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ORIGINAL RESEARCH

Cirsilineol reduces inflammation and apoptosis in an *in vitro* model of acute pancreatitis

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Abstract

Acute pancreatitis (AP) is a severe inflammatory disorder for which effective treatments are currently lacking. Cirsilineol (CSL), a flavonoid derived from Artemisia plants, is recognized for its potent anti-inflammation and antioxidant characteristics. The mechanisms underlying AP and potential therapeutic agents remain unknown. This study investigates the effects of CSL on AP and delves into its underlying mechanism. Our results indicate that CSL significantly improved cell viability in AR42J pancreatic acinar cells and influenced the levels of pro-inflammatory cytokines. Furthermore, CSL markedly decreased apoptosis markers and inhibited the Nuclear Factor kappa-B (NF-κB) activation by reducing phosphorylation of p65 and inhibitor of NF-κB alpha (IκBα). These findings underscore the powerful anti-inflammatory and anti-apoptotic properties of CSL, acting through the NF-κB pathway, indicating its promise as a therapeutic option for AP.

Keywords

Cirsilineol; Acute pancreatitis; Inflammation; Apoptosis; NF-κB signaling

1. Introduction

Acute pancreatitis (AP) is a severe type of inflammatory condition in pancreas [1]. It results from the premature activation of digestive enzymes in the pancreas, causing inflammation and significant tissue damage [2]. The manifestation of acute pancreatitis can vary from minor, self-resolving episodes to serious, necrotizing forms that lead to systemic complications [3]. The global incidence of AP has been on the rise, with mortality rates climbing from 15% to 30% and reaching over 50% in cases complicated by sepsis [4]. The development of AP is characterized by complex interactions among various mechanisms, including disrupted calcium signaling, trypsinogen activation, and the inflammatory cell influx. Despite considerable progress in comprehending the pathophysiology of AP, impactful therapeutic interventions remain scarce, primarily focusing on supportive care such as pain relief, hydration, and dietary support [3, 4]. Despite advancements in understanding the pathophysiology of AP, effective therapeutic interventions remain limited, underscoring the urgent need for new treatment strategies.

Cirsilineol (CSL), a flavonoid isolated from Artemisia plants, has attracted attention for its diverse biological activities [5, 6]. CSL has shown notable effectiveness in decreasing lung damage mediated by particulate matter (PM) 2.5, alleviating oxidative stress, and regulating levels of inflammatory cytokines [7, 8]. Moreover, CSL inhibited the Nuclear Factor kappa-B (NF-κB)/Ikappa (IKK) pathway, thereby preventing inflammation in various experimental settings [9]. Due to its potent anti-inflammatory properties, CSL is considered a hopeful option for treating AP. However, the precise ways in which CSL delivers its protective benefits in AP still require further investigation.

One of the key molecular mechanisms underlying AP is the activation of NF-κB pathway [10, 11]. NF-κB plays a crucial role as a transcription factor in the regulation of genes related to in inflammation and immune responses [10]. In the context of AP, early activation of NF-κB in pancreatic acinar cells is triggered by the degradation of its inhibitors, IkappaBα (IκBα) and IkappaBβ (IκBβ), leading to the transcription of mediators that exacerbate pancreatic injury and inflammation [11]. The suppression of NF-κB activation has demonstrated a decrease in inflammation and tissue injury severity in experimental AP models, suggesting that targeting this pathway holds promise for therapeutic intervention.

Recent studies have indicated that CSL has the potential to reduce inflammation and oxidative stress in allergic rhinitis models and other inflammatory conditions by regulating important signaling pathways, including NF-κB [8, 12]. This suggests that CSL may have a promising therapeutic potential in treating AP, a condition characterized by uncontrollable inflammation and cellular death playing a central role in the advancement of the disease. By targeting the NF-κB pathway, CSL could potentially reduce the inflammatory response and cell death associated with AP, thereby improving clinical outcomes.

This study aims to investigate the potential of CSL in treating AP. The findings from this research could provide new

insights into the application of CSL as a therapeutic agent for AP.

2. Materials and methods

2.1 Materials

All chemicals and kits used in this study were obtained from the following sources: CSL and Caerulein were purchased from Sigma-Aldrich; AR42J pancreatic acinar cells were obtained from American Type Culture Collection (ATCC, USA); DMEM (Dulbecco's Modified Eagle Medium, Gibco, 11995065, Grand Island, NY, USA) and FBS (Fetal Bovine Serum, Gibco, 16000044, Grand Island, NY, USA) were purchased from Gibco; Cell Counting Kit-8 (CCK-8) was obtained from Beyotime (C0038, Beyotime, Beijing, China); Enzyme-Linked ImmunoSorbent Assay (ELISA) Kits for interleukin (IL)-6 (PI330), Tumor Necrosis Factor-α (TNF-α, PT516), and interleukin-1β (IL-1β, PI305) were from Beyotime (PI330, PT516, PI305, respectively, Beyotime, Beijing, China); RNA Extraction Kit was obtained from Beyotime (R0027, Beyotime, Beijing, China); Reverse Transcription Kit was from Takara (PrimeScript Kit, RR037A, Osaka, Japan); Quantitative Real-time Polymerase Chain Reaction (qPCR) Reagents, including SYBR(R) GREEN I NUCLEIC ACID GEL STAIN (SYBR) Green Mix (Beyotime, D7261, Beijing, China), and custom synthesized primers; Flow Cytometry Reagents (Annexin V-Fluorescein (FITC)/Propidium (PI) Apoptosis Kit) were from Beyotime (C1062, Beyotime, Beijing, China); Protein Extraction and Western Blot Reagents including Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (Beyotime, P0013B, Beyotime, Beijing, China), Bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, P0010, Beyotime, Beijing, China), primary antibodies (cleaved-caspase3, Abcam, ab2302), cleaved-poly ADP-ribose polymerase (PARP, Abcam, ab32064), p65 (Abcam, ab16502), p-p65 (Abcam, ab86299), IκBα (Abcam, ab32518), p-IκBα (Abcam, ab133462), Horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, ab6721), and Enhanced chemiluminescence (ECL) Detection System (Beyotime, P0018S).

2.2 Cell culture and reagents

AR42J pancreatic acinar cells were obtained from ATCC (USA) and cultured in DMEM (Gibco) with the addition of 10% FBS (Gibco) at 37 °C in an atmosphere containing 5% CO_2. CSL and Caerulein were obtained from Sigma-Aldrich.

2.3 Cell viability assay

Cell viability was assessed using CCK-8 according to the previous study [7]. AR42J cells were seeded in 96-well plates and incubated overnight. Cells were then treated with CSL (0, 1.25, 2.5, 5, 10 and 20 μM) for 24 h. To induce AP, cells were pre-treated with caerulein (100 nM) for 1 h, followed by CSL (1.25, 2.5 and 5 μM) for 24 hours. After incubating for 2 hours, 10 μL of CCK-8 was added, and the optical density (OD)450 value was determined with a microplate reader from Bio-Rad, USA.

2.4 ELISA

The concentrations of pro-inflammatory cytokines IL-6, TNF-α and IL-1β in the supernatants of cell cultures were measured through ELISA kits, following protocols outlined in a prior investigation [9].

2.5 Quantitative PCR (qPCR)

Total RNA was extracted from AR42J cells using the Beyotime RNA Extraction Kit (R0027) and reverse-transcribed into cDNA using the PrimeScript Kit (Takara). qPCR was performed using SYBR Green Mix. The expression levels of IL-6, TNF-α and IL-1β mRNA were standardized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control and assessed utilizing the $2^{-\Delta\Delta Ct}$ technique, as described in a prior investigation [8]. Primer sequences were as follows: IL-6 (5′-GAGGATACCACTCCCAACAGACC-3′; 5′-AAGTGCATCATCGTTGTTCATACA-3′), TNF-α (5′-AGGCGGTGCCTATGTCTCAG-3′; 5′-GAGGAGCACGTAGTCGGGTA-3′), IL-1β (5′-CTGTCCTGCGTGTTGAAAGA-3′; 5′-TGAGTGATACTGC CTGCCTG-3′), and GAPDH (5′-TCAAGAAGGTGGTGAAG CAGG-3′; 5′-TCAAAGGTGGAGGAGTGGGT-3′).

2.6 Flow cytometry

Apoptosis was assessed by flow cytometry using the Annexin V-FITC/PI Apoptosis Kit according to the previous study [7]. AR42J cells were collected, rinsed, and labeled with Annexin V-FITC and PI. The samples were then assessed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.7 Immunoblot analysis

Protein extraction was carried out by using RIPA lysis buffer (Beyotime, P0013B) with subsequent quantification employing the BCA Kit. Following this, the samples were subjected to Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and then transferred onto Polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies: cleaved-caspase3 (1:500, Abcam, ab2302), cleaved-PARP (1:500, Abcam, ab32064), p65 (1:1000, Abcam, ab16502), p-p65 (1:300, Abcam, ab86299), IκBα (1:500, Abcam, ab32518), and p-IκBα (1:300, Abcam, ab133462). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Abcam, ab6721). The signals were detected using an ECL detection system (Beyotime, P0018S).

2.8 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad 8.0 (GraphPad Software, San Diego, CA, USA). Differences were analyzed using one-way Analysis of Variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Effect of CSL on AR42J cell viability

We first investigated the effect of CSL on AR42J cell viability. AR42J cells were treated with different concentrations of CSL (0, 1.25, 2.5, 5, 10 and 20 μM), and cell viability was assessed after 24 hours. Low level of CSL could not affect the viability of AR42J cells (Fig. 1a). However, high levels of CSL suppressed the viability of AR42J cells (Fig. 1a). In a separate experiment, AR42J cells were treated with caerulein (100 nM) to induce inflammation and AP, followed by treatment with CSL (1.25, 2.5 and 5 μM). CSL improved cell viability compared to caerulein-only (Fig. 1b). The findings suggest that CSL boosts the survival of AR42J cells in both regular and AP settings.

3.2 CSL reduces caerulein-induced inflammation in AR42J cells

To assess the anti-inflammatory properties of CSL, we measured the levels of inflammatory cytokines in AR42J cells. The cells treated with caerulein (100 nM) are used to stimulate inflammation, followed by treatment with CSL. ELISA results showed that CSL treatment decreased secretion of IL-6, TNF-α and IL-1β caused by caerulein (Fig. 2a). Additionally, qPCR analysis revealed that CSL significantly decreased the mRNA levels of these pro-inflammatory cytokines in caerulein-treated AR42J cells (Fig. 2b). These findings indicate that CSL has a significant anti-inflammatory effect on AR42J cells.

3.3 CSL reduces caerulein-induced apoptosis in AR42J cells

We next explored the impact of CSL on apoptosis in AR42J cells using flow cytometry and immunoblot analysis. Cells were treated with caerulein (100 nM) and CSL (1.25, 2.5 and 5 μM). Flow cytometry indicated a reduction in percentage of apoptotic cells with CSL treatment (Fig. 3a). Immunoblot analysis further confirmed that CSL decreased the levels of cleaved-caspase3 and cleaved-PARP in AR42J cells, suggesting the suppression of cell apoptosis (Fig. 3b). These results demonstrate that CSL mitigates apoptosis in AR42J cells induced by caerulein.

3.4 CSL inhibits NF-κB pathway in AR42J cells

To understand the mechanism behind the anti-inflammatory as well as anti-apoptotic effects of CSL, we examined the NF-κB pathway. Cells treated with caerulein and CSL were subjected to immunoblot analysis to measure the expression and p-p65 and p-IκBα. CSL significantly inhibited the phosphorylation of both p65 and IκBα, indicating suppression of the NF-κB pathway, in caerulein-induced AR42J cells (Fig. 4). This indicates that CSL demonstrates its anti-inflammatory and anti-apoptotic properties by inhibiting the NF-κB pathway.

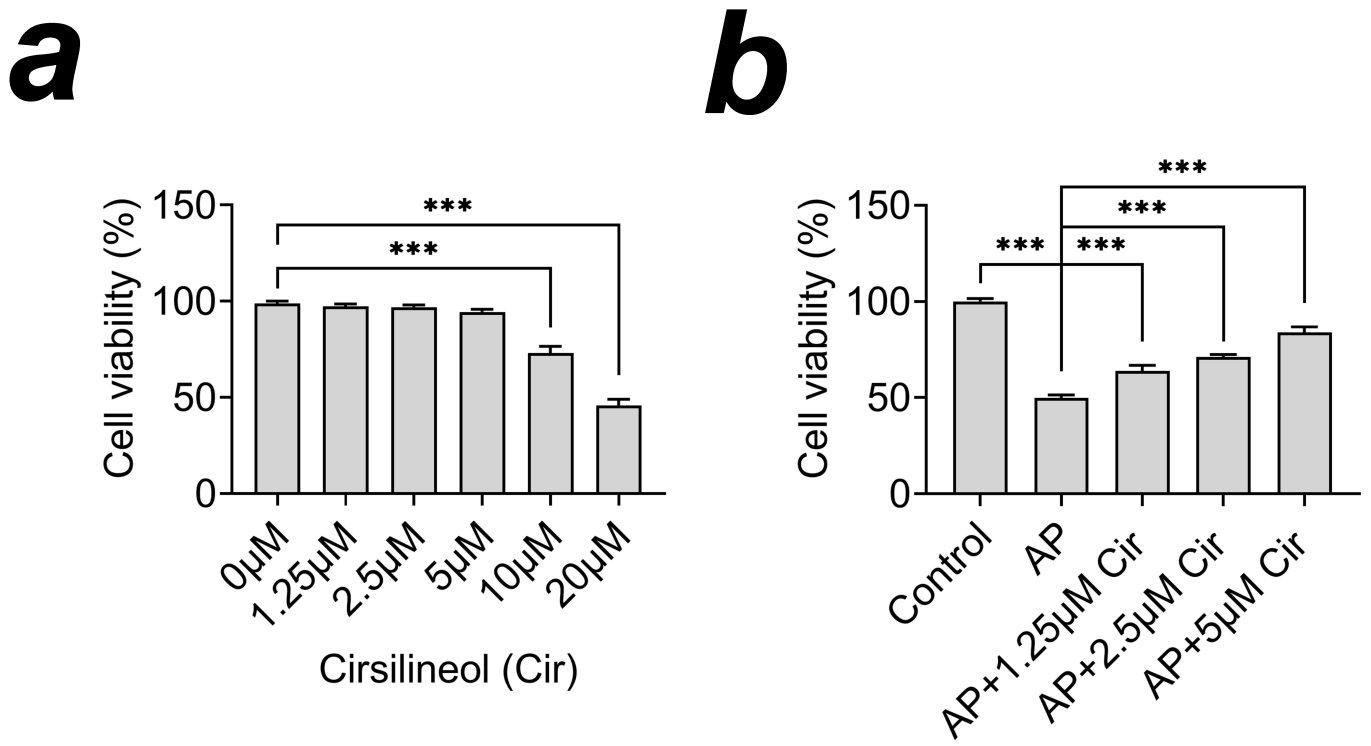

FIGURE 1. Effect of cirsilineol on AR42J cell viability. (a) AR42J cells were treated with varying concentrations of cirsilineol (0, 1.25, 2.5, 5, 10 and 20 μM). Cell viability was assessed using the CCK-8 assay after 24 hours of treatment. The OD450 value was measured. (b) AR42J cells were treated with caerulein (100 nM) to induce inflammation, followed by treatment with cirsilineol (1.25, 2.5 and 5 μM). Cell viability was assessed using the CCK-8 assay after 24 hours of treatment. The OD450 value was measured. The results are presented as the mean $\pm$ standard deviation (SD) of three independent experiments. ***$p < 0.001$. AR42J, Rat Pancreatic Acinar Cell Line; AP, Acute pancreatitis; Cir, Cirsilineol.

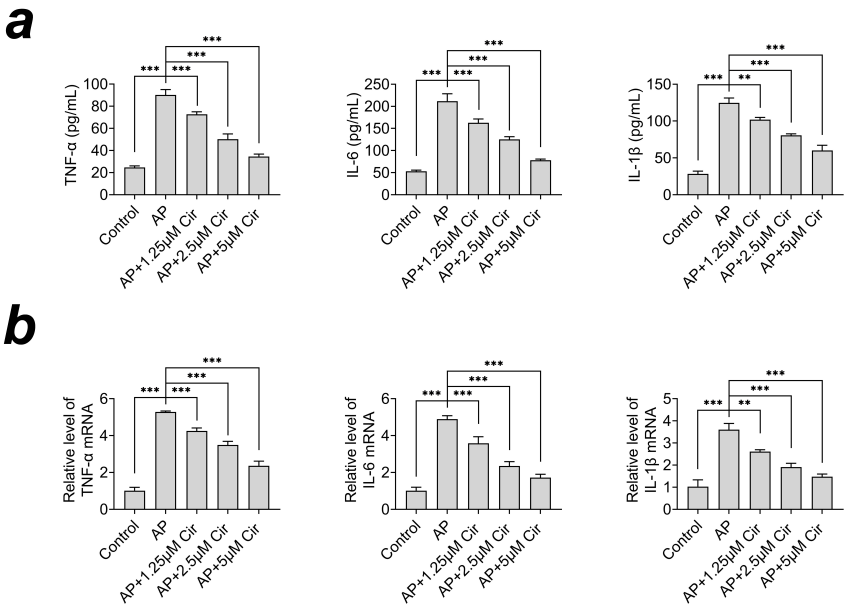

FIGURE 2. Cirsilineol reduces caerulein-induced inflammation in AR42J cells. (a) AR42J cells were treated with caerulein (100 nM) to induce inflammation, followed by treatment with cirsilineol (1.25, 2.5 and 5 μM). Levels of pro-inflammatory cytokines IL-6 (Interleukin-6), TNF-α (Tumor Necrosis Factor-alpha) and IL-1β (Interleukin-1 beta) were measured using ELISA. (b) mRNA levels of pro-inflammatory cytokines IL-6 (Interleukin-6), TNF-α (Tumor Necrosis Factor-alpha) and IL-1β (Interleukin-1 beta) were measured using qPCR. The results are presented as mean ± SD from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. AP, Acute pancreatitis; Cir, Cirsilineol.

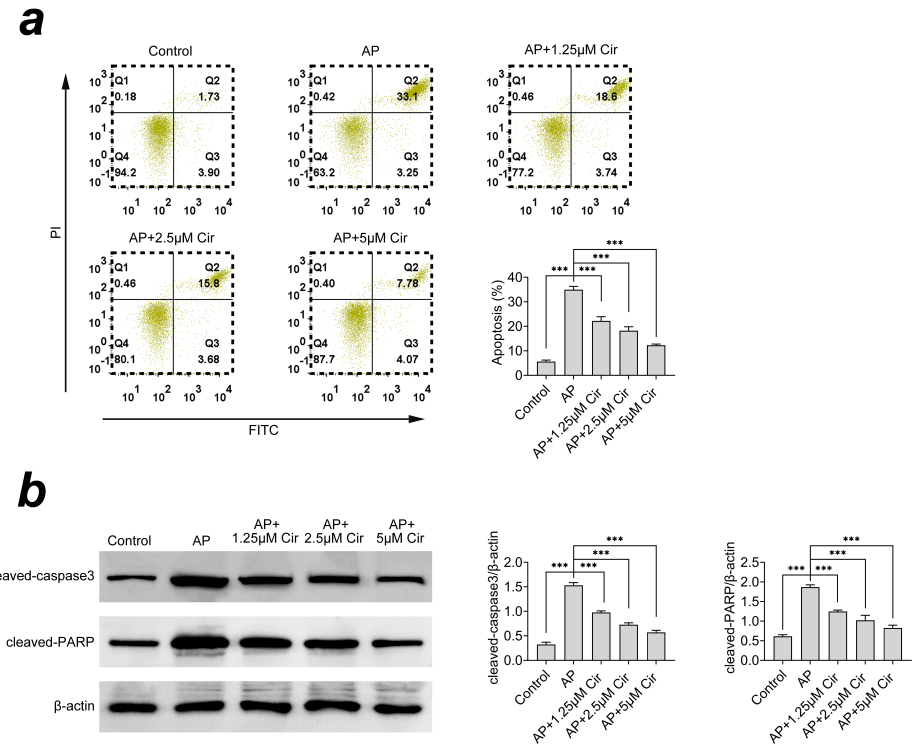

FIGURE 3. Cirsilineol reduces caerulein-induced apoptosis in AR42J cells. (a) Apoptosis in AR42J cells treated with caerulein (100 nM) and cirsilineol (1.25, 2.5 and 5 μM) was assessed using flow cytometry. The percentage of apoptotic cells was determined by Annexin V/PI staining. (b) Immunoblot analysis was performed to detect the levels of cleaved-caspase3 and cleaved-PARP in AR42J cells treated with caerulein (100 nM) and cirsilineol (1.25, 2.5 and 5 μM). The results are presented as mean ± SD from three independent experiments. $^{***}p < 0.001$. PARP, Poly (ADP-ribose) polymerase; AP, Acute pancreatitis; Cir, Cirsilineol; PI, Propidium; FITC, Fluorescein Isothiocyanate.

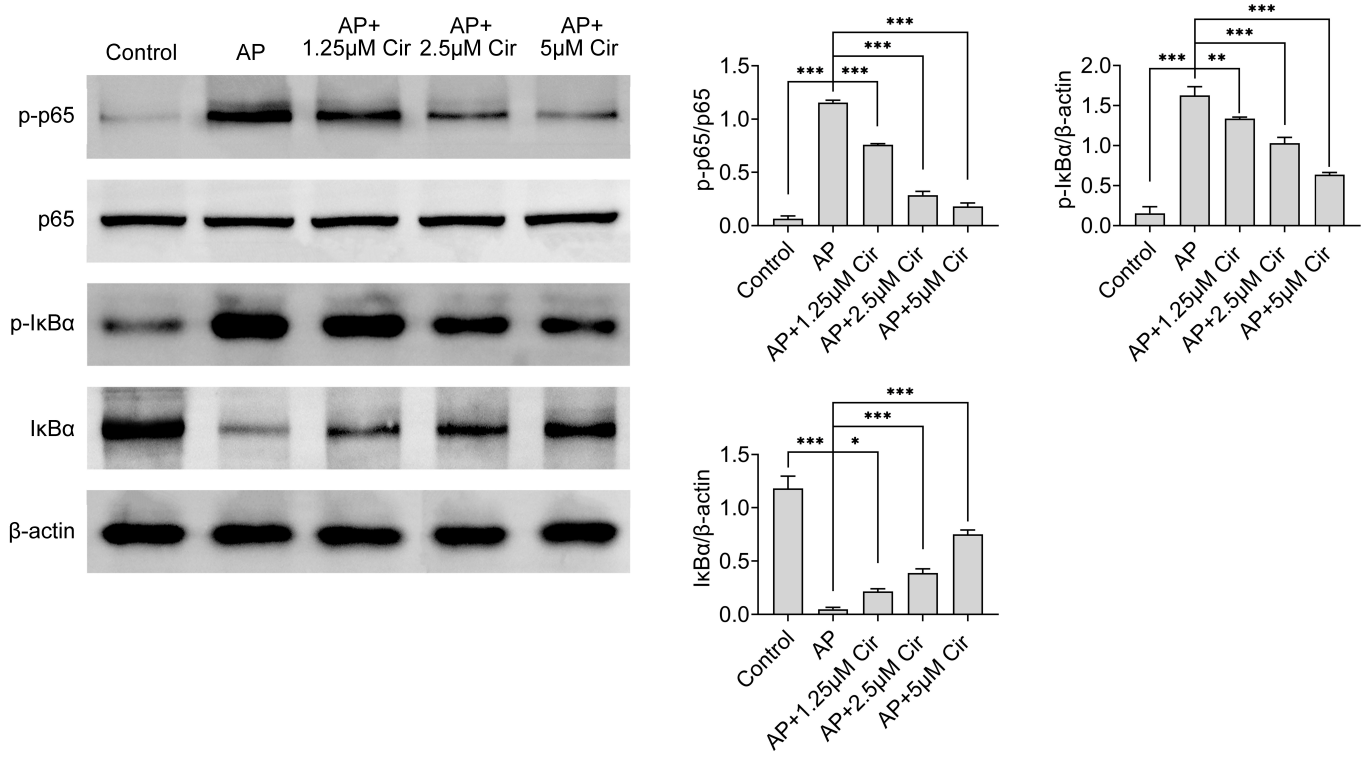

FIGURE 4. Cirsilineol inhibits NF-κB pathway in AR42J cells. AR42J cells treated with caerulein (100 nM) and cirsilineol (1.25, 2.5 and 5 μM) were analyzed for NF-κB signaling pathway activation using Immunoblot. The expression and phosphorylation levels of p65 (p-p65) and IκBα (p-IκBα) were measured. The results are expressed as mean $\pm$ SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NF-κB, Nuclear Factor-kappa B; IκBα, Inhibitor of Nuclear Factor kappa-B kinase subunit alpha; AP, Acute pancreatitis; Cir, Cirsilineol.

4. Discussion

AP is featured by digestive enzyme premature activation in pancreas, leading to inflammation as well as extensive tissue damage [13]. This disease progression is characterized by a sophisticated interplay of mechanisms, such as the disruption of calcium signaling, initiation of trypsinogen activation, and mobilization of inflammatory cells [14, 15]. Despite significant advancements in understanding the pathogenesis of AP, effective therapeutic interventions remain limited. At present, the primary approach to treatment is mainly supportive, emphasizing the management of pain, resuscitation with fluids, and providing nutritional assistance. There is currently a lack of specific pharmaceutical agents that can directly alleviate the inflammatory and apoptotic mechanisms contributing to the progression of the disease.

Inflammation as well as apoptosis is vital in the progression as well as severity of AP [10, 16]. The primary cause of inflammation in acute pancreatitis is the secretion of pro-inflammatory cytokines, leading to increased damage to the pancreatic tissue and the onset of systemic complications [10]. Apoptosis, also known as programmed cell death, exacerbates the progression of acute pancreatitis by removing injured acinar cells, thus intensifying the inflammatory response [10]. Our study demonstrated that CSL reduced these pro-inflammatory cytokine levels. Additionally, CSL markedly decreased apoptosis markers such as cleaved-caspase3 and cleaved-PARP, suggesting a dual role in modulating both inflammation and cell death.

We selected tumorigenic rat exocrine pancreas (AR42J) cells over primary isolated murine acinar cells for their consistency and reproducibility in *in vitro* studies. It is more convenient to culture and sustain AR42J cells compared to primary acinar cells, which present challenges in isolation, have a limited lifespan, and demonstrate notable variability. Additionally, AR42J cells are a well-established model in pancreatitis research, facilitating controlled investigations of the molecular mechanisms underlying CSL's effects on inflammation and apoptosis in acute pancreatitis.

CSL, a flavonoid compound extracted from Artemisia plants, is renowned for its various biological functions, such as its anti-inflammatory properties [9]. Previous studies have shown that CSL can attenuate lung injury induced by particulate matter, reduce oxidative stress, and modulate inflammatory cytokine levels [12]. In studies on allergic rhinitis models, CSL has been shown to reduce pro-inflammatory markers and oxidative stress, demonstrating its potential as a therapeutic option for a range of inflammatory conditions [5]. Our findings extend these observations to AP, demonstrating that CSL can effectively improve cell viability and reduce inflammation and apoptosis in pancreatic acinar cells.

The potential mechanisms by which CSL exerts its anti-inflammatory and anti-apoptotic effects may involve the inhibition of critical signaling pathways implicated in these biological processes [8, 12]. Our study showed that CSL signif-

icantly inhibited the NF-κB pathway, evidenced by reduced phosphorylation of p65 and IκBα. This pathway is vital for the transcriptional regulation of pro-inflammatory cytokines and apoptosis-related genes. Through the inhibition of NF-κB, CSL not only decreases the inflammatory reaction but also hinders the programmed cell death of pancreatic acinar cells. These findings align with prior research on CSL's anti-inflammatory properties in various disease models, indicating a shared fundamental mode of operation.

Besides evaluating apoptosis, we also quantified necrosis by examining PI staining, which is an indicator of cellular membrane permeability. Our data demonstrated that CSL treatment not only reduced the percentage of early and late apoptotic cells (Annexin V-positive) but also decreased the number of necrotic cells (PI-positive). In comparison to the control group, the groups treated with CSL showed a notable reduction in the percentage of necrotic cells, suggesting a protective impact on necrosis.

The NF-κB is a central mediator of inflammation and apoptosis in various diseases, including AP [17]. Activation of NF-κB results in the synthesis of pro-inflammatory cytokines and adhesion molecules, working together to stimulate the inflammatory response [18, 19]. In the setting of AP, the prompt activation of NF-κB has been linked to elevated levels of inflammation and damage to the tissue [20]. Our study demonstrated that CSL effectively inhibits NF-κB activation, thereby reducing both inflammation and apoptosis in pancreatic acinar cells.

Although this study did not utilize an *in vivo* model of AP, assessing pancreatic enzyme activity is essential to elucidate the effects of CSL on pancreatic exocrine function. In upcoming research, we plan to assess the levels of amylase and lipase activity in the AR42J model of cerulein-induced AP in order to confirm and strengthen the outcomes of our study. Previous studies have demonstrated methodologies for such assessments, and we plan to incorporate these approaches to provide a more detailed analysis of CSL's therapeutic potential. Further investigation is required to fully understand how CSL inhibits NF-κB activation. Subsequent research endeavors should prioritize delving deeper into these mechanisms and assessing the potential therapeutic benefits of CSL in real-world medical scenarios. Additionally, investigating the potential side effects and optimal dosing of CSL will be crucial for its development as a therapeutic agent.

5. Conclusions

In conclusion, our study reveals that CSL exhibits strong anti-inflammatory and anti-apoptotic properties in an *in vitro* model of acute pancreatitis by inhibiting the NF-κB pathway. These results emphasize the promising prospect of CSL as a new therapeutic option for treating AP.

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

WQZ—designed the study and carried them out. WQZ, YT, YMZ and BNL—prepared the manuscript for publication and reviewed the draft of the manuscript; supervised the data collection. WQZ, YT, YMZ—analyzed the data. WQZ, YT—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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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