

SCM-198 Can Regulate Autophagy Through the Bax/Bcl-2/TLR4 Pathway to Alleviate Renal Ischemia-Reperfusion Injury

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Abstract

Renal ischemia-reperfusion (I/R) injury is frequently observed in several clinical cases. In this study, we want to investigate that SCM-198 attenuates renal injury in the renal I/R model and find out the possible mechanisms. Wistar albino 40 male rats were classified into four groups (n=10): control, DMSO, I/R, and SCM-198 30 mg/kg. In the group 4, SCM-198 was administered intraperitoneally once at the doses of 30 mg/kg following the reperfusion. Glomerular associated proteins (PCX), tubular damage factors (NGAL, KIM-1), blood urea nitrogen (BUN), serum creatinine, inflammatory cytokines (IL-1 β , IL-18, and TNF- α), Bax/Bcl-2, TLR4, LC3B, and Beclin-1 were evaluated. SCM-198 played an essential role in mitigating kidney damage. SCM-198 alleviated tubular damage and decreased IL-1 β , IL-18, and TNF- α levels. SCM-198 reduced the apoptosis marker Bax/Bcl-2 ratio, immune system protein TLR4, and autophagy proteins LC3B and Beclin-1. In brief, our results support the notion that SCM-198 has protective effects on I/R-induced renal injury. SCM-198 therapy may be a new alternative for the prevention and treatment of renal I/R injury.

Keywords: SCM-198, Renal ischemia-reperfusion, Renal injury, Inflammatory cytokines, Autophagy, Bax, Bcl-2

Introduction

Renal ischemia-reperfusion (I/R) injury ranks among the major reasons of acute kidney injury (AKI) (1). AKI is a syndrome includes sudden and rapid loss of renal function. Renal injury impairs homeostasis leading to waste product accumulation (2-4). The incidence of AKI is increasing each year, independent of age or gender. 5-10% of the hospitalized patients and about 30% of the critically ill patients suffer from AKI. Patients with AKI can make a full recovery, but AKI; is a significant risk factor that may result in end-stage renal disease (ESRD) or chronic kidney disease (CKD) (5). Among the pathophysiological mechanisms of I/R injury, complex processes including high reactive oxygen species (ROS) levels, reduction of ATP, Na⁺-K⁺ ATPase dysfunction in the cell membrane, Ca²⁺ overload, mitochondrial dysfunction, apoptosis and cell necrosis (6, 7), cell membrane damage, and autophagy (8) are involved.

Leonurin, also called SCM-198 (4-guanidino-n-butyl syringate-C₁₄H₂₁N₃O₅), is a bioactive alkaloid extracted from *Herba leonuri* (also known as mother weed) and it is a member of the *Labiatae* family (9, 10). Previous research has revealed that SCM-198 can affect various biological processes, including oxidative stress, inflammation reaction, angiogenesis, apoptosis, and autophagy, in many different in vivo and in vitro studies (9, 11-15). However, the effect and underlying mechanisms of SCM-198 on renal I/R are still not fully understood. Therefore, we wanted to examine the effects of 30 mg/kg SCM-198 administration during bilateral renal I/R in this study.

Materials and Methods

Drugs

SCM-198 (catalog no: HY-N0741, MedChem Express, New Jersey, USA) was dissolved

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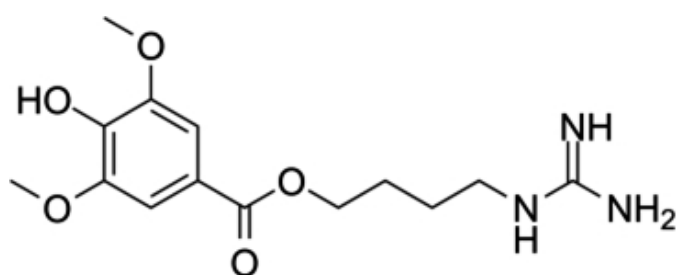


Figure 1. SCM-198 chemical structure.

in 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA).

I/R Model

In the current research, 40 healthy, 12-week-old, male Wistar albino male rats were weighed (240-260 g) and planned as 4 groups (n=10). Rats were purchased from Atatürk University Medical Experimental Application Research Center (Erzurum, Turkey). All experiments were carried out with permission from Atatürk University Animal Experiments Local Ethics Committee (protocol number: 17.09.2018-174).

Rats were anesthetized via Ketamine 75 mg/kg (Pfizer, Istanbul, Turkey) and Xylazine 8 mg/kg (Rompun™, Bayer, Turkey). In the I/R group, the renal pedicles were clamped bilaterally with microvascular clamps by reaching the retroperitoneal region from the back regions in the lateral position, and 60 minutes of ischemia was initiated. Afterward, clamps were removed from the renal pedicles, the incision areas of the animals were closed with 3.0 silk suture (Dogsan, Istanbul, Turkey), and 24-hour reperfusion was initiated (16, 17). In the control group, the renal pedicles were reached but not clamped. In the DMSO group, the kidneys were reached by incision as in the control group, and DMSO was applied. In the SCM-198 group, in addition to the procedures performed in the I/R group, 30 mg/kg SCM-198 was administered intraperitoneally (i.p.) 5 minutes before reperfusion. After the I/R process was completed, the rats were sacrificed, renal tissue and blood samples were taken.

Collection and Storage of Tissue Samples

The blood collected from the rats was centrifuged at 3,500 rpm for 15 minutes without wasting any time, and serum was obtained. Urea and creatinine (Cre) levels were measured from the serum. Renal tissues were divided into two parts at the point where the renal pedicle enters the kidney. One part was used in the preparation of the kidney homogenate. The kidneys were weighed and homogenized at a ratio of 1:20 in 0.1 M potassium phosphate buffer solution (PBS, pH 7.4). The homogenates were then centrifuged at 5,000 g for 20 minutes at 4°C. Supernatants were stored at -80°C in pre-labeled microcentrifuge tubes until they were used in biochemical analysis. Protein determination; the protein concentration were determined by

using the Lowry method using a commercial total protein kit (Sigma Aldrichs, St. Louis, MO) (18). The other kidney piece was fixed in 10% formalin. Then, the samples were embedded in paraffin. Following the paraffinization, the samples were sectioned and stained with hematoxylin and eosin to evaluate the histological changes.

Urea and Creatine Measurement

Serum Urea (catalog no: STA-382 Cell Biolabs, Inc San Diego, USA) and Cre (catalog no: E-EL-R0058 Wuhan, China) concentrations were measured using an ELISA reader (BioTEK Powerwave XS Winooski, UK).

Biochemical Analysis

Tumor necrosis factor- α (TNF- α) (catalog no: E-EL-R0019, Elabscience, Wuhan, China), interleukin-1 β (IL-1 β) (catalog no: E-EL-R0012, Elabscience, Wuhan, China), interleukin-18 (IL-18) (catalog no: E-EL-R0567, Elabscience, Wuhan, China), kidney injury molecule-1 (KIM-1) (catalog no: E-EL-R0575, Elabscience, Wuhan, China), neutrophil gelatinase-associated lipocalin (NGAL) (catalog no: E-EL-R0662, Elabscience, Wuhan, China), and podocalyxin (PCX) (catalog no: E-EL-R0764, Elabscience, Wuhan, China) levels were determined via ELISA reader (BioTEK Powerwave XS Winooski, UK) in kidney tissues. All measurements were performed adhering to the kit manufacturer's protocol.

Immunohistochemical (IHC) Method

Renal tissues of the rats were determined in 10% neutral formalin solution. Tissues were taken into paraffin blocks after routine alcohol-xylol follow-up. Sections of 5 μ m taken on polylysine slides were exposed to the xylol and alcohol series, and washed with PBS. The samples were kept in 3% H₂O₂ for 10 minutes to inactivate endogenous peroxidase. The antigen was treated with the retrieval solution at 500 watts for 2x5 minutes to reveal the antigen in the tissues. Later, tissues were incubated with LC3B (ABclonal, catalog no: ab15191, 1/200 dilution ratio), TLR4 (Santa Cruz, catalog no: 293072, 1/200 dilution ratio), Bcl-2-associated X protein (Bax) (Bioss, catalog no: bs-0127R, 1/200 dilution ratio), B-cell lymphoma 2 (Bcl-2) (Bioss, catalog no: bs-0032R, 1/200 dilution rate), and Beclin-1 (Bioss, catalog no: bs-1353R, 1/200 dilution ratio) primary antibodies at the room temperature for 20 minutes. Mouse and Rabbit Specific HRP/DAB IHC Detection Kit-Micro-polymer kit (Abcam, catalog no: ab236466) was used as the secondary antibody suggested by the manufacturer. DAB (3,3'-Diaminobenzidine) was used as chromogen. After contrast painting with Mayer's Hematoxylin, it was covered with entellan and examined under a light microscope. Immunopositivity in kidney tissues was evaluated as none (-), mild (+), moderate (++), and severe (+++).

Statistical analysis

The obtained data were analyzed with SPSS v.20 (IBM, Chicago, USA) software. In histopathological evaluations, the differ-

ence between the groups was determined by Kruskal Wallis, and the Mann-Whitney U test determined the group that made the difference. In biochemical analyses, One-way ANOVA was used to analyze the differences between groups, and then Duncan's post hoc test was used for multiple comparisons. All data are expressed as mean $\pm$ SE. $P < 0.05$ was accepted statistically significant.

Results

Serum Cre and Urea Levels

Serum Cre and Urea levels elevated in the I/R group compared to the control and DMSO groups. In the group that received SCM-198 treatment, the levels of these parameters decreased

cantly in the control and DMSO groups, Bcl-2 immunopositivity was moderate (Figures 5, 6, 7, 8, 9). While LC3B, TLR4, Bax, Beclin-1 immunopositivity was found to be moderate in the I/R group, Bcl-2 immunopositivity could not be determined. In the SCM-198 group, LC3B, TLR4, Bax, Bcl-2, and Beclin-1 immunopositivity was mild.

Discussion

In the renal I/R model, the levels of mediators indicating renal injury, such as blood urea nitrogen (BUN), Cre, KIM-1, NGAL (19), and Cystatin-C (20), are increased. While BUN and Cre provide information about tubular damage and glomerular filtration, KIM-1 is produced in proximal tubule cells

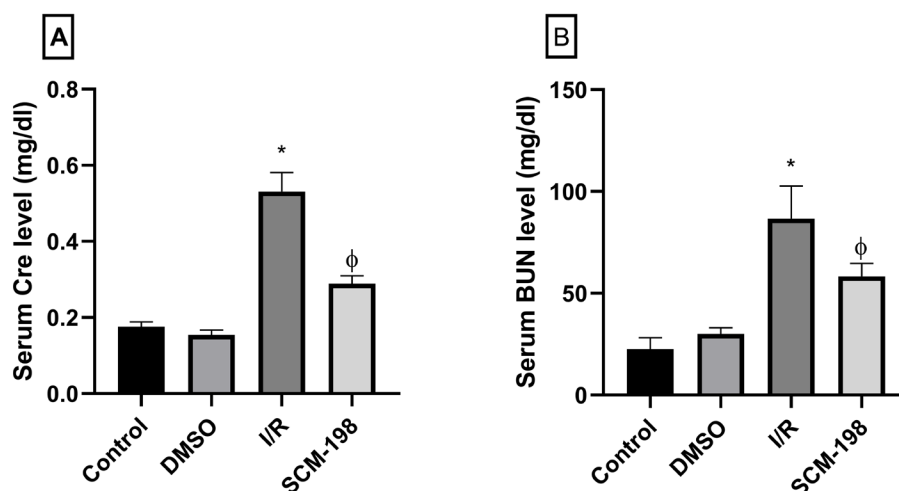


Figure 2. Effects of SCM-198 on kidney functions. (A) Urea and (B) Cre levels. * $P < 0.05$ compared to the control and DMSO groups, $^{\phi}P < 0.05$ compared to the I/R group.

(figure 2).

Inflammatory Cytokine Levels

ELISA test was used to measure the inflammatory cytokines. IL-1 β , TNF- α , and IL-18 levels were found to be high in the I/R group. TNF- α , IL-18, and IL-1 β levels declined significantly in the SCM-198 group (Figures 3A, 3B, and 3C). In the light of all data, inflammation that increased in renal I/R injury was reduced with SCM-198 administration.

Kidney Damage Biomarkers Levels

The levels of kidney damage markers (NGAL, KIM-1, and PCX) were significantly higher in the I/R group than in the control and DMSO groups. These levels were significantly lower in the SCM-198 group than in the I/R group (figure 4).

Immunohistochemical (IHC) Staining

We detected a significant difference among the groups regarding TLR4, Bax, Bcl-2, LC3B, and Beclin-1 immunopositivity in the renal tissues (Figure 10, $p < 0.05$). While LC3B, TLR4, Bax, Beclin-1 immunopositivity could not be determined signifi-

(21, 22). NGAL is expressed in the intercalated cells of the collecting channel and thick arm of the loop of Henle (23, 24). Approaching normal levels of BUN, KIM-1, Cre, and NGAL indicate reduced renal injury (19, 20, 25). Additionally, PCX is a podocyte-specific marker and membrane protein involved in regulating glomerular filtration (26). We have not previously encountered a renal I/R study in which PCX levels were investigated. Still, some evidence has been presented showing that changes in PCX levels are associated with renal injury (27, 28). We showed that PCX levels increased after our bilateral I/R model. We think that the probable cause of this increase in PCX levels may be to improve the decreased filtration after bilateral I/R. We found significant improvements in the levels of parameters indicating renal injury in the SCM-198 group.

Toll-like receptor (TLR) is a transmembrane protein structurally produced in renal cells. TLRs fall into two categories: those that are localized to the cell membrane (TLR-1, 2, 4, 5, 6, 10, 11, 12, and 13) and those that are located inside the cell (TLR-3, 7, 8, and 9) (29). TLR-4, one of the family members, is structurally expressed in the renal epithelium, and its expression increases in the renal I/R (30, 31). The activation of the

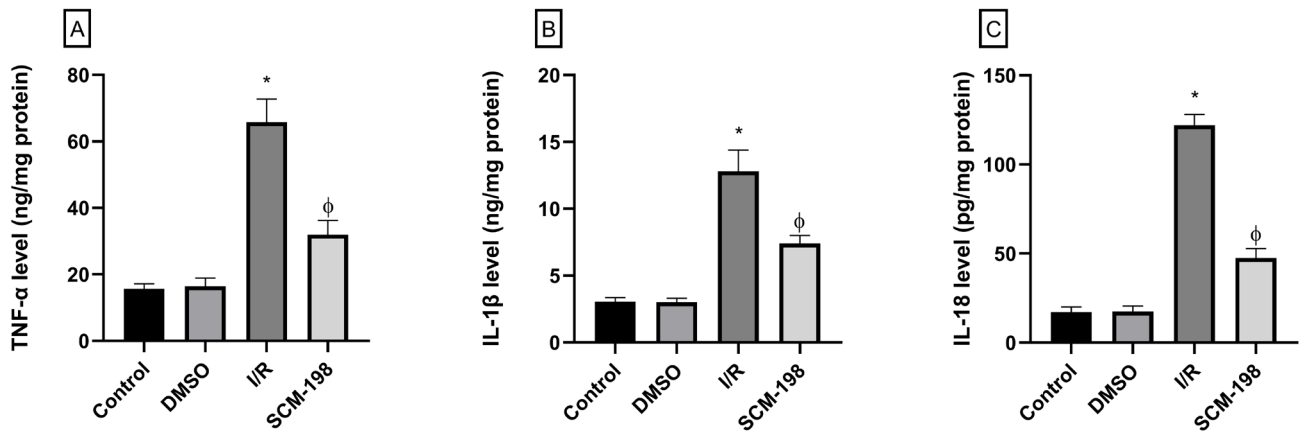


Figure 3. Effects of SCM-198 on inflammatory cytokines. (A) TNF- α , (B) IL-1 β and (C) IL-18 levels. * $P < 0.05$ compared to control and DMSO groups, $^{\phi}P < 0.05$ compared to I/R group.

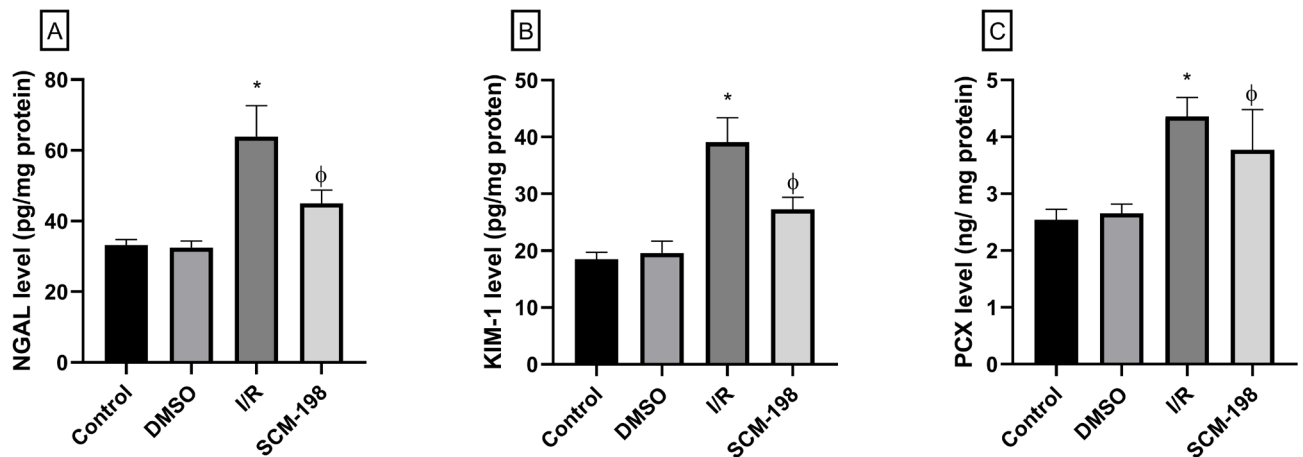


Figure 4. Changes in kidney damage biomarkers in the kidney I/R model (A) NGAL, (B) KIM-1, and (C) PCX levels. * $P < 0.05$ compared to control and DMSO groups, $^{\phi}P < 0.05$ compared to I/R group.

TLRs leads to the transport of NF- κ B from the cytoplasm to the nucleus, which increases the inflammatory cytokine levels and proinflammatory responses (30). During AKI induced by I/R injury, TLR-2 and TLR-4 were found to activate NF- κ B and AP-1 signaling pathways in renal tubular epithelial cells. Subsequently, upregulated mediators such as IL-6, IL-1 β , IL-18, MCP1, TNF- α , and IFN- γ enhance inflammatory responses that lead to acute tubulointerstitial damage (30-32). Inhibition of inflammatory cytokine release is one of the main goals in preventing I/R damage. At this point, we evaluated the effects of SCM-198 on TLR-4 mediated cytokines. Increased TLR-4 immunopositivity and cytokine levels (IL-1 β , TNF- α , and IL-18) in I/R were decreased by SCM-198 treatment. Our results are consistent with many studies. SCM-198 has been demonstrated anti-inflammatory effects by suppressing NF- κ B activity (11, 33). Besides, it has been reported to downregulate the

release of various adhesion molecules and cytokines (34, 35).

The primary pathological mechanism of AKI is apoptosis in tubular cells and accompanying renal dysfunction (25, 36, 37). There are many different findings (changes in caspase-3 activity, cytochrome c release, and Bcl-2/Bax ratio) indicating that apoptosis results from I/R (39). From the Bcl-2 protein family, Bcl-2 and Bcl-XL are the best-defined anti-apoptotic factors. Bax, Bad, or Bcl-Xs from other family members supports apoptosis (39-41). In renal I/R injury, Bax and caspase-3 levels increase, and Bcl-2 level decreases, resulting in tubular cell apoptosis (42). The Bcl-2 expression level was increased significantly, and the Bax expression level was reduced considerably in the SCM-198 group compared to the I/R group, and thus the Bcl-2/Bax ratio increased. The high Bcl-2/Bax ratio in the results explains the mechanism of the anti-apoptotic effects of SCM-198. Similar to our results, Liu et al. and Zhu et

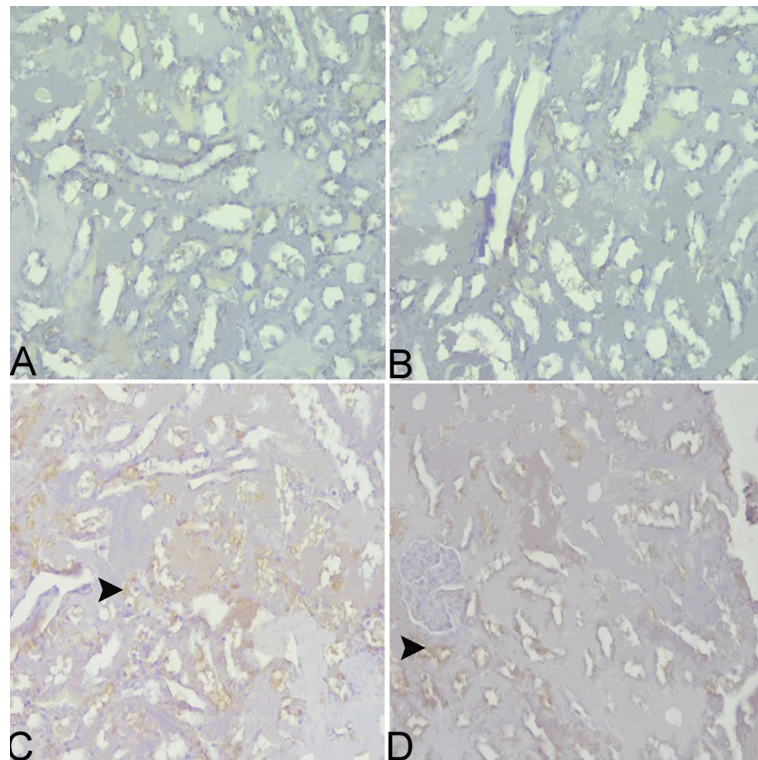


Figure 5. (A) Control group, LC3B immunonegativity (B) DMSO group, LC3B immunonegativity (C) I/R group, moderate LC3B immunopositivity was observed in renal tubule epithelial cells (arrowhead), (D) SCM-198 group, mild LC3B immunopositivity was detected in renal tubular epithelial cells (arrowhead).

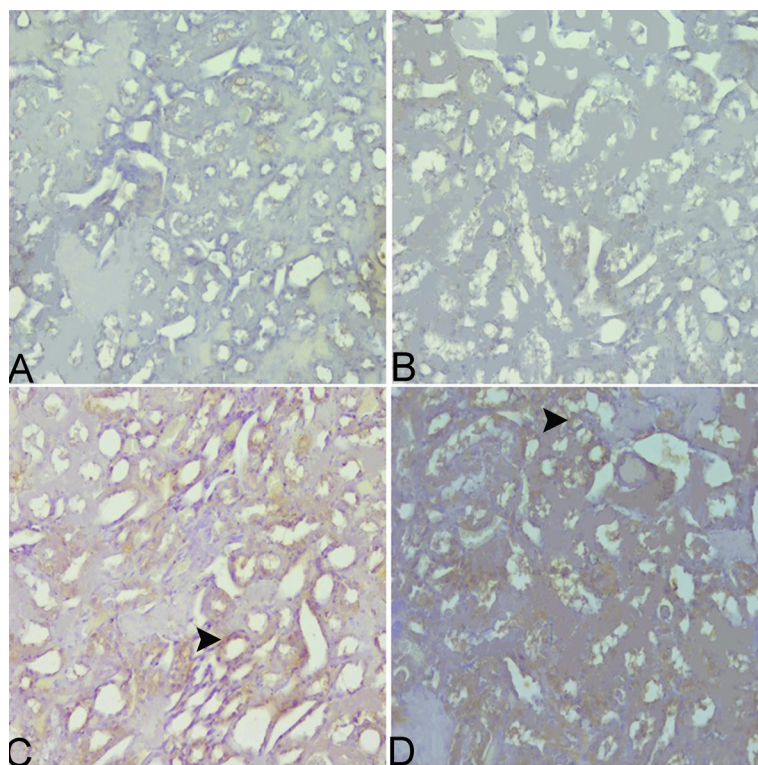


Figure 6. (A) Control group, Beclin-1 immunonegativity, (B) DMSO group, Beclin-1 immunonegativity, (C) I/R group, moderate Beclin-1 immunopositivity in renal tubule epithelial cells (arrowhead), (D) SCM -198 group, mild Beclin-1 immunopositivity in renal tubule epithelial cells (arrowhead).

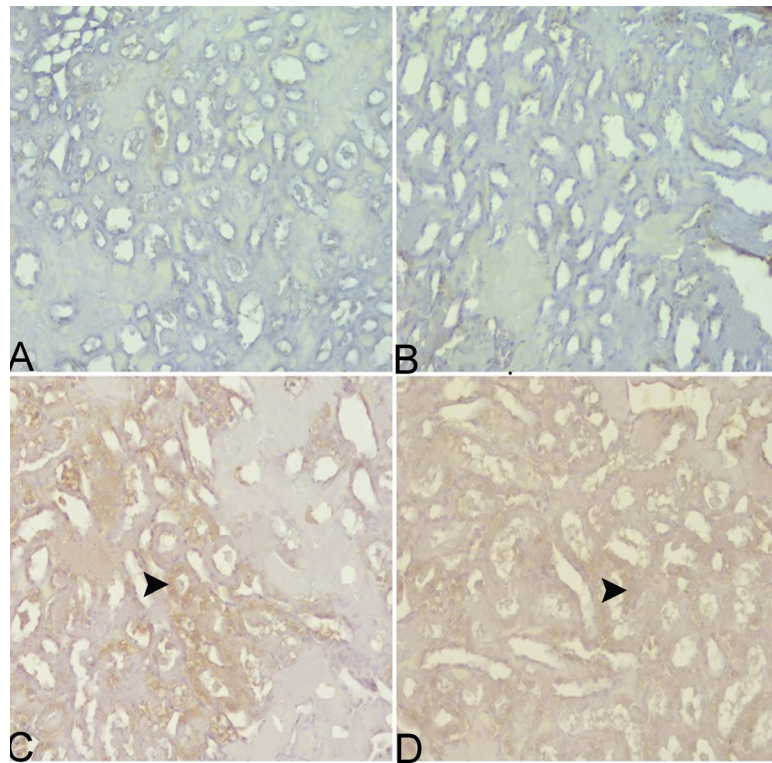


Figure 7. (A) Control group, TLR4 immunonegativity, (B) DMSO group, TLR4 immunonegativity, (C) I/R group, moderate TLR4 immunopositivity in renal tubule epithelial cells (arrowhead), (D) SCM-198 group, mild TLR4 immunopositivity in renal tubule epithelial cells (arrowhead).

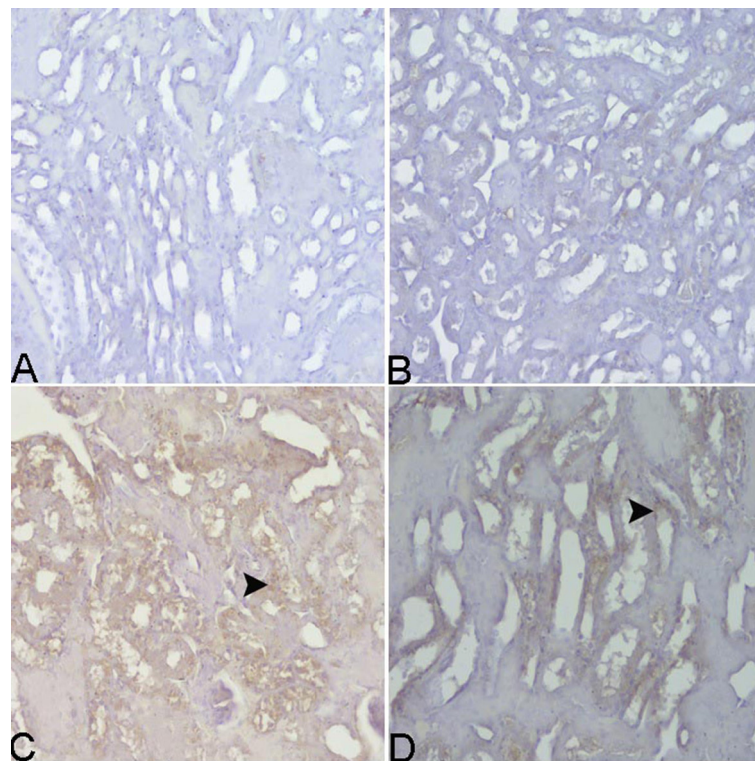


Figure 8. (A) Control group, Bax immunonegativity, (B) DMSO group, Bax immunonegativity, (C) I/R group, moderate Bax immunopositivity in renal tubule epithelial cells (arrowhead), (D) SCM-198 group, mild Bax immunopositivity in renal tubule epithelial cells (arrowhead).

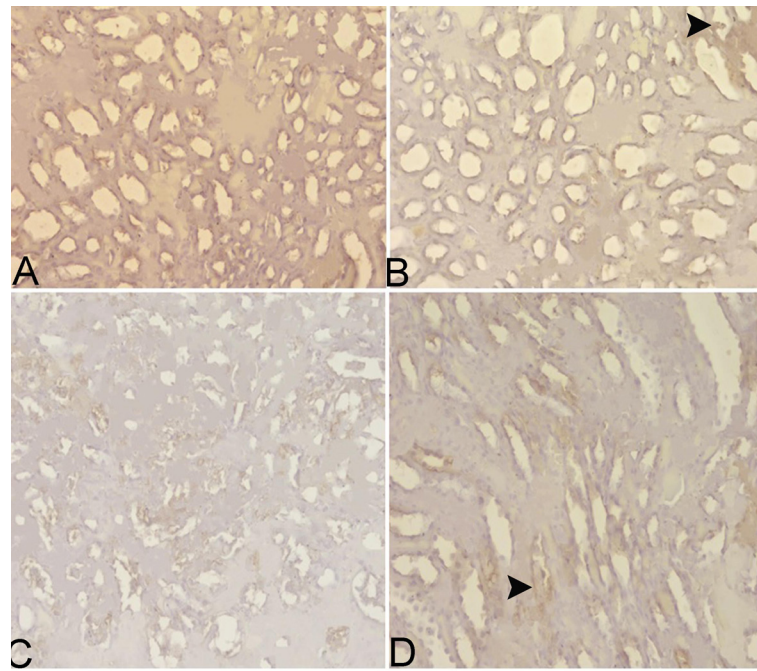


Figure 9. (A) Control group, severe Bcl-2 immunopositivity in renal tubule epithelial cells (arrowhead), (B) DMSO group, severe Bcl-2 immunopositivity in renal tubule epithelial cells (arrowhead), (C) I/R group, mild Bcl-2 immunopositivity in renal tubule epithelial cells, (D) SCM-198 group, moderate Bcl-2 immunopositivity in renal tubule epithelial cells (arrowhead).

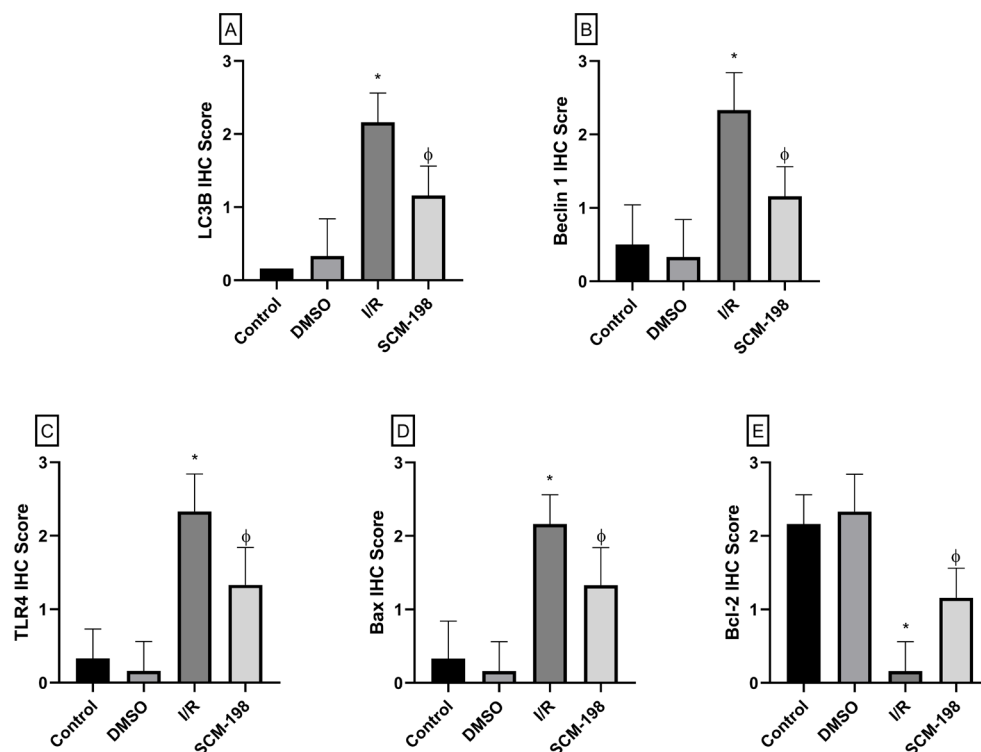


Figure 10. IHC scoring in renal tubule cells (A) LC3B, (B) Beclin-1, (C) TLR4, (D) Bax and (E) Bcl-2. *P<0.05 compared to control and DMSO groups, φP<0.05 compared to I/R group.

al. showed in their studies that SCM-198 has an anti-apoptotic effect by lowering the Bax/Bcl-2 ratio (13, 43).

Conclusion

As a result, the current study shows that SCM-198 reduces I/R-induced tubular damage and modulates inflammatory responses. The mitigating effect of SCM-198 on renal dysfunction is likely mediated by the increase of Bcl-2/Bax and the inhibition of autophagy. Our findings suggest that SCM-198 is a novel modulator of renal I/R pathogenesis and may be a novel therapeutic agent for renal I/R.

Conflict of Interest

There is no conflict of interest between the authors in the article.

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Author contributions

All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript. EE, BB, AT, MCG, YB, and SA have collected and evaluated the data; EE, AT, MCG, and BB have written the paper; EE, YB, and SA have contributed data or analysis tools; EE, SA, and YB have performed the biochemical and histopathological analysis; and EE, BB, and AT have conceived and designed the analysis.

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