DOI 10.1007/s11094-022-02554-w
Pharmaceutical Chemistry Journal, Vol. 55, No. 11, February, 2022 (Russian Original Vol. 55, No. 11, November, 2021)

SEARCH FOR NEW DRUGS

SAIKOSAPONIN-D PREVENTS ACUTE RENAL INJURY VIA INHIBITION OF NLRP3 INFLAMMASOME BY SIRT1

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Original article submitted November 10, 2020.

The chemical composition of bupleurum plant is complex. The main active components of bupleurum are saponins, volatile oils, flavonoids and polysaccharides, exerting multiple pharmacological effects. Here, we have first found that saikosaponin-d (SSD) reduced kidney injury and inflammation, induced SIRT1, and suppressed protein expression on *in vivo* mice model of acute kidney injury (AKI). In LPS-induced kidney cell inflammation model, SSD suppressed IL-1B, NLRP3, SIRT1 and ROS expression as detected after cells were treated with SSD. Furthermore, the anti-inflammation effects of SSD on inflammation and kidney injury were detected on *in vivo* mice model of ARI via NLRP3 expression. The results showed that ROS inhibitor increased anti-inflammation effects of SSD on *in vivo* mice inflammation model and *in vitro* model. Finally, NLRP3 inhibitor also reduced anti-inflammation effects of SSD on *in vivo* mice inflammation model and *in vitro* model. On the whole, these findings confirm that SSD prevents AKI via inhibition of NLRP3 inflammasome by SIRT1.

Keywords: saikosaponin-d; acute renal injury; NLRP3; SIRT1

1. INTRODUCTION

Acute renal injury (ARI) or acute kidney injury (AKI) is defined as sharply declined renal function within a few days under the impacts of pre-renal, renal, and post-renal damage [1]. AKI is microscopically manifested as necrosis of renal tubular epithelial cells, tubular formation within interstitial vessels and infiltration of interstitial cells [2]. Currently, one of the well-studied inflammasomes is NLRP3 inflammasome, including nucleotide binding oligomerization domain like receptors family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1 [3]. The interaction between NLRP3 and ASC can activate Caspase-1, which in turn promotes IL-1β release and causes inflammation [4]. In terms of kidney disease, NLRP3 is associated with the pathogenesis of various kidney diseases [5]. The expression of NLRP3 inflammasome is found in renal tubular epithelial cells and glomerular cells [5]. AKI caused by ischemia-reperfusion is associated with activation of NLRP3 inflammasome [6]. The progression of various chronic kidney diseases is also associated with NLRP3 [6].

Silent information regulator 1 (Sirt1), is a deacetylase dependent on nicotinamide adenine dinucleotide (NAD^+) and is a member of the mammalian Sirtuins family. Multiple studies have demonstrated that Sirt1 can regulate gene silencing, cell proliferation, differentiation, senescence, apoptosis, and energy metabolism by deacetylating receptor proteins (histone/non-histone), thereby playing important roles in anti-aging, tumor, glycolipid metabolism regulation and the cardiovascular system. In recent years, great attention has been paid to the roles of Sirt1 in the development of kidney disease and the underlying regulatory mechanisms. Bupleurum is a dry root of Umbelliferae plant *Bupleurum chinensis* DC. or *B. scorzonerifolium* Willd. It is a commonly used medicinal material with a long history of application, exerting anti-tumor, liver protection, anti-bacterial, antipyretic effects, etc. [7]. Saikoside is a substance with a variety of pharmacological characteristics extracted from Chinese traditional medicine bupleurum [8]. Extensive stud-

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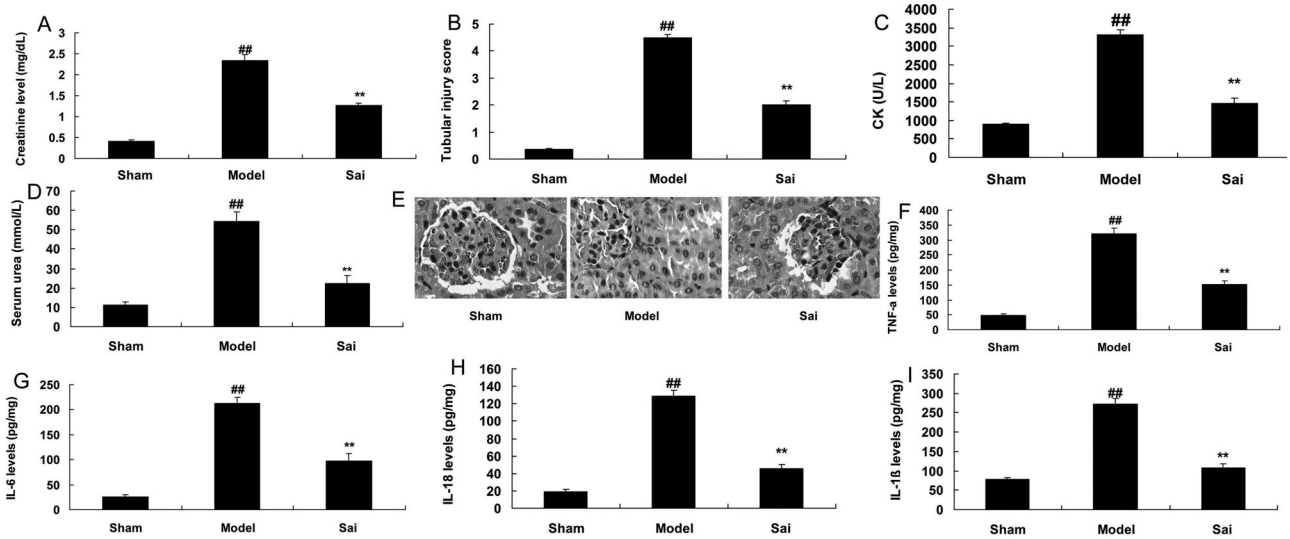

Fig. 1. Saikosaponin-d prevents AKI and reduced inflammation in mice: Creatinine (A), tubular injury score (B), CK levels (C), serum urea (D), kidney bean by HE (E), TNF-α (F), IL-1β (G), IL-6 (H) and IL-18 (I) levels. Sham, sham normal group; Model, AKI model group; Sai, SSD treatment group. $^{\#\#}p < 0.01$ compared with sham normal group; $^{**}p < 0.01$ compared with AKI model group.

ies have reported that saikoside is commonly used in cold and fever. Alternate chills and fever, malaria, liver qi stagnation, chest rib pain, rectal prolapse, uterine prolapse, irregular menstruation, anti-convulsion, anti-inflammation, anti-depression, anti-epilepsy, anti-tumor etc. [9]. This study was aimed to elucidate the effects of saikosaponin-d (SSD) on AKI and provide a possible molecular mechanism.

2. MATERIALS AND METHODS

2.1. Animals, Drugs and In Vivo Model

Male C57BL/6J mice were obtained from the Animal Resource Center of Vital River (Beijing, China). All animal procedures were approved by the Institutional Animal Care and Use Committee of Hebei General Hospital in this study. Adult (8-week-old) C57BL/6J mice were housed in temperature- (22 – 23°C) and humidity-controlled cages (40 – 50%) and fed a standard normal diet with free access to rodent food and water.

2.2. Experimental Procedures

All mice were narcotized with 35 mg/kg pentobarbital sodium and then mice were opening the abdominal cavity. Kidney was ligated by bilateral renal pedicle clamping for 30 min. Sham-operated mice narcotized with 35 mg/kg pentobarbital sodium underwent the same surgical procedures but without bilateral renal pedicle. Saikosaponin-d group mice were treated with SSD (10 mg/kg, i.p.) for three days before IR surgery. Mice of Tenovin-6 group were treated with Tenovin-6 (40 mg/kg, i.p.) and SSD (10 mg/kg, i.p.) for three days before IR surgery.

Hematoxylin and eosin staining. Renal tissue samples were fixed with 4% paraformaldehyde for 24 h and sections were deparafnized in xylene and rehydrated with degraded alcohol. Samples were subjected to hematoxylin and eosin (H&E) staining.

Inflammation and ROS production levels by ELISA. TNF-α, IL-1β, IL-6 and IL-18 levels were measured using inflammation ELISA Kits. ROS production levels were measured using inflammation ROS ELISA Kits.

Cell Lines and cell culture. Mouse renal tubular epithelial (mTE) cells were purchased from Shanghai cell bank, Chinese academy of sciences. mTE cells were cultured in DMEM with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO_2. mTE cells were treated with LPS (500 ng/mL) for 4 h at 37°C and 5% CO_2.

Western blotting. Proteins were extracted from tissues or cultured cells using RIPA buffer, and the protein concentrations were measured using BCA Protein Quantification Kit. Total proteins (100 μg per lane) were then resolved on 10% SDS-PAGE gels and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were then probed with antibodies: for specific target proteins at 4°C overnight after blocking with nonfat milk at 37°C. The membrane was washed with TBST and then incubated with anti-rabbit secondary antibody (1:5000) for 2 h at room temperature. Data were evaluated by image analysis software (ImageJ version 1.48).

2.3. Statistical Analysis

All data are presented as mean ± SD. Confidence $P < 0.05$ was considered to be statistically significant. One-

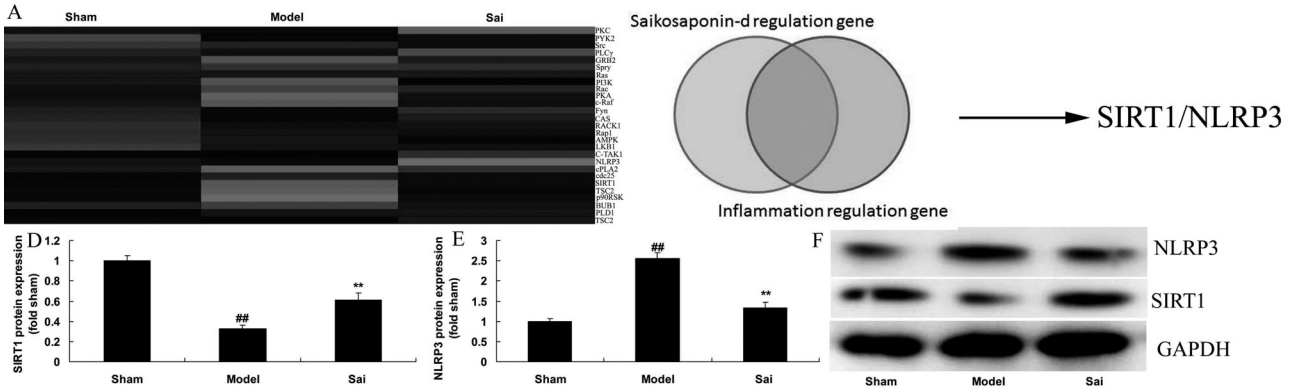

Fig. 2. Saikosaponin-d reduced inflammation in mice via NLRP3 inflammsome by SIRT1. Heat map and analysis graphics (A), SIRT1 (B) and NLRP3 (C) protein expression by statistical analysis and western blotting assays (D). Sham, sham normal group; Model, AKI model group; Sai, SSD treatment group. $^{\#\#}p < 0.01$ compared with sham normal group; $^{**}p < 0.01$ compared with AKI model group.

way analysis of variance (ANOVA) or Student's t-test was used to evaluate the significance of differences groups.

3. RESULTS

3.1. Saikosaponin-d prevented AKI and reduced inflammation in mice

In order to systematically identify the SSD effects, AKI group mice were treated with SSD and surveyed. Creatinine, serum urea and creatine kinase (CK) levels, and tubular injury score in AKI model were higher than those of sham group (Figs. 1A-1E). Meanwhile, TNF-α, IL-1β, IL-6 and IL-18 levels in AKI mice were increased as compared to sham group (Figs. 1F-1I). However, SSD reduced AKI-induced creatinine, serum urea and CK levels, and tubular injury score in AKI mice as compared to the injury model (Figs. 1A-1E).

3.2. Saikosaponin-d reduced inflammation in mice via NLRP3 inflammsome by SIRT1

In order to determine that mechanism of Saikosaponin-d on inflammation in AKI mice, we found that SSD regulated gene using gene chip. Then, SIRT1/NLRP3 maybe target spot for the SSD effects (Fig. 2A). Saikosaponin-d induced SIRT1 protein and suppressed NLRP3 inflammsome protein expression in AKI mice as compared to AKI model (Figs. 2B-2D).

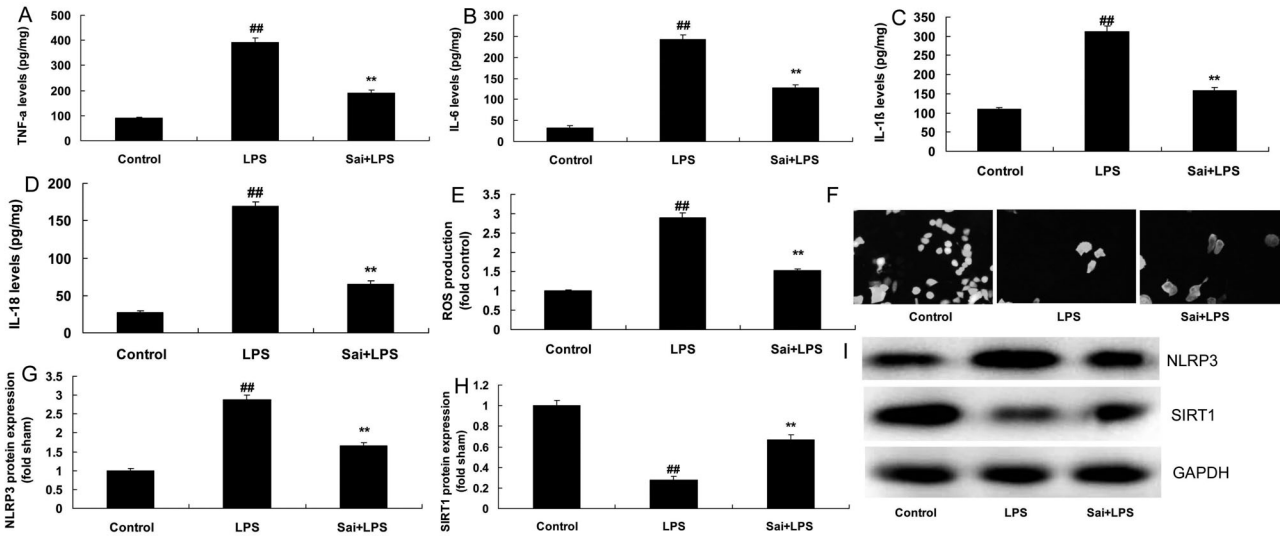

Fig. 3. Saikosaponin-d reduced inflammation and ROS production via NLRP3 inflammsome by SIRT1. TNF-α (A), IL-1β (B), IL-6 (C) and IL-18 (D) levels, ROS production (E and F), SIRT1 (G) and NLRP3 (H) protein expression by statistical analysis and western blotting assays (I). Control, control mimics group; LPS, LPS *in vitro* model group; LPS+ Sai, SSD treatment in LPS *in vitro* model group. $^{\#\#}p < 0.01$ compared with control mimics group; $^{**}p < 0.01$ compared with LPS *in vitro* model group.

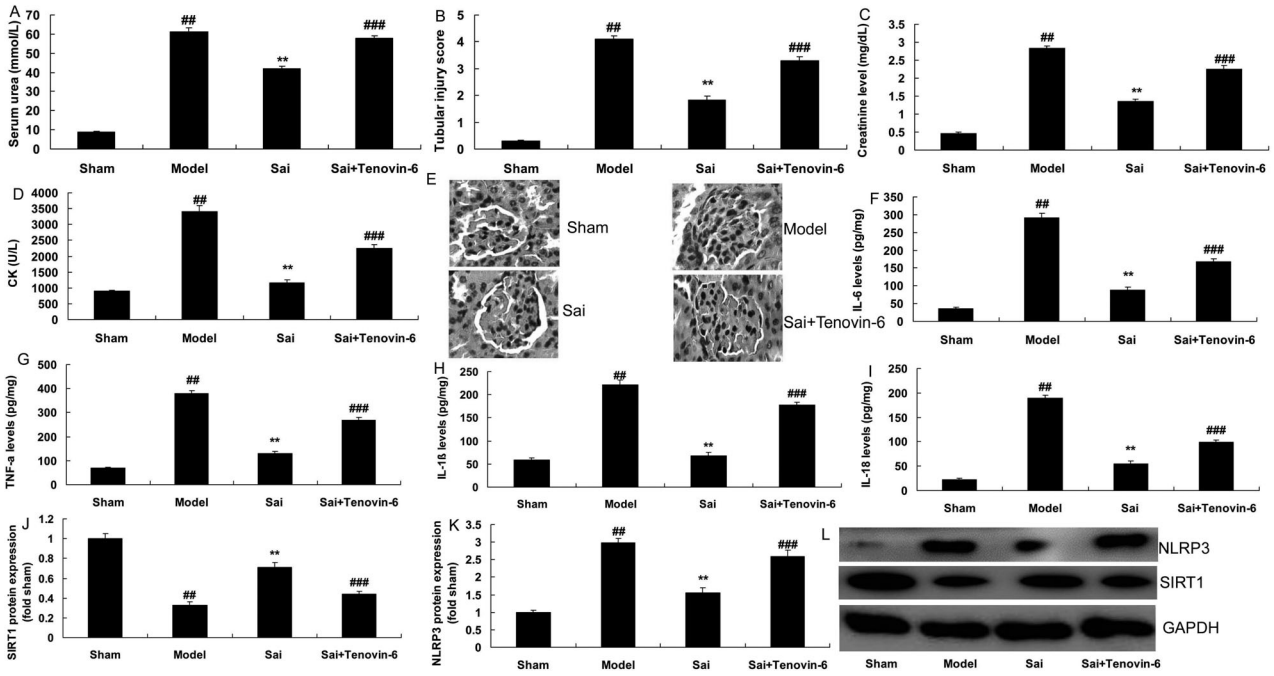

Fig. 4. SIRT1 inhibitor reduced the anti-inflammation effects of Saikosaponin-d *in vivo* model. Creatinine (A), tubular injury score (B), CK levels (C), serum urea (D), kidney bean by HE (E), TNF-α (F), IL-1β (G), IL-6 (H) and IL-18 (I) levels, SIRT1 (J) and NLRP3 (K) protein expression by statistical analysis and western blotting assays (L). Sham, sham normal group; Model, AKI model group; Sai, SSD treatment group; Sai + Tenovin-6, treatment with SSD and Tenovin-6 group. $^{\#\#}p < 0.01$ compared with sham normal group; $^{**}p < 0.01$ compared with AKI model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

3.3. Saikosaponin-d reduced inflammation and ROS production via NLRP3 inflammsome by SIRT1

In addition, *in vitro* model, SSD reduced LPS-induced TNF-α, IL-1β, IL-6 and IL-18, and inhibited ROS production levels, compared to model of LPS group (Figs. 3A-3F). However, SSD suppressed NLRP3 protein expression and induced SIRT1 protein expression in LPS-induced vitro model as compared to LPS-induced *in vitro* model group (Figs. 3G-3I).

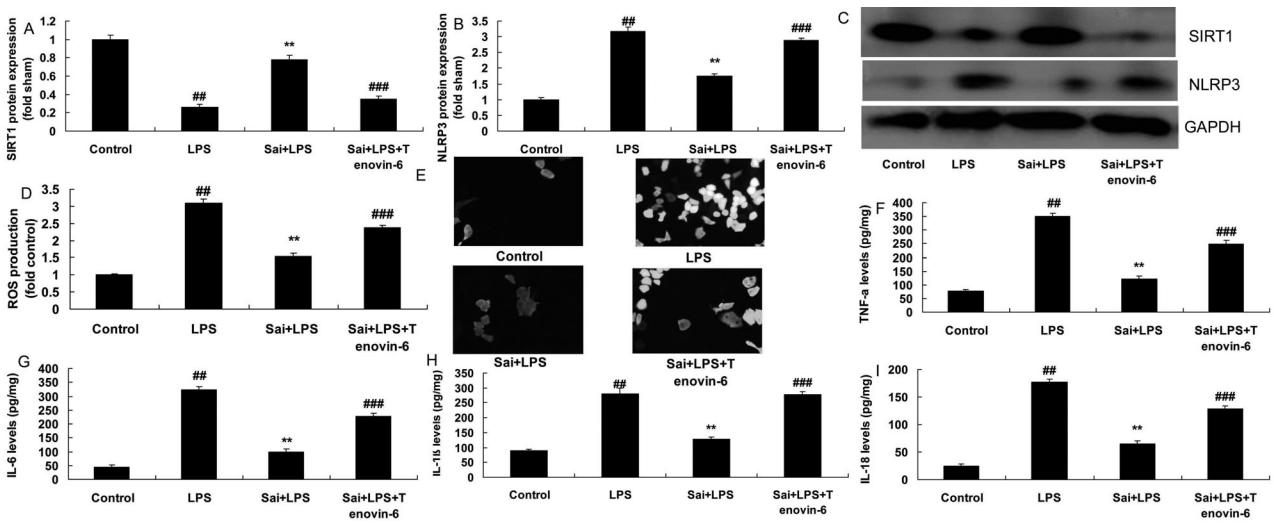

Fig. 5. The inhibition of SIRT1 reduced the effects of anti-inflammation effects of Saikosaponin-d *in vitro* model. TNF-α (A), IL-1β (B), IL-6 (C) and IL-18 (D) levels, ROS production (E and F), SIRT1 (G) and NLRP3 (H) protein expression by statistical analysis and western blotting assays (I). Control, control mimics group; LPS, LPS *in vitro* model group; LPS+ Sai, SSD treatment in LPS *in vitro* model group; LPS + Sai + Tenovin-6, treatment with SSD and Tenovin-6 in LPS *in vitro* model group. $^{\#\#}p < 0.01$ compared with control mimics group; $^{**}p < 0.01$ compared with LPS *in vitro* model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

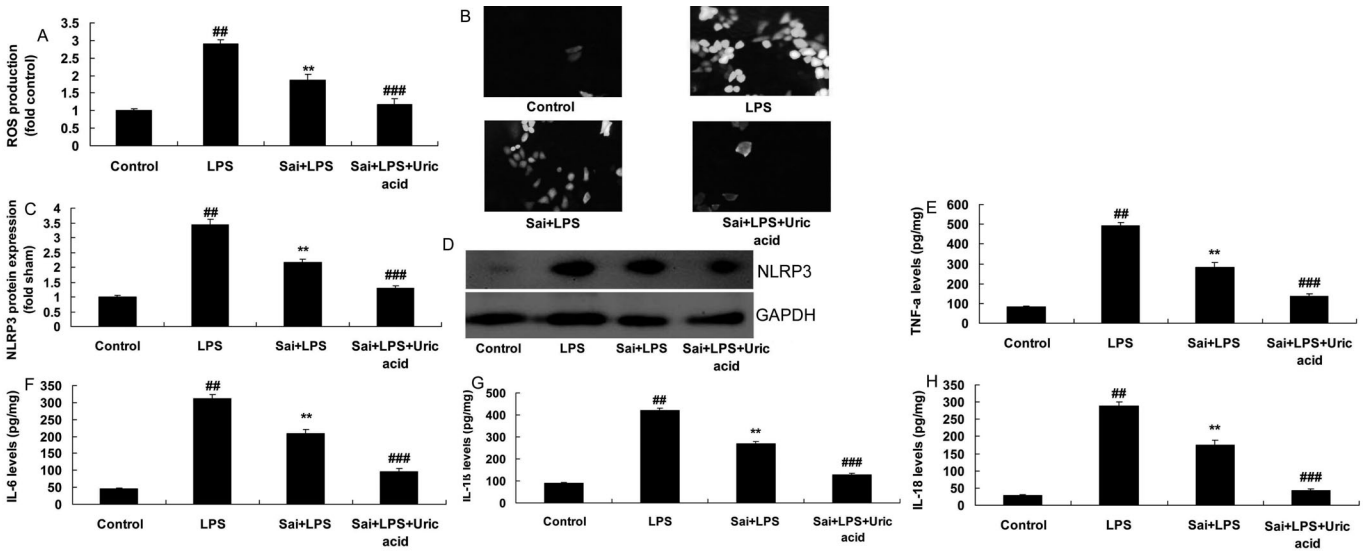

Fig. 6. ROS inhibitor increased the anti-inflammation effects of Saikosaponin-d *in vitro* model. ROS production (A and B), NLRP3 (C) protein expression by statistical analysis and western blotting assays (E), TNF-α (E), IL-1β (F), IL-6 (G) and IL-18 (H) levels. Control, control mimics group; LPS, LPS *in vitro* model group; LPS+ Sai, SSD treatment in LPS *in vitro* model group; LPS + Sai + uric acid, treatment with SSD and uric acid in LPS *in vitro* model group. $^{\#\#}p < 0.01$ compared with control mimics group; $^{**}p < 0.01$ compared with LPS *in vitro* model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

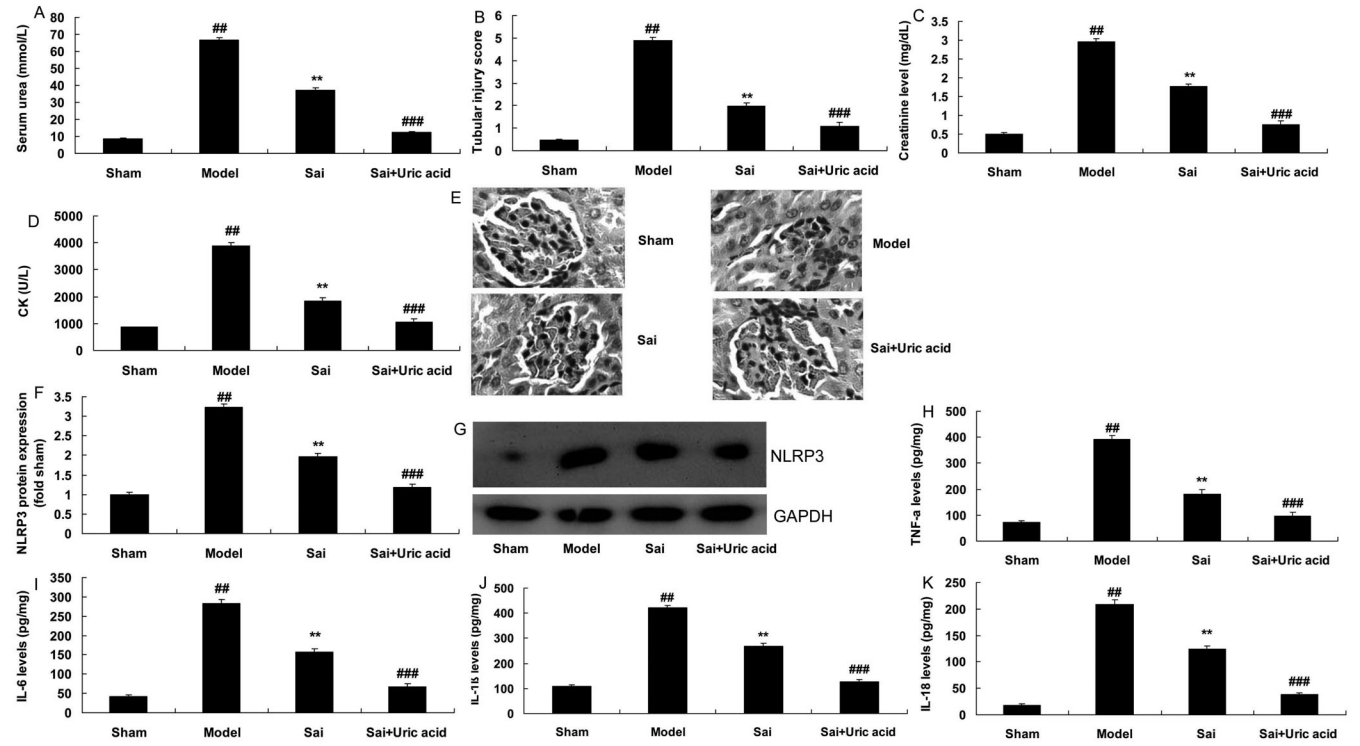

Fig. 7. ROS inhibitor increased the anti-inflammation effects of Saikosaponin-d *in vivo* model. Creatinine (A), tubular injury score (B), CK levels (C), serum urea (D), kidney bean by HE (E), NLRP3 (F) protein expression by statistical analysis and western blotting assays (G), TNF-α (H), IL-1β (I), IL-6 (J) and IL-18 (K) levels, Sham, sham normal group; Model, AKI model group; Sai, SSD treatment group; Sai + Tenovin-6 + uric acid, treatment with SSD and uric acid group. $^{\#\#}p < 0.01$ compared with sham normal group; $^{**}p < 0.01$ compared with AKI model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

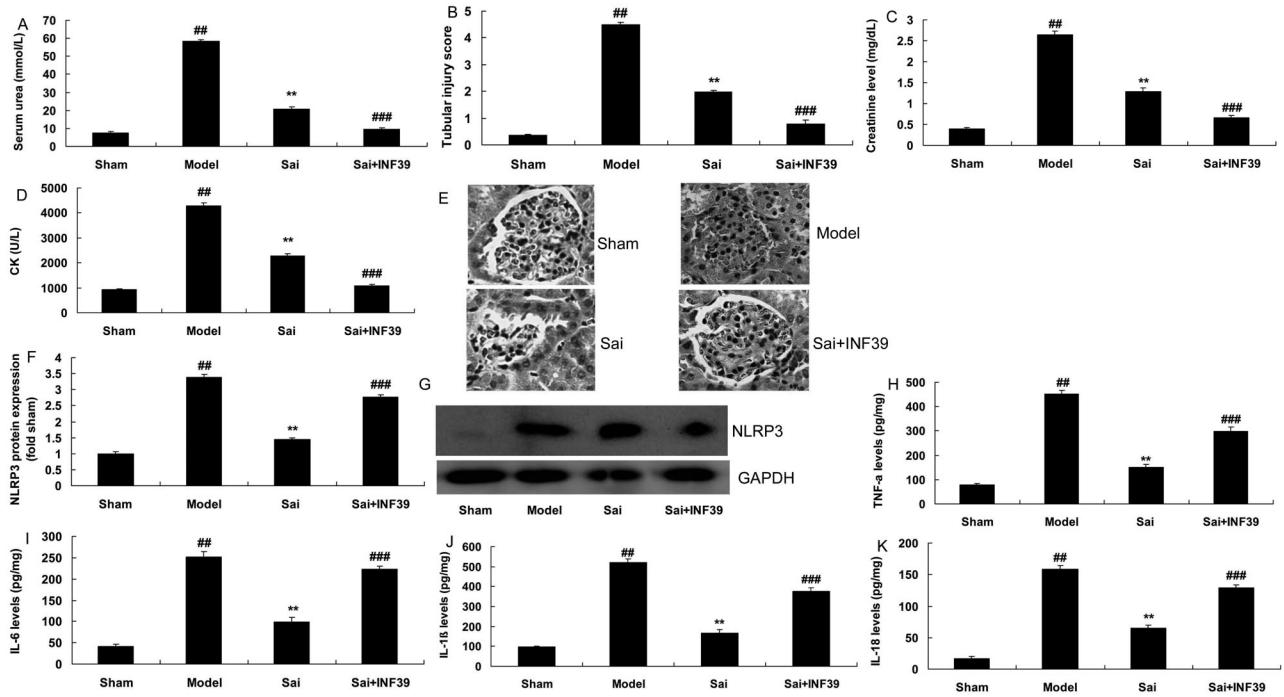

Fig. 8. NLRP3 inhibitor also increased anti-inflammation effects of Saikosaponin-d on inflammation *in vivo* mice model. Creatinine (A), tubular injury score (B), CK levels (C), serum urea (D), kidney bean by HE (E), NLRP3 (F) protein expression by statistical analysis and western blotting assays (G), TNF-α (H), IL-1β (I), IL-6 (J) and IL-18 (K) levels, Sham, sham normal group; Model, AKI model group; Sai, SSD treatment group; Sai + Tenovin-6 + INF39, treatment with SSD and INF39 group. $^{\#\#}p < 0.01$ compared with sham normal group; $^{**}p < 0.01$ compared with AKI model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

3.4. SIRT1 inhibitor reduced the anti-inflammation effects of Saikosaponin-d in vivo model

To further determine the role of SIRT1 in the anti-inflammation effects of SSD *in vivo* model, Tenovin-6 (40 mg/kg, i.p.), SIRT1 inhibitor and SSD were used to treat AKI mice. Tenovin-6 increased creatinine, serum urea and CK levels, and tubular injury score in AKI mice treated with SSD as compared to SSD treatment group (Figs. 4A-4E).

Tenovin-6 also elevated TNF-α, IL-1β, IL-6 and IL-18 levels in AKI mice treated with SSD as compared to SSD treatment group (Figs. 4F-4I). Tenovin-6 reduced SIRT1 protein expression and induced NLRP3 protein expression in AKI mice by treated with SSD as compared to SSD treatment group (Figs. 4J-4L).

3.5. The inhibition of SIRT1 reduced the anti-inflammation effects of Saikosaponin-d in vitro model

In vitro model, Tenovin-6 (10 ìM) reduced SIRT1 protein expression and induced NLRP3 protein expression in LPS-induced *in vitro* by Saikosaponin-d as compared to SSD treatment group (Figs. 5A-5C).

Tenovin-6 also increased ROS production levels, and elevated TNF-α, IL-1β, IL-6 and IL-18 levels induced by SSD in LPS *in vitro* model as compared to SSD treatment group (Figs. 5D-5I).

3.6. ROS inhibitor increased the anti-inflammation effects of Saikosaponin-d in vitro model

To explain the function of ROS in anti-inflammation effects of SSD *in vivo* model, uric acid, ROS inhibitor reduced ROA production and suppressed NLRP3 protein expression *in vitro* model by SSD as compared to SSD treatment group (Figs. 6A-6D). Then, uric acid reduced TNF-α, IL-1β, IL-6 and IL-18 levels in LPS-induced *in vitro* by SSD as compared to SSDA treatment group (Figs. 6E-6H).

3.7. ROS inhibitor increased the anti-inflammation effects of Saikosaponin-d in vivo model

Next, compared with SSD treatment group, uric acid increased the anti-inflammation effects of SSD on creatinine, serum urea and CK levels, and tubular injury score in AKI mice (Figs. 7A-7E). Uric acid suppressed NLRP3 protein expression and reduced TNF-α, IL-1β, IL-6 and IL-18 levels in AKI mice as compared to SSD treatment group (Figs. 7F-7K).

3.8. in NLRP3 inhibitor also increased anti-inflammation effects of Saikosaponin-d on inflammation in vivo mice model or vitro model

Finally, INF39 (25 mg/kg) also reduced creatinine, serum urea and CK levels, and tubular injury score in AKI

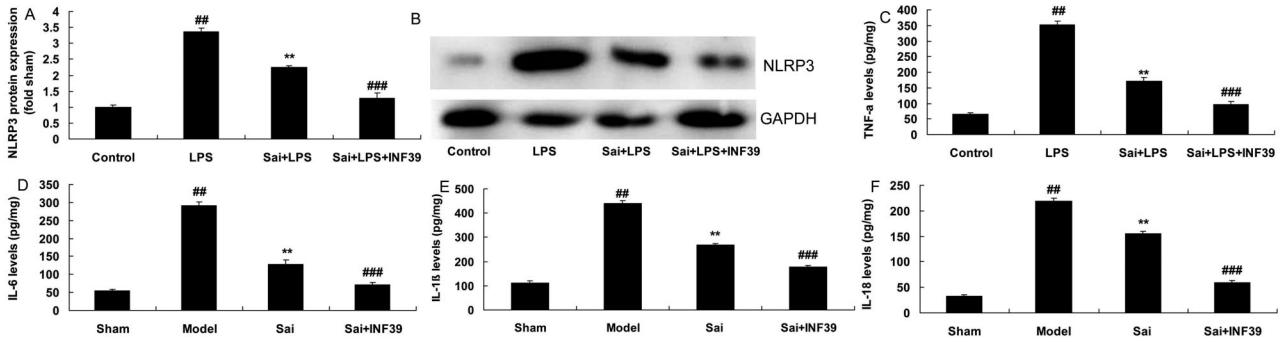

Fig. 9. NLRP3 inhibitor also increased anti-inflammation effects of Saikosaponin-d on inflammation *in vitro* model. NLRP3 (A) protein expression by statistical analysis and western blotting assays (B), TNF-α (C), IL-1β (D), IL-6 (E) and IL-18 (F) levels. Control, control mimics group; LPS, LPS *in vitro* model group; LPS+ Sai, treatment with SSD in LPS *in vitro* model group; LPS + Sai + INF39, treatment with SSD and INF39 in LPS *in vitro* model group. $^{\#\#}p < 0.01$ compared with control mimics group; $^{**}p < 0.01$ compared with LPS *in vitro* model group; $^{\#\#\#}p < 0.01$ compared with SSD treatment group.

mice by treated with SSD as compared with SSD treatment group (Figs. 8A-8E).

INF39 suppressed NLRP3 protein expression and reduced TNF-α, IL-1β, IL-6 and IL-18 levels in AKI mice as compared to SSD treatment group (Figs. 8F-8K). INF39 also suppressed NLRP3 protein expression and reduced TNF-α, IL-1β, IL-6 and IL-18 levels in AKI mice as compared to SSD treatment group (Fig. 9).

4. DISCUSSION

AKI is a common clinical acute and severe kidney disease. Patients with AKI not only have a high death rate, but also have a high risk of progression into chronic kidney disease [10]. Therefore, AKI has become a public health issue of global concern. Studies have shown that aseptic inflammation (no inflammatory response to pathogens) is mediated by inflammasome [11]. Our study confirmed that SSD reduced AKI-induced creatinine, serum urea and CK levels, tubular injury score in AKI mice as compared to AKI model. Wang, et al. [12] showed that SSD attenuates ventilator-induced lung injury in rats. Therefore, could provide protective effects against AKI.

Sirt1 could mediate gene silencing by deacetylating receptor proteins dependent on NAD^+, thereby regulating cell proliferation, apoptosis and senescence, and participating in the regulation of glucose homeostasis of the body [13]. Sirt1 plays an extremely important role in the development of kidney disease [14]. Sirt1 can attenuate the apoptosis of renal cells caused by various injuries, decrease renal inflammation, alleviate mitochondrial function and reduce oxidative stress, thereby improving diabetic nephropathy and protecting the kidney from acute injury, delaying the aging process of the kidney, and improving the prognosis of chronic kidney disease [15, 16]. This result suggested that SSD reduced inflammation and ROS production via NLRP3 inflammsome by SIRT1. Que, et al. [17] showed that SSD suppressed ROS levels in rat hepatic stellate cell activation. As such, activation of SIRT1 represents an approach to using the effects of SSD to treat renal injury.

The NLRP3 inflammasome was originally considered to be mainly present in immune cells such as neutrophils, T cells, B cells, macrophages, etc. [5, 18]. However, recent studies have found that a variety of kidney disease progression are accompanied by the activation of NLRP3 inflammasome, including chronic kidney disease, acute tubular necrosis, kidney injury caused by ischemia-reperfusion, etc. [19]. Our study also demonstrated that the inhibition of SIRT1 reduced the effects of anti-inflammation effects of Saikosaponin-d *in vitro* model via NLRP3 inflammasome. Pu, et al. [20] showed that arctigenin attenuates inflammation in dextran sulfate sodium-induced acute colitis through suppression of NLRP3 inflammasome by SIRT1. Further experiments would be required to improve the SSD effects in AKI treatment.

Thus, we have demonstrated that SSD prevented AKI development injury and reduced inflammation in mice, so that SSD may be valuable in insight into activation of AKI and its clinical application. Since precise mechanisms of SSD action on inflammation or AKI remain unclear, it is necessary to further study this issue in the future.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of the Hebei General Hospital.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Lin Kang was dedicated to the integrity of the entire study; Fan Yang carried out the study concepts, definition of intellectual content, data acquisition and manuscript editing; Xiuzhi Zhang was involved in the manuscript preparation; Jing Zhao focused on the experimental studies; Yang Liu carried out the statistical analysis; Huanfen Zhao was involved in the manuscript review; Zhijuan Hu focused on the clinical studies and data analysis; Bing Liu was dedicated to the study design; Chunnian He carried out the literature research. All authors have read and approved this article.

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