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# Sappanone A suppresses CHOP expression and exerts neuroprotective effects in epilepsy

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**ABSTRACT**

***Objective(s):*** Sappanone A (SA) is a compound derived from *Caesalpinia sappan* L that exhibits multiple biological effects, including anti-apoptotic, anti-inflammatory, and anti-oxidant activities. Because oxidative stress, cell apoptosis, and neuroinflammation are associated with the pathophysiology of epilepsy, we conducted this study to explore the role of SA in epilepsy.

***Materials and Methods:*** We used SA to treat pentylenetetrazole (PTZ)-induced mice as well as HT22 hippocampal cells exposed to $Mg^{2+}$-free solution. Seizure behavior in the mice was recorded. Nissl staining, TUNEL assay, and flow cytometry were used to evaluate neuronal damage and apoptosis. Moreover, we employed biochemical tests to assess inflammation and oxidative stress levels, and conducted quantitative reverse transcription polymerase chain reaction and western blot to detect CHOP expression.

***Results:*** Pretreatments with SA (25 and 50 mg/kg) significantly prolonged seizure latency and reduced seizure duration in PTZ-induced epileptic mice (all $P<0.001$), whereas 10 mg/kg had no significant effects. At effective doses, SA also alleviated neuronal damage, reduced inflammation, and attenuated oxidative stress (all $P<0.05$). Furthermore, SA suppressed CHOP expression, and CHOP overexpression partially reversed SA's protective effects ($P<0.05$).

***Conclusion:*** Our findings suggest that SA pretreatment exerts neuroprotective effects against epilepsy, potentially through suppression of CHOP expression. However, the causal relationship between CHOP suppression and SA-mediated neuroprotection in epilepsy warrants further investigation.

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## Introduction

Epilepsy is a prevalent chronic neurological condition defined by an enduring predisposition to recurrent seizures (1). Epilepsy affects over 70 million people globally, and active epilepsy accounts for approximately 51 million of these cases (2, 3). Epilepsy is associated with a range of physical and mental comorbidities, including heart diseases, cognitive disorders, depression and anxiety, which reduce quality of life and impose a great burden on individuals and society (4). Even though various anti-epileptic drugs (AEDs) have been developed for clinical use, approximately 30% of patients are unresponsive to the current AEDs (5). Therefore, there is a great need to develop novel agents or explore new targets for the treatment of epilepsy.

Sappanone A (SA), a homoisoflavanone isolated from the heartwood of *Caesalpinia sappan* L., exhibits anti-apoptotic, anti-inflammatory, and antioxidant properties (6). Accumulating evidence suggests that SA holds great potential for treating various disorders. Specifically, our recent review summarized its therapeutic effects in diverse inflammation-related diseases, including myocardial ischemia-reperfusion injury, liver injury, respiratory diseases, and kidney injury (7). Notably, the neuroprotective effect of SA has been increasingly acknowledged. Wang *et al.* (8) reported that in cerebral ischemia-reperfusion injury, SA exhibited neuroprotection by inhibiting neuroinflammation, oxidative stress, and cell apoptosis. A very recent study found that SA exerted neuroprotective effects in spinal cord injury by suppressing microglial neuroinflammation via activation of the Keap1/Nrf2 signaling pathway (9). Despite the well-documented therapeutic efficacy of SA in multiple neurological disease models, its specific role and underlying mechanisms in epilepsy remain poorly understood.

Endoplasmic reticulum (ER) stress refers to a potentially fatal cellular state triggered by the aberrant accumulation of unfolded or misfolded secretory proteins within the ER lumen (10). It has been implicated in the pathogenesis of epilepsy (11, 12). Interestingly, the CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), an ER stress-related protein, has been reported to mediate the neuroprotective effect of SA against cerebral ischemia-reperfusion injury (8). However, whether the therapeutic effect of SA on epilepsy involves CHOP regulation is yet to be clarified.

Thus, the current study used pentylenetetrazole (PTZ)-induced mice and HT22 hippocampal cells exposed to

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$Mg^{2+}$-free solution to explore the efficacy and mechanism of SA in epilepsy (Figure 1), which may provide a theoretical basis for the development of antiseizure medications.

## Materials and Methods

### *Animals and reagents*

Male C57BL/6 mice (6-8 weeks old, average weight: 20-30 g) were from the Experimental Animal Center of Southwest Medical University. All mice were housed in a temperature-managed environment (22-24 ˚C) and supplied with sufficient water and food. The experimental processes were performed following the guidelines of the Animal Welfare Agency and the protocol received approval from the Ethical Committee for Experimental Research of our university (Permit Number: 20230831-006). The work has been reported in line with the ARRIVE criteria (13).

SA and PTZ were purchased from Wei Ke Qi Biotechnology (Sichuan, China) and Sigma-Aldrich (Shanghai, China), respectively. SA was dissolved in dimethyl sulfoxide (DMSO) and then diluted with saline to a final DMSO concentration of 0.1% for injection, while PTZ was solubilized in 0.9 % NaCl. Doses and schedules for drug administration were determined based on the literature (6, 14, 15).

### *PTZ-induced seizure mouse model and drug administration*

After one week of adaptation to laboratory conditions, mice were randomly assigned to five groups of 10-12 mice each: Control group, PTZ group, PTZ+SA-10 (10 mg/kg) group, PTZ+SA-25 (25 mg/kg) group, and PTZ+SA-50 (50 mg/kg) group. The PTZ+SA-10, PTZ+SA-25, and PTZ+SA-50 groups received intraperitoneal injections of SA at doses of 10, 25, or 50 mg/kg, respectively, once daily for 7 days, while the control and PTZ groups received an equivalent volume of 0.1% DMSO on the same schedule to serve as vehicle controls. Subsequently, 60 mg/kg PTZ was injected intraperitoneally to induce acute epileptic seizures in the PTZ, PTZ+SA-10, PTZ+SA-25, and PTZ+SA-50 groups, while the control group received an equivalent volume of 0.9% NaCl (14). After PTZ administration, seizure behavior was observed for 30 min and evaluated using the Racine scale (16). The latency and duration of the first generalized seizure were recorded. Two hours after PTZ injection, animals were sacrificed, and brain tissues were collected for further examination.

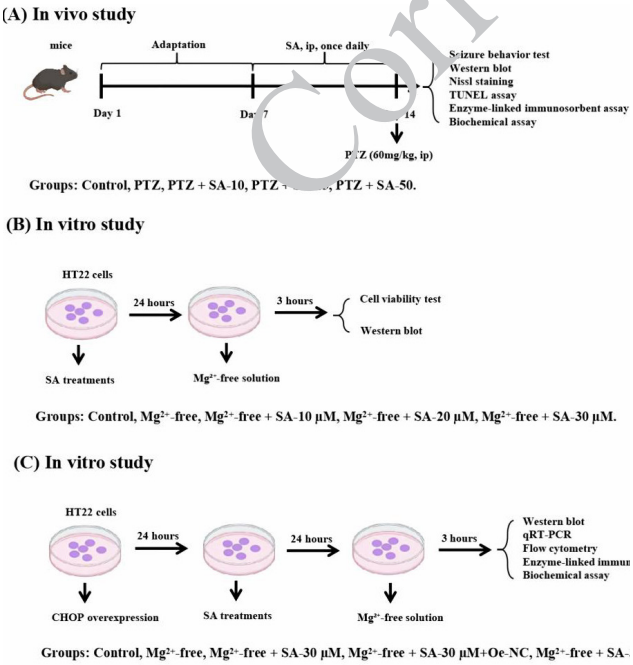


**Figure 1.** Schematic diagram illustrating the experimental design of the present study in mice and HT22 cells
SA: Sappanone A; PTZ: Pentylenetetrazole

### *Cell culture and treatment*

Immortalized hippocampal neuronal cells (HT22) were purchased from Procell Life Science & Technology Co., Ltd. (CL-0697, Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) under environmental conditions of 37 ˚C with 5% $CO_2$. The cells were randomly divided into control and $Mg^{2+}$-free groups that were treated with normal and $Mg^{2+}$-free extracellular fluid, respectively, for 3 hr. In the $Mg^{2+}$-free combined with SA intervention groups, SA was applied to pretreat cells at 10 μM, 20 μM, and 30 μM for 24 hr before treatment with $Mg^{2+}$-free extracellular fluid.

### *Cell transfection*

Before transfection, $3\times10^5$ of HT22 cells per well were seeded into a 6-well plate overnight at 37 ˚C to reach 70% confluence at the time of transfection. The plasmid for CHOP overexpression was synthesized by GenePharma (Shanghai, China), with the empty pcDNA vector (lacking CHOP) serving as the negative control. Transfection was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's standard protocol. CHOP expression was assessed by reverse transcription-quantitative PCR (RT-qPCR) and western blot at 48 hr after transfection to evaluate transfection efficiency.

### *Cell counting Kit-8 (CCK-8) Assay*

The Cell Counting Kit-8 (KGA317, KeyGen Biotec) was used to detect cell viability. In brief, cells were seeded in 96-well plates at a volume of 100 μl/well ($5\times10^3$ cells/well). After treatment, 10 μl of CCK8 solution was added to each well, and the plate was incubated for 2 hr. Lastly, cell viability was measured using a microplate reader (SuPerMax 3100, Shanghai Shanpu Biotechnology Co., Ltd) at 450 nm.

### *Nissl staining*

Brain sections were baked for one hour, then routinely deparaffinized in water and rinsed with distilled water. Subsequently, sections were stained with Methylene Blue Stain for 5 mins, washed with distilled water, transparentized with xylene, and sealed with neutral resin. Tissue morphology in the hippocampus of mice was examined under an Olympus Bx43 microscope (Olympus Corporation, Japan), and images were captured. Nissl-positive neurons in the hippocampal CA1 region were counted using Image-Pro Plus software (Media Cybernetics Corporation, USA).

### *TUNEL assay*

To assess cell death in the hippocampus, TUNEL staining was conducted using a commercial kit (Beyotime, China).

Each slide was observed using an Olympus Bx53 microscope (Olympus Corporation, Japan), and the corresponding images were obtained. The nuclei of the normal cells were dyed blue, while the nuclei of apoptotic cells displayed red staining, which were considered TUNEL-positive cells. The quantity of TUNEL-positive cells in the CA1 subfield of the hippocampus was determined using Image-Pro Plus software (Media Cybernetics Corporation, USA).

### *Flow-cytometry*

Annexin V-FITC/PI Apoptosis Kit (AP101-100-kit, MULTI SCIENCES) was used to determine the cell death of HT22 cells according to the manufacturer's instructions. A total of $1\times10^6$ cells was resuspended in binding buffer and incubated with 5 µl of Annexin V-FITC and 10 µl of PI for 10 minutes in the dark. The stained cells were analyzed using flow cytometry (NovoCyte 2060R, ACEA BIO (Hangzhou) Co., Ltd).

### *Measurement of inflammatory cytokine levels*

The contents of tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and IL-10 in culture supernatants (HT22 cells) and hippocampal homogenates (mice) were tested by applying enzyme-linked immunosorbent assay kits, which were purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China).

### *Measurement of malondialdehyde and glutathione levels*

Following the manufacturer's protocol, the levels of malondialdehyde (MDA) and glutathione (GSH) in culture supernatants (HT22 cells) and hippocampal homogenates (mice) were determined using commercially available assay kits purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China).

### *Western blot*

Animal hippocampal tissues and cultured cells were lysed in RIPA lysis buffer (Solarbio, China) to obtain whole-protein extracts, and protein concentrations were measured using the BCA kit (Beyotime, China). The protein was separated by 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were incubated overnight at 4 ˚C with the following primary antibodies: anti-CHOP (1:1000; Proteintech, USA) and anti-GAPDH (1:1000; Proteintech, USA), followed by the corresponding secondary antibodies (1:10000; Bioswamp, China) for one hour. Protein was visualized using an ECL detection kit (Smart-Lifesciences, China). TANON GIS software (TANON, China) was applied to analyze the results.

### *Quantitative realtime PCR (qRTPCR)*

Total RNA was extracted using Trizol reagent (Ambion, USA) following the manufacturer's protocol. Then, the total RNA was reversely transcribed into cDNA using the cDNA Synthesis SuperMix kit. Each cDNA (1 µl) was amplified using SYBR FAST qPCR Master Mix (KAPA Biosystems, USA) on a Bio-Rad CFX-Connect 96 RT-qPCR System (Bio-Rad, USA). The primer sequences: CHOP forward: 5'-AAAACCTTCACTACTCTTGACCCTG-3', CHOP reverse: 5'-TCTCCT GATCCTTCTCCTTCAT-3'; BIM forward: 5'-CAACACAAACCCCAAGTCCT-3', BIM reverse: 5'-CATTTGCAAACACCCTCCTT-3'; Actin forward: 5'-AGGCAAATCGTGCGTGAC-3', Actin reverse: 5'-CATACCCAAGAAGGAAG

GCT-3'. Actin was used as the reference gene. The result was calculated using the $2^{-\triangle\triangle Ct}$ method.

### *Statistical analysis*

The results were described as means±standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's test. GraphPad Prism software version 9.0 was employed for all analyses, and a probability of $<0.05$ was regarded statistically significant.

## Results

### *SA administration improved seizure-like behavior in PTZ-induced seizure mice*

The effect of different doses of SA on PTZ-induced seizures was demonstrated in Figure 2. One-way ANOVA revealed a significant effect of SA pretreatment on seizure

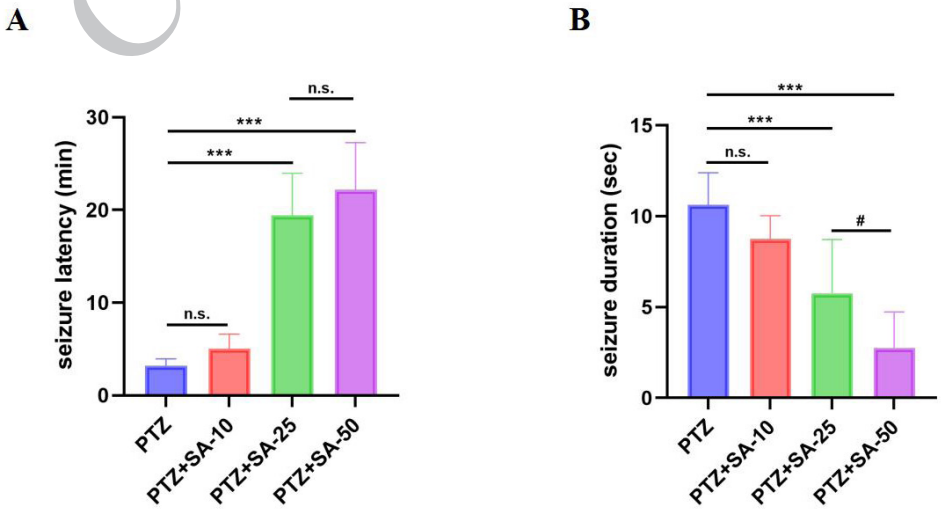


**Figure 2.** SA improved seizure behavior induced by PTZ in mice
(A) Latency to the first generalized seizure (n=8 per group). (B) Duration of the first generalized seizure (n=8 per group). ***$P<0.001$ vs PTZ group, #$P<0.05$ vs PTZ+SA-25 group, ns, not significant ($P>0.05$)
SA: Sappanone A; PTZ: Pentylenetetrazole

latency (F (3, 28)=60.99, *P*<0.001, n=8 per group). Tukey's *post hoc* test further demonstrated that the 10 mg/kg SA group (5.05±1.47 min) showed no statistically significant difference from the PTZ group (3.21±0.69 min, *P*=0.723). In contrast, the 25 mg/kg SA group (19.43±4.25 min) and the 50 mg/kg SA group (22.16±4.76 min) exhibited significantly prolonged seizure latency compared with the PTZ group (both *P*<0.001). However, no remarkable difference in latency prolongation was observed between the 25 mg/kg and 50 mg/kg SA groups (*P*=0.419).

Similarly, SA pretreatment significantly attenuated seizure duration (F (3, 28)=21.86, *P*<0.001, n=8 per group). Tukey's *post hoc* analysis revealed that the 10 mg/kg SA group (8.75±1.20 s) showed no statistically significant difference from the PTZ group (10.63±1.65 s, *P*=0.297). In contrast, the 25 mg/kg SA group (5.75±2.77 s) and the 50 mg/kg SA group (2.75±1.85 s) exhibited significantly reduced seizure duration compared with the PTZ group (both *P*<0.001). Notably, the 50 mg/kg SA group also showed a significantly greater reduction in seizure duration compared with the 25 mg/kg SA group (*P*=0.037).

Thus, our data indicated that SA exerted an antiepileptic effect in PTZ-induced seizure mice, as evidenced by a dose-dependent pattern of improvement in seizure-like behavior.

### *SA alleviated neuronal injury, inhibited inflammation, and reduced oxidative stress in PTZ-induced seizure mice*

Neuronal injury, inflammatory response, as well as oxidative stress contribute to the pathogenesis of epilepsy (17-19). We explored the role of SA in neuronal damage, inflammation, and oxidative stress in PTZ-induced seizure mice, and the results are shown in Figure 3.

Nissl staining and TUNEL assay were performed to evaluate neuronal injury in the hippocampus of mice (Figure 3). One-way ANOVA revealed significant overall effects of SA pretreatment on both the number of surviving Nissl-positive neurons (F (4, 20)=35.98, *P*<0.001, n=5 per group) and TUNEL-positive cells (F (4, 20)=10.12, *P*<0.001, n=5 per group). Tukey's *post hoc* analysis showed that the PTZ group exhibited a marked reduction in Nissl-positive neurons and a significant increase in TUNEL-positive cells compared with the control group (both *P*<0.001). Pretreatments with 25 mg/kg and 50 mg/kg SA significantly increased Nissl-positive neurons (both *P*<0.001) and reduced TUNEL-positive cells (*P*=0.049 and *P*=0.001, respectively) relative to the PTZ group. In contrast, the 10 mg/kg SA group did not significantly affect either Nissl-positive cell counts (*P*=0.612) or TUNEL-positive cell numbers (*P*=0.129).

We further evaluated inflammatory cytokine levels and oxidative stress markers in hippocampal homogenates. One-way ANOVA revealed significant overall effects of SA pretreatment on all measured parameters: TNF-α (F (4, 10)=39.08, *P*<0.001, n=3 per group), IL-1β (F (4, 10)=81.59, *P*<0.001, n=3 per group), IL-10 (F (4, 10)=18.41, *P*<0.001, n=3 per group), MDA (F (4, 25)=13.08, *P*<0.001, n=6 per group), and GSH (F (4, 25)=14.94, *P*<0.001, n=6 per group). Tukey's *post hoc* analysis revealed that PTZ treatment significantly elevated the levels of pro-inflammatory cytokines TNF-α and IL-1β, while depleting the anti-inflammatory cytokine IL-10 compared with the control group (all *P*<0.001). SA pretreatment at 25 mg/kg and 50 mg/kg significantly reversed these changes, as evidenced by reduced TNF-α and IL-1β levels (all *P*<0.001), along with restored IL-10 levels (*P*=0.005 and *P*=0.001, respectively). However, SA pretreatment at 10 mg/kg showed no significant effect on any of the measured cytokines (*P*=0.262 for TNF-α; *P*=0.083 for IL-1β; *P*=0.961 for IL-10). Similarly, PTZ treatment significantly increased MDA levels and decreased GSH levels compared with the control group (*P*=0.002 for MDA; *P*<0.001 for GSH). Both the 25 mg/kg and 50 mg/kg SA pretreatments significantly reduced MDA (both *P*<0.001) and restored GSH levels (*P*=0.039 and *P*=0.005, respectively) relative to the PTZ group, whereas the 10 mg/kg dose had no significant effect on either marker (*P*=0.380 for MDA; *P*=0.996 for GSH).

Collectively, our results demonstrated that SA exhibited

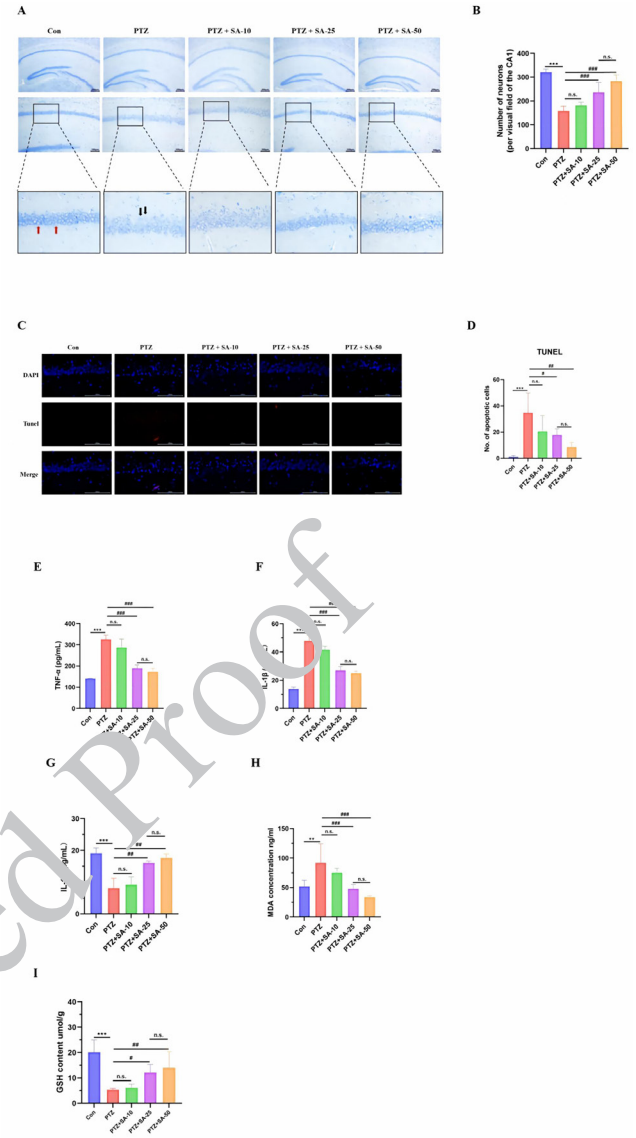


**Figure 3.** SA exerts protective effects against neuronal injury, inflammation, and oxidative stress in the PTZ-induced seizure model in mice
(A-B) Representative Nissl staining images in the hippocampus of mice (n=5 per group). The upper panels showed the whole hippocampus (bar=200 um), the middle panels showed the CA1 region (bar=100 um), and the lower panels showed higher magnification of the CA1 region. The red arrows indicate the normal neurons, and the black arrows indicate the injured cells. (C-D) Cell death in the CA1 region of the mouse hippocampus (n=5 per group). Bar=100 um. (E) Levels of TNF-α (n=3 per group). (F) Levels of IL-β (n=3 per group). (G) Levels of IL-10 (n=3 per group). (H) The levels of MDA (n=6 per group). (I) Levels of GSH (n=6 per group). $^{**}P$<0.01, $^{***}P$<0.001 vs Con group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs PTZ group; ns, not significant (*P*>0.05)
SA: Sappanone A; PTZ: Pentylenetetrazole; MDA: Malondialdehyde; GSH: Glutathione

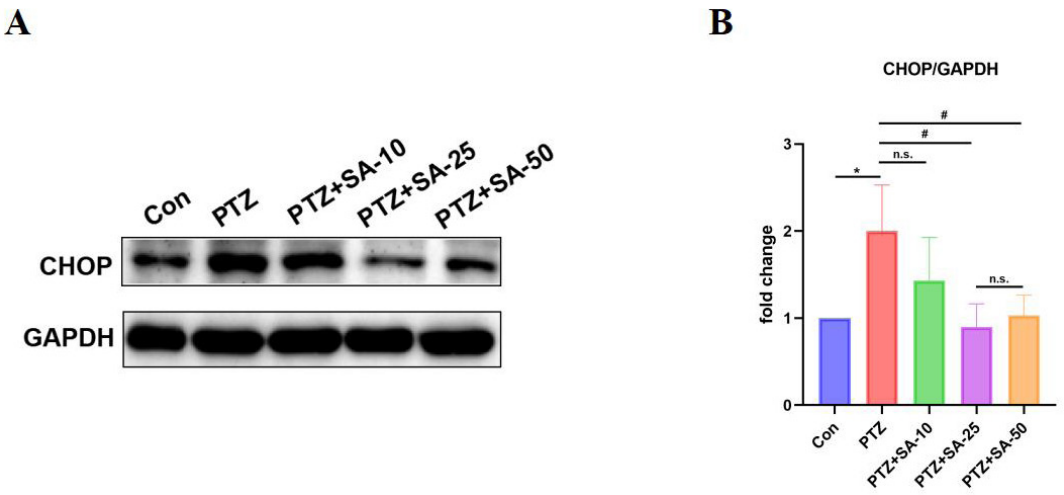


**Figure 4.** SA regulates the expression of CHOP in the PTZ-induced seizure model in mice (n=3 per group)
$^{*}P$<0.05 vs Con group; $^{\#}P$<0.05 vs PTZ group; ns, not significant ($P$>0.05)
SA: Sappanone A; PTZ: Pentylenetetrazole; CHOP: CCAAT/enhancer-binding protein (C/EBP) homologous protein

a dose-dependent trend in ameliorating neuronal damage, inflammation, and oxidative stress in PTZ-induced mice.

***SA inhibited CHOP expression in PTZ-induced seizure mice***

Prior studies have revealed that CHOP plays a critical role in regulating activation of inflammatory factors, oxidative stress, and cell apoptosis (20). We next investigated whether SA affected the expression of CHOP in PTZ-induced mice. One-way ANOVA demonstrated a significant effect of SA pretreatment on CHOP expression (F (4, 10)=5.07, $P$=0.017, n=3 per group). Tukey's *post hoc* test further demonstrated that the expression of CHOP protein was significantly increased in the PTZ-induced mice ($P$=0.0[illegible]). Both 25 and 50 mg/kg SA inhibited the expression of CHOP protein ($P$=0.022 and $P$=0.038, respectively), which was not observed in SA at 10 mg/kg ($P$=0.346)(Figure 4A-C).

Based on these results, we speculate that the protective effects of SA in PTZ mice may be involved in its inhibition of CHOP.

***CHOP overexpression abolished the neuroprotective effects of SA in HT22 hippocampal cells treated with $Mg^{2+}$-free solution***

To further explore the potential role of CHOP in mediating the neuroprotective effects of SA, we up-regulated CHOP expression in HT22 hippocampal cells treated with $Mg^{2+}$-free solution, which is an *in vitro* model of epileptiform activity. Exposure to $Mg^{2+}$-free solution reduced the viability of HT22 hippocampal cells compared to the control group, and this reduction was partially rescued by SA pretreatment in a dose-dependent trend (Figure 5A). Similar to the *in vivo* observations, up-regulated CHOP expression was observed in HT22 hippocampal cells treated with an $Mg^{2+}$-free solution (Figure 5B-C). At the same time, SA administration exhibited a dose-dependent trend in down-regulating CHOP expression (Figure 5B-C). To verify the functional role of CHOP in SA's protective effects, we transfected HT22 cells with a CHOP-overexpressing plasmid, which successfully up-regulated CHOP expression (Figure 6A-C). Moreover, we examined the mRNA expression of BIM, a well-characterized downstream target of the CHOP pathway, which revealed

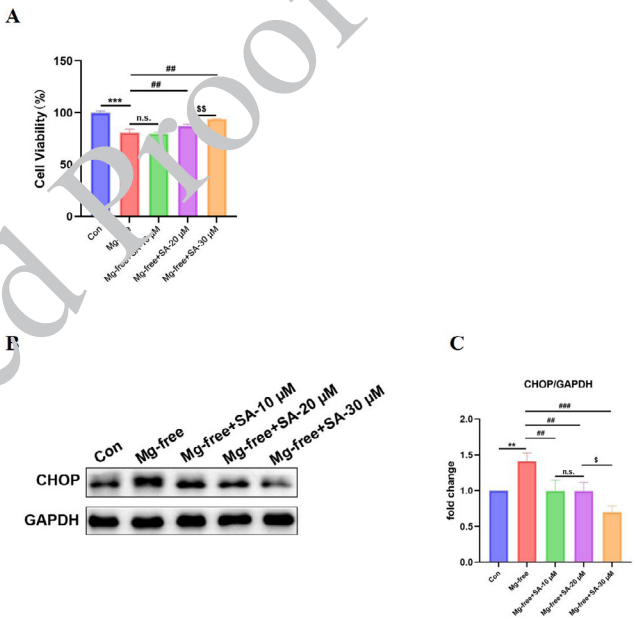


**Figure 5.** SA regulates the expression of CHOP in HT22 cells treated with $Mg^{2+}$-free solution
(A) Cell viability was assessed by using a CCK-8 kit (n=5 per group). (B-C) Expression of CHOP protein (n=3 per group). $^{**}P$<0.01, $^{***}P$<0.001 vs Con group; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs $Mg^{2+}$-free group; $^{\$}P$<0.05, $^{\$\$}P$<0.01 vs $Mg^{2+}$-free+SA-20 μM group; ns, not significant ($P$>0.05)
SA: Sappanone A; CHOP: CCAAT/enhancer-binding protein (C/EBP) homologous protein

that CHOP overexpression significantly up-regulated BIM mRNA compared to the empty vector group ($P$=0.003, Figure 6D), suggesting that the exogenous CHOP protein was transcriptionally functional and capable of driving the expression of its pro-apoptotic downstream effectors. Flow cytometry revealed that $Mg^{2+}$-free exposure significantly increased the apoptosis rate compared with the control group ($P$<0.001). One-way ANOVA showed a significant overall effect of SA on apoptotic cells (F (4, 10)=321.30, $P$<0.001, n=3 per group). Tukey's *post hoc* test indicated that SA pretreatment significantly reduced apoptosis ($P$<0.001), while CHOP overexpression obviously reversed the protective effect of SA ($P$<0.001)(Figure 6E-F). Additionally,

$Mg^{2+}$-free exposure significantly elevated TNF-α, IL-1β, and MDA (all $P<0.001$), while reducing IL-10 and GSH (both $P<0.001$). One-way ANOVA revealed significant overall effects of SA on all measured parameters (TNF-α: $F(4, 10)=173.80$, $P<0.001$, n=3 per group; IL-1β: $F(4, 10)=31.89$, $P<0.001$, n=3 per group; IL-10: $F(4, 10)=54.32$, $P<0.001$, n=3 per group; MDA: $F(4, 25)=76.80$, $P<0.001$, n=6 per group; GSH: $F(4, 25)=36.80$, $P<0.001$, n=6 per group). Tukey's *post hoc* test showed that SA significantly reversed these inflammatory and oxidative alterations compared with the $Mg^{2+}$-free group (all $P<0.001$). Notably, CHOP overexpression partially reversed the inhibitory effects of SA on TNF-α, IL-1β, and MDA ($P=0.003$, $P=0.035$, and $P=0.047$, respectively), while concurrently abolishing its promoting effects on IL-10 and GSH ($P=0.044$ and $P=0.014$, respectively)(Figure 6. G-K). All *in vitro* experiments were repeated at least three times.

These findings suggested that the beneficial effect of SA against seizure-related injury may involve, at least in part, the suppression of CHOP and its associated ER stress pathway.

## Discussion

In recent decades, SA, a compound extracted from *Caesalpinia sappan* L, has attracted increasing interest due to its therapeutic potential in various diseases, including cerebral ischemia-reperfusion injury (8), diabetic kidney disease (21), osteoarthritis (22), and myocardial ischemia-reperfusion injury (23). In the present study, we observed that SA pretreatment remarkably increased seizure latency and reduced the duration, indicating the antiepileptic activity of SA. Mechanistically, we found that SA suppressed CHOP-mediated ER stress, and that CHOP overexpression reversed SA's beneficial effects on neuronal apoptosis, inflammation, and oxidative stress. These observations implicate CHOP as a critical node in the signaling network modulated by SA.

Epilepsy can cause neuroinflammation accompanied by a boost in pro-inflammatory cytokine levels and massive inflammatory mediators, which further contributes to the recurrence of seizures (24, 25). Furthermore, inhibiting inflammation has been regarded as an effective approach for treating epilepsy (26). Several previous studies have suggested the anti-inflammatory abilities of SA in a variety of diseases. In an asthma model induced by ovalbumin, SA could attenuate the airway inflammation via modulation of the Nrf2 pathway (15). Additionally, SA could block kidney fibrosis and inflammation induced by diabetes via suppressing the NF-κB signaling pathway (21). In addition to peripheral inflammation, SA's influence on neuroinflammation has drawn increasing attention. A study by Wang *et al.* (8) indicated the anti-neuroinflammatory role of SA treatment in a rodent model of middle cerebral artery occlusion allowing reperfusion, as displayed by decreased levels of proinflammatory mediators including TNF-α, IL-1β, and IL-6. Another study by Liao *et al.* (27) found that SA inhibited microglia-mediated neuroinflammatory response via targeting inosine monophosphate dehydrogenase 2 (IMPDH2). In the present study, SA pretreatment suppressed pro-inflammatory cytokine release while concurrently promoting anti-inflammatory cytokine production in both *in vivo* and *in vitro* epileptic models, demonstrating its dual immunomodulatory profile.

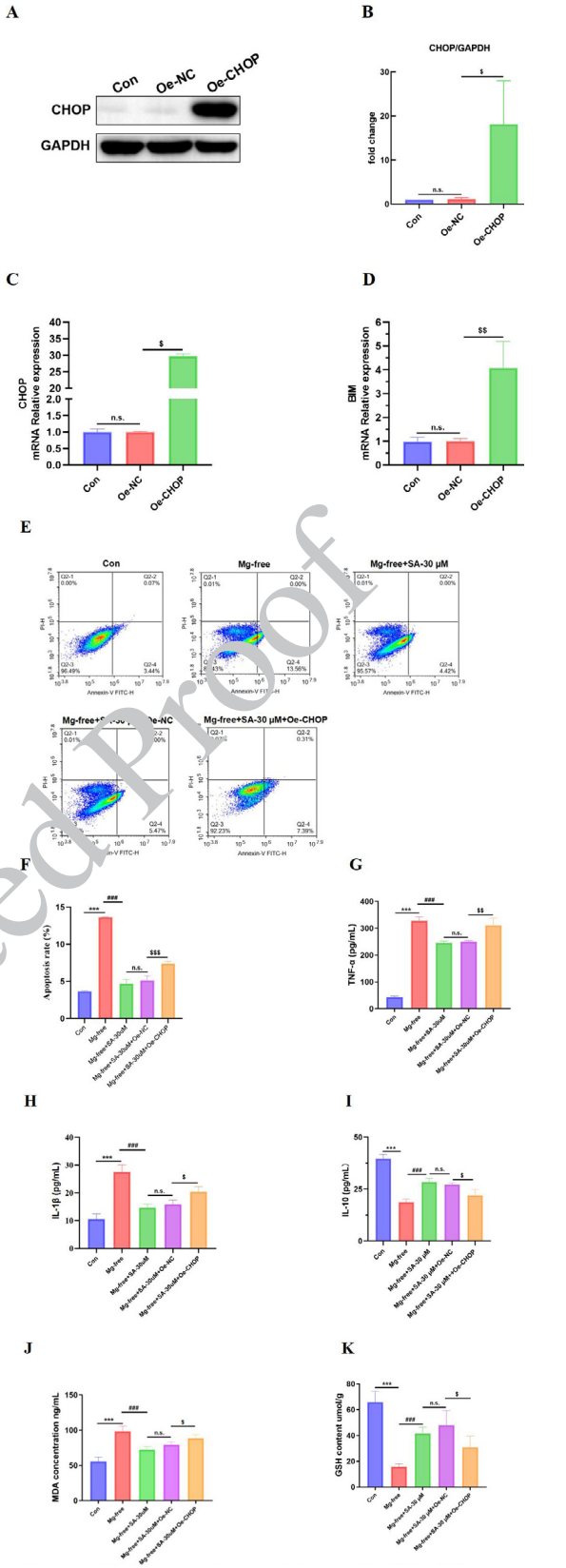


**Figure 6.** CHOP overexpression reversed the protective effects of SA on cell apoptosis, inflammation, and oxidative stress in HT22 cells treated with $Mg^{2+}$-free solution

(A-B) Expression of CHOP protein (n=3 per group). (C) Expression of CHOP mRNA (n=3 per group). (D) Expression of BIM mRNA (n=3 per group). (E-F) Cell apoptosis was detected using flow cytometry (n=3 per group). (G) Levels of TNF-α (n=3 per group). (H) Levels of IL-β (n=3 per group). (I) Levels of IL-10 (n=3 per group). (J) The levels of MDA (n=6 per group). (K) Levels of GSH (n=6 per group). $^{***}P<0.001$ vs Con group; $^{###}P<0.001$ vs $Mg^{2+}$-free group; $^{$}P<0.05$, $^{$$}P<0.01$, $^{$$$}P<0.001$ vs Oe-NC group; ns, not significant ($P>0.05$)

SA: Sappanone A; CHOP: CCAAT/enhancer-binding protein (C/EBP) homologous protein; BIM: Bcl-2 interacting mediator; MDA: Malondialdehyde; GSH: Glutathione

Oxidative stress is regarded as the disequilibrium between the generation of reactive oxygen species (ROS) and antioxidant defensive mechanisms (28). It is well established that oxidative stress contributes to the occurrence and development of epilepsy (29). Prolonged seizures induce an increase in ROS generation (30), which further leads to membrane lipid peroxidation and MDA generation (31). Furthermore, the ability of GSH, which is one of the most important antioxidants, is remarkably attenuated following epileptic seizures (32). Indeed, increased oxidative stress characterized by reduced GSH concentrations and elevated MDA levels has been reported in both epileptic patients and experimental epilepsy models (33, 34), and treatments targeting oxidative stress may exert antiepileptic and neuroprotective effects (35). Notably, SA was recently suggested to block oxidative stress responses in acetaminophen-induced acute liver injury by regulating the Nrf2 signaling pathway (6). Another investigation also observed an antioxidative effect of SA on the hypoxia/reoxygenation-induced cardiomyocyte injury through preventing mitochondrial apoptosis and suppressing the PI3K-Akt-Gsk-3β signaling pathway (36). In line with prior studies, we also observed that SA pretreatment decreased the levels of MDA and increased the contents of GSH in PTZ-induced seizure mice and HT22 hippocampal cells treated with $Mg^{2+}$-free solution, exerting a significant anti-oxidative stress activity.

Neuronal injury and apoptosis are common features of seizures and epilepsy, which may be attributed to many factors, including excitotoxicity (37), neuroinflammation (19), and oxidative stress (38). In the current study, our *in vitro* flow cytometry data confirmed that SA suppressed neuronal apoptosis induced by $Mg^{2+}$-free exposure. Meanwhile, *in vivo* TUNEL and Nissl staining results revealed that SA attenuated hippocampal neuronal damage in PTZ-induced seizure mice. Given that neuroinflammation and oxidative stress are well-established upstream inducers of apoptotic cascades, and SA exhibited concurrent anti-inflammatory and antioxidant activities in our epilepsy model, it is plausible that its anti-apoptotic effect arises, at least in part, from ameliorating these upstream pro-apoptotic milieus. Collectively, these findings support that SA exerts robust neuroprotective effects against seizure-associated neuronal cell death.

ER stress is a complex signaling cascade involved in the inflammatory response and neuronal apoptosis in epilepsy (39), and inhibition of hyperactivated ER stress is proposed to be a useful target for epilepsy intervention (12). Our study focused on the CHOP-mediated pathway, given that CHOP serves as the master switch for ER stress-driven apoptosis (40) and has been implicated as a key mediator of seizure-induced neuronal apoptosis (41). Beyond its pro-apoptotic function, CHOP has been reported to promote neuroinflammation (8). Since prolonged ER stress induced by repetitive seizures promotes the activation of pro-apoptotic factor CHOP (42), we speculate that CHOP's dual capacity to drive apoptosis and amplify neuroinflammation decreases seizure thresholds, which in turn provokes recurrent seizures, forming a "vicious circle" that is detrimental in epileptic networks. CHOP therefore represents a convergent node at which interrupting this cumulative damage process could simultaneously alleviate both apoptotic cell death and the inflammatory response that sustains seizure recurrence. In this study, we examined whether SA modulates CHOP signaling in our epilepsy models. We observed that SA pretreatment effectively suppressed CHOP up-regulation in epilepsy, and CHOP overexpression abolished SA's suppressive effects on cell apoptosis, inflammation, and oxidative stress under epileptic conditions, suggesting that CHOP may participate in SA's protective cascade. Furthermore, CHOP overexpression increased the expression of its downstream target BIM, confirming the functional competency of the overexpressed construct. While these gain-of-function data support a role for CHOP in SA's action, future studies employing loss-of-function approaches are warranted to establish the causal relationship between CHOP inhibition and the neuroprotective effects of SA in epilepsy.

The current study has some limitations. First, the dosages of SA used in this study were selected based on existing evidence. Our *in vivo* three-dose regimen (10, 25, and 50 mg/kg) demonstrated efficacy at 25 and 50 mg/kg. Still, not at 10 mg/kg, the absence of intermediate doses between 10 and 25 mg/kg precludes a formal dose-response analysis. Future studies incorporating additional intermediate doses (e.g., 15 and 20 mg/kg) are warranted to define the therapeutic window of SA better. Second, we only investigated the effect of SA on CHOP, and other ER stress-related proteins like XBP1s and ATF6 were not examined. Future research using a broader panel of ER stress markers is necessary to fully elucidate how SA modulates the integrated ER stress response in epilepsy. Third, the role of CHOP in mediating SA's effects was primarily derived from CHOP overexpression rescue experiments, without complementary loss-of-function validation. Thus, while our data identify CHOP as a critical node in SA's neuroprotective signaling against epilepsy, the causal relationship between CHOP and SA's neuroprotection awaits further validation. Fourth, we did not investigate potential sex differences in the antiepileptic effect of SA, since only male mice were used in our study. Fifth, our *in vitro* experiments used the HT22 cell line, which lacks the full glutamate receptor expression of primary neurons. Given that the $Mg^{2+}$-free model relies on NMDA receptor activation, these findings should be interpreted with caution, and future studies using primary cortical or hippocampal neurons would be valuable for validation. Moreover, only a single animal model (C57BL/6 mice) and a single seizure-inducing agent (PTZ) were used in our study, and future studies could investigate the effects of SA on other animal species and epilepsy models to determine the reliability and generalizability of our results. Despite these limitations, we still believe our study provides a new perspective.

## Conclusion

To sum up, our study demonstrated that SA pretreatment alleviated seizure behavior in PTZ-treated mice, highlighting its potential as a preventive or adjunctive strategy for seizure management. The mechanism may involve SA-mediated suppression of CHOP-driven ER stress, leading to reduced inflammation, oxidative stress, and neuronal damage.

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## Data Availability

Data will be available from the corresponding author upon request.

## Informed Consent

This study was approved by the Ethical Committee for Experimental Research of Southwest Medical University (Permit Number: 20230831-006).

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## Authors' Contributions

J F, JL L, and X C designed the experiments; J F and JL L performed the experiments and collected the data; J F and X C conducted the statistical analysis and interpreted the data; J F wrote the draft manuscript, and X C provided critical revisions. All authors read and approved the final version of the manuscript for publication.

## Conflicts of Interest

The authors report no conflicts of interest.

## Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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