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Research Article

Kaempferol Reverses Acute Kidney Injury in Septic Model by Inhibiting NF-κB/AKT Signaling Pathway

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Sepsis is the main cause of acute kidney injury (AKI), mainly due to systemic immune dysregulation. Kaempferol (KAE) is a natural flavonoid compound with multiple biological activities including anti-inflammatory, antioxidant, and antiapoptotic properties. In this study, we constructed a sepsis-induced AKI mouse model and an LPS-induced glomerular mesangial cell (HK-2) *in vitro* sepsis AKI model. We found that KAE ameliorated sepsis-induced renal pathological damage, reversed renal function damage, and inhibited p-p65 and p-AKT protein expression. In addition, KAE reversed LPS-induced proliferation and inhibited apoptosis in HK-2 cells. These studies suggest that KAE reverses sepsis by inhibiting activation of the NF-κB/AKT pathway to reverse acute kidney injury.

1. Introduction

Acute kidney injury (AKI) is a clinical syndrome of rapidly declining kidney function and kidney injury caused by multiple factors, and infection is one of the most common causes of AKI [1]. Acute kidney injury currently affects 13 million people worldwide each year and is one of the most common complications in hospitalized patients [2, 3]; its adverse consequences include kidney failure, cardiovascular events, and increased mortality [4, 5]. In China, 29 million patients are hospitalized due to AKI; approximately 40% of them die of AKI, and most of them cannot fully recover [6].

Sepsis is a systemic immune response triggered by infection and is the main killer of patients in the ICU with AKI [7, 8]. Therefore, it is a challenge to prevent and control the occurrence of AKI and reduce the morbidity and mortality of septic patients. Pathophysiological mechanisms of AKI in sepsis are complex and multifactorial, posing significant obstacles to its treatment. Among the known mechanisms of AKI in sepsis, oxidative stress responses are associated with inflammatory responses [9, 10]. Huang et al. showed that ultrasmall TWNDs have a very strong ability to eliminate a variety of ROS, which allows them to restore renal function in AKI at very low doses [11]. Ren et al. showed that fesarone prevented AKI in sepsis by reducing renal inflammation and apoptosis through inhibition of Src-mediated NF-κB p65 and MAPK signaling pathways, thereby preventing AKI in sepsis [12].

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, KAE) is a natural flavonoid compound that is present in a variety of edible plants (e.g., legumes, leeks, tea) and commonly used drugs in traditional Chinese medicines (e.g., kaempferol, cat eye grass, white hair grass) [13]. Kaempferol and its derivatives have been largely demonstrated to have anti-inflammatory, antioxidant, antibacterial, and antitumor effects [14]. Molitorisova et al. reported that kaempferol modulated allergic airway inflammation and associated asthma characteristics [15]. Similarly, Liu et al. demonstrated that kaempferol was able to reduce oxidative stress and inflammatory responses

through the downregulation of ROS-dependent MAPK/NF-κB and pyroptosis signaling pathways [16]. However, kaempferol has not been reported to treat sepsis-induced AKI. It is a product of natural origin and has fewer toxic side effects and interactions with chemical agents and biological agents. Therefore, in this study, we established *in vitro* and *in vivo* AKI models of sepsis and investigated the effect of kaempferol on sepsis-induced AKI and its mechanism.

2. Materials and Methods

2.1. Construction and Grouping of Sepsis Mouse Model. Eight-week-old male C57BL/6 mice (22–26 g, SFP grade) were purchased from Kevens Animal Laboratory Co., Ltd., Changzhou, China. Mice were maintained at room temperature ($25 \pm 2^{\circ}$C, $55 \pm 5\%$ humidity) for 3 days with free access to food and water. All animal study procedures were performed following the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and the National Institutes of Health (NIH Publication No. 86-23, Revised 1996). The animal protocol was approved by the Ethics Committee of Shanghai University. Sepsis models were established using cecal ligation and puncture (CLP) surgery [17]. Forty mice were randomly divided into the following 5 groups ($n = 8$/group): control group, AKI group, KAE 25 mg/kg group, KAE 50 mg/kg group, and KAE 100 mg/kg group. All treatment reagents were injected through the tail vein, and treatment was continuously administered to the treatment groups for 3 weeks. At the end of the study, tissues were collected from euthanized mice after weighing and anaesthetization with ether (Sigma–Aldrich).

2.2. H&E Staining. After harvesting the kidney tissues, the right kidney was washed with normal saline, fixed in 4% paraformaldehyde fixative for 48 hrs, stripped step-by-step, processed transparently, embedded in paraffin wax, cut into 5-μm-thick sections, deparaffinized, hydrated, stained with hematoxylin & eosin method, rinsed, dehydrated and cleared, mounted, and observed and photographed under a microscope (NIKON E100, Japan).

2.3. ELISA Experiments. Tissue and cell samples were processed according to TNF-α and IL-1β ELISA kit instructions (MULTISCIENCES, Hangzhou, China), and the absorbance at 450 nm was measured using a microplate reader to draw a standard curve and calculate the concentration.

2.4. RT–PCR. Total RNA was extracted using TRIzol according to the manufacturer's protocol, and recovered RNA was purified in strict accordance with the RNA recovery kit's instructions. cDNA from a reverse transcription reaction was used, and we performed real-time PCR in triplicate using Power SYBR Green polymerase chain reaction (PCR) master mix and an ABI 7500 real-time PCR system. All procedures were performed according to the manufacturer's recommendations. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the primer sequences used are shown in Table 1, with GAPDH used as an internal control. The relative expression of target genes was calculated based on the 2-Ct value.

2.5. Western Blot. Samples from each group were added to protein lysis buffer and PMSF, placed on ice for 40 min, and centrifuged at 12,000 r/min for 40 min at 4°C, and then the supernatant was taken. Protein quantification in the supernatant was performed by a Bradford assay using BSA as a standard. Twenty microgram protein samples were separated by 10% SDS–PAGE. The antibodies used were NF-κB p65 (AF5006, Affinity), p-NF-κB p65 (AF3387, Affinity), Akt (AF6261, Affinity), and p-Akt (AF0016, Affinity), and HRP-labeled goat anti-mouse IgG ($H + L$) (SA00001-1; Proteintech) was used as a secondary antibody. Band intensities were quantified using ImageJ software, and all samples were compared to GAPDH (60004-1-Ig, Proteintech) as a reference protein.

2.6. Cell Culture. The human tubular epithelial cell line HK-2 (FH0228, FuHeng Biology) was cultured in high glucose DMEM containing 10% fetal bovine serum at 37°C and 5% CO_2. HK-2 cells were treated with 10 μg/ml lipopolysaccharide (Sigma–Aldrich) for 24 h to induce a cellular model of sepsis acute kidney injury in vitro according to Sun et al. [18].

2.7. Cell Proliferation. Cells from the experimental and control groups in the logarithmic growth phase were inoculated into 96-well plates at 1×10^4 cells/well, and culture medium was added to a final volume of 100 μl in each well; the cells were cultured in 5% CO_2 at 37°C, and 6 replicate wells and 2 control wells were included. CCK-8 reagent was added, and the absorbance at 450 nm was measured using a microplate reader. Cell growth curves were plotted with control zeroing, with time as the abscissa and absorbance as the ordinate.

2.8. Flow Apoptosis. Apoptosis was detected by flow cytometry using an Annexin V-FITC Apoptosis Assay Kit (Beyotime, Shanghai, China). After removing the intervention culture medium from each group, trypsin was added to digest the cells, and DMEM complete culture medium was used to adjust the cell suspension concentration to 10^6/mL. Then, 75% ethanol was added up to 2 mL to fix the cells, and the cells were gently mixed after standing for 5 min and then placed in a low-temperature centrifuge for centrifugation at 12,000 rpg for 10 min. After the end of centrifugation, the supernatant was removed, and PBS solution was added to the cell pellet to gently wash the cells three times for 5 min each time. Annexin V-FITC/PI double staining and flow cytometer onboard detection were performed according to the manufacturer's instructions.

2.9. Statistical Analysis. Experimental data are presented as the means ± SD, and one-way analysis of variance (ANOVA) and correlation analysis were performed using GraphPad

Table 1: Primer sequences used for PCR.

Gene	Gene	Primer sequences(5′-3′)
TNF-α	Mouse	Forward: ACTCCAGGCGGGTGCCTATG Reverse: GTGAGGGTCTGGGCCATAGAA
IL-1β	Mouse	Forward: TGCCACCTTTTGACAGTGATGAG Reverse: TGATGTGCTGCTGCGAGATTT
GAPDH	Mouse	Forward: AGAACATCATCCCTGCATCC Reverse: AGTTGCTGTTGAAGTCGC
TNF-α	Human	Forward: AGCCCCCAGTCTGTATCCTT Reverse: CTCCCTTTGCAGAACTCAGG
IL-1β	Human	Forward: GCCCATCCTCTGTGACTCAT Reverse: AGGCCACAGGTATTTTGTCG
GAPDH	Human	Forward: ACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTA

Prism statistical software 7.0. Differences between the two groups were compared using Bonferroni post hoc comparisons. $p < 0.05$ was considered statistically significant.

3. Results

3.1. KAE Improves Sepsis-Induced AKI. KAE treatment of septic mice significantly increased body weight (Figure 1(a)), significantly decreased the renal index (kidney weight/body weight) (Figure 1(b)), and significantly increased serum creatinine and blood urea nitrogen (Figures 1(c) and 1(d)). Taken together, the results suggest that KAE ameliorates sepsis-induced acute kidney injury in mice.

3.2. KAE Ameliorates Pathological Changes in Mice with Sepsis-Induced AKI. We performed HE staining to observe morphological changes in the kidney. As shown in Figure 2, septic mice showed marked changes in the renal tissue, such as glomerular necrosis and hypertrophy, which were significantly improved by KAE treatment.

3.3. Anti-inflammatory Response to KAE in Sepsis-Induced AKI Mice. Inflammation is well known to contribute to the pathogenesis of AKI [19]. In this study, we detected increased IL-1β and TNF-α protein levels in renal tissue during AKI and significantly decreased expression after administration of different doses of KAE (Figures 3(a) and 3(b)). We then detected IL-1β and TNF-α expression levels in kidney tissues from each group and found consistent changes in protein expression detected by ELISA (Figures 3(c) and 3(d)).

These results suggest that KAE plays an anti-inflammatory role in AKI therapy.

3.4. Mechanism of KAE in Sepsis-Induced AKI in Mice. To further investigate the potential mechanism of KAE in sepsis-induced AKI in mice, we examined changes in NF-κB/AKT pathway expression levels within renal tissue (Figure 4(a)). We found that KAE did not affect NF-κB p65 and AKT expression, whereas levels of NF-κB p65 and AKT phosphorylation were dose-dependently decreased (Figure 4(b)). Overall, our findings suggest that KAE reverses sepsis-induced AKI by inhibiting NF-κB/AKT pathway phosphorylation.

3.5. KAE Inhibits LPS-Induced Proliferation of Glomerular Mesangial Cells. To assess the toxicity of KAE to glomerular mesangial cells, we performed viability tests with different KAE concentrations. As KAE showed no significant inhibition of cell viability at concentrations below 25 μM (Figure 5(a)), we chose KAE doses of 5, 10, and 20 μM for in vitro experiments. In addition, LPS was found to inhibit glomerular mesangial cell proliferation, which was reversed by KAE (Figure 5(b)). In conclusion, KAE can promote mesangial cell proliferation in a dose-dependent manner.

3.6. KAE Suppresses the Secretion of Inflammatory Cytokines. Administration of KAE to LPS-treatedHK-2 cells significantly decreased the secretion of the inflammatory cytokines TNF-α and IL-1β, as measured by ELISA, and the secretion of inflammatory cytokines was significantly increased in LPS-treated cells (Figure 6(a)). Subsequently, we measured the mRNA expression levels of IL-1β and TNF-α in the cells of each group (Figure 6(b)), and the results were consistent with the ELISA results. These data suggest that KAE can reverse AKI injury by inhibiting the secretion of inflammatory cytokines.

3.7. KAE Inhibits HK-2 Cell Apoptosis. LPS promoted apoptosis in HK-2 cells, and KAE treatment significantly decreased the LPS-induced increase in apoptosis (Figure 7). These results suggest that KAE reverses AKI injury by inhibiting the apoptosis of HK-2 cells.

3.8. KAE Inhibits the NF-κB/AKT Pathway. LPS activated the NF-κB/AKT pathway, causing cell damage in HK-2 cells, while KAE reversed LPS-induced NF-κB/AKT pathway activation (Figures 8(a) and 8(b)). This result suggests that KAE reverses AKI injury by inhibiting NF-κB/AKT pathway activation.

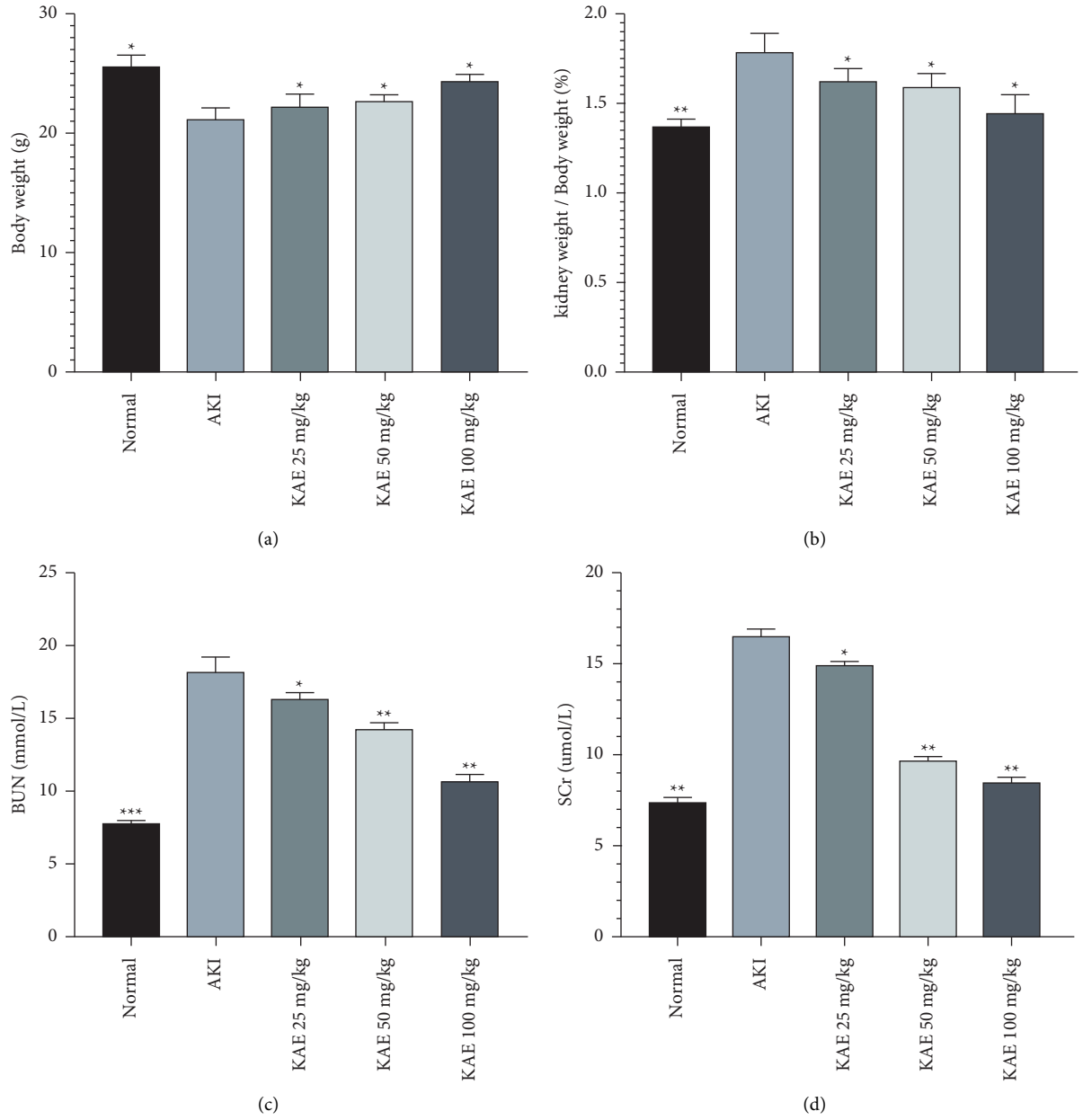


FIGURE 1: KAE improves sepsis-induced AKI effects. (a) Body weight. (b) The ratio of kidney weight to body weight. (c, d) The levels of BUN and SCr. $N = 8$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus AKI group.

4. Discussion

AKI is an acute disease that seriously threatens human life and health, and the mortality rate of AKI caused by sepsis is significantly higher than that of AKI patients without sepsis [20]. Studies have shown that AKI caused by sepsis is mostly manifested as injury caused by an inflammatory response and accompanied by apoptosis [21]. In our study, we first demonstrated through small mouse experiments that KAE can improve renal index and renal histological damage in sepsis-induced AKI, i.e., decrease BUN and SCr concentrations and TNF-α and IL-1β expression. *In vitro*, we constructed a LPS-induced sepsis injury model in HK-2 cells. The effects of KAE on cell proliferation, inflammation, and apoptosis were examined to further investigate the cytoprotective effect of KAE on septic kidney injury. The experimental results showed that KAE could reduce the expression of proinflammatory cytokines and increase the expression of anti-inflammatory cytokines in mice with suppurative AKI, thereby reversing AKI kidney

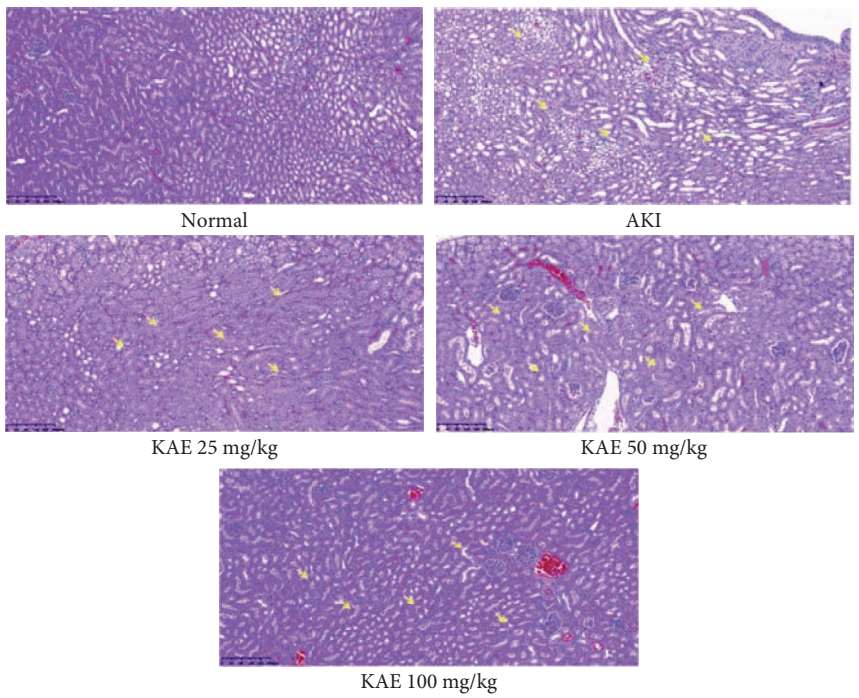
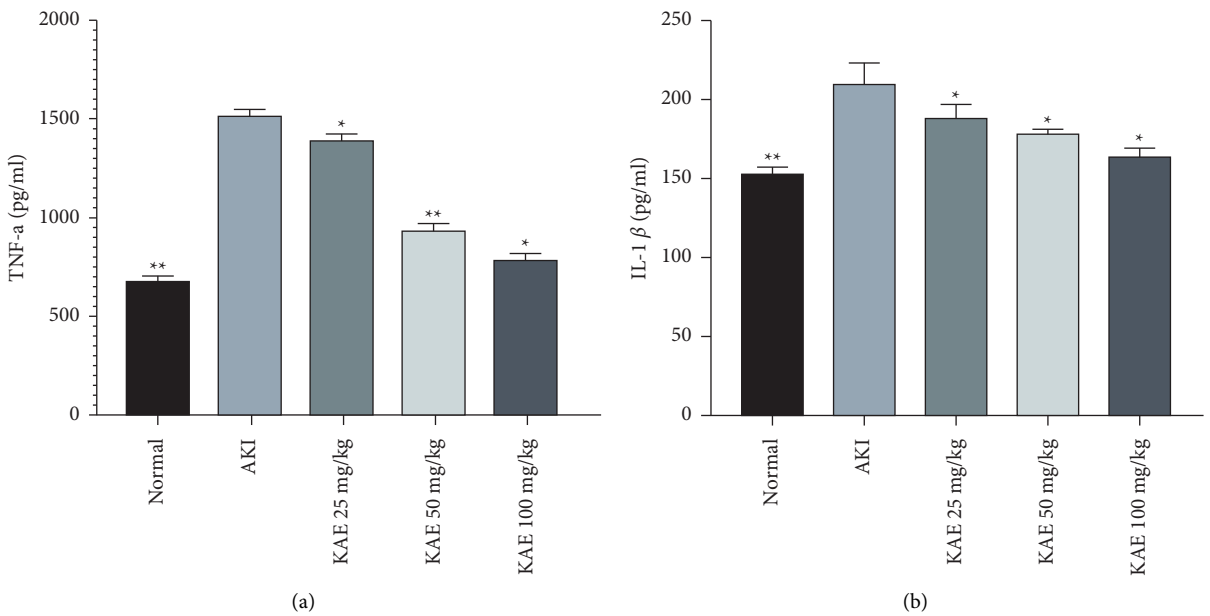


Figure 2: KAE ameliorates pathological changes in sepsis-induced AKI in mice.Representative photomicrographs showing glomerular structures (hematoxylin & eosin staining, 10×).



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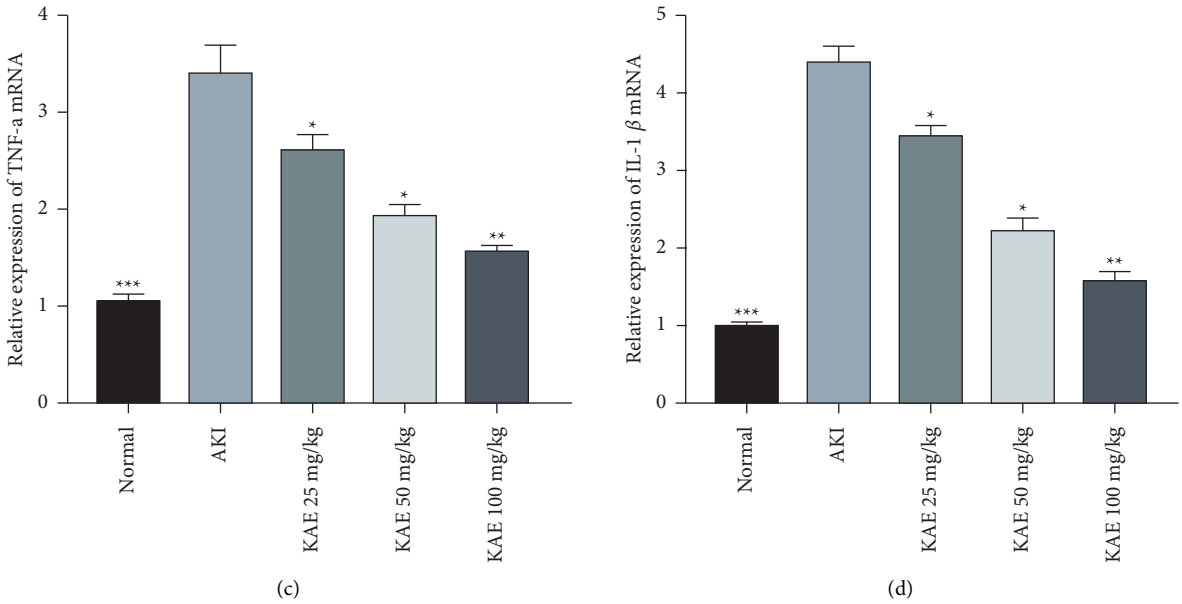


Figure 3: Inflammatory responses of KAE to sepsis-induced AKI in mice. (a, b) IL-1β and TNF-α production in the kidney cortex. (c, d) The mRNA levels of IL-1β and TNF-α in the kidney cortex. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus AKI group.

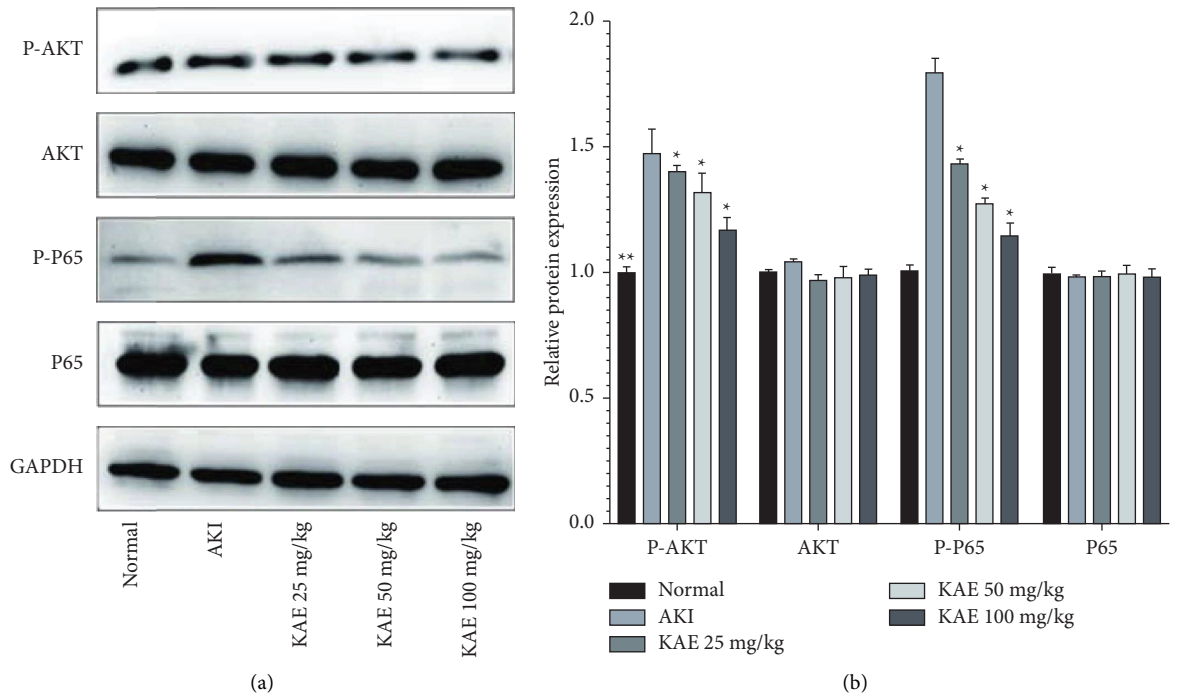


Figure 4: Mechanism of KAE on sepsis-induced AKI in mice. (a) Western blot analysis for total NF-κB p65, P-NF-kB p65, AKT and P-AKT expression in different groups. (b) In relative protein expression statistics, all of the data are expressed as the means ± SD of the data obtained in three parallel experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus AKI group.

injury. In addition, we demonstrated that KAE could modulate proliferation, apoptosis, and inflammation responses in LPS-treatedHK-2 cells.

BUN and SCr are important indicators for judging the severity of the renal injury and are commonly used to evaluate renal function. Alshehri et al. found that kaempferol decreased concentrations of BUN and SCr in a rat model of CdCl 2-induced kidney injury [22]. In this study, we found that kaempferol significantly improved the degree of kidney injury and decreased levels of injury parameters such as

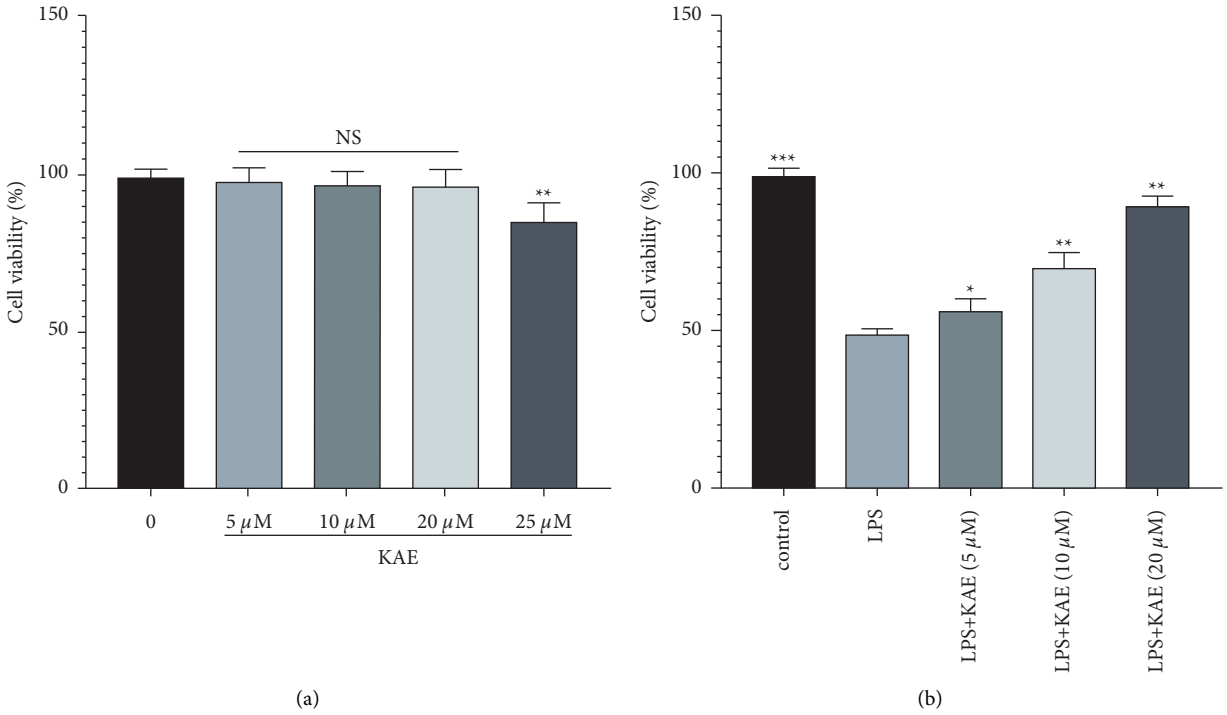
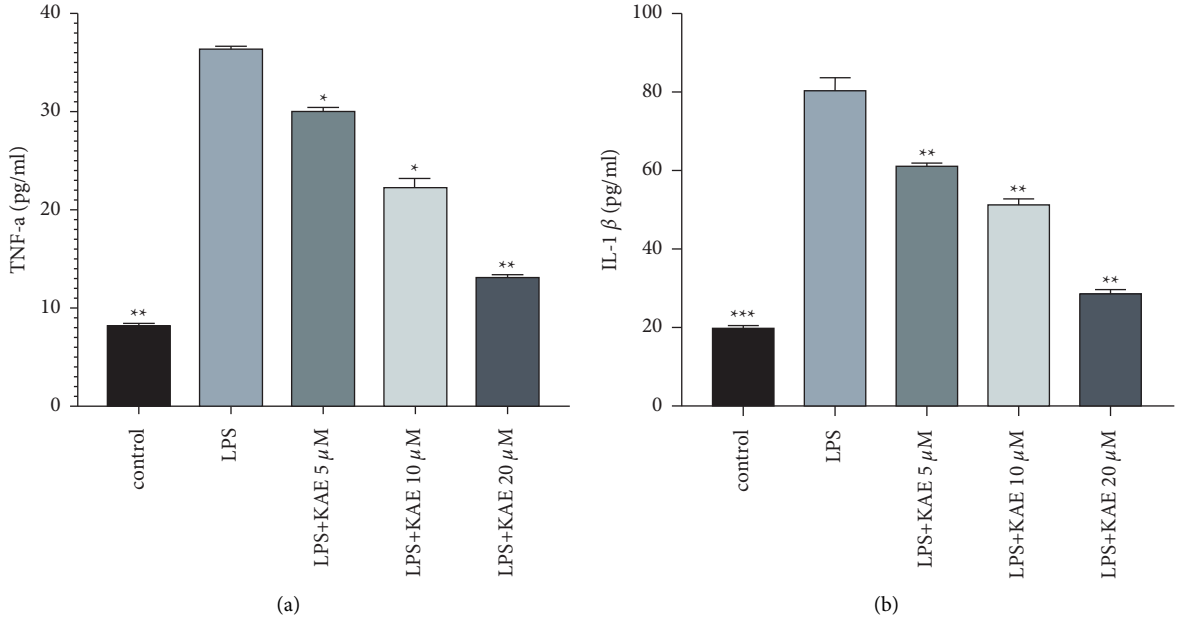


Figure 5: KAE inhibits LPS-induced proliferation of glomerular mesangial cells. (a) Cell viability of HK-2 cells treated with KAE at different concentrations (0, 5, 10, 20 and 25 μM) for 3 hrs; (b) LPS-inducedHK-2 cell viability at 24 h after treatment with KAE at different concentrations (5, 10 and 20 μM). $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus LPS group.



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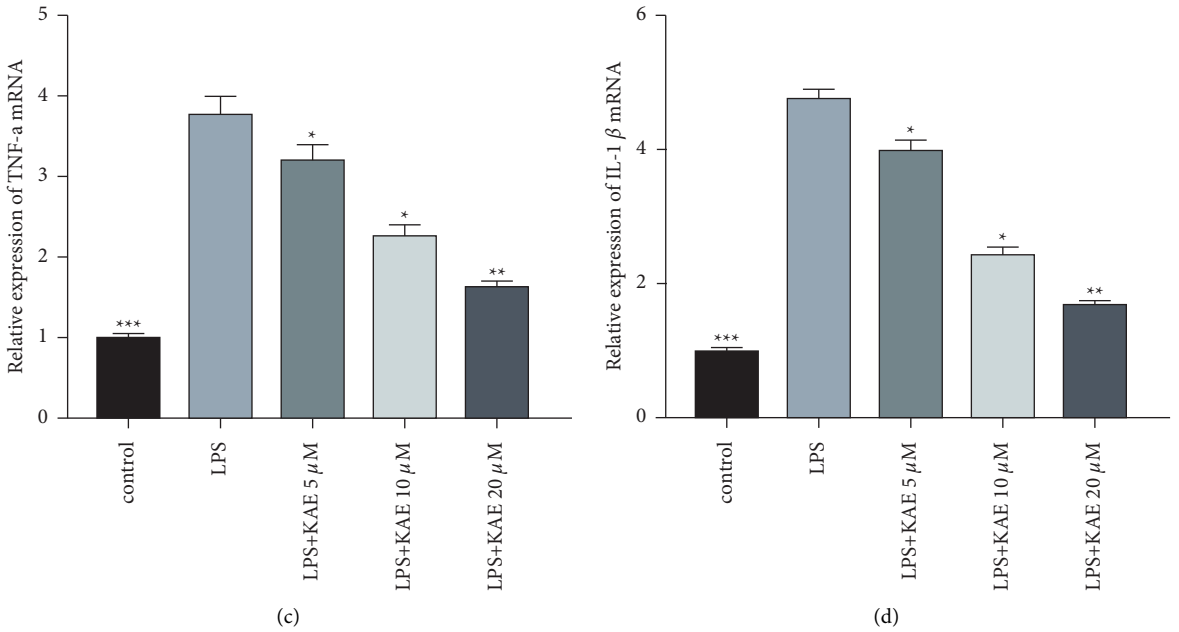

Figure 6: KAE Suppresses Secretion of Inflammatory Cytokines. (a, b) The level of proinflammatory cytokines TNF-α and IL-1β determined by enzyme linked immunosorbent assay (c, d) The mRNA level of proinflammatory cytokines TNF-α and IL-1β determined by reverse transcriptase polymerase chain reaction. $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus LPS group.

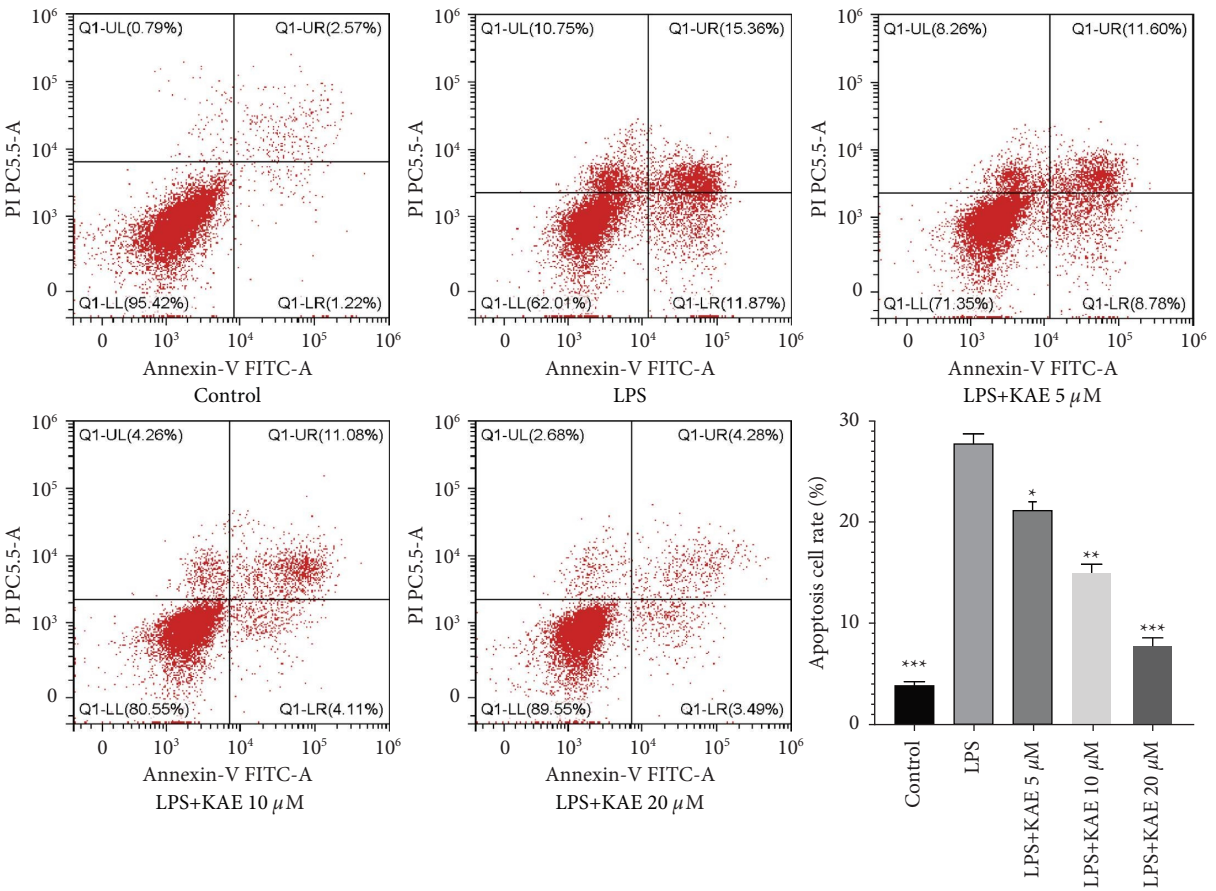

Figure 7: KAE Inhibits HK-2 Cell Apoptosis.The apoptosis rate of the cells with LPS, LPS + KAE 5 μM, LPS + KAE 10 μM and LPS + KAE 20 μM in HK-2 cells by flow cytometry.$N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus LPS group.

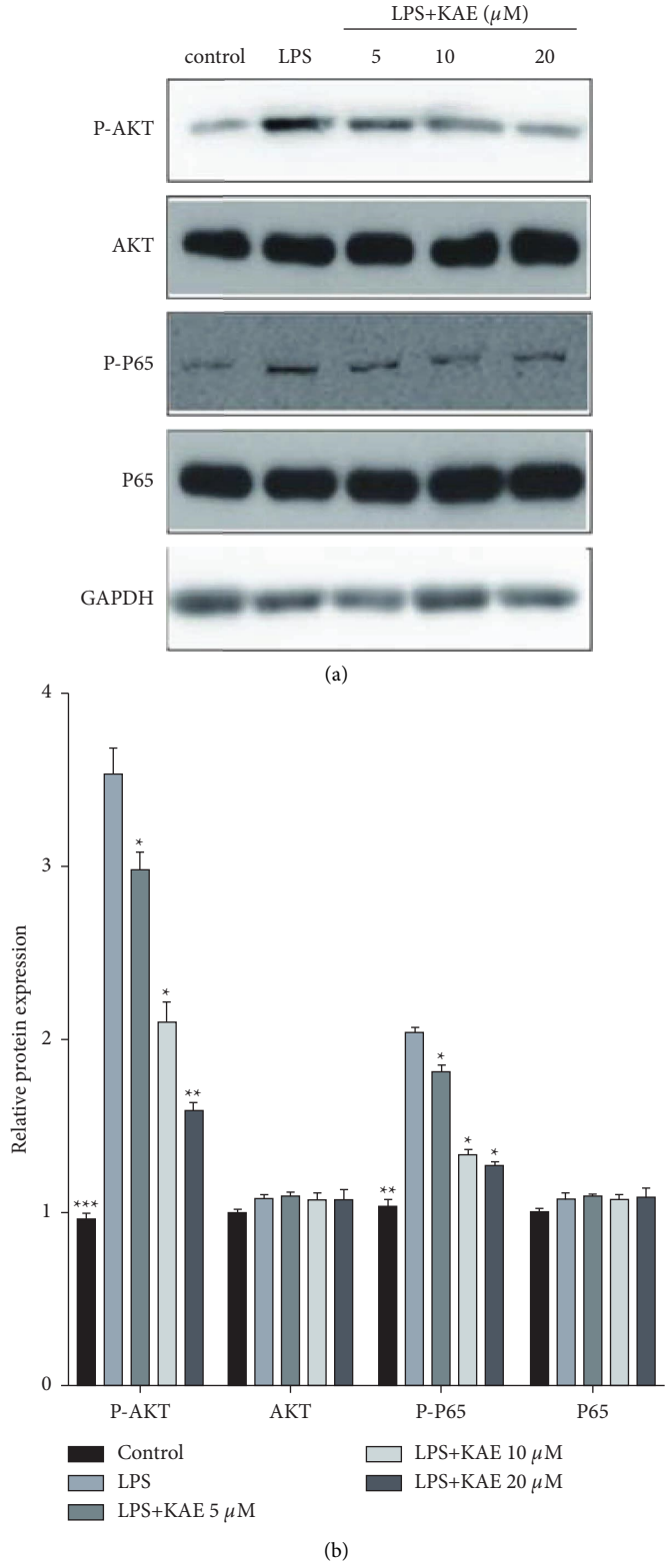

Figure 8: KAE Inhibits NF-κB/AKT Pathway. (a) The levels of protein including NF-κB p65, P-NF-κB p65、AKT and P-AKT in the cells treated with LPS, LPS + KAE 5 μM, LPS + KAE 10 μM and LPS + KAE 20 μM in HK-2 cells determined by Western blot. (b) Quantitative analysis of the protein expression. $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus LPS group.

BUN and SCr in a mouse model of septic kidney injury. This study is the first to validate the protective effect of kaempferol in the AKI model. In addition, we also investigated the expression of TNF-α and IL-1β, representative regulators of inflammation, which were similar to the results for BUN and SCr. These results suggest that

kaempferol can further explain its protective effect by inhibiting the inflammatory response caused by renal injury in sepsis.

In cellular experiments, Zhang et al. found that LPS can increase TNF-α and IL-1β expression in HK-2 cells [23]. In this study, HK-2 cells were treated with 10 μg/ml LPS, and TNF-α and IL-1β expression was increased, confirming that LPS can induce inflammatory damage in HK-2 cells. Subsequently, treatment with kaempferol at different concentrations was found to be effective in reducing inflammatory cell levels, and in addition, we found that kaempferol treatment significantly reduced LPS-induced apoptosis of HK-2 cells by flow cytometry, suggesting that kaempferol could attenuate LPS-induced inflammatory injury by inhibiting proinflammatory cytokine expression and inhibiting apoptosis.

NF-κB/AKT is an important regulator of cellular homeostasis [24]. NF-κB is a transcription factor that has been implicated in inflammation, tumors, apoptosis, and various autoimmune diseases, and it is recognized that NF-κB activation is caused by various external stimuli and is essential for the induction of various proinflammatory cytokines (e.g., IL-1β, TNF-α) [25, 26]. Inhibition of NF-κB signaling has been shown to attenuate acute kidney injury [27, 28]. AKT is a downstream target gene of NF-κB and an important functional protein in maintaining cell survival [29]. It has been demonstrated that AKT inhibition improves inflammatory responses to reverse AKI kidney injury [30]. In this study, we clarified that KAE reversed AKI kidney injury by inhibiting the NF-κB/AKT signaling pathway.

In our study, we first demonstrated that KAE lowers the expression of proinflammatory cytokines and increases the expression of anti-inflammatory cytokines in septic AKI mice, thereby reversing AKI kidney injury. Akhter N et al. reported that calcium dobesilate could reverse AKI injury in severely burned mice by inhibiting NF-κB/AKT expression [31], which is consistent with our study. Park et al. found that kaempferol was able to reduce LPS-induced inflammatory mediators by down-regulating NF-κB and AKT [32]. The above information provides an effective research strategy from our study to reverse acute kidney injury caused by sepsis. In this study, we demonstrate that kaempferol reverses sepsis-induced acute kidney injury by inhibiting the NF-κB/AKT signaling pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Fusheng Shang and Lili Lin conceived and designed the experiments; Dagui Chen, Senlin Ma, Wangzheng Ye, Wenwen Wang, and Da Sun performed the experiments; Dagui Chen, Senlin Ma, and Wangzheng Ye analyzed the data; Fusheng Shang and Lili Lin wrote the manuscript. All authors read and approved the final manuscript. Dagui Chen, Senlin Ma, and Wangzheng Ye contributed equally to this work.

Acknowledgments

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