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Research article

MFG-E8-derived peptide attenuates inflammation and injury after renal ischemia-reperfusion in mice

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ABSTRACT

Background: Renal ischemia-reperfusion (renal I/R) injury may lead to acute kidney injury (AKI). After renal I/R, proinflammatory mediators cause immune cell infiltration and further injury. Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) is a protein involved in cell-cell and cell-matrix interactions. MSP68 is an MFG-E8-derived peptide that inhibits neutrophil adhesion and migration. Here, we evaluated whether MSP68 attenuates renal I/R injury.

Materials and methods: Adult C57BL/6 mice were subjected to bilateral renal ischemia for 30 min followed by reperfusion and intraperitoneal administration of saline (vehicle) or MSP68 (5 mg/kg). Sham animals underwent laparotomy without renal I/R. The blood collected and studied for BUN, creatinine, and LDH by colorimetry. The kidneys were analyzed for IL-6 and TNFα by qPCR, ELISA, histological injury, and apoptosis by TUNEL.

Results: At 24 h after surgery, serum levels of BUN, creatinine, and LDH were markedly higher in vehicle-treated renal I/R mice than in sham mice, but significantly lower in MSP68-treated renal I/R mice. Similarly, compared to sham, renal levels of IL-6 mRNA and protein and TNFα protein were markedly higher in vehicle-treated renal I/R mice, but significantly lower in MSP68-treated renal I/R mice. Vehicle-treated renal I/R mice also had severe renal tubular histological injury, which was significantly lower in MSP68-treated renal I/R mice. Additionally, the kidneys of vehicle-treated renal I/R mice had a 93-fold increase in TUNEL-positive cells, which were reduced by 35% in mice treated with MSP68.

Conclusion: MSP68 has the potential to be developed as novel therapeutic agent for patients with AKI.

1. Introduction

Renal ischemia–reperfusion (renal I/R) injury is a common clinical complication of hypoperfusion states occurring in hypovolemic or septic shock, major cardiovascular and abdominal surgeries, renal transplantation, and severe burns [1]. Following reperfusion, kidney tissue damage occurs, causing the loss in renal function that clinically characterizes acute kidney injury (AKI) [2]. AKI occurs in a large number of hospitalized patients and is independently associated with an up to five-fold increase in mortality [3]. Additionally, AKI reduces the renal functional reserve. Hence, AKI is a significant contributor to the development of chronic kidney disease (CKD), which is associated with additional morbidity and healthcare costs [4, 5]. The current understanding of the pathophysiology of renal I/R is incomplete and, as such, no specific therapy has yet been developed to improve AKI secondary to renal hypoperfusion.

Renal I/R injury consists of a temporary impairment of blood flow resulting in tissue hypoxia followed by reperfusion leading to the initiation of inflammatory cascades and subsequent renal injury [6]. Due to the high metabolic rate, renal tubular epithelial cells are particularly vulnerable to the hypoxic insult causing them to undergo cell death and release DAMPS, which in turn activate immune cells to release proinflammatory cytokines and chemokines further amplifying the immune response [7]. Previous studies have demonstrated significant infiltration

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of neutrophils into the kidney following renal I/R, as well as a decrease in kidney inflammation when neutrophil infiltration is reduced [8]. Therefore, suppressing neutrophil infiltration may be a viable therapeutic option for AKI resulting from renal I/R injury.

Milk fat globule-epidermal growth factor-factor VIII (MFG-E8) is a secretory glycoprotein that was initially discovered in breast milk and mammary epithelial cells [9], and previously shown by our lab to attenuate inflammation and organ injury in a mouse renal I/R model [10]. MFG-E8 contains an arginine-glycine-aspartate (RGD) motif which binds integrins. Integrins on the surface of neutrophils and other leukocytes interact with ICAM-1 and VCAM-1 on endothelial cells as well as with fibronectin, vitronectin, collagen and other components of the extracellular matrix. In this way, integrins allow leukocytes to adhere to the post-capillary venular wall and migrate through the tissue parenchyma [11]. Accordingly, our lab and others have shown that MFG-E8 diminishes neutrophil infiltration in acute lung injury via its binding to $\alpha_v\beta_3$ integrin [12, 13].

In order to develop an oligopeptide capable of reducing leukocyte infiltration, we have generated MFG-E8-derived peptides and identified MFG-E8-derived short peptide 68 (MSP68) as a pentamer (VRGDV) containing the RGD motif and able to inhibit neutrophil adhesion to fibronectin and pulmonary artery endothelial cells (PAECs) *in vitro* and improve survival in sepsis [14]. We have also shown that MSP68 reduces the adhesion and migration of various other immune cells [15]. Given MSP68's ability to attenuate inflammation and organ injury in a murine model of sepsis, in this study we hypothesized that MSP68 could decrease inflammation and improve kidney injury in a murine model of renal I/R.

2. Materials and methods

2.1. Ethics approval

All experiments involving live animals were carried out in accordance with the National Institutes of Health guidelines for the use of experimental animals and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Feinstein Institutes for Medical Research.

2.2. Animal model of renal I/R injury

Adult male C57BL/6 wild-type (WT) mice (20–25 g) were housed in a temperature-controlled facility with a 12-h light cycle and fed a standard laboratory diet. The mice were randomly allocated to either the sham, renal I/R plus vehicle, or renal I/R plus treatment groups. Each animal was anesthetized with 2.5% inhaled isoflurane, positioned on a 38 °C heating pad, and the abdomen was shaved and prepped with 10% povidone-iodine. A midline incision was made and bowel was displaced to reveal renal hila bilaterally. Microvascular clips were applied to each renal pedicle (artery, vein, and ureter) and removed after 30 min. Upon visual confirmation of the reperfusion, mice were intraperitoneally instilled with 5 mg/kg filter-sterilized MSP68 peptide ($n = 7$) or an equivalent volume (100–150 μl) of vehicle (normal saline, $n = 5$). MSP68 (Genscript, Piscataway, NJ) is a pentapeptide (VRGDV) with a molecular weight of 545 g/mol. The abdomen was then closed in layers with suture, followed by subcutaneous administration of a 500-μl bolus of normal saline to compensate for the dehydration caused by the laparotomy. Sham animals underwent the exact same procedure, with the exceptions of pedicle clamping and intraperitoneal instillations. Twenty-four hours after reperfusion, animals were euthanized with carbon dioxide asphyxiation and the blood and renal tissues were collected.

2.3. Analysis of serum organ injury markers

Blood samples were centrifuged at 2,000 × *g* for 10 min to collect serum and then either analyzed for injury parameters immediately or stored at -80 °C. Blood urea nitrogen (BUN), creatinine, and lactate dehydrogenase (LDH) were measured by using commercial assay kits according to the manufacturer' instructions (Pointe Scientific; Lincoln Park, MI).

2.4. Assessment of cytokines

The protein and RNA measurements were conducted in tissue from the right kidney. Right kidney tissue was homogenized in lysis buffer (Tris-buffer saline, 1% Triton X-100, 50 mM EDTA, and 50 mM EGTA) containing protease inhibitor (Roche Diagnostics; Indianapolis, IN). Protein concentrations were determined by Bio-Rad protein assay reagent (Hercules, CA).

2.5. Quantitative real-time PCR analysis

Total RNA was extracted from renal tissue using TRIzol (Invitrogen; Carlsbad, CA) and underwent reverse-transcription into cDNA using reverse transcriptase (Applied Biosystems; Foster City, CA). The PCR reaction was performed in final volume of 20 μl containing 0.08 μmol of forward and reverse primer, 2 μl cDNA, and 10 μl SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems Step One Plus real-time PCR machine under the thermal profile of 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Mouse β-actin was used for normalization. Relative expression of mRNA was represented as fold change using the $2^{-\Delta\Delta Ct}$ method with the sham group as comparison. The primer sequences were: IL-6 Forward, CCGGAGAGGAGACTTCACAG; IL-6 Reverse, CAGAATTGCCATTGCACAAC; β-actin Forward, CGTGAAAAGATGACCCAGATCA; β-actin Reverse: TGGTACGACCAGAGGCATACAG.

2.6. Histological evaluation of renal injury and immunohistochemistry

The left kidney was fixed in 10% formalin and embedded in paraffin. Tissue was sectioned into 5-μm slices then stained with hematoxylin and eosin (H&E). Using a light microscope, the tissue was examined at 200× magnification and the degree of injury at the corticomedullary junction was assessed in a blinded fashion using the following morphological parameters: dilatation of Bowman's space, tubular epithelial flattening, interstitial inflammation, loss of tubular brush borders, and cast formation. Each parameter was scored either 0 (0% injury), 1 (<10%), 2 (10%–25%), 3 (26%–75%), or 4 (>75%). Scores were averaged for each sample over 5 randomly selected fields. Although brush border is best evaluated using the periodic acid-Schiff staining, the brush border can also be evaluated with the H&E staining, which stains intact brush borders in tubular lumen as pink.

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The number of apoptotic cells present in renal tissue sections was measured by in situ labeling of DNA fragmentation using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (Roche Diagnostics). The slides were then counterstained with 49,6-diamidino-29-phenylindole dihydrochloride (DAPI) and visualized using a fluorescent microscope (Zeiss Apotome).

2.8. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). After verification of normal distribution, data were compared via one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test for multiple group comparisons. Differences between the experimental groups reaching a *p-value* of 0.05 or less were considered statistically significant.

3. Results

3.1. MSP68 reduces serum levels of renal injury markers after renal I/R

We first examined the ability of MSP68 to improve kidney injury as determined by the serum levels of BUN, creatinine, and LDH at 24 h after surgery. Compared with sham, the levels of BUN, creatinine, and LDH in renal I/R mice treated with vehicle increased significantly by 12.1-, 4.8-, and 11.5-fold, respectively (Figure 1A-C). In renal I/R mice treated with MSP68, however, the levels of BUN, creatinine, and LDH were 28.4%, 35.0%, and 29.7%, lower than those in vehicle mice, respectively (Figs. 1A-C). These results indicate that treatment with MSP68 can attenuate renal function as measured by BUN and creatinine and cell damage as indicated by LDH after renal I/R.

3.2. MSP68 decreases kidney inflammation after renal I/R

We next assessed for evidence of decreased inflammation in the kidneys of MSP68-treated renal I/R animals. IL-6 mRNA expression was strongly induced in the kidneys of vehicle-treated renal I/R mice with a 137.3-fold increase as compared to the sham group (Figure 2A). Renal I/R mice treated with MSP68, however, had a significant 54.5% reduction in the IL-6 mRNA expression as compared to vehicle-treated mice (Figure 2A). At the protein level, renal IL-6 was 2.5-fold higher in the vehicle-treated renal I/R mice compared to sham animals. MSP68 decreased this level by 15.2% compared to the vehicle group (Figure 2B). Similar to IL-6, the renal levels of TNF-α protein were 2.8-fold higher in the vehicle-treated renal I/R mice compared to sham laparotomy animals. MSP68 decreased the renal level of TNF-α by 11.7% compared to the vehicle group (Figure 2C). Together, these data indicate that MSP68 decreases renal IL-6 and TNFα levels after renal I/R.

3.3. MSP68 attenuates kidney histological injury after renal I/R

We next compared the structural integrity of the kidney in mice subjected to sham surgery or renal I/R and treated with either vehicle or with MSP68 by histological evaluation using hematoxylin and eosin (H&E) staining. H&E staining of the kidneys of mice that underwent sham laparotomy exhibited normal histological architecture (Figure 3A). The kidneys of mice subjected to renal I/R, however, exhibited varying degrees of injury (Figure 3B, C). The average histologic injury score at the corticomedullary junction of vehicle-treated renal I/R mice was elevated (3.1 out of a maximum of 4.0 points), but in mice treated with MSP68 this score was significantly reduced by 38.3% (Figure 3D). These results show that MSP68 significantly attenuates renal tubular injury after renal I/R.

3.4. MSP68 is associated with a reduction in the number of TUNEL-positive cells after renal I/R

After renal I/R, numerous TUNEL-positive cells are typically observed in the renal tubules and parenchyma. Since apoptosis is a consequence of kidney injury and inflammation, we evaluated the effects of MSP68 on the number of TUNEL-positive cells after renal I/R. At 24 h after surgery, the kidneys of renal I/R mice treated with vehicle had a 92.7-fold increase in the number of TUNEL-positive cells, compared with those of sham mice (Figs. 4A, B). The kidneys of renal I/R mice treated with MSP68, however, had 34.9% less TUNEL-positive cells compared with the vehicle group (Figs. 4C, D). These data indicate that MSP68 significantly attenuated the induction of apoptosis in the kidneys of mice subjected to renal I/R.

4. Discussion

Most cases of AKI are the result of ischemic insult due to decreased renal blood perfusion caused by trauma-hemorrhage, sepsis, or surgical complications, which can be recreated using the murine renal I/R injury model [16]. In this study, we demonstrated the ability of a small peptide derived from MFG-E8 to mitigate kidney injury after renal I/R. MSP68 improved kidney function as measured by serum BUN and creatinine, as well as decreased cellular injury as measured by LDH. Additionally, MSP68 decreased the kidney levels of the pro-inflammatory cytokine IL-6. Furthermore, MSP68 protected mouse kidneys from histological injury and reduced apoptosis.

We have previously shown that MSP68 has the ability to impair neutrophil adhesion to human fibronectin and PAECs in vitro, as well as decrease the infiltration of neutrophils to the liver and lung in mice that underwent cecal ligation and puncture (CLP) [14]. Therefore, we inferred that MSP68 might be able to attenuate organ damage and inflammation in a murine model of renal I/R injury. The role of neutrophils in renal I/R has previously been studied. Kelly *et al.* have shown that neutrophil depletion prior to renal I/R confers significant improvement in kidney damage [17]. Recent studies have re-examined the role of neutrophils in renal I/R suggesting the formation of neutrophil extracellular traps (NETs) may play a significant role in further inducing tissue damage [18]. Therefore, inhibiting neutrophil infiltration to protect from tissue damage is a sensible strategy.

Treatment with MSP68 after renal I/R resulted in relative preservation of renal function as compared to vehicle-treated mice as shown by improvement in serum creatinine, BUN, and LDH. Clinically, creatinine is one of the most common parameters used to assess kidney function [19].

Renal I/R triggers the synthesis and release of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF-α) and chemokines, leading to activation of the innate immune system [20]. Therefore, we used PCR and ELISA to analyze the inflammatory response in post-ischemic kidneys. Our results showed significant renal upregulation of IL-6 mRNA and IL-6 protein in the vehicle group with significant improvement in the MSP68-treated group after 24 h of reperfusion. We also examined IL-1β and TNF-α gene regulation but failed to show significant upregulation in renal I/R mice compared to the sham group, which we attributed to the time point of analysis. Consistent with our conjecture regarding the importance of timing, renal protein levels of TNF-α were indeed elevated in the vehicle group as compared to the sham group. MSP68-treatment significantly reduced renal TNF-α protein levels.

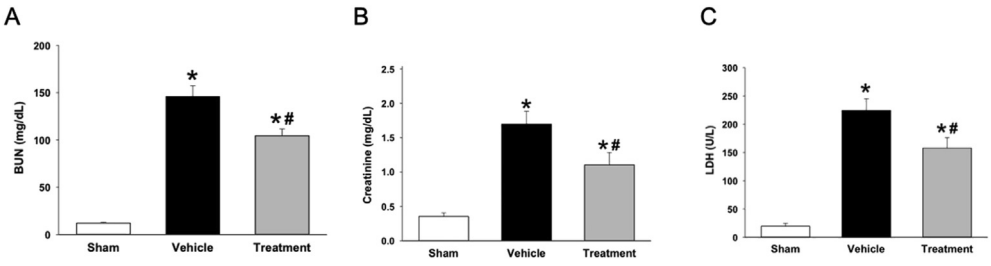

Figure 1. MSP68 reduces kidney injury markers after renal I/R. At 24 h after surgery, renal I/R caused a marked increase in the serum levels of (A) creatinine, (B) blood urea nitrogen (BUN), and (C) lactate dehydrogenase (LDH). The increase was significantly reduced in mice treated with MSP68. *Mean ± SEM (sham, n = 5 mice; renal I/R, n = 7 mice/group); *p < 0.05 vs. sham; #p < 0.05 vs. vehicle; one-way ANOVA plus SNK.*

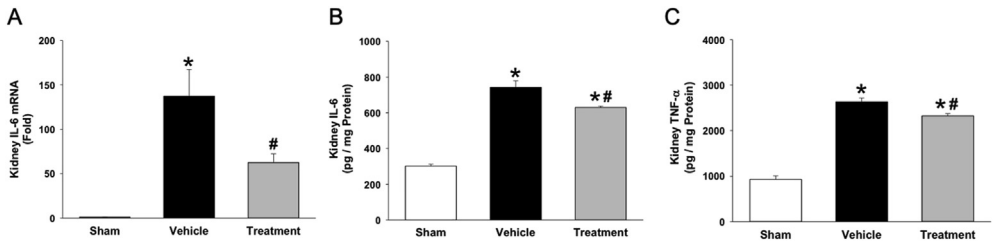

Figure 2. MSP68 decreases kidney inflammation after renal I/R. At 24 h after surgery, renal I/R caused a marked increase in the kidney levels of IL-6 (A) mRNA and (B) protein, and in the kidney levels of (C) TNF-α protein. These increases were significantly reduced in mice treated with MSP68. *Mean ± SEM (sham, n = 5 mice; renal I/R, n = 7 mice/group); *p < 0.05 vs. sham; #p < 0.05 vs. vehicle; one-way ANOVA plus SNK.*

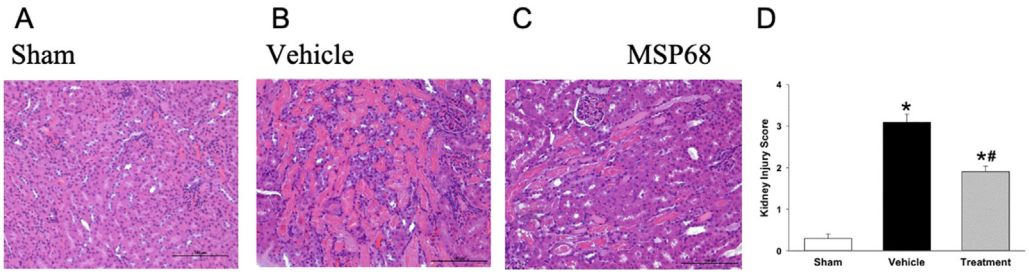

Figure 3. MSP68 attenuates kidney histological injury after renal I/R. At 24 h after surgery, (A) sham mice exhibited normal kidney histological architecture. (B) Renal I/R mice treated with vehicle had significant renal histological injury, as indicated by tubular epithelial flattening, interstitial inflammation, loss of tubular brush borders, and cast formation. (C) Renal I/R mice treated with MSP68 had a reduction in the renal histological injury. *Representative images of tissue sections with H&E staining at an original magnification of 200×.* (D) Semiquantitative score of experimental groups. *Histologic injury score in each group was graded blindly; average of five corticomedullary junction fields for each experimental group at 200 × magnification. Mean ± SEM (sham, n = 5 mice; renal I/R, n = 7 mice/group); *p < 0.05 vs. sham; #p < 0.05 vs. vehicle; one-way ANOVA plus SNK.*

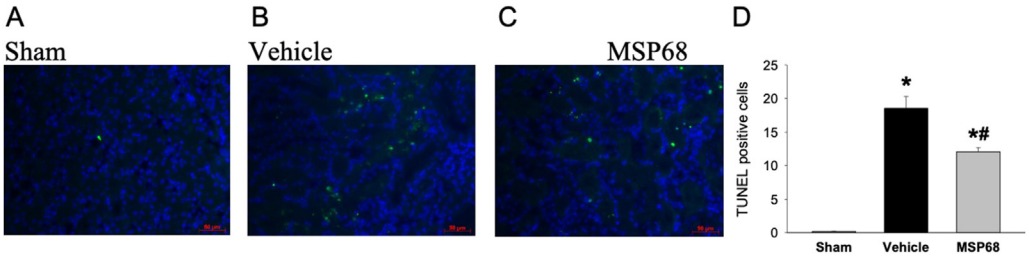

Figure 4. MSP68 is associated with a reduction in the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells after renal I/R. At 24 h after surgery, (A) sham mice exhibited rare TUNEL-positive cells. (B) Renal I/R mice treated with vehicle had a significant increase in the numbed of kidney TUNEL-positive cells. (C) Compared with the vehicle group, renal I/R mice treated with MSP68 had significantly less kidney TUNEL-positive cells. *Representative images of tissue sections with TUNEL staining at an original magnification of 200×.* (D) Semiquantitative score of experimental groups. *The number of TUNEL cells in each group was graded blindly; average of five corticomedullary junction fields for each experimental group at 200 × magnification. Mean ± SEM (sham, n = 5 mice; renal I/R, n = 7 mice/group); *p < 0.05 vs. sham; #p < 0.05 vs. vehicle; one-way sANOVA plus SNK.*

In order to assess renal damage after renal I/R, renal tissue was examined histologically. There was a significant decrease in tissue damage in the MSP68-treated group as compared to the vehicle group. Analysis was performed by assessing the corticomedullary junction using a modified previously described grading scheme [17].

Schumer *et al.* have previously shown that even brief periods of renal arterial clamping can induce significant apoptosis throughout a 48 h reperfusion window [21]. Furthermore, the inhibition of apoptosis has previously been shown by Daemen *et al.* to attenuate inflammation in post-ischemic kidneys [22]. Since MFG-E8 promotes the clearance of

apoptotic and other dying cells [12], we utilized the TUNEL assay to evaluate whether MSP68 was also able to reduce the number of apoptotic and other dying cells in renal tissue sections. In this study, we found that MSP68 treatment significantly decreased the number of TUNEL-positive cells in renal I/R.

Our study has some limitations that require consideration. MSP68 was administered intraperitoneally, which may have reduced its renal concentration due to poor absorption from the serosa to the circulation or first passage metabolism by the liver, although to our knowledge these confounding effects have not been observed with other oligopeptides. Moreover, these would be conservative biases, as we did observe reno-protective effects in renal I/R mice treated with MSP68. We used as a control saline, and not a scrambled peptide. We avoided using a scrambled peptide because it might also have confounding biological activity. Moreover, considering a typical peptide half-life in the circulation of minutes and the small amount of MSP68 administered (125 μg reflecting 230 nmol in a 25-g mouse), we deemed MSP68's colloid osmotic effects too small to be able to influence the blood's normal osmotic pressure of ~300 mOsm/L and thus not requiring the use of a peptide control. Ischemia/reperfusion-induced changes in the renal function were crudely estimated using the clinically used biomarkers creatinine and BUN rather than the more reliable creatinine clearance. The creatinine clearance, however, requires collection of the 24-h urinary output, which is technically challenging in the mouse. Besides, all mice received volume resuscitation with 1 ml saline solution, so it is unlikely that the mice would have become sufficiently dehydrated to increase the creatinine and BUN. While LDH is not per se a renal injury marker, it is a commonly used as tissue injury marker. Considering that LDH was normal in the sham mice and elevated in the renal I/R mice, the logical explanation is that renal I/R injury caused injured and dying renal cells to release LDH. Other renal injury markers such as KIM-1 and NGAL are more sensitive and are able to indicate renal injury before creatinine and BUN become elevated. In our study, however, there was sufficient time after renal injury to result in elevated blood levels of creatinine and BUN. Regretfully, we cannot compare the effects of MSP68 in the cortex and medulla because only the corticomedullary junction region was examined. While tubular epithelial cells are known to undergo apoptosis after renal I/R injury, we cannot ascertain the cell type because we did not conduct cell-specific staining. A final limitation is that we did not evaluate the mechanism of action accounting for MSP68's renoprotection.

In summary, renal injury after renal I/R remains a significant clinical and economic challenge. Acute kidney injury has been shown to increase both hospitalization length of stay and the cost of hospitalization. MSP68 represents a possible novel therapeutic to be further developed for treating patients with AKI.

Declarations

Author contribution statement

J. Last: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Brenner and N. Denning: Analyzed and interpreted the data; Wrote the paper.

H. Yen: Performed the experiments.

M. Aziz and P. Wang: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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