Da Cheng Qi Decoction Alleviates Cerulein-Stimulated AR42J Pancreatic Acinar Cell Injury via the JAK2/STAT3 Signalling Pathway

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Research

Keywords: Da Cheng Qi Decoction, Acute pancreatitis, JAK2/STAT3 signalling pathway, AR42J rat pancreatic acinar cells

DOI: https://doi.org/10.21203/rs.3.rs-48121/v1

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Abstract

**Background:** Acute pancreatitis (AP) is a common acute abdomen, characterized by the dysregulation of digestive enzyme production and secretion. Many studies have shown that Da Cheng Qi Decoction (DCQD) is a secure, effective prescription on AP. In this study, cerulein-stimulated AR42J cells damage model was established to further explore the feasibility and underlying mechanism of DCQD as a potential JAK2/STAT3 pathway inhibitor for the treatment of AP.

**Methods:** Cell viability of DCQD was measured using a Cell Counting Kit-8 assay. Pancreatic biochemical markers such as amylase, lipase and C-reactive protein production were measured by assay kits respectively. Cytokines (TNF-α, IL-6, IL-10 and IL-1β) were assayed by ELISA. Protein location was detected by immunofluorescence staining. Proteins expression was quantified by Western blotting, and gene expression was assessed by real-time PCR. For mechanistic analysis of the effect of DCQD on JAK2/STAT3 signaling pathway, selective JAK2 inhibitor (Fedratinib) and STAT3 inhibitor (Stattic) as well as STAT3 activator (Garcinone D) were used.

**Results:** DCQD protected cells by regulating cerulein-induced inflammation and reducing the secretion of pancreatic biochemical markers. The mechanisms mediating these effects may be related to the suppression of the JAK2/STAT3 pathway. Additionally, DCQD significantly attenuated cell injury and regulated the expression of downstream targets Bax and Bcl-XL. Moreover, treatment with JAK2 inhibitor Fedratinib or STAT3 inhibitor Stattic reversed the activated effect of cerulein on JAK2/STAT3 pathway. And the activation of JAK2/STAT3 pathway, via STAT3 activator Garcinone D, did exert damage on cells, which bore a resemblance to cerulein.

**Conclusion:** The activation of JAK2/STAT3 pathway may play a key role in the pathogenesis of cerulein-stimulated AR42J pancreatic acinar cell injury. DCQD could improve inflammatory cytokines and cell injury, which might be mediated by suppressing the activation of JAK2/STAT3 signaling pathway.

Background

Acute pancreatitis(AP) is a common acute abdomen, of which severe complication involves systemic inflammatory response syndrome(SIRS) and multiple organ dysfunction syndromes (MODS)(1, 2). The incidence and prevalence of AP are increasing worldwide and have a great influence on life quality and work ability. Growing evidence shows that several factors, including cholelithiasis, alcoholism and smoking, increase incidence and mortality burden(3). Currently, due to lack of available medicines, the mainstay of treatment for AP is based on surgery(4). However, poor surgical prognosis and high recurrence rate make the operation result unsatisfactory.

Nowadays, Traditional Chinese Medicine (T.C.M.), one of the most popular complementary and alternative therapy in the world, has attracted more and more attention in the treatment of AP(5). Da Cheng Qi Decoction(DCQD), a famous formula recorded in Shang Han Lun, consists of Rhei radix et rhizome (Da Huang), Magnoliae officinalis cortex (Hou Pu), Aurantii fructus immaturus (Zhi Shi) and
Natrii sulfas (Mang XIAOXiao). Over the years, many studies have shown that DCQD is a secure, effective drug on AP(6, 7).

Presently, more and more clinical studies have proven that the levels of a few pro- and anti-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and interleukin (IL)-10, are elevating early in patients with AP(8). Determining the severity of inflammatory reaction at admission do contribute to predicting clinical outcome in AP. On the other hand, as one of the principal signaling pathway for cytokines and growth factors, Janus kinase 2 signal transducers and transcription 3 (JAK2/STAT3) signaling pathway is essential for the innate immunity and suppress inflammation(9). In addition, many researchers suggested that JAK2/STAT3 signaling pathway acts an important role in the pathogenesis and development of AP(10, 11). Inflammatory cytokines can participate in the pathogenesis of AP by activating JAK2/STAT3 pathway (12). At the same time, many drugs can treat AP through JAK2/STAT3 pathway(13, 14). What's more, based on the enrichment results of KEGG (Kyoto Encyclopedia of Genes and Genomes) in network pharmacology and proteome research in our previous research, JAK2/STAT3 signal pathway may be the key of DCQD in interfering with AP.

Although a previous study sporadically showed that DCQD could regulate inflammatory cytokines and intestine injury in rats with severe acute pancreatitis via JAK2/STAT3 signaling pathway(15), the potential mechanism of DCQD participating in AP through JAK2/STAT3 is not clear. For what, a simple and feasible in vitro model of AP should be taken into consideration. Therefore, in this study, cerulein-induced AR42J cells damage model was established to further explore the feasibility and underlying mechanism of DCQD as a potential JAK2/STAT3 inhibitor for the treatment of AP.

**Methods**

**Preparation of DCQD**

Da Huang, Hou Pu, Zhi Shi and Mang Xiao were purchased from Shanghai Kangqiao Chinese Medicine Pieces Co., Ltd. (Shanghai, China). The raw materials were qualified according to analysis listed in China Pharmacopeia and authenticated by Pharmacist Yanjun Cheng (Shanghai Hongqiao Chinese Medicine Pieces Co., Ltd, Shanghai, China). Da Huang, Hou Pu, Zhi Shi and Mang Xiao were weighed according to the ratio of 12:24:12:9. Briefly, Hou Pu and Zhi Shi were boiled twice totally. For the first time, Hou Pu and Zhi Shi were soaked with cold water at a ratio of 1:6 (W/V) for 30 min, and then, boiled for 30 min. Da Huang was soaked in the hot filtrate for 10 min and filtering again. For the second time, the residues of the first decoction were further boiled with 4 times water for 20 min. Da Huang was soaked as mentioned before. Mixing these two filtrates and Mang Xiao was dissolved in the hot solution. Condensed to an appropriate volume and 95% ethanol was added two times to 60% and 75% ethanol concentration, respectively. Filtered after stored in a refrigerator at 4°C for 24 h. Finally, the aqueous extract was concentrated by reduced-pressure evaporation to 150 mL (equal to 1 g/mL of dried herbs). The extract of DCQD was diluted 20 times by culture media as the stock solution for the following experiment (the concentration was recorded as C).
Cell culture

AR42J rat pancreatic acinar cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A). Cells were cultured in RPMI-1640 (Hyclone Labs, UT, U.S.A) medium containing 10% fetal bovine serum (FBS) (GIBCO, NY, U.S.A) and 1% Penicillin-Streptomycin solution (100X) (Solarbio science & technology Co., Ltd., Beijing, China) at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability assay

For detection of DCQD cytotoxicity and cell viability, cell counting kit-8 (CCK-8) assay was applied in this study. Procedures were as follows: Firstly, AR42J cells were incubated in each well of a 96-well plate at a density of 4 × 10³ cells/well and grown for 12 hours. Thereafter, cells were pretreated with different concentrations of DCQD (C, C/2, C/3, C/4, C/5, C/10) for 2 h. Secondly, 100μL of the CCK-8 solution (Signalway Antibody LLC, Maryland, USA) was added to each well. After incubation for 1 h, absorbance for detecting cell viability was read at 450 nm on a microplate reader (iMark680; Bio-Rad Laboratories, Inc.) and converted to cell numbers with the standard curve. To observe the morphological change, the cells were observed under a light microscope (XDS-500C, Peikon, Shanghai).

Cell treatment

Based on the CCK-8 results, different doses of DCQD (C/6, C/5 and C/4) were selected for the further study.

To explore the amelioration of DCQD on AR42J cells injury induced by cerulein (CER), cells were divided into following groups: Control, cells were cultured in medium and treated with phosphate buffered saline (PBS); Model, cells were stimulated with CER (10⁻⁷ M) (Shanghai Yuanye Biotechnology Co, Ltd, Shanghai, China) as previous prescribed (16); Low dose group (LG), medium dose group (MG) and high dose group (HG), cells were treated with DCQD (C/6, C/5 and C/4 respectively) for 2 h before the stimulation with CER (10⁻⁷ M).

To further verify the effect of DQCD on JAK2/STAT3 signaling pathway, cells were divided into following groups: Control, cells were cultured in medium and treated with PBS; Model, cells were stimulated with CER (10⁻⁷ M); DCQD group (DG), cell were treated with DCQD (C/4) without stimulated with CER (10⁻⁷ M); Treatment group (TG), cell were treated with DCQD (C/4) for 2 h before the stimulation with CER (10⁻⁷ M).

To study whether DCQD ameliorated CER-induced AR42J cells injury by suppressing the activation of JAK2/STAT3 signaling pathway, cells were divided into following groups: Control, cells were cultured in medium and treated with PBS; Model, cells were stimulated with CER (10⁻⁷ M); DCQD group (DG), cells were treated with DCQD (C/4) without stimulated with CER (10⁻⁷ M); Treatment group (TG), cells were treated with DCQD (C/4) for 2 h, prior to CER (10⁻⁷ M) stimulation; Fedratinib group (FG), cells were treated
with Fedratinib (3nM) (JAK2 inhibitor) for 2 h, prior to CER (10^{-7}M) stimulation; Stattic group (SG), cells were treated with Stattic (5μM) (STAT3 inhibitor) for 2 h, prior to CER (10^{-7}M) stimulation; Garcinone D group (GG), cells were stimulated with Garcinone D (10μM) (STAT3 activator); DCQD+Garcinone D group (D+GG), cells were treated with DCQD (C/4) for 2 h, prior to Garcinone D (10μM).

The cells were harvested 8 h after the onset of treatment and stored at -20°C for later experiments. Concentrations of Fedratinib (3nM), Stattic (5μM) and Garcinone D (10μM) were adapted from previous studies showing to inhibit or activate JAK2/STAT3 signaling pathway in vitro(17-19).

**Measurement of amylase, lipase and C-reactive protein production**

Amylase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the activity of amylase in each group cells, according to the instructions provided by the manufacturer. Absorbance of each well was measured at a wavelength of 660 nm. The lipase content in cellular supernatants was detected by the lipase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the method described by the manufacturer. Total lipase content (U/L) was measured at a wavelength of 420 nm. Levels of C-reactive protein in cellular supernatants was determined with the C-reactive protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturers’ directions. The absorbance of the mixture was determined at 570 nm. C-reactive protein concentration (mg/L) was calculated by comparing the sample absorbance to a standard curve obtained by using standard purified C-reactive protein as reference.

**Enzyme-linked immunosorbent assay (ELISA)**

Inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-10 production in the supernatant of treated cells were measured using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by following the manufactures' protocols. In the end, the absorbance data of each well was measured at 450 nm. TNF-α, IL-1β, IL-6 and IL-10 concentration (ng/L) was detected using standard purified recombinant cytokine.

**Immunofluorescence staining**

Cells of each group were washed three times with 0.02M PBS, and fixed with 4% formaldehyde for 30 min at room temperature. After that, cells were permeabilized with 0.5% Triton X-100 (Solarbio science & technology Co., Ltd., Beijing, China) in PBS for 10 min. After 1 h in 1% BSA (Solarbio science & technology Co., Ltd., Beijing, China), the cells were incubated at 4°C for 12 hours with rabbit anti-p-STAT3 (1 : 1000). After three 3 min washes in PBS, the cells were incubated with a Alexa Fluor 488 conjugated goat anti-rabbit IgG (H+L) (Beyotime, Shanghai, China) antibody at room temperature for 1 h. The nuclear of cells were stained with Hoechst 33342 for 10 min. Finally, fluorescence microscope was used to capture the
images. The rabbit anti-p-STAT3 (catalog number: ab76315) was obtained from Abcam (Abcam, Cambridge, UK).

**Real-time PCR analysis**

Total RNA was isolated from treated cells by using Trizol Reagent (Invitrogen, CA, U.S.A) and was dealed with RNase-free DNase. Reverse transcription was performed with the cDNA Reverse Transcription kit (Fermentas, MA, U.S.A). For real-time PCR reactions, amplification mixture (25μL) contained 1μL of primer mix, 2μL of cDNA template, 12.5μL of SYBR green Mix (Thermo Scientific Molecular Biology, MA, U.S.A) and 9.5μL of ddH2O. Specific mRNA quantification was performed on ABI 7300 Real Time PCR instrument (Applied Biosystems, CA, U.S.A), according to the manufacturer’s guidelines. The data were analyzed by use of ABI Prism 7300 Sequence Detection System software (Applied Biosystems, CA, U.S.A). Expression levels of the mRNAs of interest were normalized to those of endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of mRNAs relative to GAPDH was calculated by the $2^{-ΔΔC_{t}}$ method. All steps were performed under RNase-free conditions. Primers for JAK2, STAT3, Bcl-XL, Bax and GAPDH in the reaction were listed in Table 1.

| Gene    | Product size, bp | Primer 5'-3'            |
|---------|------------------|-------------------------|
| JAK2    | 206              | Primer F: CAGGAATCCACCTTTCATC |
|         |                  | Primer R: CAGAGCACTCAGAGGCTTGTC |
| STAT3   | 242              | Primer F: GCCATCCTAAAGCACAAAGC |
|         |                  | Primer R: TCAGGGTAGAGGTAGACCAGTG |
| Bcl-XL  | 133              | Primer F: ATGGGGTAAACTGGGGTCG |
|         |                  | Primer R: TGGTCATTCAGGTAGGTGGC |
| Bax     | 298              | Primer F: GCGATGAATGCGACAACAAC |
|         |                  | Primer R: CCGAAGTAGAAGGGAGGC |
| GAPDH   | 237              | Primer F: GGAGTCTACTGGCGTCTCAC |
|         |                  | Primer R: ATGAGCCCTTCCACGATGC |
Western blot analysis

Treated cells of each group were lysed in RIPA buffer (Shanghai JRDUN Biotechnology Co, Ltd, Shanghai, China) containing both protease and phosphatase inhibitor mixture to extract whole cell protein. Using BCA assay kit (Thermo Scientific Molecular Biology, MA, U.S.A) to quantify the protein levels. Equal amounts of Proteins (25 μg/well) were separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. The non-specific sites on each blot were blocked with 5% skim milk for 12 hours at 4°C. Target proteins were detected by corresponding primary antibodies. After overnight incubation with the primary antibody, each blot was washed three times with TBST buffer. Blots were then labeled with anti-rabbit IgG-HRP conjugated secondary antibody. The membranes were imaged by ECL reagent. Band intensity was normalized against GAPDH by Tanon-5200. Antibodies were used at the following concentrations: anti-JAK2/p-JAK2 1:5000, anti-STAT3 1:1000, anti-p-STAT3 1:20000, anti-Bax 1:1000, anti-Bcl-XL 1:1000, GAPDH 1:2000 and anti-rabbit IgG-HRP conjugated secondary antibody (1:1000). Sources of antibodies were as followed: Rabbit monoclonal [EPR108(2)] antibodies against JAK2 (catalog number: ab108596), Rabbit monoclonal [E132] antibodies against JAK2 (phospho Y1007 + Y1008) (catalog number: ab32101), Mouse monoclonal [9D8] antibodies against STAT3 (catalog number: ab119352); Rabbit monoclonal [EP2147Y] antibodies against STAT3 (phospho Y705) (catalog number: ab76315); Rabbit monoclonal [E18] antibodies against Bcl-XL (catalog number: ab32370) and Rabbit monoclonal [E63] antibodies against Bax (catalog number: ab32503) were from Abcam (Abcam, Cambridge, UK); GAPDH (catalog number: 5174) was from CST (Cell Signaling Technology, Inc., MA, U.S.A); anti-rabbit IgG-HRP conjugated secondary antibody (catalog number: A0208) was from Beyotime (Beyotime, Shanghai, China).

Statistical analysis

Each experiment above was repeated in triplicate to ensure quantitative accuracy. Statistical analysis was performed by SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All experimental data were presented as the mean±standard deviation (S.D.) and analyzed for statistical significance by one-way ANOVA. Value of p<0.05 and p<0.01 were defined as significant and very significant difference.

Results

Cytotoxicity and Cell Proliferation in AR42J Cell Exposed to DCQD

DCQD significantly reduced cell viability at doses of C(P < 0.01) (Fig. 1A), and showed no evident cytotoxic effects from concentration C/2. Therefore, a further study about the effect of DCQD on reversing CER-induced cell proliferation inhibition were carried on. Cells were pre-incubated with different concentrations of DCQD (C/2, C/3, C/4, C/5, C/10) for 2 h and then stimulated with CER. As shown in Fig. 1B, CER stimulation significantly reduced cell viability (P < 0.01), and DCQD pre-incubation increased
cell viability in an approximately dose-dependent manner. Cell viability was highest at concentration of C/4, followed by C/5. We speculated that the most effective concentration of DCQD in cells was C/4; and selected this concentration as well as C/5 and C/6 for subsequent experiments to observe the concentration-dependent manner. In addition, under the light microscope (Fig. 1C), the AR42J cells in the control group had normal morphology and structure, gathered in clumps or lumps, and could produce appropriate amount of secretions. However, compared with the control group, the AR42J cells in the model group showed severe inflammatory reaction, of which density decreased, volume increased. More suspended cell fragments and necrotic cells could be founded. After the pre-treatment of DQCD (C/4), the morphology and structure of cells tended to be normal. In a word, DCQD could alleviate cell injury induced by CER.

Effects of DQCD on the reflective markers of severity of AP

In previous studies, the releases of amylase has been used as a marker for monitoring models of AP, while lipase and C-reactive protein are potentially useful as markers of clinical severity of AP(20–22). To further confirm the effect of DCQD on AR42J cells injury, we quantified biochemical enzymes related to AP, such as amylase, lipase and C-reactive protein. CER induction markedly increased the levels of amylase (Fig. 2A), lipase (Fig. 2B) and C-reactive protein (Fig. 2C). In contrast, the activities of amylase, lipase and C-reactive protein were significantly decreased in the DCQD pretreatment group relative to the model group, demonstrating a concentration-dependent response.

Effects of DQCD on the production of inflammatory cytokines

Changes in cytokine production was consistent with the inflammatory state of AP. ELISA was utilized to determine the production of inflammatory cytokines such as TNF-α, IL-1β, IL-10 and IL-6 in AR42J cells. It was shown that CER triggered a significant elevating secretion of TNF-α (Fig. 3A), IL-6 (Fig. 3B), IL-1β (Fig. 3C) in AR42J cells compared with that in control group (P < 0.01). DCQD significantly decreased the level of IL-6, IL-1β and TNF-α in a concentration-dependent manner (P < 0.05 or P < 0.01). On the other hand, a significant decrease in production of IL-10, a kind of anti-inflammatory cytokine, was noted in cells stimulated with CER compared to the control group (P < 0.01) (Fig. 3C). DCQD significantly increased the level of IL-10 in MG and HG group (P < 0.05 and P < 0.01). Overall, these results suggested that DCQD successfully protected against CER-induced AR42J cell injury and inflammation.

DCQD suppressed JAK2/STAT3 signaling pathway in a concentration-dependent manner
The effect of DCQD on JAK2/STAT3 signaling pathway in AR42J cells stimulated with CER was investigated. Firstly, JAK2 mRNA and STAT3 mRNA were evaluated by using Real time-PCR after treating with different concentrations of DCQD. As illustrated in Fig. 4A, JAK2 mRNA and STAT3 mRNA were significantly increased in acinar cells treated with CER compared to the control group. Pretreatment of acinar cells with DCQD decreased JAK2 mRNA and STAT3 mRNA expression as a dose-dependent manner. Next, the expression of JAK2, p-JAK2, STAT3 and p-STAT3 proteins in cells stimulated with CER in the presence or absence of DCQD were examined. These results demonstrated that CER activated JAK2/STAT3 signaling in pancreatic acinar cells. As shown in Fig. 4B, 2 h pretreatment of DCQD suppressed the activation of JAK2/STAT3 signaling pathway at the protein level. The expression of p-JAK2 and p-STAT3 proteins decreased with increasing DCQD concentrations, indicating a dose-dependent response. Immunofluorescence analysis were performed for the location of p-STAT3 in cells. Generally speaking, p-STAT3 staining was mainly present in the cytoplasm. Once JAK2/STAT3 was activated, p-STAT3 would translocate to the nucleus(23). Results confirmed that p-STAT3 protein was highly expressed in the nucleus of cells from model group, while its expression was lower in the nucleus of cells treated with DCQD (Fig. 4C). These results showed that DCQD may be beneficial in AP by inhibiting the activation of JAK2/STAT3 signaling in pancreatic acinar cells.

Effect of DCQD on downstream effectors in the JAK2/STAT3 pathway

According to the protein expression of p-STAT3 in different groups (Fig. 5B), DCQD did suppress the activation of JAK2/STAT3 signaling pathway, which was consistent with our previous experimental results (Fig. 4B). Since Bax and Bcl-XL are two of the crucial downstream effectors of STAT3, the levels of Bax and Bcl-XL were tested to further verify the inhibitory effect of DQCD on JAK2/STAT3 pathway. Refered in Fig. 5A, the mRNA level of Bax was significantly increased in CER-treated groups. Similarly, a noticeable increase was also found in protein level of Bax in CER-treated groups (Fig. 5B). DCQD blocked the effect of CER, and the expression of Bax was significantly decreased compared to model group. By contrast, the protein and mRNA level of Bcl-XL showed an opposite trend against Bax. These data further confirmed that DCQD did suppress the JAK2/STAT3 pathway and regulated the expression of JAK2/STAT3 downstream effectors, such as Bax and Bcl-XL. Furthermore, given that no significant difference was found between DG and Control groups (P > 0.05), the side effect of DCQD could be excluded.

The amelioration of DCQD was mediated by suppressing the activation of JAK2/STAT3 signaling pathway

To test the amelioration effect of DCQD depended on the suppression of JAK2/STAT3 signaling pathway, selective JAK2 and STAT3 inhibitors as well as STAT3 activator were used. Compared with the control group, no significant morphological changes of cells were observed under light microscopy in FG, SG and
TG (Fig. 6A). In addition, refered in Fig. 6B-D, the results of Control, Model and TG were in line with our previous experimental results (Fig. 2). Fedratinib (JAK2 inhibitor) and Stattic (STAT3 inhibitor) significantly reduced the releases of amylase, lipase and C-reactive protein of cells (P < 0.01). Notably, the effect of Fedratinib and Stattic on improving cells injury and reducing the reflective markers of severity of AP were more potent than that of DCQD. Further Western blotting analysis demonstrated our findings (Fig. 6E). Stimulation with CER markedly increased the p-JAK2/JAK2 and p-STAT3/STAT3 levels compared with that in the control group (P < 0.01). This increase was dramatically counteracted by DCQD and Fedratinib (P < 0.05 and P < 0.01, respectively), while Stattic only decreased the p-STAT3/STAT3 level (P < 0.05), as we expected. We reasoned that JAK2/STAT3 signaling pathway played an important role in cells injury induced by CER. What's more, we also used Garcinone D (STAT3 activator) to study whether the therapeutic target of DCQD was, at least, in part, on JAK2/STAT3 signaling pathway. As shown in Fig. 6G-I, like CER, GG had a similar damage effect on cells. The releases of amylase, lipase and C-reactive protein of cells in GG were significantly increased (P < 0.01). And the cells exhibited morphological features of injury when treated with Garcinone D compared with the control group. However, pre-treatment of DCQD (C/4) for 2 h markedly reversed the increase of amylase, lipase and C-reactive protein in cells. Besides, the morphological features showed some improvement (Fig. 6F). The volume of cells tended to be normal, and the secretions such as trypsin decreased significantly. Western blotting assays confirmed the above findings as well (Fig. 6J). In GG group, the p-JAK2/JAK2 and p-STAT3/STAT3 levels were markedly increased compared with that in the control group (P < 0.01). Yet, this increase was significantly reversed by DCQD (P < 0.05). What's more, no discernible difference was observed between the groups treated with DCQD alone and Control group (P > 0.05), which ruled out the possible effect of DCQD. Overall, we reasoned that the effect of DCQD is, at least, partially medicated through the JAK2/STAT3 signaling pathway.

**Discussion**

DCQD reportedly inhibits the cytokine's activity, promotes the gastrointestinal motility, and regulates the inflammatory response in patients with AP(24). However, the molecular mechanism underlying DCQD effect on pancreatic acinar cells is not fully understood. Here, we found that DCQD protected AR42J pancreatic acinar cells by inhibiting CER-induced inflammation and reducing the secretion of pancreatic biochemical markers, such as amylase, lipase and C-reactive protein. The mechanisms mediating these effects may be related to the suppression of the JAK2/STAT3 signaling pathway. Additionally, DCQD significantly attenuated cell injury and regulated the expression of downstream targets Bax and Bcl-XL.

At present, AR42J is one of the cell lines with the characteristics of normal pancreatic acinar cells, which has been used to study the growth, secretion and proliferation of pancreatic exocrine cells(25). CER is an ortholog of the intestinal hormone cholecystokinin, which is one of the most-characterized and widely used agents in experimental animal models of pancreatitis. CER has been reported to simulate the characteristics of human pancreatitis by inducing the death of acinar cells, the formation of edema and the infiltration of inflammatory cells into the pancreas in vivo and in vitro(26, 27). In this research, the rat pancreatic acinar cell line AR42J was used as an in vitro model to evaluate the effect of DCQD against...
pancreatic acinar cell injury induced by CER. In agreement with previous studies, our results showed that $10^{-7}$M CER notably induced cell injury(16).

Generally, AP is recognized as an excessive inflammatory response characterized by the dysregulation of digestive enzyme production and secretion(26). Indeed, the death of acinar cells due to uncontrolled inflammation may be one of the crucial factors resulting in AP. Once pancreatic acinar cells are injured or dead, cytokines will be released soon, which can trigger an inflammatory response and ultimately lead to devastating consequences(28). The release of these inflammatory factors from damaged acinar cells, as one of the early events in the development of AP, is associated with the degree of pancreatic inflammation(29). IL-6, as a multifunctional cytokine most closely associated with STAT transcription factor activity(30), is the most credible in assessing the severity of AP and predicting the risk of early complications, with a high sensitivity range (about 89%-100%)(31). TNF-α can regulate not only leukocyte adhesion molecules but also other pro-inflammatory cytokines. It can act a priming activator of immune cells as well. Recently, a number of studies have shown that TNF-α plays a pivotal role in the pathogenesis of AP, most of which come from both animal models and in vitro studies. In particular, it promotes the systemic progression of inflammation and terminal organ dysfunction, which is usually observed in severe diseases(32). Another major cytokine, IL-1β, can drive the excessive systemic inflammatory response, too. Since the levels of IL-1β and IL-6 of the pancreatic acinar cells stimulated with CER or the activated neutrophils were similar at the late stage of stimulation, it has similar accuracy to IL-6 in predicting AP upon admission and also has been used as a biomarker of disease severity(33). Moreover, in CER-stimulated pancreatic acinar cells, IL-1β could be earlier detected and more expressed at early stage of stimulation(34, 35). In contrast, IL-10, an anti-inflammatory cytokine, acts as potent suppressants. Once activated, it can prevent the extracellular killing function of macrophages(36). IL-10 is likely a primary factor in the negative feedback system, which hinders the production of pro-inflammatory cytokines like IL-6 and TNF-α in various cells(37, 38). Interestingly, in previous studies, the level of IL-10 showed different trends in various AP models. In vivo experiment, Yuan et al. found no change in the concentration of IL-10 in kidney tissues of rats with sever AP(39). But in the mice with pancreatitis-induced lung injury, Piao et al. found that the level of IL-10 in serum was significantly decreased(40). In vitro studies, an increase in IL-10 production were observed in the supernatant of pancreatic acinar cells after stimulation with TNF-α(20). A previous study also found, in CER-stimulated AR42J pancreatic acinar cells, the expression of IL-10 was markedly increased in gene level(41). However, another research demonstrated that in the supernatant of pancreatic acinar cells treated with lipopolysaccharide, the levels of IL-10 in culture supernatant decreased with the lipopolysaccharide concentration. We suspected that a reason for these discrepancies is mainly due to the different kinds of AP model or differences in detection methods and levels. Previous study has provided evidence for the effect of DCQD in the control of acute kidney injury in rats with severe acute pancreatitis by cytokine inhibition, suggesting that long-term treatment with DCQD can reduce exacerbations and complication in AP(39). This study also noticed very high levels of pro-inflammatory factors, TNF-α, IL-1β, and IL-6 as well as a lower level of anti-inflammatory factor IL-10 in the supernatant of CER-induced cells. These changes in cytokine levels were noticeably mitigated by DCQD pretreatment, which means that DCQD may play a
critical role in the regulation of the inflammatory response in AP. These results combined with the increased cell viability and improvement of cell morphology and structure indicated that DCQD might exert a therapeutic effect for AP, at least, in part via creating a balance between pro-inflammatory and anti-inflammatory factors.

In the diagnosis of AP, serum amylase and lipase remain important tests with a high specificity. However, they cannot reflect its severity (42). In this respect, C-reactive protein has been established as a prognostic variable in human AP (43). Past researches have proved both the diagnostic value of amylase and lipase as well as the prognostic value of C-reactive protein (21, 22). In this study, DCQD effectively reduced the CER-induced increase in amylase, lipase and C-reactive protein levels. Moreover, DCQD effect appeared a dose-effect relationship.

The JAK2/STAT3 signaling pathway is a pleiotropic cascade responsible for the transcription of various genes involved in immunity and inflammation. An increasing body of evidence has been shown the important role of JAK2/STAT3 pathway in the development of AP (44, 45). JAK2 can be activated by numerous cytokines or growth factors. Ligand binding induces phosphorylation of tyrosine kinases and isomerization of JAK-related receptors subunits. Activated JAK in turn phosphorylates receptors for recruitment of STAT protein (46). STAT3 is one of the pivotal JAK2 effectors and its transcriptional activity is regulated by phosphorylation of JAK2. The phosphorylated STAT3 is released from the receptor complex to form a dimer. Then, these dimers will translocate to the nucleus, where they directly bind to the promoter regions of specific target genes, thereby regulating the transcription of the target genes, such as Bcl-2 family (47). Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-XL are two of the representative members in Bcl-2 family (48). Bax and Bcl-XL can regulate each other's functions through heterodimers, and their relative concentrations may play an important role in regulating apoptosis (48). Of note, study has also shown that DCQD, as an inhibitor of the JAK2/STAT3 pathway, exerts marked effects in AP models in vivo (15). This study investigated the role of JAK2/STAT3 pathway in CER-induced AR42J cells and whether DCQD can improve the cell injury through this pathway. Firstly, consistent with previous study (49), the results showed that CER induced the phosphorylation of JAK2 and STAT3 in AR42J cells. Pretreatment with JAK2 inhibitor, Fedratinib, or STAT3 inhibitor, Stattic, reversed the activated effect of CER on JAK2 and STAT3 phosphorylation. Furthermore, the activation of JAK2/STAT3 pathway, via STAT3 activator Garcinone D, did exert damage on cells, which bore a resemblance to CER. Based on these, we reasoned that the JAK2/STAT3 signaling pathway is a potential therapeutic target in the treatment of AP. Next, we also found that DCQD exhibited dose-dependent inhibitory effects on the JAK2/STAT3 signaling pathway. DCQD could not only inhibit p-STAT3 nuclear translocation, but also decrease the expression of JAK2 and STAT3 mRNA as well as the ratio of p-JAK2/JAK2 and p-STAT3/STAT3 in protein level. Moreover, DCQD could also regulate the expression of JAK2/STAT3 downstream effectors, such as Bax and Bcl-XL. In a word, the present study provided in vitro evidence that the JAK2/STAT3 pathway is activated in AP, and that the protective effect of DCQD in AP is associated with the inhibition of this activation.
However, some questions have yet to be solved: Due to the fact that the molecular targets of DCQD may not be only dependent on the JAK2/STAT3 signaling pathway, the possibility of other mechanisms cannot be excluded. Moreover, since DCQD succeeded in regulating the apoptotic Bcl-2 family member: Bax and Bcl-XL, whether it will further modulate pancreatic acinar cell apoptosis? Further studies are required to answer these questions and explore the mechanism of action of DCQD in detail.

**Conclusion**

The activation of JAK2/STAT3 pathway may play a key role in the pathogenesis of cerulein-stimulated AR42J pancreatic acinar cell injury. DCQD could improve inflammatory cytokines and cell injury, which might be mediated by suppressing the activation of JAK2/STAT3 signaling pathway.

**Abbreviations**

DCQD: Da-Cheng-Qi-decoction; AP: acute pancreatitis; SIRS: systemic inflammatory response syndrome; MODS: multiple organ dysfunction syndromes; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-10: interleukin-10; IL-1β: interleukin-1β; JAK2: Janus kinase2; STAT3: signal transducers and transcription3; T.C.M.: Traditional Chinese Medicine; CER: cerulein; FBS: fetal bovine serum; PBS: phosphate buffered saline; CCK-8: cell counting kit-8; ELISA: Enzyme-Linked immunosorbent assay; BSA: bovine serum albumin; p-STAT3: phosphorylated-signal transduction and activators of transcription 3; p-JAK2: phosphorylated- Janus kinase2; Real-time PCR: Real-time polymerase chain reaction; cDNA: complementary DNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-XL: B-cell lymphoma-extra large; Bax: Bcl2-associated X; BCA: bicinchoninic acid; SDS-PAGE: sodium dodecylsulfate–polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; HRP: horseradish peroxidase; TBST: Tris Buffered saline Tween; RIPA: Radio-Immunoprecipitation Assay; SD: standard deviation; ANOVA: A one-way analysis of variance; ECL: enhanced chemiluminescence;

**Declarations**

**Authors’ contributions**

ZZ, YC and XW designed the study; ZZ and KL wrote the main manuscript text; ZZ, YC, WD performed the animal experiments; ZZ, YC and RA analysed the data and prepared the figures. All authors read and approved the final manuscript.

**Acknowledgements**

Not applicable.

**Competing interests**


The authors declare that they have no competing interests

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

Funding

This work was supported by Scientific research project of Shanghai Health Committee 2017401668 “Development of traditional medicine” project of Hongkou District Health Committee HGY-MGB-2018-01-08 Budget internal medicine research project of Shanghai University of traditional Chinese Medicine 2018LK0732019LK098 Scientific research project of Hongkou District Health Committee Hongwei1903-04 Shanghai “Rising Stars of Medical Talent” Youth Development Program 20200506 Construction project of Shanghai new interdisciplinary program of traditional Chinese Medicine (20181118).

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**Figures**
Cytotoxicity and cell Proliferation in AR42J cells exposed to DCQD. (A) AR42J cells were incubated with DCQD at the concentrations of C, C/2, C/3, C/4, C/5, C/10 for 2 h. Cell viability was measured by the CCK-8 assay. (B) AR42J cells were pretreated with DCQD at the concentrations of C, C/2, C/3, C/4, C/5, C/10 for 2 h, and then cells were stimulated with CER (10-7M) for 8 h. Cell viability was measured by the CCK-8 assay. (C) The morphological changes of cells were assessed by using a light microscope (400×). Results are expressed as the mean±S.D., n=3. Micrographs from one representative experiment out of three independent experiments are shown. All bar graphics:*P<0.01 vs. the control group, ##p<0.01 vs. the model group.
Figure 2

Effects of DQCD on the reflective markers of AP. The releases of amylase, lipase and C-reactive protein were detected by amylase assay kit (A), lipase assay kit (B) and C-reactive protein assay kit (C), respectively. Data are presented as the means±S.D., n=3. All bar graphics: #p< 0.05, ##p< 0.01 vs. the model group.
Effects of DQCD on the production of inflammatory cytokines. Inflammatory factor TNF-α (A), IL-6 (B), IL-10 (C) and IL-1β (D) in cellular supernatants was assayed by ELISA. Data are presented as the means±S.D., n=3. All bar graphics: #p< 0.05, ##p< 0.01 vs. the model group.
Figure 4

DCQD suppressed JAK2/STAT3 signaling pathway in a concentration-dependent manner. (A) Real time-PCR was performed to detect the mRNA expression of JAK2 and STAT3. (B) Western blot was performed to detect the protein level of JAK2, p-JAK2, STAT3 and p-STAT3. (C) Immunofluorescence staining (400×) was performed to detect the location of p-STAT3 in cells. GAPDH was as the loading control for band density normalization. Data are presented as the means±S.D., n=3. Immunofluorescence micrographs from one representative experiment out of three independent experiments are shown. All bar graphics: #p<0.05, ##p<0.01 vs. the model group.
Figure 5

Effect of DCQD on downstream effectors in the JAK2/STAT3 pathway. (A) Real time-PCR was performed to detect the mRNA expression of Bax and Bcl-XL. (B) Western blot was performed to detect the protein level of p-STAT3, Bax and Bcl-XL. GAPDH was as the loading control for band density normalization. Data are presented as the means±S.D., n=3. All bar graphics: #p< 0.05, ##p< 0.01 vs. the model group.
The amelioration of DCQD was mediated by suppressing the activation of JAK2/STAT3 signaling pathway. (A and F) The morphological changes of cells were assessed by using a light microscope (400×). The releases of amylase, lipase and C-reactive protein were detected by amylase assay kit (B and G), lipase assay kit (C and H) and C-reactive protein assay kit (D and I), respectively. (E) Western blot was performed to detect the protein level of JAK2, p-JAK2, STAT3 and p-STAT3. GAPDH was as the loading control for band density normalization. (J) Western blot was performed to detect the protein level of STAT3 and p-STAT3. GAPDH was as the loading control for band density normalization. Data are presented as the means±S.D., n=3. Micrographs from one representative experiment out of three independent experiments are shown. All bar graphics: #p< 0.05, ##p< 0.01 vs the model group.