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Levistolide a Attenuates Acute Kidney Injury in Mice by Inhibiting the TLR-4/NF-κB Pathway

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Introduction: Acute kidney injury (AKI) is characterized by a significant reduction in kidney function and the accumulation of metabolites such as Creatinine (CRE) and Blood Urea Nitrogen (BUN). Levistolide A (LA), an active component of Ligusticum chuanxiong, offers multiple therapeutic benefits, including cardiovascular and neuroprotection, antitumor and analgesic effects, as well as anti-inflammatory, antioxidant, antifibrotic, and proapoptotic actions. However, the underlying mechanism of LA in treating AKI has not been fully elucidated.

Methods: In this study, we established a glycerol-induced AKI model in mice to evaluate the protective effects of LA. Renal function was assessed by measuring levels of CRE and BUN. Histological analyses were performed to evaluate kidney tissue damage. Additionally, oxidative stress markers, apoptosis indicators, inflammatory cell infiltration, and inflammatory mediator levels were assessed. The involvement of the TLR-4/NF-κB signaling pathway was investigated through molecular assays.

Results: LA treatment significantly ameliorated glycerol-induced AKI in mice, evidenced by reduced levels of CRE and BUN. Histological examination revealed decreased renal tissue damage in LA-treated groups. LA exerted antioxidant effects by increasing the levels of Glutathione (GSH) and Superoxide Dismutase (SOD), while reducing Reactive Oxygen Species (ROS) accumulation. Apoptosis in renal tissues was attenuated, as indicated by decreased caspase-3 activation. Furthermore, LA reduced the infiltration of inflammatory cells and the release of inflammatory mediators such as TNF-α and IL-6. Mechanistically, LA suppressed the inflammatory response by inhibiting the TLR-4/NF-κB signaling pathway, as demonstrated by reduced NF-κB activation and decreased expression of TLR-4.

Conclusion: Levistolide A mitigates acute kidney injury through its antioxidative properties and modulation of the TLR-4/NF-κB signaling pathway. These findings provide valuable insights into the therapeutic potential of LA for AKI treatment and lay the groundwork for further mechanistic studies.

Keywords: acute kidney injury, levistolide A, TLR-4/NF-Kb, inflammatory response, antioxidant effect

Introduction

AKI involves a precipitous decrease in the kidney's excretory capacity. This condition progresses quickly, typically within hours to a few days, and is marked by a sharp decline in the glomerular filtration rate. This condition is further characterized by the buildup of metabolic waste products, including CRE and BUN. Clinical manifestations of AKI also include reduced urine output, accumulation of metabolic acids, and elevated concentrations of potassium and phosphorus.[1] AKI is a serious disease. Annually, approximately 13.3 million patients are diagnosed with AKI by doctors with about 1.7 million fatalities all over the world. Moreover, patients with incomplete AKI recovery are at an increased risk of progressing to chronic kidney disease (CKD) and ultimately to irreversible end-stage renal disease (ESRD).[1]

The pathological mechanisms of AKI encompass oxidative stress, apoptosis, ferroptosis, autophagy, and inflammation.[2–5] In the initial phase of AKI, the injury triggers morphological and functional alterations in the endothelial cells of blood vessels and the epithelial cells of renal tubules.[6] Tubular injury can trigger an inflammatory response, which may further contribute to

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the development of renal fibrosis.[7] Therefore, the principal strategies for mitigating kidney injury encompass reducing the incidence of inflammation, inhibiting the release of inflammatory mediators, and targeting inflammation-related signaling pathways. Quercetin reduced cisplatin-induced renal inflammation, lowered TNF-α, IL-6 and IL-1β in serum, inhibited M1 macrophages, and upregulated M2 macrophages activity. It alleviates kidney injury and reduces the activity of Mincle/Syk/NF-κB signaling in both vitro and vivo.[8]

NF-κB is a pivotal transcription factor associated with the pathogenesis of a variety of inflammatory diseases. AKI, a syndrome marked by inflammatory tubular injury, can have its onset triggered by TLR-4. In the rat model of ischemia-reperfusion-induced AKI, pretreatment with leonurine alleviated renal injury by promoting Nrf2 nuclear translocation and inhibiting the TLR-4/NF-κB pathway.[9] Furthermore, studies have demonstrated that downregulation of the NF-κB pathway can be achieved by decreasing the protein expression of NF-κB.[10] Deficiency in TLR-4 safeguards the kidneys against dysfunction and histological damage caused by renal ischemia-reperfusion by diminishing the apoptosis of granulocytes, proinflammatory cytokines, chemokines, and renal tubular epithelial cells.[11] The activation of both the MyD88-dependent and TRIF-dependent pathways is initiated by the homodimerization of the TLR-4/MD-2 complex. In the MyD88-dependent pathway, MyD88 is recruited, which subsequently engages TRAF6 and IRAK4. This cascade ultimately triggers the activation of IKK, leading to the activation of NF-κB.[12] The transcriptional regulation of numerous cellular processes, such as apoptosis, inflammation, and immune response, is profoundly influenced by the NF-κB pathway.[13] When cells are stimulated, IkB-α undergoes phosphorylation and degradation, releasing active NF-κB. NF-κB translocates to the nucleus and attaches to DNA, thereby promoting the transcription and expression of inflammation-related genes.[14]

Renal tubular apoptosis is a key pathological feature of kidney injury.[15] Evidence from clinical studies and animal models highlights the essential role of tubular apoptosis in the progression of AKI.[16,17] Apoptosis effectively eliminates damaged cells.[18] In the endogenous pathway, cellular stress causes the mitochondrial outer membrane to become permeable which leads to the release of apoptotic factors. The apoptotic factors bind to Apaf-1, activating caspase 9. Under endoplasmic reticulum (ER) stress, caspase 12 becomes activated. The extract from salt-processed Achyranthes bidensii reduces ROS accumulation and cell apoptosis in the renal. Additionally, it modulates the expression of apoptosis marker protein TLR-4, thereby alleviating kidney damage.[19]

Oxidative stress is a pivotal factor in the progression of renal disease. The balanced production of oxidants and antioxidants is beneficial to the maintenance of homeostasis.[20] Glutathione (GSH), Glutathione peroxidase (GPx1), Superoxide dismutases (SOD) and catalase are all common in vivo oxidants.[21] The molecules mediated by oxidative stress are Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS encompasses multiple reactive molecules and free radicals originating from molecular oxygen. If endogenous antioxidants are depleted or not compensated, cell damage occurs, and oxidants increase within the body. When oxidative stress becomes unbalanced, the immune system will be activated to prevent further oxidative damage.[22] Studies have shown that in the glycerol-induced AKI rat model, administration of stevioside can reduce Superoxide anion generation (SAG) and increase GSH level. In addition, reductions in glomerular and tubular dilatation, and the accumulation of cell debris in the renal tubular lumen can be observed.[23]

Numerous pharmacological studies have demonstrated that Chuanxiong exhibits significant effects in neuroprotection, anti-tumor activity, analgesia, anti-inflammation, anti-oxidation, anti-fibrosis, and pro-apoptosis.[24] LA is an active component of Ligusticum Chuanxiong which shows great potential as a therapeutic agent for the treatment of liver fibrosis. In a CCl4-induced liver fibrosis rat model, LA treatment inhibited the increase of Ang II, AT1R, the phosphorylation of ERK, and c-Jun signaling in hepatic stellate cells,[25] thus ameliorating liver fibrosis and improving liver function. Besides, LA has also been found to inhibit liver fibrosis by anti-angiogenesis, reducing sinus capillarization through the vascular endothelial growth factor signaling pathway.[26] Moreover, LA also demonstrates therapeutic potential for thromboangiitis obliterans (TAO).[27] LA directly curtailed the phosphorylation and activity of Syk, and decreased the expression of NLRP3 in human umbilical vein endothelial cells as well as the vascular tissues of vasculitis rats.[28] However, whether and how LA regulates renal function, pathology, inflammation, antioxidant and apoptosis in reducing AKI remain largely unknown. Therefore, we aimed to investigate the effects of LA on AKI and the potential mechanisms involving TLR-4/NF-κB in this process.

Materials and Methods

Animals

Eight-week-old male C57BL/6 mice were selected for this research. The animals were obtained from the Laboratory Animal Center of Guangzhou University of Chinese Medicine. All experimental protocols and animal care practices were approved by the Experimental Ethics Committee of Guangzhou University of Chinese Medicine and strictly adhered to the guidelines of the National Institutes of Health.

AKI Mouse Model and Administration

The mice were divided to four groups: a control group, an AKI group, a low-dose LA group (LA-Low, 3 mg/kg), and a high-dose LA group (LA-High, 9 mg/kg). The control group was administered 0.9% sodium chloride solution (Beyotime, China). The AKI model was established in the AKI, LA-Low, and LA-High groups by injecting a 50% glycerol solution (BioFroxx, Germany) at a dose of 0.05 mL/10 g. The LA treatment was administered intragastrically to the LA-Low and LA-High dose groups at 2, 24, and 48 hours after modeling. The control and AKI groups received 0.5% sodium carboxymethyl cellulose (Sinopharm Group Chemical Reagent, China). Blood samples were collected from a subset of anesthetized mice and tested using the kits. Another subset of mice was perfused with PBS (Bausch De BioEngineering, China), and their kidneys were fixed in paraformaldehyde (Beyotime, China) for pathological examination.

Detection of Blood Urea Nitrogen (BUN) and Serum Creatinine (CRE)

Equilibrate the BUN kits (Nanjing Jianjian Bioengineering Institute, China, C013-2-1) to room temperature after taking it out of the 4°C refrigerator. Prepare the enzyme buffer by mixing enzyme stock and diluent at a 3:1000 ratio. Mix thoroughly to make a 100 mmol/L standard stock, then dilute it to a 10 mmol/L working solution. In a centrifuge tube, add 250 μL of buffer and 20 μL of the sample, mix well, and incubate at 37°C for 10 minutes. Add phenol chromogenic reagent and sodium hypochlorite, mix, and incubate again at 37°C for 10 minutes. Transfer the mixture to a 96-well plate and measure the OD at 640 nm. Calculate the BUN results using the formula provided in the manual.

Allow the CRE assay kits (Nanjing Jianjian Bioengineering Institute, China, C011-2-1) to reach room temperature after removing it from the 4°C refrigerator. Add enzyme solution A to the wells, followed by the sample serum, standard, and distilled water. Mix thoroughly and incubate at 37°C for 5 minutes, then measure the absorbance at 546 nm (OD A1). Next, add enzyme solution B, mix again, and incubate at 37°C for another 5 minutes. Measure the absorbance at 546 nm (OD A2). Calculate the CRE results using the formula provided in the manual.

Determination of Reduced Glutathione (GSH) and Superoxide Dismutase (SOD)

The renal tissues were taken and add PBS. Kidney tissue homogenates were obtained by homogenization on ice. The tissues stood for 10 min and were centrifuged.

Following the GSH assay kit instructions (Nanjing Jianxian Bioengineering Institute, China, A006-2-1), dilute the kidney tissue homogenate with saline. Take 100 μL of the diluted sample, add 100 μL of precipitant, mix thoroughly, and centrifuge. Use the supernatant for testing. Prepare the GSH working solution by diluting the GSH stock solution with distilled water at a 1:9 ratio, and create a standard curve. For test wells, add 100 μL of supernatant, 100 μL of buffer, and 25 μL of chromogenic reagent, mixing well. For blank wells, use 100 μL of precipitant, 100 μL of buffer, and 25 μL of chromogenic reagent. For standard wells, add 100 μL of 20 μmol/L GSH, 100 μL of buffer, and 25 μL of chromogenic reagent. Let it stand for 5 minutes, measure the OD at 405 nm, and calculate the GSH concentration using the provided formula.

Operate according to the instructions of SOD detection kits (Nanjing Jianxian Bioengineering Institute, China, A001-3). Prepare the substrate working solution by mixing the substrate stock with buffer at a 1:200 ratio. Dilute the enzyme stock with enzyme diluent at a 1:10 ratio to obtain the enzyme working solution. After preliminary testing, dilute the 10% kidney tissue 100-fold. Set up the wells as follows: Control Wells: Add double-distilled water, then the enzyme working solution and substrate solution. Blank Control Wells: Add double-distilled water, then enzyme diluent and substrate solution. Assay Wells: Add the diluted kidney sample, followed by the enzyme working solution and substrate solution. Assay Blank Wells:

Add the diluted kidney sample, then enzyme diluent and substrate solution. After mixing, incubate the plates at 37 °C for 20 minutes and measure the absorbance at 450 nm. Calculate the SOD activity of the samples using the provided formula.

Kidney Tissue Section Treatment

Paraffin Section

Kidney tissues were fixed in paraformaldehyde for 72 hours and then stored at 4°C. Then the tissues were immersed in PBS, trimmed, placed in embedding boxes, and subjected to gradient dehydration. Following dehydration, the embedding boxes were removed, and the tissues were embedded in wax. The tissues were then positioned at the base of the embedding mold and briefly frozen. Liquid wax was poured into the mold and allowed to solidify on ice. Once solidified, the wax block was trimmed, and the paraffin blocks were mounted on a microtome. The slices were flattened and baked for 2 hours.

Preparation of Frozen Section of Kidney Tissue

OCTsswas purchased from Sakura Americas. The kidney tissues were removed from paraformaldehyde and stored in 30% sucrose solution at 4 °C. After 48h, the tissues were trimmed, frozen, and embedded in OCT.

Pathological Glycogen (PAS) Staining

Pathological glycogen staining solution (PAS) (Zhuhai Bso, China) was used to detect PAS. The assay was meticulously conducted in strict accordance with the manufacturer's detailed instructions.

Hematoxylin and Eosin Staining

Tissues were fixed with PFA, and embedded sections were prepared. After dewaxing, the sections were first submerged in eosin staining solution (Leigen Biotechnology, China) and then rinsed with pure water. The tissues were subsequently stained and washed again. Finally, the tissues were dehydrated, sealed, and observed.

Kidney Tissue Immunofluorescence Staining

Circles were drawn around the tissue using an IHC pen. The tissues were then placed in PBS with Triton X-100 for 20 minutes. Goat serum was added and the reaction was carried out for 30 minutes. The tissues were incubated after the application of primary antibodies (CD45, CD11b: Abcam, UK; TLR-4, NF-κB: Cell Signaling, USA). After rinsing, the tissues were incubated with secondary antibodies in the dark. Following rinse, the tissues were stained with DAPI (Beyotime, China) and incubated for about 10 minutes. Finally, the slices were sealed with glycerol and images were collected.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 was detected following the instructions provided with the ELISA kits (Beyotime, China).

TUNEL (TdT-Mediated dUTP Nick-End Labeling) Apoptosis Detection

The assay was conducted following the instructions provided with the TUNEL cell apoptosis detection kits (Beyotime, China). The renal tissues were circled with an immunohistochemistry pen and immersed in PBS solution. Triton X-100 was subsequently added, and the tissues were incubated for approximately 15 minutes. The renal tissues were then washed twice with PBS for 10 minutes each time. TUNEL test solution was added, and the tissues were incubated. Following this, the tissues were washed. The sections were co-stained with DAPI and visualized under a microscope.

Data Statistics and Analysis

SPSS 21.0 was employed for data analysis, and GraphPad Prism 8.0 was used for plotting. Experimental results were presented as mean ± SEM. P-value of < 0.05 was considered statistically significant.

Results

LA Mitigates Renal Injury and Ameliorates Pathological Damage in AKI

Renal function was assessed based on changes in CRE clearance and serum CRE levels. CRE clearance is a critical indicator for evaluating renal filtration function, as increased CRE levels indicate decreased renal function. BUN is another commonly used marker of renal function; elevated BUN levels may suggest renal insufficiency.

In this study, BUN and CRE levels were measured, and their serum concentrations fluctuated in response to alterations in the glomerular filtration rate. After 72 hours of modeling, the levels of BUN and CRE in the AKI group injected with glycerol at 5 mL/10 g were significantly higher than those in the normal group, while those in the LA group administered 3 mg/kg and 9 mg/kg decreased significantly after treatment (Figure 1A and B). Renal tissue sections were stained with HE and PAS (Figure 1C and D). The AKI group exhibited pathological changes, including renal tubular epithelial cell exfoliation, bare basement membrane formation in the renal tubules, lumen dilation, and the generation of numerous casts. In contrast, LA administration reduced tubular dilation and cast formation in the LA-Low group and LA-High group. These results indicate that LA can significantly alleviate the pathological conditions of AKI and reduce renal injury. Therefore, LA has therapeutic effects on glycerol-induced AKI.

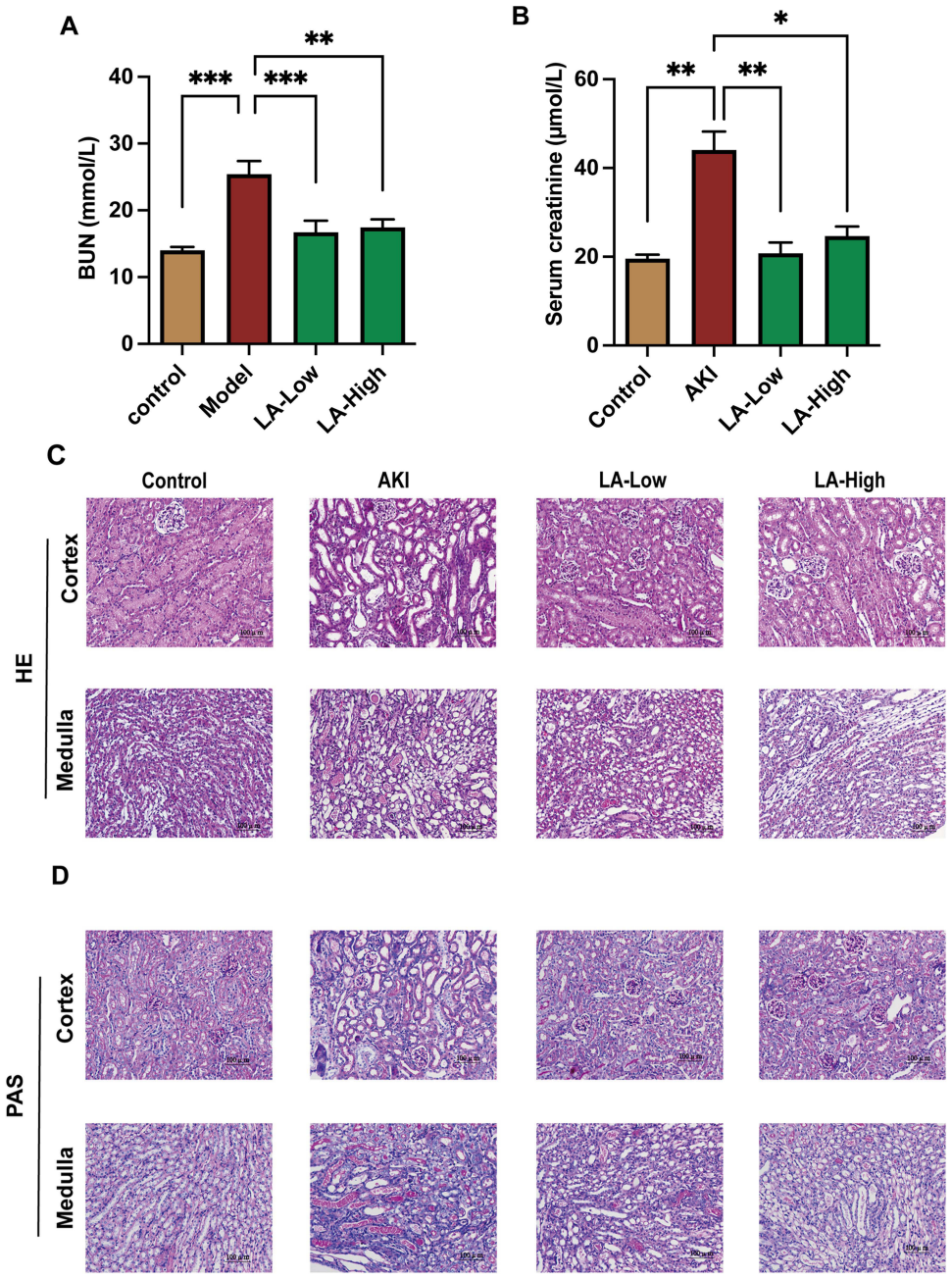


Figure 1 LA can alleviate renal injury in AKI. (**A**) Measurement of BUN following treatment with varying doses of LA (n ≥ 4 for each group). (**B**) Determination of serum CRE levels after treatment with different doses of LA (n ≥ 4 for each group). (**C**) HE staining of renal cortex and medulla following treatment with various doses of LA (20×). (**D**) PAS staining of renal cortex and medulla after administration of different doses of LA (20×). Data are presented as mean ± SEM, *P < 0.05, **P < 0.01, ***P<0.001 (compared to the model group).

LA Reduces Inflammatory Infiltration in AKI

Acute inflammation occurs in the initial stages of AKI, with the release of inflammatory chemokines and cytokines from necrotic tissue, leading to alterations in autoimmune cells. In order to investigate whether LA could reduce inflammatory infiltration following AKI, Immunofluorescence staining of renal tissue was performed.

CD45, a widely expressed lymphocyte antigen and receptor-linked protein tyrosine phosphatase, is found on all leukocytes. Macrophages are marked by the presence of CD11b and F4/80. The results demonstrates that, the AKI group exhibited increased fluorescence intensity and higher levels of CD45, F4/80, and CD11b, while the fluorescence intensity of mice treated with 3 mg/kg LA and 9 mg/kg LA decreased. Notably, macrophage infiltration was significantly reduced in the LA high dose group (Figure 2A–C).

Additionally, the content of IL-6 in the serum of the AKI group was elevated compared to the control group. After LA treatment, the IL-6 levels decreased (Figure 2D). These results indicate that glycerol-induced AKI can lead to inflammatory cell infiltration in kidney tissue and promote the expression of macrophages and neutrophils. LA can inhibit this infiltration, suggesting that LA may slow down the progression of AKI by reducing the infiltration of inflammatory cells and the expression of inflammatory factors.

LA Exhibits Antioxidant Effects in AKI

To assess whether LA has an antioxidant effect on glycerin-induced AKI, we measured levels of GSH, SOD, and myeloperoxidase (MPO). Kidney tissues were collected 72 hours after LA administration. We found that the GSH levels tended to decrease in the AKI group but increased after LA treatment (Figure 3A). Similarly, SOD levels, which were reduced in the AKI group compared to the control group, significantly increased following LA administration (Figure 3B). Immunofluorescence analysis of kidney tissue revealed that MPO content was elevated in the AKI group but decreased after LA treatment. These findings indicate that LA exerts an antioxidant effect (Figure 3C).

LA Decreases Apoptosis in AKI-Affected Renal Tissue

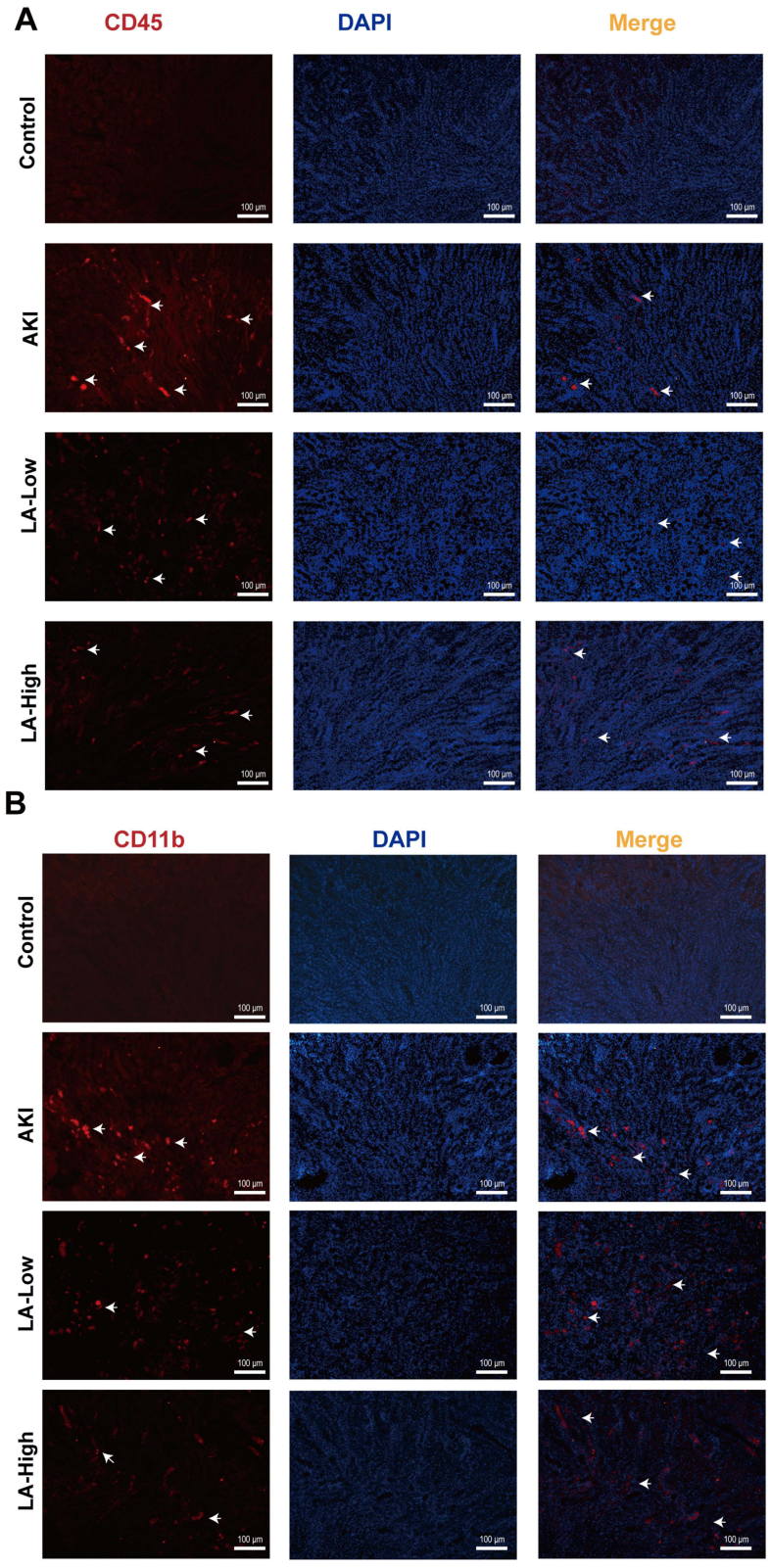
TUNEL is frequently utilized as an indicator of apoptosis. AKI leads to tubule apoptosis, necrosis, cell shedding, and peeling of the tubular basement membrane. To investigate whether LA can reduce apoptosis in renal tissue during acute renal injury, we assessed apoptosis levels in the renal tissues. These results showed that the apoptosis was significantly elevated in the AKI group compared to the control group but was reduced after 3 days of LA treatment (Figure 4A), indicating that LA can reduce apoptosis in kidney tissues.

LA Inhibits Activation of TLR-4/NF-κB Pathway in AKI Mouse Models

Given the numerous reports of TLR-4/NF-κB pathway involvement in inflammatory responses, we investigated whether the protective effect of LA in glycerol-induced AKI mouse models is correlated to this pathway. Kidney tissue samples were collected and subjected to immunofluorescence staining, which revealed an increase in NF-κB content in the AKI group compared to the control group. Following LA treatment, NF-κB levels were reduced in LA administration groups (Figure 5A). Additionally, the kidney-labeled TLR-4 levels were elevated in the AKI group compared to the control group but decreased after treatment with different doses of LA (Figure 5B).

Discussion

In this study, we demonstrated that LA could improve renal function, ameliorate the pathology of AKI, and reduce the inflammatory infiltration of AKI. Furthermore, we found that LA exhibited anti-oxidative properties, reduced apoptosis, and suppressed the activation of the TLR-4/NF-κB signaling pathway in the AKI model (Figure 6). In the AKI group, BUN and CRE levels were significantly elevated but decreased substantially following LA treatment. Additionally, in the AKI group, renal cysts were reduced, renal tubular epithelial cells were shed, bare basement membranes of renal tubules were formed, and the renal tubular lumen was dilated with numerous casts. After LA treatment, renal tubular dilatation and casting were reduced. Collectively, these results indicate that LA administration mitigates renal pathological damage in AKI.



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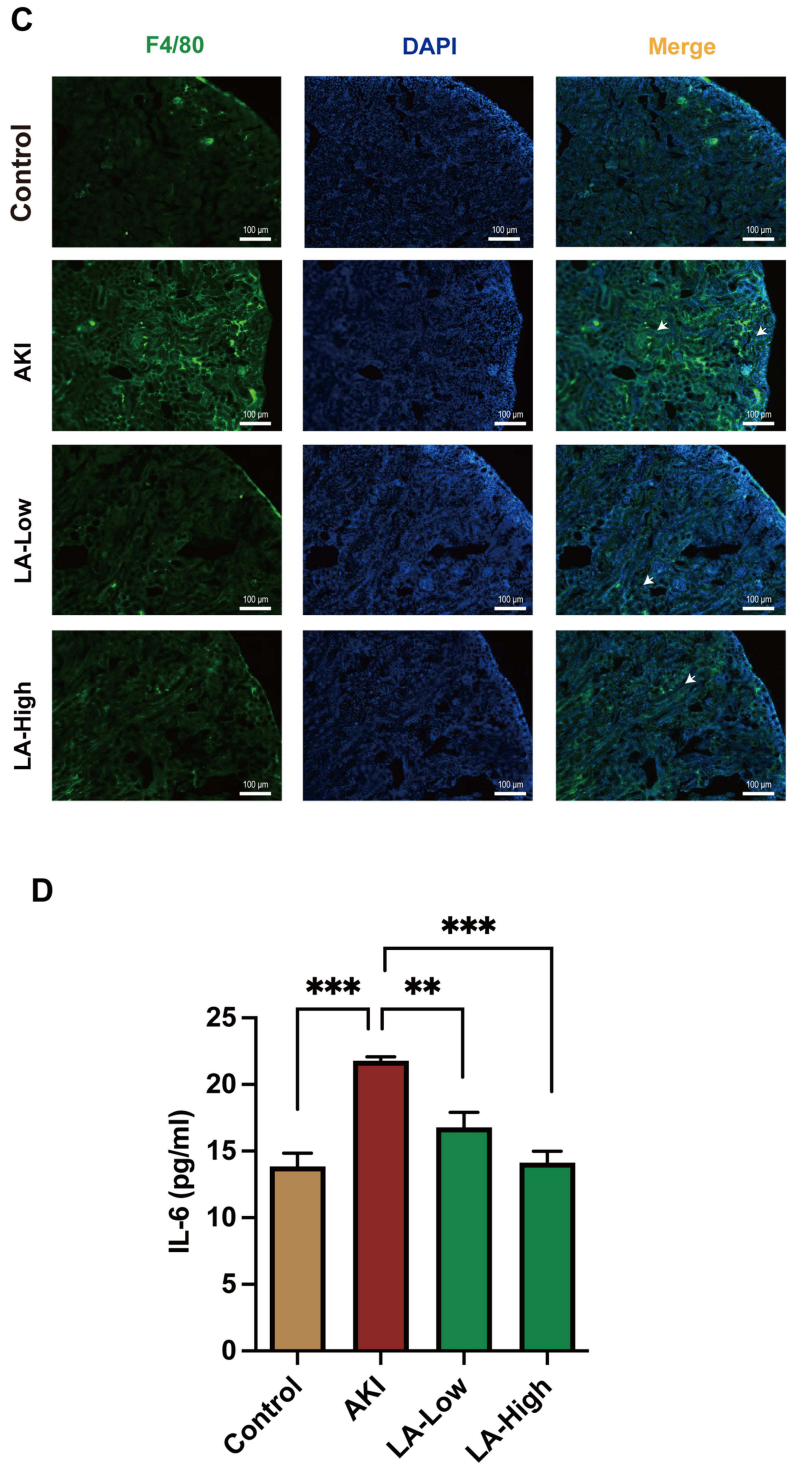

Figure 2 In the glycerol-induced AKI model, LA reduces inflammatory infiltration in renal tissue. (**A**) Immunofluorescence staining of CD45 after administration of varying doses of LA (10×). (**B**) Immunofluorescence staining of CD11b after administration of different doses of LA (10×). (**C**) Immunofluorescence staining of F4/80 following the administration of various doses of LA (10×). (**D**) ELISA analysis of IL-6 expression levels in serum after administration of different doses of LA. Data are presented as mean ± SEM, with n ≥ 4 for each group, *P < 0.05, **P < 0.01, ***P<0.001 (compared to the model group).

To the best of our knowledge, CD11b and F4/80 are markers expressed on macrophages and play crucial roles in regulating pathogen recognition, phagocytosis, and cell survival.[29] Additionally, CD11b activation significantly suppresses LPS-induced proinflammatory responses in macrophage by inhibiting the MAPKs and NF-κB signaling pathways. CD11b activation also inhibits LPS/IFN-γ-induced macrophage proinflammatory responses in vitro. CD45 is a receptor protein tyrosine phosphatase

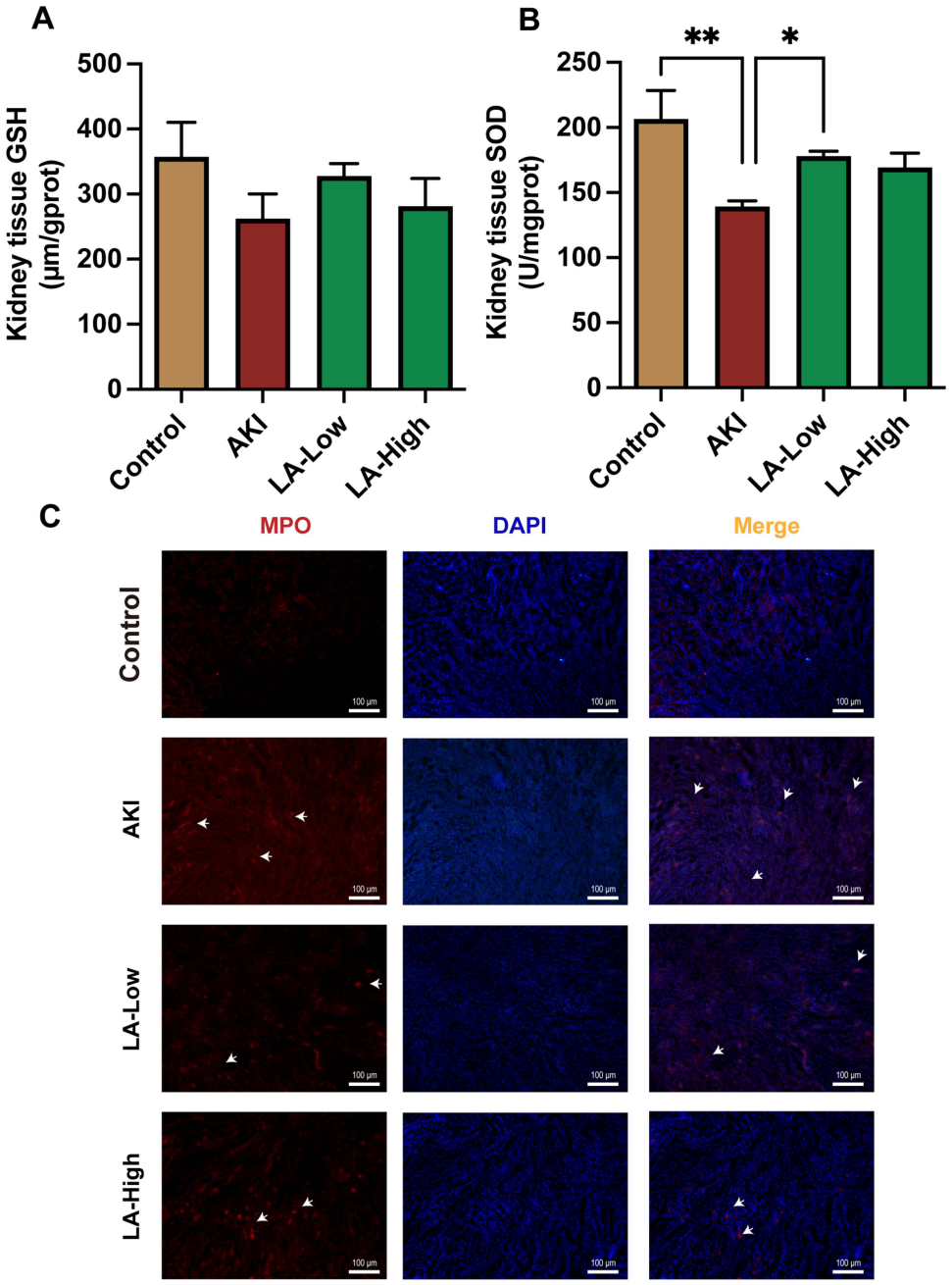

Figure 3 In AKI, LA exhibits antioxidant properties. (**A**) Compared to the model group, GSH levels increased after administration of different doses of LA. (**B**) SOD levels were elevated following the administration of various doses of LA, in comparison to the model group. (**C**) Immunofluorescence staining of MPO in each group after administration of different doses of LA (10×). Data are expressed as mean ± SEM, with n ≥ 4 for each group, *P < 0.05, **P < 0.01 (compared to the model group).

essential for antigen receptor signaling and lymphocyte development.[30] A deficiency in CD45 leads to T and B lymphocyte dysfunction, resulting in combined immunodeficiency.[31] In this study, to evaluate whether the therapeutic effect of LA on kidney injury is involved in cellular immunity and inflammation, we performed immunofluorescence staining of kidney tissues from mice. We demonstrated that LA can reduce the IL-6 content in the AKI mouse model, which confirms the previous conjecture that LA can reduce inflammatory infiltration in AKI. In addition, the content of CD45, CD11b and F4/80 increased in the AKI group, which illustrates that AKI led to macrophage lymphocytes. After LA treatment, the content of CD45, CD11b and F4/80 decreased, which illustrates that LA reduces the number of macrophages and lymphocytes. Therefore, it can be seen that LA inhibits the occurrence of immune response and inflammation, and improves kidney damage.

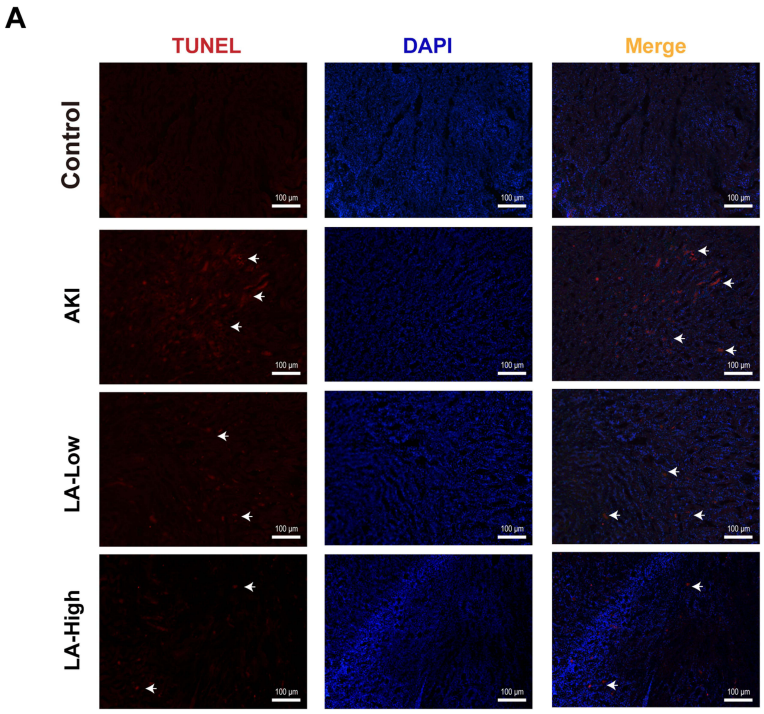

Figure 4 LA significantly reduces apoptosis in renal tissue. (**A**) Immunofluorescence staining with TUNEL was used to analyze renal tissue (10×).

Studies have established that oxidative stress and inflammation contribute to the development of AKI and are closely interconnected. To ascertain whether LA mitigates kidney injury through its antioxidant properties, we evaluated MPO and SOD levels to gauge the antioxidant effect of LA in AKI. GSH, the most abundant non-protein thiol, is a critical factor in REDOX signaling and exerts substantial anti-oxidative stress effects.[32] Extensive research has been conducted on AKI treatment using traditional Chinese medicine compounds, monomers, and active components. The studies have revealed that MPO drives oxidative stress by enhancing the generation of RNS and ROS and by modulating polarization and inflammation-related signaling pathways in microglia and neutrophils. Studies have also demonstrated that medicinal herbal compounds exhibit significant MPO inhibitory effects, showing antioxidant and neuroprotective effects. Flavonoids such as quercetin, rutin, and eriodictyol show neuroprotective and antioxidant effects associated with MPO inhibition. Polyphenolic compounds like resveratrol, carvacrol, curcumin, and Angelica sinensis exhibit potential as MPO inhibitors, useful for combating oxidative stress and inflammation to reduce cerebral ischemia-reperfusion injury.[33] It is evident that dysregulation of MPO can lead to tissue damage and is closely associated with increased oxidative stress and inflammation. Therefore, inhibiting MPO release is critical for alleviating disease phenotypes, making MPO inhibition a promising target for therapeutic intervention. SOD protects kidney cells from oxidative damage by scavenging superoxide free radicals and reducing oxidative stress responses. Additionally, SOD is a crucial anti-aging and antioxidant enzyme, receiving extensive clinical attention in the treatment of AKI. In this study, to investigate the antioxidant effect of LA, we found that MPO expression was significantly downregulated after LA treatment. SOD levels, which were decreased in the AKI group, significantly increased following LA administration, indicating that LA has a potent antioxidant effect. We further explored the potential molecular mechanisms of LA in treating AKI and renal injury. Our results suggest that LA treatment exerts a substantial antioxidant effect on AKI (Figure 6). The plots of TUNEL staining showed that TUNEL increased in the AKI group and decreased after LA treatment. Therefore, these results indicate that LA can mitigate apoptosis in kidney tissue affected by AKI.

TLR-4 responds not only to exogenous microbial motifs but also to molecules released from stressed cells, along with degradation products of endogenous macromolecules. After renal ischemia-reperfusion injury, the release of endogenous ligands interacts with TLR-4 expressed by vascular endothelial cells, renal tubular epithelial cells, and leukocytes. This

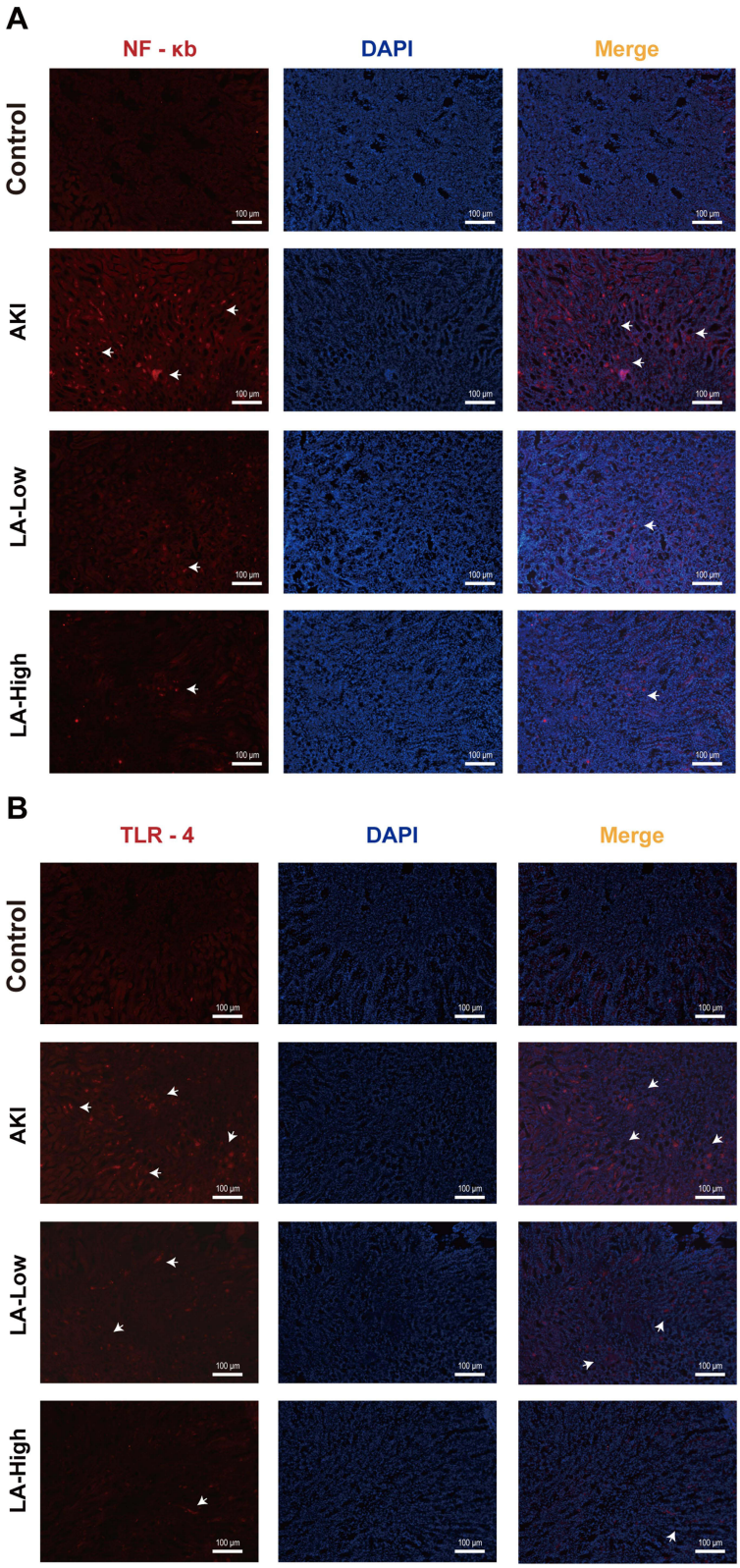

Figure 5 LA suppresses the activation of the TLR4/NF-κB pathway. (**A**) Changes in NF-κB levels after administration of varying doses of LA. (**B**) Alterations in TLR-4 levels following the administration of different doses of LA.

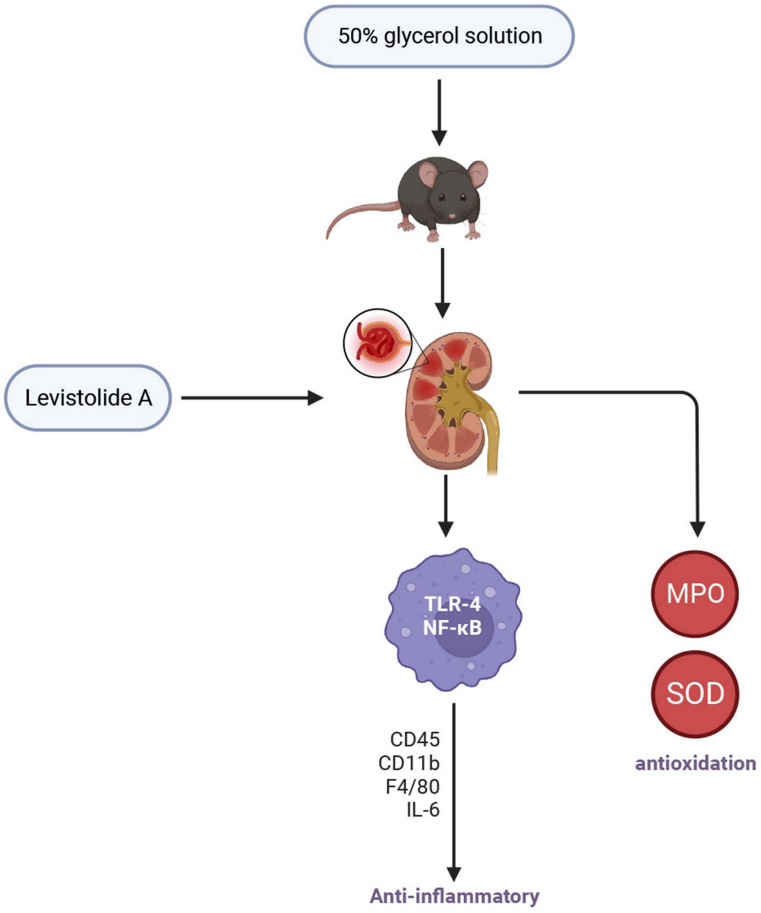

Figure 6 LA improves kidney injury by exerting antioxidant effects and suppressing inflammation.

interaction triggers TLR-4 activation, leading to the release of proinflammatory mediators, increased leukocyte migration and infiltration, and the activation of both the innate and adaptive immune systems, thereby exacerbating renal tissue injury and promoting renal fibrosis.[34] Immunofluorescence analysis revealed elevated NF-κB in the AKI group and a reduction following LA treatment. Similarly, TLR-4 content was increased in the AKI group and reduced after LA treatment, particularly at the low dose. Excessive infiltration of inflammatory cells and associated inflammatory factors can drive the progression from AKI to CKD, with the degree of infiltration correlating with the severity of kidney damage. TLR-4 appears to have a detrimental effect on renal ischemia-reperfusion injury, as TLR-4 deficiency has been shown to protect the kidney from dysfunction and histological damage associated with ischemia-reperfusion injury by reducing granulocyte apoptosis, proinflammatory cytokines, chemokines, and renal tubular epithelial cells. These findings suggest LA can ameliorate renal injury by reducing TLR-4 and NF-κB expression in renal tissue, likely due in part to its inhibition of the TLR-4/NF-κB pathway in a mouse model of AKI.

While our study presents promising results, several limitations must be acknowledged. Firstly, the timeframe for observing the long-term effects of LA was relatively short. Future studies should extend the observation period to assess the sustained efficacy and safety of LA over longer durations. Secondly, our research was conducted solely in a murine model, lacking clinical translation in human subjects. To bridge this gap, subsequent studies should focus on conducting clinical trials to validate its efficacy and safety in humans.

Moreover, the broader therapeutic potential of LA beyond AKI remains underexplored. Future research should investigate LA's effects in other kidney diseases to fully elucidate its pharmacological spectrum. Additionally, optimizing the dosage and delivery methods of LA is crucial for maximizing its therapeutic benefits while minimizing potential side

effects. Pharmacokinetic and pharmacodynamic studies are necessary to determine the optimal dosing regimen and to enhance LA's bioavailability and targeting capabilities.

Conclusion

We found that LA was protective against glycerol-induced AKI in the renal. In the AKI mouse model, renal tubules dilated and epithelial cell shedding resulted in exposure of the basement membrane which produced a large number of casts. LA treatment lowered the levels of BUN and CRE in serum, reduced the dilatation of renal tubules, and decreased the casting of renal tubules. Furthermore, LA treatment attenuated the elevation of CD45, CD11b, F4/80 and IL-6 levels. In summary, LA significantly improves renal function and reduces inflammatory infiltration. The increase in SOD levels and decrease in MPO levels further suggest that LA exerts antioxidant effects. LA alleviates AKI by inhibiting the expression of TLR-4 and NF-κB. These combined actions suggest LA's potential as a novel treatment option for kidney injury. Our experimental findings demonstrate that that LA has a significant therapeutic effect on glycerol-induced AKI in mice by reducing inflammation and providing antioxidant benefits, offering key evidence for its potential in new drug development and clinical applications.

LA has shown promising preclinical efficacy in some disease models, including AKI. These results suggest a strong therapeutic potential, but as with any preclinical finding, the next crucial step is to determine how these findings could translate to human applications.

The dose used in animal models may not directly correlate with safe and effective doses for humans due to differences in metabolism, pharmacokinetics, and toxicity. As such, future studies should focus on determining the appropriate human-equivalent dose using scaling methods, considering both the therapeutic window and potential side effects. Pharmacokinetic studies, including absorption, distribution, metabolism, and excretion (ADME) profiling, are essential to understand how LA behaves in human systems and to refine dosage recommendations. Additionally, investigating the long-term safety of LA in chronic dosing regimens is essential before considering clinical trials.

The findings from this study indicate that LA may offer protection against kidney damage, possibly through mechanisms such as modulation of oxidative stress and inflammation, inhibition of apoptosis, and regulation of the TLR-4/NF-κB signaling pathway, the therapeutic targets identified in animal models may not function in the same way in humans due to genetic, environmental, or physiological differences. To bridge this gap, future studies should first focus on validating the efficacy and safety of LA in humanized animal models.

These models can provide critical insights into how LA interacts with human-like biological systems and offer a better prediction of its performance in clinical settings.

Ethics Statement

The animal study was approved by the Animal Ethics Committee (Approval Number: 20200721005) and was conducted in accordance with local legislation and institutional requirements.

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Disclosure

The authors affirm that this research was carried out free from any commercial or financial relationships that could be perceived as a potential conflict of interest.

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