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Chaiqin chengqi decoction ameliorates acute pancreatitis in mice via inhibition of neuron activation-mediated acinar cell SP/NK1R signaling pathways

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ABSTRACT

Ethnopharmacological relevance: Chaiqin chengqi decoction (CQCQD) and its derivatives have been widely used in China for the early management of patients with acute pancreatitis (AP). Numerous studies demonstrate the anti-inflammatory and anti-oxidative effects of CQCQD and derivatives, but whether these effects can be attributed to suppressing neurogenic inflammation, has never been studied.
Aim of the study: To investigate the effects of CQCQD on substance P (SP)-neurokinin 1 receptor (NK1R) based neurogenic inflammation in an experimental AP model.
Material and methods: For AP patients on admission, pain score was accessed by visual analog scale (VAS); the levels of serum SP and expressions of pancreatic SP and NK1R were also determined. For *in vivo* study, mice received 7 intraperitoneal injections of cerulein (50 μg/kg) at hourly intervals to induce AP, whilst controls received normal saline injections. In the treatment groups, CQCQD (10 g/kg, 200 μl) was intragastrically given at the third, fifth, and seventh of the cerulein injection or the NK1R antagonist CP96345 (5 mg/kg) was intraperitoneally injected 30 min before the first cerulein administration. The von Frey test was performed to evaluate pain behavior. Animals were sacrificed at 12 h from the first cerulein/saline injection for severity assessment. Pharmacology network analysis was used to identify active ingredients of CQCQD for AP and pain. *In vitro,* freshly isolated pancreatic acinar cells were pre-treated with CQCQD (5 mg/ml), CP96345 (1 μM), or selected active compounds of CQCQD (12.5, 25, and 50 μM) for 30 min, followed by SP incubation for another 30 min.
Results: The VAS score as well as the levels of serum SP and expressions of pancreatic SP-NK1R were up-regulated in moderately severe and severe patients compared with those with mild disease. CQCQD, but not CP96345, consistently and significantly ameliorated pain, pancreatic necrosis, and systemic inflammation in cerulein-induced AP as well as inhibited NK1R internalization of pancreatic acinar cells. These effects of CQCQD were associated with reduction of pancreatic SP-NK1R and neuron activity in pancreas, dorsal root ganglia, and spinal

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cord. Baicalin, emodin, and magnolol, the top 3 active components of CQCQD identified via pharmacology network analysis, suppressed NK1R internalization and NF-κB signal pathway activation in isolated pancreatic acinar cells.

Conclusions: CQCQD ameliorated cerulein-induced AP and its associated pain via inhibiting neuron activation-mediated pancreatic acinar cell SP-NK1R signaling pathways and its active compounds baicalin, emodin, and magnolol contributed to this effect.

Abbreviations

CQCQD	chaiqin chengqi decoction
AP	acute pancreatitis
SP	substance P
NK1R	neutokinin-1 receptor
CER	cerulein
DRG	dorsal root ganglia
DAMPs	damage-associated molecular pattern
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
GPCR	G protein-coupled receptors
p38 MAPK	p38 mitogen-activated protein kinase
p-ERK	phosphorylated extracellular signal-related kinase
TCM	traditional Chinese medicine
MPO	myeloperoxidase
IL-6	interleukin-6
H&E	hematoxylin and eosin
NGF	nerve growth factor
DAPI	4′,6-diamidino-2-phenylindole
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
MAP	mild acute pancreatitis
MSAP/SAP	moderately severe and severe acute pancreatitis
PGP 9.5	protein gene product 9.5

1. Introduction

Acute pancreatitis (AP) is an inflammatory disease of the digestive system that has become increasingly prevalent worldwide (Petrov and Yadav, 2019). While the clinical course for most AP patients is usually self-limiting, around 15–20% patients (Crockett et al., 2018; Forsmark et al., 2016) will develop severe disease characterized by persistent organ failure that is often complicated with pancreatic necrosis and sepsis (Guo et al., 2014; Schepers et al., 2019; Shi et al., 2020; Sternby et al., 2019). At present, there is no specific and effective pharmacological therapy available for AP (Moggia et al., 2017; Mukherjee et al., 2019). As per published clinical guidelines, supportive measures such as analgesia, fluid therapy, and nutrition supplementation remain the primary modalities for managing AP patients (Greenberg et al., 2016; Leppaniemi et al., 2019; Yokoe et al., 2015).

Severe pain is the primary clinical feature of AP that leads to hospital admission (Schorn et al., 2015; Stigliano et al., 2017) and serves as a key criterion for diagnosing AP (Banks et al., 2013). Previous studies have shown that increased inpatient opioid requirement is strongly correlated with more severe AP (Matic et al., 2020; Parsa et al., 2019) and prolonged hospital stay (Wu et al., 2019). Therefore, opioid usage has also been suggested as a method to monitor disease progression of AP (Buxbaum et al., 2018). Additionally, administration of epidural analgesia in the clinical setting has been shown to reduce the mortality of AP patients with organ failure (Jabaudon et al., 2018) and improve arterial perfusion of necrotizing pancreas (Sadowski et al., 2015).

From a pathophysiological point of view, pain is the clinical manifestation of pancreatic inflammation; however, increasing evidence suggests that pain also plays a critical role in the progression of AP through the extensive neuron network surrounding the pancreas (Babic and Travagli, 2016; Liddle and Nathan, 2004). Anatomically, the pancreas has abundant peripheral neuron fibers responsible for regulating the organ's complex exocrine and endocrine function (Babic and Travagli, 2016). During AP, damaged pancreatic acinar cells release enzymes, cytokines (Lugea et al., 2017), and damage-associated molecular pattern (DAMPs) molecules (Kang et al., 2014; Liu et al., 2017) which stimulate adjacent neurons. In responding to these local inflammatory mediators, the primary neuron terminals release substance P (SP), a principle pain and inflammatory mediator (Steinhoff et al., 2014), to signal the perception of pain to the central nervous system via primary afferents and dorsal root ganglia (DRG) (Ji et al., 2016). Simultaneously, SP activates the surrounding cells' inflammatory signaling pathways, leading to a process commonly known as neurogenic inflammation (Steinhoff et al., 2014). In the case of AP, excess SP from the peripheral nervous system results in local neurogenic inflammation, this process has been shown in experimental animal models to increase damage to the pancreatic acinar cells and exacerbate pancreatic necrosis (Liddle and Nathan, 2004), creating an auto-amplification loop that contribute to cell death and organ failure (Linkermann et al., 2014).

SP is a classic initiator of neurogenic inflammation widely distributed in the nervous system and peripheral tissues (Babic and Travagli, 2016; O'Connor et al., 2004; Steinhoff et al., 2014). It comprises 11 amino acids and is encoded by *TAC-1* (Navratilova and Porreca, 2019; Steinhoff et al., 2014). SP executes its biological activities such as pro-inflammation, neuro-neuronal transmission, and neurogenic inflammation by binding to a range of neurokinin receptors (NKRs; NK1R, NK2R, and NK3R) that belong to the tachykinin receptor family, a sub-family of G protein-coupled receptors (GPCRs), in which NK1R has been widely used as the primary target to study SP function since it possesses the highest affinity for SP (Steinhoff et al., 2014). NK1R is ubiquitously expressed in pancreatic acinar cells, neurons, epithelial cells, endothelial cells, immune cells and can be found in both human and mice (Caberlotto et al., 2003; Koh et al., 2012). After binding with SP, the N-terminal of NK1R is phosphorylated and recruits β-arrestin1, a scaffolding recruiting (binding) protein to NK1R, resulting in NK1R internalization from the cellular membrane to cytoplasm and activation of downstream signaling pathways (NF-κB, p38 MAPK, and Src kinase family) (Steinhoff et al., 2014). In the animal model, this process has been shown to exacerbate pancreatic necrosis; on the contrary, the inhibition of SP-NK1R signaling pathways attenuates the severity of mouse AP models (Bhatia et al., 1998; Grady et al., 2000; Koh et al., 2011; Lau and Bhatia, 2006; Maa et al., 2000).

Chaiqin chengqi decoction (CQCQD) is a Chinese herbal formula modified from dachengqi decoction (DCQD). CQCQD has been safely used to treat AP patients for over 30 years in our hospital (Jin et al., 2018; Li et al., 2020a) in addition to standard AP management (Crockett et al., 2018; Working Group IAP/APA Acute Pancreatitis Guidelines, 2013). Our past research indicated that both CQCQD and DCQD were shown to reduce pancreatic necrosis and systemic inflammation in experimental AP models (Wen et al., 2020; Ma et al., 2020).

Furthermore, in our pursuit to understand the underlying protective mechanism of CQCQD, out team have shown that CQCQD is capable of reducing serum SP levels in L-arginine-induced AP in rats (Zhang et al., 2017). While providing the full explanation of how CQCQD executes its protective mechanism in AP is beyond the scope of the current work, as a natural extension of our past research, in this study, we aimed to i) establish the clinical relationship between blood SP/pancreatic SP-NK1R levels and disease severity in AP patients; ii) attest the hypothesis that CQCQD can modulate the progression of neurogenic inflammation in pancreatic acinar cells via inhibiting the SP-NK1R signaling pathway; iii) identify the active components of CQCQD responsible for attenuating both AP severity and pain whilst assessing their effect on SP-NK1R signaling pathways *in vitro*.

2. Materials and methods

2.1. Ethics, human sample collection and animals

The study protocols (2020, No. 196) were reviewed and approved by the Institutional Review Board and Biomedical Ethics Committee of West China Hospital, Sichuan University. The human sample collection and storage protocols were certified by the China Human Genetic Resources Management Office (2016, No. 406) as a part of West China Biobanks. Protocol for pain assessment using the Visual Analog Scale (VAS) is provided in Supplementary materials and methods. Peripheral blood samples (in serum separator tubes) were obtained from healthy volunteers, or AP patients who presented to our hospital within 48 h of symptom onset and samples were processed with the established protocol (Liu et al., 2017). The inclusion and exclusion criteria were reported in our previous study (Shi et al., 2020). Normal pancreata were obtained from patients who had undergone left-sided or small unobstructing pancreatic tumor resection according to a previous protocol (Murphy et al., 2008). Necrotized and surrounding inflamed pancreata were harvested from patients with infected pancreatic necrosis during necrosectomy procedure. Written informed consent was obtained from the patients or their legal representatives for all human samples used in this study. AP severity of patients was classified according to 2018 American Gastroenterological Association Guidelines (Crockett et al., 2018) as mild (no local and systemic complications), moderately severe (local complication without persistent organ failure), and severe (persistent organ failure regardless of local complication).

All animal experiment procedures were approved by the Animal Ethics Committee (2019170A). Male C57BL/6 mice (22–23 g) were purchased from Beijing Huafukang Bioscience Co., Ltd. (Beijing, China). Animals were maintained at 22 ± 2 °C with a 12 h light-dark cycle with ad libitum access to water and standard laboratory chow.

2.2. Drug preparation and reagents

Raw herbal materials of CQCQD (Supplementary Table 1) were purchased from Sichuan Hospital of Traditional Chinese Medicine (Chengdu, Sichuan, China). The botanical names of the plants were cross-referenced using information from http://www.theplantlist.org. A voucher specimen (No. 201605) of CQCQD formula with unique identification numbers (dahuang, GD-005; zhishi, GZ-008; houpu, GH-006; mangxiao, GM-005; chaihu, GC-008; huangqin, GH-014; zhizi, GZ-010; yinchen, GY-008) was preserved in the herbarium of Laboratory of Ethnopharmacology at the West China Hospital for future reference. Preparation for CQCQD was previously described (Wen et al., 2020). CQCQD solution was prepared freshly by dissolving its powder (extraction yield: 26%) in double-distilled water before each experiment (final concentration: 0.108 g/ml). Details of reagents with relevant product information are provided in the Supplementary Table 2.

2.3. Pharmacology network construction and analysis

Briefly, (1) Search Tool for Interactions of Chemicals (SITICH; http://stitch.embl.de/) and Encyclopedia of Traditional Chinese Medicine (ETCM; http://www.tcmip.cn/ETCM/) databases were used to predict the potential targets of 22 Q-markers (unpublished data) in CQCQD. AP-associated targets or pain-related targets were obtained from OMIM (https://www.omim.org/) and DisGeNET (http://www.disgenet.org). Thus, a local database of CQCQD, AP, and pain was constructed. (2) "Overlapped targets-components" network was established using Cytoscape 3.6.0 software (https://cytoscape.org). The network is a bipartite network consisting of two sets of nodes, one set of nodes represents overlapped targets, the other set indicates corresponding components. A target node and a component node are linked if they specifically correspond to each other according to local database. (3) Network scoring for the contribution of each component was based on node degree (the number of connections to target nodes).

2.4. Experimental AP model and treatments

Mice received 7 intraperitoneal injections of a cholecystokinin analogue cerulein (50 μg/kg) at hourly intervals to induce AP, this is labelled as the CER group. Controls received normal saline injections at the same regimen of cerulein and is labelled as the CON group. In the treatment groups, CQCQD (10 g/kg, 200 μl) was gavaged together with the third, fifth, and seventh of the cerulein injection (Wen et al., 2020) or NK1R antagonist CP96345 (5 mg/kg) was intraperitoneally injected 30 min before the first cerulein administration (Li et al., 2018a). Animals were sacrificed at 12 h from the first cerulein/saline injection. In separate experiments, mice received 1, 4, or 7 injections of cerulein (50 μg/kg) and were culled 1 h after the procedure to measure messenger RNA (mRNA) of SP (*Tac1*) or NK1R (*Tacr1*) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

2.5. Von Frey test

Pain-related behavior in mice was monitored using an up-down test paradigm method with calibrated von Frey filaments (Ugo Basile; Comerio, Italy) of different forces. The withdrawal threshold of mice was detected before the induction of the experiment to establish the baseline, then again measured at 4 consecutive checkpoints of 0.5, 1.5, 2.5, 3.5 h (6.5, 7.5, 8.5, and 9 h after the induction of AP) after the last (7^{th}) cerulein injection. This is consistent with our previously established protocol (Qiu et al., 2020).

2.6. AP severity assessment

Detailed protocols for pancreatic histopathology assessment and measurement of pancreatic and lung myeloperoxidase (MPO), and serum interleukin-6 (IL-6) were described in our previous studies (Du et al., 2018; Huang et al., 2017; Ma et al., 2020; Wen et al., 2020). Human serum SP levels were measured by an enzyme-linked immunosorbent assay in according to the manufacturer's instructions.

2.7. Isolation of pancreatic acinar cells

These methods are provided in Supplementary materials and methods. In brief, pancreatic acinar cells freshly isolated from mice were digested by collagenase IV using our previously established procedure (Huang et al., 2014, 2017). Cells were pre-incubated with selected compounds from CQCQD at concentrations of 12.5, 25 and 50 μM respectively, or NK1R antagonist (CP96345, 1 μM) for 30 min at room temperature followed by incubation with SP (5 μM) for another 30 min.

2.8. Histopathology, immunohistochemistry, and immunofluorescence

More details are provided in Supplementary materials and methods. The pancreata and T6-L2 spinal cord were collected and fixed in 10% formalin for 24 h before hematoxylin and eosin (H&E), immunohistochemistry, or immunofluorescence staining. SP antibody (1:100), nerve growth factor (NGF) antibody (1:100), and NK1R antibody (1:100) were used to determine the expression of these proteins on the pancreas by immunohistochemistry (Du et al., 2018; Wen et al., 2020). β-arrestin1 antibody (1:250) and c-Fos (9F6) antibody (1:100) were applied for immunofluorescence on pancreatic and spinal cord tissues, respectively. The fluorescent signals for c-Fos/β-arrestin1 (both excitation 555 nm, emission 630–693 nm) and nucleus (4′,6-diamidino-2-phenylindole [DAPI]; excitation 401 nm, emission 450–500 nm) were viewed by an epi-fluorescence microscopy ZEISS AX10 imager A2/AX10 cam HRC (Jena GmbH, Zeiss; Heilberg, Germany). NK1R antibody (1:100), NF-κB p65 antibody (1:100), and β-arrestin1 antibody (1:250) were used for immunofluorescence of pancreatic acinar cells. The fluorescent signals of NK1R/NF-κB p65 (excitation 488 nm, emission 500–550 nm) and β-arrestin1 (excitation 555 nm, emission 630–693 nm) were determined by a Nikon A1R^{+} two-photon confocal scanning microscope (Tokyo, Japan).

2.9. RT-qPCR and Western blot

Methods for extracting and detecting mRNA/protein expression were previously described (Ma et al., 2020; Wen et al., 2020) with details provided in Supplementary materials and methods and Supplementary Table 2.

2.10. Statistical analysis

Data are presented as mean ± SEM and were analyzed using analysis of variance (ordinary one-way ANOVA) or Tukey's post-hoc test for multiple comparisons with Prism 8.0 software (GraphPad Software Inc.; San Diego; CA, USA). P value <0.05 was considered to be statistically significant.

3. Results

3.1. Pancreatic SP-NK1R signaling pathways are activated in AP patients

We first determined the VAS score in AP patients (Fig. 1A). Compared with mild patients (MAP), the VAS score of moderately severe and severe patients (MSAP/SAP) was significantly increased, indicating an increase in self-reported pain. We then detected serum SP levels of healthy volunteers and a cohort of AP patients at the time of their hospital admission (Fig. 1B). The mean levels of serum SP were significantly higher in the MSAP/SAP group (435 pg/ml) compared with the MAP group (188 pg/ml) or healthy control group (182 pg/ml), while there was no difference between the latter two groups. Pancreatic immunohistochemistry analyses revealed that the expression of SP (Fig. 1C) and NK1R proteins (Fig. 1D) were markedly up-regulated in the necrotized pancreata compared with their adjacent inflamed pancreata, both were greatly higher than the normal pancreata, in which there was no discernible staining. These findings indicate that the pancreatic SP-NK1R signaling pathways were activated in AP patients and correlated with disease severity.

3.2. CQCQD alleviates AP-associated pain and inflammation in a mouse AP model

A schematic representation of all time points for the administration cerulein injection, CQCQD, CP96345 administration, and von Frey test on mice are shown in Fig. 1A and Supplementary Fig. 1A. The mean values of withdrawal threshold for von Frey test at all time points are plotted (Fig. 2B(i);Supplementary Fig. 1B(i)) and quantitively analyzed (Fig. 2B(ii); Supplementary Fig. 1B(ii)). Compared with the control group (CON; *light grey*), there was a dramatic reduction of withdrawal threshold at 0.5 h after the last cerulein injection (CER; *orange*), and this remained significantly lower at all other time points tested (Fig. 2B(i); Supplementary Fig. 1B(i)). Specific NK1R antagonist CP96345 significantly increased the cerulein-induced dropped withdrawal threshold at 1.5 h but not for other time points (Supplementary Fig. 1B(i,ii); *blue*). It did not improve cerulein-induced pancreatic edema, inflammatory cell infiltration, and necrosis (Supplementary Fig. 1C; *blue*) nor alter serum amylase, lipase, or IL-6 (data not shown). In contrast, CQCQD treatment (*green*) reverted the effects of cerulein on withdrawal threshold at all time points (Fig. 2B(ii)). The reduction of pain by CQCQD was associated with improved pancreas histopathology (Fig. 2C) as well as decreased pancreatic MPO, lung MPO, and serum IL-6 levels (Supplementary Fig. 2). These results suggest that CQCQD has analgesia effect that is associated with reduced pancreatic necrosis and systemic inflammation.

3.3. CQCQD suppresses pancreatic SP-NK1R signaling pathways

Previously, we have shown that CQCQD had direct anti-inflammation effects in AP (Wen et al., 2020). Here, based on the fact that pancreatic SP-NK1R signaling pathways were activated in AP patients (Fig. 1), we hypothesized CQCQD would reduce pain and the severity of AP at least in part via blocking neuron activation-mediated pancreatic SP-NK1R signaling pathways. To this end, we measured the changes in expression of NGF, protein gene product 9.5 (PGP 9.5), and c-Fos in AP-induced mice. NGF is a neurotrophic factor and neuropeptide in charge of regulation of growth, maintenance, and proliferation of neurons (Denk et al., 2017). The increasing expression of NGF in pancreas resulted in a higher density of peripheral innervation in mice (Robert H. Edwards et al., 1989). PGP 9.5 is a ubiquitin-carboxyl hydrolase that is mostly expressed in cytoplasm of neurons and some neuroendocrine cells (Day and Thompson, 2010). C-Fos is a proto-oncogene that is expressed within neurons following depolarization and was previously suggested as a reliable marker for peripheral stimulation-induced neural activity (Harris, 1998). The mRNA expression of pancreatic SP *Tac1 gene* significantly increased 1 h after one or four injections of cerulein, and further increased 1 h after seven injections (Fig. 3A(i)). At 6 h of the last injection of a total of 7 cerulein injections, both pancreatic *Tac1* and NK1R gene *Tacr1* were highly up-regulated (Fig. 3A(ii)). CQCQD reduced the protein expression of pancreatic SP, NK1R, NGF (immunohistochemistry; Fig. 3B) and β-arrestin1 (immunofluorescence; Supplementary Fig. 3) in the cerulein-induced AP mice. Cerulein injections induced expression of NGF, PGP 9.5, or c-Fos in the pancreas (Fig. 3C; *left panel*), DRG (Fig. 3C; *right panel*), and/or neurons in the T8-L2 spinal cord (Fig. 3D), indicating activation of peripheral and central neurons during AP. CQCQD decreased all neuron activity parameters (Fig. 3) and these findings suggest CQCQD suppressed pancreatic SP-NK1R signaling pathways via inhibition of neuron activity.

3.4. CQCQD inhibits SP-induced NK1R internalization in pancreatic acinar cells

We subsequently investigated the dynamic changes and translocation of NK1R in freshly isolated mouse pancreatic acinar cells. Under physiological and unstimulated situation, the NK1R, a membrane receptor, was evenly distributed on the membrane of pancreatic acinar cells (Fig. 4A). After stimulation with SP (5 μM), the NK1R begun to internalize into the cytoplasm over 30 min in a time-dependent manner (Fig. 4B), consistent with translocation of β-arrestin1 into the nucleus and activation of NF-κB p65 (Fig. 4C). Therefore, 30 min was chosen for assessing the effects of CQCQD and CP96345. Pre-incubation of pancreatic acinar cells with CQCQD (5 mg/ml) or CP96345 (1 μM) for

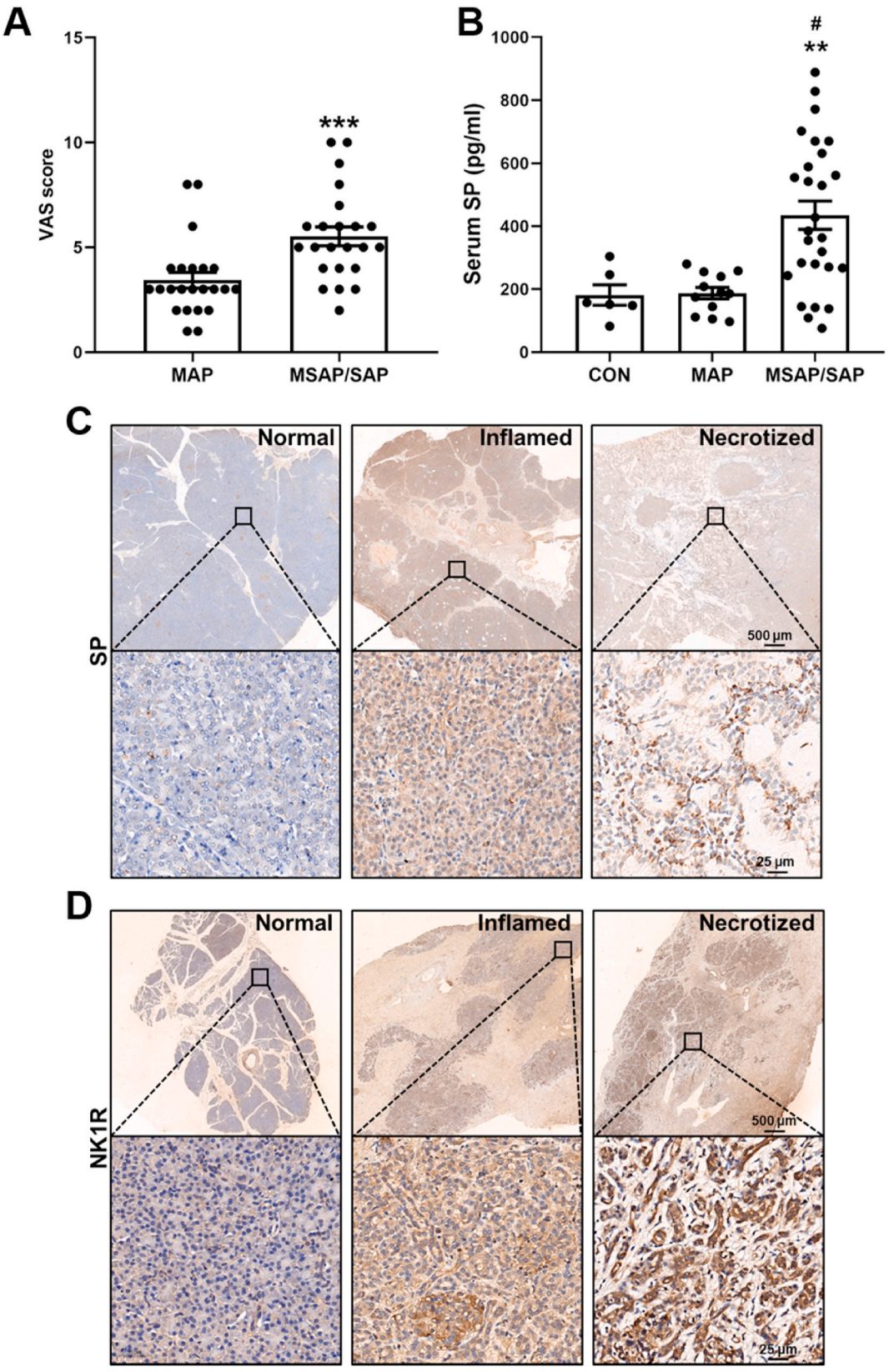

Fig. 1. Pain and SP-NK1R signaling pathway activation in AP patients. **(A)** Visual analog scale (VAS) score of AP patients was with disease severity of mild (MAP; n = 23) as well as moderately severe and severe (MSAP/SAP; n = 23). **(B)** Serum SP levels in MAP (n = 12), MSAP/SAP (n = 27), and healthy volunteers (n = 6). Representative immunohistochemistry images of pancreatic **(C)** SP and **(D)** NK1R. Data are expressed as mean ± SEM in bar plots. $^{\#}P < 0.05$ *versus* healthy volunteers; $^{**}P < 0.01$ *versus* MAP; $^{***}P < 0.001$ *versus* MAP.

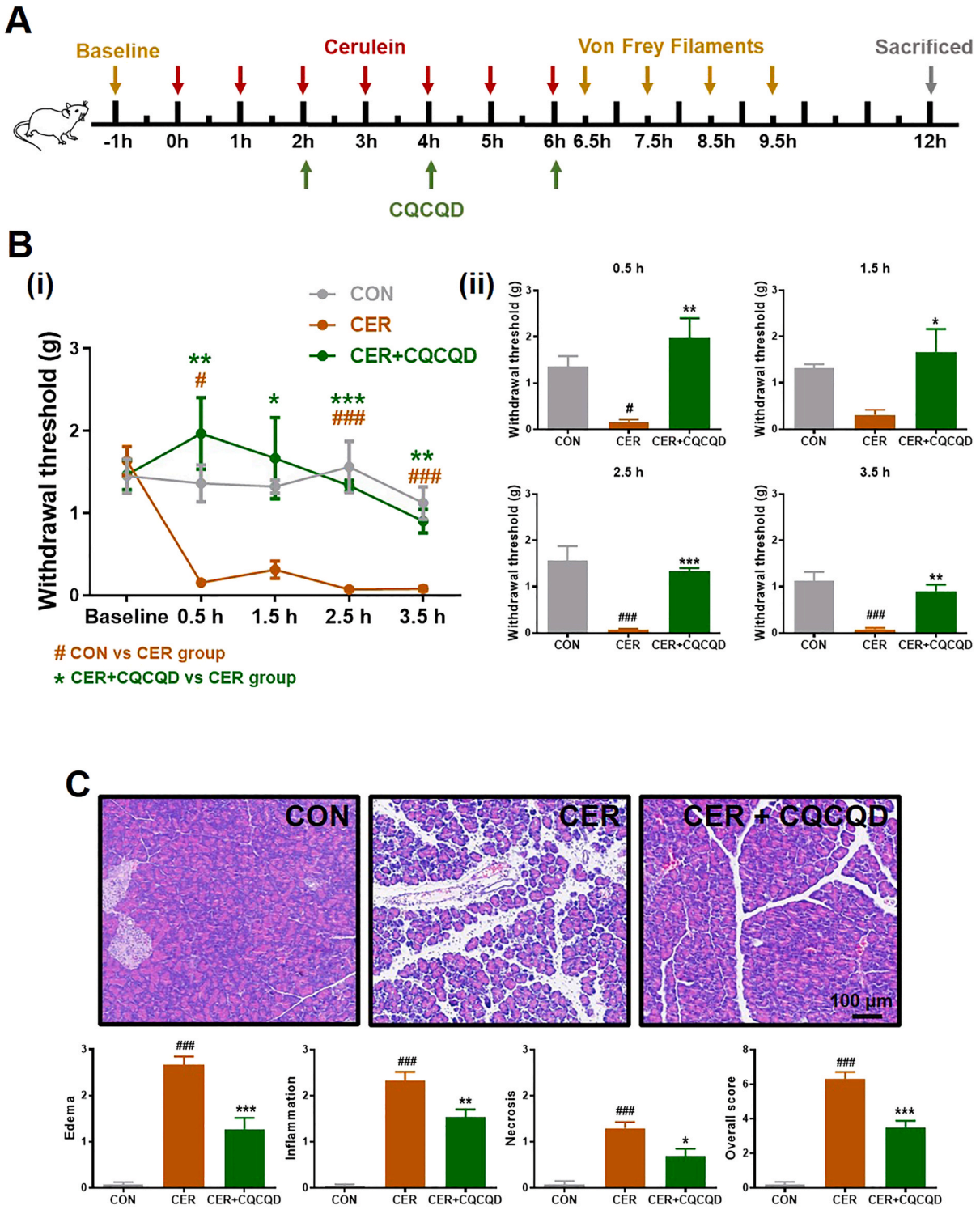

Fig. 2. CQCQD reduces pain and pancreatic necrosis in cerulein-induced AP. **(A)** The schematic representation of all the time points for the administration of cerulein (CER) and CQCQD (10 g/kg oral gavage per time) and when the von Frey test in conduct in mice. Controls (CON) received normal saline injections. Baseline was tested 1 h before first CER/saline injection. **(B)** Pain withdrawal threshold measured: (i) time-course variation recorded as the line charts and (ii) bar plots showing the results of withdrawal threshold at each time point. **(C)** Representative H&E pancreas images with histopathology scores (edema, inflammatory cell infiltration, acinar cell necrosis, and their sum value). Data are expressed as mean ± SEM in line charts (B(i)) and bar plots (B(ii) and C) from 5 to 6 mice per group. $^{\#}P < 0.05$ *versus* CON group; $^{\#\#\#}P < 0.001$ *versus* CON group; $^{*}P < 0.05$ *versus* CER group; $^{**}P < 0.01$ *versus* CER group; $^{***}P < 0.001$ *versus* CER group.

30 min markedly inhibited SP-induced NK1R internalization (Fig. 4D).

3.5. Network pharmacology analysis of active components from CQCQD for AP and pain

By searching corresponding databases, a total of 305 component-related targets, 530 AP-related targets and 689 pain-related targets

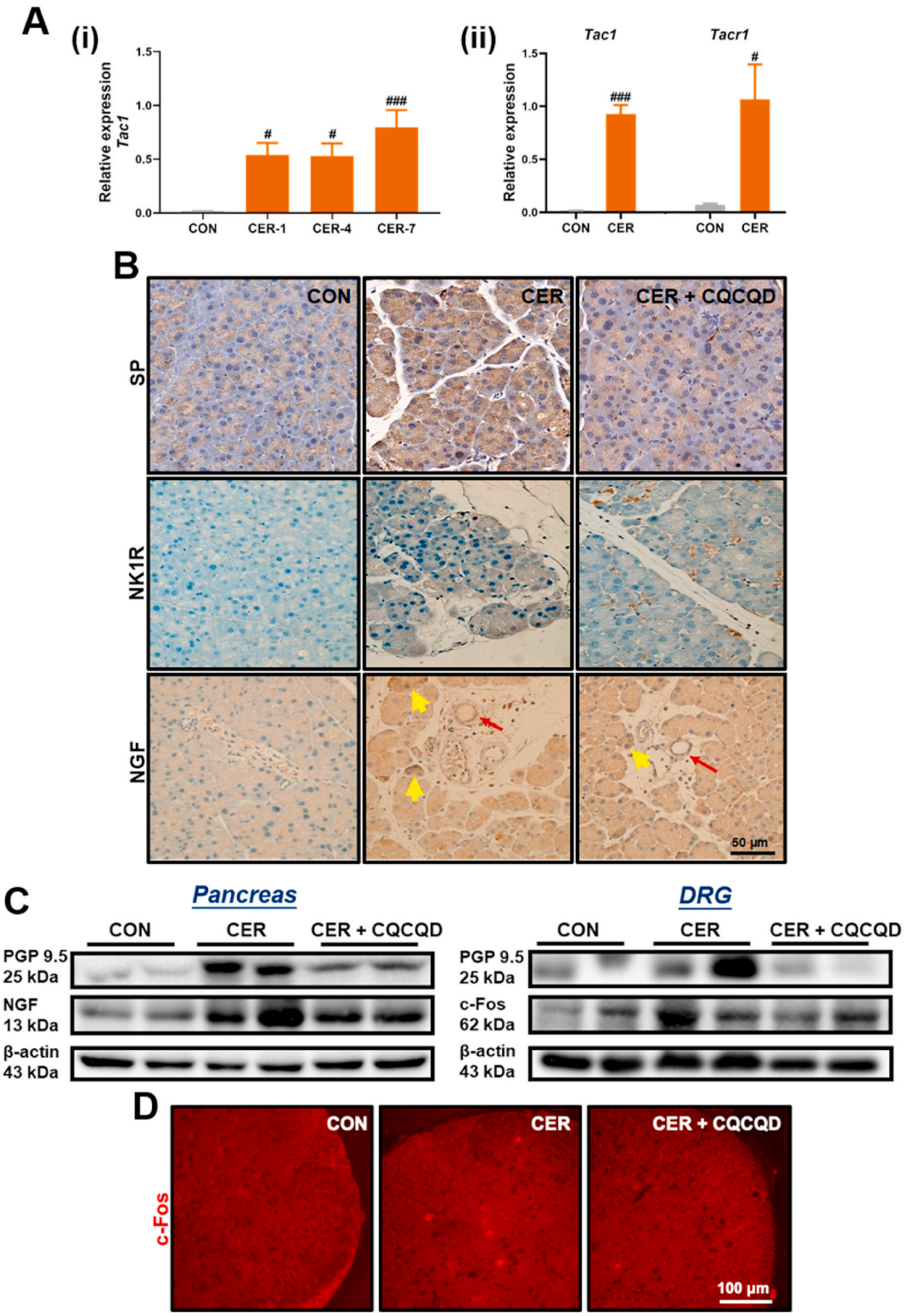

Fig. 3. CQCQD reduces neuron activation-mediated up-regulation of pancreatic SP-NK1R in cerulein-induced AP. **(A)** RT-qPCR results for pancreatic *Tac1* and *Tacr1* mRNA expression of SP and NK1R, respectively: (i) expression of *Tac1* 1 h after 1, 4, and 7 injections of cerulein (CER) in mice, (ii) expression of *Tac1* and *Tacr1* at 6 h of the last injection of a total of 7 cerulein injections. **(B)** Representative immunohistochemistry images of pancreatic SP, NK1R, and NGF (ductal cells are marked with red arrows, while injured acinar cells are marked with yellow arrow heads). **(C)** Representative Western blot images for expression of proteins representing neuron activation in the pancreas (PGP 9.5 and NGF) and dorsal root ganglia (DRG) (PGP 9.5 and c-Fos). β-actin was used as a loading control. **(D)** Representative immunofluorescent images for c-Fos labelled cells in the spinal cord. Data are expressed as mean ± SEM in bar plots (A) from 5 to 6 mice per group. $^{\#}P < 0.05$ *versus* CON group; $^{\#\#\#}P < 0.001$ *versus* CON group.

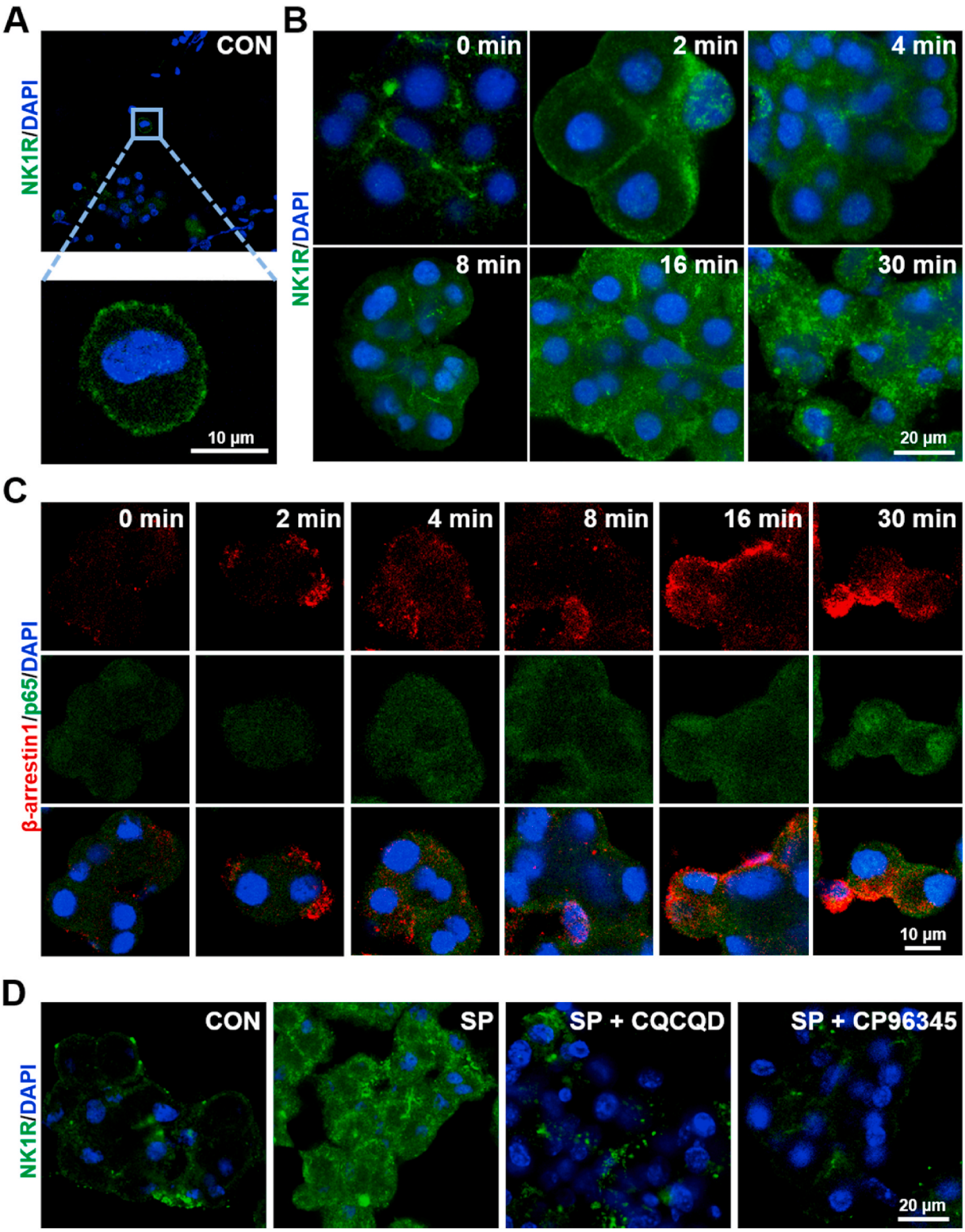

Fig. 4. CQCQD suppresses SP-induced NK1R internalization in pancreatic acinar cells. **(A)** NK1R expression on normal cells without stimuli. **(B)** Representative images of time-lapsed NK1R signals after stimulation with SP (5 μM). **(C)** Representative images of time-lapsed β-arrestin1 and NF-κB p65 signals after stimulation with SP (5 μM). **(D)** Representative images of NK1R expression after pre-treatment with CQCQD (5 mg/ml) or CP96345 (1 μM) for 30 min, followed by stimulation with SP (5 μM) for 30 min.

were obtained. Among all those targets, 26 overlapped targets were identified and kept for network construction using Cytoscape 3.6.0 software (Fig. 5A). Among the overlapped targets, 5 of them (TLR4, NF-κB, TNF, IL-6, and IL-17A) in pro-inflammatory pathways have been extensively studied; and 6 of them (HTR2A, CNR1, CNR2, PTGS2, GSTP1, GLP1R) are associated with neuron mechanisms. Further, an "overlapped targets-components" network was established, indicating that 18 corresponding components have specific interaction on these

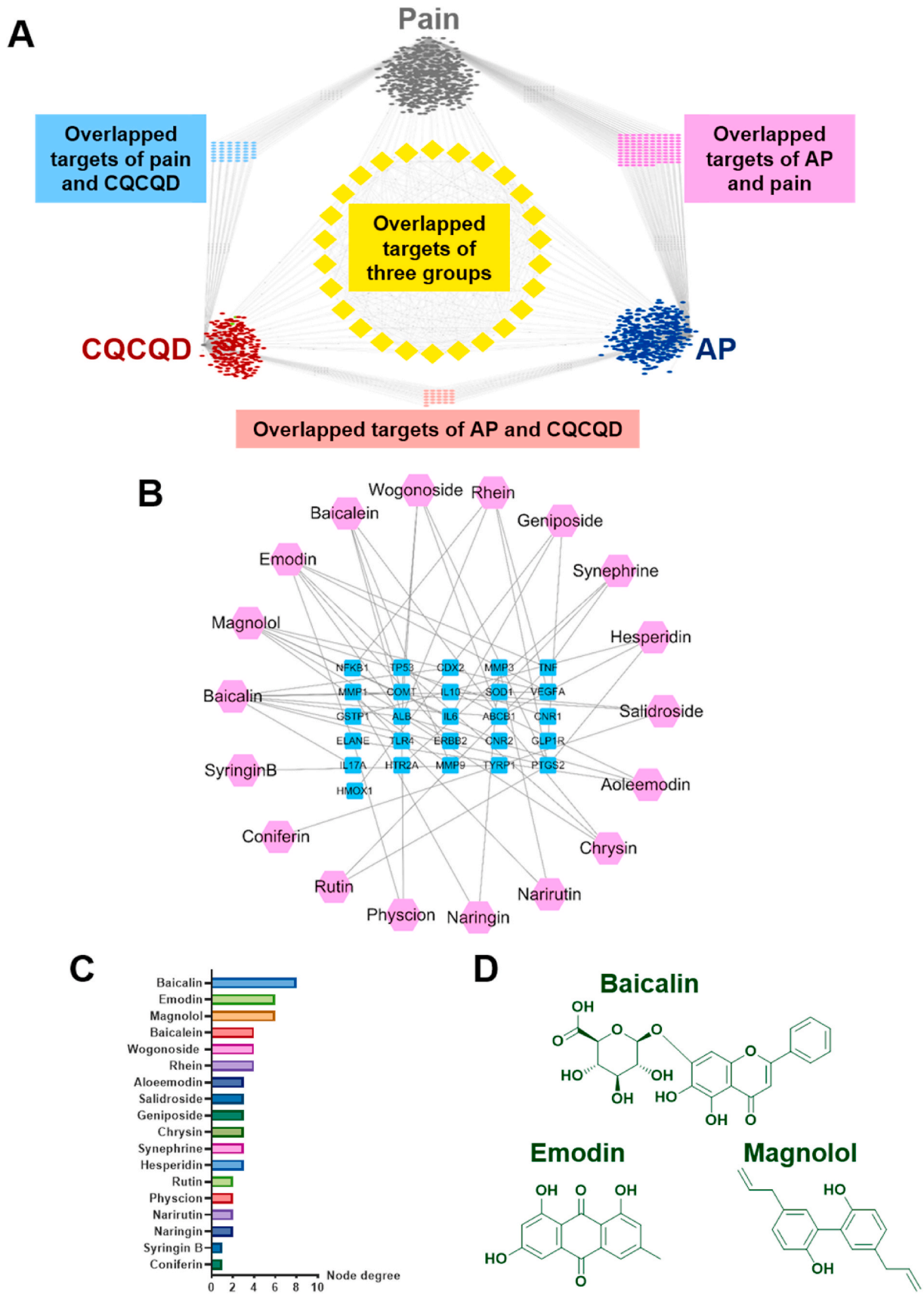

Fig. 5. Network pharmacology analysis of active components of CQCQD for AP and pain. **(A)** A total of 26 overlapped targets of AP, pain, and CQCQD were filtered out. **(B)** An "overlapped targets-components network" was constructed by connecting filtered overlapped targets (central rectangular) and predicted components (surrounding circle). **(C)** Ranking of selected components according to the efficient score. **(D)** Chemical structures of top 3 screened active compounds.

overlapped targets (Fig. 5B). Most of these components belong to flavonoids and lignans which have been reported to possess both anti-inflammatory and analgesic effects (Li et al., 2020b). Baicalin, emodin, and magnolol were the top three compounds (Fig. 5C) in the CQCQD based on the node degree (8 for baicalin and 6 for emodin/magnolol) with chemical structure shown in Fig. 5D.

3.6. Validation of screened ingredients for SP-induced NK1R internalization and NF-κB activation

We validated the effects of screened active ingredients of CQCQD in SP-induced NK1R internalization and down-stream signaling (Fig. 6). Stimulation of pancreatic acinar cells with SP (5 μM) induced NK1R internalization (Fig. 6A), increased expression of *β-arrestin1* mRNA (Fig. 6B) as well as NF-κB p65 and p-IκB proteins (Fig. 6C). Baicalin, emodin, and magnolol (all at 12.5, 25, and 50 μM) retarded SP-induced NK1R internalization and reduced expression of *β-arrestin1* (Fig. 6B), NF-κB p65, and p-IκB (Fig. 6C).

4. Discussion

Our results indicate pancreatic acinar cell NK1R activation induced by SP during initial pancreatic injury contributed to the aggravation of disease severity in experimental AP, which was attenuated by CQCQD and its active components. We have shown that admission pain score was correlated with disease severity in AP patients; furthermore, the circulating SP levels are increased in MSAP/SAP patients, which were associated with activated SP-NK1R signaling pathways in the necrotized and surrounding inflamed pancreata. Given pain and SP are the cardinal manifestation and classic indicator of neurogenic inflammation, respectively, this evidence provides the basis for future studies of the relationship between neurogenic inflammation and AP pathophysiology in clinical settings.

In acute critical illness such as SAP (Garg and Singh, 2019; Linkermann et al., 2014), inflammation and pain positively re-enforce each other, therefore, minimizing pain is considered a potential strategy to mitigate inflammation (Brierley and Linden, 2014; Grace et al., 2014). It has been shown that denervating primary sensory neurons (Nathan et al., 2002), ablation of the celiac ganglion (Noble et al., 2006), or administration of epidural anesthesia (Alp et al., 2006) were able to alleviate the severity of AP in experimental AP models. Moreover, it has been shown that genetic deletion (Romac et al., 2018; Swain et al., 2020) or pharmacological inhibition (Qiu et al., 2020; Schwartz et al., 2011) of critical ion channels (i.e. TRPA1 and TRPV1/4) that are responsible for neurogenic inflammation, not only relieved pain but also reduced pancreatic necrosis in experimental AP models. Previous attempts of translating these findings into clinical practice by the administration of non-steroidal anti-inflammatory drugs (Yekkirala et al., 2017) and opiates (Barlass et al., 2018; Basurto Ona et al., 2013; Wu et al., 2017) were with significant limitations such as Sphincter of Oddi spasm, decreasing intestinal motility, dizziness, nausea, and gastrointestinal bleeding (Meng et al., 2013; Stigliano et al., 2017). Also, epidural analgesia required invasive techniques, and intensive monitoring that may not be generally available or applicable in all hospitals (Jabaudon et al., 2018; Sadowski et al., 2015). Therefore, there is an unmet need for novel pharmacological regimes capable of suppressing neurogenic inflammation that targeting both inflammation and pain without marked side effects. SP-NK1R is one of the most studied mechanisms that provoke neurogenic inflammation, and several groups have claimed that interrupting SP-NK1R signaling pathways is an effective treatment strategy for AP (Bhatia et al., 1998; Grady et al., 2000; Koh et al., 2011; Lau and Bhatia, 2006; Maa et al., 2000).

To further emphasize the importance of SP-NK1R-mediated neurogenic inflammation in AP, we have looked into the expression of pancreatic SP-NK1R and the relevant proteins in the neurons. The sources of SP are primarily from pancreatic innervated neurons (Koh and Bhatia, 2011). Previous studies have shown that in chronic pancreatitis, pancreatic SP mRNA expression was clinically elevated (Di Sebastiano et al., 2000) and the pancreatic expression of NK1R mRNA was associated with greater pain intensity, frequency, and duration (Shrikhande et al., 2001). Our results also indicate elevated pancreatic SP-NK1R expression in AP patients and mice. Simultaneously, previous findings from L-arginine-induced rodent AP model, where the expression of pancreatic NGF (Toma et al., 2000) and spinal cord c-Fos (Wick et al., 2006) were highly up-regulated. Our observations that in a cerulein-induced AP model, the up-regulation of key proteins in the pancreatic innervated neuron (NGF, PGP 9.5), DRG (PGP 9.5, c-Fos) and Spinal cord (c-Fos) result in the activation of pancreatic SP-NK1R pathway reaffirmed the findings. We also directly showed that SP induced NK1R internalization and β-arrestin1 nuclear translocation in a time-dependent manner, resulting in NF-κB activation in freshly isolated pancreatic acinar cells *in vitro*. Overall, our data indicate an important role of SP-NK1R signaling pathways for pancreatic necrosis and systemic inflammation during AP. Our work indicates that the protective effects of CQCQD in pancreatic acinar cells are likely attributed to the inhibition of neuron activation-mediated upregulation of SP-NK1R. Using pharmacology network analysis, we have narrowed down to three likely candidates: baicalin, emodin, and magnolol in CQCQD with potential to attenuate inflammation and pain in AP. In our experiment, all three compounds were able to suppress SP-induced NK1R internalization, *β-arrestin1* expression and NF-κB p65 activation in pancreatic acinar cells. Notably, the primary downstream signaling of NK1R-*β-arrestin1* is the NF-κB pathway (Hoeppner et al., 2012), one of the most well established cellular inflammatory mechanism for AP (Saluja et al., 2019). Thus, it is reasonable to infer that, the pharmacological mechanism of CQCQD on SP-NK1R mediated inflammation in acinar cells potentially relies on the interaction between these active components and NK1R. This provides an excellent platform for our ongoing research focused on evidence-based pharmacology of traditional Chinese medicine (TCM).

The TCM theory describes AP and its associated pain as “Stagnation of qi and blockage of blood flow” (Du et al., 2016), the resulting symptoms are equivalent to those induced by inflammation and nociceptive stimulation (Brierley and Linden, 2014; Grace et al., 2014; Liddle and Nathan, 2004). DCQD, the base formula of CQCQD, is well accepted to promote qi and blood flow in TCM theory, and has been used to treat abdominal pain secondary to non-surgical intestinal obstruction that shares similar pathophysiology with AP in the context of TCM (Yang et al., 2014). Besides the anti-inflammatory ingredients, many flavonoids and lignans of DCQD have analgesic effects by inhibiting pain mediators such as prostaglandin-2, nitric oxide, TRPV1, and c-Fos (Li et al., 2020b). These active ingredients include emodin of *Rheum palmetum* L. (Gao et al., 2011; Sui et al., 2010a), magnolol and honokiol of *Magnolia officinalis* Rehder & E.H.Wilson (Lin et al., 2007, 2009), naringin (Xu et al., 2017), hesperidin (Carballo-Villalobos et al., 2017; Visnagri et al., 2014), and rutin (Azevedo et al., 2013) of *Citrus* × *aurantium* L. CQCQD is modified from DCQD by adding *Bupleurum marginatum* Wall. ex DC., *Scutellaria baicalensis* Georgi, *Gardenia jasminoides* Ellis and *Artemisia capillaris* Thunb, where these additions are known to enhance the anti-inflammatory effects of DCQD (Jang et al., 2015; Jung et al., 2008; Zhao et al., 2015). Of them, baicalin (Cherng et al., 2014; Chou et al., 2003; Li et al., 2018b; Sui et al., 2010b) and baicalein (Lai et al., 2018) of *Scutellaria baicalensis* Georgi, quercetin (Gao et al., 2016) and luteolin (Basu and Basu, 2020) of *Bupleurum marginatum* Wall. ex DC., and geniposide (Gong et al., 2014) of *Gardenia jasminoides* Ellis have been shown to possess analgesic effects in a range of experimental pain models. In this study, although we have only investigated three most likely active components based on the pharmacological network analysis, future assessment may be applied to the evaluation of other interesting compounds in the formula. One of the key benefits of CQCQD is it rarely associated with overt side effects (Xiang et al., 2017), thus making it an ideal intervention to compare with conventional analgesic

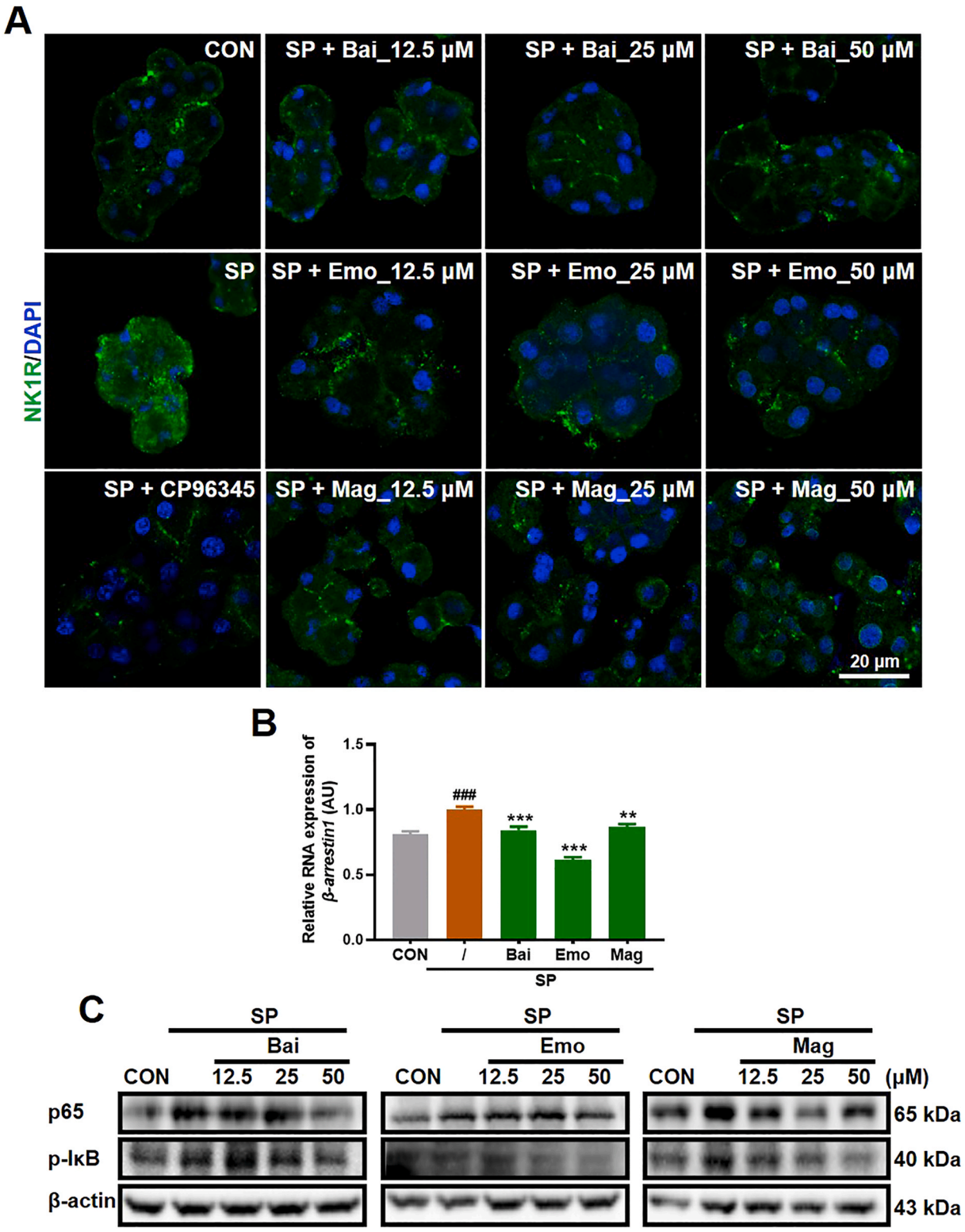

Fig. 6. Validation of top three screened ingredients of CQCQD for SP-induced NK1R internalization and NF-κB activation in pancreatic acinar cells. **(A)** Representative images NK1R protein expression induced by SP (5 μM) with or without pre-treatment with baicalin (Bai; 12.5, 25, 50 μM), emodin (Emo; 12.5, 25, 50 μM), magnolol (Mag; 12.5, 25, 50 μM), or CP96345 (1 μM) for 30 min. **(B)** Expression of *β-arrestin1* mRNA and the inhibitive effects of selected compounds (50 μM for Bai and Emo, 25 μM for Mag) after SP stimulation (5 μM) for 30 min. **(C)** Representative Western blot images for protein expression of NF-κB p65 and p-IκB. Data are expressed as mean ± SEM in bar plots in (B) from 3×10^7 cells per group of 3 or more independent experiments. $^{###}P < 0.001$ *versus* CON group; $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* SP group.

medicine in a randomized clinical trial for pain relief in AP with serum SP level as a potential biological end-point.

This study has several limitations. Firstly, while we have generated a substantial amount of evidence to demonstrate the exacerbating effect of SP-NK1R-mediated neurogenic inflammation during AP, this was only established in the pancreatic acinar cells. To fully appreciate and decipher the crosstalk between the neuron and acinar cells, assessment of cultured or primary neuron cells is necessary. Secondly, despite the fact we have demonstrated the relationship between serum SP levels and AP severity in the clinical setting, our research did not assess if CQCQD administration could reduce the amount of SP or interrupt the human SP-NK1R pathways. This question will be answered by a prospective randomized trial of CQCQD in AP patients that we are currently running. Thirdly, we did not further explore the synergetic effects of baicalin, emodin, and magnolol, nor assess the impact of the remaining compounds on other targets for inflammation, pain, and the neuron.

5. Conclusion

CQCQD ameliorated severity of cerulein-induced AP and its associated pain in mice. These effects of CQCQD at least in part were observed to be via inhibiting pancreatic acinar cell SP-NK1R signaling pathways and can be attributed to its three active compounds, baicalin, emodin, and magnolol.

Author contributions statement

TL, QX, and WH obtained funding, conceived and designed the study, and supervised the students. CH, DD, YW, JL, JY, and NS performed the experiments. CH, DD, RW, and TL analyzed the data. TJ, NS, KJ, and LD collected clinical data. XF, RM, JAW, ARP, and RS had important intelligence input. CH, WH, and TL drafted the paper. JH critically revised the manuscript. All the authors read and approved the final version of the manuscript before submission.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114029.

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