• BASIC RESEARCH •

Protective effects of rhubarb on experimental severe acute pancreatitis

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Received: 2003-10-10   **Accepted:** 2003-12-06

Abstract

AIM: To investigate the effects of rhubarb on severe acute pancreatitis (SAP) in rats.

METHODS: Severe acute pancreatitis was induced by two intraperitoneal injections of cerulein (40 µg/kg body weight) plus 5-h restraint water-immersion stress. Rhubarb (75-150 mg/kg) was orally fed before the first cerulein injection. The degree of pancreatic edema, serum amylase level, local pancreatic blood flow (PBF), and histological alterations were investigated. The effects of rhubarb on pancreatic exocrine secretion in this model were evaluated by comparing with those of somatostatin.

RESULTS: In the Cerulein+Stress group, severe edema and diffuse hemorrhage in the pancreas were observed, the pancreatic wet weight (11.60±0.61 g/Kg) and serum amylase (458 490±43 100 U/L) were markedly increased ($P<0.01$ *vs* control). In the rhubarb (150 mg/kg) treated rats, necrosis and polymorphonuclear neutrophil (PMN) infiltration in the pancreas were significantly reduced ($P<0.01$), and a marked decrease (50%) in serum amylase levels was also observed ($P<0.01$). PBF dropped to 38% (93±5 mL/min per 100 g) of the control in the Cerulein+Stress group and partly recovered in the Cerulein+Stress+Rhubarb 150 mg group (135±12 mL/min per 100 g) ($P<0.01$). The pancreatic exocrine function was impaired in the SAP rats. The amylase levels of pancreatic juice were reduced in the rats treated with rhubarb or somatostatin, comparing with that of untreated SAP group. The bicarbonate concentration of pancreatic juice was markedly elevated only in the rhubarb-treated group ($P<0.01$).

CONCLUSION: Rhubarb can exert protective effects on SAP, probably by inhibiting the inflammation of pancreas, improving pancreatic microcirculation, and altering exocrine secretion.

INTRODUCTION

Acute pancreatitis is a commonly occurring disease with self-limited course and uneventful recovery. But it can present as a severe form with significant morbidity and mortality. Up to now, the pathogenesis of acute pancreatitis still remains poorly understood and the treatment still remains nonspecific and primarily supportive. Rhubarb, a traditional Chinese herbal medicine, has been widely used in China for the treatment of many diseases. It has also been shown to have a good curative effect on acute pancreatitis in recent years[1,2]. However, fewer experimental data are available to reveal its possible mechanisms. In the present study, we investigated the effects of rhubarb on severe acute pancreatitis (SAP) in rats, and tried to elucidate the possible mechanisms of its clinically well-known therapeutic effect.

MATERIALS AND METHODS

Materials

Cerulein was purchased from BACHEM Co., Ltd. (Switzerland). The tablets of rhubarb were kindly provided by professor Dong-Hai Jiao (Shanghai Xiangshan Chinese Medicine Hospital), which is the ethanol extract from crude rhubarb. The main chemical constituents of rhubarb tablets include emodin, aloe-emodin, rhein, d-catechin, and gallic acid. Sandostatin (somatostatin) is purchased from Novartis Pharma (Switzerland) and Secretin from Eisai Co., Ltd. (Japan). Male Sprague-Dawley rats (provided by the animal center of PUMC Hospital) weighing 350-450 g were used in these studies. They were fed standard laboratory chow and tap water *ad libitum* and housed in cages in a temperature-(22±2 ℃) and humidity-(55±5%) controlled room, with a 12-h light cycle before experimentation.

Protocol I: effects of rhubarb on SAP

Animal models and experimental design SAP was induced by two intraperitoneal injections (*ip*) of cerulein (40 µg/kg body weight) at 1-hr interval and then the rats were immersed in 22 ℃ water to the level of the xiphoid in restraint cages for 5 h[3] (stress). Rhubarb (75 or 150 mg/kg body weight) was orally administered (*po*) twice, 2 and 15 h before the first cerulein injection. The doses of rhubarb were selected according to our preliminary study. Experiments were performed 5 h after the first cerulein injection because previous data had shown that at that time cerulein-induced pancreatitis in the pancreas were the most severe[4]. The time course of cerulein-induced pancreatitis had been confirmed in our early study[5].

Thirty-four rats were randomly divided into five groups and treated as shown below: Control group: normal saline (NS) *po*+*ip*, without stress (*n*=6); Rhubarb group: rhubarb (150 mg/kg) *po*+NS *ip*, without stress (*n*=6); Cerulein+Stress group: NS *po*+cerulean *ip*, with stress (*n*=9); Cerulein+Stress+Rhubarb 75 mg group: rhubarb 75 mg/kg *po*+cerulein *ip*, with stress (*n*=6); Cerulein+Stress+Rhubarb 150 mg group: rhubarb 150 mg/kg *po*+cerulein *ip*, with stress (*n*=7).
Microcirculation in pancreas Local Pancreatic blood flow (PBF) was measured with a hydrogen gas clearance technique prior to sacrifice[6,7]. All animals were anesthetized with an

intraperitoneal pentobarbital injection (40 mg/kg body weight). Body temperature was monitored by a rectal thermometer and kept at 36-37 ℃ with a heating lamp over the abdomen. The pancreas was exposed by laparotomy and then a urethane-coated platinum electrode, 80 μm in diameter with a 0.5 mm portion uncoated at its tip, was inserted into the pancreatic duct through an opening at proximal end of the duct and its tip was kept at 2.5 cm away from the duodenum. The incision was covered with a piece of wet gauze to avoid the loss of body warmth and fluid. The rats were then ventilated with 10% hydrogen in air through a nasal tube. Platinum catalyzes the oxidation of gaseous hydrogen to hydrogen ions. The process is accompanied by a release of electrons, which can be measured as current. For measuring the changing rate of the tissue hydrogen concentration, the blood flow can be determined and calculated by using the following formula: blood flow (mL/min/100 g pancreas)=100 (É.) 0.693/$T_{1/2}$. PBF value was directly provided using a MHG-Dl (Unique Medical Co., Tokyo, Japan) with a built-in computer.

Serum amylase level and pancreatic wet weight After measuring PBF, a blood sample was collected from the vena cava to determine serum amylase level by Phadebas amylase test[8]. After decapitation, the pancreas was quickly removed, trimmed of fat and lymph nodes, and weighed to determine the severity of pancreatic edema, expressed as grams pancreatic wet weight per kilogram body weight.

Histologic examination A portion of the pancreatic tail from each rat was fixed in 40 g/L neutral-buffered formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. A blinded pathologist evaluated morphologic changes microscopically. Interstitia1 edema was scored as 0=absent, 1=expanded interlobular septa, 2=expanded intralobular septa, and 3= separated individual acini. Hemorrhage was evaluated as percentage involvement of the total pancreas: 0=absent, 1=1-10%, 2=11-50%, 3=more than 50%. Vacuo1ization and parenchymal necrosis were scored as percentage involvement of the examined area: 0=absent, 1=1-10%, 2=11-25%, 3=26-50%, and 4=more than 50%. Polymorphonuclear neutrophil (PMN) infiltration was scored as 0=absent, 1=less than 20 PMNs per intermediate-power field (IPF) (at×200 magnification), 2=20-50 PMNs per IPF, 3=more than 50 PMNs per IPF.

Protocol II: effects of rhubarb on pancreatic exocrine secretion in rats with SAP

Animal models and experimental design SAP was induced as mentioned in protocol I. Rhubarb (150 mg/kg) was orally administered twice at 2 and 15 h before the first cerulein injection. Sandostatin was subcutaneous injected (*sc*) from the leg three times at 30 min before, 1 h and 2.5 h after the first cerulein injection at a dose of 15 μg/kg 0.1 mL.

Thirty-six rats were randomly divided into six groups: Control group: NS *po+sc+ip*, without stress (*n*=6); Rhubarb group: rhubarb (150 mg/kg) *po*+NS *sc+ip*, without stress (*n*=6); Sandostatin group: NS *po*+sandostatin *sc*+NS *ip*, without stress (*n*=6); Cerulein+Stress group: NS *po+sc*+cerulein *ip*, with stress (*n*=6); Cerulein+Stress+Rhubarb 150 mg group: rhubarb (150 mg/kg) *po*+NS *sc*+cerulein *ip*, with stress (*n*=6); Cerulein+Stress +Sandostatin group: NS *po*+sandostatin *sc*+cerulein *ip*, with stress (*n*=6).

Surgical Procedures Five hours after the initiation of the experiment, the rats were anesthetized and intravenously injected cerulein (0.25 μg/kg) and secretin (5 U/kg) respectively from the tail vein. A laparotomy was then performed through a midline incision, and the duodenal loop was identified. The main pancreatic duct was ligated proximal to the duodenal, and polyethylene tube (PE10) was inserted into the duct for draining pancreatic juice[9]. Pancreatic juice was collected for 30 min. The protein content of pancreatic juice was quantitated by Follin' s method. Amylase was assessed by Phadebas test and bicarbonate concentration by blood gas analyzer (ABL510, Denmark).

Statistics
All data were expressed as mean±SD. For continuous data, statistical analysis of data was accomplished by Student' s *t*-test and analysis of variance (ANOVA). Histologic data were expressed as range of the scores and mean±SD and compared by means of nonparametric tests: the Mann-Whitney for two groups and the Kruskal-Wallis for multiple groups. *P*<0.05 was considered statistically significant.

RESULTS

Part I: protective effects of rhubarb on cerulein+stress -induced SAP in rats
Severe edema and diffused hemorrhage in the pancreas were observed macroscopically in the Cerulein+Stress group. Upon microscopic examination, edema, hemorrhage, focal acinar necrosis, conspicuous vacuo1ization, and PMN infiltration in the pancreas were observed also in the Cerulein+Stress group. In the Cerulein+Stress+Rhubarb (75 or 150 mg/kg) groups, the severities of pancreatitis were alleviated with reduced scores of histology (*P*<0.01, *P*<0.05). There was no significant change in edema between the groups (Table1).

The data of pancreatic wet weight, serum amylase level, and PBF were shown in Table 2. There were no significant changes in the rats treated with rhubarb alone compared with the controls. The pancreatic wet weight and serum amylase level markedly increased in the Cerulein+Stress group. With the addition of rhubarb (150 mg/kg) to SAP model, a slight decrease in pancreatic wet weight from 11.60±0.61 g/kg to 10.65±0.77 g/kg (*P*>0.05) and an obvious reduction of 50% in serum amylase (*P*<0.01) was observed. PBF was 242±17 mL/min/100 g before the treatment. In the Cerulein+Stress group, it dropped to 93±5 mL/min/100 g (38% of the normal value) and partially recovered in the Cerulein+Stress+Rhubarb 150 mg group (135±12 mL/min per 100 g, 60% of the normal value, *P*<0.01 *vs* the SAP group).

Table 1 Effect of rhubarb on morphologic alterations in pancreas in rats with sever acute pancreatitis

Group	*n*	Edema	Hemorrhage	Vacuolization	Necrosis	PMN infiltration
Control	6	0	0	0	0	0
Rhubarb	6	0	0	0	0	0
Cerulein+Stress	9	2-3 (2.8±0.2)	2-3 (2.8±0.2)	2-3 (2.7±0.2)	2-3 (2.5±0.2)	2-3 (2.2±0.2)
Cerulein+Stress+Rhubarb 75 mg	6	3 (3.0±0)	1 (1.0±0)	2-3 (2.2±0.2)	1-2 (1.4±0.2)	1-2 (1.6±0.2)
Cerulein+Stress+Rhubarb 150 mg	7	2-3 (2.8±0.2)	1-2 (1.0±0.3)[a]	1-3 (1.8±0.3)[a]	1-2 (1.2±0.2)[b]	1-2 (1.8±0.2)[b]

Values are expressed as the range of the scores, with the mean±SD. The nonparametric test (Kruskal-Wallis method) showed [a]*P*<0.05, [b]*P*<0.01 *vs* Cn+St group; and no significant difference in edema.

Table 2 Effect of rhubarb on the changes of pancreatic wet weight, serum amylase activity, pancreatic blood flow

Group	n	Pancreatic wet weight (g/kg)	Serum amylase activity (U/L)	Pancreatic blood flow (mL/min/100 g)
Control	6	3.29±0.12	81 360±2 200	242±17
Rhubarb	6	3.34±0.14	85 370±2 910	238±18
Cerulein+Stress	9	11.60±0.61[b]	458 490±43 100[b]	93±5[b]
Cerulein+Stress+Rhubarb 75 mg	6	10.99±0.91[b]	321 710±89 800[b]	113±12[a]
Cerulein+Stress+Rhubarb 150 mg	7	10.65±0.77[b]	298 650±36 450[ad]	135±12[ad]

[a]$P<0.05$, [b]$P<0.01$ *vs* control group; [c]$P<0.05$, [d]$P<0.01$ *vs* Cn+St group.

Table 3 Effect of rhubarb and Sandostatin on the exocrine function of pancreatitis in rats

Group	n	Volume (μL/30 min)	Bicarbonate (mmol/L)	Protein (mg/mL)	Amylase (U/L)
Control	6	697.30±22.13	139.50±38.98	12.33±1.65	157 580±14 038
Rhubarb	6	704.58±29.52	108.00±38.31	12.87±2.52	154 000±18 717
Sandostatin	6	685.90±36.49	123.00±35.04	13.21±0.92	156 916±8 540
Cerulein +Stress	6	192.22±28.83[b]	130.67±41.69	3.64±0.98[b]	69 440±13 449[b]
Rhubarb+Cerulein+Stress	6	177.33±46.70[b]	403.00±68.59[bd]	2.62±0.65[b]	3 768±990[bd]
Sandostatin+Cerulein+Stress	6	216.60±19.08[b]	134.83±59.64	3.18±0.75[b]	4 285±3 148[bd]

[a]$P<0.05$, [b]$P<0.01$ *vs* control group; [c]$P<0.05$, [d]$P<0.01$ *vs* Cn+St group.

Part II: the effects of rhubarb on pancreatic exocrine function in cerulein+stress -induced SAP

After pretreatment with rhubarb or somatostatin, total volume, amylase activity, protein content, and bicarbonate concentration of pancreatic juice in both Rhubarb group and Sandostatin group did not differ significantly from the controls (Table 3). Amylase activity in Cerulein+Stress group declined to 44%. In addition, the volume and protein output reduced significantly compared with that of the control group. No significant improvement was found in the volume and protein content of pancreatic juice in the Cerulein+Stress+Rhubarb group and the Cerulein+Stress+Sandostatin group compared with Cerulein+Stress group, whereas the amylase activity of pancreatic juice in the Cerulein+Stress+Rhubarb group and the Cerulein+Stress+Sandostatin group significantly decreased than that of the Cerulein+Stress group ($P<0.01$), and were 2.4% and 2.7% of the control respectively. Interestingly, the bicarbonate concentration of Cerulein+Stress+Rhubarb group increased to 403±69 mmol/L, which was three times of the control. This phenomenon could not be seen in Cerulein+Stress+Sandostatin group.

DISCUSSION

Severe acute pancreatitis still has a comparatively high mortality due to the systemic inflammatory response syndrome leading to multiple organ failure. Molecular and pathophysiologic investigations have allowed us to get more information about the events in the initiation and the natural course of acute pancreatitis, and subsequently to know more about how to deal with it. Despite considerable experimental efforts, the complexity in the evolution of acute pancreatitis is still far from being completely understood.

Since studies of acute human pancreatitis has many limitations due to its rapid and severe clinical course, so innovative therapeutic concepts should first be clarified in animal experiments. The cerulein model of experimental acute pancreatitis has become popular for the analysis of intracellular events in the early phase of pancreatitis. The major limitation of this non-invasive model is that it can only produce a mild, self-limited disorder. In order to establish a non-traumatic, easy to induce

and reproducible experimental model of clinical relevance, some researchers had modified the animal treatment protocols. It was revealed that early microcirculatory changes, included the increased permeability of endothelial lining and an accumulation of extravasated fluid in the perilobular space, would be more severe if cold stress was added to cerulein induced pancreatitis[10]. Recently, Ding *et al*.[11] had established a mouse model of severe acute pancreatitis by co-injection of cerulein and lipopolyasccharide, which could produce the same pathological characteristics as those of severe acute pancreatitis in human. In our work, we have used a rat model, in which cerulein-induced mild acute pancreatitis could develop into severe acute pancreatitis under water-immersion stress[12]. Cerulein can cause blocking of zymogen secretion, co-localization of zymogens and lysosome, and digestive enzyme activation. Water-immersion stress can stimulate sympathetic nerve system. In this model, multiple vasoconstrictive factors are released, which contribute to a reduction of pancreatic blood perfusion and tissue hypoxia or anoxia with the consequence of damages of microvascular endothelium. Moreover, an enhanced release of cytokines in turn precipitates the microcirculatory disorders. Our study had shown that cerulein plus water-immersion model evolved a highly reproducible form of pancreatitis that was characterized by hyperamylasemia, pancreatic hemorrhage and reduction of pancreatic blood flow.

The significance of disorders of the microcirculation with the consequence of tissue hypoxia or anoxia has been under consideration in the pathogenesis of acute pancreatitis for several decades[13-15]. The hypothesis that the pathogenesis of acute pancreatitis involves ischemia - reperfusion-associated events has attracted new attention[16,17]. The decreased pancreatic capillary blood flow, reduced functional capillary density, and irregular intermittent perfusion were observed in the specimens of cerulein-induced experimental acute pancreatitis[18]. In addition, experimental studies have indicated that synthesis and release of pro-inflammatory cytokines such as TNFα, IL-1β[19], and platelet activating factor (PAF)[20,21], were responsible for local injury and systemic dispersion of the inflammation in the development of pancreatitis. There also had strong evidence that oxygen free radicals were closely associated with the severity of acute pancreatitis[22,23]. Elucidation

of these mechanisms may lead to the possibility of specific therapies aimed at reducing microcirculatory disorders, interrupting the inflammatory process, and therefore preventing tissue injury in acute pancreatitis. Unfortunately, there is still no specific compound for treatment of severe acute pancreatitis other than supportive critical care today.

Rhubarb, a Chinese herbal medicine, has a very broad spectrum of biological activities and pharmacological functions. Rhubarb is used as a laxative, antiphlogistic, and-homeostatic in the treatment of constipation, diarrhea, jaundice, and gastrointestinal hemorrhage, etc. Clinical studies have shown that rhubarb could improve the prognosis of patients with systemic inflammatory reaction syndrome (SIRS) by its antagonizing effect against inflammatory cytokines and complements[24]. As reported by Chen et al., rhubarb could reduce the leakage of oxygen radicals from the mitochondria of intestinal mucosa, exert protective effect on barrier of intestinal mucosa, and improve the gastrointestinal blood perfusion in shocked rats[25-28]. It has also been shown that rhubarb was effective in alleviating the severity of early phase of acute pancreatitis, and preventing further complications at later stages[2]. The sites of action could be related to its inhibition of pancreatic enzymic activities[29]. In experimental pancreatitis the therapy of Tong Xia purgative method, in which rhubarb was the main compound, could alleviate the degree of lung injury mediated by TNF[30]. Taken together, Chinese herbs have many pharmacological substances and therefore have multiple therapeutic effects on acute pancreatitis. This study has shown that administration of rhubarb resulted in a marked reduction of serum amylase activity, significant amelioration in the severity of SAP, and improvement of microcirculatory disturbances in pancreas. Rhubarb may play an important role in the regulation of local blood flow of pancreas by eliminating the oxygen free radicals, as well as inhibiting the release of cytokines.

Few studies have characterized the alteration in pancreatic exocrine function after the induction of experimental pancreatitis. Niederau et al.[31] evaluated basal and stimulated pancreatic secretion in vivo and in vitro in four different models of acute pancreatitis. They found that a secretory blockade during pancreatitis was strikingly similar in all models, in particular the pancreatic secretory response to CCK. This secretory blockade might at least partly explain the failure to treat acute pancreatitis effectively by inhibition of secretion. Our preliminary experiment also showed a nearly complete blockade of pancreatic exocrine secretion in rats with SAP (data was not shown). We successfully obtained the pancreatic juice sample by co-injection with CCK analog and secretin at physiological dosage. This study demonstrated a significant reduction of the volume, amylase activity, and protein content of pancreatic juice in rats with SAP. After treatment with rhubarb, bicarbonate output was distinctively increased, which has yet unknown significance. Like secretin, rhubarb might stimulate flow of bicarbonate- and electrolyte-rich ductular secretion via cAMP, and then exert a protective effect on acute pancreatitis[32], which requires further study.

Our present study showed that rhubarb with its natural complexity and typical prescription in combination exerted protective effects on SAP in rats, probably through improvement of pancreatic microcirculation, amelioration of inflammation, inhibition of pancreatic enzyme, and partial alteration of pancreatic exocrine.

ACKNOWLEDGMENT

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