Signa Vitae

ORIGINAL RESEARCH

In vitro effects of Ganoderic acid A on NF-κB-mediated inflammatory response in caerulein-stimulated pancreatic acinar cells

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Abstract

This study aimed to investigate Ganoderic acid A's (GAA) possible effects on inflammation and oxidative stress of caerulein-stimulated pancreatic acinar cells and uncover its effects on acute pancreatitis (AP). A cell model of AP was constructed by treating pancreatic acinar AR42J cells with caerulein. Cell counting kit-8 (CCK-8) assay was used to measure cell viability, while Enzyme-Linked Immunosorbent Assay (ELISA) and 2′,7′-Dichlorofluorescein (DCF) fluorescence assays were used to measure inflammation and oxidative stress. Western blot analysis was used to investigate Nuclear Factor-kappa B (NF-κB) signaling pathway inhibition, focusing on p65 and NF-kappa-B inhibitor alpha (IκBα) phosphorylation states. GAA significantly enhanced cell viability in caerulein-stimulated pancreatic acinar AR42J cells. It also significantly reduced AR42J cells' inflammatory response. Furthermore, GAA treatment mitigated oxidative stress by decreasing Reactive oxygen species (ROS) production. Lastly, GAA inhibited the NF-κB pathway, as evidenced by decreased p65 and IκBα phosphorylation. By inhibiting NF-B-mediated inflammation, GAA attenuated caerulein-induced AP.

Keywords

Acute pancreatitis (AP); Ganoderic acid A (GAA); Inflammatory response; Oxidative stress; NF-κB pathway

1. Introduction

Acute pancreatitis (AP) represents a severe pancreatic inflammatory that manifests rapidly and often causing significant morbidity and mortality [1]. The lack of a curative treatment option, despite extensive research, highlights the need for novel therapeutic strategies. According to epidemiological trends, AP incidence is on the rise, as a result of gallstones, alcohol consumption, hypertriglyceridemia, hyperparathyroidism, pancreatic cancer, obesity, endoscopic retrograde cholangiopancreatography (ERCP), trauma, infections, medications, autoimmune disorders, and genetic predispositions [2]. In AP, pancreatic enzymes are pathologically activated, triggering autodigestion and activating a sterile inflammatory response that results in pancreatic edema, hemorrhage and necrosis [3]. It is possible for this local inflammation to escalate into systemic inflammatory response syndrome (SIRS) if inflammatory mediators produced by activated leukocytes and pancreatic cells exceed the confines of the pancreas.

Inflammation and oxidative stress play a central role in AP progression [4, 5]. Damage to pancreatic acinar cells, regardless of the cause, leads to uncontrolled immune cell migration infiltration and excessive inflammatory cytokines secretion [6]. Consequently, further inflammation and oxidative stress

are precipitated, exacerbating tissue injury and edema. Nuclear factor kappa B (NF-κB), a pivotal transcription factor, orchestrates the expression of numerous inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-6 and IL-1β, which are closely associated with AP severity [7]. Additionally, AP progression is accompanied by an increase in reactive oxygen species (ROS) and a concomitant decrease in superoxide dismutase (SOD), inducing excessive oxidative stress [8, 9].

Ganoderma lucidum, commonly known as Lingzhi or Reishi mushroom, has been extensively used in traditional Chinese medicine for various ailments treatment [10]. The triterpene compound Ganoderic acid A (GAA derived from this mushroom is capable of exhibiting strong anti-inflammatory and antioxidant properties [11]. Previous studies have demonstrated that GAA mitigates inflammation by inhibiting Rho/Rho-associated protein kinase (ROCK)/NF-κB pathways, improving conditions like acute lung injury [12]. Furthermore, GAA protects hippocampal neurons from damage by enhancing SOD activity and inhibiting apoptosis [13]. GAA remains underexplored despite its known benefits when treating acute pancreatitis.

This study aims to bridge this gap by investigating GAA's inhibitory effects on the NF-κB pathway, thereby attenuating

the inflammatory and oxidative stress responses induced by caerulein in pancreatic acinar AR42J cells. Using *in vitro* experiments, we examine the effects of GAA on cell viability, inflammation, and oxidative stress under AP conditions, providing a potential new therapeutic avenue for acute pancreatitis.

2. Materials and methods

2.1 Cell culture and treatment

Human AR42J cell line (ATCC) was incubated in Roswell Park Memorial Institute (RPMI) 1640, containing 10% Fetal bovine serum (FBS), at 37 °C in a humidified atmosphere of 5% Carbon dioxide (CO_2), ensuring sterile conditions to prevent contamination. Next, they were incubated with GAA (Sigma) at 0 μM, 2.5 μM, 5 μM, 10 μM and 20 μM for 24 h. Caerulein (MCE, HY-A0190, New Jersey, USA) treated into AR42J cells upon 5 nM concentration for 24 h.

2.2 CCK-8 assay

AR42J cells were seeded into 96-well plates. For cell viability detection, CCK-8 (C0078, Beyotime, Beijing, China) was added to each well. Plates were then incubated for 4 h. Spectrophotometers calibrated to specific absorbance readings were used to measure optical density at 450 nm.

2.3 Cell apoptosis

To detect the apoptotic cell number, Annexin V/Propidium Iodide (PI) apoptosis detection (C1065M, Beyotime, Beijing, China) was performed according to the manufacturer's instructions.

2.4 Real-time polymerase chain reaction (PCR)

Total RNA from indicated cells were isolated with Trizol reagent (Invitrogen, 15596-018, Carlsbad, CA, USA). Quantitative PCR was performed with SYBR mixture (Takara, RR820A, Osaka, Japan). PCR amplification was performed in a thermocycler, with initial denaturation at 95 °C for 10 min. This was followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A quantitative assessment of the target gene's relative amount was carried out using the $\Delta\Delta$CT method. The cDNA was amplified using the primers: IL-1β: 5$'$-ACAAGGAGAAGAAAGTAATGAC-3$'$, 5$'$-GCTGTAGAGTGGGCTTAT-3$'$; TNF-α: 5$'$-CCCAGGGACCTCTCTCTAATCAG-3$'$, 5$'$-GCTGGCAC-CACTAGTTGGTTGT-3$'$; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5$'$-AGAAGGCTGGGGCTCATTTG-3$'$, 5$'$-AGGGGCCATCCACAGTCTTC-3$'$.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected for an ELISA assay to detect the IL-1β and TNF-α levels (ELISA kits, Shanghai, China).

2.6 2$'$,7$'$-Dichlorofluorescein (DCFDA)—cellular ROS assay

Fixation with formaldehyde, washed with phosphate buffer saline (PBS), permeabilized with PBS containing 0.5% Triton X-100, followed by staining with ROS detection kit (Abcam, ab113851, Cambridge, UK) were carried out. 4$'$,6-diaminyl-2-phenylindole (DAPI) was used to stain the cells. On a Zeiss fluorescence microscope, images were captured after staining and fluorescence intensity was quantified using ImageJ.

2.7 Immunoblot

We extracted proteins from cells using Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, L0025, Beijing, China). The samples were then electrophoresed with 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PolyVinylidene Fluoride (PVDF) membranes, and blocked. The membranes were then incubated with primary antibodies targeting p65 (1:500, ab32536, Abcam), p-p65 (S536, 1:500, ab76302, Abcam), IκBα (1:1000, sc-203, Santa Cruz), p-IκBα (Tyr42, 1:500, sc-101714, Santa Cruz) and β-actin (1:3000, ab8226, Abcam) for 1 h. For the final step, the membranes were conjugated for 1 h with specific secondary antibodies.

2.8 Statistics

Statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). A Student's *t*-test was used to compare two groups and an Analysis of Variance (ANOVA) followed by a *post-hoc* Tukey's test to compare multiple groups. Data were represented as mean $\pm$ standard deviation (SD) with $p < 0.05$ shows statistically significant.

3. Results

3.1 GAA significantly enhanced cell viability in caerulein-stimulated pancreatic acinar AR42J cells

To investigate GAA's effect on AP progression, we first examined its effects on AR42J cells. Through CCK-8 assays, we noticed that the treatment of GAA had modest effects on AR42J cell growth at concentrations of 2.5, 5 and 10 μM, whereas treatment with high concentrations of GAA (20 μM) suppressed AR42J cell growth (Fig. 1A). For the next experiment, a low concentration was used. AP progression was then simulated by treating AR42J cells with caerulein. As observed by CCK-8 assays, caerulein suppressed the growth of AR42J cells, whereas GAA further enhances it (Fig. 1B).

Using Flow cytometry (FCM) assays, we found caerulein treatment stimulated the apoptosis of AR42J cells. GAA treatment, however, reversed the apoptosis promoted by caerulein treatment in AR42J cells (Fig. 1C). Therefore, GAA improves cell viability in human caerulein-stimulated AR42J cells.
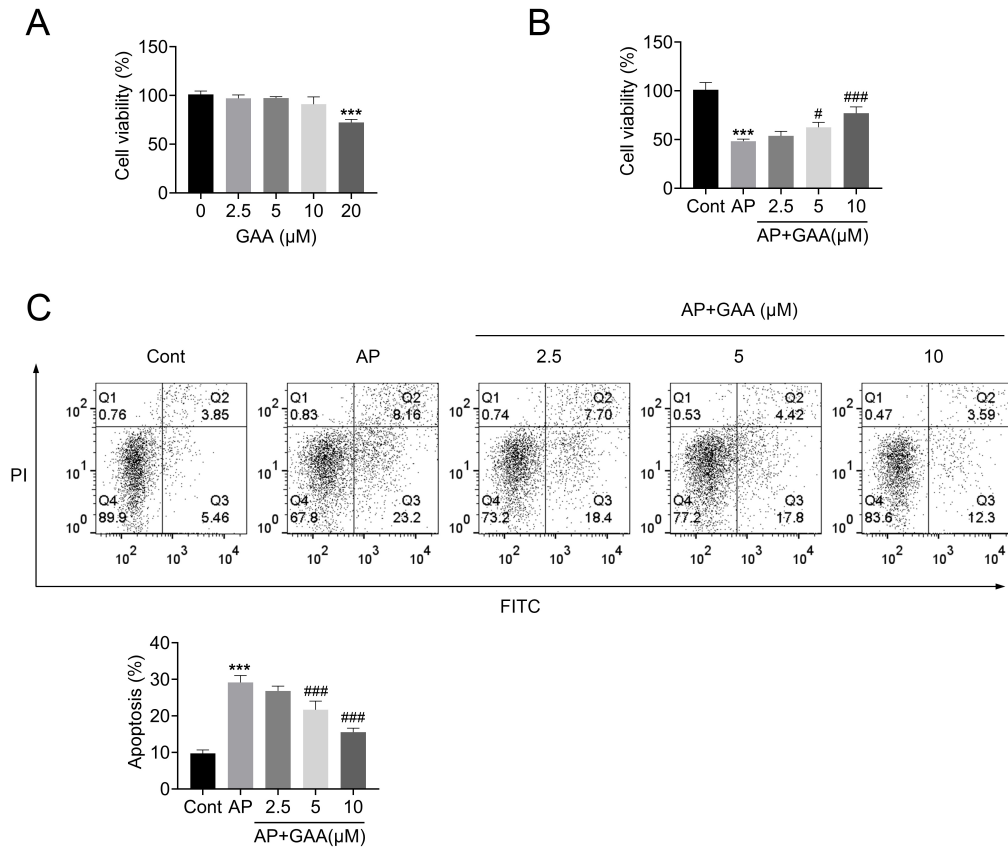
FIGURE 1. GAA significantly enhanced cell viability in caerulein-stimulated pancreatic acinar AR42J cells. (A) CCK-8 assays showed AR42J cell growth upon GAA treatment at concentrations of 2.5, 5, 10 and 20 μM for 24 h. OD450 value was measured. ***$p < 0.001$, GAA *vs*. control. (B) CCK-8 assays showed AR42J cell growth upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. OD450 value was measured. (C) FCM assays showed the apoptosis of AR42J cells upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. ***$p < 0.001$, Caerulein (AP) *vs*. control, #$p < 0.05$, ###$p < 0.001$, AP + GAA *vs*. AP. AP, acute pancreatitis; GAA, Ganoderic acid A; PI, Propidium Iodide; FITC, Fluorescein Isothiocyanate.

3.2 GAA effectively reduced the inflammatory response of caerulein-stimulated AR42J cells

Caerulein-stimulated AR42J cells were tested for inflammation by qPCR and ELISA, which detected markers TNF-α and IL-1β. As a result of qPCR assays, we found that caerulein treatment upregulated the expression of these inflammatory factors in AR42J cells, but GAA treatment further suppressed the expression, suggesting a suppression of inflammation (Fig. 2A). Similarly, ELISA showed the promotion of secretion of TNF-α and IL-1β in caerulein-stimulated AR42J cells, whereas GAA treatment further suppressed the secretion, suggesting the inhibition of the inflammatory response (Fig. 2B). GAA effectively suppresses the IL-1β-stimulated inflammatory response in caerulein-stimulated AR42J cells.

3.3 GAA mitigated oxidative stress by decreasing ROS production in caerulein-stimulated AR42J cells

The effect of GAA on ROS production, a key component of oxidative stress and AP progression, in caerulein-stimulated AR42J cells was further investigated. In caerulein-stimulated AR42J cells, ROS levels were detected using immunostaining. Interestingly, caerulein increased ROS levels in AR42J cells. However, GAA treatment further reduced the ROS levels in caerulein-stimulated AR42J cells, with decreased ROS intensity (Fig. 3). Therefore, By ROS production in caerulein-stimulated AR42J cells, GAA mitigated oxidative stress.

3.4 GAA inhibited the NF-κB pathway in caerulein-stimulated AR42J cells

To determine how GAA-mediated inflammation and oxidative stress in AR42J cells stimulated by caerulein, we detected effects of GAA on the NF-κB pathway. The NF-κB pathway affects inflammation and oxidative stress in several disease models. We noticed that caerulein treatment increased p65 and IκBα phosphorylation levels in AR42J cells, and suppressed IκBα expression (Fig. 4). However, GAA treatment further suppressed p65 and IκBα phosphorylation levels, increased IκBα expression in caerulein-stimulated cells, suggesting NF-κB pathway suppression (Fig. 4). Thus, GAA suppresses the NF-κB pathway in caerulein-stimulated AR42J cells.
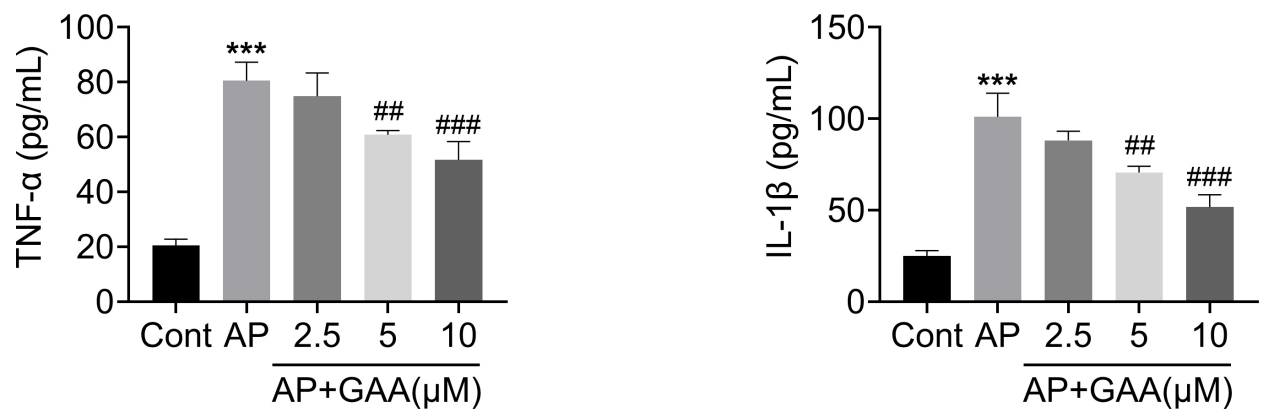
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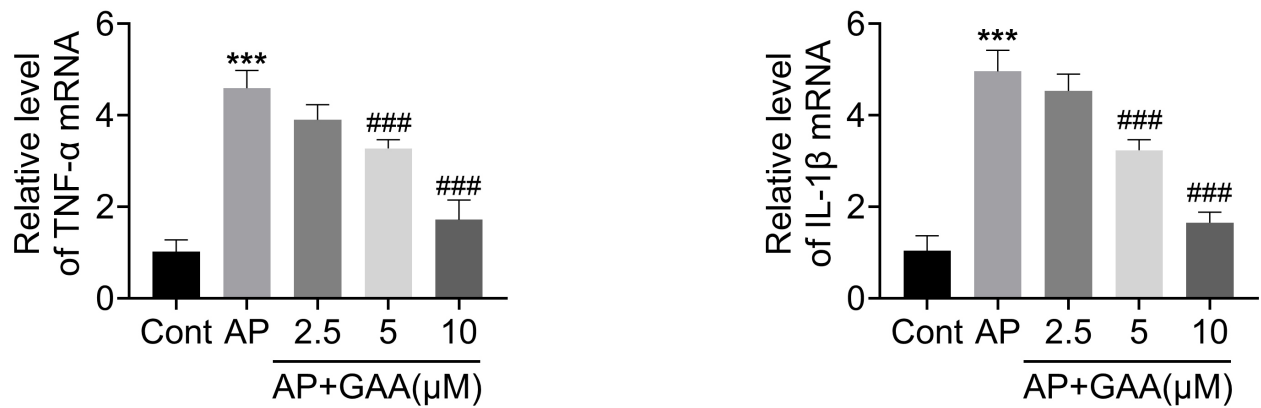
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FIGURE 2. GAA effectively reduced the inflammatory response of caerulein-stimulated AR42J cells. (A) qPCR assays indicated the mRNA levels of TNF-α and IL-1β of AR42J cells upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. (B) ELISA showed TNF-1α and IL-1β secretion of AR42J cells upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. ***$p < 0.001$, Caerulein (AP) *vs.* control, ##$p < 0.01$, ###$p < 0.001$, AP + GAA *vs.* AP. AP, acute pancreatitis; GAA, Ganoderic acid A; TNF-α, Tumor Necrosis Factor-α; IL-1β, Interleukin-1β.
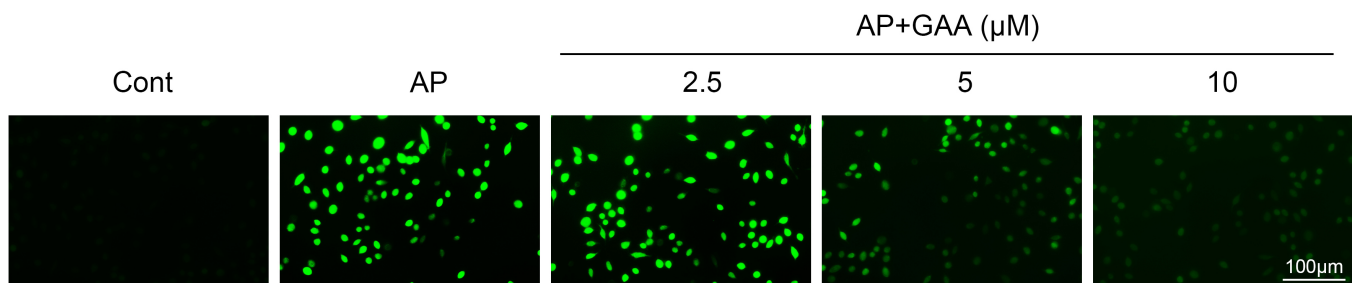

FIGURE 3. GAA mitigated oxidative stress by decreasing ROS production in caerulein-stimulated AR42J cells. Immunostaining assays showed ROS levels of AR42J cells upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. Green panel indicates ROS levels. Scale bar, 100 μm. AP, acute pancreatitis; GAA, Ganoderic acid A.
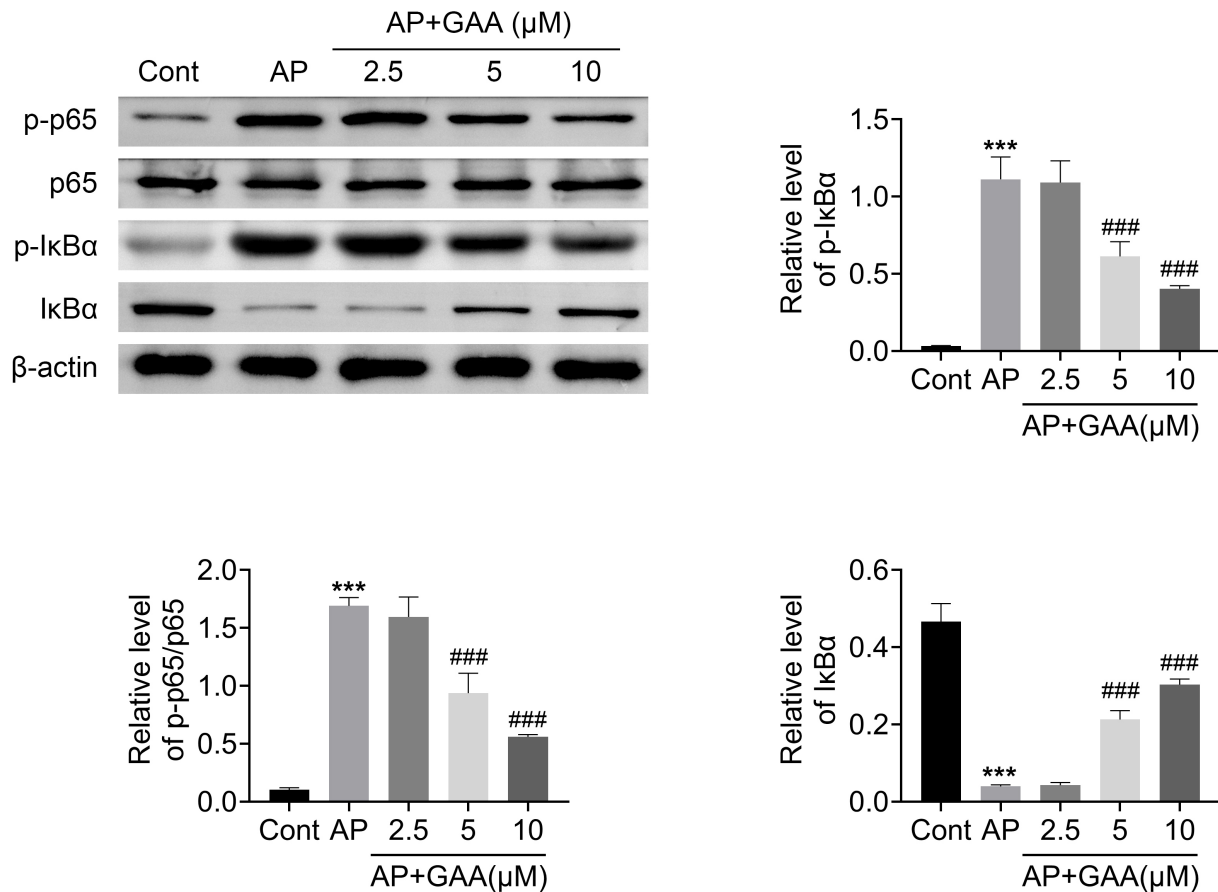
FIGURE 4. GAA inhibited the NF-κB pathway in caerulein-stimulated AR42J cells. Immunoblot assays showed p65 and IκBα expressions and phosphorylation levels of in AR42J cells upon GAA treatment at concentrations of 2.5, 5 and 10 μM and caerulein for 24 h. The relative expression of IκBα and relative phosphorylation levels of p65 and IκBα were quantified. ***p < 0.001, Caerulein (AP) *vs.* control, ###p < 0.001, AP + GAA *vs.* AP. AP, acute pancreatitis; GAA, Ganoderic acid A; IκBα, NF-kappa-B inhibitor alpha.

4. Discussion

AP occurs when pancreatic enzymes become autodigestively activated, resulting in tissue necrosis, systemic inflammation, and multiple organ dysfunctions [14]. AP is often triggered by premature activation of trypsinogen into trypsin in the pancreas, followed by inflammatory cascades and oxidative stress that exacerbate tissue damage [15]. In the absence of specific pharmacological treatments, most therapeutic options rely on supportive care, raising the urgency of targeted therapies. Novel drugs that target AP molecular pathways are essential to improving outcomes in patients. The bioactive compound GAA from Ganoderma lucidum is presented herein as a promising candidate. AP pathogenesis could be modulated by its anti-inflammatory and antioxidant properties, offering a new therapeutic avenue.

Inflammatory diseases such as acute pancreatitis (AP) have recently been linked to GAA's potential mechanisms of action. GAA has been shown to modulate a number of signaling pathways that play an important role in inflammation progression and oxidative stress that are central to AP pathogenesis. GAA inhibits NF-κB pathway activation, a critical regulator of inflammatory responses [16]. It is shown that NF-κB regulates the expression of various pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, which are significantly elevated in AP. By suppressing NF-κB signaling, GAA potentially reduces cytokines release, mitigating the inflammatory milieu associated with AP. Aside from limiting local damage to the pancreas, this effect also reduces the risk of systemic complications, which are common in severe cases of AP.

Moreover, oxidative stress plays a dual role in AP, both as a consequence and as an exacerbator of the inflammatory process. By improving the activity of antioxidants like superoxide dismutase (SOD) and by directly scavenging reactive oxygen species (ROS), GAA reduces oxidative stress [17]. It prevents the progression of damage by preserving cellular integrity and function.

Furthermore, GAA's ability to affect other inflammatory pathways, such as the Rho/ROCK pathway, suggests that it has broader therapeutic effects than just NF-κB inhibition. Inflammatory responses in AP could be modulated by effects on cellular apoptosis, migration, and adhesion.

An important role played by the NF-κB pathway is to orchestrate the inflammatory response, regulating the expression of a wide array of pro-inflammatory cytokines and chemokines that exacerbate pancreatic injury. By activating NF-κB, immune cells are recruited to the pancreas, further amplifying inflammatory cascade and causing the disease to progress.

In this study, GAA significantly inhibited NF-κB activation, attenuating NF-κB activation and consequently reducing the expression of critical inflammatory mediators such as TNF-α, IL-6 and IL-1β [18]. Thus, GAA's modulation of NF-κB activity might serve as a key mechanism to mitigate AP's destructive and inflammatory processes, highlighting its potential as a targeted therapy to interrupt the inflammatory cycle that underpins this debilitating disease.

5. Conclusions

Specifically, we used pancreatic acinar AR42J cells as an *in vitro* model, which might not fully replicate the complex intercellular interactions and physiological responses observed *in vivo* during acute pancreatitis. Without further validation, the results may not be directly applicable to clinical scenarios. As well, despite its potential to modulate inflammatory and oxidative pathways, the precise pharmacokinetic properties and potential systemic effects remain unknown in humans. GAA's efficacy and safety in treating AP will require further research involving animal models and eventually clinical trials. This study supports Ganoderic Acid A being able to attenuate inflammatory and oxidative stress responses in acute pancreatitis through the inhibition of the NF-κB pathway.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YZJ and JH—designed the study and carried them out, prepare the manuscript for publication and reviewed the draft of the manuscript. YZJ, JH and NM—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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