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Original Research Article

Effect of activating blood circulation and removing blood stasis on recurrence of alcoholic acute pancreatitis, and the possible underlying mechanism

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Abstract

Purpose: *To determine the effect of activating blood circulation and removing blood stasis on the recurrence of alcoholic acute pancreatitis (AAP), and its possible mechanism.*
Methods: *Sixty healthy rats were selected and assigned to control, model and treatment groups (n =20/group). Treatment group was given Taohong Siwu decoction, while control and model groups were given abdominal injection of 0.9 % normal saline. Serum levels of amylase, pancreatic histopathological score, col Ⅰ content, mRNA expression levels of TGF-β 1, TβRII, and Smad3, as well as sPLA2 activity, were determined.*
Results: *Serum amylase, pancreatic histopathological score and col Ⅰ content were significantly reduced in treatment group, relative to model group, while relative mRNA expressions of TGF- β 1, TβRII and Smad3 at each time point were significantly lower than those in the model group ($p < 0.05$). Serum PLA2 activity and sPLA2 - π a mRNA expression were significantly lower than those in the model group ($p < 0.05$).*
Conclusion: *Activation of blood circulation and removal of blood stasis significantly prevents the relapse of AAP in rats through regulation of PLA2 activity and TGF-β1/TβRII/Smad3 signaling pathway. Cohort studies in humans would be required to confirm the application of Taohong Siwu decoction to prevent recurrence of AAP.*

Keywords: *Alcoholic acute pancreatitis, Recurrence, Blood circulation, PLA2, Blood stasis*

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INTRODUCTION

Acute pancreatitis (AP) which occurs in young adults, is a common acute critical disease in gastroenterology [1]. Based on the etiological classification, AP is generally divided mainly into alcoholic AP, gallstone AP, familial hyperlipidemic AP, and post-operative AP. In Western countries, the incidence of AP ranks first among all types of pancreatitis [2]. In China, up to 70 – 80 % of AP cases are due to biliary tract diseases and alcohol intake [3]. With improvements in standards of living, the demand for alcohol has increased. excess alcohol intake damages

pancreatic function, resulting in the occurrence of alcoholic acute pancreatitis (AAP) [4]. At present, there is no effective treatment for AAP in clinics [5]. In recent years, the impacts of *activating blood circulation* and *removing blood stasis* method on the treatment of AAP has become increasingly significant. Clinical results have shown that these methods significantly improve the treatment effectiveness and prognosis of AAP. However, the mechanism involved is still unclear. In this investigation, a rat model of AAP rat was used to study the influence of *Huoxue Huayu* injection (*Taohong Siwu* Decoction) on the recurrence of AAP, and the associated mechanism was investigated.

EXPERIMENTAL

Animals

Sixty healthy male rats aged 45 - 55 days, with a mean age of 49.25 ± 3.81 days and mean weight of 191.08 ± 12.35g, were used. The rats were adaptively fed with standard laboratory rat chow for 7 days in a feeding room at 23 ℃. The 60 rats were assigned to control, model, and treatment groups (n = 20/group). This research was approved by the Animal Ethical Committee of Tongde Hospital of Zhejiang Province (approval no. TDH202304) and performed according to the Principles of Laboratory Animal Care [6].

Drugs and equipment

Taohong Siwu Decoction was prepared from a mixture comprising 15 g of *Angelica sinensis*, 15 g of rehmannia, 6 g of safflower, 10 g of *Paeonia alba*, 9 g of peach kernel, 10 g of *Ligusticum chuanxiong* and 65 g of the whole prescription. The mixture was decocted into a paste, ground into powder and dissolved into soup [7]. The equipment used were ultra-clean workbench, high-speed centrifuge, optical microscope, ultra-low temperature refrigerator, microplate reader, and glass slide [8].

Methods

All rats were fasted overnight before the experiment. Rats in the model group and treatment group were treated with 20 % urethane anesthesia (3 mg/kg) [9]. The animals were fixed on a board, and a pe50 medical plastic infusion tube was inserted into the right vein of each rat. The rat abdomen was cleaned and the skin was prepared. The rat skin was disinfected with 75 % alcohol. Under sterile conditions, the 2 mm incision was made on the middle of the abdomen. After opening the abdominal cavity, the duodenum was located. A 5-gauge needle was used to puncture the duodenal wall on the opposite side of the pancreaticobiliary opening, and a 0.5 cm intravenous in-dwelling needle was inserted into the biliopancreatic duct. After connecting the syringe, 2.0 % sodium taurocholate (0.1 mL/100 g) was injected retrogradely. During the operation, the pancreaticobiliary duct was temporarily clamped with a vascular clamp in order to make for bile outflow. Congestion and edema in pancreatic tissue were examined. Then, the vascular clip was removed, the seromuscular layer of the duodenal puncture port and the skin incision was sutured, and the abdominal cavity was closed [10].

The procedure for the control group was the same as described above, but the injection liquid was replaced with an equal volume of 0.9 % normal saline. Following the rat model establishment, animals in the treatment group were given *Taohong Siwu* Decoction at one-tenth of the human drug dose. The model group and the control group were injected with an equivalent volume of 0.9 % normal saline. Four rats were taken from each of the three groups at 3, 6, 12, 24 and 48 h after establishment of the model. Abdominal aortic blood (1 mL) was drawn, and the serum was separated after centrifugation for 10 min. It was stored at - 50 °C. After 6 h, part of the abdominal tissue was subjected to H & E staining, while the remaining specimens were stored at - 50 °C prior to subsequent use.

Evaluation of indices

Routine observation

Rats in each group were closely monitored for activity, mental condition, appetite, and secretion.

Assay of serum amylase

Serum amylase was assayed using an automatic Biochemical Analyzer. The higher the serum amylase level, the higher the possibility of pancreatitis.

Pathological changes in pancreatic tissue

The pancreatic tissue of each group was fixed in formaldehyde, embedded in paraffin, and sliced into 3 μm sections. The specimens were routinely stained with H & E, and finally sealed. The stained sections were studied under a light microscope, and pathological changes in intestinal mucosal tissues were photographed. The histopathological scores were evaluated with reference to the modified Schmidt method. Edema scores were 0 for no swelling; 1 point for

mild widening of lobar space; 2 points for severe widening of lobar space; 3 points for widening of acinar space, and 4 points for widened intercellular space.

The scores for necrosis area were 0 points for dead; 1 point for necrosis area of 1 - 10 %; 2 points for necrosis area of 11 – 20 %; 3 points for necrosis area of 21- 30 %, and 4 points for necrosis area in excess of 30 %.

Inflammatory cell infiltration was scored 0 - 1 point if perivascular leukocyte count was 0; 1 point for perivascular leukocyte count of 2 - 10; 2 points for perivascular leukocyte counts of 11 - 20; 3 points for perivascular leukocyte counts of 21 - 30, and 4 points for perivascular leukocyte counts in excess of 30 [11].

Col Ⅰ level

The level of col 1 in pancreatic tissue was determined using ELISA**.** Pancreatic tissue (80 mg was lysed, and 1 mL of the lysate was used to measure col 1 with ELISA kit in line with the kit manual guidelines.

mRNA expressions of TGF-βR1, TβRII and Smad3

Total mRNA was extracted from 80 mg of pancreatic tissue using TRIzol reagent, and the RNA was reverse-transcribed to cDNA, followed by PCR amplification of the cDNA using SYBR (40 cycles for 30, 5 and 30 sec at 95^{o}C; and 30 sec at 75^{o}C, and then 95^{o}C for 60, 30 and 5 sec, and 75^{o}C for 30 sec). Data on mRNA copies were re-computed per microgram of total RNA, and quantification was done using cycle threshold procedure, with β-actin as housekeeping gene. The relative expression levels of the target genes were calculated with $2^{-\Delta\Delta CT}$ method. The sequences of primers used were shown in Table 1.

Assay of sPLA2 activity

The activity of sPLA2 in serum was determined using sPLA2 kit.

Statistical analysis

Statistical Package for Social Sciences (SPSS) 23.0 was used for statistical analysis of data. All measurement data are expressed as mean ± standard deviation (SD). One-way analysis of variance was used for multi-group comparison. Count data are expressed as numbers and percentages, and SNK method was used to compare the mean number of multiple samples. Differences were considered statistically significant *at* $p < 0.05$.

RESULTS

Serum amylase activity after model establishment

Serum amylase activity in the model group peaked at 6 h after the establishment of rat model of acute pancreatitis and thereafter decreased continuously at 12, 24 and 48 h. Serum amylase level in model group at each time point was significantly increased, relative to control. However, serum amylase activity in treatment group decreased continuously from 3 to 48 h after model establishment, and the activity at each time point was significantly lower than that of model group ($p < 0.05$). These results are presented in Table 2.

Table 1: Sequences of primers used

Gene	Primer sequence (Forward) 5’ - 3’	Primer sequence (Reverse) 5’ - 3’
TGF-β	CTCAACACCTGCACAGCTCC	AGTTGGCATGGTAGCCTTG
TβRII	CCCTACTCTGTCTGTGGATGA	GACGTCATTTCCCAGAGTAC
Smad3	AGCGAGTTGGGAGACAT	AGTTGGGAGACTGGACGA
β-Actin	TTGTATAACCAACTGGGACGATATGG	GACTTGATCTTCATGGTGCTAGG

Table 2: Comparison of serum amylase activity of the 3 groups at various time-points (U/L, n=20)

Group	3h	6h	12h	24h	48h
Treatment	5308.1±401.0	4171.1±456.5	3336.3±320.5	2002.3±272.5	1436.8±160.5
Model	6127.6±458.3^{a}	7260.3±480.7^{a}	4204.1±438.7^{a}	2725.3±293.8^{a}	2973.0±235.7^{a}
Control	2406.1±210.2^{ab}	1438.7±127.6^{ab}	1438.7±124.3^{ab}	1432.7±124.3^{ab}	1387.8±110.1^{ab}
F	552.81	1116.85	386.36	143.05	999.53
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

$^{a}P < 0.05$, compared with treatment group; $^{b}p < 0.05$, compared with model group

Pancreatic histopathological scores

Table 3 shows that histological scores were consistently higher in model rats than in control rats, but at each time-point, rats in the treatment group showed significantly lower scores than model rats ($p < 0.05$).

Levels of col I after the establishment of acute pancreatitis model

The col I content in model group peaked at 6 h, decreased significantly at 12 h, and increased at 24 and 48 h. Compared with control group, model rat col Ⅰ content at each time point was significantly raised ($p < 0.05$). For rats in treatment group, col 1 content peaked at 6 h, decreased at 12 h, increased slightly at 24 h, and decreased again after 48 h. However, rats in treatment group had significantly decreased col Ⅰ levels, relative to model rats ($p < 0.05$). These data are presented in Table 4.

mRNA levels of TGF-B 1, TBRII and SMAD3

The mRNA expressions of TGF- β1, TβRII and Smad3 in model group were increased at 6 h, decreased at 12 h, and increased at 24 h and 48 h. The relative mRNA expressions of TGF β 1, TβRII and Smad3 in model group were significantly elevated at each time point, relative to control ($p < 0.05$). However, the relative mRNA expressions of TGF β 1, TβRII and Smad3 in the treatment group were significantly decreased ($p < 0.05$; Table 5).

Table 3: Pancreatic histopathological scores at each time-point for the three groups (n = 20)

Group	3h	6h	12h	24h	48h
Treatment	2.66±0.37	2.82±0.47	3.82±0.62	4.12±0.53	4.38±0.55
Model	3.66±0.60[a]	3.82±0.29[a]	5.82±0.29[a]	5.94±0.26[a]	6.21±0.23[a]
Control	0.65±0.20[ab]	0.82±0.15[ab]	1.15±0.27[ab]	1.33±0.25[ab]	1.35±0.30[ab]
F	262.62	427.48	605.79	787.07	811.62
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[a]$P < 0.05$, compared with the treatment group; [b]$p < 0.05$, compared with the model group

Table 4: Comparison of col I content of three groups of rats at each time point after model establishment (pg/g, n = 20)

Group	3h	6h	12h	24h	48h
Treatment	58.6±6.9	85.1±10.6	73.4±11.2	77.2±12.2	37.3±4.1
Model	63.6±8.8[a]	169.1±29.1[a]	85.1±19.4[a]	187.3±20.6[a]	215.6±35.2[a]
Control	20.0±1.6[ab]	23.1±3.1[ab]	14.2±2.1[ab]	17.3±1.4[ab]	17.4±1.2[ab]
F	267.68	332.54	171.24	2801.96	568.45
P-value	<0.05	<0.05	<0.05	<0.05	<0.05

[a]$P < 0.05$, compared with the treatment group; [b]$p < 0.05$, compared with the model group

Table 5: The mRNA expressions of TGF- β1, TβRII and Smad3 in each group (n = 20)

Group	Time-point (h)	TGF-β1 mRNA	TβRⅡ mRNA	Smad3 mRNA
Treatment	3	0.82±0.13	0.29±0.04	0.91±0.13
	6	1.83±0.11[a]	1.88±0.26[a]	2.73±0.11[a]
	12	1.24±0.20[ab]	1.34±0.21[ab]	2.13±0.26[ab]
	24	1.63±0.22[abc]	1.84±0.24[ac]	2.85±0.43[ac]
	48	1.74±0.26[ac]	1.24±0.25[abd]	1.20±0.18[abcd]
F		94.85	176.92	245.20
P-value		< 0.05	< 0.05	< 0.05
Model	3	0.85±0.14	0.31±0.04	1.09±0.14
	6	2.44±0.34[a]	2.14±0.34[a]	3.40±0.43[a]
	12	1.23±0.23[ab]	0.86±0.18[ab]	1.73±0.36[ab]
	24	3.03±0.43[abc]	3.17±0.42[abc]	5.23±0.64[abc]
	48	3.63±0.52[abcd]	3.26±0.53[abc]	5.43±0.69[abc]
F		215.59	293.22	319.56
P-value		< 0.05	< 0.05	< 0.05
Control	3	0.43±0.07	0.21±0.06	0.40±0.07
	6	0.33±0.06[a]	0.17±0.05[a]	0.33±0.06[a]
	12	0.22±0.05[ab]	0.22±0.05[b]	0.31±0.06[a]
	24	0.18±0.02[abc]	0.20±0.03	0.37±0.07[c]
	48	0.19±0.04[ab]	0.23±0.04[b]	0.40±0.06[bc]
F		88.85	4.77	8.11
P-value		< 0.05	< 0.05	< 0.05

[a]$P < 0.05$, compared with 3h; [b]$p < 0.05$, compared with 6h; [c]$p < 0.05$, compared with 12h; [d]$p < 0.05$, compared with 24h

Table 6: Comparison of sPLA2 activity of rats in groups after modeling

Group	Serum PLA2 Activity (μMol /min/L)	SplA2 - IIa Mrna/β-Actin grayscale ratio
Treatment	474±49	1.32±0.26
Model	706±47[a]	1.85±0.10[a]
Control	580±57[ab]	1.59±0.12[ab]
F	102.99	45.80
P-value	<0.05	<0.05

[a]$P < 0.05$; compared with the treatment group, [b]$p < 0.05$, compared with the model group

SPLA2 activity after model establishment

There were significantly higher serum PLA2 activity and sPLA2-IIa mRNA levels in model rats than in control rats following model establishment, with a significant increase in grayscale level relative to β-actin ($p < 0.05$). However, serum PLA2 activity and sPLA2-IIa mRNA in treatment group were significantly reduced, relative to values in model rats, with lower grayscale levels, relative to β-actin ($p < 0.05$; Table 6).

DISCUSSION

With improvements in the quality of life of people, demand for alcohol is on the increase, and this has resulted in significant increases in the incidence of alcoholic pancreatitis. Research has shown that the incidence of acute pancreatitis is about 12.9 % [12]. Sustained heavy drinking directly damages the pancreas and stimulates secretion of a large amount of pancreatic juice and digestive enzymes from gastric juice, pancreatic juice and duodenum. These secretions cause spasms of the sphincter of Oddi, edema of duodenal papilla, and obstruction of the pancreatic duct, leading to the occurrence of AAP.

In addition, the destruction of epithelial cells by pancreatic enzymes increases the levels of Ca^{2+} and protein in pancreatic juice, a situation that further destroys acinar tissue, resulting in inflammation and fibrosis [13]. There are two pathways of alcohol metabolism in the pancreas: oxidative and non-oxidative, with non-oxidative being the major route. The oxidative pathway depends mainly on alcohol dehydrogenase (ADH) and cytochrome P450 2E1.

Taohong Siwu Decoction, a popular TCM formulation used for activating blood circulation and removing blood stasis, has been widely used in clinics. The present study was focused on the influence of *Taohong Siwu* Decoction on the recurrence of AAP, and its possible mechanism [15]. In the treatment group, serum amylase level was gradually decreased until it returned to normal, and the pancreatic histopathological score and col Ⅰ content were also significantly decreased. At the same time, after treatment, the relative molecular expressions of TGF- β1, TβRII, and Smad3 at each time point were significantly decreased, relative to the value in model rats, indicating that *Taohong Siwu* Decoction inhibited the TGF-β1/smad3 signaling pathway. Hyperactivation of this pathway not only increases pancreatic fibrosis, but also leads to organ fibrosis, as TGF-β1 is a multifunctional cytokine which is closely related to fibrosis and is involved in the occurrence of pancreatitis [16]. In addition, sPLA2 index (serum PLA2 activity and sPLA2- IIa mRNA/ β-actin grayscale ratio) was significantly reduced, suggesting that the use of *Taohong Siwu* Decoction to enhance blood circulation and *remove blood stasis* may effectively reduce sPLA2. It can be seen that this method of *activating* blood circulation and *removing blood stasis* may significantly restore pancreatic microcirculation and perfusion, effectively inhibit pancreatic secretion, reduce pancreatic duct pressure, and reduce acinar damage. During ethanol-induced damage to tissue cells or inflammation, TGF- β1 and ECM are released from cells so as to protect pancreatic tissue. The signal transduction protein in the signal pathway, i.e., TGF-β Smad, plays an important role in signal transduction, and it promotes mRNA expression. At the same time, drug research has found that safflor yellow in safflower, ligustrazine in *Ligusticum chuanxiong*, and paeoniflorin in red peony, block the anabolic pathway of PAF and inhibit its generation. Through the sPLA2-catalyzed biosynthesis of PAF, blood-activating and stasis-removing drugs effectively reduce sPLA2, thereby improving pancreatic microcirculation and preventing the recurrence of AAP while enhancing recovery from pancreatitis.

CONCLUSION

The use of *Taohong Siwu* decoction for activating *blood circulation* and removing *blood stasis* significantly mitigates acute pancreatitis and reduces the possibility of recurrence of AAP. *Taohong Siwu* decoction may be clinically used to prevent the recurrence of AAP. However, it requires cohort studies to confirm this.

DECLARATIONS

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Ethical approval

This work was approved by the Animal Ethical Committee of Tongde Hospital of Zhejiang Province, China (approval no. TDH202304).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Shifeng Zhu conceived and designed the study. Weixing Ying collected and analyzed the data. Haicheng Dong wrote the manuscript.

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