Melatonin attenuates the inflammatory response via inhibiting the C/EBP homologous protein-mediated pathway in taurocholate-induced acute pancreatitis

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Abstract. Acute pancreatitis (AP) is a serious disease characterized by the activation of trypsin, autodigestion, edemas, hemorrhages and necrosis. However, the mechanisms of regulating the apoptosis and inflammation of acinar cells in AP remain unclear. The endoplasmic reticulum (ER) stress-related molecule, C/EBP homologous protein (CHOP), has pro-apoptotic and pro-inflammatory properties, in addition to regulating ER stress responses. In the present study, a lentivirus-mediated RNA interference (RNAi) approach was used to specifically knockdown the expression of CHOP in the pancreatic tissue of Sprague-Dawley rats to investigate the potential role of CHOP during AP, which was induced by the retrograde injection of 5% taurocholate into the biliopancreatic duct of rats. Pre-treatment with melatonin was further used to identify the potential anti-inflammatory mechanisms in AP. Pancreatic tissues were procured for western blot analysis, histological examination, reverse transcription-quantitative polymerase chain reaction and immunohistochemical staining. ER stress was rapidly activated in the early stage and increased over time in the rat AP model. However, the silencing of CHOP expression markedly inhibited apoptosis and ER stress, reducing the activation of nuclear factor-κB and inflammation injury in AP. Melatonin also exhibited anti-inflammatory and apoptotic effects, and significantly decreased the expression of CHOP. Thus, it can be concluded that the CHOP-mediated pathway serves an important role in the development of AP, and that melatonin can reduce pancreatic damage via the inhibition of CHOP expression.

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Introduction

The endoplasmic reticulum (ER) is one of the largest and most important cellular organelles, serving a vital role in the synthesis, folding and modification of proteins, and is the largest Ca^{2+} pool, participating in intracellular signal transduction. Various pathophysiological stimuli can contribute to the accumulation and aggregation of unfolded or misfolded proteins within the ER, resulting in ER stress (1). When cells are exposed to ER stress, a series of mechanisms are triggered, known as the unfolded protein response (UPR). The UPR signaling pathways are activated by three ER membrane-associated proteins, namely the PERK, ATF6 and IRE1 proteins (2), which act to meet the folding demand within the ER and promote cell survival (1,3-5). However, if cells ultimately fail to balance the folding capacity and restore homeostasis to the ER, uncontrolled ER stress will, through the UPR pathways, initiate apoptosis (6,7). Several molecules, including C/EBP homologous protein (CHOP), Caspase-12, IRE1, JNK, Bax and Bak, have been reported to be involved in ER stress-induced apoptosis (1). However, it has been demonstrated that numerous pro-apoptotic factors induced by CHOP, such as DR5, BIM, TRB3 and GADD34, promote protein synthesis and exacerbate oxidative stress in stressed cells (8-11). Furthermore, the involvement of CHOP-mediated apoptosis has been demonstrated in various diseases, including diabetes, brain and renal injury, neurodegenerative abnormalities, and even certain cardiovascular diseases (3,12-15). Notably, CHOP serves a crucial role in ER stress and cell apoptosis, and evidence suggests that CHOP is deleterious to cells when they are subjected to ER stress (16).

One of the most serious diseases of the exocrine pancreas is acute pancreatitis (AP), which is characterized by perivascular infiltration and inflammation, as well as tissue edema, hemorrhage, and acinar and fat necrosis in the pancreas (17,18).

However, the understanding on the complicated pathogenesis of this disease is currently limited. Several potential factors have been proposed to explain pancreatic injury, including pathological intra-acinar trypsinogen activation, intracellular calcium overload, inflammatory mediators, bacterial infections, apoptosis, activation of nuclear factor (NF)-κB and oxidative stress (19-22). All these factors interact with each other and form a complex network control system to mediate acinar cell injury and the inflammatory response, and certain of these are also considered to be potential causes of ER stress (2,23-25). Indeed, the pancreas is particularly vulnerable to ER stress since its cells possess particularly abundant ER to support the organ's prominent role in the synthesis of digestive enzymes (1,26,27). Recently, evidence surfaced that ER stress and UPR were activated in AP, and were indispensable in the acceleration of the development of AP.

ER stress induces significant morphological changes during AP pathogenesis (28,29). Kubisch *et al* (30) reported that ER stress-associated receptors, including IRE1, PERK and ATF6, along with their downstream signaling pathway-associated molecules, such as eIF2, XBP1, CHOP and Caspase-12, were markedly activated in the early stage of AP in a rat model induced by arginine, leading to the formation of vesicle particles inside the ER. It was also reported that PERK and phosphorylation of eIF2a were activated at an early stage (within 4 h) in cerulein-induced pancreatitis (31). In pancreatitis, excessive IRE1a activated the phosphorylation of JNK and other 'warning genes', such as p38 and NF-κB, which strongly responded to ER stress during AP injury, promoting transcription of various inflammatory genes and pancreatic neutrophil infiltration (32-34).

The aforementioned studies demonstrated that the ER stress response serves a major role in pancreatic acinar injury and AP induction. However, earlier studies indicated that ER stress had a protective role in AP; for instance, XBP1 or PERK deficiency was reported to aggravate the exocrine pancreatic dysfunction (35,36). Furthermore, in alcohol-induced pancreatitis, ER stress was attenuated by a robust UPR involving XBP1 in acinar cells, which markedly weakened eIF2 phosphorylation and the expression of PERK, ATF4 and CHOP, controlling the severity of ER stress and alleviating the AP injury (37). Thus, ER stress-response appears to regulate the injury of pancreatic acinar cells differently, by either aggravating or attenuating it, possibly depending on the balance between CHOP-mediated pro-apoptotic and pro-survival ER stress responses (3). However, the direct involvement of CHOP in the AP pathological process has not been fully explored.

Melatonin, which is secreted by the pineal gland, exerts several prominent cell-protective functions, including anti-inflammatory, antioxidant and anti-apoptotic properties (38). Studies have confirmed that pretreatment with melatonin markedly reduced lipid peroxidation, tissue edema and inflammation in cerulein-induced AP in rats (39). However, whether melatonin exhibits an anti-inflammatory effect via the CHOP-mediated pathway remains unclear.

Thus, in the present study, a lentivirus-mediated RNA interference (RNAi) approach was used to specifically knockdown the expression of CHOP to investigate whether and how the CHOP-mediated pathway contributes to the development of AP injury in the pancreatic tissue of Sprague-Dawley (SD) rats. The potential role of melatonin in the CHOP-mediated signaling pathway to decrease inflammation and apoptosis was further demonstrated in the rat AP model.

Materials and methods

Animals. A total of 40 clean-grade male SD rats, weighing 200-300 g (6 to 8-weeks-old), were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The animals were maintained under tandard conditions in a room with a 12-h light/dark cycle, and were provided free access to food and water. All animals were adapted in the animal center for at least 1 week and deprived of rat chow for 12 h before experimentation, but allowed unlimited access to water throughout the experimental period. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical University (Wenzhou, China). All the animal studies complied with the current ethical considerations and were approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University.

Animal groups and procedures. All SD rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), and randomly assigned to the negative control (NC; n=8), sham-operated (SO; n=8), AP (n=8), melatonin treatment (MT; n=8) or CHOP knockdown (CK; n=8) groups. In the SO group, the rats underwent the same surgical procedure as other experimental groups, but without infusion with 5% taurocholate. The surgical procedure performed involved cannulating the biliopancreatic duct via penetration of the duodenum with a 24-guage catheter. In the AP group, after clamping the hepatic duct using a microclip, AP was induced by an infusion of 5% taurocholate (1 ml/kg body weight; Sigma-Aldrich; Merck KGaA) into the biliopancreatic duct via a microinjection pump at a rate of 0.2 ml/min. In the MT group, melatonin (50 mg/kg body weight; Sigma-Aldrich; Merck KGaA) was administered via intraperitoneal injection 30 min before AP was induced. In the CK group, rats underwent the same surgical procedure as rats in the AP group but also underwent lentiviral transfection silencing CHOP expression prior to AP induction. All surgical procedures were performed under sterile conditions. After 9 h, the rats were sacrificed by exsanguination under anesthesia with chloral hydrate (300 mg/kg), and the pancreatic tissues were rapidly collected and divided into two parts. Part of the tissue samples was fixed in 4% paraformaldehyde and prepared for routine paraffin-embedding prior to immunohistochemical and pathological examination analyses, while the remaining tissue was stored at -80˚C for the western blot and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.

Lentivirus-mediated stable RNA interference of CHOP in pancreatic tissue. A lentivirus (vehicle: hU6-MCS-Ubiquitin-EGFP-IRES-puromycin), which carried a short hairpin (sh)RNA targeting the CHOP gene (GenBank no. NM_024134), was purchased from GeneChem Co., Ltd. (Shanghai, China). This lentivirus was used for CHOP silencing and termed LV-shCHOP. Briefly, the rats were anesthetized through intraperitoneal injection of 300 mg/kg chloral hydrate. Subsequently, LV-shCHOP (50 μl; $4x10^8$ TU/ml; GeneChem Co., Ltd.) was injected into their left gastric artery of the rats

via a microinjection pump at a rate of 0.2 ml/min, and the left gastric artery was then ligated to avoid reflux of the virus. After 5 days, the AP model was induced in rats. After 9 h of induction, the rats were sacrificed by exsanguination as aforementioned, and the pancreatic tissues were rapidly collected; some tissues were stored as frozen sections (14 μm). Subsequently, the green fluorescent protein (GFP)-positive cells in pancreatic tissue were observed with a Nikon inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan). The effect of CHOP expression silencing in the pancreatic tissues was then analyzed by western blot analysis and RT-qPCR.

Western blot analysis. Total proteins from pancreatic tissues were extracted and homogenized in ice-cold lysis buffer containing radioimmunoprecipitation assay, phosphatase and phenylmethane sulfonyl fluoride (ratio, 100:10:1) for 30 min on ice. Next, the extracts were transferred to a microcentrifuge tube and centrifuged at $1.2x10^4$ x g for 15 min (4˚C), and protein concentrations were determined using a BCA assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, 45 μg total protein was separated through 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) at 300 mA for 1 h. The membranes were blocked with 5% skim milk for 2 h at 25˚C and then incubated with primary antibodies overnight at 4˚C. The primary antibodies used in this analysis included 78 kDa glucose-regulated protein (GRP78; cat. no. ab108615; Abcam, Cambridge, UK), CHOP (cat. no. ab179823; Abcam), tumor necrosis factor α (TNF-α; cat. no. ab6671; Abcam), B-cell lymphoma 2 (Bcl-2; cat. no. ab32124; Abcam), Bcl-2-associated X protein (Bax; cat. no. ab182733; Abcam), caspase-3 (cat. no. ab32351; Abcam), phospho-NF-κB inhibitor α (p-IκBα; Ser 32; cat. no. 2859; Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-NF-κB p65 (p-p65; Ser 536; cat. no. 3033; Cell Signaling Technology, Inc.), and β-actin (Cell Signaling Technology, Inc.) at a 1:1,000 dilution. β-actin was used as an internal control. On the following day, membranes were washed three times with Tris-buffered saline/Tween-20 (TBST) and incubated for 1 h at room temperature with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5,000; Bioworld Technology, Inc., St. Louis Park, MN, USA), and then washed three times with TBST. Finally, the protein bands were visualized using a Western Bright ECL detection kit (Advansta, Menlo Park, CA, USA). The density of specific bands was quantified using Image Lab software with a ChemiDoc MP imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed in triplicate.

RT-qPCR analysis. Total RNA from pancreatic tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Next, total RNA was reverse transcribed into cDNA using an RT kit (Thermo Fisher Scientific, Inc) at 25˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min; the concentration of RNA was determined with a spectrophotometer by measuring the optical density at 260/280 nm. The qPCR procedure was subsequently conducted using a Real-Time qPCR system (Bio-Rad Laboratories, Inc.) and the Takara Power SYBR-Green PCR Master mix (cat. no. DRR820A; Takara Bio, Inc., Otsu, Japan). The PCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences were as follows: GRP78, 5'-CAAGAACCA ACTCACGTCCA-3' (forward) and 5'-ACCACCTTGAAT GGCAAGAA-3' (reverse); CHOP, 5'-CCAGGAAACGAA GAGGAAGA-3' (forward) and 5'-CTTTGGGAGGTGCTTG TGA-3' (reverse); Bcl-2, 5'-AGGATTGTGGCCTTCTTTGA-3' (forward) and 5'-CAGATGCCGGTTCAGGTACT-3' (reverse); Bax, 5'-CAGGATCGAGCAGAGAGGAT-3' (forward) and 5'-GTCCAGTTCATCGCCAATTC-3' (reverse); caspase-3, 5'-ACTGGACTGTGGCATTGAGA-3' (forward) and 5'-AAT TTCGCCAGGAATAGTAACC-3' (reverse); TNF-α, 5'-TGA TCCGAGATGTGGAACTG-3' (forward) and 5'-CGAGCA GGAATGAGAAGAGG-3' (reverse); IL-6, 5'-TACCCCAAC TTCCAATGCTC-3' (forward) and 5'-GGTTTGCCGAGT AGACCTCA-3' (reverse); β-actin, 5'-CGTGAAAAGATG ACCCAGAT-3' (forward) and 5'-ACCCTCATAGATGGG CACA-3' (reverse). β-actin was used as an internal control. The cDNA was amplified over the course of a 40-cycle program at 95˚C for 30 sec, 95˚C for 5 sec and 60˚C for 30 sec. Finally, the quantified relative gene expression levels of GRP78, CHOP, Bcl-2, Bax, caspase-3, IL-6 and TNF-α mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (40). Each experiment was performed in triplicate.

Histological analysis and pathological scores of pancreatic tissues. The pancreatic tissue samples were fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin wax and stained with hematoxylin-eosin. Alterations in the pancreatic tissue were assessed by an experienced pathologist who was blinded to the experimental protocol. Eight randomly selected visual areas in each pathological section were observed under a light microscope (Nikon Corporation) and scored by an experienced histologist using standards obtained from a study by Schmidt *et al* (41). Briefly, the pancreatic tissues were scored for edema, inflammatory cell infiltration, acinar cell degeneration and hemorrhage, each on a scale for 0-3. The maximum score for each visual area was 12. Each experiment was performed in triplicate.

Immunohistochemical staining. Pancreatic tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were deparaffinized with xylene and hydrated through a graded ethanol series, followed by microwave heat repair using citrate buffer (pH 6.0; OriGene Technologies, Inc., Beijing, China) for 15-20 min and cooling for 10 min in an ice-water bath. Tissue sections were then treated with 3% hydrogen peroxide (OriGene Technologies, Inc.) to suppress the activity of endogenous peroxidase. Next, the tissue sections were blocked with 5% goat serum (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 1 h at room temperature and then incubated with CHOP and Bcl-2 antibodies in a 1:100 dilution overnight at 4˚C. Subsequently, the sections were incubated with a goat anti-rabbit secondary IgG antibody conjugated to biotin (undiluted; cat. no. DS-0005; OriGene Technologies, Inc.) for 1 h at room temperature, followed by staining with DAB (OriGene Technologies, Inc.) and hematoxylin (Solarbio Science & Technology Co., Ltd.). Images were captured using a light microscope (Nikon Corporation) and quantified using the ImagePro-Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Each experiment was performed in triplicate.

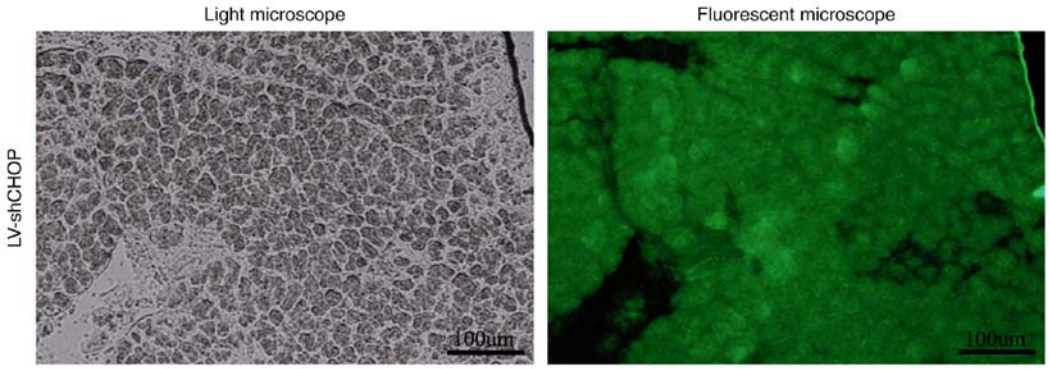

Figure 1. Efficacy of LV-shCHOP-mediated RNA interference in pancreatic tissue. The stable transduction efficiency of LV-shCHOP in pancreatic tissue was detected using light and fluorescent microscopy (original magnification, x40; bar, 100 μm). LV-shCHOP, lentiviral-mediated C/EBP homologous protein short hairpin RNA.

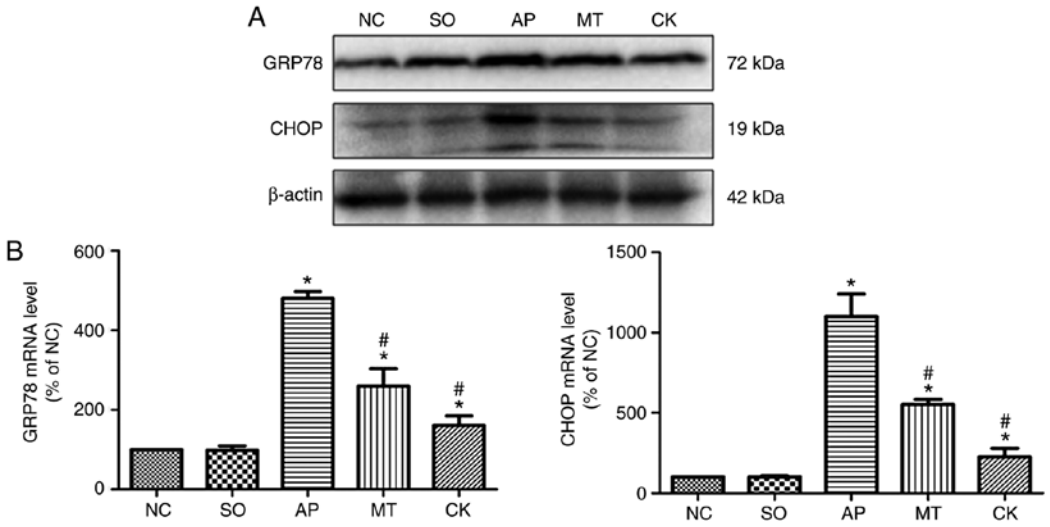

Figure 2. Expression of markers of ER stress in pancreatic tissue obtained from rats after 9 h of treatment in the NC, SO, AP, MT and CK groups. (A) GRP78 and CHOP protein expression levels in the pancreatic tissue were examined by western blot analysis, and β-actin was used as an internal control. (B) mRNA levels of GRP78 and CHOP were quantified by reverse transcription-quantitative polymerase chain reaction. Data (n=8 per group) are expressed as the mean ± standard error of at least three independent experiments. *P<0.05 vs. the SO and NC groups; #P<0.05 vs. the AP group. CHOP, C/EBP homologous protein; GRP78, 78 kDa glucose-regulated protein; NC, negative control; SO, sham-operated; AP, acute pancreatitis; MT, melatonin treatment; CK, CHOP knockdown.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. SPSS version 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis, while data comparisons were made using the analysis of variance and Newman-Keuls multiple comparison tests, as appropriate. P<0.05 was considered to denote a statistically significant difference.

Results

Transduction efficacy of LV-shCHOP-mediated RNAi in pancreatic tissue. Highly efficient transduction (>85%) of LV-shCHOP in pancreatic tissue was achieved after 5 days of infection (Fig. 1). The GFP-positive cells were also observed with a Nikon Inverted fluorescence microscope (Fig. 1).

Effects of taurocholate and treatment with melatonin on markers of ER stress in the pancreatic tissue. As illustrated in Fig. 2A and B, GRP78 and CHOP levels were markedly elevated in the AP group compared with the SO and NC groups. By contrast, the MT and CK groups exhibited significantly reduced protein levels of GRP78 and CHOP compared with the AP group. In the CK group, silencing of CHOP expression in the pancreatic tissue was achieved, which significantly attenuated the ER stress following the induction of AP, while pre-treatment with melatonin also downregulated the CHOP levels and restrained ER stress in AP.

Effects of taurocholate and treatment with melatonin on inflammation and apoptosis-associated molecules in pancreatic tissue. TNF-α, p-p65 and p-IκBα levels were markedly increased in the AP group compared with the SO and NC groups. However, the CK and MT groups exhibited a significant reduction in the levels of these molecules compared with the AP group (Fig. 3A and B). Furthermore, Fig. 4A and B demonstrates that the levels of Bax and caspase-3 were significantly elevated, while Bcl-2 level was reduced, in the AP group relative to the SO and NC groups. By contrast, the

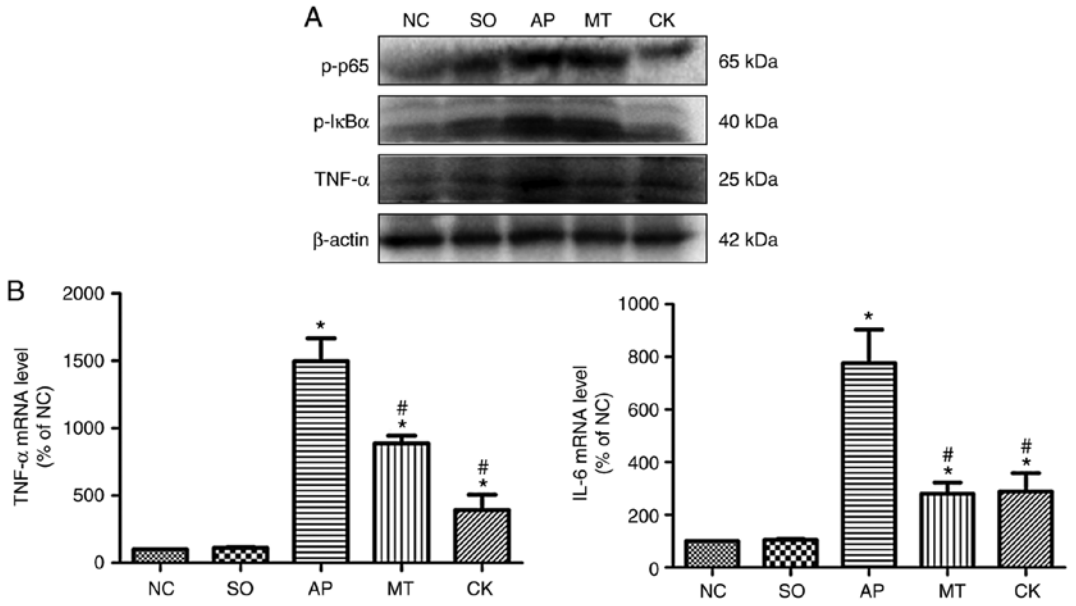

Figure 3. Expression levels of inflammation-associated molecules in pancreatic tissue collected from rats after 9 h of treatment in the different groups. (A) p-p65, p-IκBα and TNF-α protein expression levels in the pancreatic tissue, examined by western blot analysis. (B) mRNA levels of TNF-α and IL-6 were quantified by reverse transcription-quantitative polymerase chain reaction. Data (n=8 per group) are expressed as the mean ± standard error of at least three independent experiments. *P<0.05 vs. the SO and NC groups; #P<0.05 vs. the AP group. IκBα, nuclear factor-κB inhibitor α; TNF-α, tumor necrosis factor α; IL, interleukin; p-, phosphorylated; NC, negative control; SO, sham-operated; AP, acute pancreatitis; MT, melatonin treatment; CK, C/EBP homologous protein knockdown.

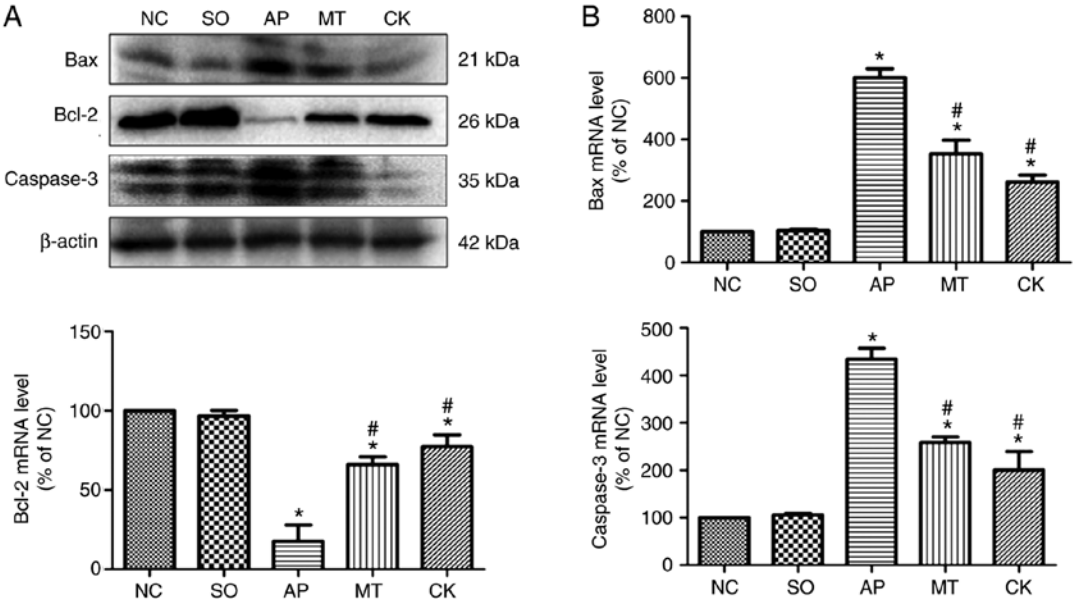

Figure 4. Expression levels of apoptosis-associated molecules in pancreatic tissue samples collected from rats after 9 h of treatment in the different groups. (A) Bax, Bcl-2 and caspase-3 protein expression levels in the pancreatic tissue were examined by western blot analysis, and β-actin was used as an internal control. (B) mRNA levels of Bax, Bcl-2 and caspase-3 were quantified by reverse transcription-quantitative polymerase chain reaction. Data (n=8 per group) are expressed as the mean ± standard error of at least three independent experiments. *P<0.05 vs. the SO and NC groups; #P<0.05 vs. the AP group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, negative control; SO, sham-operated; AP, acute pancreatitis; MT, melatonin treatment; CK, C/EBP homologous protein knockdown.

CK and MT groups demonstrated significantly enhanced protein levels of Bcl-2 and reduced protein levels of Bax and caspase-3, compared with the AP group. These levels indicated that knockdown of CHOP expression in pancreatic tissue reduced the activation of the NF-κB pathway, inflammation and apoptosis response following the induction of AP, while pre-treatment with melatonin also relieved inflammation and apoptosis in AP. Therefore, CHOP was observed to exacerbate AP injury through inducing apoptosis, and activating the NF-κB pathway and inflammation response. Besides, melatonin reduced apoptosis, and alleviated AP severity and tissue injury through CHOP-mediated pathways.

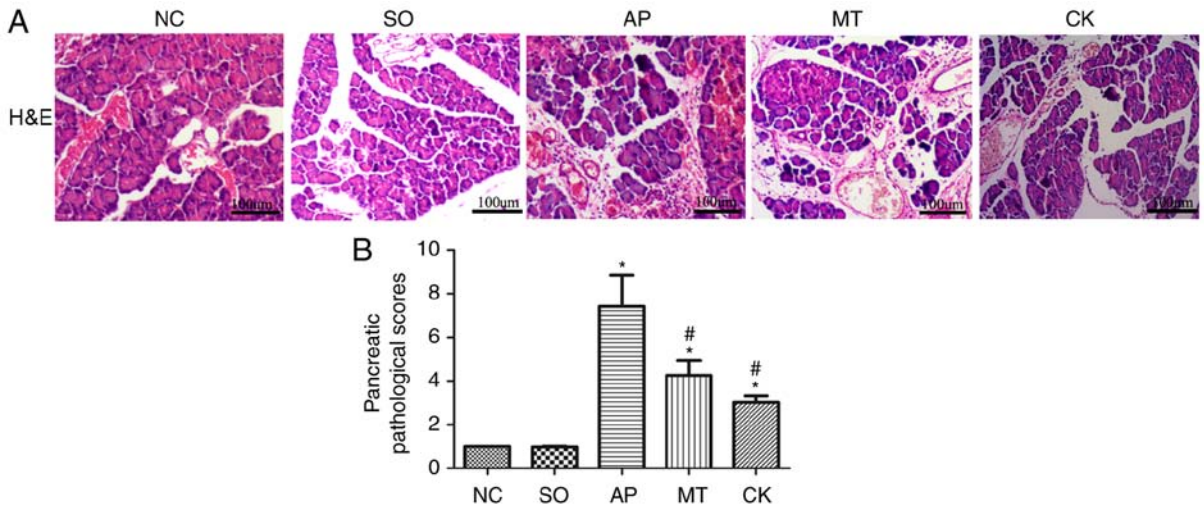

Figure 5. Histological changes and pathological scores of pancreatic tissues obtained from rats after 9 h of treatment in the different groups. (A) Histological changes were more severe in the AP group as compared with those in the SO and NC groups, but were less pronounced in the MT and CK group after 9 h of induction (H&E staining; original magnification, x40; bar, 100 μm). (B) Pathological scores of pancreatic tissues. Data (n=8 per group) are expressed as the mean ± standard error of at least three independent experiments. *P<0.05 vs. the SO and NC groups; #P<0.05 vs. the AP group. NC, negative control; SO, sham-operated; AP, acute pancreatitis; MT, melatonin treatment; CK, C/EBP homologous protein knockdown; H&E, hematoxylin-eosin.

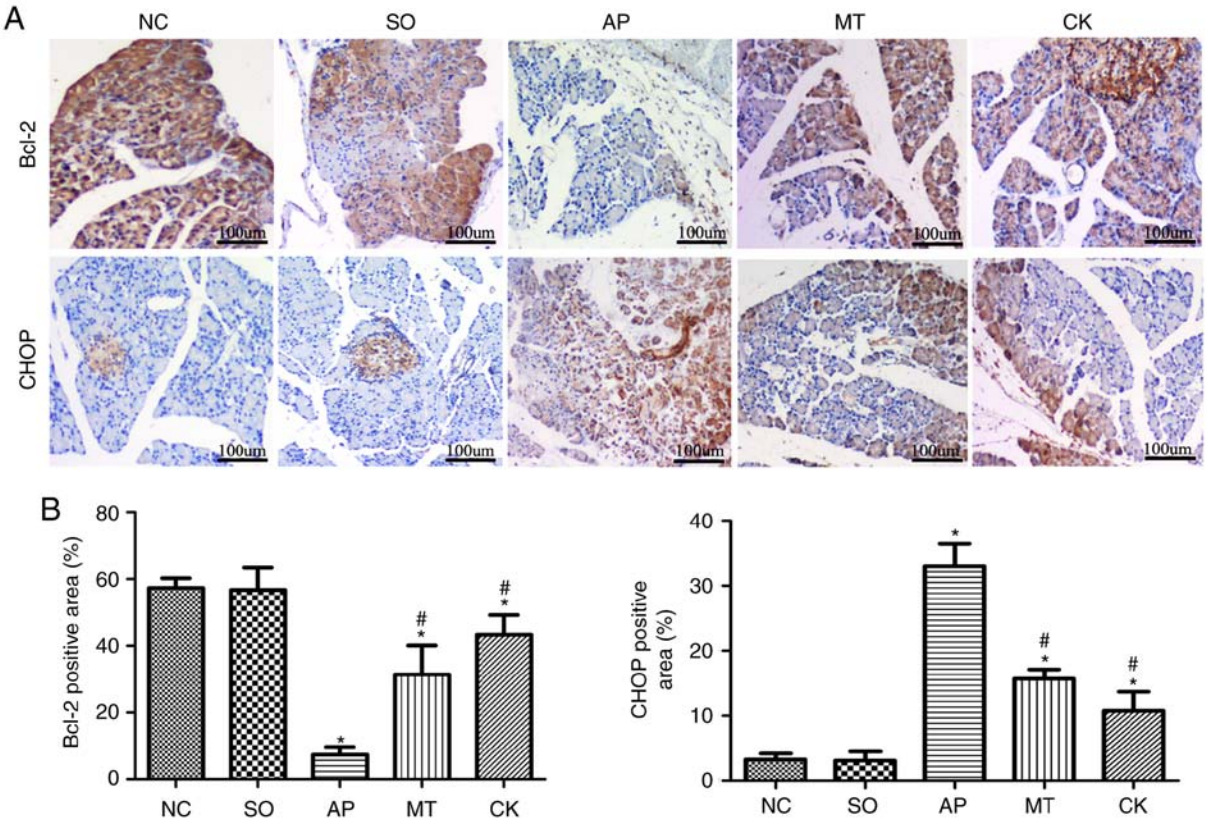

Figure 6. Expression levels of CHOP and Bcl-2 in pancreatic tissue samples from rats after 9 h of treatment in the different groups. (A) Expression levels of CHOP and Bcl-2 in the pancreatic tissue, as determined by immunohistochemistry (original magnification, x40; bar, 100 μm). (B) Image analysis of the area of CHOP and Bcl-2 staining. Data (n=8 per group) are expressed as the mean ± standard error of at least three independent experiments. *P<0.05 vs. the SO and NC groups; #P<0.05 vs. the AP group. CHOP, C/EBP homologous protein; Bcl-2, B-cell lymphoma 2; NC, negative control; SO, sham-operated; AP, acute pancreatitis; MT, melatonin treatment; CK, CHOP knockdown.

Pathological scoring and histopathological examination of pancreatic tissues. Microscopic examination of pancreatic samples from the SO group displayed a normal morphology at 9 h after surgery. The AP group tissues exhibited evident edema, hemorrhage and inflammatory cell infiltration. By contrast, the injury to pancreatic tissues in the MT and CK groups was significantly alleviated compared with the AP group (Fig. 5A). The mean pathological score of pancreatic

samples in the MT and CK groups was significantly lower compared with the AP group, although it remained markedly higher than that in the SO and NC groups (Fig. 5B).

Effect of taurocholate and treatment with melatonin on CHOP and Bcl-2 expression in pancreatic tissue by immunohistochemistry. The immunohistochemical analysis results further revealed significantly higher expression of Bcl-2 and lower expression of CHOP in the CK and MT groups, as compared with the expression in the AP group (Fig. 6A and B). This result further revealed that silencing of the CHOP gene in pancreatic tissue significantly reduced the expression of the CHOP protein; RT-qPCR and western blotting indicated that silencing CHOP expression reduced the expression of pro-apoptotic proteins, Bax and caspase-3, and increased that of anti-apoptotic Bcl-2, which suggested a reduction in apoptosis during AP. In addition, in the MT group, the expression of CHOP and apoptosis were also significantly attenuated, indicating that melatonin reduced apoptosis through CHOP-mediated pathways.

Discussion

Although AP is one of the most lethal diseases of the exocrine pancreas, the underlying mechanisms remain unclear. Therefore, various cellular and animal models have been developed in order to identify the early mechanisms of AP, which determine the ultimate severity of the disease. In recent years, studies have demonstrated that ER stress serves an indispensable role in the development and progression of AP (42). The present study demonstrated that the ER stress-induced CHOP-mediated pathway was activated in the early stage of AP inflammation and served an important role in exacerbating pancreatic injury by inducing apoptosis, NF-κB pathway activation and the expression of proinflammatory cytokines. Furthermore, the study also provided a new insight by demonstrating that melatonin protects against pancreatitis inflammation via inhibition of the CHOP-mediated pathway.

GRP78 is an important molecular chaperone (43), which is located in the ER and widely used as an ER stress marker and a key regulator of the UPR. Normally, it is expressed at a very low level, however, when responding to ER stress, GRP78 expression significantly increases and is separated from three ER transmembrane proteins (PERK, ATF6 and IRE1), thereby activating the UPR signaling pathway (1). In the current study, GRP78 was found to be upregulated early in a rat AP model, suggesting that ER stress was activated early in AP.

CHOP/GADD153 is an ER stress-specific transcription factor that belongs to the C/EBP family of basic leucine zinc finger structure transcription factors and is a signaling molecule involved in ER stress-induced apoptosis (44). Previous studies have reported that, under normal conditions, the expression of CHOP is low; however, when responding to ER stress, IRE-1, PERK and ATF6 are able to promote CHOP activation, with overexpression of CHOP resulting in cell apoptosis (1,18,45), with the PERK signaling pathway being the most important driver of this process. Ji *et al* (46) identified through mice fed with ethanol that hepatocellular apoptosis was significantly reduced in CHOP-deficient mice compared with wild-type mice, while other genes associated with apoptosis were also changed, such as Jun D, Bcl-xl and Gadd45. ER stress is addressed via UPR-activated CHOP protein, and thus far, several mechanisms have been proposed for CHOP-mediated apoptosis. Such mechanisms include the increase of the levels of pro-apoptosis proteins, such as Bax/Bak, the decrease of the antiapoptotic protein Bcl-2 levels (47), the release of cytochrome *c* from mitochondria, and the activation of apoptotic-associated caspase cascade (48,49). All of these previous studies, as well as the results of the current study, demonstrated that CHOP was activated in the early stage of inflammation and also increased over time, alongside increased Bax and caspase-3 levels and decreased Bcl-2, supporting the idea that CHOP-mediated apoptosis mainly occurs via a mitochondria-dependent pathway. Furthermore, the present study western blot and RT-qPCR results indicated that silencing of CHOP in pancreatic tissue suppressed apoptosis by inhibiting the expression of Bax and caspase-3, and activating Bcl-2 during AP. These results were also confirmed by immunohistochemistry, which revealed a significantly higher expression of Bcl-2 in the CK group as compared with that in the AP group. All these results demonstrated that CHOP served a potentially important role in AP-associated apoptosis. The results of the present study were in agreement with previous findings reporting that CHOP deficiency can protect cells from ER stress-induced apoptosis (16).

The current observations indicate that UPR can initiate an inflammatory response via various mechanisms (50). Besides its pro-apoptotic role. A number of studies have also revealed a pro-inflammatory role for the CHOP-mediated pathway (3,39,51,52), however, the mechanisms between inflammation and CHOP have not been fully explored, particularly in rat AP models. In the present study, it was proposed that the pro-inflammatory mechanisms of the CHOP-mediated pathway may occur via NF-κB activation. This was a possibility supported by the study of Allagnat *et al* (2), who reported that CHOP directly supported the activation of NF-κB pathway and subsequent expression of pro-inflammatory cytokines, which aggravated ER stress and promoted CHOP expression. In turn, CHOP promoted the degradation of IκBα and amplified the activity of the NF-κB pathway. NF-κB is a member of the transcription factor family involved in the transcriptional regulation of a variety of inflammatory genes and serves an important role in the inflammatory response (2). Such a positive feedback loop may exacerbate inflammatory responses and injury, resulting in the development of AP. Consistently, the present study proved this point, as silencing of CHOP was able to significantly reduce NF-κB activation and inflammatory cytokine levels in an *in vivo* AP model. Additionally, pathological examination and immunohistochemistry analysis results revealed that CHOP silencing significantly reduced pancreatic injury and inflammatory cell infiltration. According to the experimental results, it was concluded that the CHOP-mediated pathway effectively promoted the inflammatory response in AP. Nevertheless, the potential molecular mechanism associating the inflammatory response and the CHOP-mediated pathway is of great importance and deserves further investigation.

Previous results have indicated that melatonin prevents or attenuates the severity of experimental severe acute pancreatitis (SAP) (39). However, the mechanisms of its anti-inflammatory effects in AP are currently unclear. Recently, ER stress was reported to be involved in the development and progression

of AP, and several studies have confirmed that melatonin suppressed ER stress in different models of cell damage. For instance, treatment with melatonin resulting in attenuation of cell apoptosis after brain ischemia was associated with the reduction of ER stress (53), and similar effects were also observed in lethal fulminant hepatitis (54). The main finding of the present study was that melatonin significantly alleviated pancreatic injury, inflammation and apoptosis in the AP rat model, accompanied by downregulation of the expression of CHOP (a hallmark of ER-associated apoptosis) and GRP78 (ER stress marker). The role of CHOP in aggravating the inflammation and apoptosis in AP was confirmed earlier in the present study; therefore, it is hypothesized that melatonin can inhibit the CHOP-mediated pathway to reduce injury in AP.

In conclusion, the current study indicates that the ER stress-induced, CHOP-mediated pathway is activated soon after the induction of AP. This pathway subsequently exacerbates AP injury not only by inducing the apoptosis of pancreatic tissue, but also by enhancing tissue inflammation. Therefore, the CHOP-mediated pathway may represent a potential target for developing new protection strategies against AP injury. Furthermore, the findings of the present study provided further preclinical evidence of the pancreas-protective effect of melatonin during AP via inhibition of the CHOP-mediated pathway. Owing to its efficacy and low toxicity, it is also suggested that melatonin urgently requires further evaluation through clinical applications.

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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

QZ and JH performed the majority of the experiments and analyzed the data. HZ and JL performed the molecular investigations. QZ and QC participated equally in the treatment of animals. JW and YJ designed and coordinated the research. HY and YS performed the statistical analyses. QZ wrote the manuscript.

Ethics approval and consent to participate

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

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Kubisch CH and Logsdon CD: Endoplasmic reticulum stress and the pancreatic acinar cell. Expert Rev Gastroenterol Hepatol 2: 249-260, 2008.
2. Allagnat F, Fukaya M, Nogueira TC, Delaroche D, Welsh N, Marselli L, Marchetti P, Haefliger JA, Eizirik D and Cardozo AK: C/EBP homologous protein contributes to cytokine-induced pro-inflammatory responses and apoptosis in β-cells. Cell Death Differ 19: 1836-1846, 2012.
3. Miyazaki Y, Kaikita K, Endo M, Horio E, Miura M, Tsujita K, Hokimoto S, Yamamuro M, Iwawaki T, Gotoh T, *et al*: C/EBP homologous protein deficiency attenuates myocardial reperfusion injury by inhibiting myocardial apoptosis and inflammation. Arterioscler Thromb Vasc Biol 31: 1124-1132, 2011.
4. Wu JS, Li WM, Chen YN, Zhao Q and Chen QF: Endoplasmic reticulum stress is activated in acute pancreatitis. J Dig Dis 17: 295-303, 2016.
5. Nashine S, Liu Y, Kim BJ, Clark AF and Pang IH: Role of C/EBP homologous protein in retinal ganglion cell death after ischemia/reperfusion injury. Invest Ophthalmol Vis Sci 56: 221-231, 2014.
6. Ron D and Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8: 519-529, 2007.
7. Schröder M and Kaufman RJ: The mammalian unfolded protein response. Annu Rev Biochem 74: 739-789, 2005.
8. Ohoka N, Yoshii S, Hattori T, Onozaki K and Hayashi H: TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. EMBO J 24: 1243-1255, 2005.
9. Yamaguchi H and Wang HG: CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 279: 45495-45502, 2004.
10. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, *et al*: ER stress triggers apoptosis by activating BH3-only protein Bim. Cell 129: 1337-1349, 2007.
11. Song B, Scheuner D, Ron D, Pennathur S and Kaufman RJ: Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. J Clin Invest 118: 3378-3389, 2008.
12. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E and Mori M: Targeted disruption of the Chop gene delays endoplasmic reticulum stressmediated diabetes. J Clin Invest 109: 525-532, 2002.
13. Milhavet O, Martindale JL, Camandola S, Chan SL, Gary DS, Cheng A, Holbrook NJ and Mattson MP: Involvement of GADD153 in the pathogenic action of presenilin-1 mutations. J Neurochem 83: 673-681, 2002.
14. Paschen W, Gissel C, Linden T, Althausen S and Doutheil J: Activation of GADD153 expression through transient cerebral ischemia: Evidence that ischemia causes endoplasmic reticulum dysfunction. Brain Res Mol Brain Res 60: 115-122, 1998.
15. Prasanthi JR, Larson T, Schommer J and Ghribi O: Silencing GADD153/CHOP gene expression protects against Alzheimer's disease-like pathology induced by 27-hydroxycholesterol in rabbit hippocampus. PLoS One 6: E26420, 2011.
16. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL and Ron D: CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12: 982-995, 1998.

17. Esrefoglu M, Gul M, Ates B, Batcioglu K and Selimoglu MA: Antioxidative effect of melatonin, ascorbic acid and nacetylcysteine on caerulein induced pancreatitis and associated liver injury in rats. World J Gastroenterol 12: 259-264, 2006.
18. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM and Ron D: Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol 16: 4273-4280, 1996.
19. Song JM, Liu HX, Li Y, Zeng YJ, Zhou ZG, Liu HY, Xu B, Wang L, Zhou B and Wang R: Extracellular heat-shock protein 70 aggravates cerulein-induced pancreatitis through toll-like receptor-4 in mice. Chin Med J (Engl) 121: 1420-1425, 2008.
20. van Minnen LP, Blom M, Timmerman HM, Visser MR, Gooszen HG and Akkermans LM: The use of animal models to study bacterial translocation during acute pancreatitis. J Gastrointest Surg 11: 682-689, 2007.
21. Yu JH and Kim H: Oxidative stress and inflammatory signaling in cerulein pancreatitis. World J Gastroenterol 20: 17324-17329, 2014.
22. Tando Y, Algul H, Schneider G, Weber CK, Weidenbach H, Adler G and Schmid RM: Induction of IkappaB-kinase by cholecystokinin is mediated by trypsinogen activation in rat pancreatic lobules. Digestion 66: 237-245, 2002.
23. Xue X, Piao JH, Nakajima A, Sakon-Komazawa S, Kojima Y, Mori K, Yagita H, Okumura K, Harding H and Nakano H: Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the upr counteracts ros accumulation by TNFalpha. J Biol Chem 280: 33917-33925, 2005.
24. Lin W, Harding HP, Ron D and Popko B: Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma. J Cell Biol 169: 603-612, 2005.
25. Malhotra JD and Kaufman RJ: Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277-2293, 2007.
26. Case RM: Synthesis, intracellular transport and discharge of exportable proteins in the pancreatic acinar cell and other cells. Biol Rev Camb Philos Soc 53: 211-354, 1978.
27. Berridge MJ: The endoplasmic reticulum: A multifunctional signaling organelle. Cell Calcium 32: 235-249, 2002.
28. Deng WH, Chen C, Wang WX, Yu J, Li JY and Liu L: Effects of ORP150 on appearance and function of pancreatic beta cells following acute necrotizing pancreatitis. Pathol Res Pract 207: 370-376, 2011.
29. Hartley T, Siva M, Lai E, Teodoro T, Zhang L and Volchuk A: Endoplasmic reticulum stress response in an INS-1 pancreatic beta-cell line with inducible expression of a folding-deficient proinsulin. BMC Cell Biol 11: 59, 2010.
30. Kubisch CH, Sans MD, Arumugam T, Ernst SA, Williams JA and Logsdon CD: Early activation of endoplasmic reticulum stress is associated with arginine-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 291: G238-G245, 2006.
31. Sans MD, DiMagno MJ, D'Alecy LG and Williams JA: Caerulein-induced acute pancreatitis inhibits protein synthesis through effects on eIF2B and eIF4F. Am J Physiol Gastrointest Liver Physiol 285: G517-G528, 2003.
32. Hu P, Han Z, Couvillon AD, Kaufman RJ and Exton JH: Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol 26: 3071-3084, 2006.
33. Yun SW, Bae GS, Kim MS, Park KC, Koo BS, Kim BJ, Kim TH, Seo SW, Shin YK, Lee SH, *et al*: Melittin inhibits cerulein-induced acute pancreatitis via inhibition of the JNK pathway. Int Immunopharmacol 11: 2062-2072, 2011.
34. Murr MM, Yang J, Fier A, Gallagher SF, Carter G, Gower WR and Norman JG: Regulation of Kupffer cell TNF gene expression during experimental acute pancreatitis: The role of p38-MAPK, ERK1/2, SAPK/JNK, and NF-kappaB. J Gastrointest Surg 7: 20-25, 2003.
35. Lee AH, Chu GC, Iwakoshi NN and Glimcher LH: Xbp-1 is required for biogenesis of cellular secretory machinery of exocrine glands. EMBO J 24: 4368-4380, 2005.
36. Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD and Ron D: Diabetes mellitus and exocrine pancreatic dysfunction in Perk$^{-/-}$ mice reveals a role for translational control in secretory cell survival. Mol Cell 7: 1153-1163, 2001.
37. Pandol SJ, Gorelick FS, Gerloff A and Lugea A: Alcohol abuse, endoplasmic reticulum stress and pancreatitis. Dig Dis 28: 776-782, 2010.
38. Carloni S, Albertini MC, Galluzzi L, Buonocore G, Proietti F and Balduini W: Melatonin reduces endoplasmic reticulum stress and preserves sirtuin 1 expression in neuronal cells of newborn rats after hypoxia-ischemia. J Pineal Res 57: 192-199, 2014.
39. Qi W, Tan DX, Reiter RJ, Kim SJ, Manchester LC, Cabrera J, Sainz RM and Mayo JC: Melatonin reduces lipid peroxidation and tissue edema in cerulein-induced acute pancreatitis in rats. Dig Dis Sci 44: 2257-2262, 1999.
40. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative pcr and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
41. Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT and Warshaw AL: A better model of acute pancreatitis for evaluating therapy. Ann Surg 215: 44-56, 1992.
42. Thrower EC, Gorelick FS and Husain SZ: Molecular and cellular mechanisms of pancreatic injury. Curr Opin Gastroenterol 26: 484-489, 2010.
43. Hasnain SZ, Lourie R, Das I, Chen AC and McGuckin MA: The interplay between endoplasmic reticulum stress and inflammation. Immunol Cell Biol 90: 260-270, 2012.
44. Oyadomari S and Mori M: Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 11: 381-389, 2004.
45. Matsumoto M, Minami M, Takeda K, Sakao Y and Akira S: Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett 395: 143-147, 1996.
46. Ji C, Mehrian-Shai R, Chan C, Hsu YH and Kaplowitz N: Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. Alcohol Clin Exp Res 29: 1496-1503, 2005.
47. McCullough KD, Martindale JL, Klotz LO, Aw TY and Holbrook NJ: Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating BCL2 and perturbing the cellular redox state. Mol Cell Biol 21: 1249-1259, 2001.
48. Martinou JC and Green DR: Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2: 63-67, 2001.
49. Oltvai ZN, Milliman CL and Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. Cell 74: 609-619, 1993.
50. Zhang K and Kaufman RJ: From endoplasmic-reticulum stress to the inflammatory response. Nature 454: 455-462, 2008.
51. Suyama K, Ohmuraya M, Hirota M, Ozaki N, Ida S, Endo M, Araki K, Gotoh T, Baba H and Yamamura K: C/EBP homologous protein is crucial for the acceleration of experimental pancreatitis. Biochem Biophys Res Commun 367: 176-182, 2008.
52. Namba T, Tanaka K, Ito Y, Ishihara T, Hoshino T, Gotoh T, Endo M, Sato K and Mizushima T: Positive role of CCAAT/enhancer-binding protein homologous protein, a transcription factor involved in the endoplasmic reticulum stress response in the development of colitis. Am J Pathol 174: 1786-1798, 2009.
53. Sheng R, Liu XQ, Zhang LS, Gao B, Han R, Wu YQ, Zhang XY and Qin ZH: Autophagy regulates endoplasmic reticulum stress in ischemic preconditioning. Autophagy 8: 310-325, 2012.
54. Tunon MJ, San-Miguel B, Crespo I, Laliena A, Vallejo D, Alvarez M, Prieto J and Gonzalez-Gallego J: Melatonin treatment reduces endoplasmic reticulum stress and modulates the unfolded protein response in rabbits with lethal fulminant hepatitis of viral origin. J Pineal Res 55: 221-228, 2013.