Research Paper

Study on Mechanism of Ligustrazine Regulating microRNA-21 on Apoptosis of Acinar Cells in Pancreatitis

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The current study aimed in evaluating the efficacy of quercetin on sepsis-related acute kidney injury and explores its potential mechanisms to provide theoretical support for treatment. A total of forty mice were allocated into four groups; mock, lipopolysaccharide and two quercetin dosage groups (low and high doses; 25 mg/kg and 50 mg/kg, respectively). Kidney tissue morphology was assessed. Serum levels of the kidney function markers creatinine and blood urea nitrogen, as well as inflammatory cytokines interleukin-1 beta, tumor necrosis factor-alpha, and interleukin-6, were measured. A septic injury model was established in human kidney-2 cells using lipopolysaccharide induction, and iron content in human kidney-2 cells and mouse kidney tissues was measured. Reverse transcription-polymerase chain reaction was used to assess the expression levels of ferroptosis-related genes. Spectrophotometry measured malondialdehyde and glutathione levels in human kidney-2 cells and tissues of kidneys in mouse. Enzyme-linked immunosorbent assay was used to quantify nuclear factor erythroid 2-related factor 2 and glutathione peroxidase 4 protein levels. Quercetin intervention 25/50 mg/kg significantly improved the morphology of mouse kidney tissues and decreased serum levels of inflammatory cytokines, and kidney function markers creatinine and blood urea nitrogen, showing a dose-dependent effect. Quercetin significantly reduced the content of ferrous ions and the expression of cyclooxygenase-2 and acyl-coenzyme A synthetase long chain family member 4. The expression levels of nuclear factor erythroid 2-related factor 2 and glutathione peroxidase 4 induced by lipopolysaccharide were significantly downregulated ($p<0.001$). Quercetin can alleviate sepsis-related acute kidney injury with its mechanism likely involving the activation of the nuclear factor erythroid 2-related factor 2/glutathione peroxidase 4 pathway.

Key words: Sepsis, acute kidney injury, ferroptosis, quercetin, nuclear factor erythroid 2-related factor 2, glutathione peroxidase 4

As a pancreas disease which is caused by trypsin's self-digestion, Acute Pancreatitis (AP) appears edema, congestion, or bleeding, necrosis, and clinically presents symptoms such as nausea, abdominal pain, abdominal distension, fever and vomiting. The most frequent groups include hyperlipidemia and hypercalcemia[1,2]. Tetramethylpyrazine (TMP) is one of the major ingredients of *Ligusticum chuanxiong* Hort. It has anti-tumor, sedative and analgesic effects, anti-ischemia-reperfusion injury, antithrombotic effects, and protection of heart, liver, spleen, lung and kidney, including pancreas[3-5]. Microribonucleic Acids (miRNAs) are in close contact with AP's occurrence and development, and have a regulatory relationship in AP[6]. This experiment mainly studied the mechanism of TMP on AP acinar cell apoptosis in rats by regulating miR-21.

MATERIALS AND METHODS

Materials:

Experimental animals: 60 SPF male rats with an average weight of (225.67±18.92) g were supplied by the Experimental Animal Center of West China Medical Center, Sichuan University. License no.: SCXK (Sichuan) 2019-09.

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Drugs: TMP is provided by SPH no. 1 Biochemical and Pharmaceutical Co. Ltd., Cat no.: 020501.

Cells and reagents: We acquired pancreatic acinar AR42J cells from the American Type Culture Collection (ATCC), United States of America (USA). Bombesin was purchased from Sigma, USA. Fetal bovine serum was obtained from Gibco, USA. The RPMI1640 medium was supplied by Bio-Rad, USA. We received the Lipo2000 transfection reagent from Invitrogen, USA. Additionally, we acquired the Trizol reagent, fluorescence quantitative kit, and reverse transcription kit from Takara, Japan. The Annexin V-Fluorescein Isothiocyante (FITC) apoptosis detection kit was purchased from Beijing Solarbio Science and Technology Co. Ltd., and the Enzyme-Linked Immuno Sorbent Assay (ELISA) kit was obtained from Shanghai Mlbio Co. Ltd.

Methods:

Preparation of rat AP model: After 12 h of fasting (water administration), rats were intraperitoneally injected with bombesin 40 μg/kg at an interval of 30 min, and then the same dose of bombesin was injected.

AP pancreatic acinar AR42J cell model construction: We cultured AR42J cells in RPMI1640 medium, which contained 10 % fetal bovine serum and was placed in a 5 % CO_2, 37° constant temperature incubator. AR42J passage cells were seeded in 6-well plates at 55 000 cells/well, and 15 nmol/l bombesin was added. After shaking and mixing, the cells were cultured for another 24 h.

Cell grouping: We randomly divided 60 male rats into 6 groups (n=10). The model group rats were intraperitoneally injected with bombesin (40 g/kg). The blank control group was injected with the same dose of normal saline. The TMP Low-dose group (TMP-L) received an intraperitoneal injection of 40 mg/kg/d of TMP in AP rats. The TMP Medium-dose group (TMP-M) was given an intraperitoneal injection of 80 mg/kg/d of TMP in AP rats. The TMP High-dose group (TMP-H) was administered an intraperitoneal injection of 160 mg/kg/day of TMP in AP rats. All administrations were conducted for 10 d.

The pancreatic acinar AR42J cell model was constructed, and anti-miR-21 and anti-miR-NC were transfected into AP rat acinar cells respectively, recorded as AP+anti+miR-21 and AP+anti+miR-NC groups. miR-NC and miR-21 were transfected into acinar cells of AP rats, and then treated with 50.00 μmol/l TMP, which were recorded as AP+miR-NC+TMP group and AP+miR-21+TMP group.

Apoptosis detection with flow cytometry: The cells from different groups were collected, resuspended and washed with pre-cooled Phosphate-Buffered Saline (PBS) solution. They were then suspended with an appropriate amount of 1×binding buffer, and the cell concentration was adjusted to 5×10^5 cells/ml. Taking a 100 μl cell suspension was mixed and incubated 5 μl of FITC in the dark at room temperature, with a duration of 15 min, and add another 5 μl of Propidium Iodide (PI) staining, supplemented with 1×binding buffer to 500 μl. Apoptosis was then detected using a computer.

Detection of the expression level of miR-21 in cells by RT-q Polymerase Chain Reaction (PCR): We extracted total RNA with Trizol reagent, applied Ultraviolet (UV) spectrophotometer to detect the concentration of RNA, and then they were reversely transcribed into complementary Deoxyribonucleic Acid (cDNA) according to the reverse transcription kit. Finally, for PCR amplification we applied cDNA as template. The PCR amplification procedure was as follows, 95° for 10 min, 95° for 10 s, 60° for 30 s, 72° for 30 s, with a total of 40 cycles. The primers for miR-21 were, F 5'-TGCGCTAGCTTATCAGACTGAT-3', R 5'-CCAGTGCAGGGTCCGAGGTATT-3'; U6: F 5'-CGCTTCGGCAGACATATAC-3', R 5'-AAATATGGAACGCTTCACGA-3'. We used the $2^{-\Delta\Delta Ct}$ method to calculate miR-21's relative expression level with U6 as the internal reference.

Detection of cellular TNF-alpha (α), Interleukin-1 beta (IL-1β) and Amylase (AMY) levels by ELISA: The cell supernatant was extracted and TNF-α, IL-1β and AMY levels was detected according to the experimental steps of ELISA kit instructions.

Statistical processing:

We employed SPSS 21.0 statistical software for the data analysis, and the metrology data were expressed in ($\bar{x}\pm s$). We employed independent sample t-test for two groups' comparison, used one-way analysis of variance for multiple groups' comparison, and applied Least Significant Difference (LSD)-t test was for two groups'

comparison, with $p<0.05$ as statistically significant differences.

RESULTS AND DISCUSSION

Comparing to blank control group, in the model group the apoptosis rate was risen dramatically ($p<0.05$); comparing to the model group low, medium and high doses of TMP dramatically decreased the apoptosis rate ($p<0.05$). Therefore, the subsequent experiments were performed with high doses of TMP (Table 1 and fig. 1).

Compared with blank control group, levels of TNF-α, IL-1β and AMY in model group cells increased significantly ($p<0.05$). In comparison to the model group, high-dose TMP significantly reduced cellular TNF-α, IL-1β and AMY level ($p<0.05$) (Table 2).

Comparing to the AP+anti+miR-NC group, the apoptosis rate and the expression of miR-21 in the AP+anti+miR-21 group were obviously decreased ($p<0.05$) (Table 3).

Comparing to the AP+anti+miR-NC group, miR-21's expression in the AP+anti+miR-21 group was significantly decreased, and TNF-α, IL-1β and AMY levels were decreased dramatically ($p<0.05$) (Table 4).

Comparing to the AP+miR-NC+TMP group, the expression of miR-21 in the AP+miR-21+TMP group was increased dramatically, the apoptosis rate and levels of TNF-α, IL-1β and AMY increased dramatically ($p<0.05$) (Table 5).

TABLE 1: EFFECT OF TMP ON APOPTOSIS OF AP ACINAR CELLS IN RATS ($\bar{x}\pm s$, n=9)

Group	Apoptosis rate (%)
Blank control	28.82±5.29
Model	76±8.59*
TMP-L	62.34±6.51#
TMP-M	55.37±7.30@
TMP-H	43.62±6.27&
F	350.176
p	0.000

Note: *$p<0.05$ comparing to blank control group; #$p<0.05$, @$p<0.01$ and &$p<0.001$ comparing to the model group

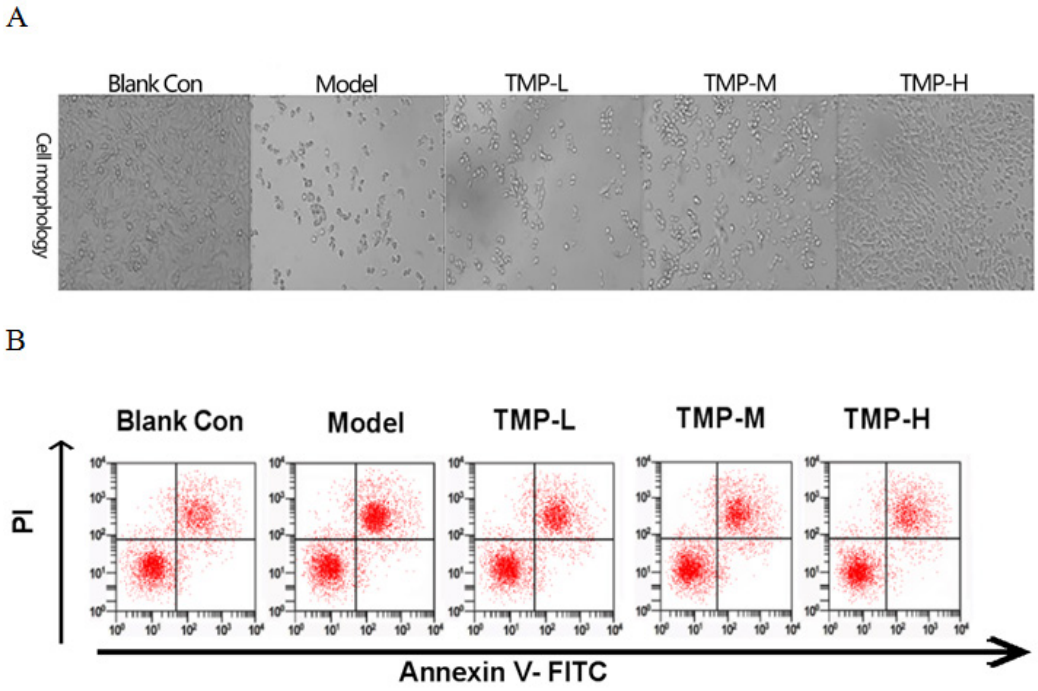

Fig. 1: Effect of TMP on apoptosis of AP acinar cells in rats. A: Pictures of cell morphology and B: Pictures of apoptosis

TABLE 2: EFFECT OF TMP ON INFLAMMATORY FACTORS OF AP ACINAR CELLS IN RATS (x̄±s, n=9)

Group	TNF-α (pg/ml)	IL-6 (pg/ml)	AMY (IU/L)
Blank control	34.21±3.57	30.94±2.24	1563.73±154.01
Model	69.37±7.52*	66.50±6.30*	7630.15±453.38*
TMP-H	46.31±4.56#	40.37±3.85#	3756.18±276.31#
F	196.321	173.425	203.672
p	0.000	0.000	0.000

Note: *p<0.05 comparing to blank control group and #p<0.05 comparing to the model group

TABLE 3: EFFECT OF INHIBITING MIR-21 EXPRESSION ON APOPTOSIS OF AP ACINAR CELLS IN RATS (x̄±s, n=9)

Group	miR-21	Apoptosis rate (%)
AP+anti+miR-NC	1.00±0.09	65.31±2.27
AP+anti+miR-21	0.30±0.03*	30.30±4.82*
t	18.861	39.621
p	0.000	0.000

Note: Compared with AP+anti+miR-NC group, *p<0.05

TABLE 4: EFFECT OF INHIBITING MIR-21 EXPRESSION ON INFLAMMATORY FACTORS IN AP ACINAR CELLS OF RATS (x̄±s, n=9)

Group	miR-21	TNF-α (pg/ml)	IL-6 (pg/ml)	AMY (IU/L)
AP+anti+miR-NC	1.01±0.09	72.31±8.62	68.30±6.62	7324.21±258.94
AP+anti+miR-21	0.33±0.04*	40.03±5.21*	42.23±5.28*	2432.01±197.65*
t	20.03	96.665	87.301	186.347
p	0.000	0.000	0.000	0.000

Note: Compared with AP+anti+miR-NC group and *p<0.05

TABLE 5: THE OVEREXPRESSION OF MIR-21 REVERSES TMP's EFFECT ON ACINAR CELL APOPTOSIS AND INFLAMMATORY FACTORS (x̄±s, n=9)

Group	miR-21	Apoptosis rate (%)	TNF-α (pg/ml)	IL-6 (pg/ml)	AMY (IU/L)
AP+miR-NC+TMP	1.02±0.11	35.61±5.80	38.07±2.49	45.61±6.67	2994.87±263.45
AP+miR-21+TMP	2.76±0.42*	69.47±7.02*	57.16±4.21*	70.52±8.33*	6831.25±196.48*
t	16.375	157.732	123.321	154.637	289.614
p	0.000	0.000	0.000	0.000	0.000

Note: Compared with AP+miR-NC+TMP group and *p<0.05

AP, as an inflammatory reaction, causes edema, pancreatic tissue digestion, hemorrhage and even necrosis followed by pancreatic enzymes activated in the pancreas due to a variety of etiologies. The severity of the lesions varies. Mild cases are mainly pancreatic edema, which is more common clinically. The condition of this disease is often self-limiting, with good prognosis, and is also known as mild AP. For severe cases, called severe AP, pancreatic hemorrhage and necrosis occur, often secondary to infection, shock as well as peritonitis, at high mortality[7,8].

This experimental study showed us that the apoptosis rate of AP group was dramatically increased comparing to the blank control group, but low, medium and high doses of TMP significantly reduced the apoptosis rate of AP cells. Comparing to the blank control group, the cellular

TNF-α, IL-1β and AMY levels in the AP group were significantly increased, but TMP obviously decreased the TNF-α, IL-1β and AMY levels of AP cells. Some research conclusions are consistent with this experiment. *Salvia miltiorrhiza* TMP has a good therapeutic effect on hyperlipidemic AP, can effectively relieve tenderness and abdominal pain, and promote the negative conversion of blood and urine AMY[9]. TMP can reduce serum AMY, IL-1β and TNF-α levels in severe AP rats, playing a therapeutic role[10]. *Salvia miltiorrhiza* TMP injection in the adjuvant treatment of patients with acute AP can decrease the inflammatory index of patients, shorten patients' recovery time, and reduce the complications' incidence[11]. *Salvia miltiorrhiza* TMP injection has a good therapeutic role on hyperlipidemic AP[12]. *Salvia miltiorrhiza* TMP can reduce the inflammatory reaction in patients with AP, with significant clinical efficacy, which is worthy of clinical reference[13].

This study found that inhibition of miR-21 expression dramatically reduced the apoptosis rate and TNF-α, IL-1 β and AMY levels were significantly reduced. The results of this experiment are consistent with others research. miR-21's relative expression level in the blood of patients with AP is correlated with the intracellular immune response, infection degree, inflammatory response and pancreatic tissue necrosis degree of patients with AP[14]. High expression of miR-21 in AP acinar cells inhibits pancreatic acinar cell apoptosis, which in turn aggravates the development of pancreatitis[15]. Comparing to the control group, serum IL-1β, IL-8 and TNF-α levels in patients with AP were dramatically higher. Serum miR-21 and serum IL-1β, IL-8 and TNF-α levels in patients with AP were significantly positively correlated[16].

The results of this experiment showed that miR-21 overexpression reversed the effect of TMP on AP acinar cells and significantly increased the apoptosis rate and TNF-α, IL-1β and AMY levels. These results showed us that TMP can inhibit AP alveolar cell apoptosis and reduce inflammatory response by inhibiting miR-21 expression. In conclusion, TMP inhibits AP alveolar cell apoptosis and reduces inflammatory response by inhibiting miR-21 expression, which provides a theoretical basis for AP treatment.

Conflict of interests:

The authors declared no conflict of interests.

REFERENCES

1. Wu Z, Wang X, Jiang X. Study on the mechanism of probucol nanosuspension on hyperlipidemic pancreatitis and regulation of blood lipid function. J Nanosci Nanotechnol 2021;21(2):1286-92.
2. Sundar V, Dutta A, Ramasamy S, Manickam V, Tamizhselvi R. Sting pathway-a futuristic therapeutic target for acute pancreatitis?. Gene 2021;778:145469.
3. Lin X, Lu XF, Hang LH. Protective effect of TMP on acute lung injury and its possible mechanism. J Southeast Univ 2020;39(6):830-4.
4. Zhu H, Zhang YW, Zhou HD. Effect of TMP on hippocampal inflammatory response in rats with sepsis associated encephalopathy. Chin J Anesth 2020;40(5):629-32.
5. Yang Q, Qi GD, Xu K. Protective effect of ligustrazine on oxidative stress injury of astrocytes induced by hydrogen peroxide. Journal Mil Med Univ 2021;43(3):226-33.
6. Jia CY, Zhang CL, Zhang XQ. Research progress of microRNAs in acute pancreatitis. Chin J Hyg Rescue 2017;3(3):180-2.
7. Qu C, Wei M, Li WQ. Extrapancreatic and pancreatic infection in acute pancreatitis. Eur J Gastroenterol Hepatol 2021;33(4):598-9.
8. Akanbi O, Adejumo AC, Soliman M, Kudaravalli P. Chronic pancreatitis patients who leave against medical advice: Prevalence, trend, and predictors. Dig Dis Sci 2021;66:424-33.
9. Zhu YF, Xie ML. Discussion on the clinical effect of *Salvia miltiorrhiza* ligustrazine in the treatment of hyperlipidemic acute pancreatitis. J Front Med 2020;10(1):204-5.
10. Wang L, Xing S. Experimental study of ligustrazine in the treatment of severe acute pancreatitis. Pract Pharm Clin Remedies 2016;19(7):842-4.
11. Wang X, Lin HX, Chen JS. *Salvia miltiorrhiza* ligustrazine injection for non-surgical treatment of acute pancreatitis. J Mudanjiang Med Univ 2019;40(2):75-6.
12. Lin FK, Jiang X, Chen HZ. Clinical observation of *Salvia miltiorrhiza* ligustrazine injection in the treatment of hyperlipidemic acute pancreatitis. Chin J Tradit Med Sci Technol 2016;23(3):349-50.
13. Gong JE. Effect of *Salvia miltiorrhiza* ligustrazine injection on clinical efficacy of acute pancreatitis. J Med Res 2015;44(1):136-8.
14. Xie ZY, Zhao YG, Zhu HW. Correlation between blood miRNA-21 expression level and inflammatory response in patients with acute pancreatitis. Journal Pract Med 2016;32(8):1292-4.
15. Liu N, Yao YF, Yuan S. Effect and mechanism of miR-21 on acinar cell apoptosis in acute pancreatitis. Chinese J Gerontol 2018;38(8):1934-6.
16. Zhang HB, Wang SF, Huang HC. The expression changes of PTX3, TFPI and miR-21 in SAP patients and their relationship with inflammatory response. EXP Lab Med 2019;37(2):75-7.