Biomedicine & Pharmacotherapy 125 (2020) 110024

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Chaiqin chengqi decoction alleviates severe acute pancreatitis associated acute kidney injury by inhibiting endoplasmic reticulum stress and subsequent apoptosis



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ARTICLE INFO

*Keywords:*
Liquid chromatography
Chaiqin chengqi decoction
Severe acute pancreatitis
Kidney injury
Endoplasmic reticulum stress
Apoptosis

ABSTRACT

*Background:* Acute kidney injury (AKI) [illegible]cterized by a[illegible]ease of serum creatinine and urea, is a severe complication of severe acute pancre[illegible](SAP) with high mort[illegible]y. Endoplasmic reticulum (ER) stress has been considered as a key pathologic p[illegible]ess in AKI. Chaiqin chengqi decoction (CQCQD) is an effective Chinese medicine formula for SAP treatme[illegible]n China and [illegible]een used for many years. Our goal is to explore the role of CQCQD on ER stress of AKI in ex[illegible]ental SAP.

*Materials & methods:* SAP was induc[illegible]rats by [illegible]grade duct injection of 5% sodium taurocholate (NaTC, 1 ml/kg), sham operati[illegible](SO) rats simu[illegible]ceived saline infusion. Intraperitoneal injection of 4-PBA (50 mg/kg, once a day [illegible]s before the surgery) or intragastric gavage of CQCQD (1 g/kg, 2 hourly × 3 after disease induct[illegible]was [illegible]eat SAP rats. All animals were humanely sacrificed 12 h after disease induction. Histopathol[illegible]scor[illegible]k[illegible]y and pancreas; serum biochemical indices and kidney protein levels of ER stress [illegible]apoptosis [illegible]were assessed. Tubular epithelial cell line (HK-2) was treated either with TNF-α (10 n[illegible]6 (10 n[illegible]) for 12 h plus either 4-PBA (0.1 M) or CQCQD (1 mg/ml) for in vitro study. Cell vi[illegible]y and [illegible]kers of E[illegible]ess and apoptosis were measured.

*Results:* Duct[illegible]erfusion o[illegible]aTC caused significant increases in serum lipase, amylase and pancreatic histopa[illegible]gy[illegible]cell infiltration, interstitial edema, and acinar cell necrosis). Kidney histopathology (tubu[illegible]ation, brush border loss, little tubular necrosis, and cast formation), serum creatine and urea levels were ra[illegible]hen compared with the SO group. Moreover, apoptotic cell death markers (caspase-9, cleaved-caspase-3, a[illegible]UNEL) and kidney ER stress proteins (BIP, IRE1-α, XBP1s, and CHOP) were elevated after NaTC administration. 4-PBA and CQCQD significantly alleviated histopathological changes of kidney and pancreas, [illegible]ammatory cytokines, biochemical markers of AKI, ER stress proteins and apoptotic cell death markers. They a[illegible]rotected HK-2 cells from injury of TNF-α and IL-6, and alleviated both ER stress and apoptosis proteins in vitr[illegible]

*Conclusion:* CQCQD may alleviate SAP-related AKI by inhibiting ER stress-related apoptosis.

## 1. Introd[illegible]on

Acute p[illegible]tis (AP), [illegible]e of the most common gastrointestinal diseases, leads [illegible]mendous physical, emotional and financial burdens [1,2]. The incide[illegible]of AP is rising, with mortality currently at approximately 2%; and [illegible]rgan failure plays a crucial role in the outcome of AP [3–5]. According to the 2013 Atlanta Classification, the presence and duration of organ failure (renal, respiratory, and cardiovascular) are used to classify AP into three categories: mild, moderate, and severe [6]. Among the three organ systems, acute kidney injury (AKI) is characterized by a decline of renal function, resulting in the accumulation of metabolic waste and toxins, and all these performances are closely related to the outcome of severe acute pancreatitis (SAP), [7–9]. The severity and duration of AKI determine outcomes of SAP, once AKI

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https://doi.org/10.1016/j.biopha.2020.110024
Received 6 January 2020; Received in revised form 11 February 2020; Accepted 13 February 2020

occurs in patients with SAP, the mortality rate rises to 75 % [10]. The prevalence rate of AKI has varied among studies [11,12]; in general, the AKI cases in SAP increased from 4.1%–11.7% from 2003 to 2012 and were accompanied by higher mortality and a longer stay in hospitals [8]. Although increasing evidence has confirmed that AKI caused by SAP is a result of systemic inflammatory response syndrome (SIRS), the mechanism by which AKI occurs has not been elucidated. There is still no drug protocol recommendation other than renal replacement therapy (RRT) for AKI in SAP patients [13]. It is therefore urgent to find effective methods to ameliorate this disease.

Available studies revealed that SAP-induced AKI was associated with initial volume depletion due to increased vascular permeability, which was followed by complex interactions between vascular, inflammatory and humor mediators [9]. Renal tubular epithelial cells (TECs) accounts for 90 % of kidney volume and is the major cell type in kidney parenchyma [14]. Apoptosis of TECs plays a crucial role in various models of AKI [15–17]. Under normal conditions, endoplasmic reticulum (ER) stress activates the adaptive signaling pathway, the unfolded protein response (UPR), restores protein folding and processing and then re-establishes normal ER function. ER stress-mediated apoptosis has been considered as a key pathologic process leading to TECs injury and loss in AKI [18]. Furthermore, 4-phenylbutyric acid (4-PBA), which is an ER stress inhibitor, protected organ damage, including kidneys, in SAP rats [19,20]. Nevertheless, there is no report focusing on the role of ER stress-mediated TECs apoptosis in AKI following SAP.

Some herbal medicine has been reported to cause kidney injury due to nephrotoxic materials, i.e., aristolochic acids [21]. However, recent attention has been paid to herbal medicine for ameliorating kidney injuries due to their antioxidant and anti-inflammatory effects [22–24]. Chaiqin chengqi decoction (CQCQD) is a well-known and widely used Chinese herb formula for SAP treatment in clinics. It plays a multi-target and comprehensive role in SAP. CQCQD can alleviate SAP by improving intestinal motility, attenuating respiratory dysfunction and protecting pancreatic acinar cell viability [25–27]. Previous studies demonstrated that CQCQD treatment shortened the duration of renal acute renal failure in severe AP patients [28]. Besides, herbal contents in CQCQD like baicalin [29], emodin [30], honokiol [31] were approved to have protective effects in the treatment of AKI. However, the active ingredients and pharmacological effects of CQCQD in the kidney are still unclear. In this study, we investigated the effect of CQCQD on SAP-induced AKI in rats and explored the underlying mechanisms by modulating ER stress to regulate apoptosis of TECs.

## 2. Materials and methods

### 2.1. Preparation of CQCQD

The herbs of CQCQD were provided by Chinese Herbs Pharmacy of West China Hospital of Sichuan University (Chengdu, China), including Chaihu (Radix Bupleuri Chinensis) 15 g, Huangqin (Radix Scutellariae Baicalensis) 15 g, Dahuang (Radix EtRhizoma Rhei Palmati) 20 g, Mangxiao (Nalrii Sulfas)20 g, Houpo (Cortex Magnoliae Officinalis) 15 g, Zhishi (Fructus Aurantii Immaturus) 15 g, Yinchen (Herba Artemisia Capillaris) 15 g, Zhizi (Fructus Gardeniae) 20 g. These herbs were first made into a decoction, the decoction was then concentrated to 300 ml. 200 ml decoction was lyophilized into powder and then stored at -80℃ (yield: 28.2 %). Before administration to animals, the lyophilized powder of CQCQD was dissolved in drinking water with a concentration of 2 g/ml of crude herbs; The rest of 100 ml CQCQD decoction was used to extract CQCQD extraction for in vitro study by adding the same volume of chloroform. After obvious stratification, the aqueous layer (AE) was collected and concentrated into a powder using an EYELA FDU-2110 lyophiliser (Tokyo, Japan). The yields of AE were subsequently calculated.

### 2.2. Analysis of High-Performance Liquid Chromatography (HPLC)

To find out the major ingredients of CQCQD, Waters 2695 system (Waters, Milford, Massachusetts, USA) which is equipped with a photodiode array detector, a quaternary gradient pump, and an autosampler, was used to perform fingerprint spectrum for CQCQD. The components were eluted with a gradient system that contains 0.2 % formic acid (mobile phase A) and LC–MS grade methanol (mobile phase B; time, min/B%: 0/98, 5/95, 10/85, 25/80, 35/70, 40/70, 50/60, 65/50,80/25,95/5 and 100/5). The chromatographic column used was Thermo Hypersll GOLD (100 × 4.6 mm). The column temperature was maintained at 30 ˚C and the mobile phase flow rate was 1 mL/min. 0.4 g CQCQD powder was ultrasonically extracted for 30 min with 5 ml methanol and filtered through a 0.22 μm filter before analysis. The 6 standards (Geniposide, Honokiol, Magnolol, Baicalin, Emodin, and Naringin; Pus biotech, China) were dissolved in methanol to prepare a standard solution.

### 2.3. Experimental groups

Sprague-Dawley rats (male, 8 week, 220 − 250 g) were purchased from the Experimental Animal Center of Sichuan University, fed with standard rat chow and sterile water ad libitum and maintained at 22 ± 2 ˚C for 1 week for acclimation. The study was approved by the Ethics Committee for Animal Experiments of Sichuan University and following the National Institutes of Health Guide for the Care. After fasting for 12 h before the experiments, the SAP model was induced by biliary-pancreatic duct injection of 5% sodium taurocholate (Sigma, USA) (1 ml/kg) using a micropump, while sham operation (SO) rats received the same volume of saline infusion [32]. 24 rats were randomly divided into SO group, SAP group, SAP + 4-PBA group (4-PBA group) and SAP + CQCQD group (CQCQD group). In the CQCQD treatment group, after SAP induction rats received intragastric gavage of CQCQD (1 g/kg, 2 hourly × 3), while before SAP induction, intraperitoneal injection of 4-PBA (50 mg/kg) was conducted once a day for three days in 4-PBA group. SO group rats were administered an equal volume of saline instead of CQCQD or 4-PBA. 12 h after the surgery, rats were reanesthetized, and samples were obtained for subsequent analysis.

### 2.4. Sample collection

Rats were sacrificed, and blood, pancreas, and kidney were collected. Blood samples were collected by intracardiac puncture and then centrifuged at 1200 *g* for 15 min. The serum was collected and stored at −80 ˚C until assayed. Pancreas and kidney tissues were fixed for 24 h with 10 % formaldehyde solution for H&E staining. Additional kidney tissues were stored at −80 ˚C for quantitative polymerase chain reaction (qPCR) analysis and western blot analysis.

### 2.5. Cell culture and drug treatment in vitro

The human renal proximal tubular epithelial cell line (HK-2) was obtained from ATCC (USA). The cells were cultured in DMEM medium (Gibco, USA) supplemented with 10 % foetal bovine serum (BI, Australia) and were maintained at 5% $CO_2$ and 37 ˚C conditions. When the cells reached 70–80 % confluence, they were treated either with TNF-α (10 ng/ml) or IL-6 (10 ng/ml) for 12 h, and they were additionally treated with either 4-PBA (0.1 M) or CQCQD (1 mg/ml) [19].

### 2.6. Cell viability measurement

Cells were washed with sterile PBS twice and then stained with Hoechst 33342(10 ng/mL) and PI (0.625 μg/mL) at 37 ˚C in the dark for 5 min. Cell images were captured using a fluorescence microscope (Tokyo, Japan) and analyzed using ImageJ software. Cell death

percentage was quantified as the percentage of PI-positive cells per 10 fields (PI-positive cells as a percentage of total Hoechst-positive cells).

### 2.7. Histological examination

Pancreas and left kidney were fixed in 4% phosphate-buffered formaldehyde and continuously sliced at 5-mm thick. The slices were then stained with eosin and hematoxylin, and assessed by 2 pathologists. The pancreatic histological assessment was performed basing on inflammation, edema and necrosis under a 200× microscope (Table 2) [33]. Tubular dilation, brush border, tubular necrosis and cast formation are presented as percentage area in ten 200 × kidney pics randomly picked in the whole corticomedullary junction of every slide (Table 3) [34].

### 2.8. Determination of serum amylase, lipase, urea, creatine, IL-6, and TNF-α levels

Serum lipase and amylase were measured using an automatic biochemical analyzer (Nanjingjiancheng, China). Concentrations of creatine and urea were measured using standard techniques with a fully automatic chemistry analyzer. Serum IL-6 and TNF-α levels were measured using ELISA kits (R&D Systems, USA).

### 2.9. Western blotting

Total protein from kidney tissues or HK-2 cells was extracted by RIPA (Abcam, UK) supplemented with 10 μg/mL PMSF and protease inhibitor cocktail (Roche, Germany). Total protein concentration was quantified using a BCA kit (Thermo, USA). The same amounts of p were separated by 10 % or 12 % SDS-PAGE and then transferred a polyvinylidene fluoride membrane. Membranes were blocked with skim milk for 2 h and then incubated with the following ibodies a ˚C overnight: XBP1s (1:1000, CST, USA), BIP (1:100 ); CHO (1:1000, CST, USA); cleaved-caspase-3 (Cle-ca e-3, 1 00, CST, USA); caspase-9 (1:1000, CST, USA); and in (1 BTT China). After rinsing with TBST (10 min 3), branes e incubated with the corresponding secon y antibod r 1 – 2 hours at room temperature. Finally, after , membran re imaged by a detection system (Bio-Rad, ) a nalyzed wit mage lab software.

### 2.10. Quantitative reverse transcription polymerase chain reaction (qPCR)

Total RNA was extra th Trizo ervicebio, China) and reversely transcri gene DNA g a Revert Aid First Strand cDNA Synth kit rimenta a). qPCR was performed using the FastS Univer SYBR Gree Master kit (Roche Diagnostics) in a CFX Conn Re tection System (Bio-Rad Laboratories) with the fo g program: pre-denaturation at 95 ˚C for 10 min, followed by 40 s of denaturation at 95 ˚C for 15 s, annealing at 53 ˚C (BIP, CHOP, A 51 ˚C (Caspase-3), 55 ˚C (XBP1s) or 48 ˚C (Caspase-9) for 1 min and extension for 1 min. The primer sequences are listed in Table 1.

**Table 2**
Pancreas histological examination.

| Condition | Score | Indication |
|---|---|---|
| Edema | 0 | Absent |
| | 1 | Focally increased between lobules |
| | 2 | Diffusely increased between loblues |
| | 3 | Acini disrupted |
| | 4 | Acini separated |
| Inflammatory infiltrate | 0 | Absent |
| | 1 | In ducts (around ductal margins) |
| | 2 | In the parenchyma (in < 20% of the lobules) |
| | 3 | In the parenchyma (in 20%-50% of the lobules) |
| | 4 | In the parench 50% of the lobules) |
| Acinar necrosis | 0 | Absent |
| | 1 | Periduct crosis (< 5% |
| | 2 | Focal p ymal necrosis 20%) |
| | 3 | Diffuse pa mal necrosi %-50%) |
| | 4 | se paren l necro 50%) |

**Table 3**
Kidney histological e natio

| Score | | 1 | | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| tubular nec | | 0–10 % | 11– % | 26–45 % | 46–75 % | 76–100 % |
| Tubular dilatation | nor | 10 % | 11–25 % | 26–45 % | 46–75 % | 76–100 % |
| loss | none | % | 11–25 % | 26–45 % | 46–75 % | 76–100 % |
| formation | none | 0–10 % | 11–25 % | 26–45 % | 46–75 % | 76–100 % |

### 2.11. TUNEL staining and analysis

After fixed, rat kidney sections were deparaffinized and rehydrated. staining was performed by using a commercially available In tu Death Detection kit (Roche, Germany) according to the manufacturer's protocol. The number of TUNEL-positive nuclei was counted in ten random renal cortical fields (200×) on each slide

### 2.12. Statistical analysis

All statistical tests were performed using SPSS 20.0 (SPSS, USA). Data are presented as the mean ± standard error (SE) for continuous variables. Differences among multiple groups were compared by one-way analysis of variance or Kruskal-Wallis. $P < 0.05$ was considered a statistically significant difference.

## 3. Results

### 3.1. Identification of the major compounds in CQCQD with HPLC

To ensure the consistency and quality control of CQCQD manufacture, major compounds of CQCQD were identified with HPLC. As can be seen from Fig. 1A, B, the baseline of mixed CQCQD sample and standard solution were stable, and the chromatographic peaks of ingredients were separated. Comparing the retention time, UV and MS spectra with reference samples, 6 major components: geniposide, honokiol, magnolol, baicalin, emodin, naringin were identified in. The sum

**Table 1**
Probe, Primer, and Product (*bp*) for qPCR.

| Gene | Upstream primer | Downstream primer | Product (*bp*) |
|---|---|---|---|
| BIP | GAGGAGGACAAGAAGGAG | TGCGGTTGCCCTGATCGTT | 124 |
| XBP1s | CTCCCAGAGGTCTACCCAG | TGGCTTCCAGCTTGGCTGAT | 88 |
| Caspase-9 | ATTGTGAACATCTTCAATGG | CTCAAAGCCATGGTCTTTCT | 104 |
| Caspase-3 | TTCTTCAGAGGCGACTACT | TCCCACTGTCTGTCTCAAT | 109 |
| β-actin | GAAGATCAAGATCATTGCTCCT | TACTCCTGCTTGCTGATCCACA | 150 |
| CHOP | CTCCAGATTCCAGTCAGAG | CCTGCTCCTTCTCCTTCAT | 132 |

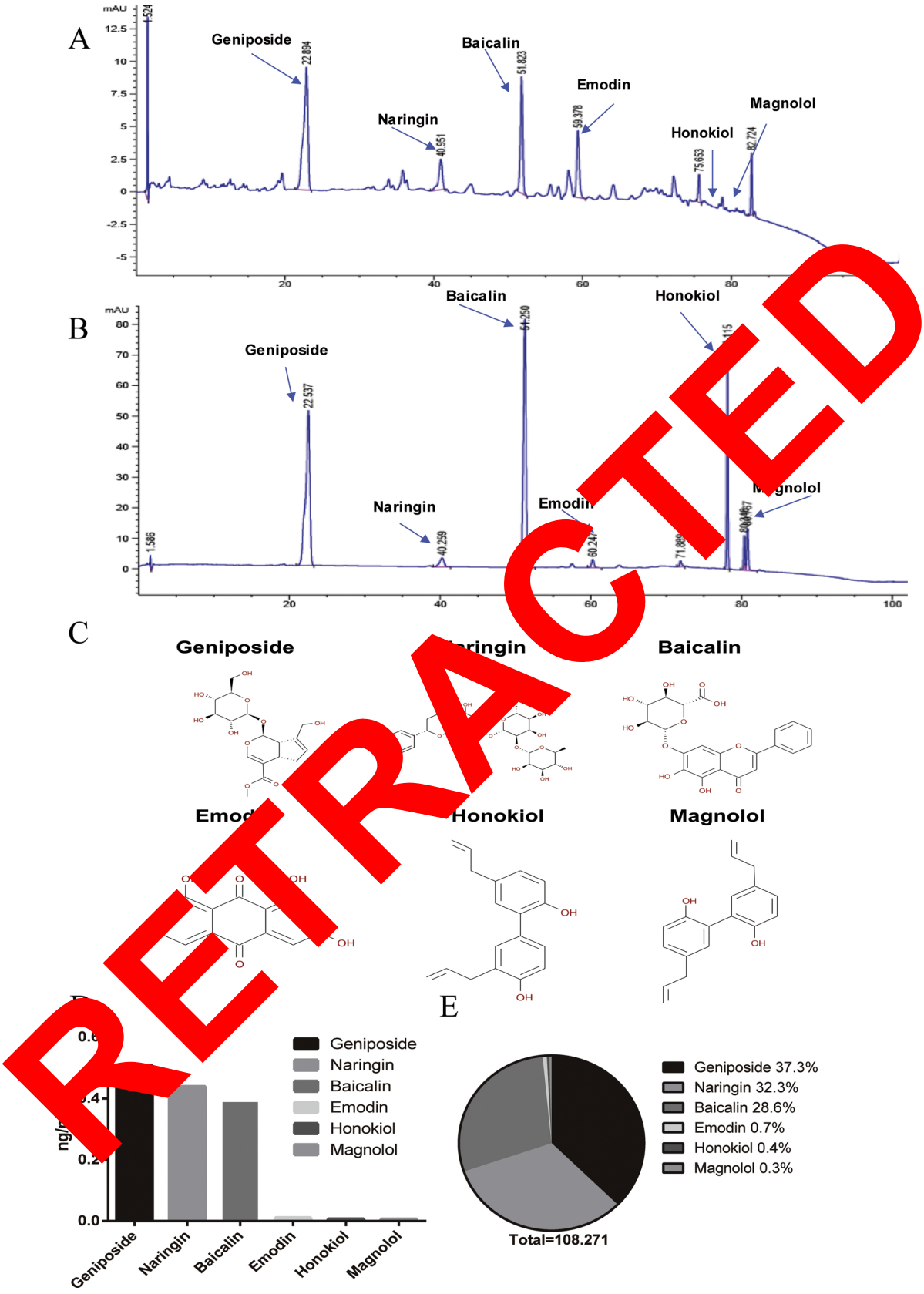




**Fig. 1.** High-Performance Liquid Chromatography (HPLC) chromatogram of 6 major ingredients in CQCQD.
A, CQCQD sample; B, Mixed standards; C Chemical structure of Geniposide, Honokiol, Magnolol, Baicalin, Emodin, Naringin; D. The content of 6 major ingredients in CQCQD; E. The percentage of each active ingredient in the total 6 major ingredients.

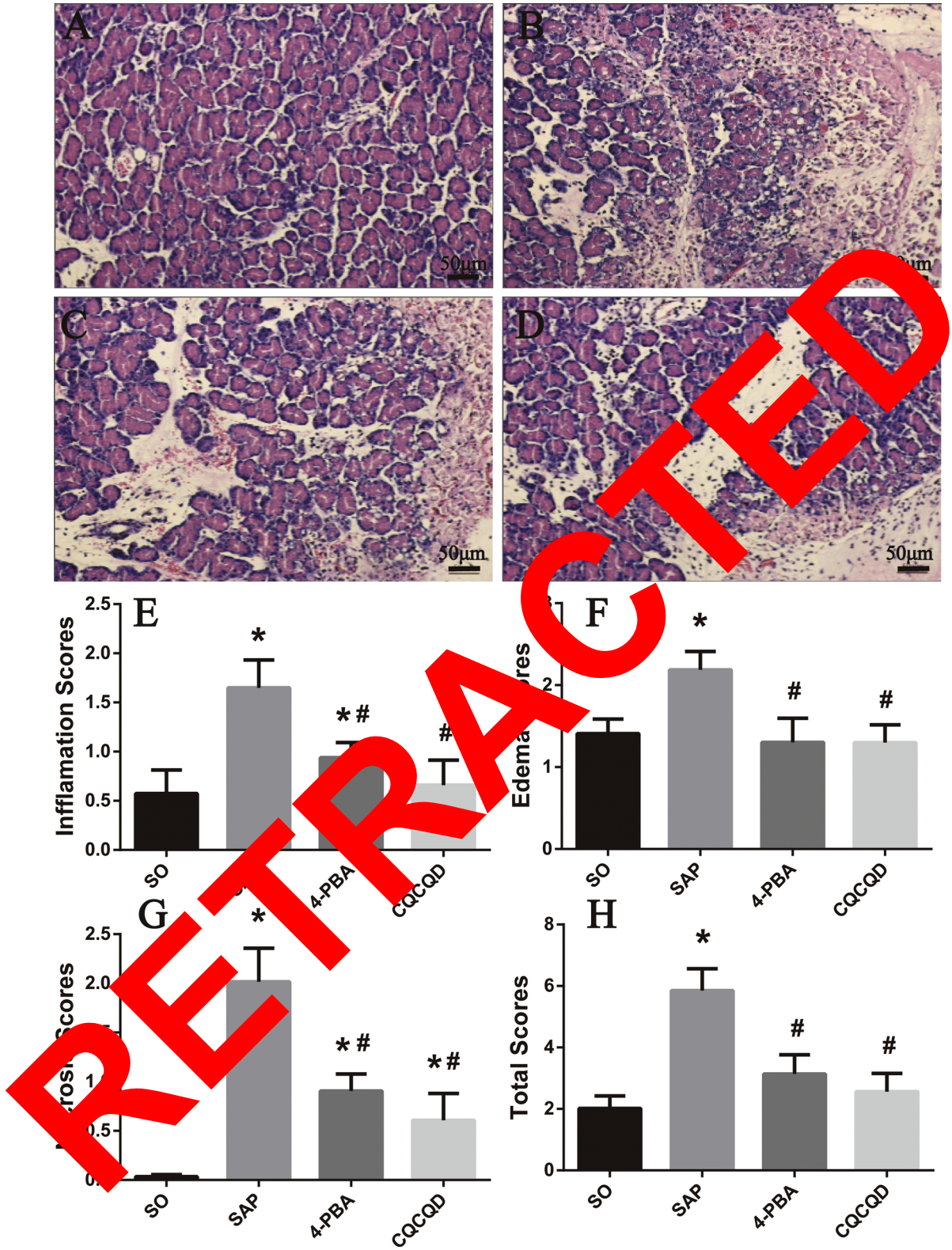




**Fig. 2.** Pancreatic histopathological changes and severity scores in different groups.
A, SO group; B, SAP group; C, 4-PBA group; D, CQCQD group (H&E stain, x 200); E, Inflammation scores; F, Edema scores; G, Necrosis scores; H, Total scores. *P < 0.05 vs. SO group, # P < 0.05 vs. SAP group (n = 6).

of naringin, geniposide, and baicalin in CQCQD accounted for 98.45 % of the total content of 6 major ingredients (Fig. 1C–E). However, they only make up 0.000135 % of the total CQCQD.

### 3.2. Morphological changes in the pancreas and kidney

Compared with the SAP group, the group treated with CQCQD and 4-PBA had significantly alleviated morphological changes of pancreas and kidney tissues (Figs. 2A–D, 3A–D), and the histopathologic scores

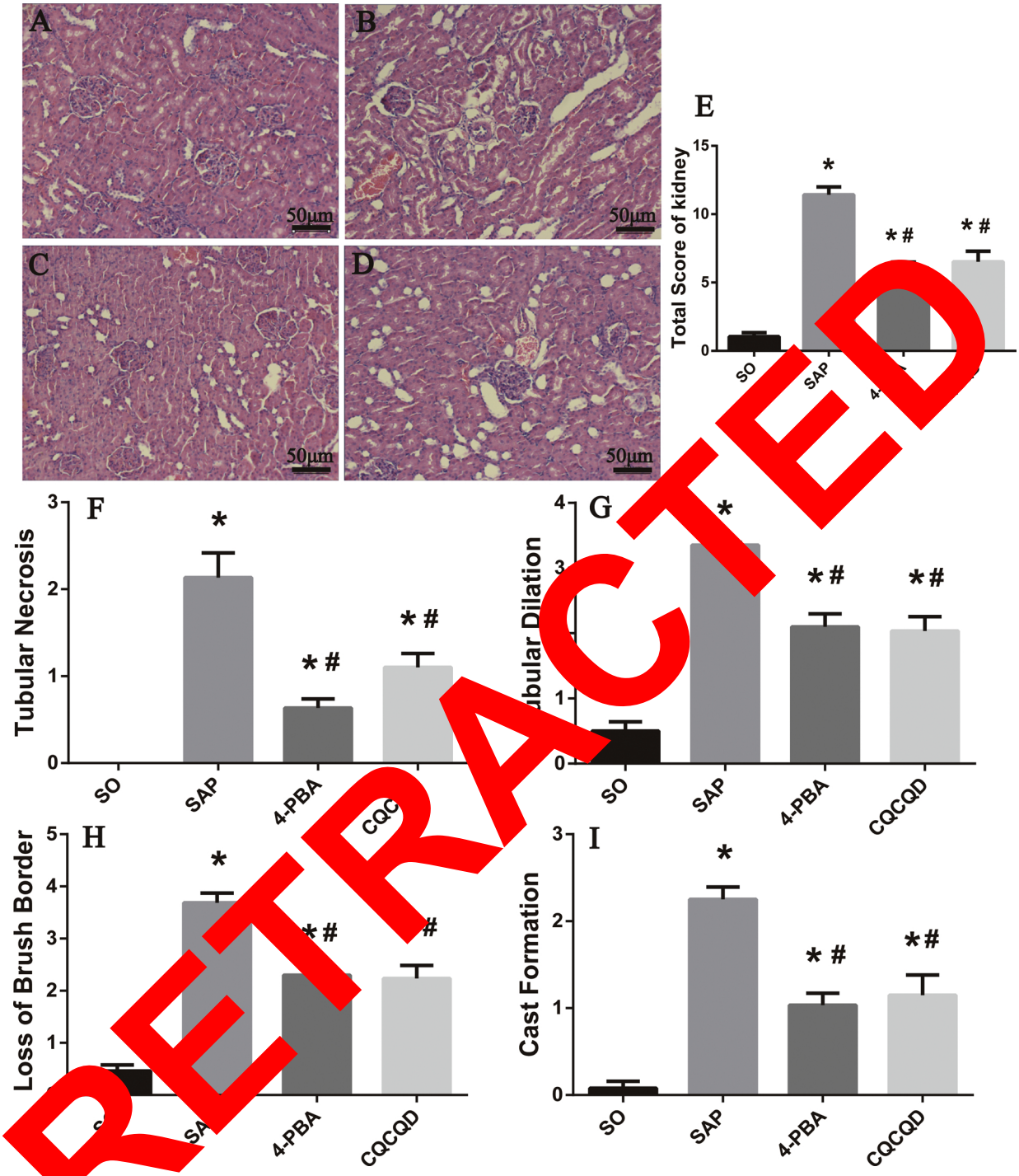




**Fig. 3.** Kidney h[illegible]thological changes and severity scores.
A, SO group; B, AP g[illegible]; C, 4-PBA group; D, CQCQD group (H&E stain, x 200); E. Total scores; F, Tubular necrosis scores; G, Tubular dilation scores; H, Loss of brush border; I, Cast formati[illegible] scores.
*P < 0.05 vs. SO group, # P < 0.05 vs. SAP group (n = 6)

declined significantly (all $P < 0.05$; Fig. 2E–H, 3E–I). There was no significant difference between the 4-PBA group and the CQCQD group.

### 3.3. The biochemical criterion in serum

Serum sample analysis revealed that there was a significant increase of amylase, lipase, IL-6, and TNF-α in the SAP group compared with the SO group (Fig. 4). With the treatment of 4-PBA and CQCQD, all of the concentrations were significantly reduced compared to the values of the SAP group (all $P < 0.05$), and no differences were observed between the 4-PBA and CQCQD groups. For creatinine and urea, 4-PBA treatment significantly decreased the increased concentrations observed in the SAP group. CQCQD treatment slightly decreased urea and creatinine in the SAP group, but these differences were not statistically significant.

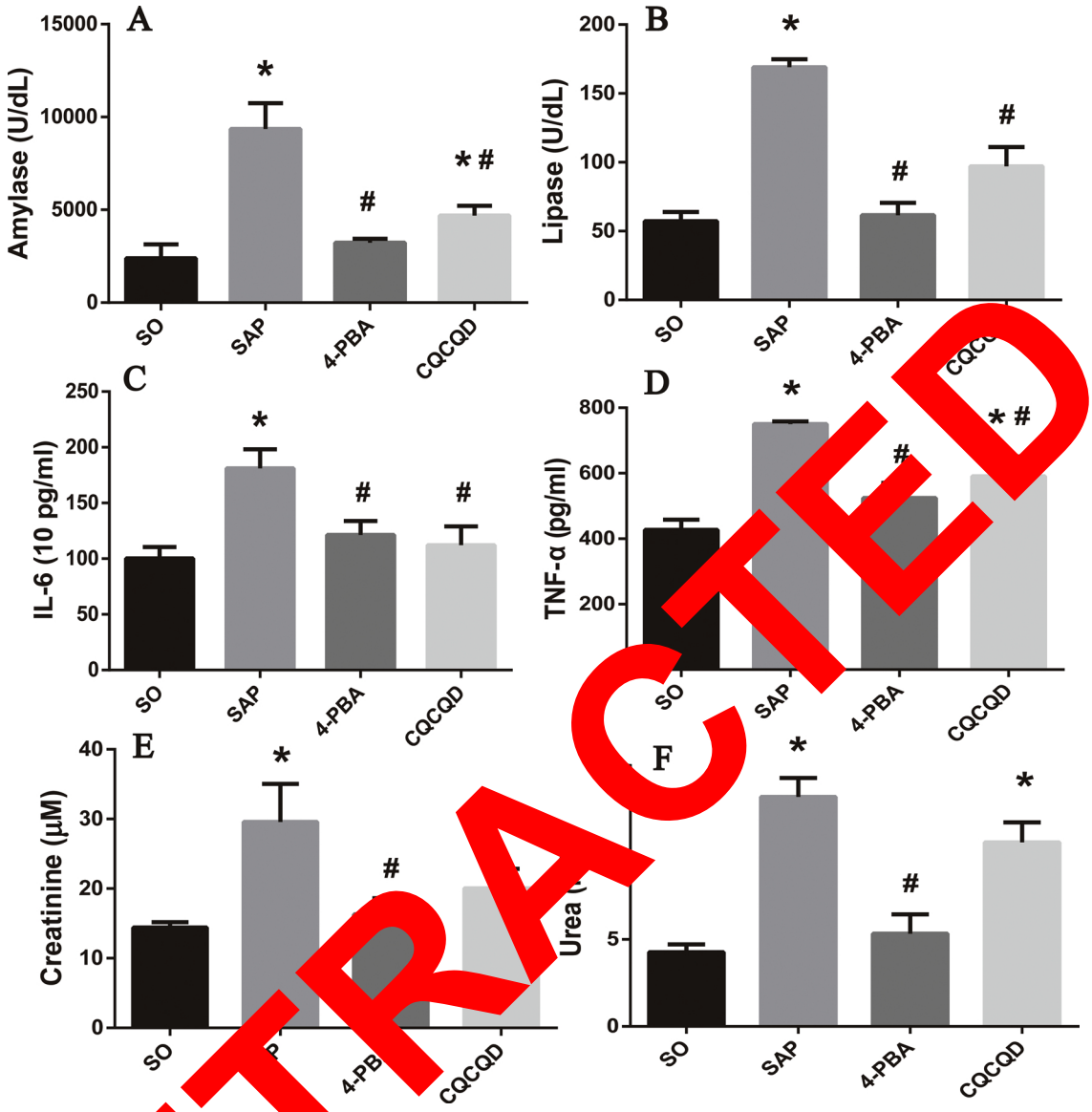




**Fig. 4.** Serum amylase, lipase, [illegible] TNF-α, creatini[illegible]d urea in different groups. *P < 0.05 vs. SO group. #P [illegible]05 vs. SAP group (n [illegible])

### 3.4. Apoptosis analysis o[illegible]ssues

As shown [illegible] compa[illegible]ats in the SO group, rats in the SAP grou[illegible]d high[illegible]TUNEL f[illegible]escence. The intensity of fluorescence w[illegible]duced [illegible]QCQD and 4-PBA treatment (Fig. 5A). We then counted th[illegible]e ina[illegible] of TUNEL-positive cells. The results showed that t[illegible]equency of TUNEL-positive cells in the SAP group was significantly [illegible]r than it was in the SO group ($P < 0.05$ Fig. 5B), while both 4-PBA a[illegible] CQCQD notably downregulated TUNEL-positive cells (both $P < 0.05$, Fig. 5B). No statistically significant difference was observed between the 4-PBA and CQCQD groups (both $P > 0.05$).

### 3.5. Expression levels of components of the ER-stress and apoptotic pathways in kidney

Considering that ER stress plays an important role in the development of AKI, components of the UPR were detected by western blotting and qPCR. As shown in Fig. 6A, BIP, CHOP and caspase-9 were higher in the SAP group than they were in the SO group, while treatments of 4-PBA and CQCQD markedly reduced their levels (all $P < 0.05$, Fig. 6A). Furthermore, XBP1s and cleaved-Caspase-3 proteins also showed higher expression levels in the SAP group (both $P < 0.05$), and these levels were alleviated in both the 4-PBA and CQCQD groups. However, only 4-PBA decreased XBP1s with a statistically significant difference ($P < 0.05$). Further analyses confirmed that the mRNA levels showed a similar tendency in their protein levels (Fig. 6B).

### 3.6. Protective effects of CQCQD on HK-2 cells

Cytokine secretion has been linked to the severity of AP. In this in vitro study, fluorescent staining of Hoechst 33342 and PI was measured to explore whether 4-PBA and CQCQD could protect HK-2 cells from the injury of IL-6 and TNF-α, two of known pro-inflammatory cytokines in SAP. As shown in Fig. 7A, B, PI was nearly undetectable in the control group, while IL-6 significantly increased the percentage of PI-positive HK-2 cells ($P < 0.05$). Both 4-PBA and CQCQD prevented HK-2 cells

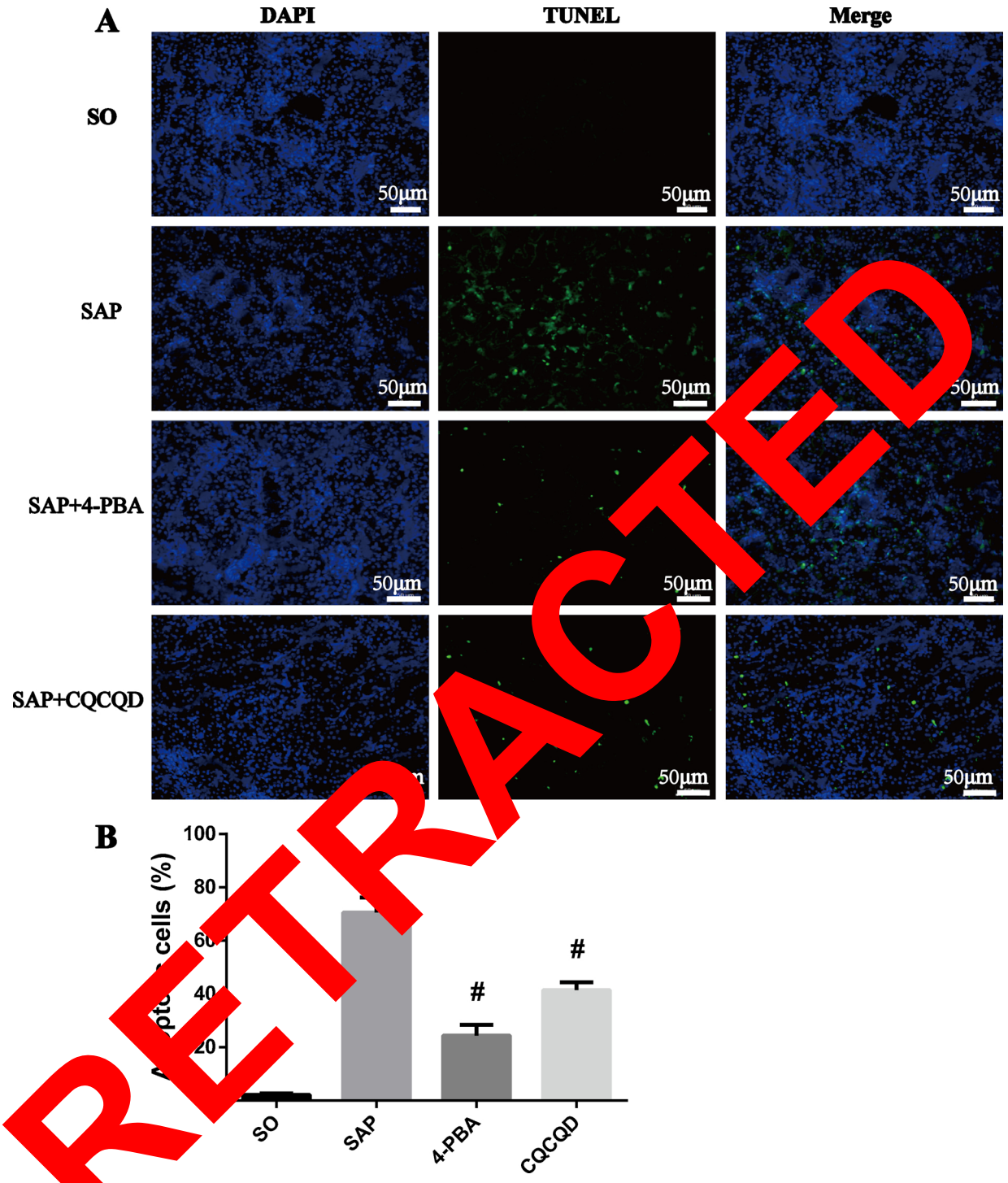




**Fig. 5.** Effect of 4-[illegible]nd CQCQD on apoptosis of kidney tissue.
A, Representative TU[illegible] photomicrographs of kidney in different groups (x 200); B The frequency of TUNEL-positive cells in different groups.
*P < 0.05 vs. SO group. #P < 0.05 vs. SAP group (n = 6)

from being damaged by IL-6 (both $P < 0.05$). There was no significant difference between the 4-PBA group and the CQCQD group. The similar protective effect of 4-PBA and CQCQD on TNF-α-induced HK-2 injury is shown in Fig. 7C, D.

### 3.7. CQCQD protects HK-2 cells by modulating ER stress

To explore the mechanism of CQCQD protection on the tubular injury, ER stress-related proteins were detected. As shown in Fig. 8, both IL-6 and TNF-α significantly increased the protein expression levels of XBP1s, BIP, CHOP, caspase-9, and Cle-caspase-3, and they decreased the expression of IRE1-α (all $P < 0.05$). Treatment with 4-PBA significantly reduced the increases in XBP1s, BIP, CHOP, caspase-9 and Cle-caspase-3 in HK-2 cells with IL-6 and TNF-α stimulation; under the same conditions, IRE1-α was upregulated (Fig. 8, all $P < 0.05$). Consistent with the 4-PBA results, CQCQD treatment showed similar effects on the expression of these proteins. However, a statistically significant difference was not obtained in all groups (Fig. 8).

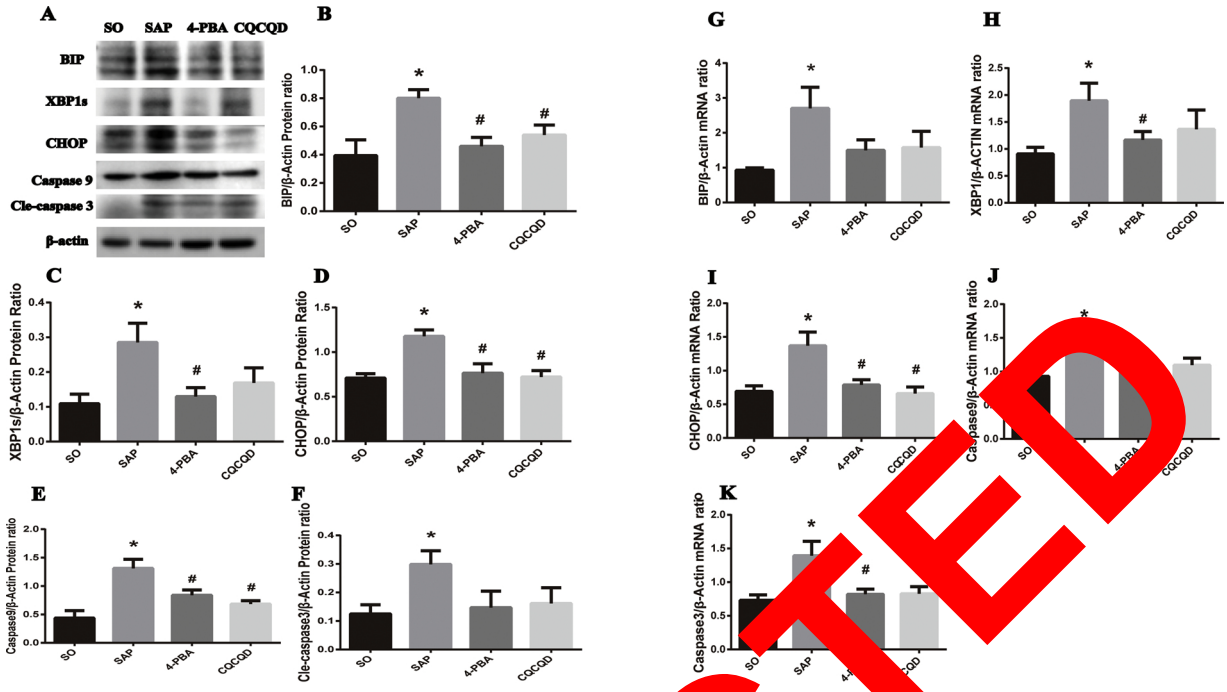


**Fig. 6.** Expression levels of ER stress proteins and apoptotic markers in kidney.
A-E, Protein levels of BIP, XBP1s, CHOP, Caspase9 and Cle-caspase3 in kidneys; F–J, mRNA levels of BIP, XBP1s, CHOP, Caspase9 and Caspase3 in kidneys
*P < 0.05 vs. SO group, #P < 0.05 vs. SAP group (n = 3).



## 4. Discussion

AKI is usually not a single disease, and it often occurs after s us diseases such as SAP [35]. In SAP, AKI usually occurs late in the cours f the disease but serves an important role in increasing the ality of SA with no effective way to cure [7,9]. Various risk factor e ted to b associated with AKI in SAP, including older age poxemi ale sex, abdominal compartment syndrome, sepsis, respi failu ary of kidney disease [8,36]. However, of these tors, be cha d. Clinically, due to the lack of kidney bio , AKI has divided into different stages (risk, injury, failure, nent) based KDIGO creatinine-based staging [35,37]. In thi udy, levels of seru creatinine and urea were chosen to assess e extent of injury. Serum creatinine and urea were increase the SAP group a significant pathologic injury of the pancr nd kid y (Figs. 2–4), which indicated the successful induction of relate d in rats.

The ER is the site ung pro synthesis, modification, folding and olig ization help p ins to perform their physiological funct ologica athological stimuli can disturb protein f g, res g in an cumulation of unfolded proteins, which ca ER st lleviate these conditions by UPR through three ER m -associa proteins (PERK, IRE1, and ATF6). They increase the f g capacity of the ER and decrease the number of proteins in the ER ssure protein quality control. However, when ER stress is severe or pr onged, the UPR switches from a pro-survival to a pro-apoptotic signal, committing the cell to death.

A close association has been proven between AP and ER stress. ER stress is an indispensable factor promoting the progression of AP [38], not only in pancreatic acinar injury [39–41] but also distant organ injury [19,42] Furthermore, some studies have treated pancreatitis via inhibiting the ER stress pathway to alleviate the inflammatory response [42–47]. 4-PBA, a fatty acid with ER chaperone properties, has been widely studied for its modulation of ER homeostasis [48–51]. In AP, Hong, et al. found that 4-PBA protected pancreas, liver, lung and kidney injury by regulating ER stress and reducing the inflammatory response to restrain cell death [19]. Therefore, we chose 4-PBA as a positive control to evaluate the effect of CQCQD on kidney injury following AP.

In th BA treatment significantly decreased the histopathological sev ity of the pancreatic and renal injuries, serum amylase, IL-6, TNF-α, creatinine, and urea levels and kidney apoptosis, h ere consistent with Hong et al's results [19]. According to our results, it further reduced the protein expression levels of BIP, XBP1s, CHOP, caspase-9, and Cle-caspase-3 in the kidney (Figs. 6 and 8). These results once again demonstrated that the inhibition of excessive ER stress could be a treatment for vital organ injury in SAP.

Injury and death of tubular cells are particularly well-known as precipitating factors in AKI [18]. Apoptosis is activated parallel to adaptive responses, and cell fate is determined by the net effect, depending on the intensity and duration of the stress [52]. In our present study, we noticed that high intensity of TUNEL fluorescence and overexpression of caspase-9 and Cleaved-caspase-3 in the kidney induced by SAP could be suppressed by 4-PBA effectively (Fig. 5); thus proving that ER stress inhibition alleviated apoptosis of kidney following SAP.

CQCQD, which is modified from Dachengqi decoction, has been applied to treat AP effectively for decades in China. Our previous study found that CQCQD downregulated IRE1-α in alveolar macrophages from dogs with SAP [53]. In this study, the effects of CQCQD treatment on pancreatic damage, serum amylase, lipase, IL-6, and TNF-α levels were the same as what we observed in our previous study [53]. Moreover, CQCQD suppressed the ER stress indicators (BIP, XBP1s, and CHOP) and caspases (caspase-9 and Cle-caspase-3) in the kidney, mitigated the pathophysiological alterations and apoptosis of the kidney, and thus restored kidney function (as shown by creatinine and urea reduction). Although not all of the aforementioned parameters were significantly alleviated in the CQCQD group, the tendencies of these results were consistent between the 4-PBA and CQCQD groups, indicating that CQCQD might attenuate SAP-related AKI partly through ER stress inhibition and cell death (apoptosis) reduction. 4-PBA was administered before the SAP model introduction, which may partly account for its better curative effect.

TCM compounds exhibit various biological activities due to diverse drugs present with multiple cellular targets. According to our unpublished research, 6 major ingredients of CQCQD were identified; geniposide, honokiol, magnolol, baicalin, emodin and naringin, which were used for

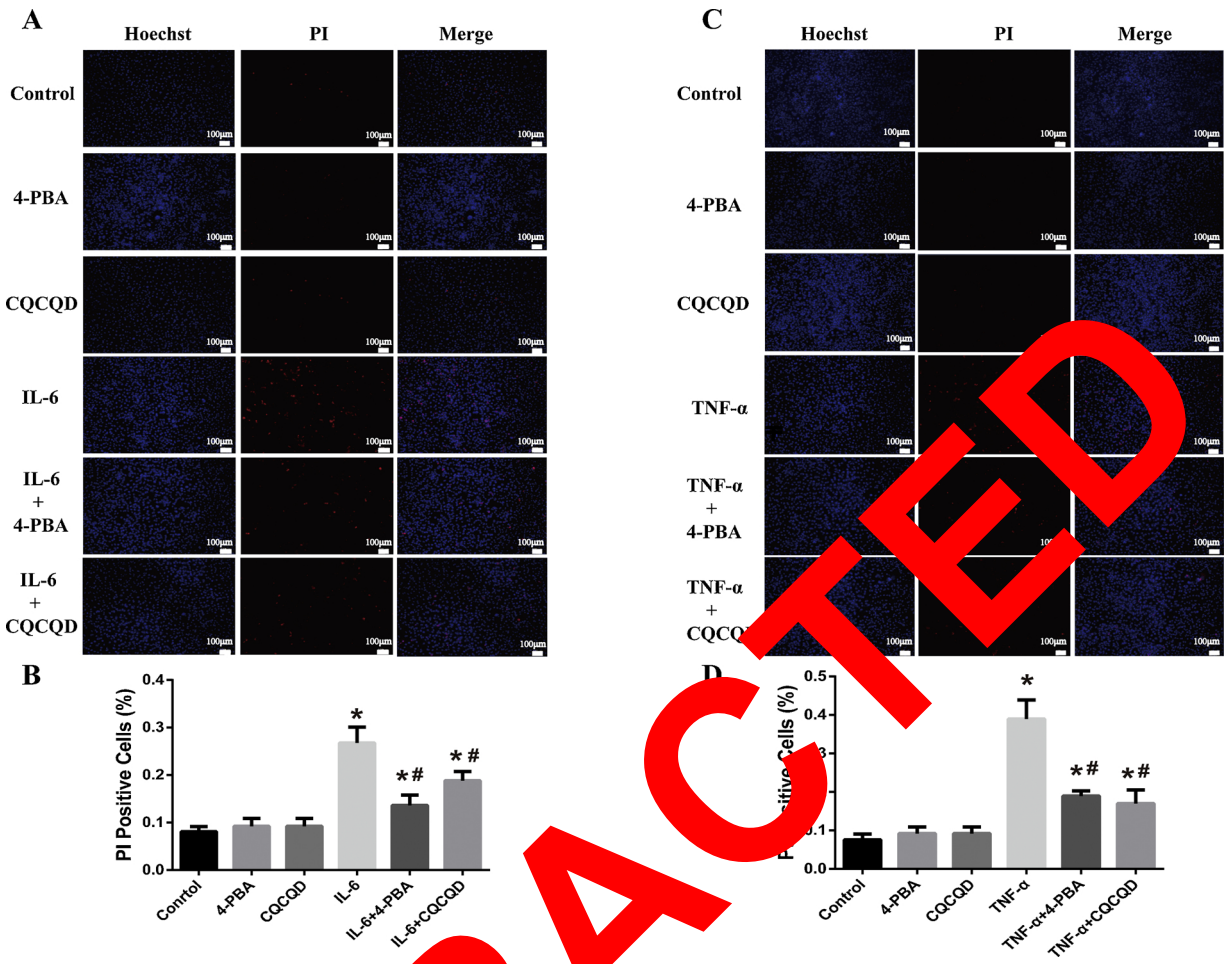


**Fig. 7.** PI-positive HK-2 Cells stimulated with IL-6 and TNF-α.
A, Representative photomicrographs of PI intake in HK-2 cells (x 100); B, Percentage of PI-positive cells stimulated with IL-6 in different groups; C, Representative photomicrographs of PI intake in HK-2 (x 100) ; D, PI-positive cells stimulated with TNF-α in different groups.
*P < 0.05 vs. Control group, #P < 0.05 vs. IL-6 group (



quality control of CQCQD manufacture. In this study, these ingredients can also be detected in CQCQD extract with HPLC. They have been proved to possess various functions, such as anti-inflammation, anti-infection, antitumor, and antioxidant activities [22,45,47,54]. Although no exact evidence has been provided to indicate they are active ingredients of CQCQD in AKI treatment, there are close relationships reported in previous researches. Geniposide inhibits palmitate-induced ER stress, reducing hepatic lipid accumulation in hepatocytes [55]. Naringin inhibits vascular endothelial cell apoptosis [56] and prevented colitis and colorectal carcinogenesis [57] by blocking the ER stress-mediated pathways. Baicalin pretreatment protects cells from tunicamycin [58] or chondrocytes from IL-2 [59] through ER stress inhibition. Emodin inhibited ER stress, restrained inflammatory reactions and thus reduced pancreatic injury of in vivo and in vitro AP models [47,60] The effects of the mixture of herbs might be the result of vast chemical compounds with synergistic or antagonistic effects. The active ingredients of CQCQD with its associated ER stress inhibition in SAP-related AKI remains unclear. Moreover, these major compounds only made up 0.000135 % of the total CQCQD extract, indicating that there are still many unknown components in CQCQD. Therefore, additional research is needed to find the active ingredients of CQCQD in SAP.

Regardless of the etiology, it is widely accepted that inflammatory mediators, such as TNF-α, IL-6, IL-1, and intercellular cell adhesion molecule-1, play a vital role in developing AP from a local inflammatory injury to systemic disease [32]. Inflammatory cytokines had been demonstrated to lead to ER stress in different cells [61,62]. This apoptotic effect is mediated in part by the increased expression of CHOP which can occur in response to activation of PERK, ATF6 and IRE1 [63,64]. In the current study, TNF-α and IL-6 stimulated HK-2 cells to explore ER stress-related apoptosis in TECs in vitro. As shown in Fig. 8 both TNF-α and IL-6 increased XBP1s, BIP and CHOP and decreased IRE1-α in HK-2 cells, indicating activation of ER stress. As expected, caspase- 9 and Cle-caspase- 3 proteins were increased and there were more PI-positive HK-2 cells in the experimental groups, which mean opoptosis actication and cell viability decrease in HK-2 cells with TNF-α and IL-6 stimulation. Moreover, treatment with CQCQD and 4-PBA restored the expression levels of ER stress indicators and caspases and alleviated HK-2 cell death. Therefore, CQCQD can attenuate TNF-α- and IL-6-induced HK-2 apoptosis by inhibiting ER stress.

Collectively, our study demonstrated that CQCQD decreases serum inflammatory cytokines, ameliorates renal histopathological changes and improves renal function in rats with SAP-induced AKI. In an in vitro study, CQCQD was also found to relieve cell death induced by TNF-α and IL-6. The therapeutic function of CQCQD may partly be attributed to its effects on TEC ER stress inhibition and subsequent apoptosis.

## 5. Conclusion

In conclusion, the present study indicates that CQCQD alleviates AKI following SAP by inhibiting ER stress and subsequent apoptosis. CQCQD is a good alternative treatment for AP-related AKI.

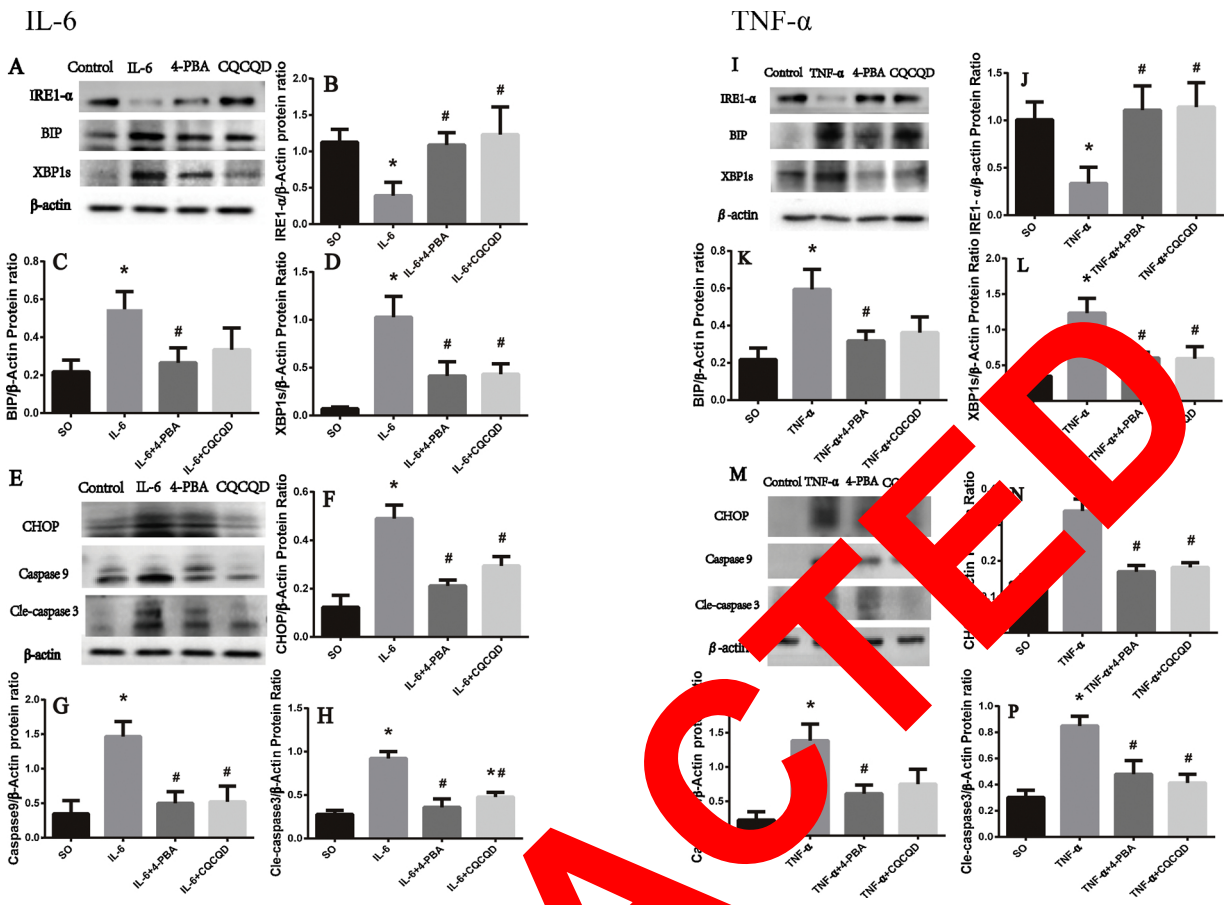


**Fig. 8.** Effect of CQCQD on ER stress proteins and apoptotic markers in H[illegible]ce[illegible]mula[illegible]ed by IL-6 or TNF-α. *P < 0.05 vs. Control group, #P < 0.05 vs. IL-6 group (n [illegible] #P < 0.0[illegible] TNF-α group (n = 3).



## Author contributions

Author contributions: Xuefei Yang a[illegible]iaoxin[illegible]g contributed equally to this study. Xuefei Yang c[illegible]cted the ex[illegible]ent, initial data analysis and the writing of th[illegible] draft; Xiaox[illegible]hang designed the study, critically revie[illegible]d and [illegible]ed the manuscript and contributed to initial data a[illegible]ysis. Jia Guo, [illegible] Lin, and Haoyang Wang provided helpful a[illegible]e for the manuscri[illegible] Xinmin Yang and Linbo Yao contributed [illegible]nitial d[illegible] analysis. Qing Xia and Ping Xue critically reviewed and [illegible]ed [illegible] manuscript, and approved the final manuscript as submitted. [illegible]thors m[illegible] the ICMJE criteria for authorship.

## CRediT [illegible]orshi[illegible]ntribution statement

**Xuefei Ya[illegible]** Data curation, Formal analysis, Writing - original draft. **Xiaoxin [illegible]:** Supervision, Writing - review & editing. **Ziqi Lin:** Supervision. **J[illegible]uo:** Supervision. **Xinmin Yang:** Data curation. **Linbo Yao:** Data curation. **Haoyang Wang:** Writing - review & editing. **Ping Xue:** Writing - review & editing, Funding acquisition. **Qing Xia:** Funding acquisition, Project administration.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

## Acknowledgments

Funded by National Natural Science Foundation of China (No. 81573766 and No. 81873107), Science and Technology Planning Program of Sichuan (No. 2019YFS0259) and Ministry of Science and Technology of the People's Republic of China (2016YFE0101800).

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