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Neoastilbin ameliorates sepsis-induced liver and kidney injury by blocking the TLR4/NF-κB pathway

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Summary. Sepsis frequently causes systemic inflammatory response syndrome and multiple organ failure in patients. Neoastilbin (NAS) is a flavonoid that plays vital functions in inflammation. This work aims to investigate the protective effects of NAS against sepsis-induced liver and kidney injury and elucidate its underlying mechanisms. The mouse model was established using cecal ligation puncture (CLP) induction. NAS was given to mice by gavage for 7 consecutive days before surgery. Liver and kidney function, oxidative stress, and inflammatory factors in serum or tissues were examined by ELISA or related kits. The expression of relevant proteins was assessed by Western blot. Hematoxylin and eosin and/or periodic acid-Schiff staining revealed that NAS ameliorated the pathological damage in liver and kidney tissues of CLP-induced mice. NAS improved liver and kidney functions, as evidenced by elevated levels of blood urea nitrogen, Creatinine, ALT, and AST in the serum of septic mice. TUNEL assay and the expression of Bcl-2 and Bax showed that NAS dramatically reduced apoptosis in liver and renal tissues. NAS treatment lowered the levels of myeloperoxidase and malondialdehyde, while elevated the superoxide dismutase content in liver and kidney tissues of CLP-induced mice. The levels of inflammatory cytokines (IL-6, TNF-α, and IL-1β) in the serum and both tissues of CLP-injured mice were markedly decreased by NAS. Mechanically, NAS downregulated TLR4 expression and inhibited NF-κB activation, and overexpression of TLR4 reversed the protective effects of NAS against liver and kidney injury. Collectively, NAS attenuated CLP-induced apoptosis, oxidative stress, inflammation, and dysfunction in the liver and kidney by restraining the TLR4/NF-κB pathway.

Key words: Flavonoids, Sepsis, Acute liver injury, Acute kidney injury, Toll-Like Receptor 4, NF-kappa B

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Introduction

Sepsis is a complicated syndrome caused by bacterial or viral infection, and is one of the leading causes of death in intensive care units (Gharamti et al., 2021). It is estimated that there are 48.9 million sepsis cases every year, with 11 million sepsis-related deaths annually, accounting for 19.7% of global deaths (Wang et al., 2023). Patients often experience systemic inflammatory response syndrome and multi-organ failure. Acute liver and kidney injury are common and serious complications of sepsis (Pool et al., 2018; Ustundag et al., 2023b), and are strongly linked to the overactivation of inflammatory factors (Fang et al., 2010; Chen et al., 2018). In recent years, the advancement of medical technologies, such as the improvement of sepsis screening tools and treatment methods (antibiotic therapy, respiratory support, fluid therapy, and organ function support), has promoted the diagnosis and therapy of sepsis (Evans et al., 2021). However, clinical approaches to prevent and treat sepsis remain restricted (Lin et al., 2018). Therefore, researching the mechanisms and discovering effective medications will contribute to the treatment of septic injury.

The initial acute response of the host to invasive pathogens typically leads to macrophages phagocytosing pathogens and the production of several pro-inflammatory cytokines. The inflammatory response imbalance is generally the primary and most essential basis for the pathogenesis of sepsis (O'Leary et al., 2010; D'Elia et al., 2013). During the early stage of sepsis, excessive activation of the inflammatory response and dysregulated expression levels of inflammatory factors are the main causes of multiple organ dysfunction, such as liver and kidney (Cohen, 2002, 2015). In the process of sepsis, oxidative stress activates a series of transcription factors, which subsequently activate various genes, including several proinflammatory cytokines. Pathologically damaging factors result in abnormal and excessive cell apoptosis, which leads to microvascular dysfunction and organ failure (Cao et al., 2019). Based on these facts, it is reasonable to believe that simultaneous regulation of inflammatory factors,

apoptosis, and oxidative stress would be more efficient in preventing organ damage than a treatment that simply targets one of these factors.

In sepsis, the toll-like receptor 4 (TLR4)/Nuclear Factor-kappa B (NF-κB) pathway is a classical signaling pathway triggered by pathogen-related molecular patterns or danger-related molecular patterns. Numerous studies have shown that NF-κB exerts important functions in organ damage caused by sepsis (Aiga et al., 2006; Kawai and Akira, 2007). Acute liver and kidney injury caused by sepsis is triggered by TLR4 activation through bacterial products or cytokines, such as TNF-α or IL-1β. This may further activate the transcription factor NF-κB, increasing the transcription of proinflammatory, apoptotic, and other sepsis-related inflammatory factors (Liu et al., 2015). Neoastilbin (NAS), a flavonoid isolated from the rhizome of *Smilax glabra*, exhibits a variety of anti-inflammatory properties. NAS has been reported to have a critical regulatory role in gouty arthritis by regulating the NF-κB pathway (Xu et al., 2022). However, the effect of NAS on sepsis-induced organ damage is still unclear. Thus, we hypothesized that NAS might affect sepsis-induced organ damage by regulating the NF-κB pathway.

Cecal ligation puncture (CLP)-induced sepsis more closely mimics human sepsis than other sepsis models, and is referred to as the "gold standard" rodent model for abdominal sepsis (Drechsler and Osuchowski, 2021). In this investigation, we used the CLP sepsis mouse model to evaluate the impacts and mechanisms of NAS on septic organ injury. The findings demonstrated that NAS exerted anti-apoptotic, anti-inflammatory, and anti-oxidative stress effects in septic mice, and reduced pathological liver and kidney injury caused by CLP. Moreover, the protective effects of NAS on septic mice were at least partly through regulating the TLR4/NF-κB pathway.

Materials and methods

Establishment of the animal model

BALB/C mice (20±2 g, Shanghai Slac Laboratory Animal Co., Ltd, China) were maintained in polycarbonate cages in a standard room (21±2°C, 45-65% relative humidity, 12 h light/dark cycle) for one week prior to the experiment. The animals were fed a regular laboratory diet and had unrestricted access to water. The standard substance NAS (dry powder, HPLC≥98%) was commercially purchased from Shanghai Yuanye Biotechnology Co., Ltd (China). NAS was dissolved in saline solution for subsequent experiments. The mice (a total of 12) were randomly assigned into sham (n=6) and 50 mg/kg NAS groups (n=6) to examine the hepatotoxicity and nephrotoxicity of NAS in mice. Then, a total of 48 mice were randomly divided into sham, adenovirus-negative control (ad-NC), adenovirus-TLR4 (ad-TLR4), CLP, CLP+12.5 mg/kg NAS, CLP+25 mg/kg NAS, CLP+50 mg/kg NAS, and CLP+50 mg/kg NAS+ad-TLR4 groups (n=6 per group). The animal experimental protocol is shown in Figure 1. The concentrations of NAS administered were referred from prior research (Xu et al., 2022). CLP surgery was conducted in accordance with previous work (Aziz et al., 2018). The CLP model was created using inhalation anesthesia with 2% isoflurane. After shaving the abdomen, a 2-cm incision was performed to expose the abdominal viscera. The cecum was isolated and ligated with a 3-0 silk ligature 0.5 cm from the end. A 22-gauge needle was used to penetrate the cecum, and a small amount of fecal content was extruded. After returning the cecum to the abdominal cavity, the abdominal incision was sutured. Mice in the sham, 50 mg/kg NAS, ad-NC, and ad-TLR4 groups underwent open surgery with exposed cecum but without ligation or perforation. One mL of normal saline was injected subcutaneously immediately after surgery. Mice in the 50 mg/kg NAS, CLP+12.5 mg/kg NAS, CLP+25 mg/kg NAS, CLP+50 mg/kg NAS, and CLP+50 mg/kg NAS+ad-TLR4 groups were given designated doses of NAS by gavage for 7 consecutive days before surgery (Xu et al., 2022). Gateway LR Clonase II (Invitrogen, Carlsbad, CA, USA) was utilized to transfer mouse TLR4 cDNA or a negative control to the adenovirus vector (Lee et al.,

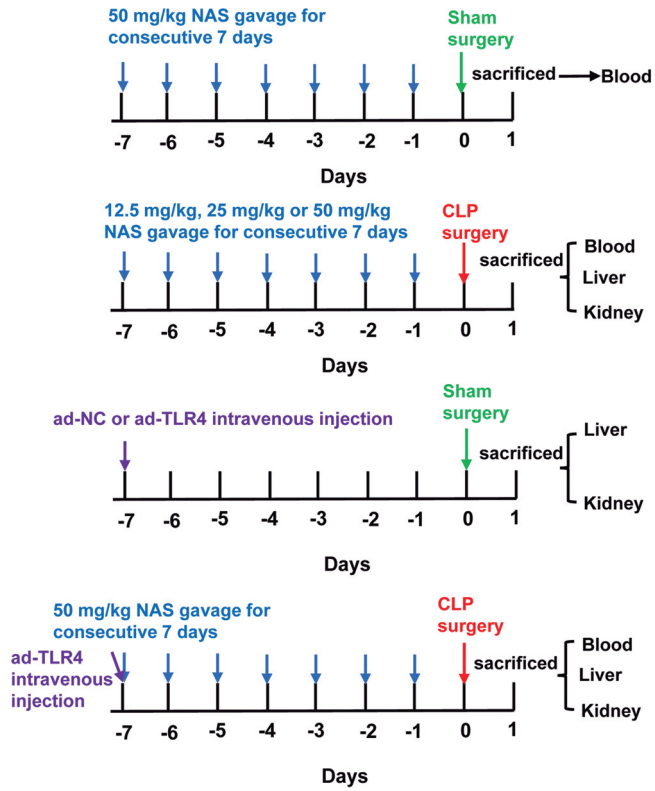

Fig. 1. The animal experimental protocol of this study. NAS by gavage for 7 consecutive days before surgery. TLR4 adenovirus (ad-TLR4, 10^7 particles/μL) or its control (ad-NC) were administered by intravenous injection one week before the operation. Then, 24h after surgery, mice were euthanized and samples were collected.

2009), and mice in the ad-NC, ad-TLR4, and CLP+50 mg/kg NAS+ad-TLR4 groups received 20 μL of negative control or TLR4 adenovirus vectors (10^7 particles/μL) by intravenous injection one week before the operation (Hao and Wei, 2022). Subsequently, 24h after CLP, all animals were euthanized by cardiac exsanguination under anesthesia (Alves et al., 2022). The blood samples were collected and centrifuged at 3000 g for 15 min to obtain serum. Sera were stored at -20°C until analysis. The liver and kidney tissues were all collected for further examination. The animal experiments were approved by the Ethics Committee of the Beijing Tongren Hospital Affiliated to Capital Medical University (Approval number: TREC2020-KY013) and were conducted in accordance with Chinese legislation on the use and care of laboratory animals.

Biochemical indicator tests

The characteristic indexes in liver and kidney tissues or serum from each group were measured. The tissues were cut into small pieces and homogenized in nine times the volume of normal saline. After centrifugation at 2500 rpm for 10 min, the supernatant was collected. The protein concentration was determined with the bicinchoninic acid assay (Solarbio, Beijing, China). The aspartate aminotransferase (AST) assay kit (C010-2-1), Alanine aminotransferase (ALT) assay kit (C009-2-1), Blood urea nitrogen (BUN) assay kit (C013-2-1), Creatinine assay kit (C011-2-1), Myeloperoxidase (MPO) assay kit (A044-1-1), Superoxide Dismutase (SOD) assay kit (A001-3-2), and Malondialdehyde (MDA) assay kit (A003-1-2) were utilized to test the levels of these indicators. All the above kits were provided by the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The experimental protocols were carried out according to the manufacturer's instructions.

Inflammatory factor tests

The serum levels of IL-6, TNF-α, and IL-1β from each group at 24h after CLP were measured with mouse IL-6 (ml098430), TNF-α (mIC50536-1), and IL-1β (ml098416) ELISA kits (all from MLBIO Biotechnology Co., Ltd, Shanghai, China), respectively, following the corresponding protocol. The absorbance values were read within 15 min with a microplate reader (Thermo Fisher, Vantaa, Finland) at 450 nm. The results were obtained by comparing them with the standard product in the designated kit. The contents of IL-6, TNF-α, and IL-1β were represented as pg/mL.

Cell apoptosis detection

The liver and kidney tissues were fixed in 10% formalin and paraffin-embedded. Then, the paraffin sections of 5-μm thickness were dewaxed for 10 min in xylene before being rehydrated in gradient ethanol (Zhao et al., 2022). Proteinase K (20 g/mL, without DNase) was applied to the sections and kept at 37°C for 20 min. After washing three times with PBS, the slices were stained in the dark at 37°C for 1h with 50 μL of One Step TUNEL Apoptosis assay kit (Beyotime, Shanghai, China), and DAPI was used to stain the nucleus. A fluorescent microscope (Shanghai Optical Instrument Factory, Shanghai, China) was used to examine the nuclear alterations and apoptosis of the tissues. Finally, the apoptotic index was calculated as the percentage of positively stained cells from six random fields from each slide.

Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining

Kidney and liver tissues were fixed in 4% paraformaldehyde overnight at room temperature. They were paraffin-embedded and sliced into 4 μm-thick sections. To perform H&E staining, the sections were stained with hematoxylin (Solarbio) for 5 min, followed by eosin (Solarbio) for 1 min. Under a light microscope (Sigma-Aldrich, St. Louis, MO, USA), the pathological alterations in the kidney and liver tissues were observed, and the injury scores were calculated. Histological changes were scored on a scale of 0 (no damage), 1 (1-10% damage), 2 (11-25% damage), 3 (26-45% damage), 4 (46-75% damage), and 5 (>76% damage), and four variables were summed to represent the organ injury according to a previous study (Gao et al., 2022). The four variables in the liver tissues include congestion, edema, infiltration of polymorphonuclear leukocytes and monocytes, and necrosis (Malkoc et al., 2020). The features in the kidney tissues include loss of brush border of renal tubular epithelial cells, tubular epithelial swelling and vacuolization, inflammatory infiltration, and tubular necrosis (Sun et al., 2021; Chi et al., 2022).

For PAS staining of the kidney, the 4-μm sections were stained with PAS for 10 min at room temperature. Images were observed under a light microscope.

Western blot

At the 24-hour time point, after washing three times with ice-cold PBS, the liver and kidney tissues were collected and immediately stored at -80°C. One week later, the frozen tissues were thawed, cut into small pieces, and homogenized in RIPA lysis buffer (Elabscience, Wuhan, China) containing protease inhibitor (PMSF) and phosphatase inhibitor cocktail. After centrifugation at 13,200 g for 30 min, the supernatant was collected to analyze the protein content using a bicinchoninic acid assay (Solarbio). The protein samples (25 μg) were separated using 15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF, Beyotime) membranes before being blocked with 5% skimmed milk. The membranes were then incubated overnight at 4°C with primary antibodies against Bcl-2 (1:2000, ab182858, Abcam, Cambridge, MA, USA),

Bax (1:1000, ab32503, Abcam), IL-6 (1:2000, ab290735, Abcam), TNF-α (1:2000, ab183218, Abcam), IL-1β (1:2000, ab254360, Abcam), TLR4 (1:2000, ab22048, Abcam), NF-κB (1:2000, #8242, Cell Signaling Technology, Beverly, MA, USA), p-NF-κB (1:1000, sc-166,748, Santa Cruz, Dallas, TX, USA), and β-actin (internal control, 1:3000, WL01372, Wanlei, Shenyang, China). The membranes were treated with secondary antibodies conjugated to horseradish peroxidase for 1h after washing three times with TBST. The image was visualized with enhanced chemiluminescence (ECL, Beyotime) and quantitatively analyzed with Image J 1.51 (National Institutes of Health, Bethesda, MD, USA). All antibodies were previously tested for specificity and sensitivity.

Statistics

All data were analyzed by GraphPad Prism 8.0 statistical software (USA) and presented as mean±standard deviation (SD). The difference between the two groups was calculated by the t-test, and by One-way ANOVA followed by Dunnett's post hoc test in multiple groups. $P<0.05$ was defined as statistically significant. At least three replicates were set for each experiment.

Results

50 mg/kg of NAS has no hepatotoxicity or nephrotoxicity in mice

The liver and kidney function indexes were determined to evaluate the hepatotoxicity and nephrotoxicity of NAS in mice. There were no significant differences in serum levels of AST, ALT, BUN, and Creatinine between the sham and 50 mg/kg NAS groups (Fig. 2A-D). These data suggested that 50 mg/kg of NAS did not have any hepatotoxicity or nephrotoxicity in mice.

NAS improves liver function and attenuates liver injury in a CLP-induced sepsis mouse model

Subsequently, 12.5 mg/kg, 25 mg/kg, and 50 mg/kg of NAS were administered to CLP-induced mice to examine the effects of NAS on liver function and injury. All mice had survived at the 24h time point. Compared with the sham group, CLP group mice exhibited piloerection, lethargy, and immobility. While these abnormal behaviors were improved in the CLP+25 mg/kg NAS and CLP+50 mg/kg NAS groups. The concentrations of AST and ALT were sharply increased under CLP induction, while their levels were gradually reduced with the increase in NAS concentration (Fig. 3A,B). When compared with the sham group, the liver tissues of CLP mice showed an accumulation of hepatocyte degeneration, inflammatory cellular infiltrates (lymphocytes and Kupffer cells), and localized necrosis (Fig. 3C). The liver tissue injury score was considerably higher than in the sham group (Fig. 3D). However, the NAS therapy groups showed better liver pathological alterations, exhibiting less hepatocyte degeneration, inflammation (lymphocytes and Kupffer cells), and necrosis, than the CLP group (Fig. 3C). The liver injury scores of the NAS treatment groups were

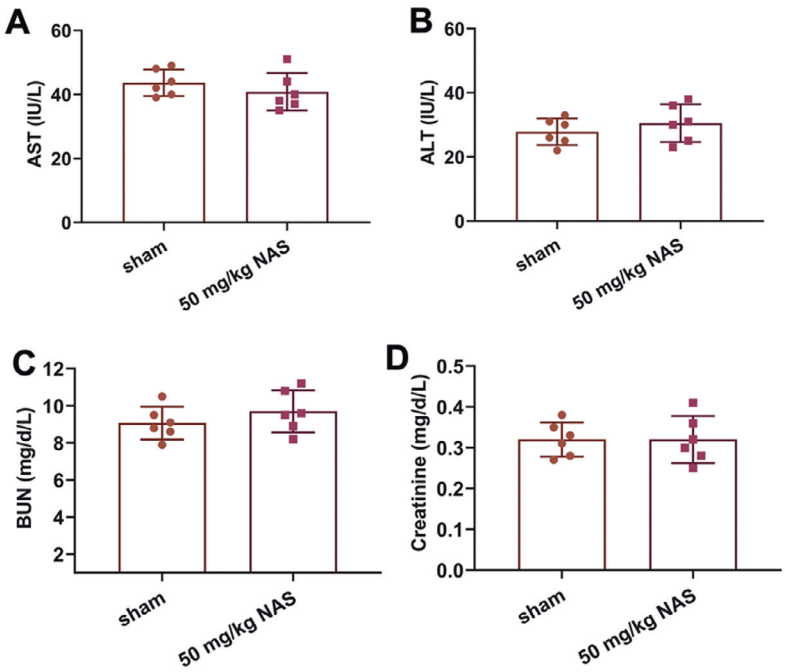

Fig. 2. 50 mg/kg of NAS has no hepatotoxicity or nephrotoxicity in mice. The levels of AST **(A)**, ALT **(B)**, BUN **(C)**, and Creatinine **(D)** in the serum of mice in the sham and 50 mg/kg NAS groups were detected with biochemical kits.

lower than those of the CLP group in a dose-dependent manner (Fig. 3D). These results revealed that NAS could improve liver function and attenuate liver injury in mice with CLP-induced sepsis.

NAS protects kidney function and reduces kidney injury in a CLP-induced sepsis mouse model

The effects of NAS on kidney function and injury were also evaluated. CLP surgery significantly increased the concentrations of BUN and Creatinine, which were gradually reduced by NAS in a dose-dependent manner (Fig. 4A,B). Renal histology by H&E and PAS staining revealed that the CLP group had glomerular edema and injury, as well as inflammatory cell infiltration in the glomerulus, as compared with the sham group. In addition, tubular injury was seen in the CLP group, including loss of the tubule brush border, tubular edema, and intratubular casts. Nevertheless, NAS administration reduced glomerular edema and injury and decreased CLP-induced tubular injury (Fig. 4C). The renal pathological score of the CLP group was notably higher than that of the sham group. Also, renal tissue injury scores in the NAS treatment groups decreased dramatically compared with CLP-induced mice (Fig. 4D). Taken together, NAS treatment could improve kidney histological changes and reduce kidney injury in septic mice.

NAS reduces apoptosis and oxidative stress in the liver and kidney of a CLP-induced sepsis mouse model

To clarify the protective mechanism of NAS against CLP-induced liver and kidney injury in mice, TUNEL labeling was performed to examine apoptotic cells in the tissues (Fig. 5A). Compared with the sham group, the number of TUNEL-positive cells in the CLP group was prominently increased. With increasing concentrations of NAS, the number of TUNEL-positive apoptotic cells gradually reduced (Fig. 5B,C). Moreover, the expression of anti-apoptotic protein Bcl-2 was decreased, whereas pro-apoptotic protein Bax was increased in the liver and kidney tissues after CLP surgery, which was restored by

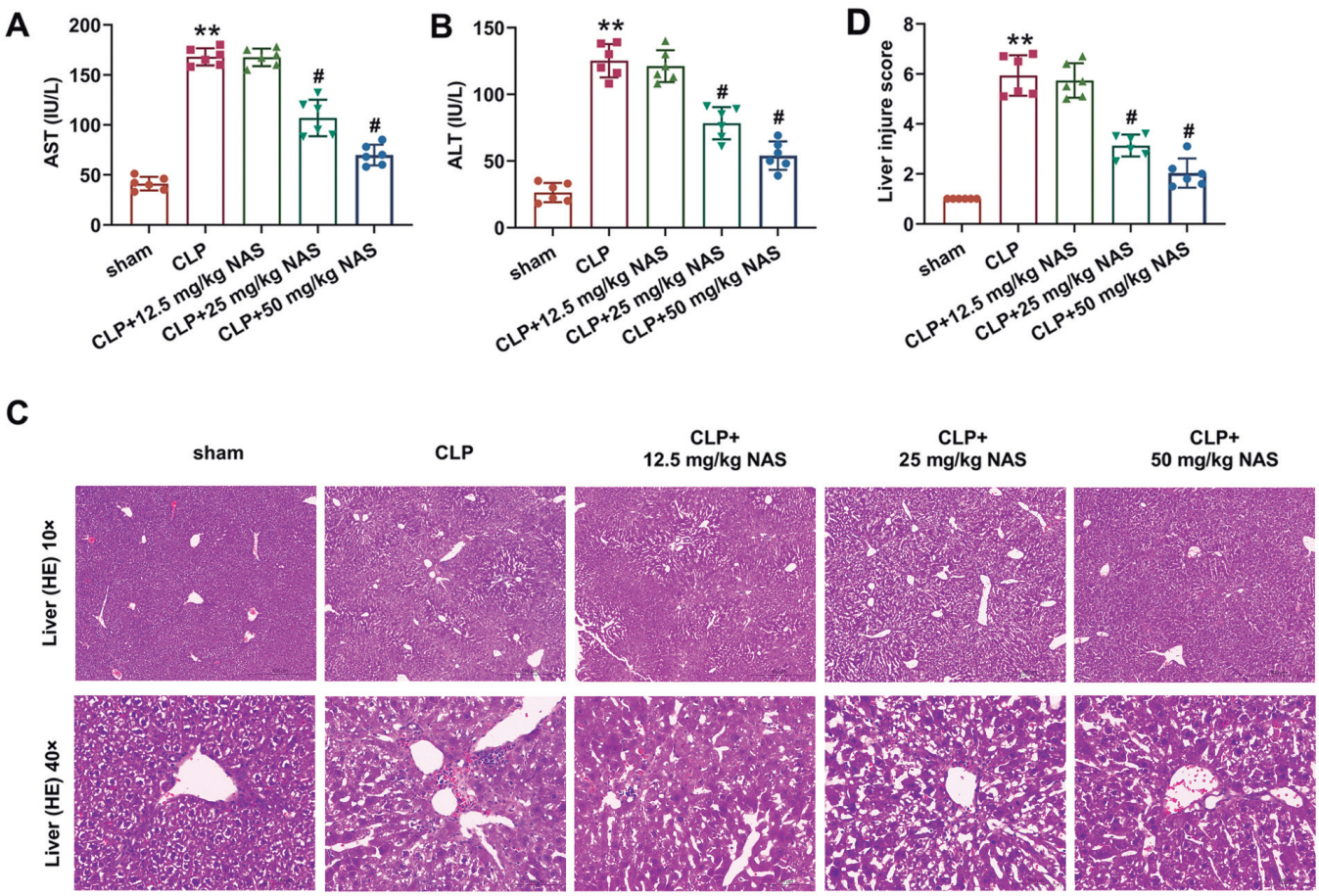

Fig. 3. NAS improves liver function and attenuates liver injury in CLP-induced sepsis mouse models. The levels of AST **(A)** and ALT **(B)** in the serum of mice from different groups were detected with biochemical kits. **C.** The liver tissues of mice from each group were detected by H&E staining and tissue injury scores were calculated. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group. C, × 100 and × 400.

NAS in a dose-dependent manner (Fig. 5D,E). Besides, the levels of MPO and MDA in liver and kidney tissues were markedly elevated, however, the levels of SOD were notably reduced after CLP. The administration of NAS reversed all these trends caused by CLP (Fig. 5F-H). These results indicated that NAS therapy alleviated apoptosis and oxidative stress in the liver and kidney of CLP-treated mice.

NAS reduces inflammation in the liver and kidney of a CLP-induced sepsis mouse model

To understand the effects of NAS on inflammation, serum levels of IL-6, TNF-α, and IL-1β were measured. As depicted in Figure 6A, these levels were notably higher in the CLP group than in the sham group. With increasing concentrations of NAS, the levels of these inflammatory factors gradually decreased (Fig. 6A). Similar results were found in the Western blot assay. The protein expression of IL-6, TNF-α, and IL-1β both in the liver and kidney of CLP-treated mice was markedly reduced by NAS administration (Fig. 6B,C). These data indicated that NAS treatment reduced the production of inflammatory cytokines in septic mice.

NAS regulates the TLR4/NF-κB pathway in CLP-injured mice

To explore the mechanism of NAS in CLP-induced injury, the protein expression of TLR4, p-NF-κB, and NF-κB in liver and kidney tissues of septic mice was examined. Compared with the sham group, TLR4 expression and the ratio of p-NF-κB to NF-κB was prominently elevated in the CLP group, which was notably reduced after treatment with NAS (Fig. 7A,B). To further demonstrate the effect of NAS on this pathway, we performed rescue experiments using adenovirus injection to elevate TLR4 expression. Figure 7C illustrated that TLR4 expression in the liver and kidney of mice was successfully enhanced by adenovirus injection. Likewise, the decrease in TLR4 expression and the ratio of p-NF-κB to NF-κB caused by NAS treatment in the liver and kidney of CLP-induced mice was notably reversed by the TLR4 adenovirus (Fig. 7D,E). Our results suggested that NAS might ameliorate CLP-induced injury by regulating the TLR4/NF-κB

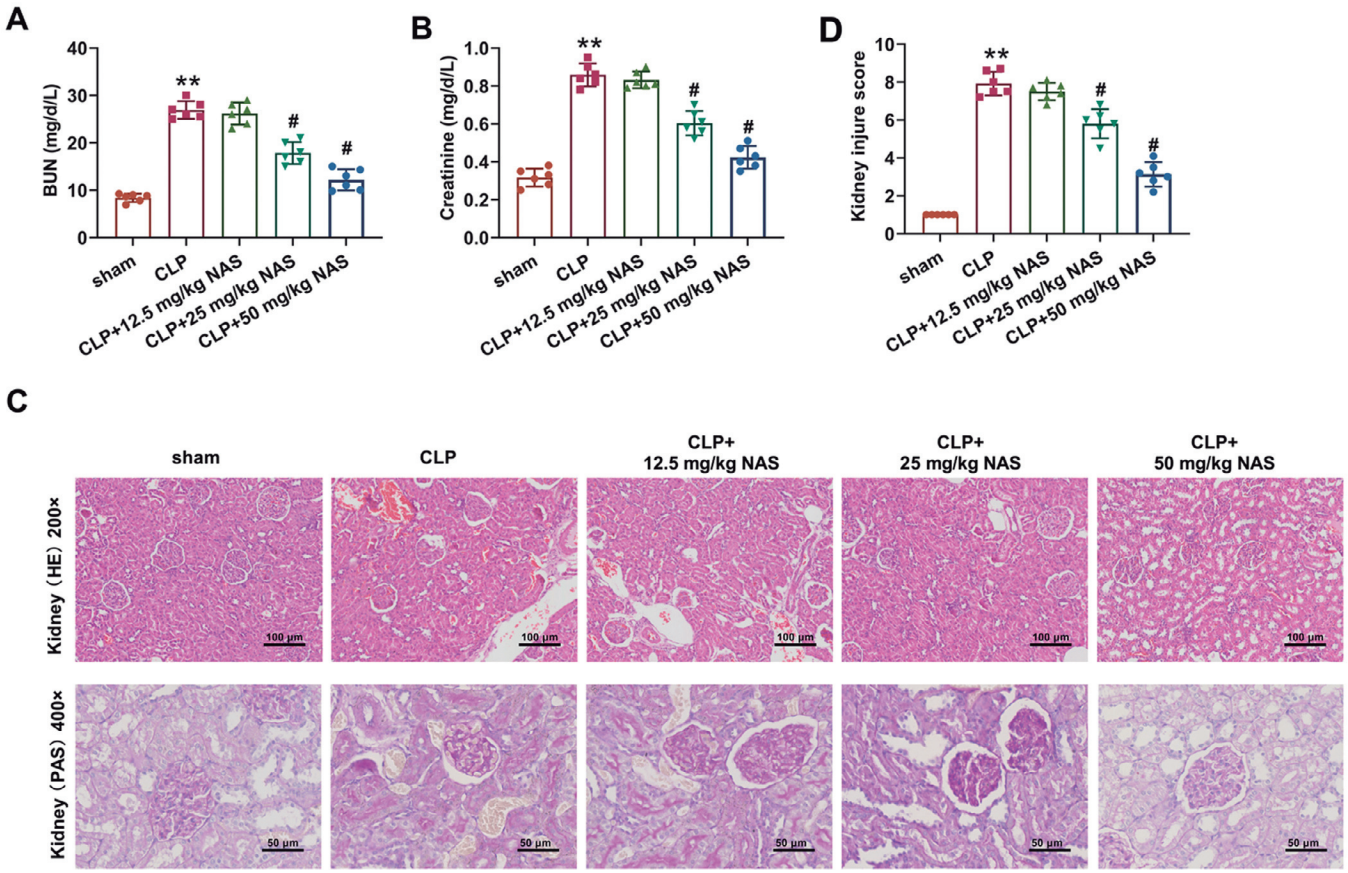

Fig. 4. NAS protects kidney function and reduces kidney injury in CLP-induced sepsis mice. **A.** The levels of BUN and Creatinine in the serum of mice from different groups were detected by biochemical kits. **C.** The kidney tissues of mice in each group were detected by H&E and PAS staining. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group. C, × 200 and × 400.

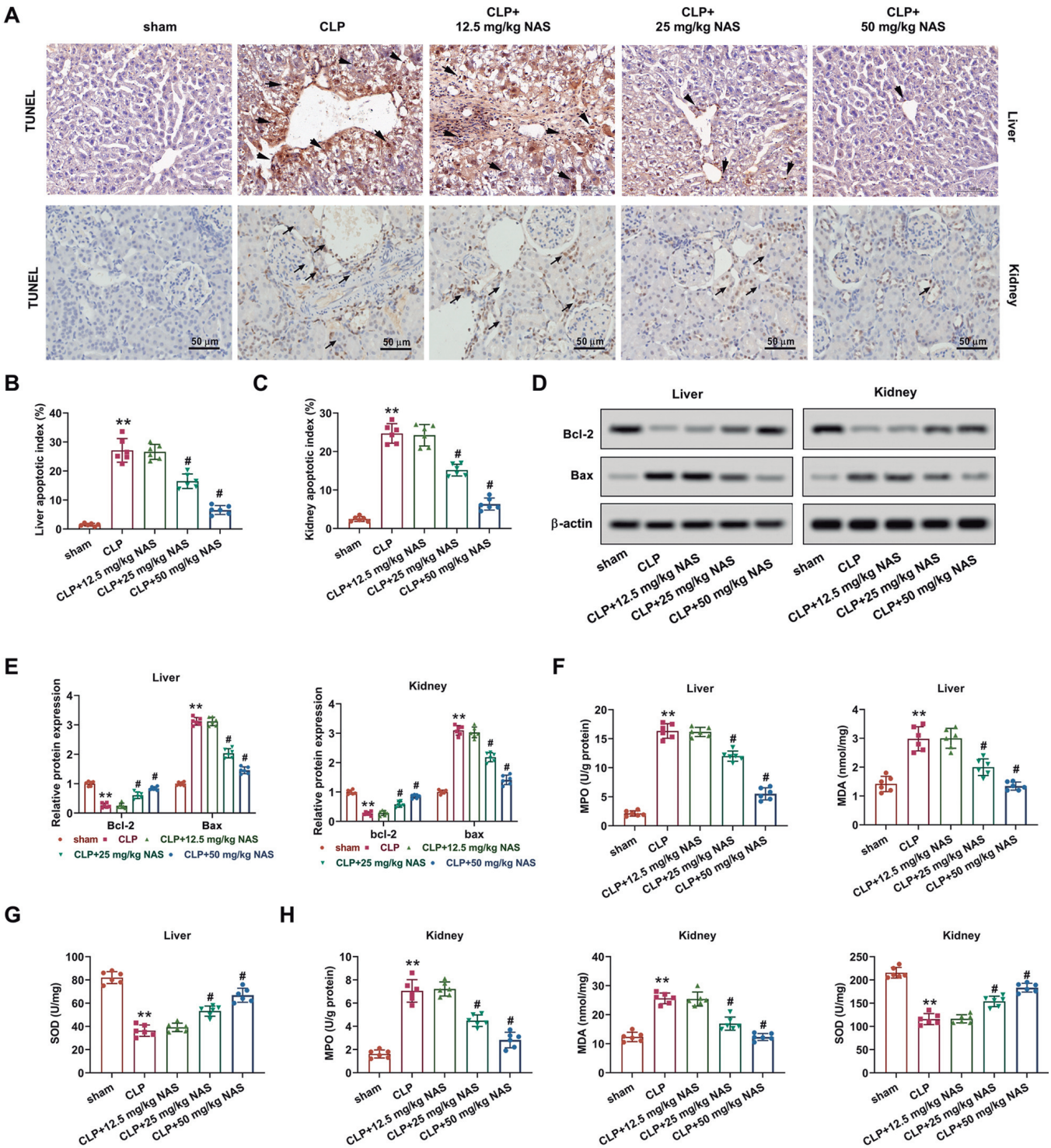

Fig. 5. NAS reduces liver and kidney tissue apoptosis and oxidative stress in a CLP-induced sepsis mouse model. **A.** Apoptosis in liver and kidney tissues was examined by TUNEL staining. Black arrows indicated positive staining for apoptotic cells. **B, C.** Quantification of TUNEL-positive liver and kidney cells in sections from each group. **D, E.** Protein levels of Bcl-2 and Bax were detected by Western blot in liver and kidney tissues. **F, G.** Activities of MPO, MDA, and SOD contents in liver tissues from the different groups. **H.** Activities of MPO, MDA, and SOD contents in kidney tissues from the different groups. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group. A, × 400.

pathway.

NAS reduces injury and apoptosis in CLP-induced septic mice by regulating the TLR4/NF-κB pathway

As indicated in Figure 8A, the liver and kidney tissue injury scores in CLP mice decreased with 50 mg/kg NAS treatment, however, overexpression of TLR4 caused significant increases in injury scores. In addition, the TUNEL assay revealed that the level of apoptosis in the liver and kidney of septic mice was partially increased by TLR4 overexpression when compared with the CLP+50 mg/kg NAS group (Fig. 8B,C). These data indicated that NAS reduced injury and apoptosis of the liver and kidney in septic mice by regulating the TLR4/NF-κB pathway.

NAS reduces oxidative stress and inflammation in CLP-induced septic mice by regulating the TLR4/NF-κB pathway

To further prove the effects of NAS on the sepsis-related TLR4/NF-κB pathway, the levels of oxidative stress indicators and inflammatory cytokines were measured. Here, we examined the levels of MPO, MDA, and SOD in the liver and kidney of septic mice from each group. MPO and MDA levels were lowered and SOD contents were increased by NAS, however,

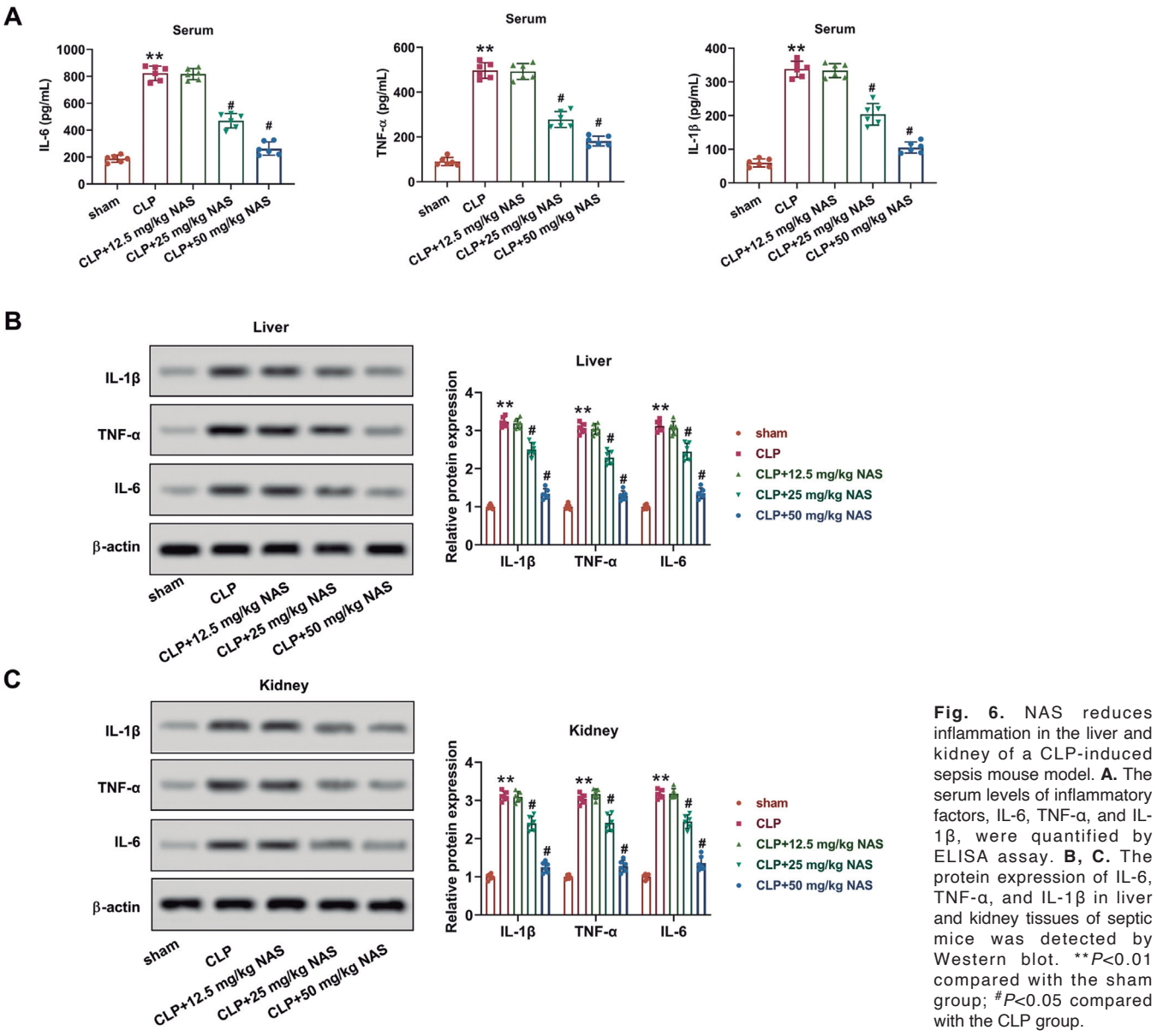

Fig. 6. NAS reduces inflammation in the liver and kidney of a CLP-induced sepsis mouse model. **A.** The serum levels of inflammatory factors, IL-6, TNF-α, and IL-1β, were quantified by ELISA assay. **B, C.** The protein expression of IL-6, TNF-α, and IL-1β in liver and kidney tissues of septic mice was detected by Western blot. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group.

overexpression of TLR4 partially reversed their levels (Fig. 9A-C). Then, inflammatory cytokines, IL-6, TNF-α, and IL-1β in the serum of septic mice were examined by ELISA. All these parameters were markedly elevated after TLR4 overexpression compared with the CLP+NAS (50 mg/kg) group (Fig. 9D,E). Similarly, compared with the CLP+NAS (50 mg/kg) group, the levels of IL-6, TNF-α, and IL-1β in liver and kidney tissues were significantly increased by TLR4 adenovirus (Fig. 9F,G). These results suggested that NAS reduced oxidative stress and inflammatory factors in septic mice by regulating the TLR4/NF-κB pathway. The general idea of this study is represented in Figure 9H.

Discussion

Sepsis is a systemic inflammatory response syndrome that damages cells and tissues and disrupts metabolism, resulting in the failure of numerous essential organs (Minasyan, 2017; Venet and Monneret, 2018). Severe sepsis is known for causing multiple organ failure, including organ histopathological parameters as well as biochemical and hematological abnormalities (Minasyan, 2017). Hence, it is particularly critical to protect organs from injury and alleviate organ damage after sepsis.

Flavonoids, one of the secondary metabolites in plants, have key pharmacological activities including anti-oxidant, anti-inflammation, etc. (Tsai et al., 2018; Zhang et al., 2020). They also exert vital protective effects against organ damage caused by sepsis. For instance, isorhamnetin had a potential therapeutic effect on Escherichia coli-induced sepsis (Chauhan et al., 2019). Pilloin markedly suppressed the production of pro-inflammatory cytokines in RAW 264.7 macrophages and septic mice (Tsai et al., 2018). Fisetin ameliorated multiple organ dysfunction in septic mice (Zhang et al., 2020). However, the effects of NAS on CLP-induced sepsis are still unclear. In our investigation, NAS did not affect liver and kidney functions at the concentration of 50 mg/kg, which indicated that it was a safe agent with no noticeable harmful or adverse effects at this dose. Hence, 12.5, 25, and 50 mg/kg of NAS were used to investigate the role of NAS in CLP-induced sepsis in this study. NAS treatment improved histological damage in the liver and kidney of septic mice and lowered the

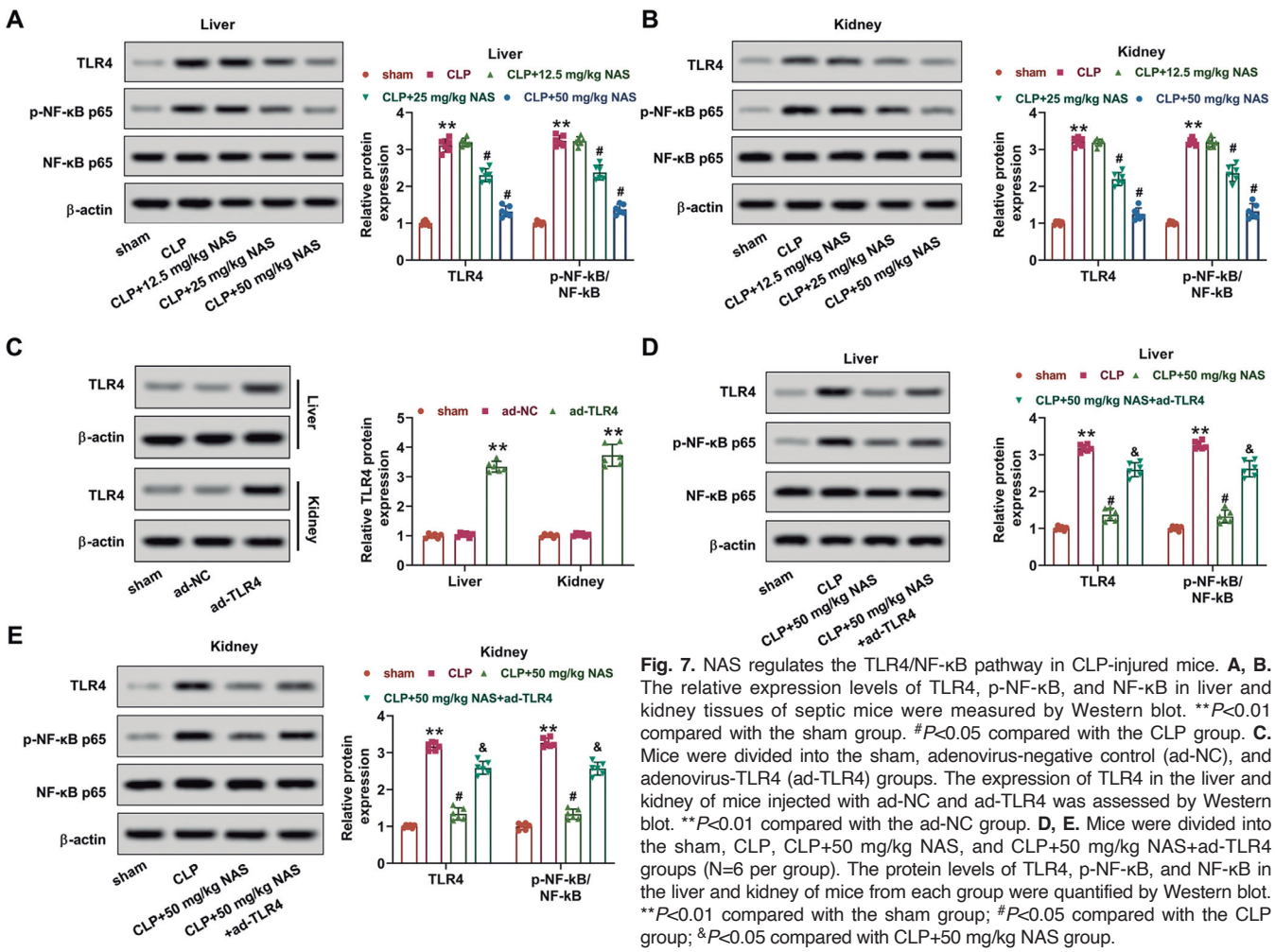

Fig. 7. NAS regulates the TLR4/NF-κB pathway in CLP-injured mice. **A, B.** The relative expression levels of TLR4, p-NF-κB, and NF-κB in liver and kidney tissues of septic mice were measured by Western blot. $^{**}P<0.01$ compared with the sham group. $^{\#}P<0.05$ compared with the CLP group. **C.** Mice were divided into the sham, adenovirus-negative control (ad-NC), and adenovirus-TLR4 (ad-TLR4) groups. The expression of TLR4 in the liver and kidney of mice injected with ad-NC and ad-TLR4 was assessed by Western blot. $^{**}P<0.01$ compared with the ad-NC group. **D, E.** Mice were divided into the sham, CLP, CLP+50 mg/kg NAS, and CLP+50 mg/kg NAS+ad-TLR4 groups (N=6 per group). The protein levels of TLR4, p-NF-κB, and NF-κB in the liver and kidney of mice from each group were quantified by Western blot. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group; $^{\&}P<0.05$ compared with CLP+50 mg/kg NAS group.

serum levels of ALT, AST, BUN, and creatinine in a dose-dependent manner. These findings revealed that NAS effectively improved liver and kidney functions in mice with sepsis.

Some factors, such as apoptosis, oxidative stress, and inflammation, can induce multiple organ failure (Heung and Koyner, 2015). CLP-induced apoptosis is involved in the development of sepsis in the context of organ damage and inflammation (Heung and Koyner, 2015). In previous research, the flavonoid fisetin relieved apoptosis in septic mice with acute kidney injury (Ren et al., 2020). Treatment with baicalein protected against the liver injury caused by sepsis by reducing hepatic apoptosis (Liu et al., 2015). In our study, NAS treatment alleviated apoptosis of the liver and kidney in septic mice.

Oxidative stress activates several transcription factors, which subsequently promote the expression levels of many genes encoding proinflammatory cytokines (Zhang et al., 2022). MPO is a common hemoglobin found in neutrophils and is involved in immune surveillance and host defense mechanisms (Tabrizi et al., 2021). Moreover, its presence implies neutrophil infiltration and the intensity of inflammation (Gwozdzinski et al., 2021). MDA is the end-product of free radicals in lipid peroxidation. Its aberrant expression causes membrane system damage, worsens tissue damage, and promotes cell degeneration or necrosis. Numerous investigations have documented the existence of oxidative stress and antioxidant deficiency in sepsis patients (Heyman et al., 2011; Ustundag et al., 2023a). SOD is an antioxidant enzyme that is helpful in the prevention of inflammatory disorders. During sepsis, the SOD level is reduced and major organ systems, such as the liver and kidney, suffer from oxidative damage (Sidonia et al., 2020). Similarly, in this work, the content of SOD was abnormally decreased, while levels of MPO and MDA were elevated in CLP-induced septic mice. The anti-oxidative stress effects of NAS have been reported in LPS-stimulated RAW246.7 cells (Liu et al., 2015). Phenolic compounds have been shown to have better antioxidant effects than ascorbic acid *in vitro* (Celep et al., 2012). Flavonoids are one of the major polyphenolic components of plants. Since their hydroxyl

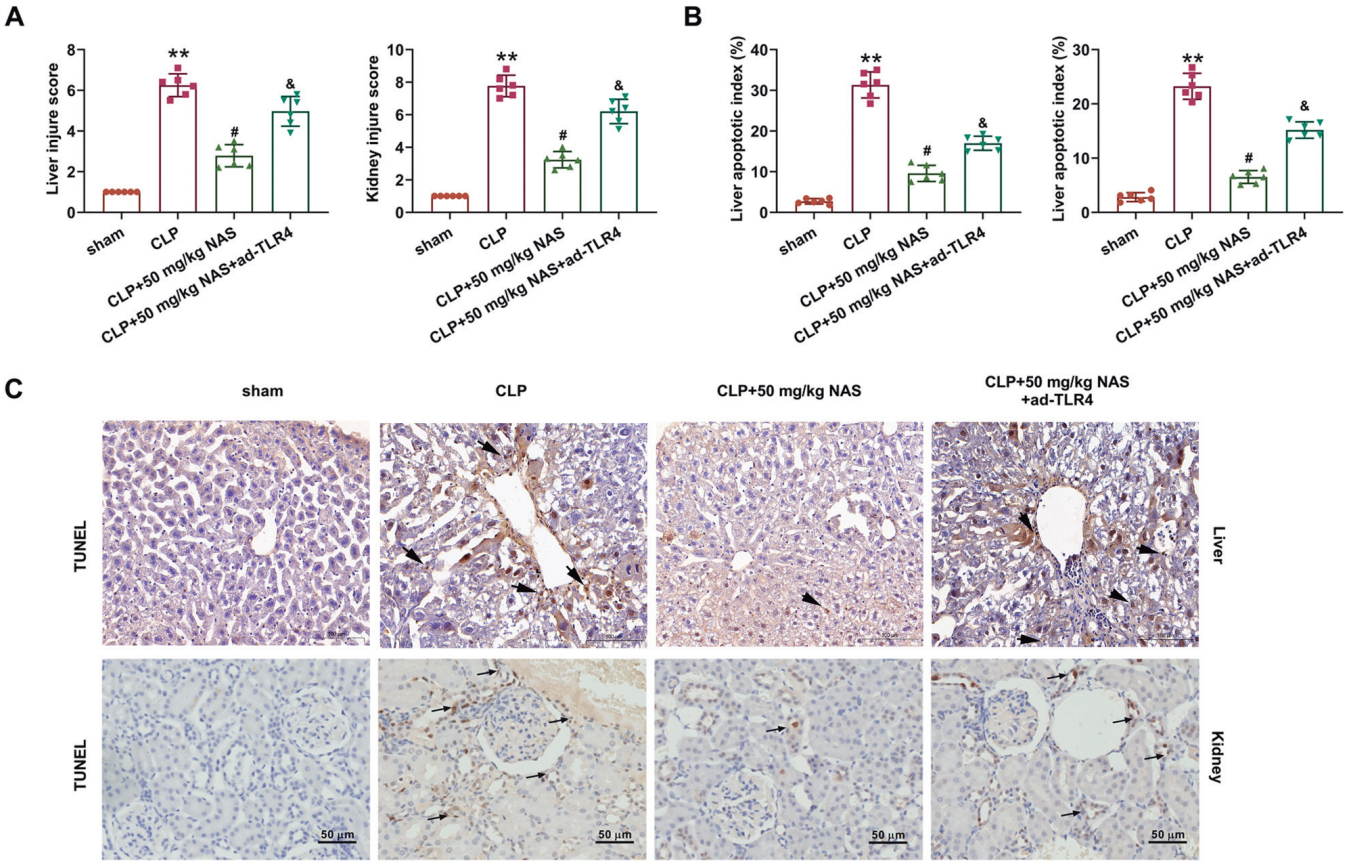

Fig. 8. NAS reduces injury and apoptosis of the liver and kidney in septic mice by regulating the TLR4/NF-κB pathway. **A.** The injury scores of the liver and kidney in mice from different groups were quantified after H&E staining. **B, C.** Level of apoptosis in the liver and kidney of mice was observed using TUNEL staining. Black arrows indicated TUNEL-stained positive cells. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group; $^{\&}P<0.05$ compared with the CLP+50 mg/kg NAS group. C, × 400.

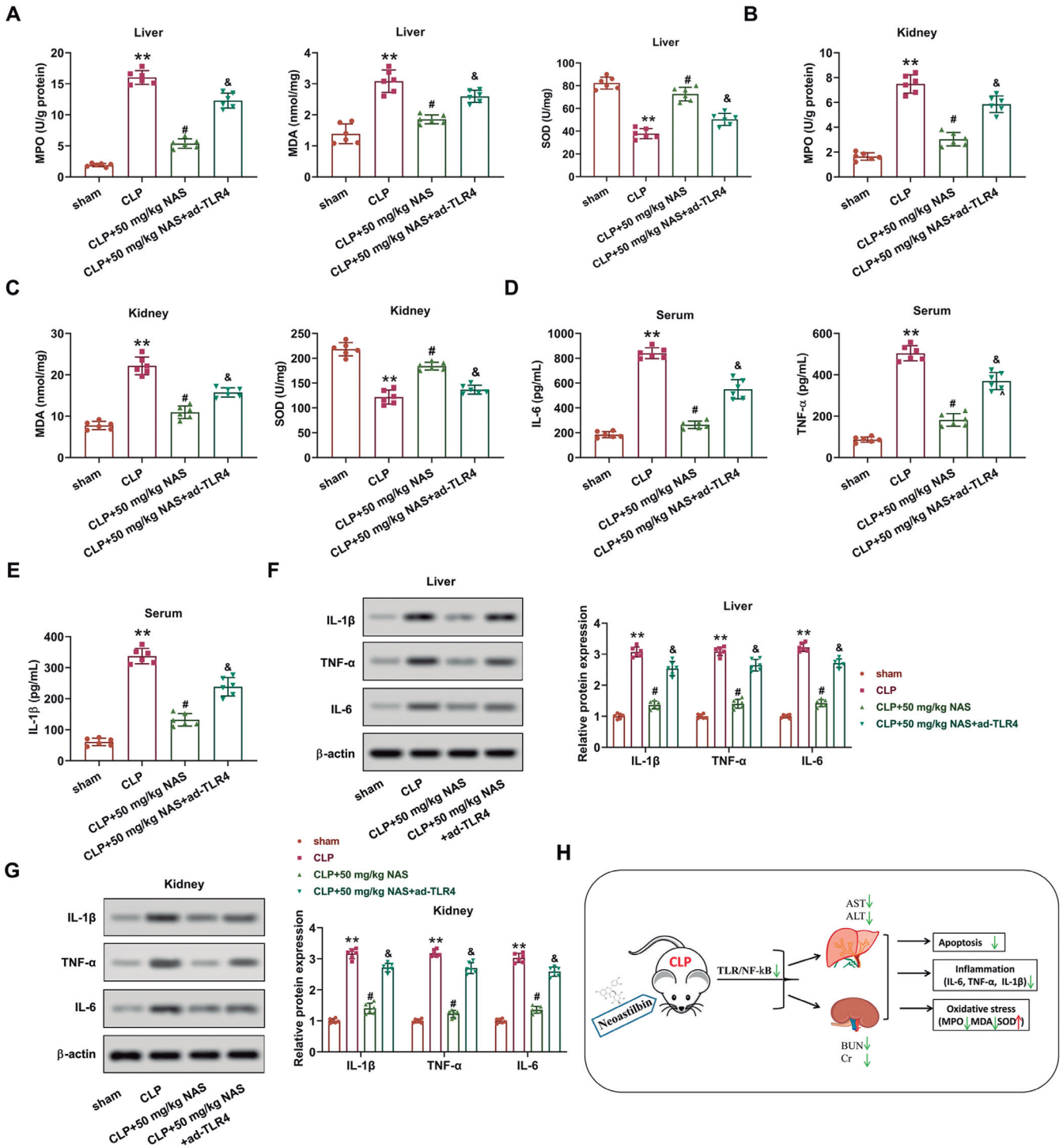

Fig. 9. NAS reduces oxidative stress and inflammatory factors in a CLP-induced sepsis mouse model by regulating the TLR4/NF-κB pathway. **A-C.** The levels of MPO, MDA, and SOD in the liver and kidney tissues of septic mice in each group were detected by the corresponding kits. **D, E.** Serum levels of IL-6, TNF-α, and IL-1β in septic mice from each group were tested by ELISA. **F, G.** The protein expression of IL-6, TNF-α, and IL-1β in the liver and kidney tissues was detected by Western blot. **H.** The general idea of this study. NAS reduces CLP-induced liver and kidney injury in septic mice through the TLR4/NF-κB pathway, as indicated by decreases in AST, ALT, BUN, and Creatinine, as well as the reduction of apoptosis, inflammatory factor levels, and oxidative stress caused by CLP. $^{**}P<0.01$ compared with the sham group; $^{\#}P<0.05$ compared with the CLP group; $^{\&}P<0.05$ compared with CLP+50 mg/kg NAS group.

groups are located at different positions, they have efficient free radical scavenging activity (Procházková et al., 2011). Our study has also proved this point. After NAS treatment, the degree of oxidative stress in septic mice was clearly ameliorated, indicating that NAS notably alleviated the oxidative stress induced by sepsis in mice.

The excessive accumulation of inflammatory factors is a common mechanism of sepsis. To protect against infection, the organism activates an inflammatory response and secretes proinflammatory mediators such as TNF-α, IL-6, and IL-1β at the early stage of CLP-induced sepsis (Gao et al., 2022). The inflammatory response is useful in fighting infection; however, excessive inflammation and systemic inflammatory response syndrome can result in multiple organ failure or death (Gao et al., 2022). For example, TNF-α recruits inflammatory cells, causes proinflammatory cytokine release and accumulation, and induces necroptosis via local autoamplification loops, leading to solid organ failure (Lukacs et al., 1995; Linkermann et al., 2014). The utilization of anti-TNF-α antibodies can significantly reduce the inflammatory response in patients with severe sepsis and improve their survival rate (Newham et al., 2014). IL-1β is involved in modulating immunological responses and prompting macrophages to produce inflammatory cytokines (Wu et al., 2014). After CLP surgery, macrophages release a large number of inflammatory cytokines such as IL-6, TNF-α, and IL-1β (Durairaj et al., 2015; Ge et al., 2019). It has been proven that NAS dramatically diminished the secretion of inflammatory factors, such as TNF-α, IL-1β, and IL-6 in mouse models of gouty arthritis (Xu et al., 2022). Here, we observed that the levels of IL-6, TNF-α, and IL-1β in the serum and liver and kidney tissues were notably reduced by NAS pretreatment in septic mice, which is in line with prior reports of the anti-inflammatory effects of other flavonoids, such as larches and naringenin (Hausenloy et al., 2016; Salehi et al., 2019). Our findings demonstrated that NAS effectively reduced the secretion of inflammatory cytokines in mice with sepsis.

TLR4 is a critical signal transduction receptor that activates NF-κB to regulate the immunological response and the release of various inflammatory factors (Hausenloy et al., 2016). Several studies showed that TLR4/NF-κB signaling was activated during sepsis (Tak and Firestein, 2001; Zhou et al., 2006). NF-κB activation elevated various pro-inflammatory factors, of which TNF-α was not only associated with liver necrosis but also aggravated liver failure in sepsis (Wang et al., 1995). TLR4/NF-κB inhibition was linked to the reduction in LPS-induced cytokine production and protection against liver tissue damage caused by the overactive immune response (Watson et al., 2008; Zhang et al., 2014). Furthermore, inhibition of TLR4 and TLR4-mediated signaling has been considered as a method to control sepsis (Opal et al., 2013). NAS has been shown to inhibit the inflammatory response in gouty arthritis in mice via the NF-κB pathway (Xu et al., 2022). Our results also suggested that increases in TLR4 expression and NF-κB phosphorylation by CLP were restrained by NAS treatment in a dose-dependent manner. To further explore the role of TLR4/NF-κB signaling in the protection of NAS in sepsis-induced liver and kidney injury, TLR4 adenovirus was used to enhance TLR4 expression. The results demonstrated that overexpression of TLR4 attenuated the protective effects of NAS on CLP-induced liver and kidney injury, including apoptosis, oxidative stress, and inflammation. These findings further confirmed that NAS reduced liver and kidney injury in septic mice by regulating the TLR4/NF-κB pathway.

Conclusion

Collectively, NAS reduced the pathological damage, apoptosis, oxidative stress, and inflammation, and restored impaired liver and kidney function in a dose-dependent manner. Furthermore, NAS exerted the protective function, at least in part, via restraining the TLR4/NF-κB pathway. It is undeniable that our work still has several limitations. Firstly, this study is based on a small sample size and did not measure the survival rate after 24 h. Secondly, our research experiments did not further investigate the effect of NAS in the CLP model using TLR4 KO mice. Thirdly, the effects of NAS on other aspects of septic mice, such as ferroptosis, mitochondrial function, fibrosis, etc., need further study. Fourthly, other pathways regulated by NAS will be investigated in the following work. Additionally, further studies are needed to evaluate whether the findings in mice can be generalized to humans.

Conflict of Interest. The Authors declare that they have no conflict of interests.

Data availability statement. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Author contributions. Qiumei Cao designed the study. Ruiming Xu, Dawei Wang, Zhengyi Shao, and Xiangbo Li performed the experiments and analyzed the data. Ruiming Xu wrote the manuscript. All authors read and approved the submitted version.

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