Chaiqin Chengqi decoction inhibits inflammatory mediators and attenuates acute pancreatitis through deactivation of janus kinase/signal transducer and activator of transcription signaling pathway

Dong Jianxia, Chen Xiaoshuang, Song Yi, Fei Xiaofan

Methods: AP was induced by caerulein both in AR42J cells and in mice. AR42J cells were divided into five groups: the control group, the AP group, the CQCQD group, JAK/STAT signaling pathway inhibitor AG490 group, and the CQCQD and AG490 group. After induction, cellular supernatant of five groups were collected for measuring the concentrations of amylase, as well as inhibited expression of several inflammatory cytokines such as IL-6, TNF-α, IL-1β, NF-κB. Administration of CQCQD significantly inhibited JAK-2 activation and down-regulated phosphorylation of downstream substrate STAT-3 the same as AG490, resulting in inhibition of inflammatory mediators and amelioration of pancreatitis.

Conclusion: The results suggested that CQCQD exerted anti-inflammatory effects on AP via reducing expression and phosphorylation of JAK and STAT.

Keywords: Pancreatic diseases; Anti-inflammatory agents; Janus kinase 2; STAT transcription factors; Chaiqin Chengqi decoction

Introduction

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas that has a high risk of developing into systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS).1 AP can be lethal unless specific therapeutic approaches are taken which prompt the need to find effective therapeutics to treat AP. Chaiqin Chengqi decoction (CQCQD), a Chinese prescription from "Dachengqi decoction", may serve as promising therapy for the treatment of AP. CQCQD, prepared with famous formula documented in On Harm Caused by Cold by Zhang Zhongjing, has shown efficacy in treating AP.
Growing evidences show that the monomers isolated from CQCQD can inhibit some pro-inflammatory signaling pathways to ameliorate inflammatory cascade reaction. Previous studies revealed that CQCQD could effectively reduce AP associated lung injury by inhibiting the induction of IL-6 and increasing the expression of IL-1β. While many clinical trials have showed that CQCQD can suppress the release of the inflammatory cytokines, such as IL-6, TNF-α, amylase and so on, but the precise molecular mechanism of CQCQD to regulate these inflammatory cytokines was still unknown.

The Janus kinase (JAK)/Signal transducer and activator of transcription protein (STAT) signaling pathway plays essential roles in a number of biological processes, including anti-inflammation, cell proliferation, immune response, JAK family, comprising JAK1, JAK2, JAK3 and TYK2, which are extremely important in pro-inflammatory cytokine-mediated signaling process. JAK-2 is a common member in JAK family, which participates in immune regulation and inflammation reaction. STAT, including STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6, participate in the aspect of regulation of inflammatory cytokines, especially STAT-3. Activation of JAK-2 is thought to require phosphorylation of JAK-2 and its activation results in the phosphorylation of their downstream substrate — STAT-3. Transforming the phosphorylation state of JAK-2 and STAT-3 leads to transcription of target genes and release of some pro-inflammatory cytokines.

In this study, we aimed to investigate CQCQD effect on the related proteins of JAK/STAT signaling pathway in vitro and in a mouse model of AP.

MATERIALS AND METHODS

Animals and cell culture
Sprague-Dawley rats (male, 6 months, body weight: 220-250 g) were purchased from Institute of Laboratory Animals of Sichuan Provincial People’s Hospital (Chengdu, China). Rats were maintained in a germ-free environment and allowed free access to food and water. All animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of Sichuan University in accordance with the requirements of the National Act (People’s Republic of China). AR42J cells (from the rat exocrine pancreas) were obtained from Drug Delivery System Lab of Sichuan University (Chengdu, China). AR42J cells were cultured in DMEM with high glucose (GIBCO, NewYork, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, NewYork, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, MO, USA). Cells were maintained at 37 ℃ in an atmosphere containing 5% CO₂. Anti-JAK-2, anti-phospho-JAK-2, anti-STAT-3, anti-phospho-STAT-3, anti-β-actin were all ordered from Santa Cruz Biotechnology, USA.

Preparation of CQCQD formulation
All Chinese medicinals used to prepare CQCQD were provided by the West China Hospital of Sichuan University (Chengdu, China): Chaihu (Radix Bupleuri Chinensis) 15 g, Huangqin (Radix Scutellariae Baicalensis) 15 g, Houpu (Cortex Magnoliae Officinalis) 15 g, Zhihui (Fructus Aurantii Immaturus) 20 g, Zhizhi (Fructus Gardeniae) 15 g, Dahuang (Radix Et Rhiza Rhei Palmae) 20 g, Mangxiaojie (Naibii Sulfas) 20 g, Yanhusuo (Rhizoma Corydalis Yanhusuo) 15 g, Chuangxiang (Rhi zoma Chuangxiang) 20 g, and Muxiang (Radix Auchlandiae) 15 g. CQCQD was prepared at a concentration of 2.0 g crude herbs per milliliter, according to the standardized preparation procedure.

Medicated Serum of CQCQD
Medicated serum was prepared according to protocols. Briefly, 10 rats were randomly divided into two groups with five rats in each group, the control group and the CQCQD group. Rats in the CQCQD group were treated with CQCQD (12 mL/kg × 1 time/4 h, 2 times/d, 5 d) by intragastric administration, while rats in control group only received normal saline. Blood was collected from the femoral artery at 1 h after the last administration and centrifuged with 5000 rpm for 15 min to obtain medicated serum of CQCQD and control serum. Finally, the serums were sterilized by filtration prior to use.

Toxicity assay of medicated serum of CQCQD
MTT assay was performed to evaluate anti-proliferative effect of medicated serum of CQCQD in AR42J cells. All of serum from different groups was dialyzed with DMEM culture. Briefly, cells were placed on 96-well-plates at a density of 1 × 10⁴ cells per well and incubated for 24 h. The cells were treated with different concentrations (0%, 10%, 20%, 50% and 100%) of CQCQD-mediated serum and control serum for 24 h. Subsequently, MTT (20 µL, 5 mg/mL, MTT in PBS, Sigma, San Francisco, CA, USA) was added to each well for additional 4 h incubation. The formazan dissolved in DMSO (150 µL) was estimated by the absorbance at 570 nm with a microplate reader (Biotech, power wave, Los Angeles, AL, USA). All experiments were carried out independently and repeated in triplicate. The relative viability rate was calculated as follows:

\[
\text{Relative viability rate} = 1 - \frac{\text{OD}_{\text{control/med}} - \text{OD}_{\text{experiment/med}}}{\text{OD}_{\text{control}}} \times 100\%.
\]

Effects of medicated serum of CQCQD in vitro
AR42J cells were placed on 6-well-plates and divided into five groups: (a) normal group: pretreated with 10% normal rat serum diluted by DMEM culture for 1 h, and then added the DMEM with 10% FBS and incubated for another 12 and 24 h; (b) AP group: pre-
treated with 10% normal rat serum diluted by DMEM culture for 1 h, and then added $10^{-8} \text{ M caerulein}$ and incubated for another 12 and 24 h; (c) CQCQD group: pretreated with 10% CQCQD-mediated serum diluted by DMEM culture for 1 h, and then added $10^{-8} \text{ M caerulein}$ and incubated for another 12 h and 24 h; (d) AG490 inhibitor group: pretreated with 10% normal rat serum diluted by DMEM culture for 1 h, and then added 50 µM AG490 and $10^{-8} \text{ M caerulein}$ and incubated for another 12 and 24 h; (e) CQCQD and AG490 group: pretreated with 10% CQCQD and AG490 group; pretreated with 10% CQCQD-medicated serum diluted by DMEM culture for 1 h, and then added 50 µM AG490 inhibitor and $10^{-8} \text{ M caerulein}$ and incubated for another 12 and 24 h. The aim of CQCQD and AG490 group was to investigate the coefficient of CQCQD and AG490.

**Animal models and administration of CQCQD**

After one week accustomed to the new environment, all the fifty healthy SD rats (220-250 g) were randomly divided into five groups: control group (normal mice + normal saline, $n = 10$), AP group (AP model + normal saline, $n = 10$), treatment group (AP model + CQCQD, $n = 10$), positive control group (AP model + AG490, $n = 10$), and CQCQD and AG490 group (AP model + AG490 + CQCQD, $n = 10$). Experimental model of AP was established as described.21 AP was induced by intraperitoneal injection of supramaximal concentrations of the stable cholecystokinin analogue caerulein at a dose of 50 µg/kg at intervals of 1 h for 6 times, while the control group was injected with normal saline. At 1 h before injection, rats in CQCQD group were treated with CQCQD (12 mL/kg) by intragastric administration, and the AP and control groups were administrated with normal saline. The AG490 group was treated by intraperitoneal injection at the dose of 5 mg/kg.25 At 6 h after the final caerulein injection, blood and pancreatic tissues from each group were collected for analysis. Pancreatic tissues were evaluated by HE analysis.

**Pathological examination and scoring of pancreatic tissues**

Pancreatic tissues were harvested and fixed in 10% neutral buffer formaldehyde in phosphate-buffered saline (PBS, pH = 7.4) overnight. Tissues were cut into 5 µm slices and stained with hematoxylin and eosin (HE) and observed under light microscopy (Olympus, Japan). Pathological grading and scoring criteria26 were shown in Table 1. For each pathological section, 10 visual fields under a high-power microscope ($\times 400$) were randomly selected and scored. The mean score of 10 visual fields from one pathological section was calculated as the pathological score.

**Determination of concentrations of cytokines in pancreatic acinar cells**

The levels of amylase, IL-6, TNF-α, IL-1β and NF-κB of pancreatic acinar cells and pancreatic juice were measured by enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA, USA). The expressions of JAK-2 and STAT-3 were detected by Western blot assay.27 Briefly, the cellular supernatant was collected and the concentration of total protein was deter-

### Table 1 Pathological scoring criteria for pancreatic tissues of AP rats

<table>
<thead>
<tr>
<th>Item</th>
<th>Pathological change</th>
<th>Score</th>
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<tbody>
<tr>
<td>Edema</td>
<td>Focal expansion of interlobular septa</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Same as 1+ diffuse interlobar expansion; septa/diffuse expansion of interlobar septa</td>
<td>2</td>
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<tr>
<td></td>
<td>Same as 2+ expansion of interacinar septa</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Same as 3+ expansion of intercellular spaces</td>
<td>4</td>
</tr>
<tr>
<td>Inflammation and perivascular infiltrate</td>
<td>2-10 intralobular or perivascular leukocytes/HPF</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11-20 intralobular or perivascular leukocytes/HPF</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>21-30 intralobular or perivascular leukocytes/HPF</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt;30 leukocytes/HPF or confluent microabscesses</td>
<td>4</td>
</tr>
<tr>
<td>Acinar necrosis</td>
<td>Diffuse occurrence of 1-4 necrotic cells/HPF</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Diffuse occurrence of 5-10 necrotic cells/HPF</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Diffuse occurrence of 11-16 necrotic cells/HPF (foci of confluent necrosis)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt;16 necrotic cells/HPF (extensive confluent necrosis)</td>
<td>4</td>
</tr>
<tr>
<td>Hemorrhage and fat necrosis</td>
<td>1-2 focus</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3-4 focus</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5-6 focus</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt;7 focus</td>
<td>4</td>
</tr>
</tbody>
</table>

Note: HPF: high power field.
mined using BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA). An identical amount of proteins (50 μg) was denatured, heated for 5 min and subjected to 10% SDS-PAGE. The gel was then transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was then incubated in blocking buffer (5% fat milk in PBS) for 1 h at room temperature (RT), washed with PBS for three times with 10 min each time, and incubated in PBS overnight at 4 °C with JAK-2 antibody (1: 200), p-JAK-2 antibody (1: 500), STAT-3 antibody (1: 200) and p-STAT-3 antibody (1: 200), respectively. After washing membranes with PBS, an HRP-conjugated antibody (goat anti-rabbit, 1: 2000) was used as the secondary antibody and incubated at RT for another 1 h. The membranes were then processed using the enhanced chemiluminescence method (Thermo, Waltham, USA), and the protein bands were visualized by Gel imaging (Bio-Rad, Gel Doc XR⁺, Hercules, CA, USA).

Statistical analysis
Data are expressed as mean ± standard deviation ( x ± s). Data were analyzed with SPSS 25.0 (International business machines corporation, New York, NY, USA). Analysis of variance (ANOVA) followed by multiple comparison tests was performed to test the differences between groups. A P < 0.05 was considered to be statistically significant.

RESULTS
Screening the concentration of CQCQD-mediated serum
The viability rate of AR42J cells at different concentrations of CQCQD-mediated serum was shown in Figure 1. The result indicated that CQCQD-mediated serum began to cause significantly cell death when the concentration was above 10% in comparing with control AR42J cells. Thus, we investigated the effect of CQCQD-mediated serum at the concentration of 10% with viability rate of 98.48%.

Expression of cytokines in CQCQD treated AR42J cells and pancreas
The cellular supernatant and pancreatic juice were collected and the concentrations of amylase, IL-6, TNF-α, IL-1β and NF-κB were detected by ELISA kit, respectively. As shown in Tables 2 and 3, the trend of active constituents of pancreatic juice similar to that of active constituents in serum. The concentrations of amylase, IL-6, TNF-α, IL-1β and NF-κB in AP group were remarkably higher than that in normal group. However, the concentrations of amylase, IL-6, TNF-α, IL-1β and NF-κB in CQCQD group, almost closing to AG490 inhibitor group, were significantly decreased compared with that in AP group (Table 2). We found that the levels of amylase, IL-6, TNF-α, IL-1β and NF-κB at 24 h were slightly higher than that of at 12 h. In conclusion, CQCQD showed significantly efficacy in regulating the inflammatory cytokines in AP (Figure 2).

Histological assessment of pancreas
Pancreatic tissue structure of control group was clear. Most of the acinar leaflets were normal without interstitial edema, inflammatory cell infiltration, bleeding and necrosis (Figure 3A). The pancreatic tissue of AP group appeared interstitial edema and inflammatory cell infiltration, such as neutrophils and mononuclear cells around the necrotic area (Figure 3B). The pathologic alterations of CQCQD group (Figure 3C), AG490 group (Figure 3D) and CQCQD and AG490 group (Figure 3E) were decreased with slight hemorrhage exudates and infiltration of inflammatory. The result illustrated that CQCQD group had similar efficacy with AG490 group. The result of pathological scores (Figure 3F) were consistent with histological assessment with the 4 score of CQCQD group and 6 score of AP group.

JAKs and STATs phosphorylation status in vivo
The expression of these predicted target proteins were required to reveal the molecular mechanism. Western blot analysis was performed. AP group induced high expression of p-JAK-2 and p-STAT-3 compared with control group. However, the pretreatment with CQCQD remarkably inhibited caerulein-induced expressions of p-JAK-2 and p-STAT-3, similar to the treatment of AG490 inhibitor (Figure 4). The pretreatment of CQCQD successfully imitated the actions of the selective JAK/STAT signaling pathway inhibitor with inhibiting caerulein-mediated expressions of p-JAK-2, p-STAT-3 proteins. Therefore, inhibitive eff ects on expressions of p-JAK, p-STAT in AP were the key molecular mechanism for CQCQD.

DISCUSSION
CQCQD is a therapy for AP documented in On Harm Caused by Cold. Our study demonstrated that CQCQD ameliorated pancreatitis injury and reduced the level of serum amylase and inflammatory mediators in vitro and in vivo. Our study also demonstrated that
CQCQD could deactive JAK and STAT in AP. These results suggest that CQCQD is a safe and effective Traditional Chinese Medicine with great potential in clinical application in treating AP. IL-6, TNF-α, and IL-1β are pro-inflammatory cytokines in AP. Mononuclear macrophages and endothelial cells secrete high level of IL-6 participating in development of AP. The concentrations of pro-inflammatory cytokines, amylase, IL-6, TNF-α, and NF-κB were detected by enzyme-linked immuno-sorbent assay kit in AR42J cells. AP: acute pancreatitis; CQCQD: Chaiqin Chengqi decoction; IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor κB. AP group versus normal group, *p < 0.05; CQCQD group versus AP group, †p < 0.05.

Table 2 Pro-inflammatory cytokines release in AR42J cells (x ± s)

<table>
<thead>
<tr>
<th>Index</th>
<th>Normal group (n = 10)</th>
<th>AP group (n = 10)</th>
<th>CQCQD group (n = 10)</th>
<th>AG490 group (n = 10)</th>
<th>CQCQD and AG490 group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase (IU/L)</td>
<td>11.90 ± 2.10</td>
<td>1530.83 ± 233.45</td>
<td>1329.01 ± 141.98</td>
<td>2050.23 ± 119.84</td>
<td>1991.83 ± 210.63</td>
</tr>
<tr>
<td>IL-6 (ng/L)</td>
<td>2540.25 ± 175.51</td>
<td>193.22 ± 0.85</td>
<td>193.22 ± 0.85</td>
<td>178.23 ± 1.32</td>
<td>178.23 ± 1.32</td>
</tr>
<tr>
<td>IL-1β (ng/L)</td>
<td>1999.80 ± 120.10</td>
<td>1529.19 ± 138.22</td>
<td>1480.80 ± 101.39</td>
<td>1338.67 ± 97.63</td>
<td>1338.67 ± 97.63</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td>129.61 ± 5.03</td>
<td>197.19 ± 9.81</td>
<td>150.07 ± 2.10</td>
<td>139.84 ± 8.27</td>
<td>127.60 ± 1.58</td>
</tr>
<tr>
<td>NF-κB (ng/L)</td>
<td>345.63 ± 19.84</td>
<td>276.70 ± 14.05</td>
<td>270.50 ± 12.64</td>
<td>256.42 ± 20.43</td>
<td>256.42 ± 20.43</td>
</tr>
</tbody>
</table>

Notes: normal group: treating with normal saline; AP group: induced by 50 μg/kg caerulein; CQCQD group: intragastrically with 20 mL/kg CQCQD; AG490 group: intraperitoneal injection with 50 μM AG490 inhibitor; CQCQD and AG490 group: intragastrically with 20 mL/kg CQCQD and 50 μM AG490 inhibitor. The concentrations of pro-inflammatory cytokines, amylase, IL-6, TNF-α, IL-1β and NF-κB were detected by enzyme-linked immuno-sorbent assay kit in AR42J cells. AP: acute pancreatitis; CQCQD: Chaiqin Chengqi decoction; IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor κB. AP group versus normal group, *p < 0.05; CQCQD group versus AP group, †p < 0.05.

Figure 2 Expressions of five pro-inflammatory cytokines in five groups

A: serum amylase; B: IL-6; C: IL-1β; D: TNF-α; E: NF-κB. The concentrations of these cytokines were detected by enzyme-linked immuno-sorbent assay kit in AR42J cells. Normal group: treating with normal saline; AP group: induced by 50 μg/kg caerulein; CQCQD group: intragastrically with 20 mL/kg CQCQD; AG490 group: intraperitoneal injection with 50 μM AG490 inhibitor; CQCQD and AG490 group: intragastrically with 20 mL/kg CQCQD and 50 μM AG490 inhibitor. AP: acute pancreatitis; CQCQD: Chaiqin Chengqi decoction; IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor κB. Compared with normal group, *p < 0.05; compared with AP group, †p < 0.05.
In conclusion, our findings suggest that CQCQD has a protective effect on pancreatic tissue by down-regulating pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β and NF-κB. AP group versus normal group, *P < 0.05; CQCQD group versus AP group, **P < 0.05.

**Table 3 Pro-inflammatory cytokines release in pancreatic tissues ( x ± s )**

<table>
<thead>
<tr>
<th>Index</th>
<th>Normal group (n = 10)</th>
<th>AP group (n = 10)</th>
<th>CQCQD group (n = 10)</th>
<th>AG490 group (n = 10)</th>
<th>CQCQD and AG490 group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase (IU/L)</td>
<td>1833.5±200.3</td>
<td>3240.8±117.5</td>
<td>2761.3±102.0</td>
<td>2550.8±89.3</td>
<td>2091.2±190.6</td>
</tr>
<tr>
<td>IL-6 (ng/L)</td>
<td>156.6±10.0</td>
<td>352.6±18.6</td>
<td>279.3±1.6</td>
<td>238.6±8.2</td>
<td>198.2±3.3</td>
</tr>
<tr>
<td>IL-1β (ng/L)</td>
<td>1172.5±105.7</td>
<td>2189.8±117.1</td>
<td>1849.5±120.1</td>
<td>1548.1±91.9</td>
<td>1428.7±101.2</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td>135.2±5.8</td>
<td>247.2±9.7</td>
<td>188.6±3.0</td>
<td>152.8±8.5</td>
<td>139.6±1.5</td>
</tr>
</tbody>
</table>

Notes: normal group: treating with normal saline; AP group: induced by 50 μg/kg caerulein; CQCQD group: intragastrically with 20 mL/kg CQCQD; AG490 group: intraperitoneal injection with 50 μM AG490 inhibitor; CQCQD and AG490 group: intragastrically with 20 mL/kg CQCQD and 50 μM AG490 inhibitor. The concentrations of pro-inflammatory cytokines, amylase, IL-6, TNF-α, IL-1β and NF-κB were detected by enzyme-linked immunosorbent assay kit in pancreatic tissues. IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor kB. AP group versus normal group, *P < 0.05; CQCQD group versus AP group, **P < 0.05.

**REFERENCES**

The expression changes in Janus kinase (JAKs) and signal transducer and activator of transcription (STATs) phosphorylation status in vivo.

A: the Western blot assay of p-JAK-2 and p-STAT-3 proteins. 1: normal group; 2: AP group; 3: CQCQD group; 4: AG490 group; 5: CQCQD and AG490 group. Normal group: treating with normal saline; AP group: induced by 50 μg/kg caerulein; CQCQD group: intragastrically with 20 mL/kg CQCQD; AG490 group: intraperitoneal injection with 50 μM AG490 inhibitor; CQCQD and AG490 group: intragastrically with 20 mL/kg CQCQD and 50 μM AG490 inhibitor. AP: acute pancreatitis; CQCQD: Chaiqin Chengqi decoction. B: the ratios of JAK-2 and p-JAK-2 proteins compared with β-actin. C: the ratios of STAT-3 and p-STAT-3 proteins compared with β-actin.