{6} blood oxygen level dependent imaging,\textsuperscript{7} arterial spin labelling,\textsuperscript{8} longitudinal and transverse relaxation time measurements,\textsuperscript{9, 10} dynamic contrast enhanced imaging,\textsuperscript{11} hyperpolarized magnetic resonances spectroscopy,\textsuperscript{12} and sodium MRI,\textsuperscript{13} to assess renal function and pathophysiology beside anatomical information. All these MRI-based methods may be useful to determine renal pathology by quantification of renal water diffusion, oxygenation, perfusion, tissue water content and viability.\textsuperscript{14-16}

In the context of the different physiological parameters that one may image in vivo, renal pH appears of outmost relevance, since the kidneys hold the key role of regulating the acid-base balance. In fact, survival of animals is heavily dependent on the acid-base homeostasis, since changes in pH could have marked outcomes on biological processes at several levels (cellular, tissue and whole animal). As a consequence, the rapid decline in kidney function following AKI will result in derangements in acid-base homeostasis.\textsuperscript{17} Thus, imaging techniques able to report in vivo accurate pH measurements and longitudinal pH alterations would have a great clinical relevance not only for early identification of AKI but also for providing reliable predictions for further outcomes.
In recent years, the development of responsive MRI contrast agents that report on tissue pH have attracted considerable attention, owing to the altered production of acids that occur in several pathologies. Chemical exchange saturation transfer (CEST) is a novel MRI approach in which the contrast is generated by saturating specific exchangeable protons on either endogenous or exogenous molecules. Owing to the sensitivity of the proton exchange rate to a variety of chemical and physical factors, including pH, several CEST pH-responsive probes have been demonstrated to measure tissue pH. Among them, iopamidol is a clinical approved radiographic agent that was demonstrated to report accurate renal pH values, independently from the concentration, both at preclinical and clinical levels. Notably, altered renal pH values have been observed in a glycerol-induced murine model of ATN involving both kidneys by exploiting MRI-CEST pH mapping with iopamidol.

Considering that ischemia is the main aetiology in human AKI, and that pH may represent a good biomarker for its detection, it was deemed of interest whether MRI-CEST renal pH mapping can detect early renal damage and distinguish AKI recovery from irreversible damage in a unilateral kidney ischemia reperfusion injury (KIRI) model. The unilateral ischemia reperfusion injury model was used because of the advantage that only one kidney is damaged, whereas the contralateral kidney is not affected. Consequently, in this model of post-ischemic AKI, serum biomarkers are minimally affected, since the contralateral kidney can compensate for the reduced renal functionality. Moreover, as the entity of the damage is dependent on the time of ischemia, this model appears suitable to fairly reproducing different human conditions of post-ischemic damage evolution, since some patients may spontaneously recuperate normal renal functionality after few days, whereas for others the initial insult did not resolve and progressively evolves to the end-stage kidney disease. The aim of this study was to assess if MRI-CEST pH mapping can detect the early onset of acute kidney damage and distinguish between the recovery and persistence of the damage following unilateral ischemia reperfusion injury in mice.

**METHODS**

**Animal model**
BALB/c mice were purchased from Charles River Laboratories (Calco, Italy) and maintained in specific pathogen-free conditions. All animal experiments were approved by the University Ethical Committee and performed in accordance with the European guidelines under directive 2010/63. The model of normothermic unilateral renal ischemia reperfusion injury was performed as follows. Briefly, after anaesthesia with a mixture of tiletamine/zolazepam 20 mg/kg (Zoletil 100; Virbac, Milan, Italy) and 5 mg/kg xylazine (Rompun; Bayer, Milan, Italy) injected intramuscular, mice were submitted to a midline laparatomy to expose the left renal pedicle, then it was occluded with a vascular clamp for 20 min (n=14 mice) or 40 min (n=15 mice). For longitudinal MRI-examinations, n=6 animal with moderate AKI and n=7 animals with severe AKI were used. Some on the animals were killed on day1 (n=4 with 20 and 40 min KIRI) and on day 7 (n=4 with 20 and 40 min KIRI) for histology work up. Sham operation was performed on the right vascular pedicle for comparison (n=6 mice). Interventions were performed under a thermic lamp to maintain constant the mice body temperature. At the end of the ischemic injury mice were allowed to recovery and supplemented with 1 ml of sterile 0.9% NaCl solution administered subcutaneously. Animals were examined by MRI before and 1, 2 and 7 days after renal re-flow.

**In vivo magnetic resonance imaging studies**

*Chemical exchange saturation transfer pH imaging*

MRI images were acquired with a 7T MRI micro-imaging vertical scanner (Avance 300, Bruker Biospin, Germany). A 30 mm birdcage resonator was used for both transmission and receiving. During imaging, each mouse was anesthetized by injecting a mixture of tiletamine/zolazepam 20 mg/kg (Zoletil 100; Virbac, Milan, Italy) and 5 mg/kg xylazine (Rompun; Bayer, Milan, Italy). Respiratory rate was continuously monitored using a respiratory air pillow (SA Instruments, Stony Brook, NY; USA).

Kidneys were localized with axial and coronal T2-weighted images covering the whole kidneys. A high spatial resolution T2-weighted image (field of view of 3x3 cm, matrix of 256x256, slice thickness of 1.5mm, in-plane resolution of 117 µm) was acquired for placing the region of interests (ROI) for correct delineation of the whole kidney. A single-shot RARE sequence (TR = 6s, TE = 4.1ms, Rare Factor = 96, centric
encoding = 1) preceded by a 3µT cw presaturation pulse for 5 s and by a fat-suppression module was used to acquire Z-spectra. The CEST spectrum was acquired in the frequency offset range ±10 ppm with 37 offsets unevenly separated. An acquisition matrix of 96x96 was reconstructed to 128x128 with a field of view of 3x3 cm (in-plane spatial resolution = 234 µm) and a slice thickness of 1.5 mm. The acquisition time for a single MR-CEST spectrum was ca. 4 min. MR-CEST images were acquired before and after i.v. injection of iopamidol (dose = 1.0 g iodine / kg body weight).

**CEST analysis**

All CEST images were analyzed using a Matlab-based home-made script (The Mathworks, Inc., Natick, MA, USA). The Z-spectra were interpolated by cubic splines, B₀-shift corrected and saturation transfer efficiency (ST%) was measured by punctual analysis. A threshold value of 2% was set, based on the ΔST variations between multiple pre-contrast ST maps (ca. 1.2% at 4.3 - 4.4 ppm) to discriminate between enhancing and not-enhancing pixels. pH values were estimated *in vivo* by applying the ratiometric approach on manually-defined region of interests. The renal pH parametric maps were superimposed onto the anatomical reference image. Filtration fraction was calculated as the percent of detected pixels within the kidney region.

**Histology and Renal Function**

Following the completion of MRI experiments, mice were euthanized and kidneys were removed immediately for histological analysis. Sagittal sections of both kidneys were overnight fixed in 4% buffered formaldehyde solution. One experienced pathologist (JCC with more than 20 years of experience) evaluated the extent of renal damage as it was previously reported. Histological examination were performed on de-waxed 5 µm PAS stained sections to evaluate the following parameters of tubular cell injury: loss of brush border (partial or complete loss of the brush border), vacuolar degeneration (presence of more than three cells with cytoplasmic vacuoles or blebs formation protruding into the lumen of the tubules), tubular dilatation (dilatation was considered when the tubular lumen was increased at least 20% versus normal correspondent), cell detachment (presence of necrotic or morphological well preserved isolated cells in the
tubular lumen), necrosis (presence of three or more cells with signs of coagulative necrosis, such as loss of cell boundaries with marked eosinophilia or extreme swelling together with nuclear changes consisting in pyknosis or chromatolysis or karyorrhexis), and formation of casts (presence of granule-hyaline, mucous or proteinaceous material in the tubular lumen). Five non consecutive fields from the cortex and the outer medulla were evaluated (magnification 200x). Renal injury scores were determined by the percentage of tubules involved: 0 = 0; 1 = up to 10%; 2 = 11–20%; 3 = 21–40%; 4 = 41–60%; 5 = more than 61%. Overall histological score was obtained by summing all the individual parameters of injury.

Blood was collected to measure serum creatinine (sCr) and blood urea nitrogen (BUN) levels in SHAM and KIRI groups at different time points. In addition, urinary pH values were measured by collecting urine just after the MRI experiments.

**Statistical analysis**

Renal pH values were measured before and after ischemic reperfusion injury for both kidneys in each group. All values were expressed as mean ±SD unless otherwise stated. Analysis of variance (ANOVA) and Bonferroni’s multiple comparison test was employed to compare the differences between groups. The Pearson product moment correlation test was used to search for correlation between parameters. A value of P < 0.05 was considered statistically significant. The statistical analysis was performed by using the GraphPad Prism 5 program package (GraphPad Inc, San Diego, California, USA).

**RESULTS**

**Histological findings confirm reversible and irreversible renal damage**

Histological evaluation confirmed extensive injury in the clamped kidneys as soon as 1 day after the induced ischemia, whereas in the contralateral ones a negligible damage was observed (Figure 1A). Anyway, different morphological pattern of damage was observed after 20 and 40 min of ischemia. At one day of re-flow upon 40 min of ischemia, kidneys showed severe morphological alterations in comparison with the
kidneys undergone to 20 min of ischemia (Figure 1B). Most of the proximal convoluted tubules throughout
the major part of the outer medulla, exhibited a higher score of ATN consisting in loss of brush borders,
tubular dilatation, cast deposition, cell detachment and tubular necrosis, in contrast to kidneys submitted to
20 min of ischemia (Figure S1). Even seven days after I/R injury, still high scores of tubular damage was
observed for the 40 min clamped kidneys, whereas the 20 min ischemic injured kidneys showed practically a
full recovery of the normal morphology. In view of these results, we like to propose herein this model of
AKI as a well-defined condition of renal reversible and irreversible injury that can be followed by non-
invasive imaging approaches to assess single kidney loss of morphology and functionality.

**Serum biomarkers are not able to detect acute renal damage**

There were no major differences between sCr levels before and after 20 min of ischemia/reperfusion injury
in all animals (Figure 2A). There were no significant differences also for the BUN levels for the 20 min KIRI
group (Figure 2B). This may be attributable to the well-functioning contralateral kidney and to the reduced
damage in the clamped one, that overall indicates an unsuitability of serum biomarkers to assess the natural
progression of mild renal damage upon ischemia/reperfusion injury. The 40 min KIRI group showed a
moderate increase in both sCr and BUN levels at both one and seven days following reperfusion injury, with
statistically significance only after 7 days for the BUN levels (31.4 ±3.0 vs 19.9 ±2.2, P<0.01, for KIRI and
SHAM groups, respectively).

Urine pH values between animals with moderate AKI and SHAM were not significantly different at both day
1 and day 7 (Figure 2C). In the acute phase, at day 1 after severe AKI injury, urine pH values were higher
than the sham group (pH=5.8 ±0.3 vs 5.0 ±0.5). In contrast to the moderate AKI group, at day 7 after surgery
urine pH values in the severe AKI group were still increased. These results confirm the inability of current
biomarkers to assess the early onset of single kidney damage.

**CEST pH detects KIRI acute damage and recovery upon 20 min ischemia injury**
After moderate AKI, MRI-CEST pH mapping detected elevation of renal pH values in the clamped kidney indicating renal damage that was most pronounced at 1 day and 2 days post ischemia (Figure 3A). pH values were significantly higher at day 1 (pH=7.0 ±0.2, P<0.01) and at day 2 (pH=6.9 ±0.2, P<0.01) compared to baseline (pH=6.7 ±0.2) for clamped kidneys. A significant increase of the pH values was observed in comparison to contralateral control kidneys without AKI at day 1 (6.7 ±0.2 vs 7.0 ±0.2, P<0.05). No significant changes of pH values were observed in the contralateral kidney without AKI compared with baseline. Examples of T₂w anatomical images and corresponding pH maps at different time points after the moderate AKI are given in Figure 4A and 4B.

**CEST pH detects KIRI acute and persistent damage upon 40 min ischemia injury**

The pH values were increased after the severe AKI onset at day 1, with more conspicuous variations at day 2 (Figure 3B). At day 1, the pH values increased to 7.0 ±0.1 in comparison to baseline values (6.7 ±0.2, P<0.01) and peaked at day 2 (pH=7.0 ±0.1, P<0.01). In contrast to moderate AKI, the pH values remained significantly elevated until day 7 (pH=6.9 ±0.2, P<0.01). A significant difference between the moderate and severe AKI was observed at this time point (P<0.01). In the contralateral kidney without AKI, pH values were unchanged throughout the entire observation period with a slight increase of pH values at day1. Examples of T₂w anatomical images and pH maps of one animal with severe AKI are shown in Figure 4C and 4D.

**CEST renal filtration fraction distinguishes between kidney damage recover and progression**

Since the pH-responsive contrast agent is completely excreted by kidneys, with no secretion or reabsorption, its detection in renal compartments can be considered an indirect measurement of renal filtration and changes in detection may reflect alterations in the glomerular filtration. Significant impairment of renal filtration measured by means of the filtration fraction estimate compared with values before surgery was detectable since 1 day after moderate and severe AKI (Figure 5). For animals with moderate AKI, filtration fraction of clamped kidney (0.56 ±0.21) was found to be significantly lower (P<0.01) than that of contralateral kidney.
(0.84 ±0.16) at 1 day after injury. After moderate AKI, renal filtration nearly returned to baseline by day 7. After severe AKI, filtration impairment of clamped kidneys persisted until day 7 (0.57 ±0.05, 0.52 ±0.11 and 0.38 ±0.12 after 1, 2 and 7 days, respectively; P<0.01).

**Correlation of renal pH values with histological damage**

MRI-CEST derived pH values significantly correlated with histological scores (r=0.87, P<0.01; Figure 3C).

**DISCUSSION**

The herein reported results show, for the first time, that MRI-CEST pH mapping allows the noninvasive detection and monitoring of renal function impairment after unilateral ischemia-induced AKI. The derangement of pH regulation was correlated to the decay of renal filtration function and to the severity of the morphological damage.

In this study, CEST pH imaging revealed significantly higher renal pH values indicating renal function impairment, in the clamped kidneys compared with the control ones, even after 1 day, independently of the duration of the ischemic injury. At this time point serum biomarkers were unable to detect renal damage and, based on AKIN criteria, animals could not be classified as having AKI. On the other hand, histopathological damages at this time point were minor for the moderate AKI, with limited cast deposition and tubular dilatation (Figure S1). Therefore, our data would suggest that MRI-CEST pH mapping can be a promising tool for assessing single kidney injury and severity of AKI.

We already established and validated the MRI-CEST pH mapping methodology for assessing AKI in mice induced by glycerol injection. Here, the unilateral model was chosen without nephrectomy since the renal function of the contralateral kidney is not compromised, leading to a milder and minimally invasive damage to identify physiological changes earlier than elevation of serum creatinine. In addition, the contralateral non-ischemic kidney may be used as a self-internal control for each animal due to the absence of functional and morphological damages. Furthermore, ischemia times of 20 and 40 min result in different kidney time
course damage evolution, with shorter times that allows for recovery, whereas prolonged ischemia times evolve in end-stage kidney disease.\textsuperscript{34, 35}

In this study, renal pH-values of the injured kidney significantly increased after AKI relative to the contralateral one, at both the ischemia times. This might be related to the impaired regulation of acid-base balance. In fact, the systemic $\text{HCO}_3^-$ concentration is regulated by kidneys through the absorption of all the filtered $\text{HCO}_3^-$ and the production of new $\text{HCO}_3^-$ to replace that consumed by non-volatile acids upon protein metabolism.\textsuperscript{17} The production of new $\text{HCO}_3^-$ is actually done by net acid excretion. It follows that with the reduction in number of functioning nephrons, the capacity of the kidney to excrete acids into urine or reabsorb bicarbonate is compromised, hence resulting in an increase of renal pH values. In addition, elevated renal pH values occurred just 1 day after AKI and persisted until 1 week in the the severe AKI model. Moreover, renal pH evolution significantly correlated with histopathologic-based scores, with a recovery of the renal damage after 7 days for the moderately injured kidneys, whereas a persistent damage was observed for the prolonged ischemia time (40 min). Therefore, MRI-CEST pH mapping allowed the distinction of moderate from severe AKI.

Considering that functional renal impairment often precedes anatomical changes, imaging modalities are of interest owing to their capability of non-invasive and longitudinal assessment of renal function. Early functional changes have previously been investigated in several models of ischemia-reperfusion AKI following the administration of Gd-based contrast agents. Most of these studies addressed the quantification of renal perfusion and estimation of glomerular filtration rate both at preclinical and clinical levels.\textsuperscript{6, 36-39} DCE-MRI methods are less time-consuming and costly than methods based on the clearance of injected tracers, and do not involve radiation issues, as in radionuclide renography.\textsuperscript{40} All these methods exploit the reduced filtration function and changes in renal microvasculature to report on the ischemia reperfusion damage. Here we exploited a FDA-approved radiographic contrast agent that, sharing similar size and distribution/elimination properties with Gd-based extracellular fluid agents, allows indirect detection of reduced renal function. In fact, as a consequence of the decreased glomerular ability to filter the injected contrast agents, a marked decrease in the filtration fraction estimate was observed following the damage. Despite this simple estimate does not provide information on glomerular filtration rate or on renal blood
flow, it was found to be correlated with the severity and with the evolution of the kidney damage. In addition, proof-of-concept studies have demonstrated that iopamidol and other radiographic agents can also provide perfusion estimates.\textsuperscript{41, 42}

Nevertheless, a major limitation of all these approaches relies in the assessment of only one function of the kidneys that is their filtration capability, while the balance of the acid-base homeostasis has never been addressed. It is important to recognize that disordered acid-base homeostasis contributes to the progression of chronic kidney disease and to increased morbidity and mortality in dialyzed patients.\textsuperscript{43} Interestingly, chronic kidney disease progression can be tempered throughout normalization of acid–base homeostasis.\textsuperscript{44} It follows that the CEST-pH mapping approach may play a fundamental role in assessing pH changes in kidney-related pathologies and in monitoring treatment response. Notably, the combined evaluation of two main factors that report on renal dysfunction, i.e. filtration fraction and renal pH values, is feasible only within this approach.

A limitation of this study was the reduced kidney coverage by acquiring only the central slice. However, also MRI-based measurements of glomerular filtration rate are commonly acquired in few representative slices. Several approaches for increasing volume coverage within the MRI-CEST acquisition have been recently proposed.\textsuperscript{45, 46}

To conclude, we have demonstrated that MRI-CEST pH mapping can be a novel promising biomarker for diagnosis and monitoring of renal function, allowing an early detection of the occurrence of renal pathology and to distinguish moderate or severe AKI.

ACKNOWLEDGEMENTS

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REFERENCES


Figure legends

Figure 1. Renal histological features. (A) Histopathological damage score was assessed at baseline, and at 1 and 7 days after moderate (20 min) and severe (40 min) ischemia/reperfusion injury in mice. (B) Representative images of the outer external medulla region from H&E stained kidney sections of sham and after moderate or severe ischemia reperfusion injury. (Magnification 200x).

Figure 2. Clinical biomarkers of ischemia-reperfusion injury. Clinical biomarker levels of (A) serum creatinine, (B) blood urea nitrogen and (C) urinary pH values for sham and renal ischemia-reperfusion injured mice were assessed at baseline, and at 1 and 7 days after moderate (20 min) and severe (40 min) ischemia/reperfusion injury in mice.
Figure 3. MRI-CEST pH assessment of ischemia-reperfusion injury and evolution. (A) Bar graph showing renal pH values in clamped and contralateral kidneys before and after moderate ischemia (20 min) reperfusion injury at several time points. (B) Bar graph showing renal pH values in clamped and contralateral kidneys before and after severe ischemia (40 min) reperfusion injury at several time points. (C) Correlation of histological score and MRI-CEST based pH values in mice imaged after moderate and severe ischemia reperfusion injury at day 0, day 1 and day 7. Significant differences compared with the contralateral kidney without AKI at the same time point and with baseline (PRE) are indicated as */*, respectively. * = P<0.05, ** = P<0.01.

Figure 4. MRI-CEST pH mapping detects renal pH changes and regional distribution of damage after moderate and severe unilateral AKI. (A,C) Representative T2-weighted anatomical images before and after moderate (A) and severe (C) AKI at different time points (day 1, day 2 and day 7) showing clamped (right) and contralateral normal kidney (left). (B,D) Representative MRI-CEST pH maps overimposed onto anatomical images before and after moderate (B) and severe (D) AKI at different time points (day 1, day 2 and day 7) showing pronounced alkalinazation and reduced filtration (not coloured pixels within the renal region) of the pH-responsive contrast agent in clamped kidney in comparison to contralateral kidney.

Figure 5. MRI-CEST filtration fraction assessment of ischemia reperfusion injury and evolution. (A) Bar graph showing filtration fraction values in clamped and contralateral kidneys before and after moderate ischemia (20 min) reperfusion injury at several time points. (B) Bar graph showing renal filtration fraction values in clamped and contralateral kidneys before and after severe ischemia (40 min) reperfusion injury at several time points. Significant differences compared with the contralateral kidney without AKI at the same time point and with baseline (PRE) are indicated as */*, respectively. * = P<0.05, ** = P<0.01, *** = P<0.001.
Figure 1
Figure 2
Figure 3
**Figure S1.** Average histological scores of renal damage. Clamped and control kidneys are indicated in red and blue, respectively. Values are presented as the mean ± standard deviations.
A bar chart showing the comparison between control and clamped conditions across different time points: control PRE, clamped PRE, control 1DAY, clamped 1DAY, control 2DAY, clamped 2DAY, control 1W, clamped 1W. The y-axis represents the filtrating fraction, ranging from 0.0 to 1.0. The chart includes error bars, and statistical significance is indicated with asterisks: ** for significance at p < 0.01 and *** for p < 0.001. The chart title and axes labels are not included in the provided text.