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Original Paper

Dachengqi Decoction Attenuates Inflammatory Response via Inhibiting HMGB1 Mediated NF-κB and P38 MAPK Signaling Pathways in Severe Acute Pancreatitis

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Key Words
Dachengqi decoction • Severe acute pancreatitis • HMGB1 • TLRs • NF-κB signaling pathway • MAPK signaling pathway

Abstract
Background/Aims: Severe acute pancreatitis (SAP) is a sudden inflammation of the pancreas. The traditional Chinese medicine formula Dachengqi decoction (DCQD) is proven to be beneficial in the comprehensive treatment for pancreatitis patients in clinical practice. However, the molecular mechanism of DCQD on SAP remains unclear. High mobility group box 1(HMGB1) that functions as a damage-associated molecular pattern molecule (DAMP) has attracted much interest. ***Methods:*** In this study, we used lipopolysaccharide (LPS) and cerulein to induce severe acute pancreatitis in C57BL/6 mice with subsequent administration with low, medium and high dose (2.3 g/kg, 7 g/kg and 21 g/kg, respectively) of DCQD. ***Results:*** DCQD treatment improved the pathological score and decreased serum amylase and lipase in a dose-dependent manner. In addition, it suppressed the immune cell-induced secretion of HMGB1 and its translocation from the nucleus to the cytoplasm, thus repressing the expression of IL-6 and TNF-α. Further, pretreatment with DCQD decreased responses of TLRs, and suppressed the activation of NF-κB and p38 MAPK pathway. ***Conclusion:*** Decreasing the secretion of HMGB1 could reduce pro-inflammatory cytokines, which may help cutting down the risks of development from localized pathological changes to a systemic inflammatory response syndrome and even lead to multiple organ failure.

Z. Cheng and Y. Chen contributed to the study equally.

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Introduction

Severe Acute pancreatitis (SAP), induced by multiple etiologic factors, is the activation of trypsin, which leads to inflammation responses of self-digestion, edema, hemorrhage or even necrosis of the pancreas [1]. The pathomechanism of SAP has not been fully investigated, whatever the reason of its pathopoiesis, SAP would eventually induce regional and systematic inflammation responses, as well as excessive production of pro-inflammatory cytokines and waterfall-like cascade reaction triggered by cytokines. Uncontrolled inflammation responses usually result in systemic inflammatory response syndrome (SIRS) and multipleorgan dysfunction syndrome (MODs), which have contributed to the high mortality in SAP [2]. Therefore, discovering new treatments or drugs to control the release of inflammatory factors is urgent.

More and more researches have shown that damage associated molecular patterns (DAMPs), including high mobility group box 1(HMGB1) and TOLL-like receptors (TLRs) that are secreted by impaired or necrotic pancreatic acinar cells, have played an important role in the pathogenesis of SAP, and is pivotal in the progress to develop from regional to systematic inflammation [3]. HMGB1 exists primarily in the nucleus. When cells are under stress, HMGB1 will be released out from nucleus to extracellular, then promote monocytes and macrophages to secret pro-inflammatory factor, which would in turn promote HMGB1 secretion. In this way, a positive feedback loop is established to amplify the inflammatory response [4]. Toll-like receptors are a series of pattern-recognition receptors (PRRs) that show important function in many diseases. Many researches have revealed that TLR4 responds to DAMP, and TLR9 participates in the development of SAP as well [5]. TLRs mainly activate MyD88-dependent pathway, TNF-associated factor 6 and MAPK signal pathway, which then activate NF-κB or AP-1 and promote the transcription of pro-inflammatory genes [6]. NF-κB is shown to be activated in the early stage of SAP and leads to up-regulated cytokine and adherence factor expression, infiltration of inflammatory cells in pancreatic tissues and thus exacerbates the inflammation [7]. In that case, inhibiting the function of NF-κB would relieve the condition of severe SAP.

Dachengqi decoction (DCQD) is a classical prescription in SAP therapy written in Shang Han Lun (Treatise on Febrile Diseases). It has been published that DCQD can decrease intra-abdominal pressure, relieve abdominal pain, improve recovery of intestinal function, and decrease complications [8]. Its active ingredients include rhein, emodin, chrysophanol, aloeemodin, physcion, magnolol, honokiol, hesperidin, naringin, and so forth. Recent research indicates its pharmacological effect of promoting intestinal motility and reducing intra-abdominal pressure, protecting immunological barrier of the intestinal mucosa and improving intestinal function [9]; it can also regulate the expression of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-18, reduce oxygen radical and ameliorate lipid emulsion [10], and promote the apoptosis of pancreatic acinar cells [11]. However, the specific mechanism and signaling pathway of its protective effects on acinar cells still requires further investigation.

In our research, we demonstrated DCQD's regulation on NF-κB and MAPK pathway and responding effects of HMBG1-TLRs in LPS-cerulein-induced SAP animal models. We also proposed the possible mechanism of its inhibition in inflammatory factors and therapeutic effects in SAP.

Material and Methods

Drugs and Reagent

DCQD is composed of *Dahuang (Radix et Rhizoma)*, *Houpu (Cortex MagnoliaeOfficinalis)*, *Zhishi (FructusAurantiiImmaturus)* and *Mangxiao (NatriiSulphas)* (Shanghai Hua Yu Chinese Herbs Co., LTD., China). 1200 g *Dahuang*, 750 g *Mangxiao*, 900 g *Zhishi*, 900 g *Houbu* were pulverized and then percolated in 60% ethanol for 24 hours. After 24 hours, 3000 ml of percolated liquid was harvested in 1-3 ml/min and

an additional 60% ethanol was added for full active reagent extraction. *Mangxiao* was ground and filtered by 80-mesh sieve and *Zhishi* and *Houpu* were boiled for 2 hours for the first time and 1.5 hours for the second time, and were then harvested, filtered and concentrated. Then 95% ethanol (3 × (3%) were added and left for 24 hours. Finally, the pharmaceuticals were filtered and mixed with *Dahuang* percolate. Ethanol was reduce- pressure-distillated and mixed with *Mangxiao*, filtered by 10-mesh sieve, dried to powder. The extraction rate is 13.4%.

Reagents were purchased as follows: Cerulein, LPS, Collagenase IV, trypsin inhibitor (Sigma, USA); 100 μm filter (Beckton Dickinson, USA); Hank's Balanced Salt Solution (Gibco, USA); Goat-antirabbit antibody (Cell Signaling Technology, USA); ECL developing liquid (Billerica, USA); Immunohistostaining Kit, DAB Staining Kit (Boster, China).

Animal model and DCQD administration

This research was approved by the Ethical Committee of Shanghai Putuo Central hospital affiliated to Shanghai University of Traditional Chinese Medicine. Ninety male C57BL/6 mice (weighed 18 ± 2g) were purchased from Slac Laboratory Animal Co., Ltd (Shanghai, China) and raised in specific pathogen-free (SPF) animal room. The mice were adaptively fed for one week and divided into ControlGroup, Acute Pancreatitis Group (SAP Group), Low-dose DCQD-SAP Combined Group (L-DCQD Group) (DCQD = 2.3 g/kg), Medium-dose DCQD-SAP Combined Group (M-DCQD Group) (DCQD = 7 g/kg) and High-dose DCQD-SAP Combined Group (H-DCQD Group) (DCQD = 21 g/kg). DCQD, obtained according to protocol above, was dissolved in sterile water to different concentrations at 0.3 g/ml, 0.9 g/ml and 2.7 g/ml. The mice were gavaged every 8 hours with DCQD and were fasted 1 day before the administration. SAP was induced by 4 times of abdominal injection ofcerulein (50 μg/kg/h) and 2 times of LPS-cerulein combined injection (cerulean = 50 μg/kg/h, LPS = 7.5 mg/kg LPS). 18 hours after the injection, the mice were sacrificed and the blood was collected via retro-orbital plexus, and pancreas was harvested, fixed with 10% formalin for pathology and immunohistochemistry for one part, with the other part preserved in -80° for protein assays.

SerumAmylase and Lipase activity assay

Blood wascollected via retro-orbital plexus, left for 1 h and centrifuged (2000 rpm, 4°C) for 15 min. Amylase and Lipase concentration was analysed with Amylase activity colorimetric assay kit and Lipase activity colorimetric assay kit (BioVision, Inc., California, USA). Instructions were provided by the manufacturer.

Serum IL-6 and TNF-αassay

IL-6 and TNF-α concentration was determined by Mouse IL-6 ELISA Kit and mouse TNF-α ELISA Kit (Cusabio Biotech Co., Ltd., China) according to manufacturer's protocol.

Histology and immunohistochemistry

Pancreatic tissue was fixed in 10% neutral formalin for 24 hours, and was sliced in 5μm-sizeddices for HE (hematoxylin and eosin) staining. Double-blind microscopic examination was performed by specialists. Five random high power fields were selected from each slice to observe the pathological changes according to pathologists.

The ABC method of immunohistochemistry for TLR4 and TLR9 assay was performed. Primary antibodies (1:100) of TLR4 and TLR9 (Abcam, Cambridge, MA, USA) were added and incubated in 4°C overnight and then incubated in 37°C for 1 hours and washed by 0.01 ml/L PBS. Goat-anti-rabbit secondary antibody was added and incubated at 37°C for 20 min. After DAB staining for 10 min, hematoxylin staining was performed for 30 seconds. Immunohistochemistry sections were observed in 10 × and 40× microscopy and Image Pro Plus was used for calculating average optical density and then data were statistically analyzed.

Immunofluorescence

Fresh pancreatic tissues were frozen sectioned and then incubated in 5% FBS for 30min. HMGB1 antibody (1:500) (Abcam, Cambridge, MA, USA) was incubated overnight and secondary antibody was added (Cy3-AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch, USA)) and incubated at room temperature for 1 hour. The nucleus was stained by DAPI and sections were sealed by glycerinum. 40 x microscopy was used for observation.

Western blot

RIPA lysate was added to pancreatic tissues (1 ml lysate for 1 ml tissue) on ice for 1 hour and then centrifuged at 12,000 rpm for 15 min. Supernatant was harvested and protein concentration were measured according to BCA Kit. 30 μg protein of each sample was used for SDS-PAGE and then blocked by 5% BSA sealing fluid for 1 h at room temperature. Primary antibodies were added: HMGB1 (1:2000), TLR4 (1:1000), TLR9 (1:1000), GAPDH (1:1000) (Abcam, Cambridge, MA, USA), NF-κB, iκB, ERK, p-ERK, JNK, p-JNK, p38, p-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA), diluted at 1:1000 ratio. After 4°C incubation overnight and 10-min washing by TBST buffer for three times, ECL developer was added and figured by Bio-rad Gel Dol EZ. Grey value analysis of target bars was performed by Image J software.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad Prism software Inc., San Diego, CA, USA) was used for statistical analysis and one-way ANOVA was used for multi-group comparison. $P < 0.05$ was considered to be statistically significant.

Results

Pathological and functional changes of pancreas by DCQD treatment

HE stain results showed that in Control Group, the pancreatic lobule was intact with well-defined borders and no obvious edema, infiltration or necrotic lesions observed (Fig. 1a). While in SAP Group, the lobule structural was damaged and lobular spaces became widen. Hemorrhage, coagulative necrosis and adiponecrosis were observed (Fig. 1b). Statistical significance of pathological score existed between SAP Group and Controls (Fig. 1f). Similar alterations were also observed in L-DCQD Group and no statistical significance was observed compared with SAP group (Fig. 1c). In M-DCQD group, overall pathological changes were greatly improved than SAP group (Fig. 1d). In H-DCQD Group, no obvious damage was observed in lobule structure and mild edema with little infiltration of inflammatory cells

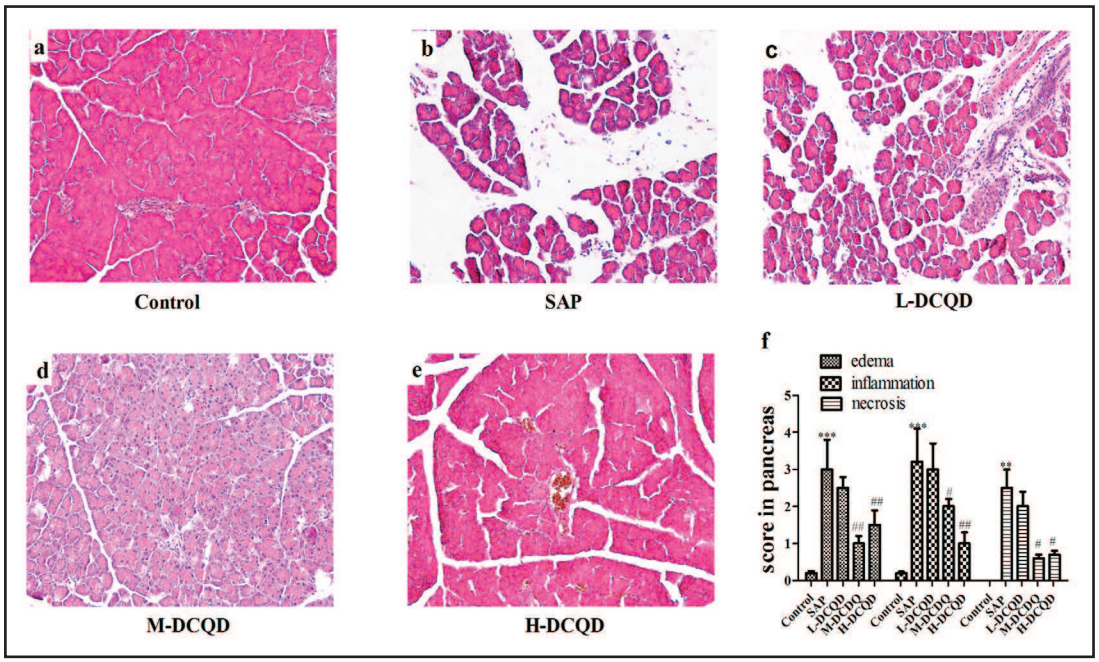

Fig. 1. Pathological changes of pancreas by DCQD treatment. (a-e) HE staining of Control group, SAP group, L-DCQD group, M-DCQD group and H-DCQD group); (f) Pathological score of edema, inflammation and necrosis. *** $P<0.001$, ** $P<0.01$, compared with Control group; # $P<0.05$, ## $P<0.01$, compared with SAP group. n=12-15.

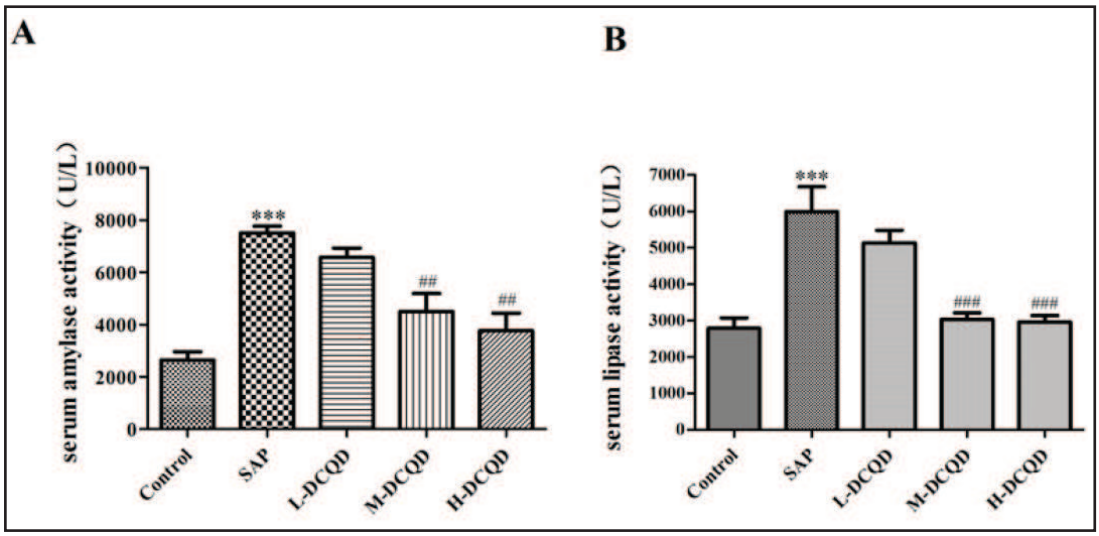

Fig. 2. Functional changes of pancreas by DCQD treatment. (A-B) ELISA results of serum amylase and lipase. *** P<0.001, ** P<0.01, compared with Control group; # P<0.05, ## P<0.01, compared with SAP group. n=12-15.

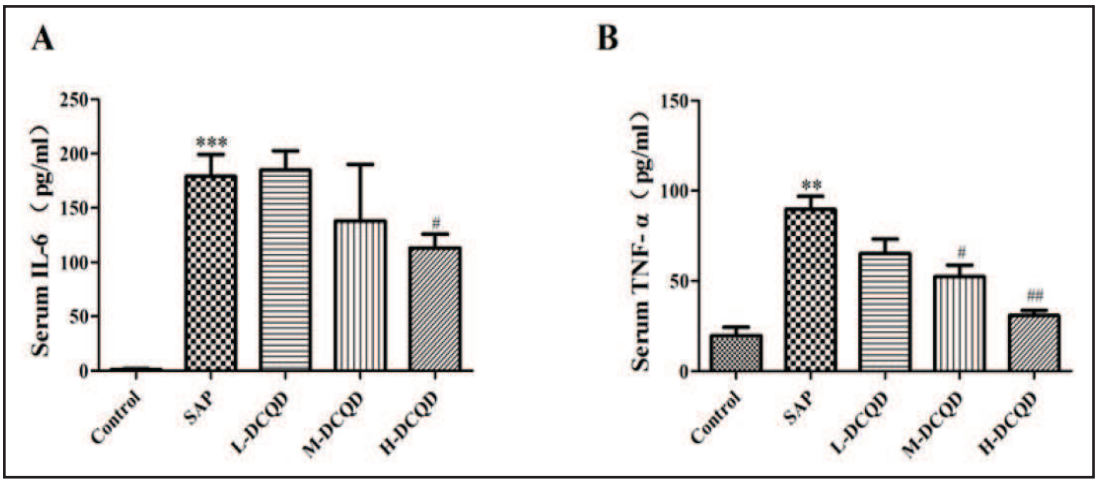

Fig. 3. Effect of DCQD treatment to IL-6 and TNF-α expression. (A-B) ELISA results of serum IL-6 and TNF-α. *** P<0.001, ** P<0.01, compared with Control group; # P<0.05, ## P<0.01, compared with SAP group. n=12-15.

(Fig. 1e). The score of H-DCQD and M-DCQD group was significantly decreased compared to SAP group and pancreatic damage was relieved (Fig. 1f).

The results of serum amylase and lipase assay also indicated that in SAP group, amylase and lipase were significantly increased compared to Controls, which indicated that the mouse SAP model induced by cerulein and LPS was successful. In M-DCQD an H-DCQD group, the expression of amylase and lipase were enormously inhibited in a dose-dependent manner (Fig. 2). Combine with Fig. 1 showed the protective effects of DCQD in pathological improvement, amylase and lipase secretion inhibition, and finally maintained the function of the pancreas.

DCQD suppressed the expression of IL-6 and TNF-α

Serum concentration of IL-6 is regarded as an index to evaluate severity and changes of SAP [12]. Meanwhile, TNF-α is a key intrinsic pro-inflammatory cytokine that is released primarily in inflammatory responses [13]. Thus, we determined the concentration of IL-6

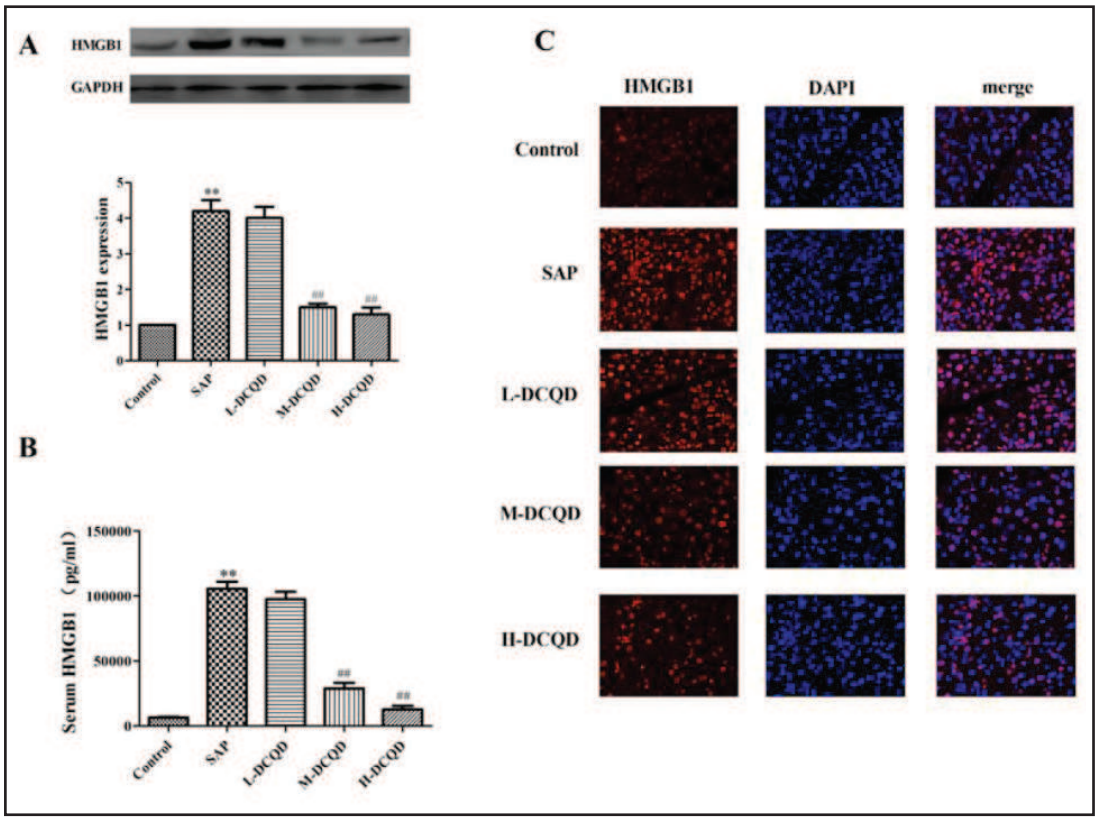

Fig. 4. Effect of DCQD treatment to HMGB1 expression. (A) Western blot analysis and statistical results for HMGB1 expression in pancreatic tissues (B) ELISA results of serum HMGB1 expression after DCQD treatment. (C) Immunofluorescence results of HMGB1 translocation after DCQD treatment. ** $P<0.01$, compared with Control group; ## $P<0.01$, Compared with SAP group. n=3-5.

and TNF-α after DCQD administration. As shown in Fig. 3a, IL-6 was highly elevated in SAP group whereas absent in Control group. After DCQD administration, though limited changes were found in L-DCQD group, IL-6 was decreased in M-DCQD group and inhibited almost half of which in H-DCQD group compared with SAP group. Similar expression pattern was also observed in TNF-α, whose expression was inhibited in a dose-dependent manner following DCQD treatment.

Effect of DCQD on expression of HMGB1in different tissues

We next analyzed the expression of HMGB1 expression in pancreatic tissues and serum. Nuclear HMGB1 is a DNA binding protein of high affinity that can directly interact with nucleosome to maintain the structure, function and stability of chromatin. The secretion of extracellular HMGB1 could promote waterfall-like cascade in inflammation responses [14]. As shown in Fig. 4a, HMGB1 expression was highly increased in SAP group compared with Controls but it was decreased in M-DCQD and H-DCQD group. We further analyzed the serum HMBG1 concentration after DCQD treated. The results (Fig. 4b) showed that HMGB1 was significantly increased in SAP group and with the increasing concentration of DCQD treatment, HMGB1 was gradually decreased, in H-DCQD group almost approached to that in Controls. Additionally, we located the expression of HMGB1 through immunofluorescence and found that HMGB1 expression in cytoplasm or extracellular matrix was increased. But after the DCQD treatment, HMGB1 was decreased and was mainly expressed in the nucleus (Fig. 4c). So DCQD could suppress the secretion of HMGB1 and its translocation from the nucleus to the cytoplasm.

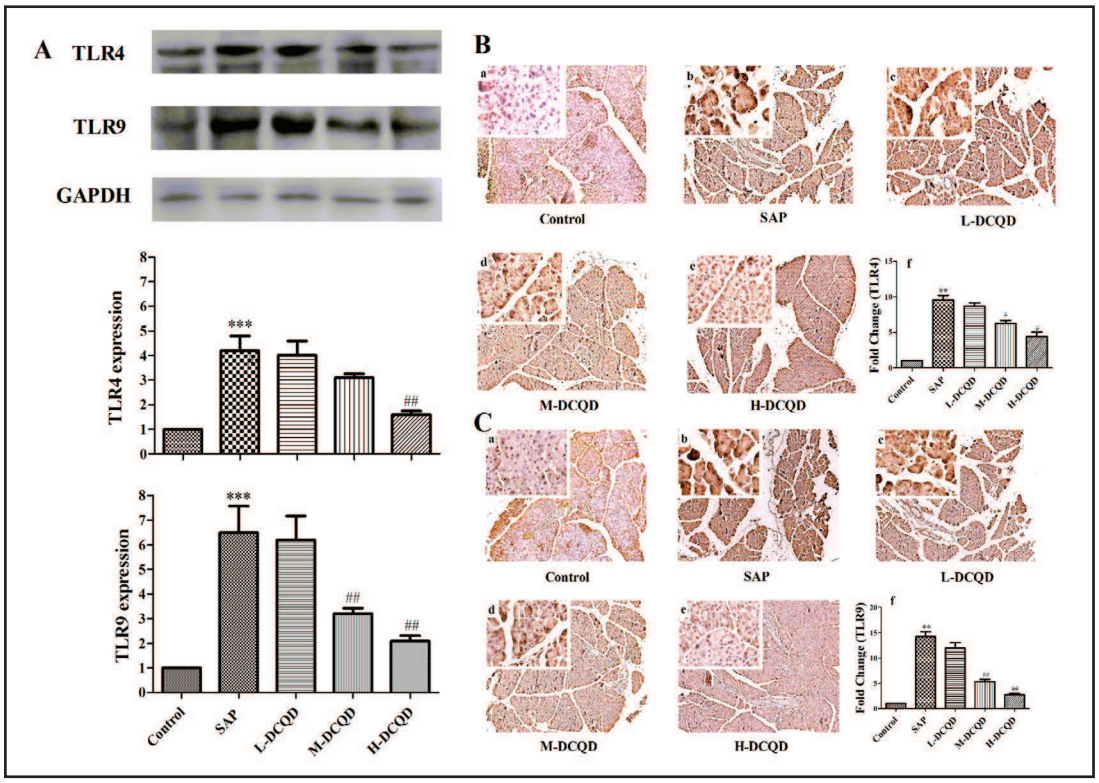

Fig. 5. Effect of DCQD treatment to TLRs expression. (A) Western blot analysis and statistical results for TLR4 and TLR9 expression in pancreatic tissues; (B –C) immunohistochemistry assay and statistical results for TLR4 and TLR9 expression, Image Pro Plus (iPP) software was used for analyzing the immunohistochemistry results ; *** P<0.001, compared with Control group; # P<0.05, ## P<0.01, compared with SAP group. n=3-5.

DCQD suppress the responses of TLRs

TLRs are the initial step and core of antigen recognition and signal transduction in inflammatory immune responses [15]. We further analyzed the expression of TLR4 and TLR9 in pancreatic tissues through Western blot and immunohistochemistry. As shown in Fig. 5a, TLR4 and TLR9 were highly expressed in SAP group with statistical significance compared with Controls. After treatment of L-, M- and H-DCQD, TLR4 and TLR9 expression were decreased in a dose-dependent manner. In addition, results of immunohistochemistry showed strong-positive expression in SAP group, mainly located in cytoplasm and extracellular. The expression was then suppressed after the DCQD treatment, which is similar to results of Western blot (Fig. 5b-c).

DCQD inhibits the activation of NF-κB and MAPK pathways

In the end, we demonstrated the influence to TLRs down-stream signal pathway in DCQD treatment. NF-κB is an important transcription factor to regulate the expression of pro-inflammatory cytokines. As shown in Fig. 6, NF-κB was more than four-times elevated in SAP group and its inhibitory factor iκB, was degraded after phosphorylated, showing decreased expression. However, after the DCQD treatment, the activation of NF-κB as well as the degradation of iκB were suppressed and returned to normal expression feature with a dose-dependent manner compared with Controls. Moreover, by analyzing three signal pathway of MAPK, we found that DCQD mainly inhibited the phosphorylation of p38 but didn't influence ERK or JNK pathway (Fig. 7). Thus, we propose DCQD treatment may inhibit the pro-inflammatory cytokines expression through suppressing p38 pathway and the activation of NF-κB.

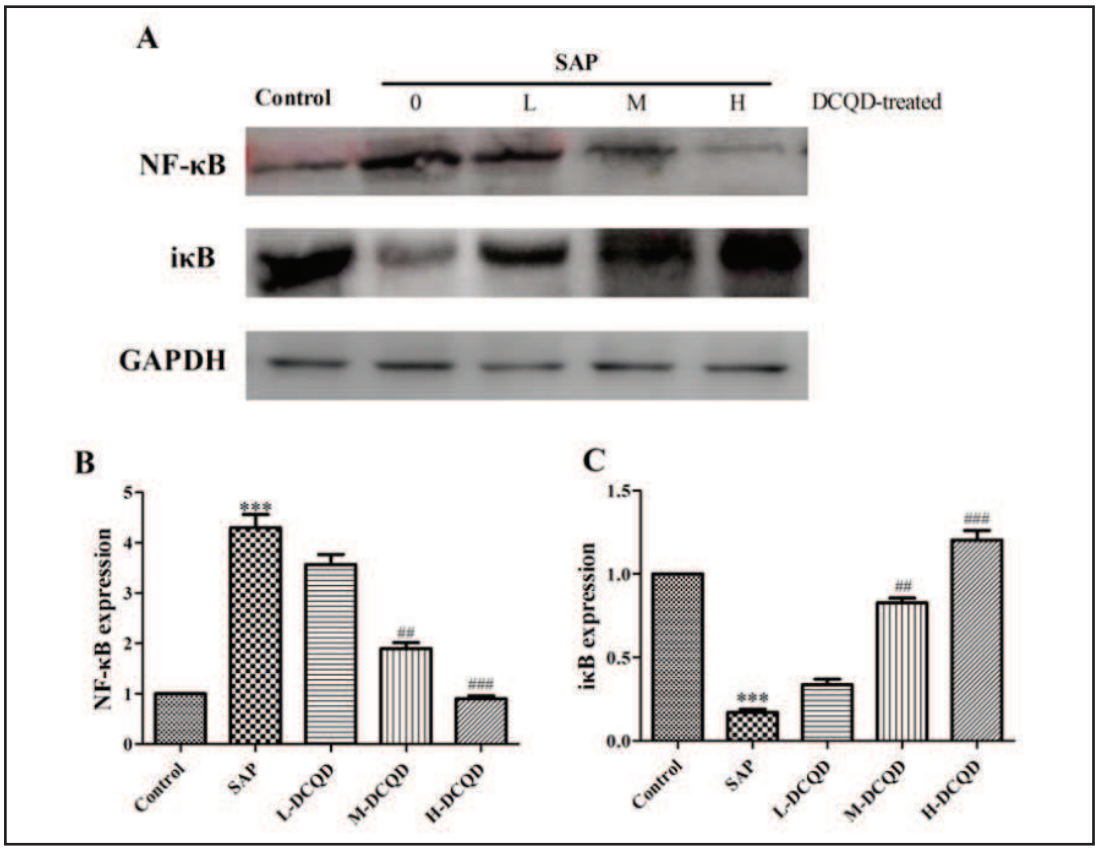

Fig. 6. Effect of DCQD treatment to NF-κB signal pathway. (A) Western blot results of NF-κB and iκB expression in pancreatic tissues. GAPDH was used as a loading control. Western blots were representative of three independent experiments. (B-C) Statistical results of A. Scanning densitometry was used for semi quantitative analysis in compared to control group levels; *** P<0.001, compared with Control group; # P<0.05, ## P<0.01, compared with SAP group. n=3-5.

Discussion

DCQD for SAP therapy has a long history and has recently attracted many attentions. In our study, we used cerulein and LPS to induce mouse model with SAP and found that DCQD treatment improved the pathological score and pancreatic function in a dose-dependent manner. In addition, it suppressed the immune cell-induced secretion of HMGB1 and translocation from the nucleus to the cytoplasm, decreased responses of TLRs receptor, suppressed the activation of NF-κB and p38 MAPK pathway, thus decreasing the secretion of pro-inflammatory cytokines and reducing the risks of developing from local pathological changes to SIRS and MODs. In addition, a research by Jia Wang [16] found that DCQD could regulate iNOS and ROS, increase the apoptosis ratio of acinar cells and protect pancreatic acinar cells from cellular injury [17]. Thus, DCQD treatment is regarded as a possible key therapy for SAP.

In our research, we proved the therapeutic effects of DCQD to SAP and found after DCQD treatment, edema, necrosis and inflammation were reduced in pancreatic tissue. Serum amylase and lipase were significantly inhibited, especially in H-DCQD group (Fig. 1-2). However, we also found in H-DCQD group, the general condition of mouse was worse than M-DCQD group. This could result from that high dose medication may induce potential toxicity and we believe that's why the dose used in clinical medicine is mainly equivalently to Medium dose in these experiments.

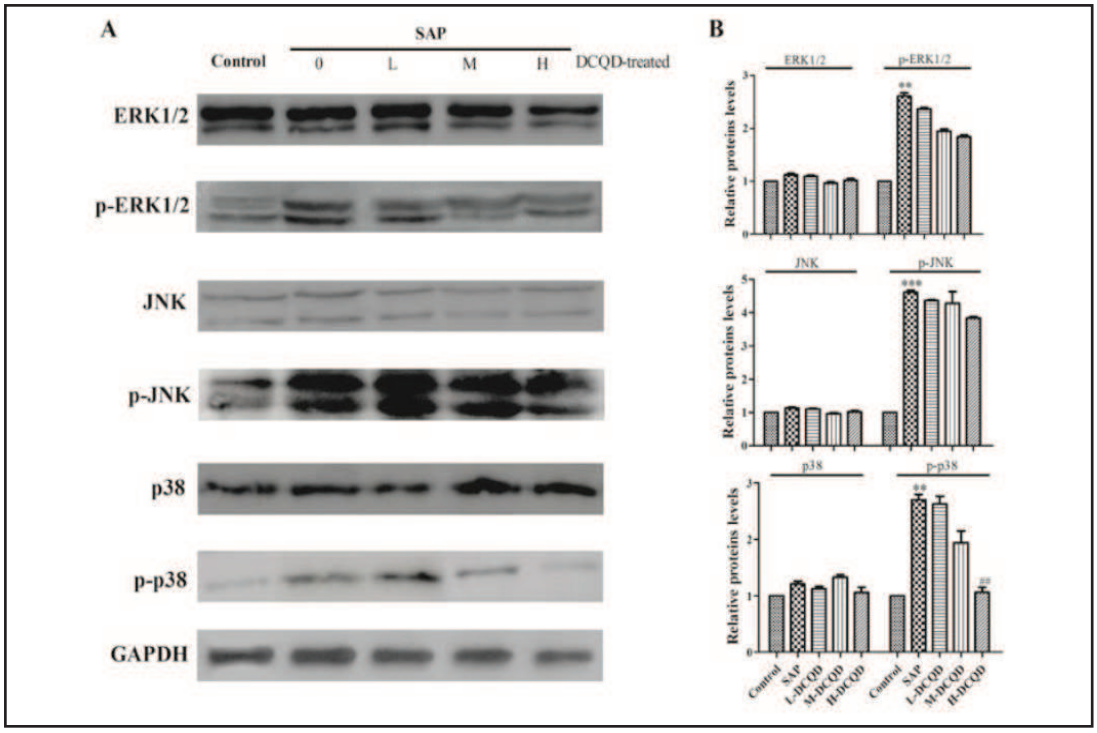

Fig. 7. Effect of DCQD treatment to MAPK signal pathway. (A) Western blot results of ERK, p-ERK, JNK, p-JNK, p38, p-p38 expression after DCQD treatment GAPDH was used as a loading control. Western blots were representative of three independent experiments l. (B) statistical results of A. Scanning densitometry was used for semi quantitative analysis in compared to control group levels;** P<0.01, *** P<0.001, compared with Control group; ## P<0.01, Compared with SAP group. n=3-5.

HMGB1 is a DNA binding protein to maintain gene stability, regulate DNA transcription and repair [18]. Moreover, HMGB1 could also function as DAMP to interact with PRR receptor like RAGE (receptor for advanced glycation end products), TLR and CXCL12 that further mediates inflammatory responses, angiogenesis and tumor growth and metastasis through multi-pathways including activation of CDC42, myD88/TRAF6, NF-κB, MAPK and anti-apoptosis protein, c-IAP [19-21]. In AP, NF-κB expression was in positive correlation with HMGB1 serum concentration, suggesting decreasing serum HMGB1 is a crucial way to block waterfall-like cascade in clinical treatment of severe SAP [22]. In our study, we analyzed pancreatic tissues and serum concentration and proposed that DCQD treatment could suppress the express and secretion of HMGB1, decrease the translocation from the nucleus to the cytoplasm and reduce inflammation response, and subsequently regulate the expression of HMGB1 (Fig. 4), which may become a new target for DCQD treatment in SAP.

Toll like receptor (TLRs) is the portal protein in inflammatory responses and is regarded as regulatory gate for inflammatory waterfalls cascade [23]. TLR4 is the first TLR found that is widely expressed on cytomembrane to recognize LPS [15] And TLR9 is a TLR identified later that expressed in cells to media the recognition of CpG structure of virus or bacteria [24]. In SAP group, we found that TLR4 expression was highly elevated in pancreatic and intestinal mucosal cell and is positive correlated with serum amylase level and up-regulation of TNF-α, IL1β and IL-6 [25]. Our research demonstrated that TLR4/9 was mainly expressed in cytoplasm of acinar cells and inflammatory cells. We also observed positive expression in pancreatic duct epithelial cells. DCQD treatment could significantly inhibit TLRs responses but showed stronger inhibitory effects in TLR9 than TLR4 (Fig. 5). On one hand, we believe that this is mainly because DCQD could also regulate pancreatic duct mucosa barrier damage [26], and TLR9 is usually regarded as a regulator for down-stream signal transduction as well as inflammatory factor in dysfunction of pancreatic duct mucosal barrier [27]. On the other

hand, TLR9 signal pathway could activate the expression of TNF-α [28], which also explained indirectly the finding that the more significant regulation in TNF-α of DCQD treatment than IL-6.

NF-κBand MAPKs pathway were proven to be activated in the early stage of SAP and regulate multiple pro-inflammatory genes [6, 29] .In our research, we found DCQD treatment inhibited cerulein-induced p38 and NF-κB activation but didn't influence ERK or JNK pathways (Fig. 6-7). However, we didn't establish another SAP animal model, which used retrograde injection of deoxycholic acid to the biliopancreatic duct to induce SAP, to analyze the effect of DCQD treatment on three pathways of MAPKs. We believe this still requires further researches.

To conclude, DCQD not only improved pancreatic pathological changes, decreased serum concentration of amylase, lipase, IL-6 and TNF-αin cerulein-induced mouse SAP model, but also inhibited HMGB1-TLRs responses to suppress the activation of NF-κB and p38 MAPK pathway, thus, reducing the risks of developing SIRS and MODs. However, DCQD treatment failed to reverse the expression of pro-inflammatory cytokines totally in SAP model. In that case, investigating how to intervene the waterfall-like cascade of pro-inflammatory factor should be a prior research in the treatment of SAP.

Acknowledgements

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Disclosure Statement

None.

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