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LABORATORY STUDY



# S-nitrosoglutathione inhibits pyroptosis of kidney tubular epithelial cells in sepsis via the SIRT3/SOD2/mtROS signaling pathway

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**ABSTRACT**

**Objectives:** Pyroptosis is considered to play an important role in the occurrence, development and prognosis of septic acute kidney injury (SAKI). We aimed to explore the specific molecular mechanism of S-nitrosoglutathione (SNG) regulating pyroptosis of kidney tubular epithelial cells (KTECs).

**Methods:** By constructing a mice model of sepsis, we pretreated them with SNG and used biochemical methods to detect the levels of serum inflammatory factors and mitochondrial reactive oxygen species (mtROS), assessed the severity of kidney injury and KTECs mitochondrial damage, and detected the expression of KTECs pyroptosis-related proteins and sirtuin 3 (SIRT3)/superoxide dismutase 2 (SOD2) pathway proteins.

**Results:** The kidney injury caused by sepsis was significantly aggravated, and the levels of IL-1β, IL-6, IL-18, TNF-α, malondialdehyde (MDA) and mtROS were all increased, accompanied by the decrease of SIRT3 and SOD2 proteins, while NOD-like receptor with pyrin domain 3 (NLRP3), gasdermin D (GSDMD), Caspase-1 proteins expression and the number of KTECs apoptotic cells were all increased. However, after SNG pretreatment, the levels of IL-1β, IL-6, IL-18, TNF-α, MDA and mtROS were all significantly decreased, the expression of SIRT3 and SOD2 proteins were increased, NLRP3, GSDMD, Caspase-1 proteins expression and the number of KTECs apoptotic cells were decreased.

**Conclusions:** SNG protects SAKI by regulating the SIRT3/SOD2/mtROS signaling pathway to inhibit the pyroptosis of KTECs.

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## 1. Introduction

Septic acute kidney injury (SAKI) is a common syndrome characterized by a rapid decline in kidney function caused by sepsis in a short period of time, with or without oliguria or anuria [1]. Its high incidence and poor prognosis make it one of the important factors of chronic kidney disease [2]. Therefore, it is crucial to understand the pathogenesis, diagnosis and therapeutic intervention of SAKI. In order to maintain the morphological function of tissues, cell death occurs at any time during the growth and development of biological individuals [3]. Pyrotosis is a programmed cell death mediated by gasdermin D (GSDMD) and dependent on Caspase [4]. The inflammasome of the NOD like receptor (NLRP3) of the pyrin domain 3 activates the precursor of Caspase-1 and promotes the release of inflammatory factors, which is an important mechanism of SAKI pathogenesis [5,6].

Dysfunction of kidney tubular epithelial cells (KTECs) is key to SAKI. Sun et al. found that USF2 regulates the TGF-β/Smad3 pathway, which in turn induces KTECs pyroptosis and promotes the occurrence of SAKI [7]. Zhou et al. found that lncRNA 9884 regulates Smad3 activation of the NF-κB pathway, mediating KTECs pyroptosis [8]. Moreover, multiple pathways mediate KTECs pyroptosis and play important roles in SAKI, such as NEK7/NLRP3, NLRP3/caspase-1, caspase-4/5/11, etc [9–11]. During the SAKI process, KTECs necrosis, shedding, imbalanced proliferation, and immune-mediated damage are the core of disease progression. Intervening in the regulation of cell apoptosis pathways through drugs or other means may directly or indirectly affect cell death, improve inflammatory response pathways, and thus protect kidney function.

S-nitrosoglutathione (SNG) is a small S-nitrosoglutathione and a synthesizer of nitric oxide, which can affect the phosphorylation state and change the downstream signaling pathway by regulating the structure and function of related proteins [12]. In septic animal models, SNG protects organs through anti-inflammatory and antioxidant

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mechanisms [13]. Sirtuin 3 (SIRT3) belongs to the sirtuins family and directly or indirectly participates in regulating mitochondrial biosynthesis, respiratory chain, glucose metabolism, and other processes by regulating the deacetylation levels of multiple proteins. It is crucial for maintaining cellular energy balance. In present study, a mice model of sepsis was established, and SNG was used to intervene in the mice with sepsis. By observing the changes of various indicators, it was verified whether SNG could inhibit the kidney tubular epithelial cells (KTECs) pyroptosis mediated by SIRT3 in septic mice, and provided new treatment ideas for patients with SAKI.

## 2. Materials and methods

### 2.1. Animals and groups

BALB/c mice were purchased from Zhejiang University (Hangzhou, China), and SIRT3 knockout (KO) mice purchased from Jackson Laboratory (Jackson Medical Technology Co., Shanghai, China). Wild type BALB/c mice and SIRT3 KO mice, gender unlimited (random selection), specific pathogen free, weighing (21 ± 3) g, 6–8 weeks old. After 2-3 weeks of adaptive feeding, mice were used for subsequent experiments. Experimental animals were randomly divided into 4 groups, namely the Control, Sham, cecal ligation and puncture (CLP) and CLP+SNG pretreatment group. The CLP specific protocol is as follows: Mice were fasted for 8-12 h, anesthetized with 2% sodium pentobarbital, and the abdomen was prepared, disinfected, and covered with towels. The vertical incision in the midline of the abdomen was about 2 cm long, and the incision was made layer by layer to the abdominal cavity. The cecum was found and ligated at a distance of 3-5 cm from the end. An 18-gauge needle was used to perforate the intestinal canal twice at the end of the cecum to squeeze out a small amount of intestinal content, which was then returned to the abdominal cavity and then closed. Normal saline (20 mL/kg) was performed postoperatively for fluid resuscitation, including Sham, CLP and CLP+SNG groups. In the Sham group, the cecum was only turned over during laparotomy. In the CLP+SNG pretreatment group, we injected different doses of SNG (12.5 mg/kg, 25 mg/kg, 50 mg/kg) through the tail vein of mice for three consecutive days, and performed CLP 8-12 h after the last day of intravenous injection.

Mice were sacrificed 48 h after CLP, and their blood was collected, centrifuged at 13400 × g (4 °C) for 8-10 min, the supernatant was collected, and stored at −80 °C until relevant biochemical indicators were detected. Bilateral kidney tissues were taken, the upper pole of the right kidney was fixed in 4% paraformaldehyde for 12-24 h, and the left kidney was placed in an ultra-low temperature refrigerator at 80 °C until it was used in subsequent experiments. All our animal experiments were approved by the Laboratory Animal Ethics Committee of Ningbo University.

### 2.2. H&E staining

The upper pole tissue of right kidney was immersed in 4% paraformaldehyde for 12-24 h. The tissue was dehydrated with graded ethanol, embedded in liquid paraffin, and sliced at 2-3μm. Xylene and ethanol were treated for dewaxing, H&E dyeing and sealing with neutral gum. Following our previous experience, we observed the kidney tissue pathological changes and performed semi-quantitative analyses based on the tubular injury severity, the tubular injury ≤ 25%, 1; 25% < the tubular injury ≤50%, 2; 50% < the tubular injury ≤75%, 3; the tubular injury ≥ 75%, 4 [14]. We calculated the area of renal tubular injury under high magnification (×400), and randomly selected one slice from each pathological section.

### 2.3. Assessment of renal function

We detected the levels of neutrophil-associated apolipoprotein (pNGAL, Boster Co., Wuhan, China), serum creatinine (SCr, Boster Co., Wuhan, China), and kidney injury molecule-1 (pKIM-1, Boster Co., Wuhan, China) according to the kit instructions, and then assessed renal function.

### 2.4. Detection of inflammatory factor

We used ELISA (Boster Co., Wuhan, China) to measure the serum levels of TNF-α, IL-6, IL-1β, and IL-18 in mice. The specific operations were carried out in strict accordance with the instructions.

### 2.5. Western-blotting

We chopped the upper pole tissue of the left kidney, used the BCA method to quantify the required volume of the sample with 50 μg of total protein per well, prepared 5% stacking gel and 10% separating gel, and loaded the sample. We carried out the electrophoresis for 30 min, and then carried out at 130 V for 90 min, transferred to PVDF membrane and blocked for 1 h. We incubated NLRP3 (1:1000, Catalog # SAB5700723, Sigma Co., Shanghai, China), Caspase-1 (1:1000, Catalog # AB1871, Sigma Co., Shanghai, China), GSDMD (1:2000, Catalog # SAB5702259, Sigma Co., Shanghai, China), SIRT3 (1:1000, Catalog # PA5-1155904, Invitrogen Co., Shanghai, China), superoxide dismutase 2 (SOD2, 1:1000, Catalog # AB10346, Sigma Co., Shanghai, China) and GAPDH (1:2000, Catalog # MAB374, Sigma Co., Shanghai, China) overnight at 4 °C, and incubated the Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody (1:5000, Catalog # A16072, Thermo Fisher Scientific Inc., Shanghai, China) for 1h, and then washed 3 times with TBS-T again.

### 2.6. Mitochondrial morphology

We placed the lower pole of the right kidney in 2.5% glutaraldehyde pre-cooled at 4 °C overnight, and fixed with 1% osmic acid for 2 h. Gradient alcohol dehydrated, uniformly

sprayed by vacuum sprayer, and observed the morphology and structure of mitochondria under transmission electron microscope (TH-F120, Guoyi Quantum Technology Co., Hefei, China).

### 2.7. Detection of oxidase in kidney tissue

We took 100 mg of lower pole tissue of the left kidney and chopped it up and added it to a homogenizer together with 900 µL of normal saline. Then, at 2 °C, we treated it with 8000 × g centrifuge for 25 min, and kept the supernatant. We used spectrophotometry to measure SOD and malondialdehyde (MDA) levels, immunofluorescence to detect reactive oxygen species (ROS), and mitochondrial ROS (mtROS) detection assay kit (Qiyue Co., Xi'an, China) to measure the level of mtROS.

### 2.8. TUNEL staining

Paraffin sections of the upper pole of the right kidney were taken. Proteinase K was added and incubated at 37 °C for 20 min, then washed with PBS; TUNEL solution (Pricella Biotechnology Co., Wuhan, China) was added dropwise at 37 °C for 1 h, and the apoptotic cells were observed after washing with PBS. Color development, observed under the microscope. Counterstained with DAPI, differentiated and returned to blue, dehydrated with gradient ethanol, transparent, mounted.

### 2.9. Survival analysis

BALB/c mice were randomly divided into 6 groups ($n = 20$ in each group), namely the Control, Sham, CLP, CLP+SNG (12.5 mg/kg), CLP+SNG (25 mg/kg) and CLP+SNG group (50 mg/kg). The diet and drinking water were resumed immediately after the operation, and the survival of the mice within 7 days was observed.

### 2.10. Statistical analysis

We conducted a normal distribution test on all data, analyzed the data using GraphPad Prism 6.02 software, and represented the data as the mean ± standard deviation (mean ± SD) based on a normal distribution. We used the one-way ANOVA to compare between multiple groups, and used Tukey's *post hoc* tests to make the further comparison between two groups. $p < 0.05$ was considered as statistically significant difference.

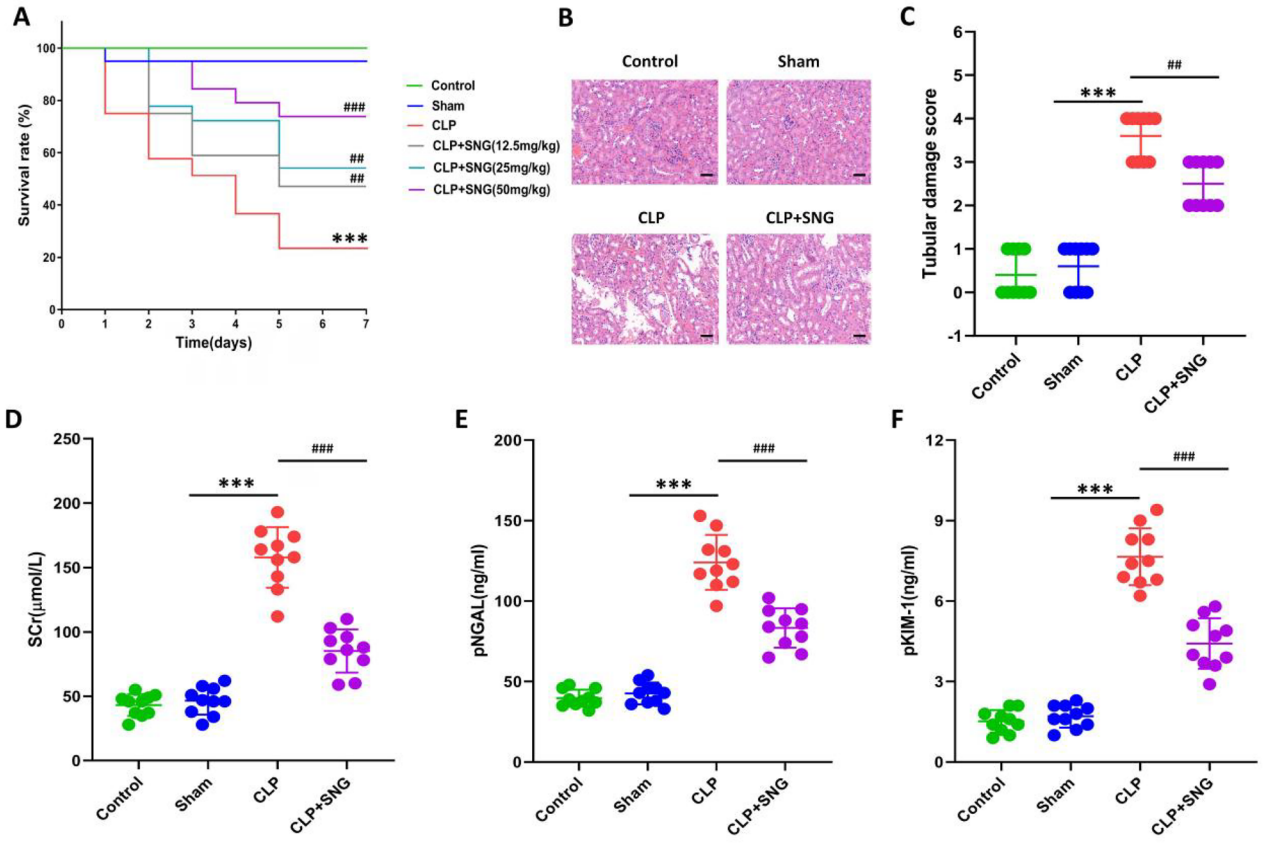


Figure 1. Protective effect of SNG on SAKI. (A) survival analysis of septic mice ($n = 20$ in each group); (B) kidney tissue pathological damage of mice in each group ($n = 10$ in each group); (C) kidney tissue pathological damage score ($n = 10$ in each group); (D) the level of SCr ($n = 10$ in each group); (E) the level of pNGAL ($n = 10$ in each group); (F) the level of pKIM-1 ($n = 10$ in each group). The ratio of female mice to male mice in each group is 1:1. Compared with the sham group, $^{***}p < 0.001$; Compared with the CLP group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

## 3. Results

### 3.1. Protective effect of SNG on SAKI

Our previous study indicated that SNG may have a protective effect on SAKI [15]. To confirm this conclusion, we pretreated septic mice with different doses of SNG, and observed the survival of mice in each group within 7 days. We found that CLP resulted in a mortality rate of 75.0%, and the mortality rates of mice pretreated with different doses of SNG were 50.0% (12.5 mg/kg), 40.0% (25 mg/kg) and 25.0% (50 mg/kg) respectively, which were significantly lower than those in the CLP group (Figure 1A.). 50 mg/kg of SNG reduced the mortality of mice most significantly, so this dose was used in our subsequent experiments. Further, we observed the pathological changes of the kidney tissue of the mice in each group. We found that the glomerular capsule of the mice in the CLP group was ruptured, KTECs fell off, and interstitial inflammatory cells were infiltrated, while the glomerular capsule of the CLP+SNG group was intact, and KTECs were smooth (Figure 1B.). Compared with the mice in the CLP group, the renal tissue pathological damage score of the mice in the CLP+SNG group was significantly reduced (Figure 1C.). We also observed that the levels of pKIM-1, pNGAL and SCr in the CLP+SNG group were lower than those in the CLP

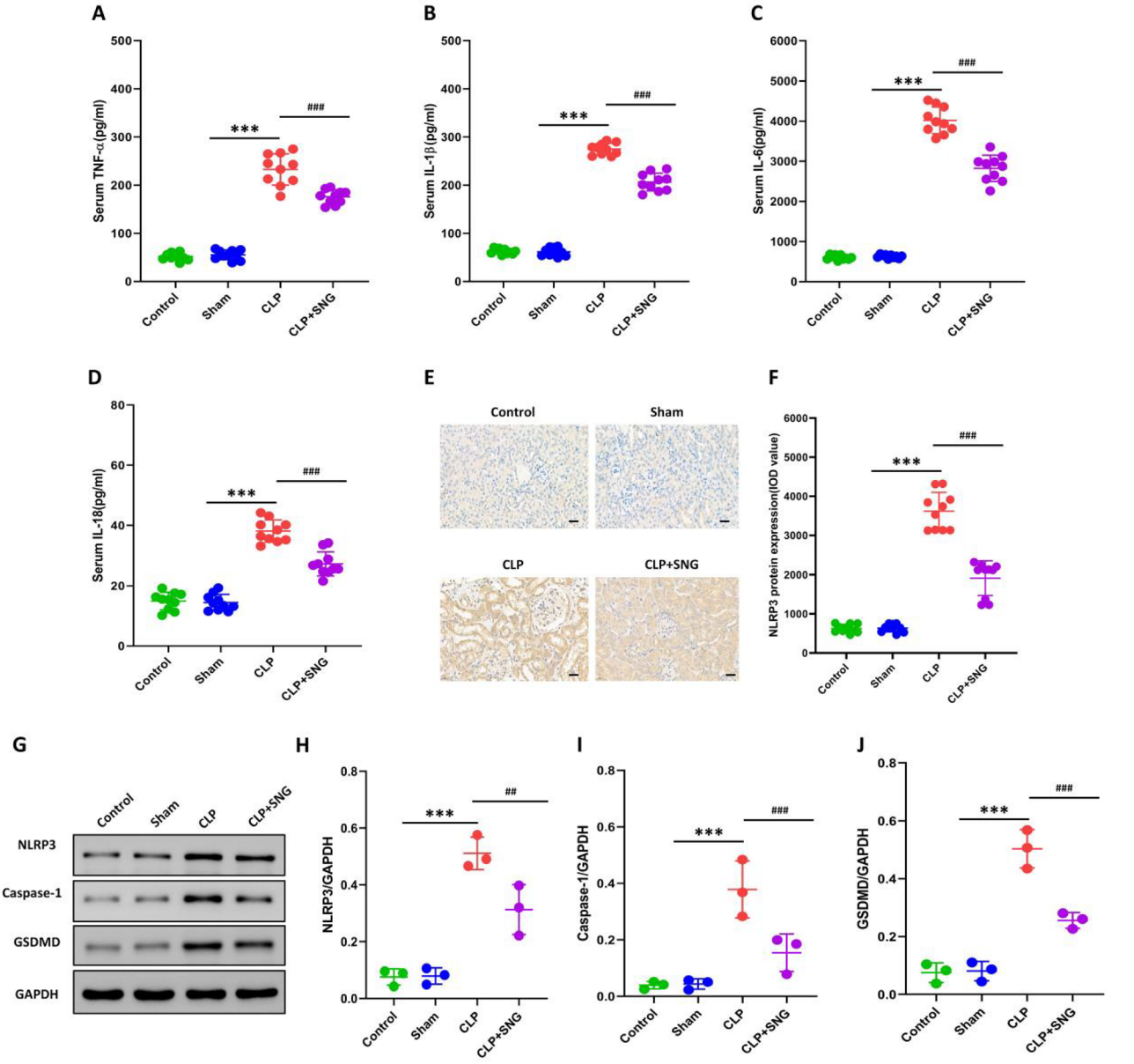


Figure 2. SNG Inhibits the release of inflammatory factors in septic mice. (A) the level of TNF-α ($n = 10$ in each group, female/male = 1:1); (B) the level of IL-1β ($n = 10$ in each group, female/male = 1:1); (C) the level of IL-6 ($n = 10$ in each group, female/male = 1:1); (D) the level of IL-18 ($n = 10$ in each group, female/male = 1:1); (E) NLRP3 protein expression ($n = 10$ in each group, female/male = 1:1); (F) the IOD values of NLRP3 protein ($n = 10$ in each group, female/male = 1:1); (G) Pyroptosis-related protein expression ($n = 3$ in each group, female/male = 1:2); (H) the level of NLRP3 ($n = 3$ in each group, female/male = 1:2); (I) the level of caspase-1 ($n = 3$ in each group, female/male = 1:2); (J) the level of GSDMD ($n = 3$ in each group, female/male = 1:2). Compared with the sham group, $^{***}p < 0.001$; compared with the CLP group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

group (Figure 1D-F.). The above results suggested that SNG could reduce the mortality of septic mice, improve kidney function, and have a significant protective effect on SAKI.

### 3.2. SNG inhibits the release of plasma inflammatory factors in septic mice

Studies have confirmed that the inflammatory response runs through the entire pathogenesis of sepsis, so the level of inflammation is a key indicator of the severity of the disease [16–18]. Our experiments also confirmed this theory, we found that CLP induced an increase in the levels of inflammatory factors IL-6, IL-18, TNF-α and IL-1β in mice, while SNG could significantly reverse this phenomenon (Figure 2A-D.). In order to clarify the important role of pyroptosis in the process of SAKI, we also detected the protein expression changes of NLRP3, GSDMD and Caspase-1 in kidney tissue. We found that CLP resulted in significant upregulation of NLRP3, GSDMD and Caspase-1 proteins, whereas SNG significantly decreased NLRP3, GSDMD and Caspase-1 protein levels (Figure 2E-J.). It could be seen that SNG protected the renal function of septic mice by inhibiting pyroptosis.

### 3.3. SNG inhibits mitochondrial oxidative stress and apoptosis

Mitochondrial oxidative stress injury and apoptosis in KTECs are important molecular biological mechanisms leading to SAKI [19,20]. To clarify the effect of SNG on mitochondrial protection and apoptosis of KTECs, we detected mitochondrial density, oxidase and apoptosis levels. We found that CLP resulted in abnormal mitochondrial morphological structure and decreased density, while the levels of MDA and ROS were significantly increased, the level of antioxidant enzyme SOD was decreased, and the apoptotic rate of KTECs was increased (Figure 3A-G.). Mitochondrial density was significantly increased after pretreatment with SNG, accompanied by a decrease in the levels of MDA and ROS, an increase in the level of the antioxidant enzyme SOD, and a decrease in the apoptotic rate of KTECs (Figure 3A-G.). These results indicated that SNG could significantly reverse CLP-induced mitochondrial oxidative stress damage and apoptosis in KTECs.

### 3.4. SNG inhibits SIRT3/SOD2/mtROS-mediated pyroptosis

To clarify the regulatory effect of SNG on SIRT3/SOD2/mtROS signaling pathway in KTECs, we detected the expression of related pathway proteins. We found that CLP-induced SIRT3 and SOD2 protein in KTECs significantly down-regulated, while mtROS increased, and SIRT3 and SOD2 protein expression increased and mtROS expression decreased after SNG pretreatment (Figure 4A-D.). Furthermore, to clarify the effect of SNG on the pyroptosis of KTECs through the SIRT3/SOD2/mtROS signaling pathway, we used SIRT3 knockout (KO) mice and validated

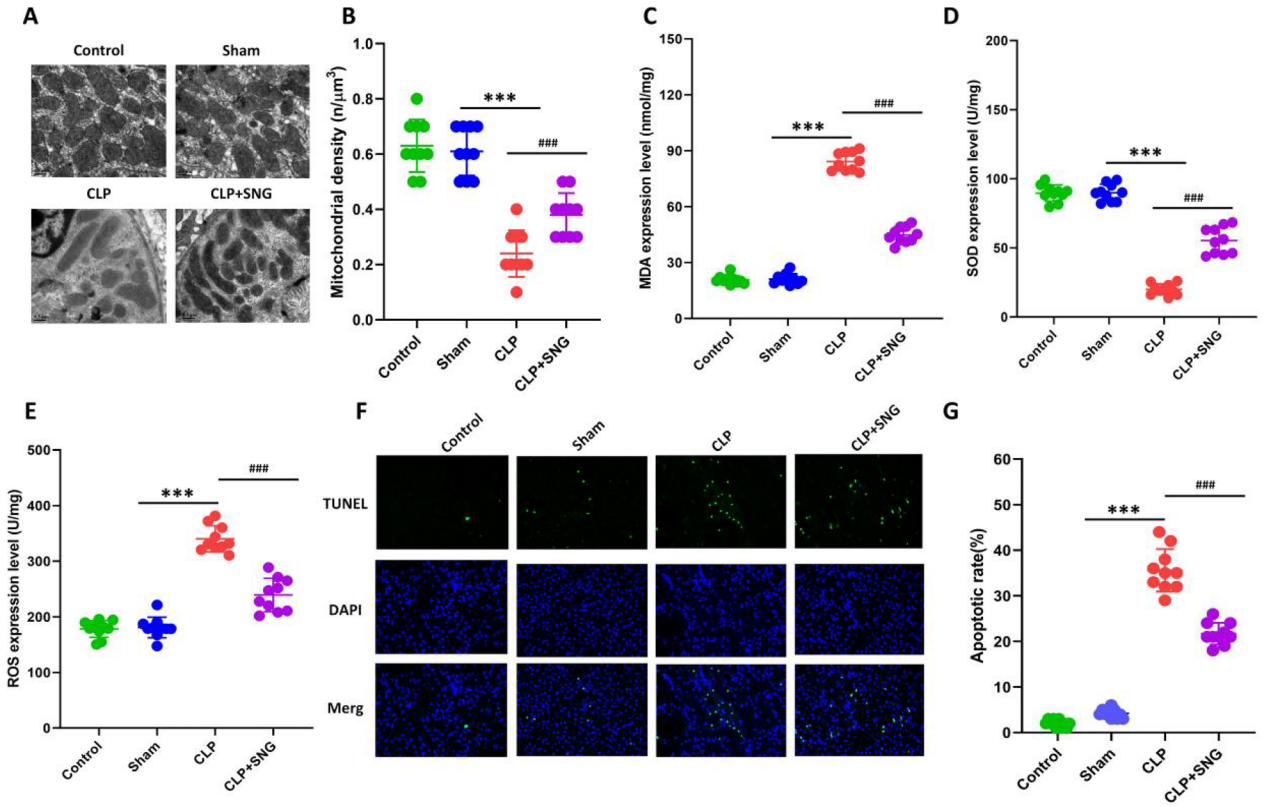


Figure 3. The effect of SNG on mitochondrial protection and apoptosis of KTECs. (A) changes in mitochondrial morphology ($n=10$ in each group); (B) changes in mitochondrial density ($n=10$ in each group); (C) the level of MDA ($n=10$ in each group); (D) the level of SOD ($n=10$ in each group); (E) the level of ROS ($n=10$ in each group); (F) apoptosis of KTECs ($n=10$ in each group); (G) apoptotic rate in KTECs ($n=10$ in each group). The ratio of female mice to male mice in each group is 1:1. Compared with the sham group, $^{***}p<0.001$; compared with the CLP group, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ ($n=10$ in each group).

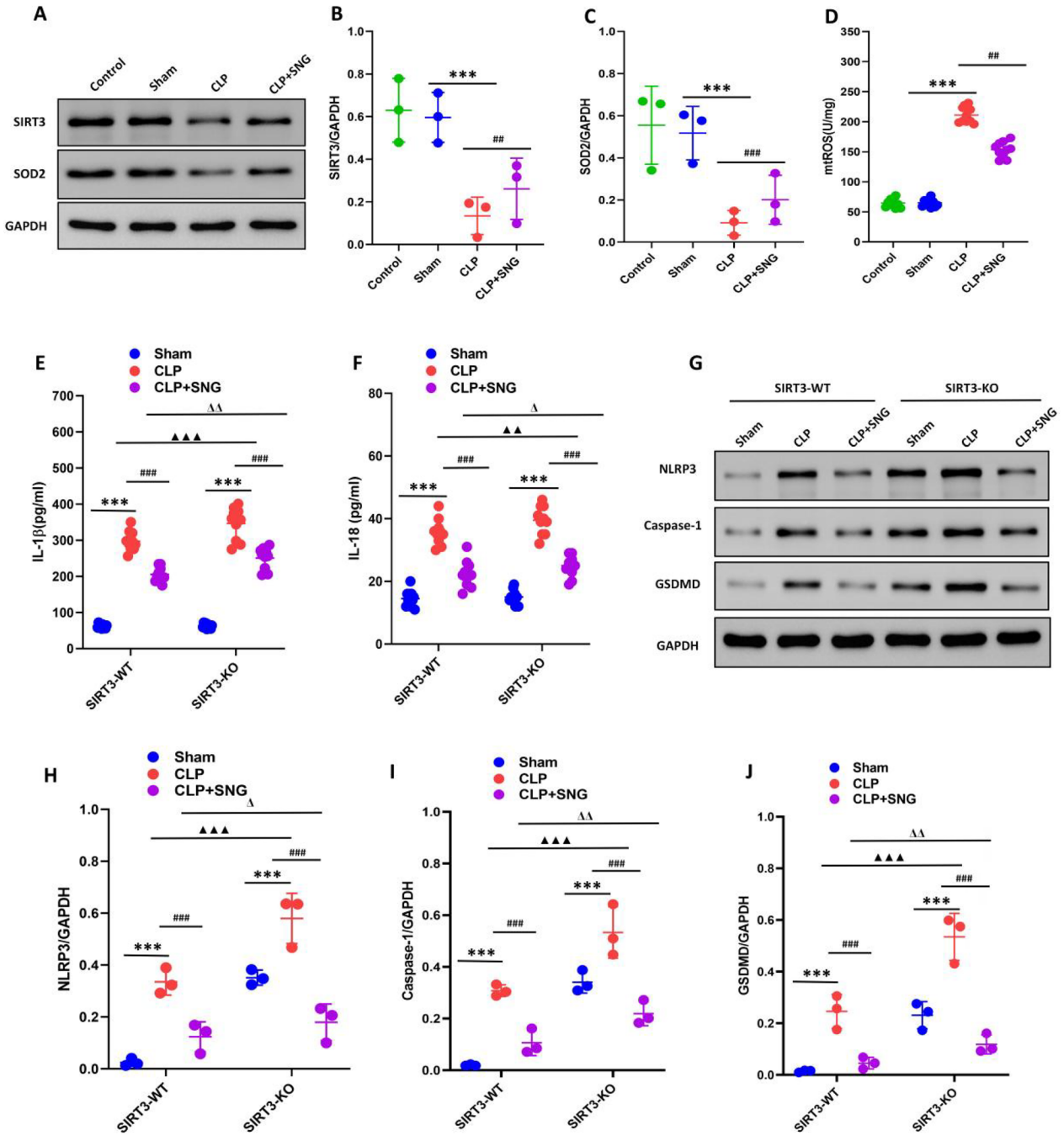


Figure 4. SNG inhibits pyroptosis by regulating SIRT3/SOD2/mtROS. (A) SIRT3/SOD2/mtROS pathway protein expression ($n = 3$ in each group, female/male = 1:2); (B) the level of SIRT3 protein ($n = 3$ in each group, female/male = 1:2); (C) the level of SOD2 protein ($n = 3$ in each group, female/male = 1:2); (D) the level of mtROS ($n = 10$ in each group, female/male = 1:1); (E) the level of IL-1β ($n = 10$ in each group, female/male = 1:1); (F) the level of IL-18 ($n = 10$ in each group, female/male = 1:1); (G) the effects of SIRT3 gene deletion on pyroptosis ($n = 3$ in each group, female/male = 1:2); (H) the level of NLRP3 ($n = 3$ in each group, female/male = 1:2); (I) the level of caspase-1 ($n = 3$ in each group, female/male = 1:2); (J) the level of GSDMD ($n = 3$ in each group, female/male = 1:2). Compared with the sham group, $^{***}p < 0.001$; compared with the CLP group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with the WT+CLP group, $\blacktriangle\blacktriangle p < 0.01$, $\blacktriangle\blacktriangle\blacktriangle p < 0.001$; compared with the WT+CLP+SNG group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$.

whether SIRT3 was successfully knocked out through polymerase chain reaction [14]. We found that SIRT3-KO significantly increased the levels of inflammatory cytokines IL-1β and IL-18, as well as NLRP3, GSDMD and Caspase-1 protein expressions, which were partially reversed by SNG pretreatment (Figure 4E-J.). The above results suggested that SNG might inhibit KTECs pyroptosis by regulating the SIRT3/SOD2/mtROS signaling pathway.

## 4. Discussion

Although the proposal of precision medicine and other advances have made us have a deeper understanding of the diagnosis and treatment of sepsis. However, the studies showed that 5.1 million people die each year from sepsis, and as the population ages, the incidence of sepsis will continue to increase, and there is an urgent need to increase

awareness of the disease [21,22]. The latest data indicated that the fatality rate of sepsis patients with acute kidney injury (AKI) has increased to 70% [23,24]. Therefore, renal dysfunction has become the primary concern for organ dysfunction after sepsis.

SNG has biological functions such as anti-inflammatory, antioxidant, anti-tumor, and hypoglycemic and lipid-lowering. Our study showed that SNG could significantly extenuate kidney tissue damage, reduce IL-1β, IL-6, IL-18, TNF-α, MDA and ROS, decrease the apoptosis of KTECs, but significantly increase the level of SOD. Our previous study showed that SNG can scavenge and inhibit ROS, providing an effective defense against oxidative stress [25]. Our present study showed that SNG could also play anti-inflammatory and anti-oxidative damage effects in SAKI, and these effects were related to the enhancement of SNG antioxidant capacity and alleviation of inflammatory response. In addition, the mechanism of SNG mediating inflammation, oxidative stress and apoptosis may be related to inhibiting Bax and other apoptotic proteins [15]. We speculated that SNG could effectively inhibit the expression of pro-inflammatory and pro-oxidative factors and promote the activity of antioxidant factors, thereby regulating inflammation and oxidative stress.

Pyroptosis is different from other cell death methods such as apoptosis. After a certain degree of activation, it can resist external damage, but excessive activation can also lead to serious complications [26,27]. In recent years, more and more studies showed that the related factors of the classical pathway NLPR3 process involved in pyroptosis are highly expressed in the body, and knocking out the related genes can reduce the level of inflammatory, and will also alleviated organ function injury to a certain extent [28,29]. Our study showed that CLP induced a significant increase in renal tissue ROS levels and NLRP3, caspase-1 and GSDMD protein expressions, and SNG pretreatment could significantly reverse this phenomenon.

When sepsis occurs, it can induce a large amount of ROS synthesis, which promotes the dissociation of TXNIP and thioredoxin, and then TXNIP binds to NLRP3 to activate NLRP3. Caspase-1 promotes the production of multiple inflammatory factors, and cleaves the N-terminal domain of GSDMD to mediate pyroptosis [30]. Studies showed that inhibiting the NLRP3 pathway can inhibit the occurrence of pyroptosis [31–33]. Tan et al. [34] showed that the NLRP3 inflammasome activation and the high degree of cell pyroptosis, is the cause of inflammatory infiltration and other damage in the kidney tissue. Li et al. [35] pointed out that NLRP3-mediated pyroptosis is involved in the occurrence of SAKI inflammatory diseases. Our study speculated that SNG might alleviate SAKI by inhibiting renal tissue pyroptosis.

Previous studies have shown that the well-characterized mechanism of NLRP3 inflammasome activation is ROS, and inflammasome activators can increase ROS production, thereby promoting the binding of thioredoxin-interacting proteins to NLRP3 and inflammasome activation [5,36,37]. SIRT3, the most important mitochondrial acetyllysine deacetylase, and which mainly regulates mtROS levels by altering the deacetylation of mitochondrial antioxidant enzymes, including SOD2, isocitrate dehydrogenase2, and glutathione peroxidase, among which SIRT3 can directly bind to deacetylated SOD2, resulting in an increase in SOD2 activity, which subsequently significantly affects mtROS homeostasis and NLRP3 inflammasome activation [38–40]. Therefore, the mitochondrial SIRT3/SOD2/mtROS signaling pathway may be an important pathway for regulating NLRP3 inflammasome activity.

In present study, we found that CLP-induced SIRT3 and SOD2 protein in KTECs significantly down-regulated, while mtROS increased, and SIRT3 and SOD2 protein expression increased and mtROS level decreased after SNG pretreatment. Therefore, SNG might inhibit KTECs pyroptosis by regulating the SIRT3/SOD2/mtROS signaling pathway. Moreover, the protective effect of SNG on SAKI did not depend on the lack of SIRT3, especially in the process of KTECs pyroptosis. Even in the deficiency of SIRT3, SNG could regulate the pyroptosis pathway of KTECs. However, the protection strength of SNG against SAKI seemed to be partially dependent on the SIRT3.

Our study has the following limitations. Firstly, we only used mice for research, and further molecular mechanisms require cell or other methodological experiments. Secondly, the number of animals used in the study is limited, especially in the study of cell pyroptosis pathways, which requires further cytological experiments for verification. Thirdly, although we tried our best to ensure consistency in modeling of septic mice, there were still inevitable loophole that could affect the results. Fourthly, the age of the mice may be an important factor affecting the results of this experiment. Finally, besides our findings, further research is needed to demonstrate whether SNG plays other pharmacological roles in sepsis.

In conclusion, the pathogenesis of SAKI is complex, and SNG has a significant protective effect on it. The specific mechanism may be closely related to the regulation of SIRT3/SOD2/mtROS signaling pathway to inhibit cell pyroptosis, reduce inflammatory factors, and alleviate mitochondrial oxidative stress.

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## Ethics approval and consent to participate

All procedures relating to animals confirmed to the National In stitutes of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Laboratory Animal Ethics Committee of Ningbo University (AEWC-2017-33).

## Authors' contributions

Heng Fan and Min Sun did the experiments, interpreted of data and wrote the first draft of the manuscript. Heng Fan and Jian-hua Zhu participated in conception, design and providing critical revisions. All authors read and approved the final manuscript.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Data availability statement

All data generated or analyzed during this study are available in this article.

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