



Original Research

Effect and mechanism of Angelic Shaoyaoan mediated AMPK/SIRT1 positive feedback loop to promote autophagy and regulate the systemic inflammatory response in acute pancreatitis

Peijun Sun¹, Miao Nie^{2*}

¹ Department of traditional Chinese medicine, Qingdao Jiaozhou Central Hospital, Jiaozhou, Shandong Province, China

² Department of Gastroenterology, Jiaozhou People's Hospital, Jiaozhou, Shandong Province, China

*Correspondence to: niemiao2021@163.com

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Abstract: This research was carried out to investigate the effect and mechanism of Angelic Shaoyaoan mediated AMPK/SIRT1 positive feedback loop to promote autophagy and regulate systemic inflammatory response in acute pancreatitis. In this study, the rat pancreatic acini AR42J cells were chosen as the research object, the application of hyla induced pancreatic acinar cells made model for acute pancreatitis, application of different concentrations of angelica peony spread effect on building cells, thus divided into control group, built in the module, the low concentration group, concentration and high concentration groups, determined by MTT method was applied to explore the above categories in cell proliferation, cell apoptosis was measured by flow cytometry, the expression of inflammatory factors in cell supernatant was determined by enzyme-linked immunoassay, and the expression of autophagy marker proteins LC3- ii and P62 was determined by Western-Bolt method. In order to explore the relationship between AMPK and SIRT1, immunoco-precipitation method was used to determine the interaction between AMPK and SIRT1, and dual luciferase experiment was used to explore the effect of AMPK on SIRT1. The AICAR group, BLM-275 group and negative control group were established. To explore the effect of SIRT1 on AMPK, we established SRT 1720 group, EX-527 group and control group. Direct binding between AMPK and SIRT1 should be determined by chromatin co-precipitation assay. In order to further explore the effect of AMPK/SIRT1 positive feedback loop on the systemic inflammatory response of acute pancreatitis, this study selected the medium-concentration Danggui Shaoyajiao SAN group as the control group (group C), and applied AMPK inhibitor BLM-275 and SIRT1 inhibitor EX 527 to the effect of medium-concentration Danggui Shaoyajiao SAN cells, respectively. The expression of autophagy marker proteins LC3- ii and P62 in groups A and B were determined by the Western-Bolt method. Results showed that compared with the control group, the cell survival rate, the expression of AMPK, SIRT1 and LC3-II in the model group were decreased, and the apoptosis rate of iNOS, IL-2, TNF- α , P62 and apoptosis were increased in the model group ($P < 0.05$). the levels of iNOS, IL-2, TNF- α , P62 and cell survival rate in low, medium and high concentration groups decreased gradually, while the expressions of AMPK, SIRT1, LC3-II and cell apoptosis rate increased ($P < 0.05$). The levels of iNOS, IL-2 and TNF- α in the three groups were gradually decreased with the increase of the concentration ($P < 0.05$). Immunoprecipitation showed that AMPK and SIRT1 could bind to each other in cells. The double luciferase experiment indicated that the reporter gene containing the SIRT1 binding site was constructed. The luciferase activity was increased in THE AICAR group and decreased in the BLM-275 group ($P < 0.05$). The reporter gene containing the AMPK promoter binding site was constructed. The luciferase activity in SRT1720 group was increased, while that in EX-527 group was decreased. SIRT1 could directly bind to the AMPK promoter. SIRT1 and LC3- ii protein expressions in group A were down-regulated, and P62 protein was increased ($P < 0.05$). The protein expressions of AMPK and LC3- ii in group B were down-regulated, and the protein expression of P62 was increased ($P < 0.05$). It concluded that AMPK can directly bind to activate SIRT1 expression, and SIRT1 expression can also activate AMPK, forming a positive feedback loop between the two. Therefore, Angelic Shaoyaoan decoction can mediate AMPK/SIRT1 positive feedback pathway to promote autophagy and regulate systemic inflammatory response in acute pancreatitis.

Key words: AMPK/SIRT1 positive feedback loop; Acute pancreatitis; Inflammatory response; Angelica peony powder.

Introduction

Acute pancreatitis (AP) is a digestive system disease characterized by pancreatic self-digestion and necrosis. Its etiology is complex, and it is usually associated with pancreatic duct obstruction, increased pancreatic duct pressure, and insufficient pancreatic blood supply and a risk factor for pancreatic cancer (1). According to relevant epidemiology, the case fatality rate of AP is as high as 30%, which is a clinically common severe acute abdomen (2). After AP occurs, a variety of inflammatory cells are activated and carcinogenic embryonic antigen is high (3), and il-6, IL-10 and other inflammatory

factors are released, leading to the initiation of systemic inflammatory response and apoptosis of pancreatic tissue cells. It is of great significance to control inflammatory response and apoptosis in AP patients, which can effectively reduce poor prognosis and increased mortality. Acute pancreatitis, which belongs to the categories of "abdominal pain", "spleen heartache" and "hate of pancreas" in Chinese traditional medicine, is usually caused by alcohol abuse or arbitrary eating of fat and sweet, spicy, or emotional stimulation, or secondary gallstones, resulting in damp-heat accumulation and irritation, and injury to the spleen and stomach. In the early stage of the treatment of acute pancreatitis,

traditional Chinese medicine mainly used to relieve heat and heat through the fu-organs, and to attack, and also to invigorate qi, Yin and blood circulation. In the later stage, according to the patient's symptoms or tongue and pulse conditions, the prescription such as Angelica Peony Powder and Sini powder was used to promote blood circulation and nourish blood, invigorate the spleen and dehumidify the liver and spleen. Angelica Peony Powder is a classic prescription commonly used in clinical practice, which mainly regulates the pathological changes of human blood and body fluid through the liver and spleen. The powder had been used in treatment of cancer alone or combined with other chemotherapy agents such as Cisplatin (4-8). In the past, this prescription was used for abdominal pain. However, the existing research results indicate that the application of Angelica Shaoyao powder can achieve a good effect for the etiology of blood deficiency and water deficiency in the late stage of acute pancreatitis (9). Autophagy, also known as type II programmed death, is an important way for cells to remove excess and damage organelles and provide energy for cell metabolism (10) and engage many cytogenetic events in cancer and malignant transformation process (10-15). In recent years, some studies have shown that autophagy is closely related to the occurrence of acute pancreatitis, for example, Biczko *et al.* (16) showed that mitochondrial dysfunction and damaged autophagy, as the main downstream effectors, play a central role in the development of AP. At present, there is no research at home and abroad to explore the specific mechanism of Danggui Shaoyao Powder (DSP) in the treatment of acute pancreatitis, and whether it is related to autophagy. Therefore, in this study, rat pancreatic acinar AR42J cells were selected as the research object, and pancreatic acinar cells were modeled as acute pancreatitis cells induced by raclamins, so as to explore the effect and mechanism of Angelica Shaoyao on the AMPK/SIRT1 positive feedback loop mediated by AMPK/SIRT1 to promote autophagy and regulate the systemic inflammatory response of acute pancreatitis.

Materials and Methods

Research materials

Drugs

angelica peony extract angelica sinensis by classical prescription proportion: radix paeoniae alba: sichuan dome: atractylodes, tuckahoe, alisma = 3:16:8:4:4:8 configured, using 75% ethanol preparation is 1.72 g/ml crude drug concentration backup solution, used in the preparation of 1 g/ml of liquids, visible Fan Shengnan specific configuration method, etc. The results of the study.

Cells and reagents

Rat pancreatic acinar AR42J cells; Tree frog; Fetal bovine serum; Rabbit anti-rat AMPK, SIRT1, LC3- ii, P62 monoclonal antibody; AMPK activator AICAR; AMPK inhibitor BLM-275; SIRT1 activator SRT1720; SIRT1 inhibitor EX-527; MTT kit; Immunoprecipitation kit; Dual-Luciferase reporting Kit; Chromosomal immunoprecipitation Kit; INOS, IL-2, TNF- α ELISA Kit; Protein imprinting luminescence system.

Cell culture

RPMI1640 medium containing 10%FBS was cultured in an incubator with a volume fraction of 5%CO₂ and a humidity of 97% at 37°C, and the medium was changed once every 2d. When the cells were fused to about 80%~90%, PBS solution was used for cleaning, 0.25% trypsin was added for digestion and subculture.

Study groups

The control group was cultured with rat pancreatic acinar AR42J cells. In the model group, rat pancreatic acinar AR42J cells were cultured in 108mol/L hydrorarin medium for 8h. In the low concentration group, rat pancreatic acinar AR42J cells were cultured in the medium containing 108mol/L hydrantin and angelica paeoniaosan 2.5mg/mL for 8h. In the medium concentration group, rat pancreatic acinar AR42J cells were cultured in the medium containing 108mol/L hydrantin and 5mg/ml angelica shaoyaoan for 8h. In high concentration group, rat pancreatic acinar AR42J cells were cultured in the medium containing 108mol/L hydrantin and 10mg/mL Angelica Shaoyaoan for 8h.

Observation Indicators

Cell proliferation was measured by MTT

The cells in the logarithmic growth phase were inoculated in 96-well plates with 5×10^3 cells per well. After the cells were attached to the wall, the above groups were divided into control group, model group, low concentration group, medium concentration group and high concentration group. After 8h of culture, 20 μ lMTT was added to each well and cultured for 4h in the incubator. After completion, the medium was discarded, dimethyl sulfoxide was added to each well, and the mixture was shaken and mixed. The absorbance value (A value) was measured at 490nm with A microplate reader, and the cell survival rate was calculated.

Flow cytometry was used to determine apoptosis

The cells at the logarithmic growth stage were inoculated in a 24-well plate with 2.5×10^4 cells per well. After cell adhesion, the control group, the model group, the low-concentration group, the medium-concentration group and the high-concentration group were divided into groups and cultured for 8h. After that, the cells were washed with PBS solution and digested with 0.25% trypsin, and then 1×10^6 cells were collected. 400 μ l 1 \times Binding Buffer was added for cell suspension, then Annexin V-FITC (5 μ L) was added for blending, and incubated at room temperature for 15 min in dark, then 5 μ lPI was added for staining, and incubated for 5min. Add 100 μ l 1 \times Binding Buffer again for mixing, and then check on the computer.

Immunoprecipitation experiment

Pancreatic acinar AR42J cell precipitates were collected, cells were lysed with cell lysate and total protein was extracted. AMPK primary antibody or SIRT1 primary antibody was used for immunoprecipitation, protein agarose beads were added and coupled with the antibody, and 2 \times SDS loading buffer solution was used to break the coupling at high temperature to obtain the co-precipitated protein. The proteins of AMPK and

SIRT1 were determined by the western-bolt method.

Fluorescence reporter activity assay

In this study, AMPK promoter region sequence and SIRT1 binding region sequence were retrieved on PubMed, and corresponding primers were designed according to the restriction site of luciferase reporter plasmid. AMPK promoter-luciferase reporter plasmids and SIRT1 binding luciferase reporter plasmids were constructed by the Shanghai Shiman Company. Then, the AMPK promoter-luciferase reporter plasmid and SIRT1 binding luciferase reporter plasmid were transfected into pancreatic acinar AR42J cells. AMPK inhibitor or activator and SIRT1 inhibitor or activator were applied to the transfected cells, and the corresponding control group was set up. After 48h, the AMPK promoter-luciferase reporter plasmid was transfected into pancreatic acinar AR42J cells. Double luciferase activity detection kit was used to detect the luciferase activity and the luciferase activity of sea kidney, and the ratio of luciferase activity was used as reporter gene activity.

Groups: AICAR group, BLM-275 group and negative control group for the effect of AMPK on SIRT1; The effects of SIRT1 on AMPK were SRT 1720 group, EX-527 group and control group.

Chromosomal immunoprecipitation experiment

The specific operation of this experiment was carried out by referring to the chromatin immunoprecipitation kit, that is, pancreatic acinar AR42J cells were selected and incubated with 1% formaldehyde for 10min at 37 °C, the DNA binding protein was cross-linked with DNA, and then 0.125m glycine was used for cross-linking to terminate, and then the cells were collected and the DNA was shattered by ultrasound. Then the SIRT1 antibody was fed, combined with the smashed DNA, eluted and cross-linked, and the DNA fragment was recovered. Then the specific primers for the SIRT1 binding site were designed, and PCR amplification was applied to determine whether SIRT1 binds to the AMPK promoter region.

Serum levels of inflammatory factors were measured by ELISA

The cells in logarithmic growth phase were inoculated with 2.5×10^4 cells per well and 24-well plates. After cell adhesion, the groups were divided into control group, model group, low-concentration group, medium-concentration group and high-concentration group, and the cells were cultured for 8h. After that, the cell supernatant was collected and centrifuged at 3500r/min for 15min. Then, the levels of iNOS, IL-2 and TNF- α , the inflammatory indicators, were measured by ELISA. The specific steps were as follows: (1) Set up blank well, standard well and sample well to be tested, in which 100ul sample diluent was added to the blank well, and standard and sample to be tested were added to the blank well respectively, gently shaken and shaken, and then incubated with the upper cover of the elisa plate at 37 °C for 2h; (2) After discarding the liquid, add 100PL test solution A to each well, add film covering, and incubate at 37°C for 1h, discard the liquid again, wash the plate for 3 times, soak for 1~2min each time, then add test solution B100pl after discarding, cover film covering, and

incubate at 37 °C for 1h; (3) Discard the liquid, shake dry and wash the plate for 5 times, add 90PL substrate solution to each well, cover the plate with film, incubate, and add 50ul termination solution if the gradient blue. After the above, the optical density of each hole at the wavelength of 450nm was determined by microplate analyzer.

Determination of autophagy marker proteins and other related proteins by Western-Bolt method

The expression of AMPK, SIRT1, LC3- ii and P62 in rat pancreatic acinar AR42J cells was determined by Western-Bolt method. Wash with PBS buffer for 3 times, centrifuge at 10000r/min for 10min to precipitate the cells, discard the supernatant, add the cell lysate, and then shake with a low-temperature operation homogenizer. After full lysis for 30min, transfer the cells to the centrifugal tube with a pipette. Centrifugation at 3500r/min at 4°C for 5min, then the supernatant was taken, and the protein concentration in the supernatant specimen was detected by using the protein concentration of BCA, and then the protein concentration was adjusted to be consistent. After boiling for 10min, 50ug total protein was taken in sodium dodecyl sulfate-polyacrylamide gel for electrophoresis, and the classification target protein was transferred to PVDF membrane by wet membrane transfer device, and then 5% skim milk powder solution was given to seal the membrane at 4°C overnight. Then, primary antibodies, namely AMPK, SIRT1, LC3- ii and P62 antibodies, were added to the membrane 2MI with TNST buffer solution, and sealed for 2h at room temperature. After 3 times of cleaning with TBST solution, the secondary antibody was added and incubated in darkness for 1 hour, and then 3 times of cleaning with TBST solution, 10min each time. After that, GAPDH protein was used as the internal reference protein, and the absorbance of the bands was analyzed by gel imaging system. The relative expression of the protein is the ratio of absorbance value of target band to absorbance value of internal reference protein

Statistical methods

SPSS20.0 statistical software was used for data processing, and all data were tested for normality and homogeneity of variance. Univariate analysis of variance was used for comparison between groups. SNK-q test was used for further comparison between groups. Measurement data were expressed as N or percentage, and a chi-square test was applied. $P < 0.05$ was considered statistically significant.

Results

Effects of Danggui Shaoyao Powder on systemic inflammatory response in acute pancreatitis

Cell proliferation and apoptosis in the five groups

The results showed that compared with the control group, the survival rate of cells in the model group was significantly decreased, and the apoptosis rate was significantly increased, with statistical significance ($P < 0.05$). Compared with the model group, the survival rate of cells in the low-concentration, medium-concentration and high-concentration groups decreased gradually, and the apoptosis rate increased significantly, with statisti-

Table 1. Cell proliferation and apoptosis in the five groups.

Group	Control group	Model group	Low concentration group	Medium concentration group	High concentration group
Cell survival rate	100.25±10.23	78.42±8.32	62.85±6.14	48.12±5.32	40.56±4.11
Apoptosis rate	8.12±1.06	13.16±1.33	16.53±1.37	20.18±1.64	25.39±1.25

Table 2. Expression of inflammatory factors in the supernatant of cells in the five groups.

Group	Control group	Model group	Low concentration group	Medium concentration group	High concentration group
iNOS	150.62±15.26	275.2±23.14	225.18±22.15	192.36±24.32	160.53±32.13
IL-2	49.52±15.28	142.16±17.34	120.13±5.42	90.82±6.73	65.55±3.27
TNF-α	60.15±20.13	285.42±34.16	176.45±16.19	115.32±20.18	80.47±4.16

Table 3. Expression of autophagy marker proteins and other related proteins in the five groups.

Group	Control group	Model group	Low concentration group	Medium concentration group	High concentration group
AMPK	8.15±3.12	6.52±2.15	6.93±2.47	7.48±3.54	8.07±4.15
SIRT1	5.62±1.15	2.11±0.82	2.85±0.39	3.56±0.85	4.32±0.97
LC3-II	0.51±0.18	0.43±0.16	1.41±0.35	2.13±0.41	3.12±0.38
P62	10.53±2.18	12.19±1.16	10.16±3.17	9.05±2.12	8.32±2.15

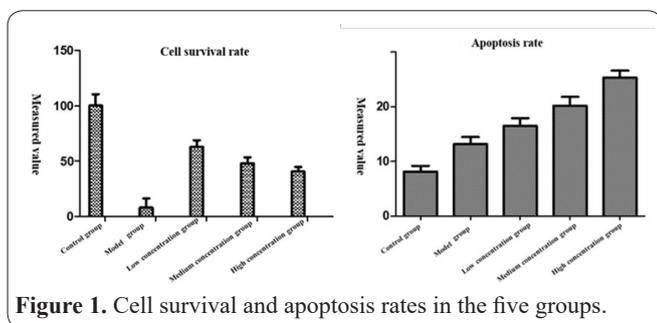


Figure 1. Cell survival and apoptosis rates in the five groups.

cal significance ($P < 0.05$). The concentration among the three groups was negatively correlated with cell survival rate ($P < 0.05$), and positively correlated with cell apoptosis rate ($P < 0.05$), as shown in Table 1 and Figure 1.

Expression of inflammatory factors in the cell supernatant of the five groups

The results showed that compared with the control group, the levels of iNOS, IL-2 and TNF-α in the model group were significantly increased, and the difference was statistically significant ($P < 0.05$). Compared with the model group, the levels of iNOS, IL-2 and TNF-α in the low concentration, medium concentration and high concentration groups were significantly decreased, and the difference was statistically significant ($P < 0.05$). The levels of iNOS, IL-2 and TNF-α in the three groups decreased gradually with the increase of the concentration, and the differences were statistically significant ($P < 0.05$), as shown in Table 2 and Figure 2.

Expression of autophagy marker proteins and other related proteins in the five groups

The results showed that compared with the control group, the expressions of AMPK, SIRT1 and LC3-II in the model group were significantly decreased, while the expressions of P62 were significantly increased ($P < 0.05$). Compared with the model group, the expressions of AMPK, SIRT1 and LC3-II in the low-concentration, medium-concentration and high-concentration groups were significantly increased. P62 was significantly decreased with statistical significance ($P < 0.05$),

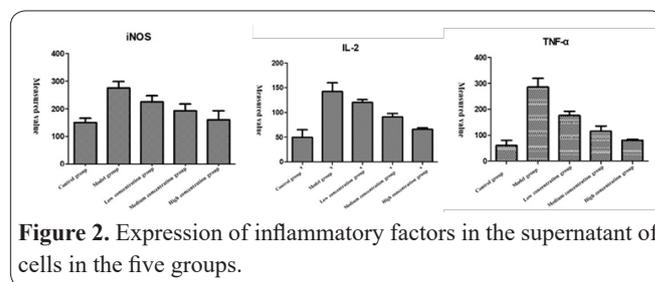


Figure 2. Expression of inflammatory factors in the supernatant of cells in the five groups.

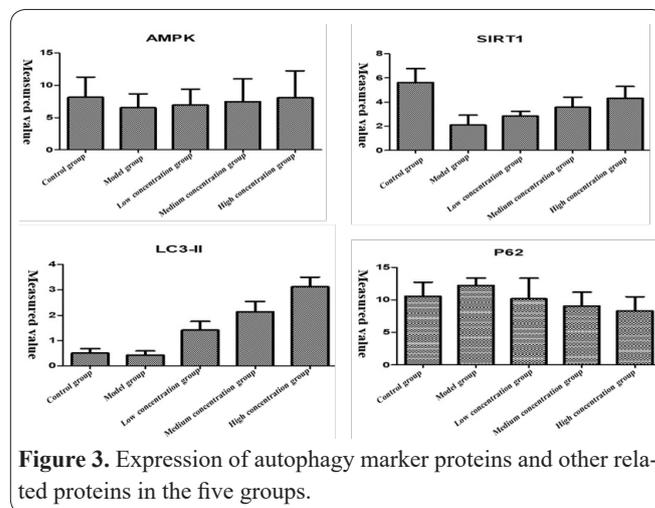


Figure 3. Expression of autophagy marker proteins and other related proteins in the five groups.

and the concentration among the three groups was positively correlated with the expression levels of AMPK, SIRT1 and LC3-II ($P < 0.05$), and negatively correlated with P62 ($P < 0.05$), as shown in Table 3. and Figure 3.

Determination of the positive feedback pathway between AMPK and SIRT1

Immunoprecipitation experiment

The immunofluorescence co-precipitation method showed that AMPK and SIRT1 could bind with each other in cells, suggesting that AMPK and SIRT1 could interact with each other.

Fluorescence reporter activity detection

The results showed that by building the reporter

Table 4. Effects of AMPK activators and inhibitors on SIRT1 gene activity.

Group	AICAR	BLM-275	control group	F	P
Luciferase activity	1.54±0.62	0.82±0.12	1.03±0.04	10.534	0.013

Table 5. Effects of SIRT1 activators and inhibitors on AMPK gene activity.

Group	SRT 1720	EX-527	control group	F	P
Luciferase activity	1.49±0.72	0.76±0.08	1.02±0.05	10.317	0.012

Table 6. Effect of AMPK/SIRT1 positive feedback loop on the systemic inflammatory response of acute pancreatitis treated with Danggui Shaoyaosan.

Group	A	B	C	F	P
SIRT1	2.11±0.45	1.05±0.15	3.72±0.85	8.416	0.002
AMPK	3.12±0.34	4.13±1.52	7.92±3.54	10.532	0.001
LC3-II	0.72±0.27	0.51±0.41	2.13±0.41	7.418	0.008
P62	14.36±1.25	15.19±1.15	9.05±2.12	8.145	0.004

gene containing the SIRT1 binding site, the AMPK activator AICAR could significantly promote the activity of the reporter gene, and the AMPK inhibitor BLM-275 could significantly inhibit the activity of the reporter gene. Specifically, compared with the control group, the luciferase activity in the AICAR group was significantly increased. The luciferase activity of BLM-275 group was significantly decreased, and the above difference was statistically significant ($P < 0.05$), as shown in Table 4. Figure 4 a.

By construction of reporter genes containing AMPK promoter binding sites, it was found that SIRT1 activator SRT1720 could significantly promote the activity of reporter genes, and SIRT1 inhibitor EX 527 could significantly inhibit the activity of reporter genes. Specifically, luciferase activity in the SRT1720 group was significantly increased compared with the control group. The luciferase activity of ex-527 group was significantly decreased, and the above difference was statistically significant ($P < 0.05$), as shown in Table 5. Figure 4 b.

Chromosomal immunoprecipitation experiment

The chromatin immunoprecipitation experiment was further carried out. The results showed that SIRT1 could directly bind to the AMPK promoter, and combined with the luciferase reporting assay and immunoprecipitation assay results, indicating a positive and negative regulatory pathway between AMPK and SIRT1.

Effect of AMPK/SIRT1 positive feedback loop on the systemic inflammatory response of Acute pancreatitis treated by Danggui Shaoyaosan

To further determine the effect of AMPK/SIRT1 positive feedback loop on the treatment of acute pancreatitis inflammatory response by using AMPK inhibitor and SIRT1 inhibitor, respectively, and the cells treated by Danggui Shaoyajiao powder. The results showed that compared with group C, the protein expression of SIRT1 and LC3- ii in group A inhibitor was significantly down-regulated, and the protein expression of P62 was significantly increased, with statistical significance ($P < 0.05$). In group B, AMPK and LC3- ii protein expressions were significantly down-regulated, while P62 protein was significantly increased, with statistical significance ($P < 0.05$), indicating that Angelic Shaiyajiao Powder promoted autophagy through AMPK/SIRT1

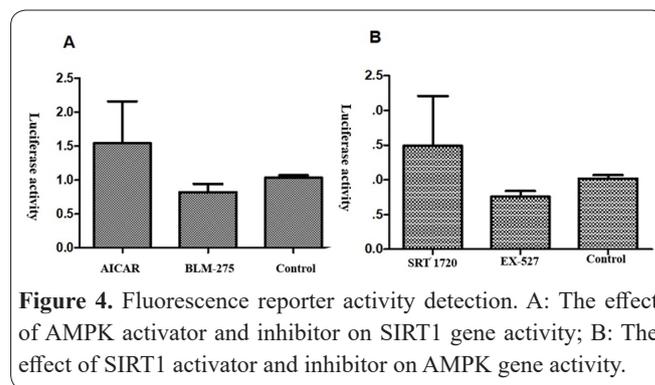


Figure 4. Fluorescence reporter activity detection. A: The effect of AMPK activator and inhibitor on SIRT1 gene activity; B: The effect of SIRT1 activator and inhibitor on AMPK gene activity.

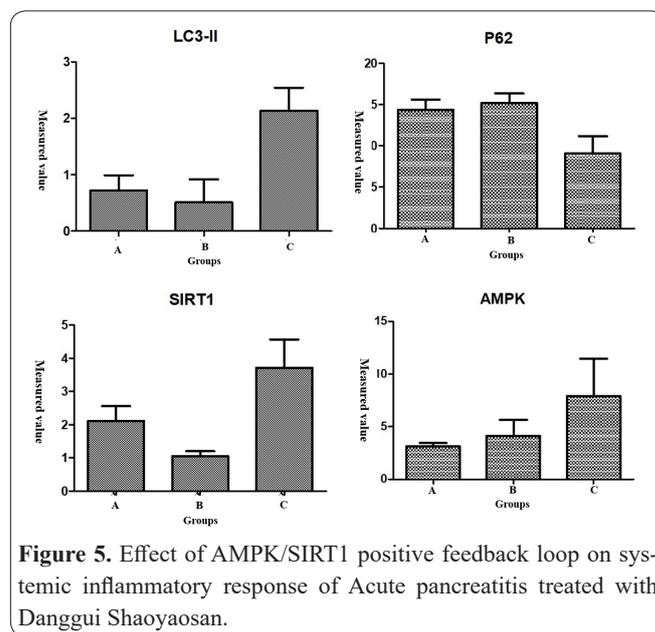


Figure 5. Effect of AMPK/SIRT1 positive feedback loop on systemic inflammatory response of Acute pancreatitis treated with Danggui Shaoyaosan.

positive feedback loop to regulate the systemic inflammatory response of acute pancreatitis, as shown in Table 6 and Figure 5.

Discussion

AP is a clinically common acute abdominal disease with many causes, including biliary, alcohol, overfeeding, and triacylglyceremia. The abnormal activation of pancreatic enzymes in the patient's pancreas leads to local inflammatory reactions such as self-digestion, edema and bad things in the pancreatic tissue. If the

disease gets worse, it can lead to a cascade effects of inflammatory mediators, leading to multiple organ failure syndromes, malignant transformation and death of the patient (17). According to the theory of Chinese Traditional Medicine, AP is a symptom of dampness and heat, which can lead to middle coke, spleen and stomach injury, and qi and blood stasis. And angelica peony powder is a classic prescription for treating women's abdominal pain, and later doctors think that the prescription for blood deficiency and water sheng. In the later stage of AP, the spleen is maladjusted, the grain and water can not be transported to the whole body, and the Yin and blood can not be transformed into blood deficiency, which leads to the loss of the muscles, joints and meridians in the whole body, so angelica Peony powder can be used in the later treatment of AP patients. Some research results have confirmed the significant effect of Danggui Shaoyao Powder on AP (18), and this study will explore the mechanism as follows in combination with the above research results.

Autophagy, also known as type II cell death, is a process in which cells use lysosomes to degrade damaged organelles and macromolecules under the regulation of autophagy-related genes (19). When human cells inside and outside conditions change, autophagy can play a positive role, such as can include through the use of the material supplies for the degradation of the cell cycle, or through the cell material and organelles updates and regulation in order to maintain stability and balance in the body, through the clear cytoplasm and damaged organelles in cells in order to protect the damaged cells (20). Current studies have identified 34 autophagy-related genes and 18 autophagy-regulating genes (12, 13, 21). Under normal circumstances, LC3-II mainly exists on the surface of autophagy vesicles and participates in the formation of autophagosomes, which also leads to the expression of LC3, indicating the occurrence of autophagy (22). P62 is a ubiquitin-binding protein involved in the selective autophagy process of cells. It binds to the ubiquitinated protein and then binds to LC3.-II protein to form a complex, which is degraded in autophagy lysozyme. Therefore, P62 can react as one of the markers of autophagy activity (23).

In experiment 1 of this study, the effect of Danggui Zhaoyao Powders on the systemic inflammatory response of acute pancreatitis showed that compared with the control group, the cell survival rate and the expressions of AMPK, SIRT1 and LC3-II in the model group were significantly decreased, while the apoptosis rate of iNOS, IL-2, TNF- α , P62 and apoptosis rate were significantly increased, suggesting that the model AP cells were successfully created by Rana in this study. Subsequently, the results suggested that the application of Angelicaoneu powders significantly reduced the inflammatory response, while the cell survival rate decreased and the apoptosis rate increased. The low expression of P62 and high expression of LC3-II confirmed that Angelicaoneu powders promoted autophagy to regulate the inflammatory response, while the increased AMPK and SIRT1 indicated that they were related to autophagy (24). Lycopene can improve pancreatitis by preventing oxidative stress-induced autophagy injury and/or directly activating autophagy in pancreatic acinocytes (25). Atg5-mediated autophagy targets NF- κ B signaling in

G2/M stasis proximal tubular epithelial cells after renal injury to prevent renal inflammation. The above results are consistent with the results of this study, confirming that the activation of autophagy can regulate the inflammatory response, possibly because the occurrence of autophagy is closely related to the clearance of inflammatory bodies and apoptotic cells. As for the relationship between autophagy and inflammasome, some studies have suggested that autophagy can also regulate the inflammatory response through the degradation of the inflammasome, but other studies have shown that autophagy can down-regulate ROS levels, thus reducing the generation of the inflammasome (26, 27). Recent studies have also found that autophagy activation exists in the process of apoptosis, and inhibition of autophagy will lead to necrosis of cells and increase the level of inflammatory response. In conclusion, autophagy can serve as a negative regulatory mechanism for inflammatory response (28). However, some studies have shown that the activation of autophagy can lead to the increase of inflammation level, which is related to excessive autophagy (29, 30). In this study, the increase of AMPK and SIRT1 also means that the activation of autophagy and regulation of inflammatory response by Angelic Shaoyaojiao Powders is related to the AMPK/SIRT1 signaling pathway, and the specific mechanism is described below.

In this study, immunoprecipitation, dual luciferase assay and chromatin co-precipitation assay were used to determine whether AMPK and SIRT1 are directly related to explore the relationship between AMPK and SIRT1. The results indicated that AMPK and SIRT1 could bind to each other in cells by immunoprecipitation assay. The double luciferase assay indicated that the reporter gene containing the SIRT1 binding site was constructed. Compared with the control group, the luciferase activity was significantly increased in the AICAR group and significantly decreased in the BLM-275 group. The reporter gene containing the AMPK promoter binding site was constructed. Compared with the control group, the luciferase activity in the SRT1720 group was significantly increased, while that in the EX-527 group was significantly decreased. Chromatin immunoprecipitation assay indicated that SIRT1 could bind directly to AMPK promoter, suggesting that AMPK could activate SIRT1 through direct binding, and the activated SIRT1 could also regulate and enhance the expression of AMPK. Therefore, a positive feedback loop was formed between AMPK and SIRT1 (31). AMPK and SIRT3 regulate each other and their expression and activity are consistent in chondral cells, suggesting the existence of ampK-SIRT3 positive feedback loop (PFL), although the exact mechanism has not been fully elucidated and needs further verification. The results of this study are consistent with those of the above studies to a certain extent, confirming the emergence of the AMPK/SIRT1 positive feedback loop. However, there are still some defects in this study. Immunofluorescence localization was not used to determine the intracellular movement of the interaction between AMPK and SIRT1, which needs further exploration.

AMPK is a widespread serine/threonine-protein kinase in eukaryotes. A large number of studies have confirmed that overexpression of AMPK significantly

inhibits inflammation. AMPK knockout can promote the increase of inflammatory factors, and the specific molecular and cellular mechanisms are closely related to p53, FxoO3a, SREBP and other signal factors (32-34). A comprehensive study of the genome, as well as gene expression, is important in the study of diseases and disorders (35, 36). SIRT1 is a metabolic regulatory enzyme related to the metabolic status of the body. A series of research results have confirmed that the overexpression of SIRT1 can significantly inhibit the expression of inflammatory genes. Currently, it is believed that this factor plays an anti-inflammatory effect mainly by down-regulating the acetylated modification levels of NF-KB, AP-1 and histone (37-39). Meanwhile, BOTH AMPK and SIRT1 are enzymes sensitive to energy metabolism in the body, so they usually play a synergistic effect to jointly maintain energy homeostasis in the body. For example, Velagapudi *et al.* (40) showed that thymoquinone inhibited the production of ROS in LPS-activated BV2 microglia. It also suggested that activation of AMPK and NAD⁺/SIRT1 might contribute to the anti-inflammatory activity of the compound in BV2 microglia, but did not have antioxidant activity.

In order to further determine the effect of AMPK/SIRT1 positive feedback loop on the inflammatory response of acute pancreatitis, AMPK inhibitor and SIRT1 inhibitor were used to treat cells with Danggui Shaoyao powder, respectively. The results showed that compared with group C, SIRT1 and LC3-ii protein expression in group A was significantly down-regulated and P62 protein expression was significantly increased, while AMPK and LC3-ii protein expression in group B was significantly down-regulated and P62 protein expression was significantly increased. These results demonstrated that Angelical-shaoyaosan promoted autophagy through the AMPK/SIRT1 positive feedback loop to regulate the systemic inflammatory response in acute pancreatitis. Metformin regulates the sirtuin-1 (SIRT1)-foxO1 (FoxO1) pathway through adenosine phosphate-activated protein kinase (AMPK)/silent mating type information to alleviate glucose and lipid metabolism disorders, renal function injury and enhance autophagy in diabetic rats (41). Sirt1 promotes autophagy through AMPK activation and reduces hypoxia-induced apoptosis through the IRE1 α pathway to protect cardiocytes from hypoxic stress. The above results are consistent with the results of this study, confirming that Danggui Shaoyaosan mediates AMPK/SIRT1 loop to activate autophagy to regulate the systemic inflammatory response in AP patients (42).

In conclusion, AMPK can directly bind to activate SIRT1 expression, and SIRT1 expression can also activate AMPK, forming a positive feedback loop. Therefore, Angelic Shaoyaosan mediates AMPK/SIRT1 positive feedback pathway to promote autophagy and regulate the systemic inflammatory response in acute pancreatitis.

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