

Melatonin attenuated inflammatory reaction by inhibiting the activation of p38 and NF- κ B in taurocholate-induced acute pancreatitis

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Abstract. The aim of the present study was to investigate the protective mechanism underlying of melatonin in severe acute pancreatitis (SAP). A total of 64 male Sprague-Dawley rats were randomly divided into four groups: The sham operation (SO) group, SAP group, melatonin treatment (MLT) group and p38 inhibitor (SB203580) treatment (SB) group. Acute pancreatitis was induced by 5% taurocholate through retrograde infusion into the biliopancreatic ducts. The melatonin and SB203580 treatment groups were administered with MLT and SB 30 min before operation the induction of SAP. Rats in each group were euthanized at 6 and 12 h following SAP induction. Blood and pancreatic tissues were removed for inflammatory examination. Peripheral blood mononuclear cells (PBMCs) were isolated following sacrifice to measure the phosphorylation of p38 and nuclear factor- κ B (NF- κ B was measured as p65 and phosphorylation of p65). The pretreatment of melatonin significantly attenuated the severity of pancreatitis. In addition, melatonin also reduced serum amylase and proinflammatory cytokine levels, including tumor necrosis factor- α , interleukin (IL)-1 and IL-6. The mean pathological scores for pancreatic tissues in the MLT group were higher than those for samples in the SO group, but were lower than those for samples in the SAP group at each time-point. Phosphorylation of p38 and p65 levels in the melatonin treatment group were lower than that in the SAP group, and higher in the SAP group than in the SO group, and the

SB203580 treatment group. Furthermore, melatonin significantly inhibited the activation of p38 and NF- κ B in PBMCs. The authors revealed that melatonin may attenuate inflammatory reactions by inhibiting the activation of p38 MAPK and NF- κ B in both acute pancreatitis rats and PBMCs. SAP is a severe disease with a high risk of morbidity and mortality. It is important to attenuated inflammatory reaction in acute pancreatitis. Thus, the authors studied melatonin, which is synthesized by the pineal gland and released into the blood. Previous studies have shown that melatonin serves a protective role in the early course of human acute pancreatitis, and melatonin concentration variations are closely related to the severity of acute pancreatitis. It may be concluded that melatonin may attenuates inflammatory reactions by inhibiting the activation of p38 MAPK and NF- κ B in both acute pancreatitis rats and PBMCs.

Introduction

Acute pancreatitis is a severe disease with a high risk of morbidity and mortality. The pathophysiology of acute pancreatitis exhibits edema, perivascular infiltration, acinar necrosis and hemorrhage in the pancreas (1). Inflammatory mediators are synthesized and secreted by pancreatic cells, thus entering the blood circulation, spreading to other organs. These inflammatory mediators give rise to systemic inflammatory response syndrome (SIRS), which is the leading cause of high mortality of the disease (2,3).

Previous studies have not completely revealed the molecular mechanisms of acute pancreatitis. Auto digestion of the gland is widely accepted, however, the initiating factor, which turns pro enzymes into their active forms, is still obscure. Previous study has focused on investigating the initiators of this systemic disease, including interleukin (IL)-1, interleukin-6 (IL-6) and tumor necrosis factor (TNF)- α . IL-1 and TNF- α , which are the primary inducers of IL-6, are the primary components of the inflammatory cytokine family. Understanding the pathway of the activation of proinflammatory cytokines is the key to explain the disease pathogenesis in the early phase.

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In previous years, several signaling pathways have been identified to serve a critical role in the process of acute pancreatitis (4-6). Mitogen-activated protein kinases (MAPKs) are known to be activated by numerous of stimuli, such as cytokines, neurotransmitters, growth factors and many different kinds of stress (7). NF- κ B is a key regulator of the immune system (8), presenting in the cytoplasm, p65 and p50 are the most common heterodimer complexes of NF- κ B, and p65 is the functional component involving the activation of NF- κ B. However, their roles in regulation of cytokines like IL-1, IL-6 and TNF- α in acute pancreatitis have not been completely elucidated.

Melatonin (*N*-acetyl-5-methoxytryptamine) was first isolated from the pineal gland successfully by Lerner *et al* (9). It is primarily known for its regulation of circadian and annual cycles (10). Subsequent research reveal melatonin not only as a potent antioxidant, but also a non-enzymatic free radical scavenger (11). Moreover, melatonin serves an important role in protecting lipid membranes against lipid peroxidation phenomenon (12).

Melatonin has been identified as a potential molecule in the therapy of inflammatory diseases (13,14). In the present study, the authors aimed to estimate its protective effects and possible mechanisms of exogenous administration of melatonin in the rat models of acute pancreatitis induced by taurocholate.

Materials and methods

Animals. A total of 64 male Sprague-Dawley rats, weighing 200-250 g, were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). They were kept at constant room temperature of 25°C in a 12 h light-dark cycle with access to standard rat pellets and water chow *ad libitum*. All animals were acclimatized in the laboratory for at least one week and were deprived of food for 12 h prior to the experiment initiation, but water was allowed free access. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical University. All the animal studies complied with current ethical considerations with the approval of the Laboratory Animal Ethics Committee of Wenzhou Medical University (Wenzhou, China).

Induction of severe acute pancreatitis. All rats were anesthetized by administration of 4% chloral hydrate (300 mg/kg; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The rats were randomly divided into the sham operation group (SO, n=16), the severe acute pancreatitis group (SAP, n=16), the melatonin treatment group (MLT, n=16) and the SB203580 treatment group (SB, n=16). In the SAP, SB and MLT groups, the biliopancreatic duct through the papilla was reached and cannulated by penetrating with a 24-gauge catheter after a micro clip clamping the hepatic duct. Acute pancreatitis was induced by 5% taurocholate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) through retrograde infusion of a dose of 1 ml/kg via a microinjection pump at a rate of 0.2 ml/min. Rats in the SO group were underwent to the same surgery but without infusion. The MLT group was treated with 50 mg/kg of melatonin (Sigma-Aldrich; Merck KGaA) intraperitoneally 30 min prior to operation. The SB group received 0.5 mg/kg SB203580 via caudal vein 30 min before

operation. Following each operation, the abdomen was closed in two layers. All procedures were conducted using sterile techniques.

Sample collection and detection of serum amylase and ELISA. At 6 and 12 h following SAP induction, rats were anesthetized with 4% chloral hydrate (300 mg/kg body weight). Abdominal aorta puncture was used to obtain 6 ml blood from each animal, 2 ml of each sample was collected and stored in tubes without anticoagulants (Generay Biotechnology Co., Ltd., Shanghai, China). Blood samples (1 in 2 ml) was centrifuged at 1,200 x g for 5 min, the serum was collected for determination of amylase activity with a fully automatic biochemical analyzer (Hitachi Corporation, Tokyo, Japan). The other 1 ml of each sample was centrifuged at 3,000 x g for 15 min, and the serum undiluted was for the ELISA assay. The remaining 4 ml blood was treated with sodium heparin anticoagulant and used to isolate peripheral blood mononuclear cells (PBMCs). The pancreatic tissues were harvested immediately, then placed in 40 g/l paraformaldehyde and prepared for routine paraffin embedding prior to pathological examination. The rats were anesthetized with 4% chloral hydrate (300 mg/kg body weight) and subsequently euthanized by exsanguination after the experiments.

Histological analysis and pathological scores of pancreatic tissues. Samples of pancreatic tissues were fixed in 40 g/l paraformaldehyde, dehydrated embedded in paraffin. Paraffin sections were cut at 4 μ m-thick sections. Pancreas sections were stained with hematoxylin and eosin, and observed using light microscopy (Nikon Corporation, Tokyo, Japan). Using the standards set by Schmidt *et al* (1), tissues were examined by two experienced histologists blinded to the experimental protocol. The pancreatic sections, presenting a minimum of six fields, were examined for each sample and scored on a scale of 0-3 (0 being normal and 3 being severe) on the basis of edema, inflammatory cell infiltration, acinar cell degeneration and parenchymal hemorrhage.

Isolation of PBMCs. PBMC isolation was performed with density gradient centrifugation. The blood sample (4 ml) was diluted with an equal volume of PBS. Then, the diluted blood was carefully laid over 4 ml ficoll (Beijing Solarbio Science and Technology Co., Ltd.). The PBMCs layered on the white band at the medium interface after centrifugation, were carefully removed into a new tube. The harvested PBMCs were diluted in PBS and the cells were pelleted by another centrifugation. Then PBMCs were immediately stored in liquid nitrogen until use.

ELISA. ELISAs for TNF- α , IL-1 and IL-6 (R&D Systems Inc., Minneapolis, MN, USA) were carried out in duplicate with 40 μ l of samples in each well of 96-well plates (Nunc, Roskilde, Denmark), which had been coated with 100 μ l aliquots of either anti-rat IL-1 (cat. no. MAB501), IL-6 (cat. no. MAB506) or TNF- α (cat. no. MAB510; all 1:500; all from R&D Systems Inc., Minneapolis, MN, USA) antibodies in PBS (at pH 7.4) at 4°C overnight. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 10% fetal bovine serum for 2 h. Following additional washes,

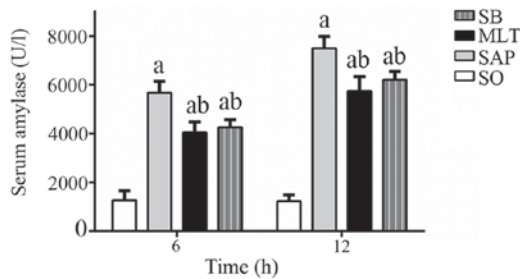


Figure 1. Effect of melatonin on serum amylase levels following taurocholate-induced pancreatitis. Data are presented as mean \pm standard deviation (n=6). ^aP<0.01 vs. SO group; ^bP<0.01 vs. SAP group. SO, sham operation group; SAP, severe acute pancreatitis group; MLT, melatonin treatment group; SB, SB203580 treatment group.

the standards and the serum were added to the plates and incubated at room temperature for 3 h. Following incubation, the wells were washed and a goat anti-mouse secondary IgG HRP conjugated secondary fluorescent antibody [cat. no. GAM007, 1:5,000; Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd., Hangzhou, China] was added to each well. Incubation continued at room temperature for 1 h. Following washing the wells again, the TMB substrate [Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd.] was added. Each were had sulfuric acid (2 mol/l) added to terminate the reaction. Color development was measured with an automated microplate ELISA reader at 450 nm. Standard curves were obtained for each sample through serial dilutions of recombinant IL-1, IL-6 or TNF- α .

Western blot analysis. Western blotting was used to measure the protein expression of β -actin, phospho-specific p38 and p65, total p38 and p65. PBMCs were completely lysed in radio-immunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Roche Diagnostics GmbH, Basel, Switzerland) at a cell density of 1×10^7 cells/100 ml. Protein concentration was determined by the bicinchoninic acid assay method (BCA kit; Beyotime Institute of Biotechnology, Haimen, China). From each sample, 50 mg of total protein separated by 12% SDS-PAGE and the resolved proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). After being blocked with 5% BSA in TBS-0.1% Tween-20 (TBST) for 2 h at room temperature, membranes were incubated overnight at 4°C with specific primary antibodies. The following antibodies were used: p-p38 (Thr180/Tyr185 Rabbit mAb, cat. no. 9215); p38 (D13E1 Rabbit mAb, cat. no. 8690); p-p65 (Ser536 rabbit mAb, cat. no. 3033); p65 (D14E12 rabbit mAb, cat. no. 8242); β -actin (13E5 rabbit mAb, cat. no. 4970; all 1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA). Then the membranes were washed three times for 10 min each with TBST, then incubated with a goat anti-rabbit secondary IgG HRP conjugated anti-rabbit secondary fluorescent antibody [cat. no. GAR0072, 1:1,000; Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd.] for 1 h at room temperature. After another 3 times washing with TBST, the enhanced chemiluminescence method (WesternBright Enhanced Chemiluminescence Substrate kit; Advanta Inc., Menlo Park, CA, USA) was used as recommended by the manufacturer to measure the quantity of protein. Protein

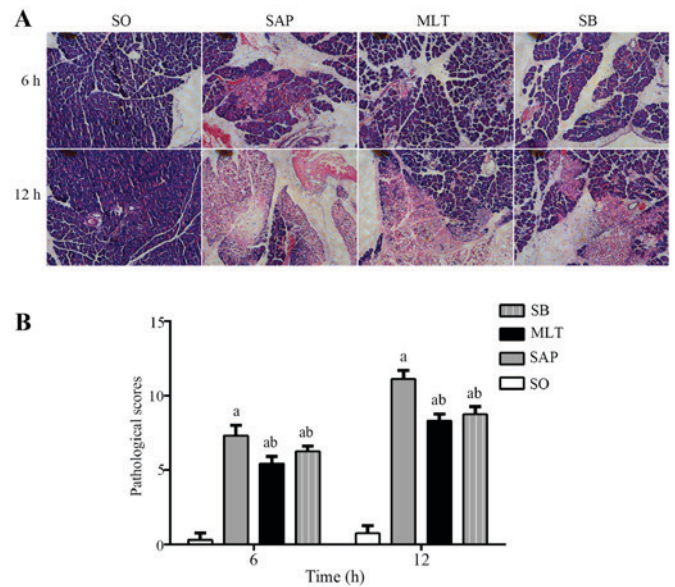


Figure 2. Protective effect of melatonin on pancreatic morphology. (A) SO groups present the normal structures of pancreas tissues at 6 and 12 h using hematoxylin and eosin staining magnification, x400. Pancreatic edema, parenchyma hemorrhage and neutrophil infiltration were observed in SAP groups at 6 h, and became worse at 12 h (magnification, x400). The MLT and SB groups had more mild edema, parenchyma hemorrhage and neutrophil infiltration (magnification, x400). (B) The pathological scores of pancreas tissues. Data are presented as mean \pm standard deviation (n=6). ^aP<0.01 vs. SO group; ^bP<0.01 vs. SAP group. SO, sham operation group; SAP, severe acute pancreatitis group; MLT, melatonin treatment group; SB, SB203580 treatment group.

expression levels were normalized against β -actin. In western blotting, the authors randomly selected three samples in each group to repeat the experiment in triplicate. Images of the blots were captured using the ChemiDoc MP system and the density of specific bands was quantified using Image Lab software version 4.1 (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were carried out using the SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA). All data are expressed as means \pm standard deviation. One-way analysis of variance followed by Newmann-Keuls post hoc test was used to identify differences between means. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of melatonin on serum amylase. Serum amylase levels were substantially elevated following the induction of pancreatitis, which further confirmed the diagnosis of pancreatitis. The levels of serum amylase in SAP groups were significantly increased at 6 and 12 h compared with SO groups (P<0.01). Pretreatment of pancreatitis rats with 50 mg/kg melatonin (MLT groups) suppressed the amylase elevation at both 6 and 12 h compared with SAP groups (Fig. 1). SB groups (0.5 mg/kg) either declined the levels of serum amylase vs. SAP groups at both time-points. Though the levels were decreased in both MLT and SB groups, but were still higher compared to SO groups at both time-points.

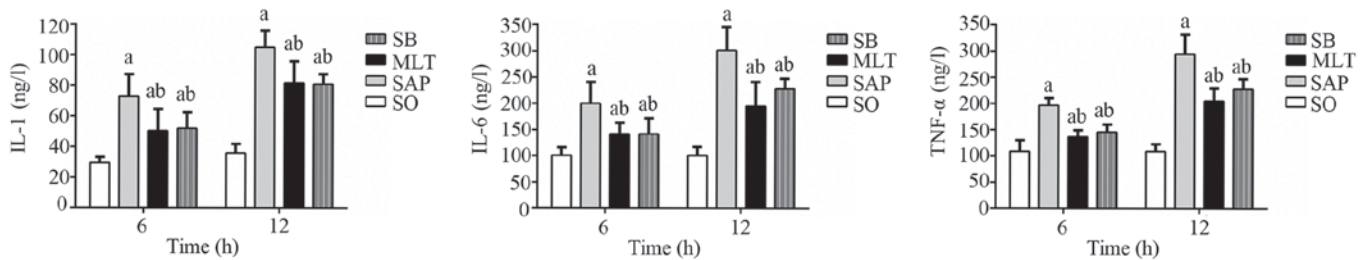


Figure 3. Effect of melatonin on inflammatory cytokines in acute pancreatitis. The induction of pancreatitis significantly elevated IL-1, IL-6 and TNF- α levels at both time-points. Pretreatment with melatonin and p38 inhibitor successfully subdued the elevation of these cytokines at 6 and 12 h. Data are presented as mean \pm standard deviation (n=6). ^aP<0.01 vs. SO group; ^bP<0.01 vs. SAP group. SO, sham operation group; SAP, severe acute pancreatitis group; MLT, melatonin treatment group; SB, SB203580 treatment group; IL, interleukin; TNF- α , tumor necrosis factor- α .

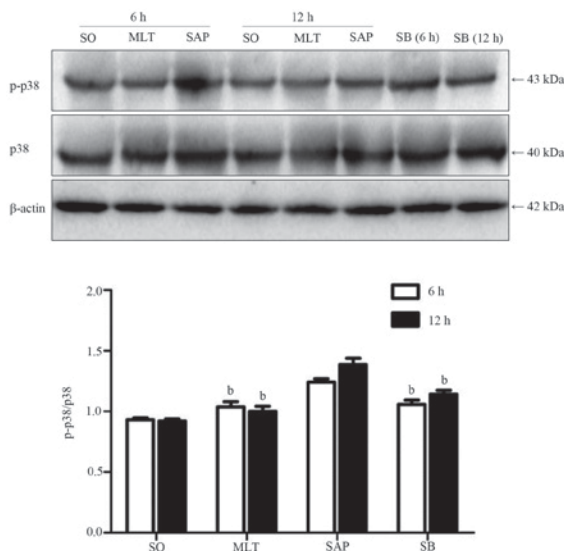


Figure 4. Effect of melatonin on the activation of p38 MAPK in PBMCs. SAP groups exhibited an appreciable phosphorylation of p38. Treatment with melatonin and p38 inhibitor resulted in a decline in phosphorylated p38 MAPK. Data are presented as mean \pm standard deviation (n=3). ^bP<0.01 vs. SAP group. SO, sham operation group; SAP, severe acute pancreatitis group; MLT, melatonin treatment group; SB, SB203580 treatment group; MAPK, mitogen-associated protein kinase; PBMCs, peripheral blood mononuclear cells.

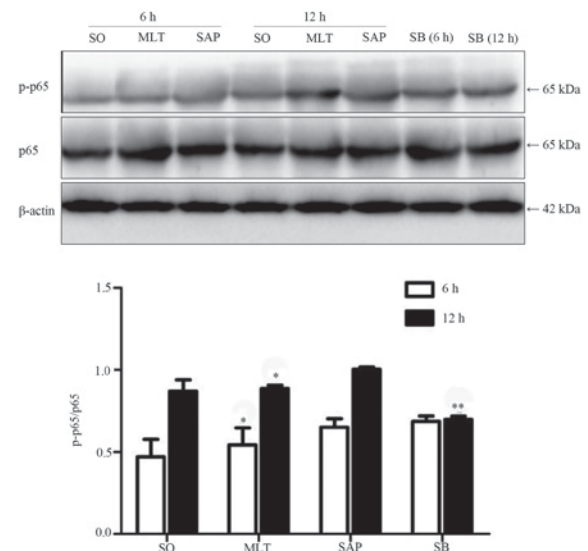


Figure 5. Effect of melatonin on nuclear factor- κ B in PBMCs. The SAP group presented a significant phosphorylation of p65. Treatment with melatonin downregulated the phosphorylated p65 in PBMCs, intervention of p38 inhibitor succeeded in suppressing phosphorylated p65 at 12 h, but failed at 6 h. Data are presented as mean \pm standard deviation (n=3). ^{*}P<0.05, ^{**}P<0.01 vs. SAP group. SO, sham operation group; SAP, severe acute pancreatitis group; MLT, melatonin treatment group; SB, SB203580 treatment group; PBMCs, peripheral blood mononuclear cells.

Effect of melatonin on pancreatic histopathology. Normal tissue morphology of pancreas was observed in SO groups. Edema, parenchyma hemorrhage and inflammatory infiltration of neutrophils in pancreatic tissues were observed in SAP group at 6 h and the changes became more severe as time prolonged. While more mild edema, parenchyma hemorrhage and inflammatory infiltration of neutrophils into the pancreatic tissues were identified in MLT groups and SB groups (Fig. 2A), the pathological scores of pancreas tissues declined considerably in both groups (Fig. 2B). The mean pathological scores for pancreatic tissues in the MLT group were significantly higher ($P<0.01$) than those for samples in the SO group (5.44 ± 0.50 vs. 0.31 ± 0.46 following 6 h; 8.31 ± 0.46 vs. 0.75 ± 0.54 following 12 h), but were significantly lower ($P<0.01$) than those for samples in the SAP group at each time-point (5.44 ± 0.50 vs. 7.31 ± 0.70 following 6 h; 8.31 ± 0.46 vs. 11.13 ± 0.58 following 12 h).

Effect of melatonin on IL-1, IL-6 and TNF- α production. Serum IL-1, IL-6 and TNF- α levels all increased in the SAP

group at 6 h compared with the SO group and did not have a significant difference as time prolonged. Compared to the SAP group, both MLT and SB groups had significantly lower levels of IL-1, IL-6 and TNF- α at both 6 and 12 h, nonetheless, were still higher at both time-points compared with SO group (Fig. 3).

Effect of melatonin on activation of p38 in PBMCs. Phosphorylation of p38 was observed following the induction of acute pancreatitis. However, both melatonin and the p38 inhibitor had a significant suppression on the activation of p38 MAP kinase when compared with SAP group at 6 and 12 h, the phosphorylation of p38 was remarkably subdued in MLT and SB groups at both time-points (Fig. 4).

Effect of melatonin on NF- κ B in PBMCs. Phosphorylation of p65 was found after the induction of acute pancreatitis. Melatonin had significantly subdued the phosphorylation of p65 when compared with the SAP group at both time-points.

Pretreatment with the p38 inhibitor suppressed the expression of phosphorylated p65 at 12 h, but not at 6 h (Fig. 5).

Discussion

In the present study, the authors have investigated the effect of melatonin on suppressing the development of taurocholate-induced AP. Administration of exogenous melatonin significantly inhibited serum amylase production, IL-1, IL-6 or TNF- α levels. Furthermore, melatonin inhibited the activation of p38 and NF- κ B. Jung *et al* (15,16) indicated that the role of NF- κ B suppression in response to melatonin treatment. But they primarily researched oxidative liver injury and pancreas samples in AP. In the current study, the authors have discussed NF- κ B in peripheral blood mononuclear cells (PBMCs) in SAP.

Pancreatic digestive enzymes including amylase and lipase contribute to necrosis of acinar cells at an early stage, and consequently, lead to the inflammation of the pancreas. Nevertheless, cytokines and oxidative stress may lead to the major factors for development of SIRS (17). Therefore, blockade of IL-1, IL-6 and TNF- α receptors soon after induction of pancreatitis are associated with decreased production of inflammatory cytokines and alleviated pathological changes (18). Previously, accumulating evidences suggested that melatonin suppresses inflammatory cytokines expression such as IL-1, IL-6 and TNF- α in rats with ventilator-induced lung injury and azoxymethane/dextran sodium sulfate-induced large bowel oncogenesis (19,20). In an experimental model of pancreatitis, pretreatment with melatonin prevented the increased levels of amylase in serum (Fig. 1) and the elevations of IL-1, IL-6 and TNF- α were inhibited (Fig. 3), suggesting that melatonin may be effective in controlling excessive exocrine enzyme secretion and inflammatory cytokines during severe acute pancreatitis.

The MAPKs are a large family of serine/threonine kinases, regulating kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs involved in cell proliferation, differentiation and death (21). NF- κ B is a transcription factor, which serves a critical role in immunity, inflammation, cell proliferation and cell death. Activation of NF- κ B target genes requires phosphorylation of NF- κ B proteins; p65 is the functional component involving the activation of NF- κ B (22). Oxidative stress and proinflammatory cytokines trigger common signal transduction pathways that result in an inflammatory cascade, especially through activation of MAPKs, as well as NF- κ B (23). The potential contaminating source to induce cellular cytokine productions may serve an important role in the activation of p38 MAPK and NF- κ B in various pathological conditions, such as acute pancreatitis (24). In addition, it has been also reported that the SB203580, a selective p38 MAPK inhibitor, virtually prevents cerulein-induced cell death and generation of inflammatory cytokines, suggesting that p38 MAPK may have detrimental role in cerulein-induced AP (25). Kim *et al* and Bae *et al* (26,27) reported that the inhibition of p38 MAPK and NF- κ B may inhibit IL-1, IL-6 and TNF- α productions. Meanwhile, melatonin has been reported to inhibit p38 MAPK and NF- κ B in various animal models (28-30). In the current study, the models of taurocholate-induced AP exhibited

increased serum amylases, IL-1, IL-6 and TNF- α levels, yet p38 MAPK and NF- κ B activation were detected in the PBMCs (Figs. 4 and 5). Therefore, the authors concluded that the levels of inflammatory cytokines in taurocholate-induced SAP were inhibited by suppression of p38 MAPK and NF- κ B activation in PBMCs.

In conclusion, the present study demonstrated that melatonin attenuates the severity of taurocholate-induced AP may through suppressing pro-inflammatory cytokine production by deactivating p38 MAPK and NF- κ B in PBMCs. Therefore, melatonin exerts potent anti-inflammatory effects in AP.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YC performed the experiments and wrote the manuscript; JW designed the study; QZ and QC analyzed the data; YZ and BS collected the data; and YJ advised on assay performance. All authors evaluated and approved the final manuscript.

Ethics approval and consent to participate

All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical University. All the animal studies complied with current ethical considerations with the approval of the Laboratory Animal Ethics Committee of Wenzhou Medical University (Wenzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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