Research Paper

Curcumin Suppresses JAK2/STAT3 Pathway to Ameliorate Pancreatic Injury Induced by Sepsis in Rats

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The occurrence of pancreatic injury is an important pathological change of sepsis. Curcumin was used to treat organ injury by sepsis, but its effect in pancreatic injury induced by sepsis still unclear. In this study, the animal model of pancreatic injury in sepsis was constructed *via* tail vein injection lipopolysaccharide into rats, and the tissues and serum of rats were collected for further investigation. The pathological changes of pancreatic injury were presented by hematoxylin and eosin staining. The concentration of inflammatory factors (interleukin-6, interleukin-1 beta and tumor necrosis factor-alpha) and oxidative stress related factors (superoxide dismutase, malondialdehyde, and reactive oxygen species) were detected by enzyme-linked immunosorbent assay kits. The terminal deoxynucleotidyl transferase dUTP nick end labeling assays and Western blot were carried out to detect apoptotic rate and apoptotic-related proteins (B-cell lymphoma 2, Bcl-2-associated X protein, caspase-3 and cleaved caspase-3). The expression level of Janus kinase 2/signal transducer and activator of transcription 3 pathway was verified by Western blot. The septic pancreatic injury rats' model were successful constructed with obvious pathological changes and enhanced expression of inflammatory factors. Curcumin could reduce the level of inflammation, oxidative stress, and apoptosis in pancreatic tissue of septic rats. In addition, curcumin ameliorate pancreatic injury induced by sepsis by inhibiting Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway, and the inhibition effect of curcumin can be reversed by adding Janus kinase 2 activator coumermycin A1. Curcumin suppresses Janus kinase 2/signal transducer and activator of transcription 3 pathway to ameliorate pancreatic injury induced by sepsis in rats, including attenuating inflammatory reaction, oxidative stress, and apoptosis. This study was supposed to provide a new research idea for the prevention and clinical treatment of pancreatic injury in sepsis.

Key words: Sepsis, pancreatic injurzy, curcumin, Janus kinase 2/signal transducer and activator of transcription 3

Sepsis is a severe systemic inflammatory response syndrome caused by the host's maladjusted response to infection, which eventually leads to multiple organ failure and septic shock, and is the main cause of death from severe infection[1]. It is the main cause of death from severe infection[2]. The incidence rate of sepsis patients is increasing, and the annual medical expenses for sepsis treatment account for 5.2 % of the hospital related expenses[3]. Despite the continuous upgrading of antibiotics and the continuous progress of intensive care technology and equipment, the mortality of sepsis is still higher than 30 %[4]. At present, the research of common sepsis organ dysfunction mainly involves lung, kidney, liver and other organs, but pancreatic injury is also common in sepsis patients and sepsis model animals[5,6], and the degree of injury is positively related to the death rate and mortality[7]. It is suggested that pancreatic injury is an important pathological change of sepsis, and its severity can be used as an indicator to judge the prognosis of sepsis. The treatment of pancreatic injury caused by sepsis may be of great significance to improve the prognosis and reduce the mortality.

Curcumin is a polyphenol extracted from turmeric plants, such as Zingiberaceae and Araceae, which has extensive pharmacological effects[8]. Curcumin is a natural compound with anti-inflammatory, anti-oxidant and anti-cancer properties[9]. Research shows

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that curcumin has broad application prospects in the treatment of inflammatory diseases with no obvious side effects[10]. Previous studies have been reported to use the curcumin to treat the chronic lung injury by sepsis [11], acute lung injury induced by sepsis[12,13], liver injury[14] and kidney injury[15,16]. However, at present, the biochemical basis and pathway of anti-inflammation of curcumin in pancreatic injury have not been clarified, and whether curcumin can be used as a therapeutic drug for pancreatic injury in sepsis needs further evaluation.

In this study, we were supposed to explore the effect of curcumin on pancreatic injury induced by sepsis. The animal model of pancreatic injury in sepsis was constructed *via* tail vein injection Lipopolysaccharide (LPS) into rats, and the tissues and serum of rats were collected for further investigation. The pathological changes of pancreatic injury were present by Hematoxylin Eosin (HE) staining. The concentration of inflammatory factors (Interleukin (IL)-6, IL-1 beta (β) and Tumor Necrosis Factor-Alpha (TNF-α)) and oxidative stress related factors (Superoxide Dismutase (SOD), Malondialdehyde (MDA), and Reactive Oxygen Species (ROS)) were detected by Enzyme-Linked Immunosorbent Assay (ELISA) kits. The Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assays and Western Blot (WB) (B-Cell Lymphoma 2 (Bcl-2), Bcl-2 Associated X-protein (BAX), caspase-3 and cleaved caspase-3) were carried out to detect apoptotic rate. The significant role of Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3) signaling pathway was verified by WB. This study was supposed to provide a new research idea for the prevention and clinical treatment of pancreatic injury in sepsis.

MATERIALS AND METHODS

Construction of pancreatic injury model in sepsis:

Experimental animals: A total of 20 male Wistar rats (8 w, weight 320±30 g) were fed with basic diet at room temperature of 18°-25°, relative humidity of 65 %-70 %, and light cycle of 12:12 h in Specific Pathogen Free (SPF) animal room. This experiment was approved by the ethics committee of our hospital (No.12345).

Animals grouping: After 7 d, the rats were divided into 4 groups with 5 rats in each group; control group (normal saline), model group (LPS), curcumin treatment group (LPS+Cur) and curcumin+coumermycin A1 (LPS+Cur+CA1) treatment group. The LPS (Sigma-Aldrich, United States of America (USA)), coumermycin A1 (Yeasen Biotechnology (Shanghai) Co., Ltd.), and curcumin (Biochem Partner) were purchased and prepared. The normally bred Westar male rats were forbidden to eat but not drink water 12 h before modeling. The rats were fixed with fixators during tail vein injection.

Control group: Tail vein injection of the same dose of normal saline.

Model group: Tail vein injection of LPS (10 mg/kg) [17].

Curcumin treatment group: Tail vein injection of LPS (10 mg/kg); after 1 h, the curcumin (100 mg/kg) was orally administered by drinking water to the model rats[18].

Curcumin+coumermycin A1 treatment group: Tail vein injection of LPS (10 mg/kg); after 1 h, the curcumin (100 mg/kg) was orally administered by drinking water to the model rats and coumermycin A1 (100 μg/kg) was intraperitoneal (i.p.) injection to the model rats[19].

Samples collection:

Blood was taken 24 h after LPS administration. Put the collected blood sample in the Eppendorf (EP) tube, mark the group and number, and centrifuge under the condition of 3000 rpm 5-10 min; suck out the supernatant, sub package it in 1.5 ml EP tubes, and mark the group and number keep in the refrigerator at 80° for standby.

After blood collection, the mice were killed by spinal dislocation method, and the mice were sterilized with alcohol. The surgical scissors were used to open the abdomen. The spleen and duodenum were first found in the left upper abdomen. The white adipose tissue between the duodenum and spleen was the pancreas. Store half of the pancreas in a refrigerator at -80° and soak the other half in 10 % formalin. Mark the group and number respectively.

Paraffin embedding:

The pancreas tissue samples were fixed in new formalin solution for 24 h. The tissue block was placed in 70 %, 80 %, 90 % and 100 % ethanol for 1 h respectively for gradient dehydration. After dehydration, it is immersed in xylene for transparent treatment. Take the tissue block treated in the previous step and place it in the embedding box for

fixation. Immerse it in the preheated paraffin. After the liquid paraffin cools and solidifies for about 5 min, the embedding is completed.

HE staining:

Trim the wax block containing tissue with a blade, and drain and dry it with a slide after slicing. In dewaxing and rehydration; the dried tissue sections were treated with xylene for 5-10 min respectively. Soak 100 % ethanol for 5 min, 90 %, 80 %, 70 % ethanol and distilled water for 2 min in sequence. The tissue sections were stained with hematoxylin for 5 min. After the differentiation water was washed back to blue, they were dyed in eosin for 2 min, and then the excess dye was washed away with tap water. The sections after dying were soaked in 70 %, 80 %, 90 % and 100 % ethanol for 10 s respectively; transparent with xylene for 5 min each; after transparent, use a pipette gun to suck 0.3 ml of neutral plastic fat back cover. The sections were observed under microscope.

ELISA assays:

The ELISA kits IL-6 kit (H007-1-2), IL-1β kit (H002-1-2), TNF-α kit (H052-1-2), SOD kit (A001-1-2), MDA kit (A003-1-2), ROS kit (E004-1-1) were purchased from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd. Add standard samples of different concentrations into the standard hole according to the instructions in the manual. Add the diluent of the standard in the blank hole, and add the sample to be tested in the remaining holes. The enzyme label plate was covered with film and closed, and incubated at 37° for 1 h. Take the enzyme label plate out of the incubator, open the sealing membrane, discard the liquid in the hole, throw it dry and add the washing liquid, leave it for 3 min and discard the liquid in the hole, repeat the last process to wash the plate for 4 times. Add liquid A 100 µl, cover the enzyme label plate with membrane and seal it, after 37° for 1 h, wash the plate for 3 times and add liquid B 100 µl. The enzyme label plate was covered with membrane and closed, and the reaction was conducted at 37° for 1 h. Add 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate 90 µl. Cover the enzyme label plate with a membrane and seal it, color it away from light at 37°. After the color in the hole changes in a gradient, stop the solution 50 µl that stop color rendering. The Optical Density (OD) value was measured by the microplate reader at 450 nm wavelength, and the expression amount was calculated.

TUNEL assay of apoptosis:

Take slices from sample collection section, dewax with xylene, add 100 % ethanol for 5 min, 90 %, 70 % ethanol and distilled water for 2 min respectively; protease K without DNase was incubated at 25° for 30 min. Phosphate Buffer Solution (PBS) was fully developed for 3 times, and each time was cleaned for 5 min. 50 µl TUNEL test solution, incubate in dark at 37° for 1 h. Observe and take photos under fluorescence microscope after sealing.

WB:

In preparation of protein sample; cut the pancreas on ice, put it into EP tube after PBS rinsing, add lysate, and then split it on ice for 30 min, 4°, 12 000 rpm, 10 min centrifugation, and collect the supernatant for standby. The protein was quantified by Bicinchoninic Acid (BCA) assay method, and then Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to transfer the membrane. Then incubate the first antibody and the second antibody for antibody hybridization. ECL is used for development. After development, the pictures were analyzed by ImageJ software. Secondary antibodies were goat anti-mouse Immunoglobulin (Ig) G+IgM H&L (HRP) preadsorbed (1:5000, ab47827 abcam). The primary antibodies were as follows; Bcl-2 (1:1000, ab32124, abcam), BAX (1:5000, ab32503, abcam), caspase-3 (1:2000, ab184787, abcam), cleaved caspase-3 (1:5000, ab214430, abcam), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:5000, ab8245, abcam), pJAK2/JAK2 (1:1000, ab32101/ab39636, abcam) and pSTAT1/ STAT1 (1:1000, ab30645/ ab31369, abcam).

Statistical analysis:

GraphPad Prism 8.0.1 software was used to analyze the data obtained. The data with normal distribution were compared between groups using Student's t-test. $p<0.05$ served as statistically significant.

RESULTS AND DISCUSSION

Firstly, we constructed the model of septic pancreatic injury rats by LPS for investigation. Compared with the control group, the rats in model group showed lethargy, chills, slowness of movement, reduced hair combing activity and severe diarrhea. Compared with the model group, the curcumin treatment group rats had less lethargy and chills, more hair combing activities, less diarrhea, and less feces. The HE staining demonstrated the pathological changes of pancreatic

injury induced by sepsis in rats, the pancreas in model group showed tissue edema, inflammatory cell infiltration, hemorrhage and necrosis compared with the control group. With the treatment of curcumin, the inflammatory cell infiltration, hemorrhage, and necrosis were significantly reduced (fig. 1A). What's more, the expression of inflammatory factors (IL-6, IL-1β and TNF-α) was remarkably increased in model group rat serum compared with control group, while the expression of inflammatory factors was also significantly attenuated by the curcumin ($p<0.01$, fig. 1B-fig. 1D). These results indicated that the curcumin improved the pathological damages and inflammation of septic pancreatic injury rats.

In the next step, the apoptosis and oxidative stress of pancreatic injury induced by sepsis in rats were detected. The TUNEL assay was conducted to show the apoptosis rate in pancreatic tissue. As shown in fig. 2A, compared with the control group, the apoptosis of pancreatic cells in model group was remarkably increased ($p<0.001$); compared with model group, apoptosis in curcumin treatment group decreased remarkably ($p<0.01$). The apoptosis-related proteins, including Bcl-2, BAX, caspase-3 and cleaved caspase-3 were measured by WB. As demonstrated in fig. 2B, the expression of apoptosis inhibitor Bcl-2 was decreased in model group but increased in curcumin treatment group ($p<0.01$); the expression of apoptosis regulator BAX and caspase-3 were enhanced in model group but inhibited in curcumin treatment group ($p<0.01$). The curcumin could mitigate the apoptosis rate in model group. Furthermore, the expression level of SOD, the scavenger with free radical as substrate, was suppressed in model group while enhanced in curcumin treatment group ($p<0.01$, fig. 2C). Nevertheless, the curcumin attenuated the expression of oxidative stress related factors in pancreatic tissue in model group, including MDA and ROS ($p<0.01$, fig. 2D). The results above indicated that curcumin suppressed the apoptosis and oxidative stress level of pancreatic injury induced by sepsis in rats.

The JAK2-STAT3 pathway is an important signal pathway involved in ischemia, hypoxia and oxidative stress of various tissues and organs[20]. The curcumin was reported to suppress inflammation and cell apoptosis *via* JAK2/STAT-3 in kidney injury[16], but there none related research in pancreatic injury induced by sepsis. Hence, we focus on the JAK2/STAT3 pathway in the following investigation. The WB was conducted to explore the expression level of key proteins in JAK2/STAT3 pathway. As presented in fig. 3, the expression level of JAK2/STAT3 proteins was not significantly changed under different condition. The relative expression level of phospho-JAK2 and phospho-STAT3 were remarkably enhanced in model group, and remarkably suppressed by curcumin ($p<0.01$). Interestingly, when the coumermycin A1 (the JAK2 pathway activator) was added, the expression level of JAK2/STAT-3 pathway showed recovering compared to curcumin treatment group and model group ($p<0.05$). The evidences above suggest that curcumin inhibited JAK2/STAT3 pathway in pancreatic injury induced by sepsis in rats.

Next, the TUNEL assay was carried out to present the apoptosis rate in groups of control, model, model treated with curcumin, model treated with curcumin and coumermycin A1. In consistent with the previous studies, the curcumin could mitigate the apoptosis rate in model group ($p<0.001$, fig. 4A). It should be noticed that when the coumermycin A1, the apoptosis rate of added was recovered completely compared to the curcumin treatment group ($p<0.001$). As for the apoptosis-related proteins, the expression trend of Bcl-2, BAX, caspase-3 and cleaved caspase-3 were consistent with the previous results. The same as the TUNEL results, coumermycin A1 recovered the expression of apoptosis-related proteins compared to the curcumin treatment group ($p<0.05$, fig. 4B). These results revealed that curcumin inhibited JAK2/STAT-3 pathway to ameliorate apoptosis of pancreatic injury induced by sepsis in rats.

In this study, LPS was injected into the tail vein of rats to establish a septic pancreatic injury model, and the effect of curcumin on septic pancreatic injury was observed at the whole animal, tissue, and molecular levels. Curcumin could reduce the level of inflammation, oxidative stress, and apoptosis in pancreatic tissue of septic rats. In addition, curcumin ameliorate pancreatic injury induced by sepsis by inhibiting JAK2/STAT3 signal pathway in rats, and the inhibition effect of curcumin can be reversed by adding JAK2 activator coumermycin A1. The aim of this study was to elucidate the mechanism of curcumin in the treatment of pancreatic injury in sepsis, and to provide a new research idea for the prevention and clinical treatment of pancreatic injury in sepsis.

In animal experiments, LPS injection is a commonly used method to build septic injury models, and LPS

is also often used to build pancreatitis models[21]. Although LPS injection often has changes in dose and administration route, it is more popular in sepsis research because of its simple and repeatable, acute, highly controlled, and standardized inducing response[22]. Besides, LPS can induce autophagy in sepsis, but it is usually accompanied by autophagy damage, leading to the accumulation of autophagy causing pancreatic injury, which is similar to pancreatitis[21,23]. Sepsis is a self-destructive disease caused by the release of proinflammatory factors and the overexpression of other inflammatory mediators[24]. Inflammatory reaction of the body can often lead to failure of important organ functions of the body, and the mortality rate is as high as 80 %~90 %[25]. In our study, we injected 10 mg/kg LPS into tail vein to constructed the septic pancreatic injury model in rats. The level of inflammation, oxidative stress, and apoptosis in pancreatic tissue of septic rats were significantly enhanced compared to control group, which indicated the successful construction of the animal model.

Recent studies have revealed that curcumin has effects of anti-inflammatory[26], antioxidant[10], anti-fibrosis[27] and anti-cancer[28]. The curcumin has been used to treat the sepsis induced organ injury in previous studies, such as lung injury, liver injury and kidney injury induced by sepsis[12,14,29]. The mechanisms involved in treatment function of curcumin include; regulating the expression of proinflammatory and anti-inflammatory cytokines, inhibiting or eliminating oxygen free radicals, and inhibiting apoptosis[30]. The essence of pancreatic injury induced by sepsis is inflammatory reaction, and the imbalance between systemic inflammatory reaction and compensatory anti-inflammatory reaction plays a key role in its pathogenesis[31]. Therefore, it is reasonable to believe that curcumin has a protective effect on pancreatic injury in sepsis. In our results, we verified that the curcumin attenuated the inflammation (IL-6, IL-1β and TNF-α), oxidative stress (MDA and ROS), and apoptosis level (TUNEL assay, Bcl-2, BAX, caspase-3 and cleaved caspase-3) and ameliorate pancreatic injury induced by sepsis in rats.

In sepsis, most inflammatory cytokines mediate their biological effects through some signal transduction methods[32]. Previous research indicated that JAK2/STAT3 pathway participated in the occurrence and development of sepsis[33]. JAK/STAT signaling is composed of JAKs and STATs protein family. JAK is activated by tyrosine phosphorylation and coupled with cytokine receptor. STAT is the substrate of JAK, which can play a role in transcriptional regulation by coupling with DNA through tyrosine phosphorylation signal pathway. The phosphorylated STAT protein mediates the growth, differentiation, proliferation, and apoptosis of cytokines and plays a key role in the development of sepsis[34]. The activated STAT protein enters the nucleus in the form of dimer and binds to the target gene, inducing target gene transcription, thereby affecting the release of a series of inflammatory factors[35]. Among them, JAK2/STAT3 signal pathway is one of the common cellular pathways of many inflammatory cytokines, which is associated with the multiple organ dysfunction in sepsis[36], such as acute lung injury[37] and kidney injury[38]. Curcumin blocks inflammatory reaction by inhibiting JAK2/STAT3 pathway, thus protecting organ function, such as kidney[16] and pancreas[39]. Our results showed that by inhibiting the key molecules in the JAK2/STAT3 pathway *via* curcumin, it cannot only block the excessive inflammatory response in sepsis, thus protecting the body's function.

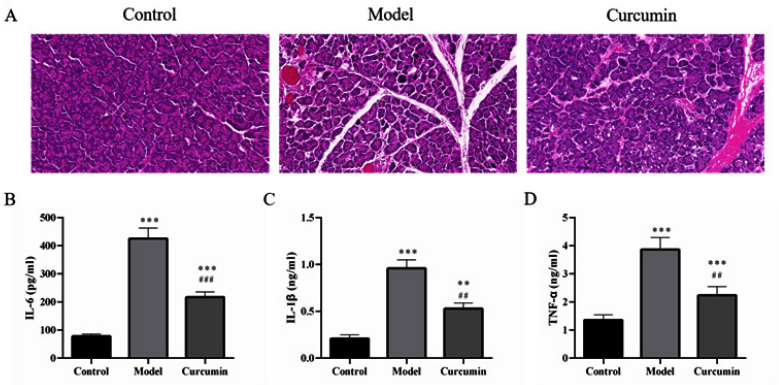

Fig. 1: Curcumin improves inflammation of pancreatic injury induced by sepsis in rats, (A): The HE staining showed the pathological differences of pancreatic injury induced by sepsis in rats; (B-D): The ELISA assays were performed to detect the expression of inflammatory factors in rat serum, including (B): IL-6, (C): IL-1β and (D): TNF-α

Note: ***p<0.001, **p< 0.01 compared with control group and ###p<0.001, ##p<0.01 compared with model group; n=3

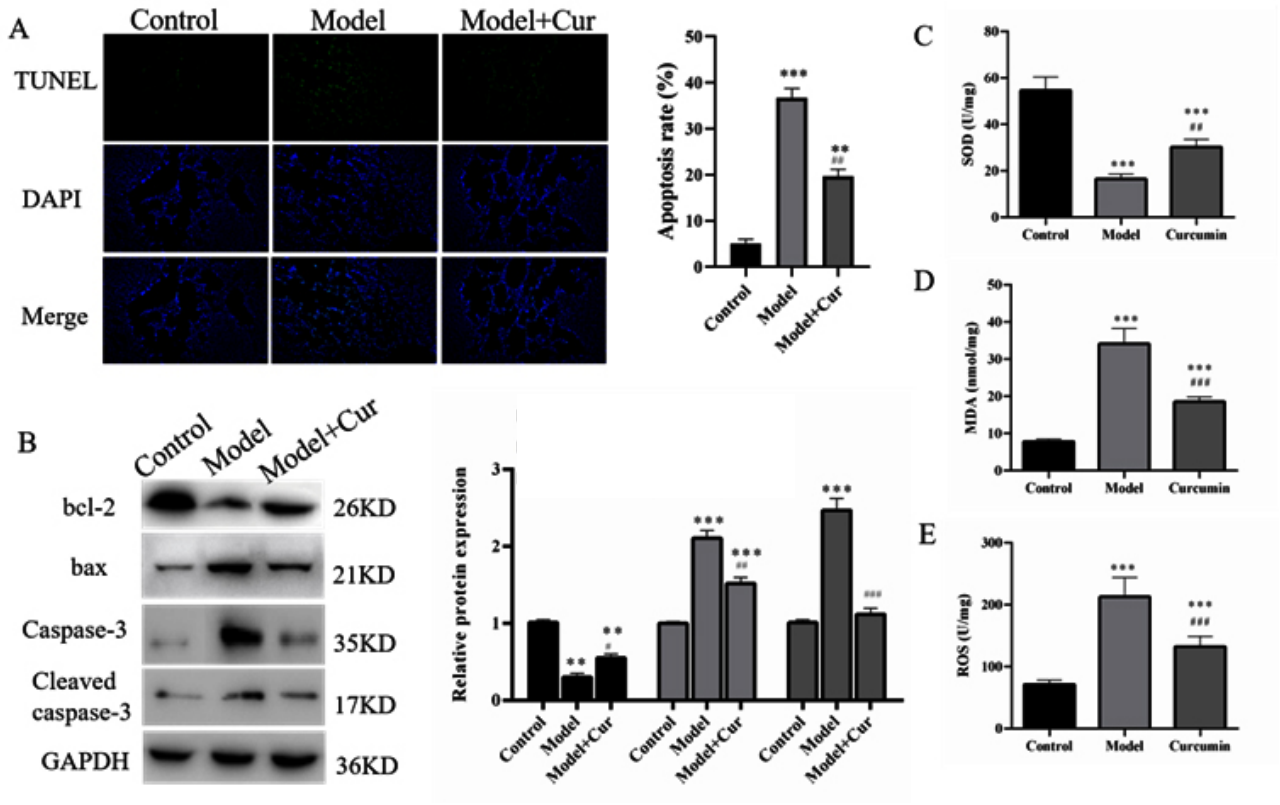

Fig. 2: Curcumin suppresses the apoptosis and oxidative stress of pancreatic injury induced by sepsis in rats, (A): The TUNEL assay was carried out to present the apoptosis rate in control, model, and model treated with curcumin groups; (B): The WB was performed to detect the relative protein expression level of apoptosis related proteins, including Bcl-2, BAX, caspase-3 and cleaved caspase-3; (C): The ELISA assays were performed to detect the expression level of oxidative stress related factors in pancreatic tissue, including SOD, MDA (D): ROS and (E): The curcumin attenuated the expression of oxidative stress related factors in model group

Note: ***p<0.001, **p<0.01 compared with control group; ###p<0.001, ##p<0.01, #p<0.05 compared with model group and n=3, ( ): Bcl-2/GAPDH; ( ): BAX/GAPDH and ( ): Cleaved-caspase-3/caspase-3

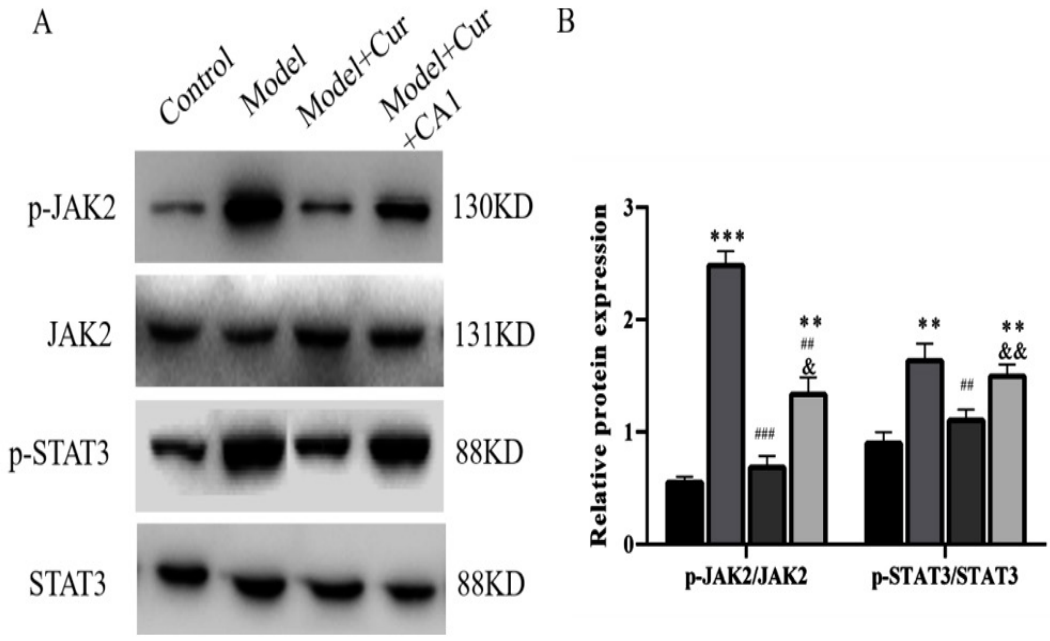

Fig. 3: Curcumin inhibits JAK2/STAT3 pathway in pancreatic injury induced by sepsis in rats, (A and B): The WB was performed to detect the relative protein expression level of phospho-JAK2 (p-JAK2), JAK2, p-STAT3 and STAT3 under different condition

Note: ***p<0.001, **p<0.01 compared with control group; ###p<0.001, ##p<0.01, compared with model group; &&p<0.01, &&p<0.05 compared with model+curcumin group and n=3, ( ): Control; ( ): Model; ( ): Model+Cur and ( ): Model+Cur+CA1

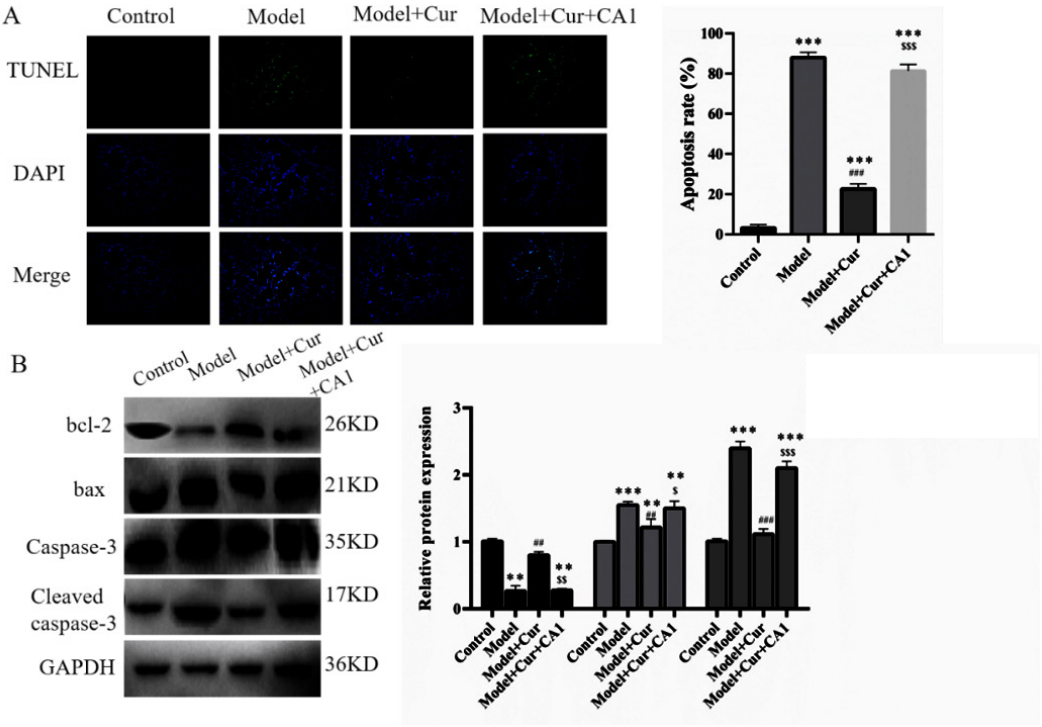

Fig. 4: Curcumin suppresses JAK2/STAT-3 pathway to ameliorate apoptosis of pancreatic injury induced by sepsis in rats, (A): The TUNEL assay was carried out to present the apoptosis rate in groups of control, model, model treated with curcumin, model treated with curcumin and CA1 and (B): The WB was performed to detect the relative protein expression level of apoptosis related proteins in different groups, including Bcl-2, BAX, caspase-3 and cleaved caspase-3

Note: ***p<0.001, **p<0.01 compared with control group; ###p<0.001, ##p < 0.01, #p<0.05 compared with model group; $$$p<0.001, $$p<0.01, $p<0.05 compared with model+curcumin group and n=3, (■): Bcl-2/GAPDH; (■): BAX/GAPDH and (■): Cleaved caspase-3/caspase-3

There are still shortcomings in this study. If the upstream signal pathway of curcumin improving LPS induced pancreatic injury in sepsis is not explored, and multiple time points and multiple doses of curcumin are not set for observation of pancreatic injury in sepsis, this will be the next research direction of our research group.

In summary, curcumin can significantly ameliorate the pancreatic injury induced by sepsis in rats by inhibiting inflammatory reaction, reducing oxidative stress, and attenuating apoptosis. Curcumin can improve pancreatic injury in septic rats by inhibiting JAK2/STAT3 pathway. This study provides a new molecular target and theoretical basis for the clinical prevention and treatment of sepsis.

Funding:

This research was supported by Fujian Natural Science Foundation Project (2019J01154).

Conflict of interests:

The authors declared no conflict of interests.

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This article was originally published in a special issue, "Drug Development in Biomedical and Pharmaceutical Sciences" Indian J Pharm Sci 2023:85(5) Spl Issue "14-21"