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Research Article

Effects of Flos Carthami on the aquaporin 1 of acute necrotizing pancreatitis rats

Lu Wang[1]

[1]*Inner Mongolia Medical University, Hohhot, Inner Mongolia, 010110, P. R. China*

OBJECTIVES: To clarify the effects of Flos Carthami on the aquaporin 1 (AQP1) of acute necrotizing pancreatitis (ANP) rats.

METHODS:60 male SD rats were randomly divided into a control group, an ANP group, a 0.9% normal saline (NS) group and a Flos Carthami group. 5 rats in each group were killed 3h, 6h and 12h after establishing the model. The contents of serum amylase and AQP1 were determined. The pancreatic tissues that were subjected to HE staining were graded by the Schmidt method. The capillary permeability was measured by Evans blue (EB) dye extravasation. The expression of AQP1 was measured by immunohistochemical methods.

RESULTS: The ascitic fluid amounts of the Flos Carthami group were significantly lower than those of the ANP group ($P<0.05$). The serum amylase levels of the ANP group at each time interval were higher than those of the control group ($P<0.01$). The pancreatic tissue EB contents of the ANP group were significantly higher than those of the control group ($P<0.05$). The AQP1 contents of the ANP group were lower than those of the control group ($P<0.05$). Compared to the ANP group, Flos Carthami significantly lowered the levels of serum amylase ($P<0.05$), pancreatic tissue EB ($P<0.05$) and AQP1 ($P<0.05$). The pretreatment of Flos Carthami mitigated the symptoms of ANP rat model, and reduced the capillary permeability, which may be associated with the up-regulated expression of AQP1.

Keywords: acute necrotizing pancreatitis; Flos Carthami; pancreatic aquaporin 1; capillary extravasation

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Introduction

Severe acute pancreatitis (SAP), a common surgical disease with multiple complications and high mortality rates, accounts for 10%-20% of acute pancreatitis [1]. The overall mortality rate of SAP is still high up to approximately 17% despite the surgical treatment development [2]. SAP induces pathological changes including pancreatic tissue edema, hemorrhage and necrosis by increasing the vascular permeability, leading to considerable exudation that may involve many organs [3]. The aquaporin 1 (AQP1) of organism capillary endothelial cells participates in the changes of capillary permeability and the vascular water transport [4]. Traditional Chinese medicine tends to treat SAP by ascribing it to abdominal angina that results from blood stasis, which can be cured by recirculating the blood [5]. Flos Carthami, one of the common blood circulation-activating traditional Chinese drugs [6], is able to obviously dilate blood vessels, mitigate microcirculation disorders, reduce capillary permeability, and inhibit the releases of thromboxane A2 (TXA2), platelet-activating factor (PAF) and 5-hydroxytryptamine, etc. [7,8]. In this study, we

* Correspondence: Lu Wang. Inner Mongolia Medical University, Hohhot, Inner Mongolia, 010059, P. R. China. Tel: 86-4713451717. E-mail:WangLuhhs@163.com

dynamically observed the effects of Flos Carthami on acute necrotizing pancreatitis (ANP) treatment and AQP1 using an experimental animal model.

Time	Control	ANP	NS	*Flos Carthami*
3 h	0	5.21±1.39	4.81±0.26	3.49±1.25#
6 h	0	7.19±1.42	6.21±1.30	3.78±1.49△
12 h	0	7.01±2.90	5.93±0.37	3.69±1.11#

Table 1. Changes of ascites amounts ($\bar{x}$ ±s, n=6, mL)

Compared to the ANP group, #P<0.05, △P<0.01.

Materials and Methods

Experimental animals and grouping

60 sterile healthy SD rats weighing 190-230 g were provided by the Animal Experiment Center of our institution. The rats were divided into a control group, an ANP group, a 0.9% saline (NS) group and a Flos Carthami group (15 rats each) by the random number method.

Reagents and apparatuses

The reagents and apparatuses used in this study include: Flos Carthami (Sinopharm Group Co., Ltd., place of origin: Jinxiang Province), natrium taurocholicum (Shanghai Heng Yuan Biotechnology Co., Ltd.), Evans blue (EB) (Aladdin),

PUZS-300 automatic biochemical analyzer (Perlong Medical Equipment Co., Ltd.), 752 UV spectrophotometer (Nanjing Qilin Analyzer Instrument Co., Ltd.), pathological section cutter (SLEE, Germany), pathological dying machine (Changzhou Centralpower Electronic Co., Ltd.), AQP1 rabbit anti-mouse polyclonal antibody (Chemicon, USA), ELISA kit (R&D, USA), S-415 cell and tissue total protein extraction kit (Sangon), and High Resolution Cytologic Image Analysis System (Wuhan Champion Image Technology Co., Ltd.).

Methods

Decoction containing 10g/kg Flos Carthami was prepared daily. The dosage for rats was calculated according to the equation dB=dA×RB/RA×(WA/WB)1/3, where dA and dB are the dosages of human and rat per kilogram body mass (mg/kg), RA and RB are the shape coefficients of human and rat (RA = 100, RB = 59), and WA and WB are the body masses of human and rat (kg). 60 kg and 200 g were utilized as the standard body masses of human and rat, respectively.

Model establishment and sample preparation

The rats were fasted 12 h before experiment without being abstained from water. They were then intraperitoneally injected with 2.5% pentobarbital for anesthesia, and the pancreatic duct hepatic portal was clipped horizontally by a noninvasive vascular clamp. Then they were injected with 5% natrium taurocholicum based on 0.1 mL/100 g body mass. The vascular clamp and the puncture needle were removed 10 min later, and the duodenum puncture hole was sutured. The ANP model was eventually established after continuously suturing the abdominal wall. The NS group and the Flos Carthami group were perfused with 2 mL of 0.9% saline and Flos Carthami decoction 48 h, 24 h and 2 h before modeling, and the models were established similarly to the ANP group. The control group merely underwent gentle duodenum and pancreata movement after abdomen open. The rats were executed 3 h, 6 h and 12 h after modeling (5 rats at each time interval). 1 h before execution, the rats were intravenously injected (penis) with 2% EB by the ratio of 0.1 mL/100 g body mass. 1 h later, the rats were intraperitoneally injected with 2.5% pentobarbital for anesthesia. Thereafter the ascites and abdominal aortic blood were sampled after cutting in the middle of abdomen that had been sterilized by benzalkonium bromide. The amount of ascites was recorded, and the pancreatic tissue samples were collected

Time	Control	ANP	NS	*Flos Carthami*
3 h	1304±734	9083±2159 #	8013±2179 #	4386±4723 △△
6 h	1065±512	8801±1585 #	8717±1403 #	5190±3927 △
12 h	1314±757	9402±1487 #	9072±776 #	6152±3693 △△

[9].

Table 2. Pathological grading at different time intervals ($\bar{x}$±s, n=6)

Compared to the ANP group, #P<0.01.

Determinations of serum amylase and AQP1

The contents of serum amylase and AQP1 were determined by the enzymatic method and the ELISA method, respectively.

Pathological observation

The pancreatic tissues were fixed in routine neutral formalin and embedded by paraffin, which were sliced into 4 μm sections that were subjected to HE staining. The pathological changes were observed under an optical microscope and graded according to the severity.

Determination of capillary permeability

The pancreatic tissue capillary permeability was determined by the EB staining method. EB binds to the albumin in blood after entering blood vessels, which will be extravasated to tissue interspaces with increasing vascular permeability. Thus, capillary permeability can be reflected by measuring the EB content in tissues [10]. Methods: 1 mL of methanamide solution was added to 300 mg tissue sample, and the resulting solution was incubated at 60°C for 24 h and then centrifuged at 2000 g/min for 30 min, from which the supernatant was collected. The OD values were recorded by determining the UV absorbances at 620 nm. Standard curves were plotted by the known EB value. Then the EB contents were calculated according to the OD values and the standard curves [11].

Time	Control	ANP	NS	*Flos Carthami*
3 h	0	6.34±0.63	6.12±1.01	3.37±0.61#
6 h	0	6.32±1.05	6.26±0.51	4.30±0.72#
12 h	0	7.29±0.80	7.05±1.02	5.51±0.62#

Table 3. Serum amylase levels ($\bar{x}$±s, n=6, U/L)

Compared to the control group, #P< 0.01.

compared to the ANP group, △P< 0.05, △△P< 0.01.

Determination of AQP1 expression by immunohistochemical analysis [12]

The expression of AQP1 was analyzed by an Elivision plus broad spectrum kit. AQP1 is mainly expressed in the membrane of capillary endothelial cells, and that of the positive cells is distributed with diffusive brown-yellow or brown particles. The immunohistochemical staining results were transformed into computer data for quantitative analysis by the High Resolution Cytologic Image Analysis System. The three microscopic fields of vision with the highest positive rates of each section were selected to

determine the interspace gray scale and the positive tissue gray scale. Utilizing interspace gray scale as the standard, the net gray scales of three fields of vision were averaged and used as the experimental value: net gray scale = background gray scale - positive gray scale [13].

Statistical analysis

All data were expressed as $\bar{x}\pm s$ and analyzed by SPSS 17.0. The differences between groups were analyzed by one way ANOVA. $P<0.05$ was considered statistically significant.

Table 4. Capillary permeability (EB content) changes ($\bar{x}\pm s$, n=6, μg/mL)

Compared to the control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Compared to the ANP group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$.

Results

Changes of ascites

No ascites was found in the control group at each time interval. Highest amounts of pale-yellow or red bloody ascites were found in the ANP group 6h after modeling, and the ascites amounts of the Flos Carthami group 3 h, 6 h and 12 h after modeling were significantly lower than those of the ANP group (Table 1).

Pancreatic pathological changes

No obvious pathological changes were observed in the control group. The ANP group were subjected to discernible pale-yellow or red bloody ascites, lipid saponification, pancreas swelling, extensive hemorrhage and necrosis. The Flos Carthami group underwent less pathological changes than the ANP group did. Pathological changes under an optical microscope: The pancreatic tissues of the control group were clearly structured with complete acinar leaflets, mild interspace hyperemia and edema, and occasional inflammatory cell infiltration. The acinar leaflets of the ANP group were ill-defined, and the pancreatic tissues underwent considerable hemorrhage and necrosis, interspace edema, and obvious inflammatory cell infiltration. The pathological changes of the NS group were similar to those of the ANP group, and the changes of the Flos Carthami group were less severe than those of the ANP group (Figure 1). The pathological grading is listed in Table 2.

Table 5. Immunohistochemical grayscale values of AQP1 expression ($\bar{x}\pm s$, n=6)

Time	Control	ANP	NS	*Flos Carthami*
3 h	206.16±33.04	272.29±24.14$^{\#\#}$	267.17±25.98$^{\#}$	214.65±60.09$^{\triangle}$
6 h	140.89±26.87	252.76±30.91$^{\#\#}$	256.25±41.42$^{\#\#}$	161.75±68.12$^{\triangle\triangle}$
12 h	97.05±26.71	220.96±32.12$^{\#\#}$	231.08±44.97$^{\#\#}$	120.21±54.76$^{\triangle\triangle}$

Compared to the control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$;

Compared to the ANP group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$.

Contents of serum amylase

The serum amylase changes are summarized in Table 3. The serum amylase levels of the ANP group and the NS group are higher than those of the control group, and the amylase levels of the Flos Carthami group were significantly lower than those of the ANP group ($P < 0.01$).

Time	Control	ANP	NS	*Flos Carthami*
3 h	114.61±8.01	79.25± 14.67$^{\#\#}$	90.17± 4.98$^{\#\#}$	113.25± 7.69$^{\triangle\triangle}$
6 h	121.46±8.18	110.25± 4.08$^{\#\#}$	113.25± 5.42$^{\#}$	124.33± 4.75$^{\triangle\triangle}$
12 h	145.94±6.83	104.27± 10.83$^{\#\#}$	95.08± 15.97$^{\#\#}$	141.53± 7.63$^{\triangle\triangle}$

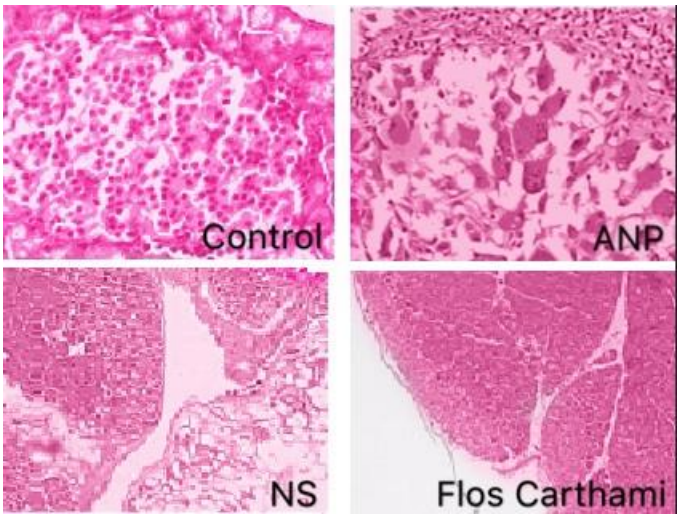

Fig. 1 Pathological changes under the optical microscope (HE)

Capillary permeability

The pancreatic tissue capillary permeability changes are listed in Table 4. The EB contents of the ANP group and the NS group at each time interval are all significantly higher than those of the control group, and the contents of the Flos Carthami group are significantly lower than those of the ANP group ($P<0.05$).

AQP1 expression

The AQP1 expression of the ANP group was significantly down-regulated compared to that of the control group ($P< 0.05$), and the expression of the Flos Carthami group was significantly up-regulated compared to that of the ANP group ($P< 0.05$). At each time interval, the positive AQP1 expression and the grayscale values of the ANP group and the NS group are significantly lower than those of the control group ($P<0.01$), and the values of the Flos Carthami group are significantly higher than those of the ANP group ($P<0.01$) (Figure 2 and Table 5).

Discussion

The non-surgical treatment methods at the early stage

of SAP include timely and sufficient fluid resuscitation, pancreatic rest and protection, gastric tract function improvement, and pathogenic factor elimination, etc. [14]. In China, SAP is mainly treated by combining traditional Chinese and Western medicine [15]. Traditional Chinese medicine tends to treat SAP by circulating flatulence and preventing liver-Qi stagnation syndrome, thereby eliminating the symptoms of nausea and vomiting, abdominal distention and constipation, comprehensively repairing spleen and gastric damages, and restoring normal functions [16].

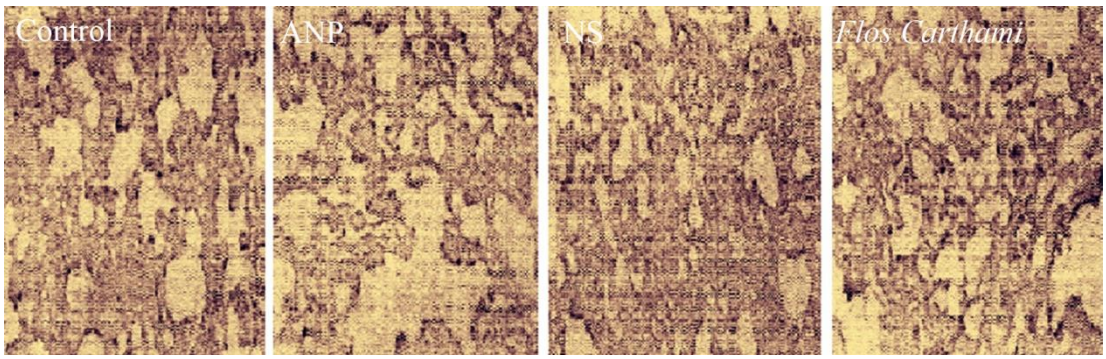

Fig. 2 AQP1 expression (immunohistochemical analysis)

Early-stage SAP has been effectively treated with Flos Carthami [17], which apparently shortened the time of abdominal pain and abdominal distention, alleviated tissue edema [18], and remarkably reduced the incidence of complications. In animal experiments, clinical mild SAP is commonly simulated by acute edema pancreatitis model [19], and SAP is simulated by ANP [20]. In this study, a rat ANP model was established by injecting 5% natrium taurocholicum through the common bile duct [21]. The results show that the pathological changes of pancreatic tissue after modeling were obviously mitigated, the amounts of ascites were reduced, and the serum amylase levels were lowered by pretreating the ANP model with Flos Carthami decoction [22]. The results indicate that Flos Carthami can effectively prevent and treat ANP, which is consistent with those reported by Zhu et al. [23].

In this study, the pancreata EB contents of the ANP group were significantly higher than those of the control group 3 h after modeling ($P < 0.01$), suggesting the significantly elevated capillary permeability. The results are in accordance with the considerable edema and ascites formation hinted by the pathological detection [24]. The significantly lower EB contents of the Flos Carthami group than those of the ANP group, inferring that Flos Carthami effectively avoided capillary extravasation. The immunohistochemical analysis indicates that Flos Carthami could reversely up-regulate the expression of AQP1, facilitate the water transport, promote the absorption of tissue edema, and mitigate the relevant symptoms.

In summary, the pretreatment utilizing Flos Carthami could prevent the pathological changes of ANP rat pancreatic tissues and decrease the capillary permeability, which may be associated with the up-regulated AQP1. The mechanisms concerning the effects of AQP1 on Flos Carthami-treated ANP capillary extravasation will provide experimental evidence for clarifying the treatment efficacy of traditional Chinese medicine.

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Conflict of interest statement

I declare that I have no financial and personal relationships with other people or organizations that can inappropriately influence my work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled. I confirm that the mentioned received grants in the "Acknowledgement" section, did not lead to any conflicts of interest regarding the publication of this manuscript.

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