Protective effect of glycyrrhizin, a direct HMGB1 inhibitor, on post-contrast acute kidney injury

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Abstract

Post contrast-acute kidney injury (PC-AKI) is defined as the deterioration of renal function after administration of iodinated contrast media. The purpose of this study was to investigate the association between HMGB1 and PC-AKI and the protective effect of glycyrrhizin, a direct inhibitor of HMGB1, in rats. Rats were divided into three groups: control, PC-AKI and PC-AKI with glycyrrhizin. Oxidative stress, mRNA expressions of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6 and TNF- α) and kidney injury markers (Kim-1, NGAL and IL-18) were assessed. In addition, the serum and intracellular protein levels of HMGB1 were analyzed. Moreover, serum creatinine (SCr), blood urea nitrogen (BUN) and lactate dehydrogenase (LDH) levels were assessed. Oxidative stress, pro-inflammatory cytokines, kidney injury markers and LDH were significantly higher in PC-AKI compared to the controls, but were lower in PC-AKI with glycyrrhizin. Intracellular and serum HMGB1 levels significantly increased after contrast media exposure, whereas they markedly decreased after glycyrrhizin pretreatment. SCr and BUN also decreased in PC-AKI with glycyrrhizin compared to PC-AKI. Our findings indicate that HMGB1 plays an important role in the development of PC-AKI and that glycyrrhizin has a protective effect against renal injury and dysfunction by inhibiting HMGB1 and reducing oxidative stress.

1. Introduction

Post-contrast acute kidney injury (PC-AKI) occurs in approximately 5–6% of patients after administration of intravenous contrast media and this incidence steeply increases in patients with impaired renal function.¹ PC-AKI is the third most common cause of acute renal failure in hospitalized patients^{2,3} and can cause long-term adverse effects including loss of kidney function and increased morbidity and mortality.⁴ Although the pathogenesis of PC-AKI is not yet fully elucidated, suggested causes of PC-AKI are the direct toxicity of contrast media, contrast media-driven reactive oxygen species and/or renal hypoxia due to vasoconstriction or high viscosity of the contrast media itself.^{2,3}

The high-mobility group box 1 (HMGB1) exists in the nuclei of mammalian cells and is a novel member of the damage-associated molecular pattern (DAMP) family. HMGB1 can be actively released from the nucleus into the cytoplasm and extracellular space in response to sterile inflammation and infection when cell membrane receptors interact with microbe-associated molecular patterns, pathogen-associated molecular patterns and inflammatory mediators such as tumor necrosis factor (TNF- α), IL-1 and interferon- γ . HMGB1 can also be passively released from necrotic or apoptotic cells.^{5,6} After excretion from the cell, HMGB1 acts as a pro-inflammatory mediator when it binds to cell surface receptors including advanced glycation end products (RAGE) or toll-like receptors (TLR) and stimulates immunocompetent cells to produce pro-inflammatory cytokines such as TNF- α , IL-1, IL-6 and IL-8, resulting in symptoms such as fever, anorexia and other inflammatory responses.^{5,6} HMGB1 also plays an important role in the pathogenesis of various diseases including sepsis, gastrointestinal inflammation, respiratory disorders, autoimmune diseases, hemorrhagic shock, cerebral ischemia and myocardial

infarction.⁶ HMGB1 is also involved in renal disease including acute kidney injury because HMGB1 can promote kidney injury through the TLR4 pathway.^{7,8}

Glycyrrhizin is derived from licorice root extract and binds directly to the high-mobility group 1 (HMGB1) box in HMGB1, consequently inhibiting the chemoattractant and mitogenic activity of HMGB1^{9–11}. In AKI caused by ischemia-reperfusion injury, glycyrrhizin is thought to attenuate renal injury through this inhibition.¹² As PC-AKI is classified as acute kidney injury, HMGB1 might be a factor in the development of PC-AKI. However, to our knowledge, no studies have explored the role of HMGB1 and the protective effect of glycyrrhizin against PC-AKI. So, the purpose of this study was to investigate the association between HMGB1 and PC-AKI and the protective effect of glycyrrhizin.

2. Results

2.1 Glycyrrhizin-mitigated oxidative stress in PC-AKI

We confirmed the effect of glycyrrhizin on reactive oxygen species (ROS) in the PC-AKI group. The MDA levels were higher in the PC-AKI group compared to the controls ($P < 0.001$), whereas MDA levels were lower in the PC-AKI with glycyrrhizin group compared to the PC-AKI group ($P < 0.001$) (Fig. 1A). In addition, in H₂DCFDA staining, the fluorescence intensity of the PC-AKI group was markedly higher than the controls, whereas it was lower after glycyrrhizin pretreatment (Fig. 1B).

2.2 Effect of glycyrrhizin on the protein expression of HMGB1 in PC-AKI

We evaluated whether glycyrrhizin could inhibit the protein expression of HMGB1. The protein expression of both intracellular ($P < 0.001$) and cytoplasmic ($P = 0.010$) HMGB1 increased in the PC-AKI group compared to the controls, whereas the PC-AKI with glycyrrhizin group showed lower protein expression levels than the PC-AKI group (intracellular, $P = 0.004$; cytoplasmic; $P = 0.008$) (Fig. 2A). Serum HMGB1 concentrations were also higher in the PC-AKI group compared to the controls ($P < 0.001$). Serum HMGB1 concentrations were lower in the PC-AKI with glycyrrhizin group than the PC-AKI group ($P < 0.001$) and similar to that of the controls (Fig. 2B).

2.3 Effect of glycyrrhizin on the mRNA expression of pro-inflammatory cytokines in PC-AKI

To examine the protective effect of glycyrrhizin on the inflammatory process, we assessed mRNA expression levels using RT-PCR. The mRNA expressions of IL-1 α , IL-1 β , IL-6 and TNF- α were all significantly upregulated in the PC-AKI group compared to the controls (IL-1 α ; $P = 0.09$, IL-1 β ; $P < 0.001$, IL-6; $P = 0.020$ and TNF- α ; $P = 0.004$). As expected, the expression of all pro-inflammatory cytokines decreased in the PC-

AKI with glycyrrhizin group compared to the PC-AKI group. (IL-1 α ; P = 0.040, IL-1 β ; P = 0.040, IL-6; P = 0.040 and TNF- α ; P = 0.003) (Fig. 3A-D).

2.4 Glycyrrhizin-mitigated kidney injury markers in PC-AKI

Next, we evaluated the mRNA expression levels of kidney injury markers using RT-PCR. The expressions of KIM-1, NGAL and IL-18 were higher in the PC-AKI group compared to the controls (P < 0.001).

Pretreatment with glycyrrhizin seemed to reduce the expression levels of these genes compared to the PC-AKI group (KIM-1 and NGAL P = 0.020, IL-18 P = 0.001) (Fig. 4A-C). In addition, serum LDH was assessed as an early renal damage biomarker. LDH concentrations were higher in the PC-AKI group compared to the controls (P < 0.05). On the other hand, LDH levels were lower in PC-AKI pretreated with glycyrrhizin (P = 0.040) (Fig. 4D).

2.5 Effect of glycyrrhizin on renal function in PC-AKI

We also confirmed the protective effect of glycyrrhizin on renal function in PC-AKI. As shown in Fig. 5, SCr and BUN were higher in the PC-AKI group compared to the controls (P < 0.001). However, both SCr and BUN were lower in the PC-AKI with glycyrrhizin group than in the PC-AKI group (P < 0.001) (Fig. 5A, B).

2.6 Effect of glycyrrhizin on renal histopathology in PC-AKI

Histological appearance was compared between the controls, PC-AKI and PC-AKI with glycyrrhizin in Fig. 6. In H&E staining, prominent tubular dilatation was noted in the PC-AKI group. In the PC-AKI with glycyrrhizin group, tubular dilatation was less prominent (Fig. 6A). On TEM, vacuolization was noted in the cytoplasm of tubular cells in PC-AKI and the PC-AKI with glycyrrhizin group had a lower degree of vacuolization than the PC-AKI group (Fig. 6B).

3. Discussion

Our results demonstrated that oxidative stress and inflammatory cytokines including IL-1, IL-6 and TNF- α increased in the PC-AKI model. Kidney injury makers such as Kim-1, NGAL and IL-18 also increased after exposure to contrast media. Furthermore, cytoplasmic and serum HMGB1 increased along with total cellular HMGB1 in the PC-AKI model. In this model, we could see that glycyrrhizin effectively mitigated kidney injury after contrast media exposure. Renal function impairment and histologic change was also attenuated with glycyrrhizin pretreatment. These results suggest that HMGB1 plays an important role in the development of PC-AKI and that glycyrrhizin has a protective effect against PC-AKI.

HMGB1 contributes to the development of many renal diseases including ischemic reperfusion injury, renal fibrosis due to chronic kidney disease, diabetic nephropathy, and granulomatous nephritis.^{7,8,13} HMGB1 is also thought to be involved in the development of PC-AKI as increased levels of HMGB1 were found in the supernatants of previous cell experiments after cells were exposed to contrast media.¹⁴

These results were comparable to our animal study which also found increased serum HMGB1 in the PC-AKI group. We also showed that total cellular HMGB1 as well as cytoplasmic and serum HMGB1 increases after the administration of contrast media, suggesting that HMGB1 might be actively translocated from the nucleus to the cytoplasm after contrast media exposure. In previous studies, the extracellular release of HMGB1 upregulated the release of pro-inflammatory cytokines.^{6,15,16} This was also noted here as pro-inflammatory cytokines including IL-1 α , IL-1 β , IL-6 and TNF- α significantly increased in the kidney tissue of the PC-AKI group compared to the controls.

Glycyrrhizin directly binds to HMGB1 by interacting with two concave shallow surfaces and inhibits the chemotactic function of HMGB1.¹⁷ Through this, glycyrrhizin can ameliorate sepsis-induced AKI, renal ischemic-reperfusion injury, gentamicin-induced AKI and diabetic kidney disease.^{10,12,18–20} In our results, pro-inflammatory cytokines decreased in the PC-AKI with glycyrrhizin group and SCr and BUN were significantly lower in the PC-AKI with glycyrrhizin group compared to the PC-AKI group. These results suggest that the deterioration of renal function after contrast media exposure could be mitigated with glycyrrhizin by blocking HMGB1 and subsequently, the pro-inflammatory cytokines.

PC-AKI is thought to be caused by both renal hemodynamic changes and direct renal parenchymal damage.²¹ The latter might be due to the direct toxicity of iodine contained in the contrast media to the tubular epithelial cells and endothelial cells or due to enhanced production of ROS by the contrast media, resulting in increased oxidative stress.^{2,21} Hence, the cytotoxicity of contrast media might be ameliorated by reducing oxidative stress. Glycyrrhizin is one well-known antioxidant.²² According to previous studies, oxidative stress can be reduced by glycyrrhizin in sepsis- or gentamicin-induced AKI, preventing further renal injury.^{18,19} We also demonstrated that glycyrrhizin could reduce oxidative stress after the administration of contrast media and this might partly contribute to the protection of renal function in PC-AKI.

SCr has two innate drawbacks that limit its role in the early diagnosis of AKI. Changes in SCr become apparent slower than benign acute kidney injury due to its large volume of distribution and SCr has a narrow time window for the detection of kidney injury due to its sharp decrease after GFR recovery. Thus, other biomarkers such as NGAL, KIM-1, IL-18 and LDH have been suggested instead of SCr.^{23,24} In a previous study, these early biomarkers of AKI also increased in PC-AKI.^{24–26} In our study, not only SCr and BUN, but also early biomarkers including the mRNA expression levels of KIM-1, NGAL, IL-18 and serum levels of LDH increased significantly in the PC-AKI group, while significantly decreasing after glycyrrhizin pretreatment. These results suggested that the administration of glycyrrhizin could mitigate kidney injury caused by contrast media.

The vacuolization of tubular epithelial cells is one of the histopathological change of PC-AKI. It is known as an early sign of PC-AKI, although the degree of vacuolization does not correlate with the deterioration of renal function.^{21,27} In our study, we observed that prominent vacuolization of tubular cells that was noted in the PC-AKI model was mitigated after glycyrrhizin pretreatment.

There were a couple of limitations to this study. First, the expression of RAGE, TLR2 and CXCR4 which are known as receptors of HMGB1 on the cell surface, and NF- κ B which is a downstream factor of HMGB1 were not evaluated in this study.¹⁴ Second, the physiology and anatomy of rats and humans differ and these differences will need to be addressed before our findings can be applied in clinical-practice. And lastly, only male rats were used in this experiment.

In conclusion, this study showed that HMGB1 plays an important role in the development of PC-AKI and that glycyrrhizin has a protective effect on renal function by inhibiting HMGB1 and reducing oxidative stress.

4. Material And Methods

4.1 Animal preparation

The experiment for animal research were approved by the department of Laboratory Resources of Yonsei University and the Animal Ethical Committee with an ID number of 2019 – 0137, and all methods were carried out in accordance with relevant guidelines and regulations. Additionally, the animal experiment procedures were performed according to the ARRIVE guidelines. Twenty-four Sprague-Dawley male rats were used in this study (body weight: 200–220 g). Rats were housed in an Animal Laboratory and maintained in a sterile 12 h light and 12 h dark experimental animal environment with $50 \pm 100\%$ of humidity and 22 ± 2 °C of temperature during the experiment.²⁸ Rats were classified into 3 groups: the control group (n = 8), PC-AKI group (n = 8) and PC-AKI with glycyrrhizin group (n = 8). There was no significant difference in body weight among the three groups ($P = 0.190$). For the PC-AKI model, water was restricted for 16–24 h. Then, 10 mg/kg of indomethacin (Sigma-Aldrich, St. Louis, MO) and 15 mg/kg of L-NAME (Cayman Chemical, Ann Arbor, MI) were administrated via intraperitoneal (IP) injection. After 20 min of administration, 10 ml/kg of iodinated contrast media (Pamiray 370, Dongkook Lifescience, Seoul, South Korea) was administrated by intravenous (IV) injection through the tail vein. In the PC-AKI with glycyrrhizin group, 30 mg/kg of glycyrrhizin (Selleckchem, Huston, TX) dissolved in saline was intraperitoneally administered 2 h before administrating of indomethacin and L-NAME. All rats were euthanized after 24 h using 10%/ min of CO₂ gas in the chamber and the kidney tissues were harvested.

4.2 Kidney function evaluation

After euthanasia, blood was collected without anticoagulants from a cardiac puncture of rats through the right atrium with a 26-gauge syringe needle and centrifuged at 3000 rpm for 20 min to obtain the serum. The serum levels of blood urea nitrogen (BUN), serum creatinine (SCr) and lactate dehydrogenase (LDH) were measured using a Cobas C502 autoanalyzer (Roche, Mannheim, Germany).

4.3 Quantitative real-time polymerase chain reaction

Total RNA was extracted from the kidney tissue using a commercial kit according to the manufacturer's instructions (Hybrid-R kit 305 – 101, GeneAll Biotechnology, Seoul, South Korea).²⁹ 1 μ g of the total RNA

was reverse transcribed with amfiRivert cDNA synthesis (GenDEPOT, Huston, TX) according to the manufacturer's instructions. mRNA expression levels of HMGB1, KIM-1, NGAL, IL-1 α , IL-1 β , IL-6 and IL-18 were assessed using the SYBR-Green reagent (GenDEPOT, Huston, TX) with the ABI7500 real-time polymerase chain reaction (RT-PCR) system (Applied biosystem, Foster city, CA) and normalized to β -actin. Gene expression was quantified using β -actin as the internal loading control. All RT-PCR reactions were duplicated and threshold (Ct) values were analyzed by the $2^{(-\Delta\Delta Ct)}$ method.

4.4 Western blotting

The harvested kidney tissue was homogenized and lysed in a pro-prep extraction solution (iNtRON Biotechnologist, Seong-nam, Korea) followed by protein quantitation with the Bradford method.²⁹ In addition, cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific, Rockford, IL, USA). Lysates were fractioned on 10–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies against HMGB1 (1:1000, #ab18256, Abcam, Cambridge, MA), and β -actin (1:10000, #LF-PA0207, AbFrontier, Seoul, South Korea). After the membranes were washed three times in 1xTBS-T for 15 min each, they were incubated with HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP, 1:10000 #SA002-500, GenDepot, Houston, TX) for 1 h at room temperature. Then the membranes were washed three times in 1xTBS-T for 15 min again. The blotted membranes were visualized by ECL reagents and exposed to X-ray film. The results were normalized to the β -actin loading control and band density was measured using Image J software (National Institutes of Health, Bethesda, MD, <https://imagej.nih.gov/ij>)²⁹

4.5 Enzyme-linked immunosorbent assay (ELISA)

To measure serum HMGB1, we used the Rat HMGB1 ELISA commercial kit (E-EL-R0505, Elabscience, Houston, Tx) according to the manufacturer's instructions. Absorbance levels of the HMGB1 concentrations were measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

4.6 Determination of MDA concentration

Membrane lipid peroxidation was assessed by measuring malondialdehyde (MDA), a product of membrane lipid peroxidation, using 2-thiobarbituric acid reactive substances. The MDA levels were calculated as MDA equivalents using a commercial kit Cat#10009055 (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.²⁹

4.7 Histology, immunofluorescence and H₂DCFDA staining

Kidney tissue was fixed with 10% buffered formalin embedded in paraffin, and cut into 4 μ m sections. The sectioned slide were deparaffinized with xylene and rehydrated by gradually increasing the ethanol concentration. Then, H&E staining was performed using the Leica Autostainer. For H₂DCFDA staining, the sectioned slide was washed twice with 1X phosphate-buffered saline (PBS) and incubated with 10 μ M

H₂DCFDA for 15 min in a 37°C incubator. The stained slides were observed with a fluorescence microscope (IX71/DP71, Olympus, Tokyo, Japan).

4.8 Transmission Electron Microscopy (TEM)

Kidney tissue was fixed overnight in 0.1 M phosphate buffer (PH7.4) containing 2% glutaraldehyde, 2% paraformaldehyde and 0.5% CaCl₂. The samples were washed twice with 0.1 M phosphate buffer for 30 min and were then fixed for 2 h with 1% OsO₄ dissolved in 0.1 M phosphate buffer, followed by dehydration through a series of increasing ethanol concentrations (50–100% with increments of 10%) with 10 min being allocated for each concentration. Specimens were embedded by poly/Bed812 kit (Polyscience, Inc., Warrington, PA) and polymerized at 65°C in an electron micro-oven (TD-700, DOSAKA, Kyoto, Japan) for 24 h.²⁸ Blocks were cut using an ultramicrotome (LEICA EM UC-7, Leica Microsystem, Vienna, Austria) and observed with TEM (JEM-1011, JEOL, Tokyo, Japan).

4.9 Statistical analysis

All results were presented as means ± standard errors of the mean (SEM). Statistical analyses were performed using Prism 9.0.0 (Graphpad, San Diego, CA). One-way analysis of variance (ANOVA) and post-hoc comparisons (Bonferroni test) were performed to compare the 3 groups. A *P* value of less than 0.05 was considered to indicate a statistically significant difference. All *P*-values were two-sided.

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6. Declarations

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Author Contribution

H.Oh performed the animal experiments, analyzed data, and drafted the manuscript. N.E Seo helped with the experimental design and analyzed data. J.S.Lim and J.S.You provided constructive suggestions on the experimental design. Y.E.Chung designed the study and edited the manuscript. All authors reviewed and approved the final manuscript.

Additional information

Competing Interest: The authors declare that they have no competing interests to declare.

Figures

Figure 1

Glycyrrhizin attenuates oxidative stress. Oxidative stress was evaluated with MDA levels in the serum (A) and H₂DCFDA staining of kidney tissue (B). Oxidative stress was significantly higher in the PC-AKI group compared to the controls, whereas it was mitigated in the PC-AKI with glycyrrhizin group. Results were expressed as means $\pm$ SEM. N=8 for each group. Statistical significance: ***P<0.001 Con vs PC-AKI, ###P<0.001 PC-AKI vs GL+PC-AKI, Magnification=20X. Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin

Figure 2

Effect of glycyrrhizin on HMGB1 protein expression. (A) Representative western blot bands of HMGB1 with intracellular total protein and cytoplasmic protein isolation. Expression levels were normalized to β -actin. The expressions of intracellular and cytoplasmic HMGB1 were significantly increased in the PC-AKI group compared to the controls, and were significantly reduced in the PC-AKI with glycyrrhizin group. (B) The serum HMGB1 measured by ELISA was significantly higher in the PC-AKI group compared to the controls, but was significantly lower in the PC-AKI with glycyrrhizin group. Results were expressed as means $\pm$ SEM. N=8 for each group. Statistical significance: ** P <0.01 and *** P <0.001 Con vs PC-AKI and PC-AKI vs GL+PC-AKI, # P <0.05 and ## P <0.01 PC-AKI vs GL+PC-AKI. Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin

Figure 3

Effect of glycyrrhizin on the mRNA expression of pro-inflammatory cytokines. (A-D) Relative expressions of IL-1 α , IL-1 β , IL-6 and TNF- α mRNA were analyzed using RT-PCR. All pro-inflammatory cytokines were higher in PC-AKI compared to the controls with/without statistical significance, whereas they were lower in the glycyrrhizin pretreatment group. Results were expressed as means $\pm$ SEM. N=8 for each group. Statistical significance: * P <0.05 and *** P <0.001 Con vs PC-AKI and Con vs PC-AKI. # P <0.05 and ## P <0.01 PC-AKI vs GL+PC-AKI. Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin

Figure 4

Effect of glycyrrhizin on the mRNA expression of kidney injury markers and serum lactate dehydrogenase (LDH). (A-C) Relative expressions of KIM-1, NGAL and IL-18 mRNA were measured using RT-PCR. The mRNA expression of kidney injury markers was significantly higher in the PC-AKI group compared to the controls, whereas it was significantly lower in the PC-AKI with glycyrrhizin group. (D) Compared to the control group, serum LDH levels significantly increased in PC-AKI, whereas they decreased with glycyrrhizin pretreatment. Results were expressed as means $\pm$ SEM. N=8 for each group. Statistical significance: * $P < 0.05$ and *** $P < 0.001$ Con vs PC-AKI and Con vs PC-AKI. # $P < 0.05$ and ## $P < 0.01$ PC-AKI vs GL+PC-AKI. Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin

Figure 5

Effect of glycyrrhizin on renal function. (A-B) Compared to the controls, SCr and BUN significantly increased in the PC-AKI group, whereas they decreased in the PC-AKI with glycyrrhizin group compared to the PC-AKI group. Results were expressed as means $\pm$ SEM. N=8 for each group. Statistical significance: *** $P < 0.001$, Con vs PC-AKI and Con vs GL+PC-AKI ### $P < 0.001$, PC-AKI vs GL+PC-AKI. Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin

Figure 6

Effect of glycyrrhizin on kidney histology. (A) Histological appearance of kidney tissue after H&E staining. Compared to the controls, renal tubular dilatation was prominent in PC-AKI, whereas it was mitigated in the PC-AKI with glycyrrhizin group. Magnification=20X, Scale bar=100 μ m. (B) Transmission electron microscopy (TEM) images show multiple large vacuoles in the cytoplasm of the PC-AKI group, but this phenomenon was alleviated in the PC-AKI with glycyrrhizin group. Magnification=5K, Scale bar=5000nm (B-C). Abbreviation: PC-AKI; post-contrast acute kidney injury, GL; glycyrrhizin