Reviewer's code: 03866293

Major concerns: 1. More experiments are needed to make the findings more solid.

Reply: The results preliminary confirmed that QYD significantly restrained CaN and NFATc3 gene expression in the intestine, ameliorated IEC apoptosis associated with SAP, and decreased the LPS-induced elevation in intracellular free Ca^{2+} levels and cell death. The findings suggest that the protective effects of QYD might be mediated, at least partially, by restraining IEC apoptosis via the CaN/NFATc3 pathway.

Further cytological studies on the function and regulatory mechanism of CaN/NFATc3 pathway in IEC apoptosis and the intervention mechanism to IEC apoptosis by the traditional Chinese medicine QYD will be carried out through means of cell biology, molecular biology, electrophysiology, and other advanced methods. Thus we can provide a related theoretical grounding for treating pancreatitis for integrative medicine.

Minor concerns: 1. What is the source of QingYidecoction used in this study? An HPLC-based chem profile has to be provided.

Reply: QingYi decoction used in the study was obtained from the Chinese Medicine Preparations Division, First Affiliated Hospital of Dalian Medical University.

Li et al. [1] have verified that a total of 201 compounds could be extracted from Qingyi Granules using HPLC-UV-HRMS/MS method, which is an empirical formula optimized from Qingyi Decoction. It includes 76 flavonoids, 21 alkaloids, 35 terpenoids, 14 amino acids, 21 phenylpropanoids, 7 anthraquinones, 15 organic acid compounds, and 13 other compounds.

Among these compounds, Geniposide, Paeoniflorin, Paeonol, anthraquinone, and Magnolol have been proved to play a major role in the treatment of pancreatitis and have been extensively studied. Recent research has suggested that anthraquinone could inhibit the NLRP3/Caspase-1 pathway, and regulate Th1/Th2 imbalance, thereby playing an efficient role in multi-organ

protection in pancreatitis[2]. It has been reported that Paeonol could relieve oxidative damage of acute pancreatitis by activating the Nrf2/HO-1 signaling pathway[3]. According to Wang (2020), Magnolol could both improve the intestinal barrier function by functionally blocking the intestinal lymphatic circulation and reduce lung injury by inhibiting the HMGB1-TLR4/NF-κB signal transduction pathway[4]. Paeoniflorin has been proved to alleviate acute renal injury following severe acute pancreatitis by inhibiting inflammatory responses and renal cell apoptosis[5]. Si and Zhuo found that Geniposide could increase the serum levels of GSH and SOD and decrease the serum levels of MDA[6].

2. Some figures could be combined into one figure, such as Fig.2 and Fig.3, as well as Fig.5 and Fig.6.

The pictures have been modified in the original text according to the comments.

3. Some typos are needed to be corrected, such as NFATc3Expression in page 11.

The spelling mistakes have been modified in the original text according to the comments.

Reviewer's code: 03976790

Pages 5, 6: In the paragraph "QingYi decoction (QYD), a Chinese herbal medicine composed of Radix Bupleuri (Chaihu, Bupleurum scorzonerifoliumWilld.), Scutellariae Radix (Huangqin, Scutellariabaicalensis Georgi), AucklandialappaAucklandiae Radix (Muxiang, AucklandialappaDecne.), Corydalis Rhizoma (Yanhusuo, Cordalisambigua Cham.et Schlecht.), CoptidisRhizoma (Huanglian, Coptis chinensis Franch.), Paeoniae Radix Alba (Baishao, Paeonia lactiflora Pall.), Rhei Radix Et Rhizoma (Dahuang, Rheum palmatum, L.) and Natrii Sulfas (Mangxiao, Mirabilite) has been used in China for many years. ", Write the names of the genus and species, respecting the rules of nomenclature: names of the genus and species in italics, a capital letter at the beginning of the name of the genus and a lower case at the beginning of the name of the species.

The names of the genus and species have been modified according to the rules of nomenclature. Radix Bupleuri (*Chaihu, Bupleurum scorzonerifolium* Willd.), Scutellariae Radix (*Huangqin, Scutellaria baicalensis* Georgi), Aucklandiae Radix (*Muxiang, Aucklandia lappa* Decne.), Rhizoma Corydalis (*Yanhusuo, Corydalis acropteryx* Fedde), Coptidis Rhizoma (*Huanglian, Coptis chinensis* Franch.), Radix Paeoniae Alba (*Baishao, Paeonia lactiflora* Pall.), Rhei Radix Et Rhizoma (*Dahuang, Rheum palmatum* L.) and Natrii Sulfas (*Mangxiao,* Mirabilite)

Page 6: in the sentence "impact on AP through multiple pathways", what is AP? Acute pancreatitis? Write in full.

AP refers to acute pancreatitis, and the full name has been added in the original text.

Page 7, "Measurement of serum amylase, IL-6, TNF-a, D-lactic acid, and DAO": Provide minimal explanations for each detection method used. To

refer to the kit supplier's instructions does not seem sufficient to me for a scientific article.

Serum amylase was detected using a spectrophotometric method with a commercial kit (Jiancheng, Nanjing, China). Briefly, 0.5 ml of starch reagent and 0.10 ml of serum samples were added to a 5 ml graduated tube. After incubating for 7.5 min at 37 °C, 0.5 ml of iodine reagent and 3.0 ml water were added immediately. The absorbance was measured at 660 nm. Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α (Lengton, Shanghai, China), D-lactic acid (Goybio, Shanghai, China), DAO (Jiancheng, Nanjing, China), and IL-6 (Lengton, Shanghai, China) were used to evaluate the levels in rat serum according to the manufacturer's instructions.

Page 7, Histological Examination: Specify which fixative was used. What was the method of dehydration of the fixed tissues?

Pancreatic and intestinal tissues were fixed in 10% formaldehyde. After dehydration in gradient alcohol and transparentizing in xylene, the tissues were embedded in paraffin and cut into 5 μm thick slices.

Page 8, Apoptosis Detection: What was the fixative used? The same than for histological study? Please, specify.

Fixed rat intestinal sections with 10% formaldehyde were deparaffinized in xylene and rehydrated through a graded ethanol series.

Page 8, "according to the manufacturer's protocol": quickly specify the method.

Briefly, rat intestinal sections were treated with 50 μL of the TUNEL reaction mixture and then incubated at 37 °C for 60 min. After rinsing with PBS, tissue sections were stained with DAPI and observed with fluorescence microscopy.

Page 8, immunofluorescence analysis: "according to the manufacturer's

protocol": idem, quickly specify the method. How were the negative controls carried out in order to verify if the reaction is indeed due to the application of the specific antibody and not an artefact? (Removal of the first antibody, or replacement, etc.)

For immunofluorescence analysis, intestinal sections were incubated in a xylol-ethanol gradient to remove the paraffin. The slides were incubated with primary antibody against NFATc3 (Santa Cruz, CA, USA) and subsequently incubated with Alexa Fluor goat anti-rabbit IgGs (Life Technologies), and then observed using fluorescence microscopy following staining of the nuclei with DAPI (Sigma–Aldrich, St. Louis, MO). The specificity of the signals was monitored by blank staining without primary antibodies.

Page8, western blot analysis: "Western blotting was performed as previously described": idem, quickly specify the method.

Total protein from intestinal tissue was extracted with a protein extraction kit (KeyGen, Nanjing, China). Protein was separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. After the nonspecific binding was blocked, the membranes were incubated with primary antibodies against calcineurin A(1:2000, Abcam, UK), calcineurin B (1:500, Abcam, UK), and β-actin (1:1000). Immunoreactive proteins were visualized using enhanced chemiluminescence detection (Bioworld Technology, Nanjing, China). ImageJ software was used for statistical analyses of the band intensities normalized to β-actin.

Figure 1: The explanation of the figure is very brief. Explain what the pictures represent. Add a scale bar on each image. What is shown in Figures B and C? Thanks to explain.

Figure 1. Histological changes in pancreatic and intestinal tissues (A). Slight edema was detected (pancreas CON group); broad necrosis of acinar cells and interstitial edema were observed (pancreas SAP group); only local necrosis,

slight interstitial edema, and few inflammatory cell infiltrates were observed (pancreas QYD group). Slight interstitial edema was detected (intestine CON group); irregular villi, mucosal erosion, and inflammatory infiltration were observed (intestine SAP group); edema and mild mucosal erosion were observed (intestine QYD group). Histological scores for the pancreas (B) and intestine (C). The histological scores for the pancreas and intestine were significantly ameliorated in the QYD treatment group compared with the SAP group.[a]$P< 0.05$, 100×. CON: control; QYD: Qingyi decoction; SAP: severe acute pancreatitis. Scale bar has been added to each image.

Figures 2, 3, 4, 6, 8, 10: Add "CON: Control; QYD: decoction of Qingyi; SAP: Severe acute pancreatitis"

The pictures and legends have been modified in the original text according to the comments.

Figures 5: Give some explanations of these three figures. Add a scale bar on each image. An insert with a control without first antibody (or other, see materials and methods) would be useful.

Figure 5. Immunofluorescence (A) and semiquantitative analysis (B) of NFATc3 expression. NFATc3 was highly expressed in the SAP group compared with the control group but significantly downregulated under QYD treatment. [a]$P< 0.05$. BLANK: control without primary antibody; CON: control; QYD: Qingyi decoction; SAP: severe acute pancreatitis. Scale bar has been added to each image.

Figure 7: Give some explanations of these three figures. Add a scale bar on each image. An insert with a control without first antibody (or other, see materials and methods) would be useful.

Figure 7. TUNEL assay to assess ICE apoptosis. Compared with that in the SAP group, the level of ICE apoptosis in the QYD groups was significantly

lower. 200×. BLANK: control without primary antibody; CON: control; QYD: Qingyi decoction; SAP: severe acute pancreatitis. Scale bar has been added on each image.

Figure 9: Explain figure 9A.

Figure 9. Fluorescence images of $[Ca^{2+}]i$ changes measured using a confocal microscope (A) and the relative mean optical density (B). Treatment with QYD serum significantly attenuated the increase in Ca^{2+} levels observed in response to LPS-induced injury. $^{a}P< 0.05$. QYD: Qingyi decoction.

Reviewer's code: 03034197

This is correctly conducted, well-designed, interesting animal study regarding the role of the calcineurin/NFATc3 pathway in the apoptosis of intestinal epithelial cells (IECs) in SAP and the potential mechanisms underlying the therapeutic effect of QingYi decoction (QYD). Manuscript should be published.

Thanks for your comments.

Reviewer's code: 03866293

All the questions were well addressed.

Thanks for your comments.

Reference

1 Li WH. Studies on Chemical Constitutions and Metabolism in vivo of Qingyi Granule. *Dalian University of Technology*, 2021

2 Yuxia X, Li C, Ling F, Lulu W, Yejiang Z, Dalian Q, Qin S, Jianming W, Shousong C. Free Total Rhubarb Anthraquinones Protect Intestinal Injury via Regulation of the Intestinal Immune Response in a Rat Model of Severe Acute Pancreatitis. *Frontiers in pharmacology* 2018; 9

3 Zhang P, Li SF, Wu SZ, Jin SQ, Wang JQ, Guo WJ. Effects of Paeonol on the Oxidative Damage of Acute Pancreatitis in Mice Induced by L-Arginine. *Acta Veterinaria et Zootechnica Sinica* 2021; 52(07): 1983-1990

4 Wang Y, Qi WJ, Zeng YW, Gu PY, Miao B. Mechanism of action of magnolol in the treatment of acute lung injury in a rat model of severe acute pancreatitis. *Journal of Clinical Hepatology* 2020; 36(12): 2782-2787

5 Zeng M, Yang F. Protect effects of paeoniflorin on severe acute pancreatitis in rats. *Journal of Clinical and Experimental Medicine* 2017; 16(09): 841-844

6 Si LZ, Zhuo Y. Effects of geniposide on the contents of GSH and SOD in pancreatic tissue of rats with acute pancreatitis. *Journal of Qiqihar University of Medicine* 2015; 36(08): 1101-1102