Research Paper

Effects of Pioglitazone Pretreatment on the Expression of NF-κB, ICAM-1 and p38MAPK Pathway in Pancreatic Tissue of Rats with Acute Pancreatitis

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To investigate the effects of pioglitazone pretreatment on the expression of nuclear factor kappa B and intercellular adhesion molecule-1 and p38 mitogen activated protein kinase pathway in pancreatic tissue of rats with acute pancreatitis. 72 specific pathogen-free adult female Sprague–Dawley rats and male Sprague–Dawley rats were randomly divided into sham operation group, model group and pioglitazone pretreatment group (pretreatment group), with 24 rats in each group. Compared with sham operation group, the amount of ascites in model group was significantly increased, the levels of serum and ascites amylase, serum tumor necrosis factor-alpha, interleukin-6, pancreatic gross score, pancreatic histological score, nuclear factor kappa B and intercellular adhesion molecule-1 in pancreatic tissue were significantly increased. Compared with the model group, the amount of ascites in the pretreatment group was significantly reduced and the levels of serum and ascites amylase, serum tumor necrosis factor-alpha, interleukin-6, pancreatic gross score and pancreatic histological score, the expression levels of nuclear factor kappa B, intercellular adhesion molecule-1 and phospho-p38 mitogen activated protein kinase in pancreatic tissue were significantly decreased ($p<0.05$). In the sham operation group, the pancreatic tissue structure was clear with occasional inflammatory cells or mild hyperemia and edema and the acinar lobules were intact; in the model group, the pancreatic stroma showed hyperemia and edema, even necrosis, with obvious inflammatory cell infiltration and acinar lobule structure disorder and the pathological changes were gradually aggravated with the extension of time; in the pretreatment group, the pancreatic tissue inflammatory cell infiltration and other pathological changes were observed compared with the model group, it was significantly improved. Pioglitazone pretreatment can inhibit the inflammatory response, reduce the level of amylase, inhibit the expression of nuclear factor kappa B and intercellular adhesion molecule-1 and inhibit the activity of intercellular adhesion molecule-1 signaling pathway in rats with acute pancreatitis.

Key words: Pioglitazone, acute pancreatitis, nuclear factor kappa B, intercellular adhesion molecule-1, p38 mitogen activated protein kinase

Acute pancreatitis is one of the common diseases of the digestive system in clinic and its pathogenesis is more complicated. Many scholars at home and abroad have put forward various theories such as "oxygen free radical damage theory", "trypsin digestion theory", "apoptosis", "white blood cell over activation theory" and "inflammatory mediator-cytokine theory". Among them, the "leukocyte over activation theory" is generally accepted by people and it is believed that the excessive release of pro-inflammatory factors is an important reason for the development of the disease[1]. Therefore, effectively controlling the inflammatory response and reducing the production of pro-inflammatory factors are the keys to the deterioration of acute pancreatitis. In recent years, studies have found that Peroxisome Proliferator Activated Receptor Gamma (PPARγ) activators play an important role in inhibiting the release of inflammatory factors, reducing inflammation and tissue damage[2]. Pioglitazone and other thiazolidinedione drugs are PPARγ synthesis activators and are highly selective for PPARγ[3]. However, the relevant mechanism of pioglitazone's anti-inflammatory effect is still unclear. In this group of studies, rats were used as the research object to establish a rat model of

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acute pancreatitis. The aim was to analyze whether the anti-inflammatory effect of pioglitazone was through the inhibition of Nuclear Factor kappa B (NF-κB) and Intercellular Adhesion Molecule-1 (ICAM-1) protein expression and the inhibition of p38 Mitogen Activated Protein Kinase (p38MAPK) pathway activity.

MATERIALS AND METHODS

Experimental materials:

72 Specific Pathogen-Free (SPF) grade adult female Sprague–Dawley (SD) rats and male SD rats were randomly selected (Guangzhou University of Traditional Chinese Medicine, production license SCXK (Guangdong) 2019-0047, use license SYXK (Guangdong) 2019-0202), weight (213±27) g. In this experiment, the temperature was 23°±2°, the humidity was 50 %±10 % and they were free to eat, drink and feed themselves for a week under the conditions of 12 h day and night.

Main instruments and reagents:

Low-temperature high-speed centrifuge (Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., model: TGL-17M); electron microscope (Yikang Optical Measuring Instrument Co., Ltd., model: XTL-3400); -80° ultra-low temperature refrigerator (Beijing Airis Biotechnology Co., Ltd., model: DW-86L626); electronic balance (Shanghai Sunny Hengping Scientific Instrument Co., Ltd., model: JA12002); reverse transcription kit (Shanghai Yucan Biotechnology Co., Ltd.); mouse anti-human phospho (p)-p38MAPK monoclonal antibody (Shanghai Hengfei Biotechnology Co., Ltd.); mouse anti-human NF-κB monoclonal antibody (Nanjing Laifuse Biotechnology Co., Ltd.); mouse anti-human ICAM-1 monoclonal antibody (Beijing Taize Jiaye Technology Development Co., Ltd.); pioglitazone (Shanghai Zhaohui Pharmaceutical Co., Ltd., production batch number: 20180060, specification: 15 mg×7 s/box).

Experimental method:

Experimental grouping: The rats were randomly divided into three groups; sham operation group, model group and pioglitazone pretreatment group (pretreatment group), with 24 rats in each group.

Establish a rat model of acute pancreatitis: The rat is fasted for 10 h, and anesthetized and is placed in a supine position. The rat makes an incision in the middle of the upper abdomen and clamps the pancreaticobiliary duct at the hepatic hilum. Gently puncture the confluence of the pancreaticobiliary duct with a syringe and inject 5 % sodium taurocholate solution 0.1 ml/100 g per minute retrograde. When the pancreas shows congestion, edema and other changes, withdraw the needle and open the arterial clamp after about 3 min, reset the pancreas and the abdomen was closed by suture. Pay attention to heat preservation after the operation and drink freely. The sham operation group was injected with the same dose of normal saline into the pancreaticobiliary duct; the sham operation group and the model group were injected with 10 % dimethyl sulfoxide solution 0.2 ml/100 g in the femoral vein 30 min before modeling and in the pretreatment group, the same dose of pioglitazone dissolved in 10 % dimethyl sulfoxide was injected into the femoral vein 30 min before modeling.

Observation indicators:

Eight rats in each group were sacrificed at 4 h, 8 h and 12 h after modeling.

Ascites volume measurement: Use a 10 ml syringe to collect ascites and record.

Serum and ascites amylase measurement: Take 3 ml of abdominal aortic blood, centrifuge to take the supernatant and use an automatic biochemical analyzer to determine the serum and ascites amylase levels of rats in each group.

Determination of serum inflammatory factor levels: Take the supernatant, the serum levels of Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) were determined by enzyme-linked immunosorbent assay. The operation was carried out strictly in accordance with the procedures of the operation kit.

Pathological changes of the pancreas: Hematoxylin and Eosin (HE) staining was used to observe the pathological changes of the pancreas in each group. Take pancreatic tissue, dehydrated with 70 %-100 % ethanol, transparent xylene, embed in paraffin and solidify overnight. Take the paraffin mass of lung tissue and store it at 4° for 25 min at low temperature, use a paraffin microtome to cut into 5 μm-thick paraffin sections, spread them in a 60° water bath and bake them overnight at 37°. The paraffin sections were dewaxed with xylene, dehydrated with gradient alcohol, washed with distilled water, stained with hematoxylin dye, washed with phosphate buffer, hydrochloric acid alcohol differentiation, ammonia water to blue, distilled

water washing, staining with eosin dye, washing with distilled water, dehydration with gradient alcohol again, transparent xylene and mounting with neutral gum.

Pancreas general score: Hughes criteria were used to evaluate the general pancreas in terms of edema, hemorrhage and fat necrosis. Each aspect was scored from 0 to 3 points and the total score was 9 points. The lower the score, the better the pancreas.

Pancreatic histology score: Kusske criteria were used to evaluate pancreatic histopathological changes from edema, infection, hemorrhage and necrosis. Each aspect was scored from 0 to 4 points and the total score was 16 points. The lower the score, the better the pancreatic tissue.

Determination of the expression levels of NF-κB, ICAM-1 and p-p38MAPK in pancreatic tissues: Western blotting was used to determine the expression levels of NF-κB, ICAM-1 and p-p38MAPK in the pancreatic tissues of rats in each group. Take the pancreatic tissue, homogenise and lyse thoroughly, transfer the lysate to a 1.5 ml Eppendorf (EP) tube, centrifuge, take the supernatant, configure the Bicinchoninic Acid (BCA) working solution, add it to each well and mix well; use a spectrophotometer to determine the protein concentration; heat to denature the protein; prepare the separation gel and concentrated gel; charge the sample; An electrophoresis was performed; Transfer the membrane and seal it with skimmed milk powder. Before scanning and analyzing, incubate the primary antibody at 4°, the secondary antibody at room temperature, the developing solution, and the gel system.

Statistical methods:

In this group of studies, ascites volume, serum and ascites amylase, serum inflammatory factor levels, pancreatic gross score and pancreatic histological score, NF-κB, ICAM-1, p-p38MAPK expression levels and other measurement data were compared with normal distribution. Multi-group comparisons are made by single-factor multi-sample mean comparison and pairwise comparisons are made by independent-sample t-test, which are all expressed as ($\bar{x}\pm s$). In this group of studies, Statistical Package for the Social Sciences (SPSS) 24.0 software package was used for statistical data analysis and the statistical results $p<0.05$ were regarded as statistically significant.

RESULTS AND DISSCUSION

Compared with the sham operation group, the amount of ascites in the model group was significantly increased ($p<0.05$); compared with the model group, the amount of ascites in the pretreatment group was significantly reduced ($p<0.05$) as shown in Table 1.

Compared with the sham operation group, the serum and ascites amylase levels in the model group were significantly increased ($p<0.05$); compared with the model group, the serum and ascites amylase levels in the pretreatment group were significantly decreased ($p<0.05$) as shown in Table 2.

Compared with the sham operation group, the levels of serum IL-6 and TNF-α in the model group were significantly increased ($p<0.05$). Compared with the model group, the levels of serum IL-6 and TNF-α in the pretreatment group were significantly reduced ($p<0.05$). Among them, the level of TNF-α reached the highest 8 h after surgery as shown in Table 3.

In the sham operation group, the structure of the pancreas is clear, inflammatory cells or mild congestion and edema are occasionally seen and the acinar lobules are intact; in the model group, the pancreatic interstitial was obviously congested, edema and even necrotic, with obvious inflammatory cell infiltration and acinar lobular structure disorder and the pathological changes gradually aggravated with time. Pathological changes such as inflammatory cell infiltration in the pancreatic tissue of rats in the preconditioning group were significantly improved compared with the model group.

Compared with the sham operation group, the gross pancreas score and pancreatic histology score of the model group were significantly higher ($p<0.05$); compared with the model group, the gross pancreas scores and pancreatic histology scores of rats in the pretreatment group were significantly reduced ($p<0.05$) as shown in Table 4.

Compared with the sham operation group, the expression levels of NF-κB, ICAM-1 and p-p38MAPK in the pancreatic tissue of the model group were significantly increased ($p<0.05$). Compared with the model group, the expression levels of NF-κB, ICAM-1 and p-p38MAPK in the pancreatic tissue of the pretreatment group were significantly reduced ($p<0.05$) as shown in Table 5.

Acute pancreatitis is a process of acute pancreatic inflammation caused by the imbalance between protective enzymes and stress signals produced by different mechanisms. It is mainly characterized by activation of pancreatic enzymes and secondary local inflammation of the pancreas[4]. The pathogenesis of

acute pancreatitis is more complicated. At present, excessive activation of white blood cells is considered to be the main cause of aggravation, multiple organ failure and even death in patients[5]. Therefore, understanding the pathogenesis of the disease, early diagnosis, timely treatment and inhibiting the excessive release of inflammatory factors are important ways to improve the quality of life and improve the prognosis of patients. PPARγ is a type of ligand-dependent nuclear transcription factor, which can regulate glucose and lipid metabolism, induce macrophage apoptosis and inhibit inflammation. Studies have found that PPARγ agonists may have a negative regulatory effect on the body's inflammatory response by inhibiting the expression of NF-κB[6]. Huang *et al.*[7] found in the study of caerulein-induced pancreatitis that troglitazone pretreatment can significantly reduce serum amylase levels. Reduce tissue damage and inhibit pancreatic inflammation and it is dose-dependent with PPARγ agonists. However, there are few reports on the effect of pioglitazone on acute pancreatitis. This group of research mainly explores the related effects and mechanisms of pioglitazone pretreatment on acute pancreatitis.

Numerous studies at home and abroad have found that inflammatory mediators play an important role in the occurrence and development of acute pancreatitis[8,9]. The imbalance between the body's pro-inflammatory cytokines and anti-inflammatory cytokines is an important factor that causes aggravation of the disease. IL-6 is a lymphokine produced by activated T cells and fibroblasts. It can act on a variety of target cells and stimulate inflammation by regulating mature inflammatory cells. Studies have found that IL-6 levels are significantly related to disease severity[10]. TNF-α is a class of small molecule peptides and an initiating factor in acute pancreatitis. It is considered to be the initiator of the inflammatory response and can be detected in the early stages of the disease[11]. The results of this study found that the degree of inflammation in the model group was severe and pioglitazone can effectively reduce the levels of TNF-α and IL-6, inhibit inflammation and slow down tissue damage.

NF-κB is a nuclear transcription factor, which is necessary for the expression of inflammatory mediator genes. Xie *et al.*[12] found that when inflammation occurs, a large number of activated inflammatory cells participate in it. Inflammatory mediators such as TNF-α, C-Reactive Protein (CRP) and cytokines are all transcripts regulated by NF-κB may be the key to regulating the inflammatory response. Inhibiting the activation of NF-κB in the body can significantly reduce the expression of a variety of downstream pro-inflammatory cytokines and prevent multi-organ damage induced by lipoproteins or cytokines. ICAM-1 is a single-chain transmembrane protein that can be expressed on the surface of a variety of cells. Under normal circumstances, it is slowly expressed on the surface of endothelial cells and epithelial cells. When an inflammatory reaction occurs, the level of cytokines such as TNF-α is stimulated to increase significantly and ICAM-1 will trigger an increase in the number of polymorphonuclear neutrophils and aggravate the inflammatory response[13]. According to related reports, in acute pancreatitis, leukocytes, especially polymorphonuclear neutrophils, migrate from the circulating blood to the inflamed area to accumulate, which is the central link that causes and aggravates pancreatic damage and causes complications in remote organs[14]. The results of this study found that the expression levels of NF-κB and ICAM-1 in the pancreatic tissue of the model group were significantly increased. This may be because NF-κB can regulate inflammatory mediators such as TNF-α, causing a large amount of inflammatory mediators to be released, thereby initiating and expanding the inflammatory cascade and aggravating the development of the disease. Pioglitazone pretreatment can significantly inhibit the expression of NF-κB and ICAM-1.

The p38MAPK signaling pathway is a pathway composed of protein kinase family. Studies have found that when the p38MAPK signaling pathway is activated, it can migrate into the nucleus. And by activating a variety of transcription factors Signal Transducer and Activator of Transcription (STAT) and other transcription factors to enhance its transcription activity, thereby regulating the expression of its downstream target genes TNF-α, IL-6, etc.[15]. Bao *et al.*[16] found in the study of pancreatitis *in vitro* model that the activity of p38MAPK in pancreatic acinar cells was significantly increased during pancreatitis. And produce TNF-α, IL-6 and other cytokines, while the anti-inflammatory effect of NF-κB inhibitors is weak. It is suggested that p38MAPK and NF-κB are the main ways to regulate the production of pancreatic cytokines during the pathogenesis of acute pancreatitis. In addition, Zhu *et al.*[17] further research confirmed that activation of p38MAPK and NF-κB signaling pathways can promote the expression of a variety of pro-inflammatory factors in monocytes/macrophages and p38MAPK may be involved in the activation of NF-κB. The results of this study found that pioglitazone

pretreatment can inhibit the expression of p38MAPK. It shows that inhibiting the activity of the p38MAPK signaling pathway can inhibit the inflammatory response to a certain extent, which may be an effective method for early control of acute pancreatitis.

In summary, pioglitazone pretreatment can inhibit the inflammatory response of acute pancreatitis rats, reduce the level of amylase, inhibit the expression levels of NF-κB and ICAM-1 and inhibit the activity of the ICAM-1 signaling pathway.

TABLE 1: CHANGES IN THE AMOUNT OF ASCITES IN EACH GROUP OF RATS (x̄±s)

Group	Cases	Ascites volume (ml)		
		4 h	8 h	12 h
Mock surgical group	8	0.00±0.00	0.00±0.00	0.00±0.00
Model group	8	4.38±1.02[a]	6.67±1.15[a]	9.95±1.51[a]
Pretreatment group	8	3.41±0.74[ab]	4.56±1.09[ab]	7.37±1.17[ab]
F		2.177	3.767	3.82
p		0.047	0.002	0.002

Note: [a]p<0.05 compared with the sham operation group and [b]p<0.05 compared with the model group

TABLE 2: COMPARISON OF SERUM AND ASCITES AMYLASE IN EACH GROUP OF RATS (x̄±s)

Group	Time	Mock surgical group (n=8)	Model group (n=8)	Pretreatment group (n=8)	F	p
Serum amylase	4 h	555.28±157.35	6583.15±1031.38[a]	5162.46±1466.48[ab]	73.58	<0.001
	8 h	513.46±157.14	6739.62±1285.54[a]	4493.58±1486.25[ab]	61.4	<0.001
	12 h	511.84±165.38	7368.74±1426.48[a]	3438.55±1253.35[ab]	78.2	<0.001
Ascites amylase	4 h	0.00±0.00	52358.47±2548.49[a]	41584.25±12584.59[ab]	2.429	0.029
	8 h	0.00±0.00	58248.24±11584.14[a]	32158.48±12546.58[ab]	4.321	0.001
	12 h	0.00±0.00	71324.58±11265.84[a]	24361.64±9647.44[ab]	8.956	<0.001

Note: ap<0.05 compared with the sham operation group and bp<0.05 compared with the model group

TABLE 3: COMPARISON OF SERUM INFLAMMATORY FACTOR LEVELS IN EACH GROUP OF RATS (x̄±s)

Group	Time	Mock surgical group (n=8)	Model group (n=8)	Pretreatment group (n=8)	F	p
IL-6 (pg/ml)	4 h	386.88±35.87	905.35±242.84[a]	733.05±118.36[ab]	22.53	<0.001
	8 h	384.62±102.48	936.25±158.19[a]	658.68±126.87[ab]	35.37	<0.001
	12 h	396.58±52.07	949.66±228.02[a]	573.57±155.37[ab]	24.28	<0.001
TNF-α (pg/ml)	4 h	70.53±17.35	325.69±66.52[a]	182.58±79.07[ab]	35.76	<0.001
	8 h	83.85±14.12	618.15±84.25[a]	259.82±53.25[ab]	175.6	<0.001
	12 h	69.71±19.82	394.85±57.29[a]	147.32±34.38[ab]	142.28	<0.001

Note: [a]p<0.05 compared with the sham operation group and [b]p<0.05 compared with the model group

TABLE 4: COMPARISON OF PANCREAS GROSS SCORE AND PANCREATIC HISTOLOGICAL SCORE OF RATS IN EACH GROUP (x̄±s)

Group	Time	Mock surgical group (n=8)	Model group (n=8)	Pretreatment group (n=8)	F	p
4 h	4 h	0.46±0.41	7.46±1.24[a]	4.26±1.58[ab]	70.14	<0.001
	8 h	0.16±0.15	6.58±1.02[a]	4.45±1.04[ab]	119.67	<0.001
	12 h	0.12±0.27	7.28±0.87[a]	4.56±0.83[ab]	206.43	<0.001
4 h	4 h	1.24±0.74	11.12±1.85[a]	9.54±1.02[ab]	134.92	<0.001
	8 h	2.45±1.58	12.98±1.47[a]	9.08±0.87[ab]	125.63	<0.001
	12 h	3.28±1.99	13.79±0.86[a]	1.08±0.69[ab]	213.95	<0.001

Note: [a]p<0.05 compared with the sham operation group and [b]p<0.05 compared with the model group

TABLE 5: THE EXPRESSION LEVELS OF NF-KB, ICAM-1 AND P-P38MAPK IN THE PANCREATIC TISSUE OF RATS IN EACH GROUP ($\bar{x}$±s)

Group	Time	Mock surgical group (n=8)	Model group (n=8)	Pretreatment group (n=8)	F	P
NF-κB	4 h	0.22±0.07	0.70±0.17[a]	0.52±0.18[ab]	21.32	<0.001
	8 h	0.17±0.05	1.12±0.20[a]	0.78±0.33[ab]	36.73	<0.001
	12 h	0.16±0.04	1.43±0.34[a]	0.64±0.26[ab]	53.41	<0.001
ICAM-1	4 h	0.20±0.09	0.75±0.28[a]	0.51±0.20[ab]	14.43	<0.001
	8 h	0.23±0.20	0.94±0.32[a]	0.63±0.29[ab]	13.43	<0.001
	12 h	0.15±0.11	1.37±0.55[a]	0.79±0.44[ab]	17.59	<0.001
p-p38MAPK	4 h	0.33±0.06	0.87±0.09[a]	0.72±0.07[ab]	112.34	<0.001
	8 h	0.26±0.05	0.74±0.08[a]	0.63±0.08[ab]	99.19	<0.001
	12 h	0.17±0.03	0.27±0.04[a]	0.22±0.04[ab]	14.63	<0.001

Note: [a]p<0.05 compared with the sham operation group and [b]p<0.05 compared with the model group

Conflict of interests:

The authors declared no conflict of interest.

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