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# Protective role of AGK2 on thioacetamide-induced acute liver failure in mice

Fang-Zhou Jiao, Yao Wang, Wen-Bin Zhang, Hai-Yue Zhang, Qian Chen, Chun-Xia Shi, Lu-Wen Wang, Zuo-Jiong Gong*

*Department of Infectious Diseases, Renmin Hospital of Wuhan University, Hubei, Wuhan, China*

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ABSTRACT

*Aims:* The aim of the present study was to investigate the protective effects of AGK2 as a selective SIRT2 inhibitor on thioacetamide (TAA)-induced acute liver failure (ALF) in mice and its potential mechanism.

*Main methods:* All male C57BL/6 mice were separated into control, TAA, AGK2 + TAA, and AGK2 groups. The histological changes were observed by hematoxylin and eosin (HE) staining. The apoptosis cells of liver tissues were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used to evaluate the damage of liver function. The inflammatory cytokines of iNOS, TNF-α, IL-1β was detected by Western blotting and RT-PCR assay. The expression of mitogen-activated protein kinase (MAPK), NF-κB, and apoptosis pathways was determined by Western blotting.

*Key findings:* AGK2 improved the damage of TAA-induced liver pathology and function. AGK2 pretreatment also reduced the levels of pro-inflammatory cytokines in ALF liver tissues. AGK2 improved the TAA-induced survival rate. Moreover, AGK2 administration suppressed the increase of phosphorylation NF-κB-p65 and the activation of MAPK pathway. In addition, pretreatment alleviated TAA-induced the liver cells apoptosis.

*Significance:* AGK2 improve TAA-induced survival rate in mice with ALF, suppress the inflammatory responses by inhibition of MAPK and NF-κB signaling pathways, and decrease the hepatocyte necrosis by inhibition of apoptosis. Pharmacologic inhibition of SIRT2 may be a promising approach for the treatment of ALF.

## 1. Introduction

Acute liver failure (ALF) is a life-threatening condition with high mortality, the causes of death including multi-organ failure, hemorrhage, infection, and cerebral edema [1,2]. ALF is an unusual clinical syndrome of often rapidly progressive with multiple unpredictable complications. There is no effective medical therapy for this syndrome. In this condition, emergency liver transplantation is lifesaving treatment option [1]. However, liver transplantations are only performed on small number of patients, due to scarcity of liver donors and high costs. Therefore, it is urgent to explore a novel solution for ALF.

The typical pathological characteristic of ALF is overwhelming hepatocyte death, with massive infiltration of inflammatory cells [3]. Mounting evidence suggest that liver inflammation plays a pivotal role in the pathogenesis and prognosis of ALF [4]. Several signaling pathways involved the inflammatory process, such as mitogen-activated protein kinase (MAPK), nuclear factor kappa β (NF-κβ), protein methylases [5]. MAPK pathways consists of p38 kinase (P38), c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK). Activation of MAPK pathways and NF-κβ result in release of proinflammatory factors (tumor necrosis factor-alpha (TNF-a), and interleukin (IL)-1β) [6,7].

Epigenetic changes involve a variety of medical areas, such as cancer, neurodegenerative, inflammatory, metabolic, cardiovascular, infectious diseases. As a main way of epigenetic changes, posttranslational histone modifications include histone methylation, histone acetylation, histone phosphorylation, and histone ubiquitination [8]. The most frequent ones are acetylation in these modifications, and it regulate gene transcription by changing nucleosome structures [9]. Acetylation of histones is mediated by histone acetyltransferases (HATs) and counteracted by histone deacetylases (HDACs) [10]. There are four class of HDAC enzyme, including Class I, II, III, and IV. While, class III of HDACs (sirtuins) are special because their enzyme needs nicotinamide adenine dinucleotide (NAD+) [11].

There are seven numbers in mammalian sirtuins, from SIRT1 to SIRT7. Several studies support that the course of inflammation is linked with immune response and metabolism networks; sirtuins could regulate these networks [12]. Evidence supports that SIRT2 is a

* Corresponding author at: Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, China.
*E-mail address:* zjgong@163.com (Z.-J. Gong).

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cytoplasmic protein but it also is found in the nucleus [13]. In addition, SIRT2 could deacetylate histone in nucleus and non-histone proteins in cytoplasm. Mounting evidence supports that SIRT2 involve in various physiological processes, including cell cycle progression, tumorigenesis [14], and inflammatory responses [15]. A previous study evaluated the anti-inflammation effect of SIRT2 in lipopolysaccharide (LPS)-stimulated macrophages. The results showed that deficiency of SIRT2 reduced NO production, iNOS expression, and repressed phosphorylation of NF-κβ-p65 in macrophages after LPS stimulation [16]. Our previous studies have shown that the class I and II HDAC inhibitor trichostatin A could alleviates liver damage in rats with acute-on-chronic liver failure by inflammatory inhibition [17]. However, it is still unclear that inhibition of SIRT2 could improve the inflammation in ALF.

The current study, thioacetamide (TAA)-induced ALF mice model was conducted. The selective SIRT2 inhibitor (AGK2) was used, and its possible hepato-protective potential and anti-inflammatory effect was investigated during ALF.

## 2. Materials and methods

### 2.1. Chemicals and drugs

Thioacetamide (TAA) were purchased from Sigma-Aldrich (USA). AGK2 were purchased from Selleckchem (USA). Anti-iNOS (#2982), TNF-α (#11948), IL-1β (#12426), phospho-p38 (#4511), p38 (#8690), phospho-JNK (#4668), JNK (#9252), phospho-ERK (#4370), ERK (#9102), phospho-NF-κB-p65 (#3033), NF-κB-p65 (#8242), Bax (#5023), Cleaved caspase 3 (#9664), SIRT2(#12650), α-tubulin (#2125), and acetyl-α-tubulin (#5335) were from Cell Signaling Technology (USA). Bcl-2 (ab196495) were from Abcam (UK).

### 2.2. Animals

Male C57BL/6J mice (6–8 weeks of age; weighing 20–25 g) were obtained from Beijing Vital River 0Laboratory Animal Technology (Beijing, China). The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Remmin Hospital of Wuhan university (WDRM (F) 20181018). Mice were kept in standard cages with light-controlled condition (12 h light-dark cycle) and maintained in 25 °C and had free access to food and water. All animals received care in accordance with the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### 2.3. Experimental model and protocols

As a common hepatotoxin, TAA has been extensively used animal models of acute liver failure [18,19]. In this study, 45 mice randomly assigned into 4 groups: control, TAA, AGK2 + TAA and AGK2 group. The dosage of AGK2 (1 μmol/mouse) was selected according to a previous published study of Wang et al. [20]. The mice model of ALF was performed by intraperitoneal injection with TAA (600 mg/kg). The drug of AGK2 (5 mg) was dissolved in DMSO and formed 10 mM mother liquor. Then, the solution was diluted in normal saline (NS) and formed final concentration of AGK2 (400 μM, 4% DMSO). For AGK2 + TAA group and AGK2 group, 2.5 ml AGK2 (400 μM) was injected into abdominal cavities of each mouse. Meantime, the same volume of 4% DMSO solution was injected in TAA group and control group. After 2 h, TAA (600 mg/kg) was given for each mouse in TAA group and AGK2 + TAA group. While, the same volume of NS was given in control group and AGK2 group. The 24 h survival rate of each group was observed after TAA injection. All mice were sacrificed and the liver tissues were harvested at 24 h time point after TAA administration.

### 2.4. Biochemical analyses

Blood samples were collected when mice were anesthetized. The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by using a Hitachi Automatic Analyzer (Japan).

### 2.5. Histopathological examination

Fresh liver tissues were fixed in 10% neutral-buffered formalin for 24 h. The liver specimens were sliced into 5 μm thickness. The sections were stained hematoxylin and eosin (HE) according to standard histological methods. The pathological changes of liver were observed and evaluated under light microscope (Olympus, Japan). The liver histological score was used to determine the degree of liver injury, consist of inflammation and necrosis scores [21]. In the scoring system, no inflammation was counted as 0, mild inflammation (< 10% of liver section) as 1, moderate inflammation (10–50% of liver section) as 2, and severe inflammation (> 50% of liver section) as 3. no necrosis was counted as 0, necrosis area < 10% was counted as 1, 10–25% was counted as 2, and > 25% was counted as 3.

### 2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Hepatic cell death was detected by TUNEL kits (Roche, USA). Briefly, the liver sections were incubated with proteinase K (Roche, USA) for 15 min at 37 °C and then sections were washed with phosphate-buffered saline (PBS). The sections were incubated with a TUNEL reaction mixture for 1 h at 37 °C. Nuclei were stained with DAPI for 5 min (Beyotime, China). The TUNEL-positive cells were observed and analyzed by fluorescence microscope (Olympus, Japan).

### 2.7. Quantitative RT-PCR

Total RNA was isolated from liver tissues by using TaKaRa MiniBEST Universal RNA Extraction Kit (Dalian, China). the RNA (1 μg) samples were reversely transcribed into cDNA according to the instruction of Prime Script™ RT reagent Kit (Takara, Dalian, China). Real-time polymerase chain reaction (RT-PCR) was performed using the SYBR Green Kit (Takara, Dalian, China) on a 7500 Sequence Detection system (Applied Biosystems, USA). The PCR conditions was carried out as follows: Initial activation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and at 60 °C for 30 s. The gene expression was calculated with the $2-\Delta\Delta Ct$ method. The primers were synthesized by Aidlab Biotechnologies Co., Ltd. (Beijing, China), and the sequences were summarized in Table 1.

### 2.8. Western blotting

The liver tissues were homogenized in RIPA lysis buffer (Beyotime, China) containing PMSF (Beyotime, China) and protease and phosphatase inhibitors and (Beyotime, China). Following centrifugation, protein concentrations of the resulting supernatants were detected by

**Table 1**
The primers sequence.

| Gene | | Primer sequence |
|---|---|---|
| β-actin | Forward | 5′-CACGATGGAGGGGCCGGACTCATC-3′ |
| | Reverse | 5′-TAAAGACCTCTATGCCAACACAGT-3′ |
| iNOS | Forward | 5′-TTGGCTCCAGCATGTACCCT-3′ |
| | Reverse | 5′-TCCTGCCCACTGAGTTCGTC-3′ |
| TNF-α | Forward | 5′-CGTCAGCCGATTTGCTATCT-3′ |
| | Reverse | 5′-CGGACTCCGCAAAGTCTAAG-3′ |
| IL-1β | Forward | 5′-TCAGGCAGGCAGTATCACTC-3′ |
| | Reverse | 5′-AGCTCATATGGGTCCGACAG-3′ |
| IFN-γ | Forward | 5′-CTCAAGTGGCATAGATGTGGAA-3′ |
| | Reverse | 5′-GACCTCAAACTTGGCAATACTC-3′ |

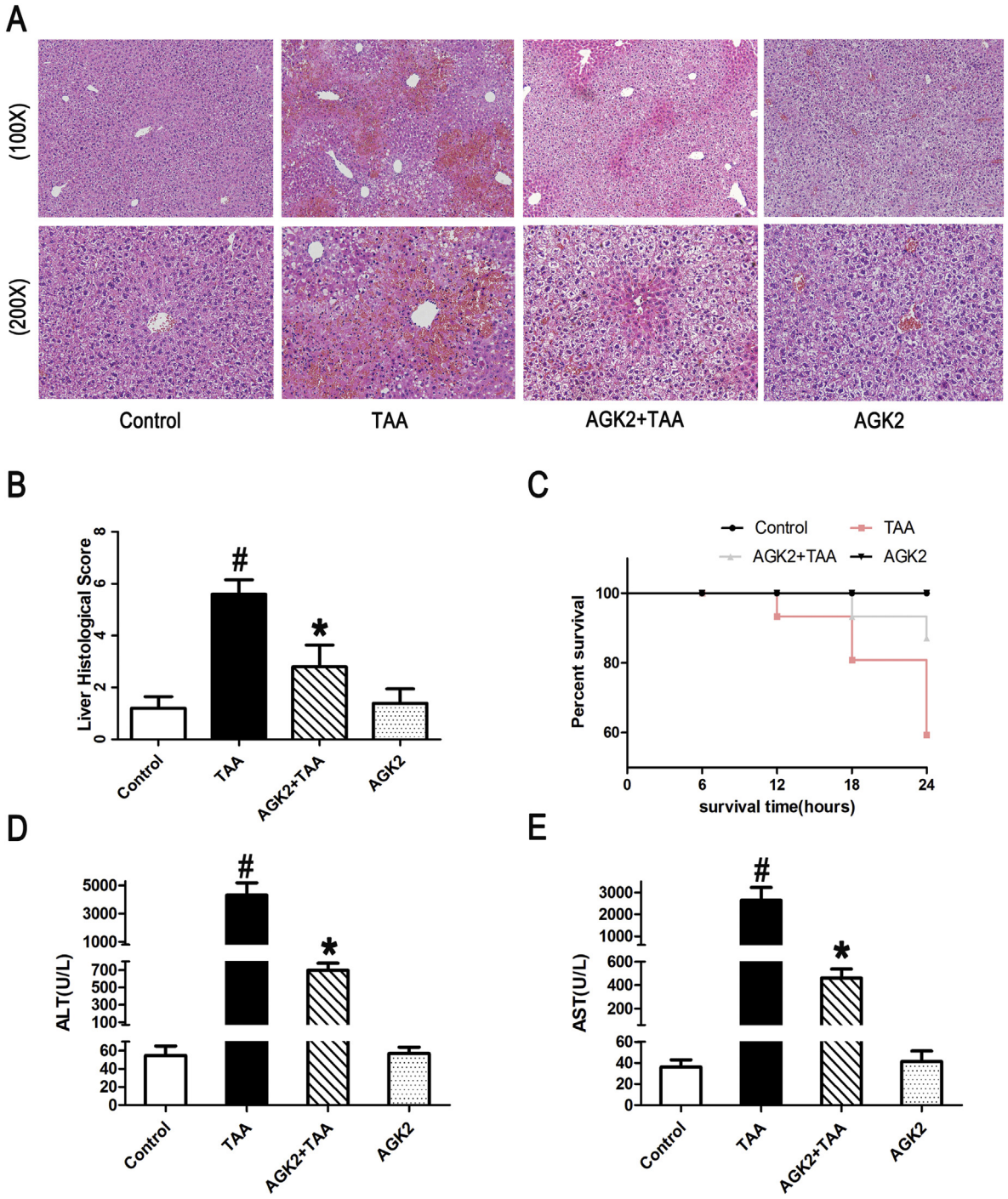


**Fig. 1.** Effect of AGK2 on liver structure, histological score, survival rate, and liver function in mice with ALF. (A) The histological changes of liver in each group were represented with HE staining. (B) The liver histological score of liver in each group. (C) The 6 h, 12 h, 18 h, 24 h survival rates of mice in each group were observed. (D) The plasma levels of ALT were measured in each group. (E) The plasma levels of AST were measured in each group. #$P < 0.05$, compared with the control group. *$P < 0.05$, compared with the TAA group. Abbreviations: alanine aminotransferase, ALT; aspartate aminotransferase, AST.

bicinchoninic acid (BCA) protein assay kit (Beyotime, China). 30 μg protein were subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, USA). Membranes were blocking with 5% non-fat milk for 1 h. Membranes were incubated with anti-iNOS, TNF-α, IL-1β, SIRT2, α-tubulin, acetyl-α-tubulin, phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK, phospho-NF-κB-p65, NF-κB-p65, Cleaved caspase 3, Bcl-2, and Bax at 4 °C overnight. Subsequently, membranes were incubated with the secondary antibody (LI-COR, USA) in the dark for 2 h. The blot was analyzed using the Odyssey Infrared Imaging system (LI-COR, USA). GAPDH was set as additional loading controls.

### 2.9. Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses were performed with SPSS 12.0 software. Statistical differences of the results between groups were determined by one-way analysis of variance (ANOVA). *P* value lower than 0.05 was considered statistically distinct.

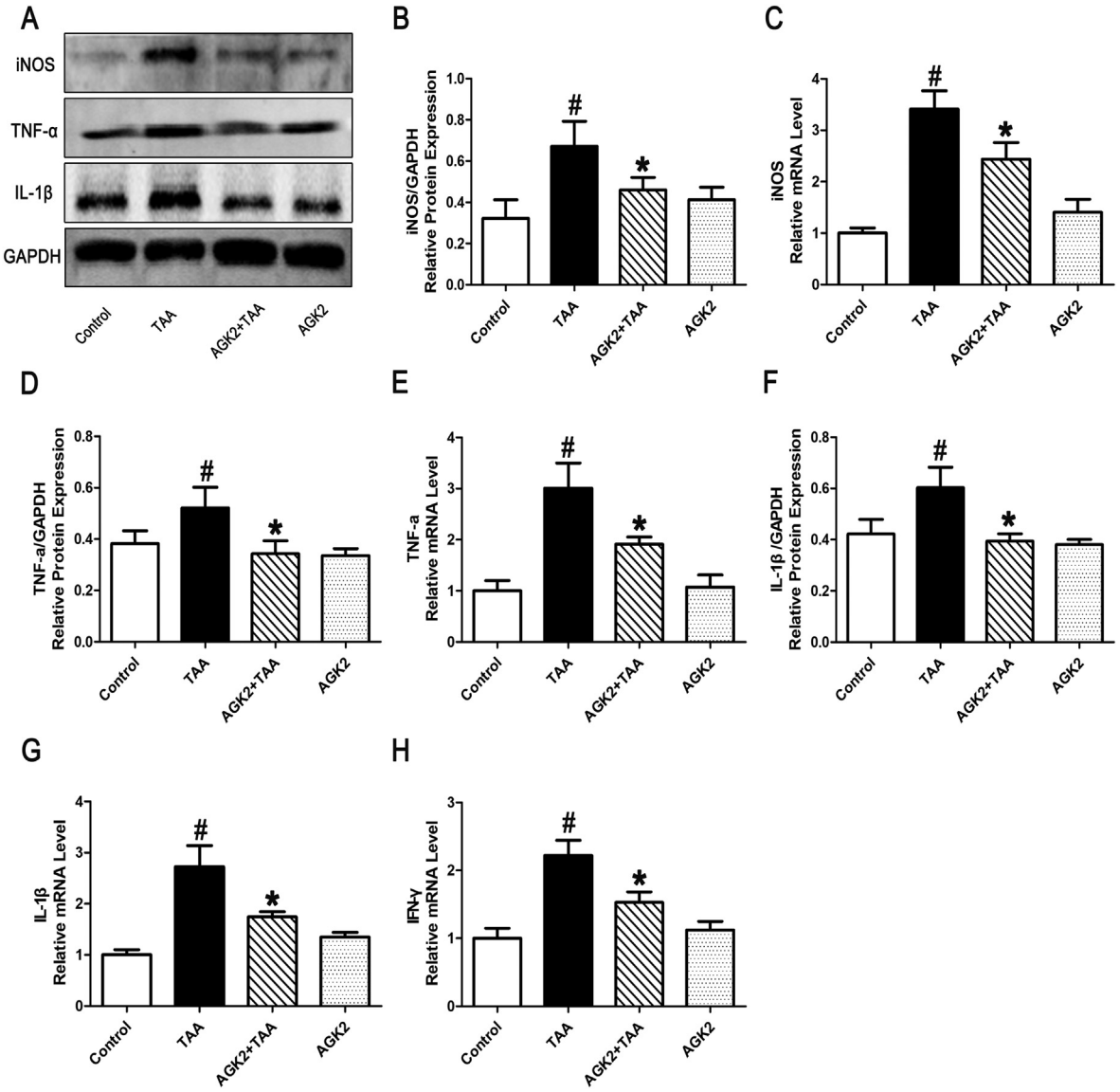


**Fig. 2.** Effect of AGK2 on the expression of inflammatory cytokines in TAA-induced ALF. (A) The proteins expression of proinflammatory cytokine iNOS, TNF-α, and IL-1β were detected by western blotting. (B) Densitometric analysis of iNOS. (C) RT-PCR analysis of iNOS mRNA levels. (D) Densitometric analysis of TNF-α. (E) RT-PCR analysis of TNF-α mRNA levels. (F) Densitometric analysis of IL-1β. (G) RT-PCR analysis of IL-1β mRNA levels. (H) RT-PCR analysis of IFN-γ mRNA levels. #P < 0.05, compared with the control group. *P < 0.05, compared with the TAA group.

## 3. Results

### 3.1. AGK2 improved the survival rate and damage of liver function and histopathology in ALF mice

The histopathological examination of the liver revealed the hepatic structure in each group. In control group, the liver cells regularly arrayed around the central vein without hepatocyte necrosis and inflammatory cell infiltration. In AGK2 group, liver tissues shown slight tissue damage compared with control group. However, TAA group displayed massive hemorrhagic necrosis, liver lobules with disturbed architecture, remarkable inflammatory cells infiltration. Whereas, in AGK2 + TAA group, the hepatocyte necrosis was improved obviously, and the numbers of infiltrative inflammatory cells were significantly reduced (Fig. 1A). Similarly, the histological score of liver in AGK2 + TAA group was lower than TAA group (Fig. 1B). In addition, the 24 h survival rate of TAA-induced mice was observed in each group. The results showed that 86.7% of mice survived in AGK2 + TAA group, whereas only 60.0% in TAA group (Fig. 1C). Finally, we assessed the plasma levels of hepatic enzymes in each group. TAA group showed significant increases in levels of ALT and AST. However, treatment with AGK2 significantly decreased the abnormal increase of AST and ALT levels (Fig. 1D, E).

### 3.2. AGK2 reduced the expression of inflammatory cytokines in ALF mice

The proteins and mRNA levels of inflammatory cytokines were measured in each group. As shown in the Fig. 2A–G, compared with control group, the iNOS, TNF-α, and IL-1β proteins and mRNA were obviously elevated in TAA group. Meanwhile, these cytokines levels were significantly suppressed in the mice treated with AGK2 and TAA (Fig. 2A–G). Similarly, AGK2 reduced the TAA-induced increase of IFN-γ mRNA levels (Fig. 2H).

### 3.3. AGK2 inhibited the MAPK and NF-κB pathways in ALF mice liver tissue

Next, we investigated whether AGK2 exerted the anti-inflammatory

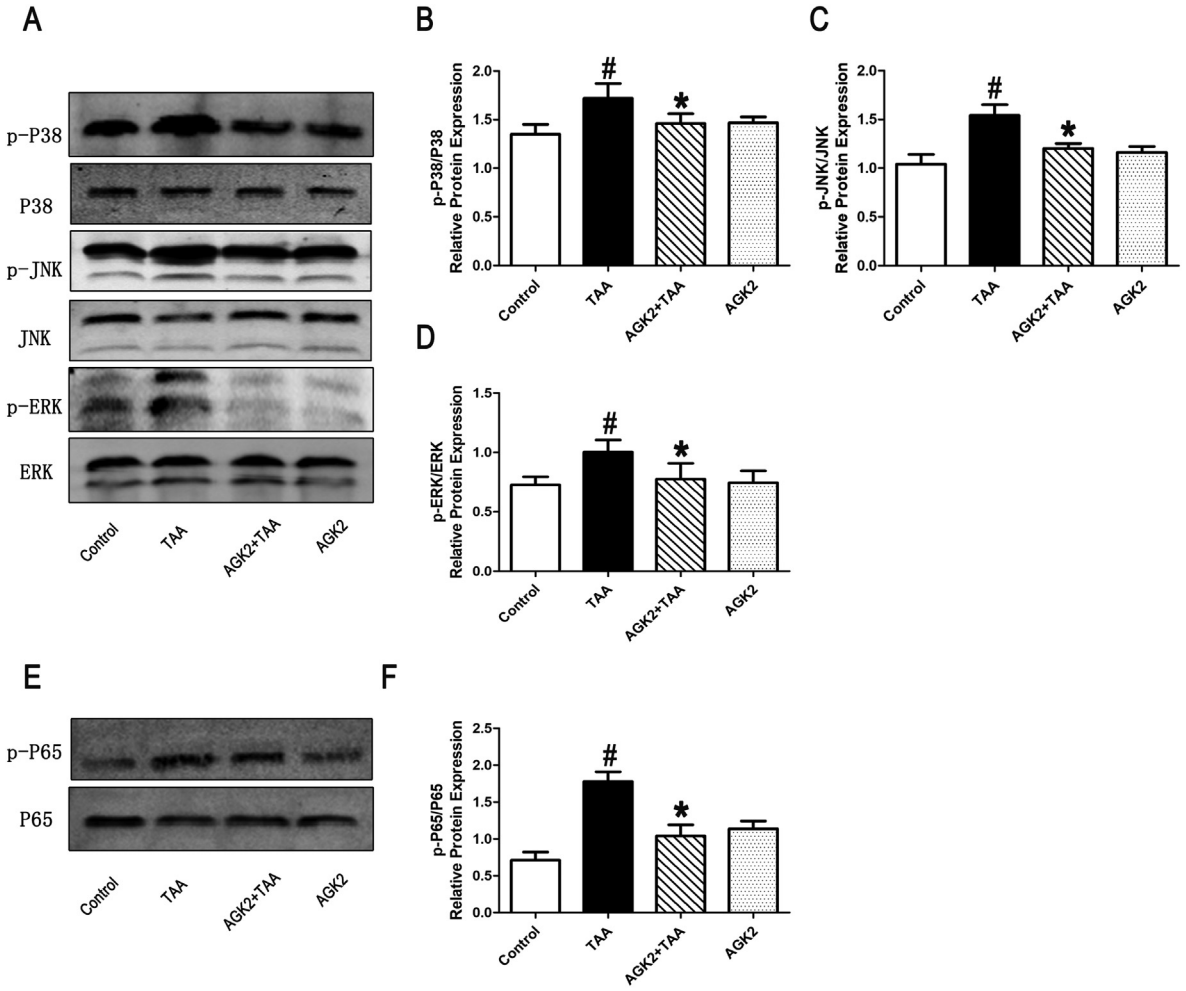


**Fig. 3.** Effect of AGK2 on MAPK and NF-κB signaling in the liver tissue in mice with ALF. (A) The protein expressions of p-P38, P38, p-JNK, JNK, p-ERK, and ERK were analyzed by western blotting. (B) The quantitative blots of p-P38/P38. (C) The quantitative blots of p-JNK/JNK. (D) The quantitative blots of p-ERK/ERK. (E) The protein expressions of p-P65 and P65 were analyzed by western blotting. (F) The quantitative blots of p-P65/P65. #P < 0.05, compared with the control group. *P < 0.05, compared with the TAA group. Abbreviations: p-, phosphorylated.

effects via MAPK and NF-κB pathway. In comparison with the control group, the levels of phosphorylation of all three MAPKs (p-P38, and p-JNK, p-ERK) were increased in TAA group. On the contrary, AGK2 pretreatment result in the decrease of this phosphorylation (Fig. 3A–D). Similarly, we observed similar changes in NF-κB pathways. AGK2 administration could inhibit the TAA-induced increase of phosphorylation of NF-κB p65 (Fig. 3E–F).

### 3.4. AGK2 decreased the hepatocyte apoptosis in ALF mice

In order to assess whether AGK2 exhibited anti-apoptosis effect in TAA-induced liver injury, we detected apoptosis cells in liver tissues by TUNEL staining. As shown in the Fig. 4A, the TUNEL-positive cells were significantly increased in TAA group. Whereas, the number of TUNEL-positive cells were obviously decreased in AGK2 + TAA group. To further confirm the anti-apoptosis effect of AGK2, the apoptotic proteins of Cleaved caspase 3, Bcl-2, and Bax were measured by western blotting. TAA group showed that the proapoptotic proteins of Cleaved caspase 3 and Bax were obviously upregulated, antiapoptotic proteins of Bcl-2 was significantly downregulated. However, treatment with TAA and AGK2 resulted in the decrease of Cleaved caspase 3 and Bax, and the increase of Bcl-2 (Fig. 4B–D).

### 3.5. AGK2 decreased the SIRT2 and increased the acetylation of α-tubulin in liver tissue

Finally, we detected the expression of SIRT2 and acetylation of α-tubulin in each group. There was no change of SIRT2 protein between control group and TAA group. However, the expression of SIRT2 was inhibited in AGK2 + TAA group and AGK2 alone group (Fig. 5A–B). Similarly, there was no difference in α-tubulin acetylation between control group and TAA group. While, the acetylation of α-tubulin was increased in AGK2 + TAA group and AGK2 alone group (Fig. 5C).

## 4. Discussion

In the current study, we investigated the protective effects of selective SIRT2 inhibitor AGK2 on ALF mice and the mechanisms underlying. The ALF model was induced by intraperitoneal injection with 600 mg/kg TAA. As expected, administration of TAA lead to obviously impairment of hepatic integrity, massive necrosis, and inflammatory cells infiltration, along with significant increases in levels of ALT and AST. However, AGK2 improved survival rate and liver histopathology and function after induction of TAA. Besides, the abnormally elevation in pro-inflammatory cytokines iNOS, TNF-α, IL-1β, and IFN-γ could be markedly suppressed by AGK2 in TAA-treated mice. Moreover, AGK2 could decrease TAA-induced the activation of MAPK and NF-κB signaling pathways in liver tissue. Finally, we detected the anti-apoptosis

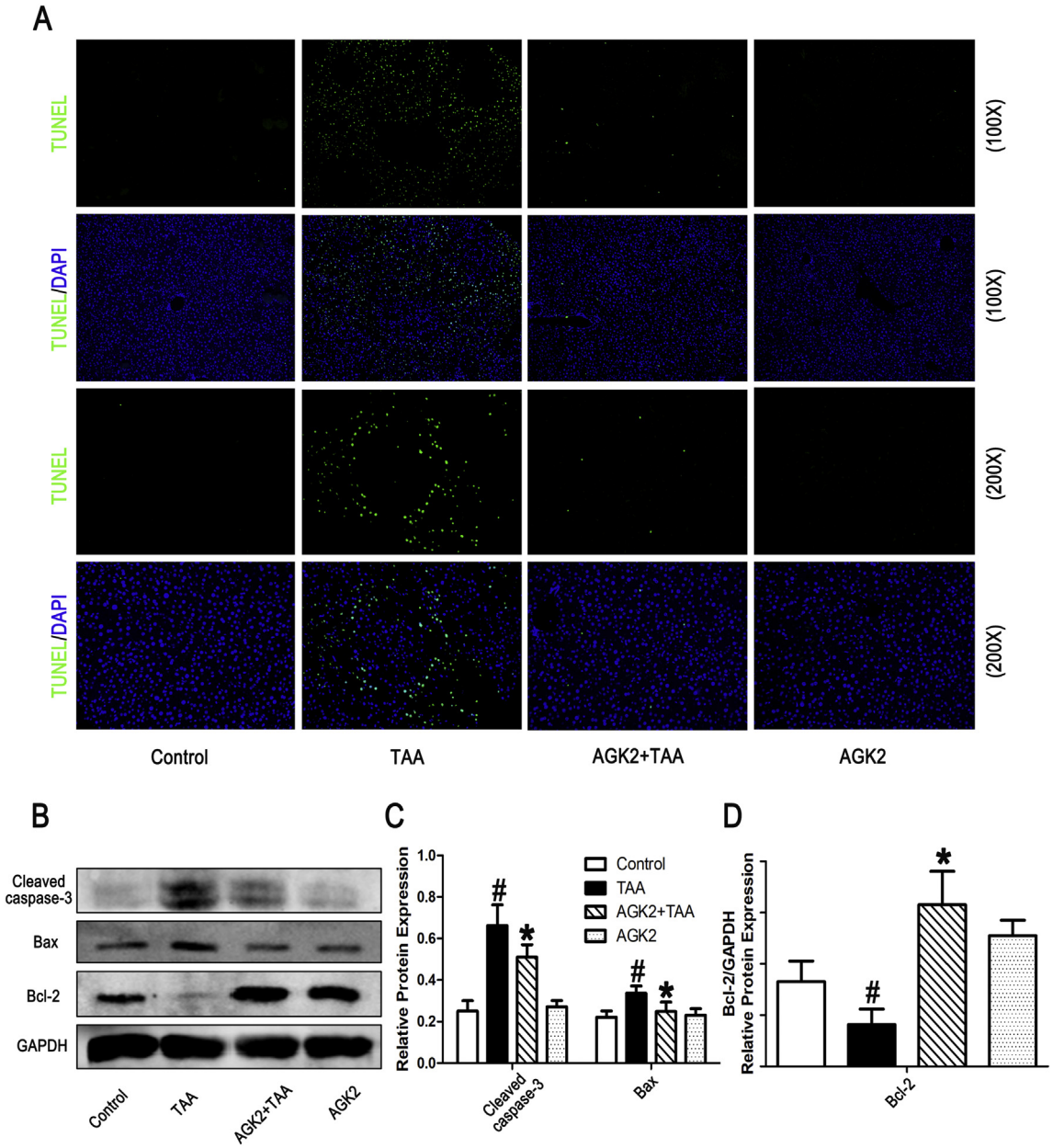


**Fig. 4.** Effects of AGK2 on hepatocyte apoptosis in ALF mice. (A) The number of apoptosis cells in liver tissues were observed by TUNEL staining. (B) The protein expressions of Cleaved caspase 3, Bcl-2, and Bax were analyzed by western blotting. (C) The quantitative blots of Cleaved caspase 3 and Bax. (D) The quantitative blots of Bcl-2. #P < 0.05, compared with the control group. *P < 0.05, compared with the TAA group.

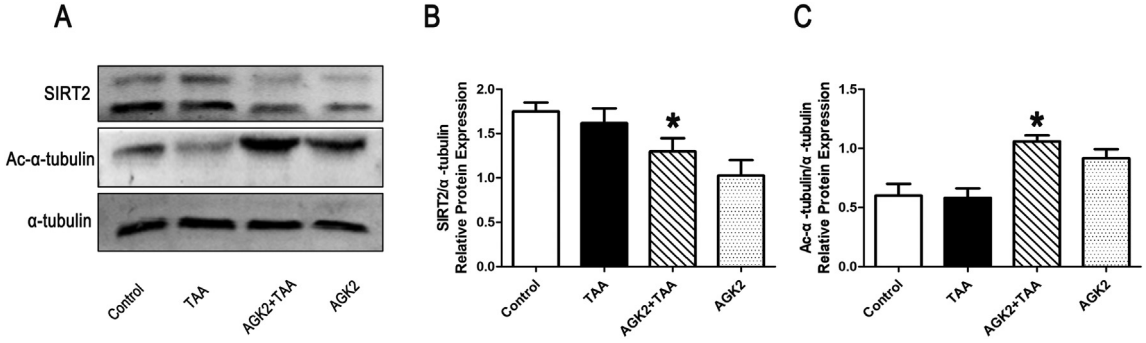


**Fig. 5.** Effect of AGK2 on SIRT2 and acetylation of α-tubulin in ALF mice. (A) The proteins levels of SIRT2, α-tubulin, and Ac-α-tubulin were analyzed by western blotting. (B) Densitometric analysis of SIRT2. (C) Densitometric analysis of Ac-α-tubulin/α-tubulin. #P < 0.05, compared with the control group. *P < 0.05, compared with the TAA group. Abbreviations: Ac-, acetyl-.

effect of AGK2 in TAA-induced hepatic injury, and found that AGK2 alleviated TAA-induced the apoptosis of liver cells. Taken together, AGK2 has protection effects on TAA-induced ALF mice.

As a main member of the sirtuins family, SIRT2 is widely expressed in multiple tissues, especially in liver, brain, muscle, adipose tissue, kidneys, and pancreas [14]. Increasing evidence has demonstrated that SIRT2 is involved in inflammatory responses. Zhao et al. reported that the inhibition of SIRT2 by AGK2 could reduce the serum levels of inflammatory cytokines (TNF-α and IL-6), improved outcomes in cecal-ligationand- puncture (CLP)-induced septic mice [22]. Similarly, in a study about renal inflammatory injury, SIRT2 knockout decreased infiltration of neutrophil and macrophage in kidney tissues, down-regulated the expressions of pro-inflammatory cytokines (TNF-a, IL-1β, and IL-6) after LPS stimulation [23]. In this study, our results indicated SIRT2 inhibitor AGK2 significantly lowered the increase of iNOS, TNF-α, IL-1β, and IFN-γ in ALF mice. In addition, AGK2 ameliorated the hepatocyte necrosis and improved liver function in TAA-induced mice. These results are accordance with a previous study that ablation of SIRT2 improved acetaminophen (APAP)-induced liver injury and decreased abnormal levels of liver enzymes [24].

MAPKs are a highly conserved cytoplasmic serine/threonine enzymes families, it links with a wide variety of biological processes [25]. Activation of MAPK regulates the inflammatory response via controlling the expression of multiple genes [26]. Several studies indicated that the upregulation of phosphorylation of MAPKs (P38, JNK, and ERK) was observed in various drug-induced liver injury mice models [27–29]. Similar to these results, our results showed that TAA-induced ALF lead to the increase of phosphorylation of MAPKs. She DT et al. demonstrated that SIRT2 inhibitors AK1 and AGK2 downregulated the levels of phosphorylation of MAPK pathway in ischemic stroke [30]. Wang J et al. reported that SIRT2 knockout decreased the phosphorylation of three MAPKs (P38, JNK, and ERK), and SIRT2 overexpression increased these phosphorylations in hepatic ischemia-reperfusion injury [31]. Consistent with the findings, our results showed that the activation of phosphorylation of MAPKs was suppressed by SIRT2 inhibitor AGK2. Nuclear factor-κB (NF-κB) is a key transcription factor that regulate the gene transcription of proinflammatory factors. Our previous study reported that broad-spectrum HDAC inhibitor Trichostatin A decreased the phosphorylation of NF-κB p65 was increased in acute-on-chronic liver failure rats [17]. In this present study, we also found that AGK2 inhibited the phosphorylation of NF-κB p65.

The apoptosis and necrosis of hepatocytes are one of pathological characteristics of ALF liver tissues. According to the HE staining results, AGK2 obviously improved hepatocyte necrosis. Therefore, we considered whether that AGK2 may also alleviate the liver injury by inhibition of apoptosis. Wang Y et al. reported that SIRT2 inhibitor AGK2 decreased renal cells apoptosis in renal ischemia/reperfusion [32]. Another study demonstrated that SIRT2 inhibitor reduced cochlear cell apoptosis after noise exposure [33]. These studies suggested that SIRT2 may play a significant role in the process of cell apoptosis. As expected, our results showed that AGK2 obviously reduced the number of the TUNEL-positive cells, inhibited the proapoptotic proteins of Cleaved caspase 3 and Bax, and enhanced the antiapoptotic proteins Bcl-2.

In order to test the effect of AGK2 on SIRT2 and acetylation, we measured the protein expression of SIRT2 and acetylation of α-tubulin. Our data indicated that AGK2 effectively reduced the expression of SIRT2, enhanced the acetylation of α-tubulin in TAA-induced liver injury. This finding is in agreement with a previous study that ablation of SIRT2 result in tubulin hyperacetylation [13].

In summary, SIRT2 inhibitor AGK2 improve TAA-induced survival rate in mice with ALF, suppress the inflammatory responses by inhibition of MAPK and NF-κB signaling pathways, and decrease the hepatocyte necrosis by inhibition of apoptosis. Pharmacologic inhibition of SIRT2 may be a promising approach for the treatment of ALF.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

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