Original Article
Da-Cheng-Qi Decoction attenuates inflammatory response and apoptosis via inhibiting NF-κB and PUMA mediated signaling pathways in acute pancreatitis

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Abstract: Objective: Da-Cheng-Qi Decoction (DCQD), a famous preparation of traditional Chinese medicine has used in treatment of acute pancreatitis (AP) for many years. However, the understanding at the molecular level of governing its action remains elusive. In this study, we investigated how DCQD alleviates the severity of AP and explores its mechanisms. Methods: AP was induced by infusion of the taurocholate into pancreatic duct of mice. Animals were treated with DCQD (20 g/kg, body weight) 2 hours of induction of pancreatitis. Serum IL-6, IL-8 and TNF-αlevels was measured by ELISA. NF-κB activity of pancreas was detected by EMSA. NF-κB p65, PUMA and activated caspase-3 was detected by western blot assay. Apoptosis was evaluated using a TUNEL assay. Results: Treatment of AP rats with DCQD significantly attenuated the inflammation in pancreas of AP rats; ELISA assay showed a considerable decrease in serum IL-6, IL-8 and TNF-α levels in DCQD treated AP rats compared to the saline-treated rats. DCQD significantly suppressed apoptosis and prevented activation of nuclear factor (NF)-κB and inhibited the expression of PUMA and activated-caspase-3 in the pancreas of AP rats. Conclusion: This study indicates that DCQD exhibits an anti-inflammatory and anti-apoptotic effect in cases of AP through inhibition of NF-κB activation and PUMA expression.

Keywords: Acute pancreatitis, Da-Cheng-Qi Decoction, NF-κB, PUMA

Introduction

The most serious complication during acute pancreatitis (AP) is the occurrence of multisystem organ failure (MOF) during the early stages. Mortality from acute pancreatitis is closely related to the development of early systemic complications, and the death rate of patients with such pathological conditions has been reported to reach 20-50% [1]. Several mediators such as activated pancreatic enzymes [2], cytokines [3], endotoxins [4], superoxides [5] and cell apoptosis [6] play important roles in the pathogenesis of AP, however, it remains difficult to develop an effective therapy.

NF-κB is a ubiquitously expressed transcription factor [7]. NF-κB-dependent gene expression plays an important role in a number of biological processes of major medical importance, including immune, inflammatory, and anti-apoptotic responses [8]. Many of the pro-inflammatory cytokines and proapoptotic proteins are known to be regulated by the transcription factor NF-κB [9-11]. Blockade of NF-κB has shown beneficial effects in animal models for these acute inflammatory diseases, including AP [12-14].

Da-Cheng-Qi Decoction (DCQD), a famous preparation of traditional Chinese medicine used in treatment of digestive diseases, is composed of *Dahuang* (Caulis Fibraureae), *Houpu* (Cortex Magnoliae Officinalis), *Zhishi* (immature bitter orange) and *Mangxiao* (Natrii Sulphas). DCQD

has been used to treat acute pancreatitis in the clinic [15-18]. A large, retrospective, placebo-controlled clinical trial indicated that DCQD can significantly reduce mortality in AP patients [19]. Recent studies using a rat model of AP have shown that DCQD induces beneficial apoptosis and attenuates histopathological changes in the pancreas of AP [20, 21].

Zhao et al. has found that DCQD could alleviate pancreatic, intestinal, and lung injury of AP by altering levels of anti-inflammatory (interleukin-4 and interleukin-10) and proinflammatory markers (tumor necrosis factor α and interleukin-6) and ameliorating the pathological damage in target tissues [22]. Wang et al. has reported that DCQD protected pancreas of AP via inducing apoptosis of injured acinar cells, decreasing necrosis, which help to avoid the release of digestive enzyme and various inflammatory mediators, significantly attenuating the progression of pancreatic injury [21]. However, the mechanisms and signaling of its protective of DCQD on AP is not clear.

Recent studies have shown that activated NF-κB could induce apoptosis by PUMA upregulation in vitro and in vivo [23-25]. The aim of the present study was to investigate the effects of DCQD in regulating cell apoptosis and the inflammatory response and explored the possible regulation mechanism of DCQD.

Materials and methods

Rats

Male Sprague-Dawley rats, weighing 200 to 250 g, were obtained from the Center of Experimental Animals of Shanghai, China. The animals were kept at room temperature and 12 h light dark cycles, and with free access to water.

Preparation of DCQD

The spray-dried drug powders of DCQD (Dahuang, Houpu, Zhishi, and Mangxiao) were purchased from Qingdao traditional Chinese medicine Limited by Share Ltd. (Qingdao, China). The spray-dried powder was mixed and reconstituted with sterile distilled water [22]. Extraction yield was approximately 12.6%. Before being orally administered to rats, the spray-dried DCQD was reconstituted with water to a concentration of 1 g/ml.

Taurocholate-induced pancreatitis

All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the affiliated hospital of Qingdao University. Rats were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium (35 mg/kg body weight) and the operation was performed under aseptic conditions. AP models were prepared according to the method reported by Kahl [26]. *Briefly,* after entering the abdomen via median epigastric incision, the bile-pancreatic duct, hepatic hilus and common hepatic duct were identified, and the duodenal papilla inside the duodenum duct wall was identified. A segmental epidural catheter was inserted into the duodenum cavity and then inserted into the bile-pancreatic duct towards the direction of papilla in a retrograde manner; two microvascular clamps were used to nip both ends of the bile-pancreatic duct, then 1.5 ml/kg body weight of sodium taurocholate (TAC) was injected into the bile-pancreatic duct with a microinfusion pump. Five minutes after injection, the microvascular clamp and epidural catheter were removed. After ensuring that there was no active bleeding in the abdominal cavity, the abdomen was closed. Intraductal perfusion of saline was applied to the sham-operated group.

DCQD administration

The AP animals were intragastrically administered DCQD (20 g/kg/BW) according to the previous report [21]. After treatment with DCQD for 48 h, the blood of vena caudalis was acquired and then centrifuged to obtain the serum for amylase examination. The pancreatic tissues were rapidly collected for pathological and apoptotic examinations as well as for PUMA and NF-κB activity assay.

Measurement of serum inflammatory

Serum inflammatory L-6, IL-8 and TNF-α levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (B&C Co.). All samples were tested in duplicate and expressed as the means.
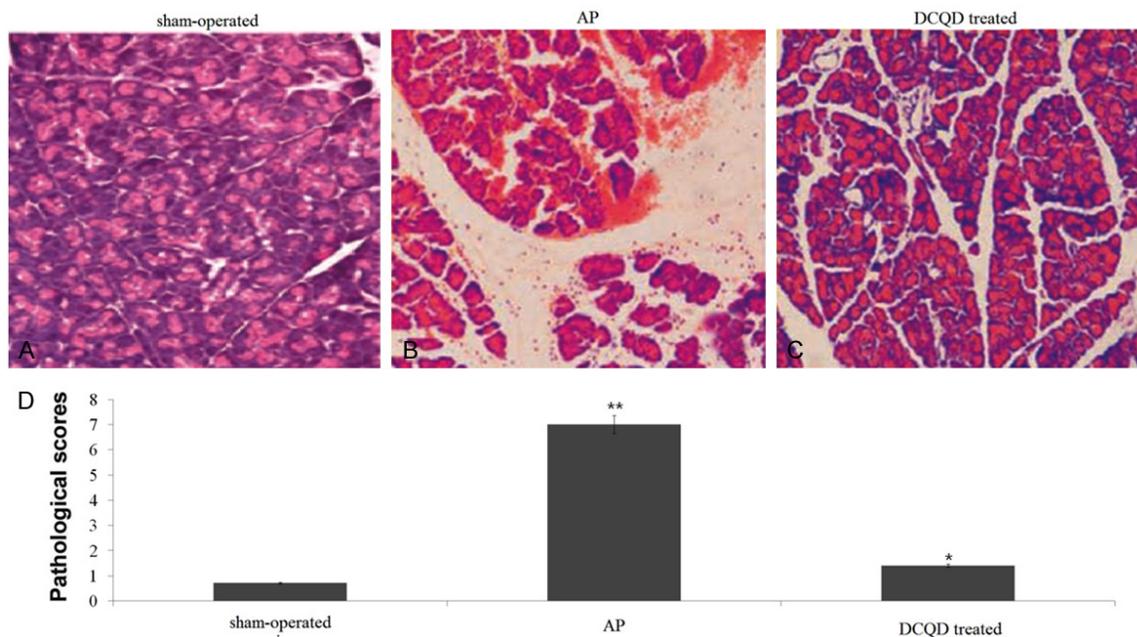
Figure 1. DCQD alleviated acute pancreatitis-associated tissue damage. Rats (n=20 per group) were given DCQD (20 g/kg body weight) 2 h after operation. After 48 h, the pancreatic tissues were collected for pathological examinations. A. HE staining of pancreas tissue in sham-treated groups (×200); B. AP group rats showed a severe degree of pancreatic damage with edema, hemorrhage, necrosis, pancreatic acinar cell vacuolization and infiltration of inflammatory cells (×200). C. In DCQD-treated group, rats showed a reduction of edema, hemorrhage, necrosis, and inflammatory cells infiltration (×200). D. Pathological scores of pancreatic injury. Vs AP, or VS sham-treated groups, *P<0.01, **P<0.01.

Histologic examination

According to routine procedures, paraffin sections of the pancreas tissue samples were prepared by HE staining, and histologic alterations of pancreas tissue were observed by light microscopy.

TUNEL staining

Sections (5 um) were subjected to hematoxylin and eosin (H&E) staining for histological analysis. TUNEL staining was performed using ApopTag kit (Chemicon International, Temecula, Shanghai, China) according to the manufacturer's instructions. The nuclei of the apoptotic cells exhibited brown staining, while those of non-apoptotic cells and the negative control were stained blue. The number of apoptotic cells was counted in each group and the apoptotic index (apoptotic cell number/total cell number ×100%).

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from pancreas (100 mg) tissues were prepared using a nuclear extraction

kit, according to the manufacturer's instructions. Briefly, 100 fmol of biotin end-labeled probe was incubated with 15 μg of nuclear extract, and then electrophoresed on a native polyacrylamide gel. The DNA was transferred to a nylon membrane, UV cross-linked, probed with the streptavidin-horseradish peroxidase conjugate, and chemiluminescent substrate was used for detection. 5'-biotin labeled NF-κB oligo, 5'-AGTTGAGGGGACTTTCCCAGGC-3', was purchased from Sigma-Aldrich. For competition experiments, 100-fold molar excess of unlabeled oligo was used.

Western blot assay

Western blotting was performed as previously described [30]. Antibodies included those against human PUMA, human phosphop65 (Ser536) and active caspase-3.

Statistical analysis

Student's t test or two way ANOVA was used for the statistical analysis of the results. The differences were considered to be significant when $P<0.05$.
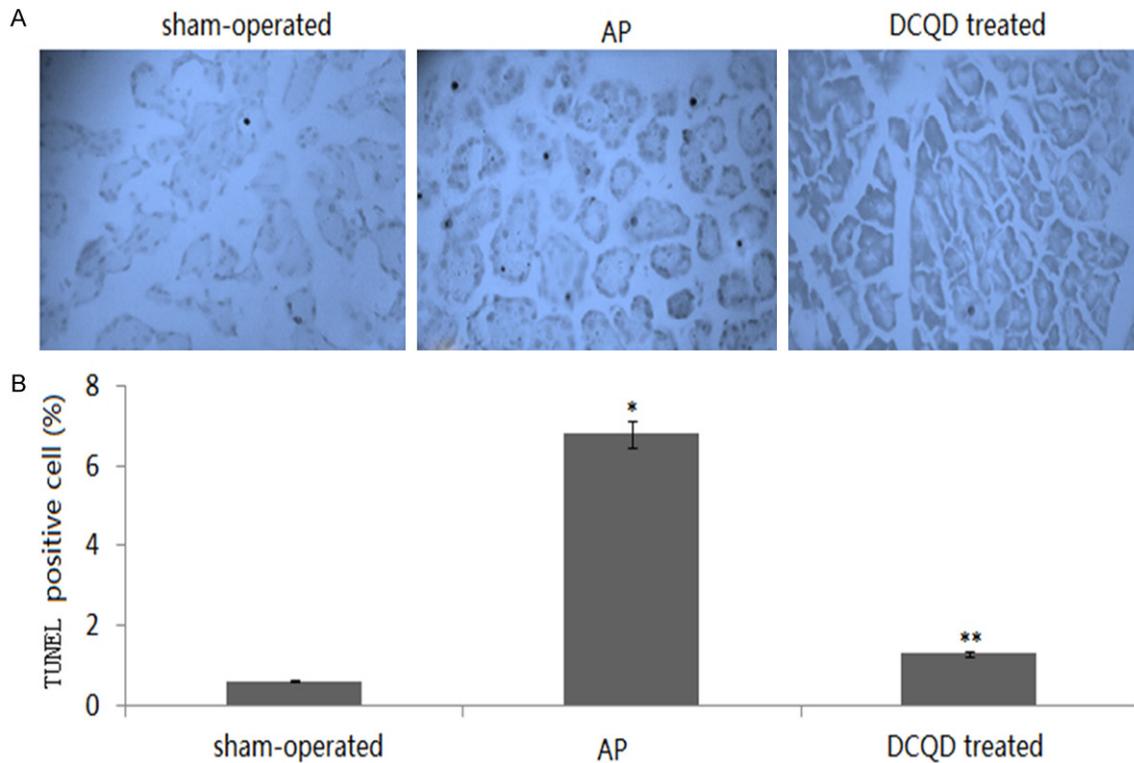
Figure 2. Effects of DCQD on acinar cell apoptosis in severe acute pancreatitis. Apoptotic acinar cells were detected by TUNEL, TUNEL-positive cells showed dark brown staining of the nucleus. A. sham-treated groups, AP and DCQD treated groups. B. Number of apoptotic cells in 10 fields countered at 200 magnification. *P<0.01 versus sham-treated group. **P<0.01 versus AP group.

Table 1. levels of IL-6, IL-8, and TNF-a in pancreatic tissue

	Sham-treated groups	AP groups	DCQD-treated groups
IL-6 (pg/ml)	140.5 ±23.8	773.0±34	201±27.4*
IL-8 (pg/ml)	126.4 ±14.5	638.7±24.3	136.4±27.4**
TNF-a (U/ml)	23.5 ±8.3	102.6±11.4	32.6±12.7**

Vs Sham-treated groups,*P<0.01, **P<0.05.

Results

Effects of DCQD on experimental acute pancreatitis

As shown in **Figure 1A**, intraperitoneal saline injections did not cause any significant histopathological changes of the pancreas; while intraductal administration of TCA induced typical features of AP, such as haemorrhagic necrotizing pancreatitis with massive haemorrhage, severe infiltration of inflammatory cells, and large areas of necrosis in the pancreas. However, in the DCQD-treated groups, reductions in pancreatic edema, hemorrhage, inflam-matory cell infiltration, and acinar cell necrosis was found compared to the TCA-groups. In addition, histopathology score were reduced significantly more in the DCQD-treated groups (**Figure 1B**). In the sham-operated group, there was no obvious change of histopathology compared to the normal pancreas (**Figure 1A**).

DCQD reduced the cell apoptosis of AP tissues

Analyzing apoptosis in situ by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling staining revealed significant cell apoptosis in the AP tissues. However, cell apoptosis was significantly decreased in the DCQD treated groups (**Figure 2A**, **2B**). These data show that DCQD can effectively inhibit pancreas cell apoptosis of AP.

DCQD reduced serum inflammatory of AP

To determine the effect of DCQD on the serum levels of inflammatory, serum IL-8, IL-6, and TNF-alpha, were measured using ELISA. Compared with the sham group, the serum lev-
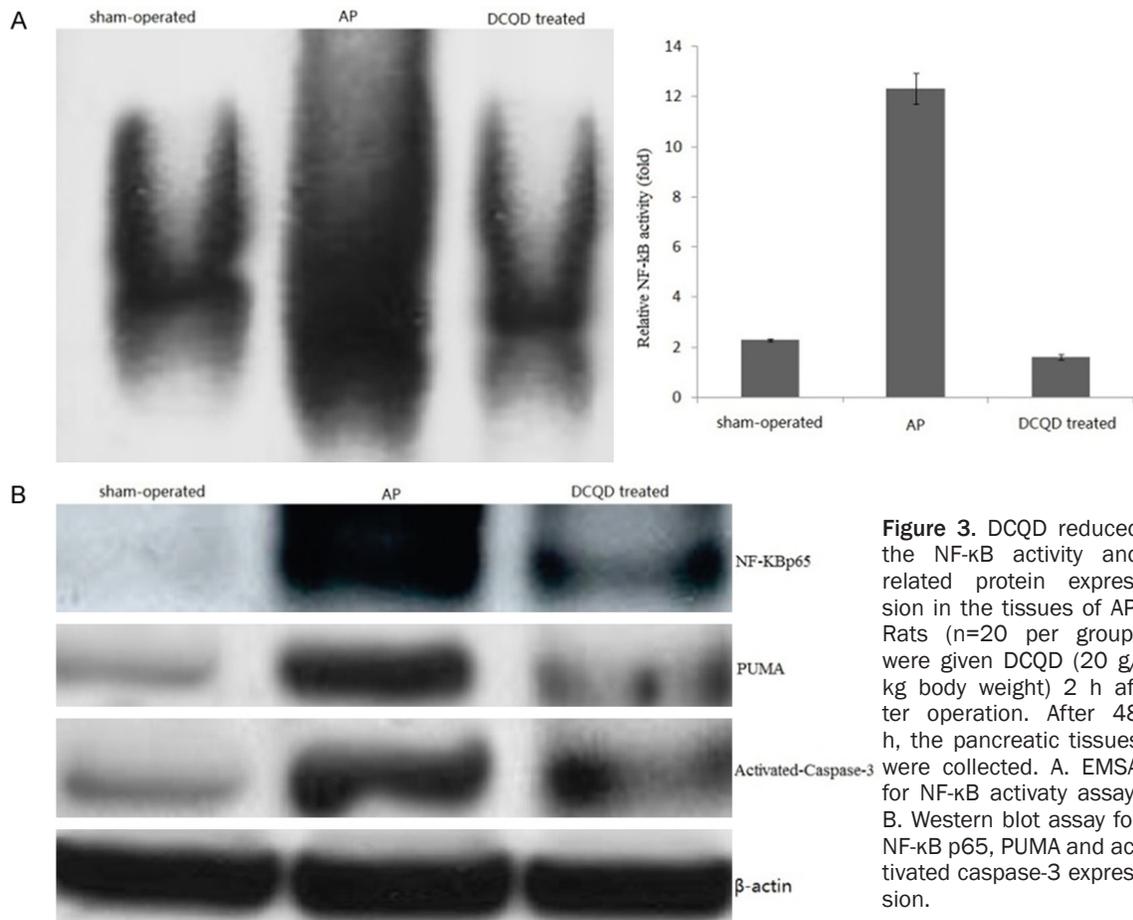
Figure 3. DCQD reduced the NF-κB activity and related protein expression in the tissues of AP. Rats (n=20 per group) were given DCQD (20 g/kg body weight) 2 h after operation. After 48 h, the pancreatic tissues were collected. A. EMSA for NF-κB activaty assay; B. Western blot assay for NF-κB p65, PUMA and activated caspase-3 expression.

els of IL-8, IL-6, and TNF-alpha, especially the proinflammatory cytokine IL-6, in the AP groups increased significantly (**Table 1**). However, the levels of IL-8, IL-6, and TNF-alpha in DCQD groups were significantly lower compared with those in the AP group (**Table 1**). These results indicated that AP increased the levels of IL-8, IL-6, and TNF-alpha in the serum; however, this increase was inhibited by administration of DCQD.

DCQD inhibits NF-κB activity

The NF-κB activity was markedly higher in AP groups (11.3±2.4) than sham operation group (defined as 1) at 48 h (*P*<0.01). Treatment with DCQD significantly inhibited the NF-κB activity in AP groups (2.5±0.6) (**Figure 3A**).

DCQD inhibited NF-κB p65, PUMA and activated caspase-3 expression

Western blot analysis showed that the NF-κB p65, PUMA and activated caspase-3 expres-

sion was notably increased in the AP groups compared with the sham operation groups (**Figure 3B**). To determine the contribution of NF-κB p65, PUMA and activated caspase-3 in AP, we treated mice with AP using DCQD. We observed that treatment with DCQD reduced the protein expression above (**Figure 3B**).

Discussion

Acute pancreatitis (AP) is a serious medical disorder with no current therapies directed to the molecular pathogenesis of the disorder. Inflammation, inappropriate intracellular activation of digestive enzymes, and parenchymal acinar cell death by necrosis are the critical pathophysiologic processes of acute pancreatitis [27-29]. It is becoming increasingly clear that activation of intense inflammatory signaling mechanisms in acinar cells is crucial to the pathogenesis of pancreatitis, which may explain the strong systemic inflammatory response in pancreatitis [28].

Activation of NF-κB within the acinar cell could lead to AP. Its significance is only recently becoming apparent [30]. Although activation of NF-κB is related with the pathophysiologic processes of AP, p65/RelA silencing in the myeloid cells alone did not lead to any difference in pancreatitis response compared with that in wild type mice [31], suggesting there is broad spectrum of NF-κB -dependent processes. Besides as mediator of the immune and inflammatory response, NF-κB pathways also contribute to cell adhesion, differentiation, proliferation and protection against apoptosis [32].

DCQD for AP therapy has a long history and has recently attracted many attentions. In this study, we observed whether DCQD alleviates the severity of AP by blocking the activation of NF-κB. In this study, we found that NF-κB was activated at 48 h following the induction of AP. However, DCQD significantly reversed p65 expression and NF-κB activity during AP, followed by the reduction of the edema, necrosis, inflammation and cell apoptosis in pancreatic tissue of AP, suggesting that DCQD could improve the pancreatic pathological changes and effectively protect pancreatic tissue of AP through inactivation of NF-κB.

It has proved that local and systemic complications and organ failure is responsible for the initial mortality of the patients with AP [33]. The main mechanisms responsible for this pathophysiologic processes are pro-inflammatory cytokines and chemokines [34, 35]. As an initiator in the occurrence of AP, TNF-α is one of important cytokines affecting AP and plays a proinflammatory role in AP at early stage. TNF-α is the primary member of the inflammatory cytokine family, primary inducers of IL-6 and IL-8 production, and known to initiate and propagate nearly all of the detrimental consequences of severe sepsis [33, 34]. Nearly all experimental models of pancreatitis have implicated TNF-α, IL-6 and IL-8 as a major pathologic cytokine associated with local and systemic tissue destruction. In this study, we found that IL-8, IL-6, and TNF-alpha was significantly increased in the AP groups. Treatment with DCQD significantly decreased IL-8, IL-6, and TNF-alpha in the AP groups, suggesting that DCQD protected pancreas from inhibition of proinflammatory cytokine.

PUMA (p53 upregulated modulator of apoptosis) as a BH3-only Bcl-2 family protein that plays an important role in p53-dependent and -independent apoptosis [36, 37]. Upon transcriptional induction in response to DNA damage, PUMA functions through inducing mitochondrial dysfunction and caspase activation [38]. Our study showed that PUMA and its downstream caspase-3 protein was rapidly induced in the pancreas of AP, followed by increased cell apoptosis. DCQD treatment blocked PUMA and caspase-3 activity and inhibited cell apoptosis in the pancreas of AP models, suggesting that DCQD induces apoptosis through inhibition of PUMA expression.

Conclusion

In conclusion, our data demonstrate that DCQD attenuates the severity of TAC-induced experimental AP in rats. DCQD exhibits an anti-inflammatory and anti-apoptotic effect in cases of AP through inhibition of NF-κB activation and PUMA expression.

Acknowledgements

Disclosure of conflict of interest

None.

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