

## Original Article

# Role of the Chemokine Fractalkine in a Rat Model of Acute Necrotizing Pancreatitis and the Interventional Effect of Ulinastatin

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## Abstract

**Background:** Severe acute pancreatitis (SAP) is a serious systemic disease with high mortality. This study aims to investigate the role of the chemokine, fractalkine (FKN), in the pathogenesis of SAP and the effects of intervention by ulinastatin on FKN expression in an SAP rat model.

**Methods:** We randomly divided 72 Sprague Dawley rats into the following groups: SAP, ulinastatin treatment (UT), and control (C). The SAP model was induced by retrograde infusion of 4% sodium taurocholate into the bili-pancreatic ducts of the rats. Rats in the UT group were injected with ulinastatin immediately after establishment of the SAP model. Serum FKN levels were detected by ELISA at various time points. Histopathological analyses of the pancreas and lung were performed. Expressions of FKN mRNA in the tissues of the pancreas and lung were detected by real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) at various time points for each group.

**Results:** Serum levels of FKN at 3 h after surgery in the SAP subgroup were significantly higher than those in the C group ( $P < 0.05$ ). There were no significant differences between the UT and C groups observed at various time points. Expression levels of FKN mRNA in the pancreatic tissues of the SAP group increased gradually. Although we observed no difference between the SAP and C groups ( $P > 0.05$ ) at 1 hour h after surgery, mRNA levels of FKN in the lung tissues at 3, 6, and 12 h post-surgery in the SAP subgroups were significantly higher than those in the C group for the same time points ( $P < 0.05$ ). Pathological injury of the pancreatic tissues was more remarkable in the SAP group compared to the UT group.

**Conclusion:** FKN may play an important role in the pathogenesis of SAP and SAP-related acute lung injury (ALI). Ulinastatin efficiently interferes with SAP and SAP-related ALI and may be related to inhibition of FKN expression.

**Keywords:** Chemokine, gene expression, pancreatitis, ulinastatin

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## Introduction

Severe acute pancreatitis (SAP) is a serious systemic disease with high mortality.<sup>1-3</sup> Its severity and multiple organ dysfunction are closely related to a variety of cytokine cascades.<sup>4</sup> However, the role of chemokines in the pathological process of SAP is not yet clear. Chemokines are small molecule secreted proteins involved in chemotaxis, whose main role is to activate white blood cells involved in inflammation. They have been divided into the two major subfamilies on the basis of the arrangement of the two N-terminal cysteine residues, CXC and CC. And two other classes of chemokines have been described: lymphotactin (C) and fractalkine (CX3C).<sup>5</sup> Fractalkine (FKN) is currently accepted as the only member of the CX3C family,<sup>6,7</sup> which is mainly expressed in the human activated endothelial cell surface, can exist in a soluble form, and plays a role in inflammation such as the induction of cell aggregation and maintaining a steady-state.

In this study, we have established a rat model of SAP, followed

by intervention with ulinastatin. The purpose was to explore the role of FKN in the pathogenesis of SAP with the intent to further improve options for diagnoses as well as to provide a new experimental and theoretical basis for the treatment of acute pancreatitis (AP) in the clinical setting.

## Materials and Methods

### Experimental animals

We obtained 72 healthy male Sprague Dawley rats that weighed  $200 \pm 30$  g from the Experimental Animal Center of Nantong University. Rats were randomly divided into three groups ( $n = 24$  for each group): SAP, ulinastatin treatment (SAP + ulinastatin, UT group), and physiological saline control (C group).

### Reagents

Reagents were purchased from the following companies: Amylase Assay Kit (Sichuan Mike Technology Co., Ltd., China); Rat FKN ELISA Kit (Shanghai Xitang Biotech Company China); and total RNA extraction reagent, Reverse Transcriptase Kit, and Real-time Quantitative PCR Kit [TaKaRa Biotechnology (Dalian) Co., Ltd., China]. Designed FKN and GAPDH primers were synthesized by Shanghai Bocai Biotechnology Co., Ltd.

### Animal model

Preoperative rats fasted for 12 h and had no water for 6 h prior to surgery. Rats were anesthetized with intraperitoneal injections

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of 2% sodium pentobarbital (50 mg/kg). After an incision into the abdomen and clamping the opening of the duodenum bile duct with an injury-free metal clip, we then inserted a syringe needle into the opening of the duodenum bile duct. We injected 4% sodium taurocholate (1 mL/kg) into the bile duct at the rate of 0.1 mL/min in the SAP group. After 5 min, the needle was removed the metal clips released and the abdomen closed. Next, ulinastatin (3000U/100g) was immediately injected subcutaneously into the thigh. In the C group we injected the same amount of sterile saline (1 ml/kg) as sodium taurocholate. All groups were randomly divided into four subgroups of six rats each. At 1, 3, 6, and 12 h post-surgery, rats were killed and their sera collected and maintained at 4°C for the detection of amylase and ELISA analysis. Simultaneously, we obtained pancreatic and lung tissue specimens for RT-qPCR and pathology.

#### Detection of serum and fractalkine (FKN)

Serum amylase levels were determined by the iodine-starch method, using an American Vitros-250 automatic biochemical analyzer. Serum FKN as detected by ELISA was measured by a Therm MK3 microplate reader at 450 nm absorbance.

#### Detection of real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

According to the operation manual, total RNA of pancreas and lung tissues were extracted and quantified by UV spectrophotometry. cDNA were synthesized using the PrimeScript™ RT Kit. The RT system included 1 µL total RNA, 2 µL 5×RT buffer, 0.5 µL oligo dT, 0.5 µL PrimeScript™ RT Enzyme Mix I, followed by the addition of diethylenetriamine water to a total volume of 10 µL. Reaction conditions were as follows: 37°C for 15 min, then 85°C for 5s. We designed the rat CX3CL1 and GAPDH primer pairs. The upstream primer for FKN was 5'-TGCCACAAGATGACCTCGCCA-3' and its downstream primer was 5'-TCCAGGTGCTTCATGGC-GTCT-3'. The amplified product was 164 bp. The upstream primer for GAPDH was 5'-AAGGTGGTGAAGCAGGCGGC-3' and its downstream primer was 5'-GAGCAATGCCAGCCCCAGCA-3'. The amplified product was 130 bp. We next extracted 2 µL cDNA as a template to RT-qPCR, added 12.5 µL SYBR premix Ex Taq and 0.5 µL primers, after which we added sterile distilled water to a reaction volume of 25 µL. A two-step PCR reaction was used, with the following conditions: pre-denaturation at 95°C for 30s, 95°C for 5 s, and 60°C for 30s, for 40 cycles. Then detected the melting curve, and finally calculated  $\Delta C_t$  and RQ values.

#### Histopathological examination

Rat pancreatic and lung tissues underwent conventional dehydration, then were embedded in paraffin, and sectioned into 5 µ sections, after which they were stained by Hematoxylin and eosin (H&E) staining for histopathological observation. Histopathology of the pancreas and lungs were scored and classified by two professional pathologists using a double-blind method. Each group randomly selected three slices; for each slice 10 high-power fields of vision were again randomly selected, and finally the extent of pancreatic tissue damage by edema, infection, hemorrhage, and necrosis were evaluated. The pathological score for pancreatic tissue was calculated according to Rongiones' standards<sup>8</sup> as a reference with a minimum score of 0 and the highest score of 4. The pathological grade for lung tissue was according to Lei<sup>9</sup> as a reference with a minimum grade of 0 and the highest grade of III.

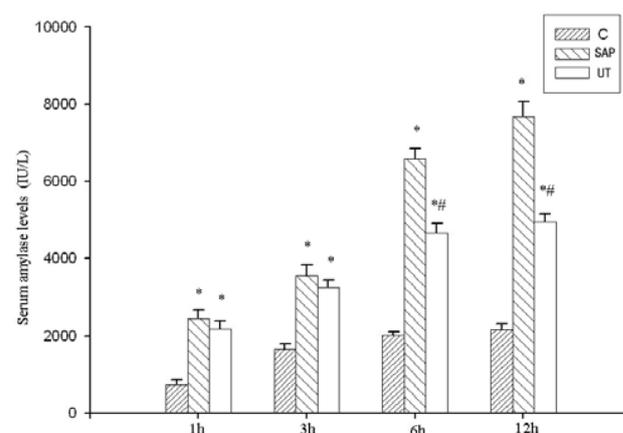
#### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using the SPSS 13.0 software package. Differences between groups were assessed by the chi-square test and histopathological score by the rank sum test.  $P < 0.05$  was considered statistically significant.

## Results

#### Changes in serum amylase activity

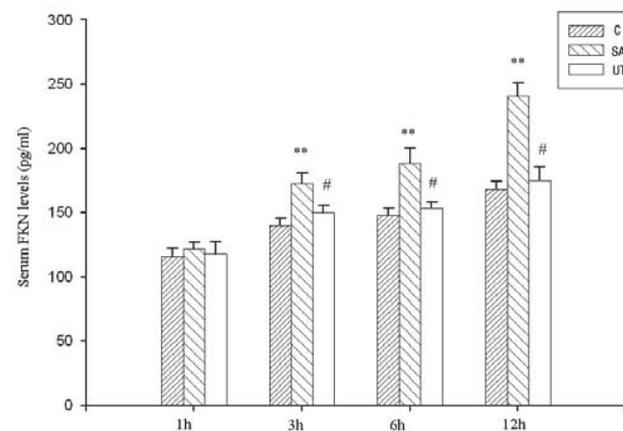
The SAP and UT subgroups of 1, 3, 6, and 12 h had significantly higher serum amylase levels compared with those of the C group ( $P < 0.01$ ). There was no statistically significant difference between the UT 1 h and 3 h subgroups compared with those of the SAP group ( $P > 0.05$ ). However, serum amylase levels in the 6 h and 12 h UT subgroups reduced significantly more than those of the SAP group ( $P < 0.01$ ; Figure 1).



**Figure 1.** Serum amylase levels of each group at various time points. Mean  $\pm$  SD. \*Compared with C;  $P < 0.01$ ; #Compared with SAP;  $P < 0.01$ .

#### Changes in fractalkine (FKN) serum concentrations

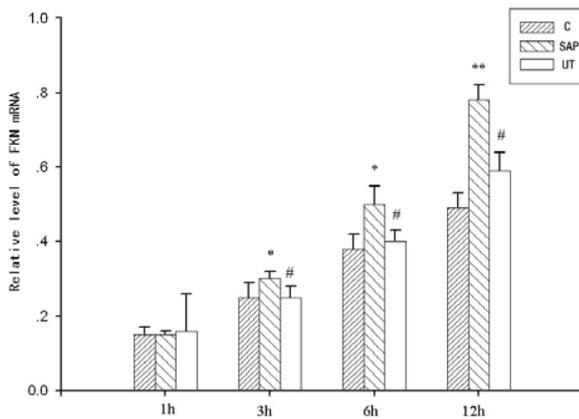
Serum FKN levels of the SAP group gradually increased over time; at 3, 6, and 12 h serum FKN concentration was higher than those in the C group ( $P < 0.05$ ), while serum levels of FKN between the UT and C groups did not significantly differ ( $P > 0.05$ ). Between the SAP and UT groups, there were significant differences in the 6 h and 12 h subgroups ( $P < 0.05$ ) as seen in Figure 2.



**Figure 2.** Serum FKN levels of each group at various time points. Mean  $\pm$  SD \*\*Compared with C.  $P < 0.01$ . #Compared with SAP.  $P < 0.01$ .

Expression of fractalkine (FKN) mRNA in pancreatic tissue

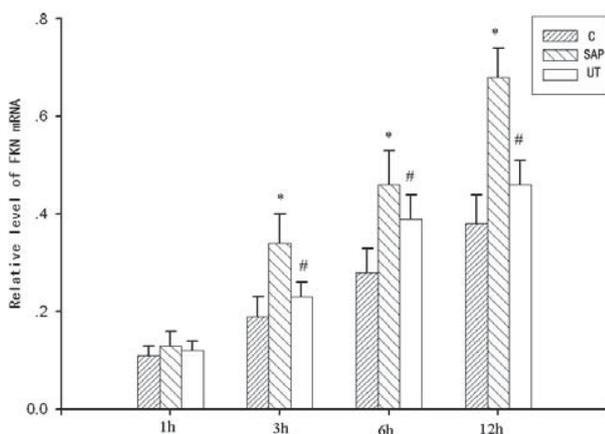
The expression levels of FKN mRNA in pancreatic tissues from the SAP group gradually increased over time. The 1 h subgroup showed no significant difference compared with that of the C group, while the expression level of the 3 h subgroup was higher ( $P < 0.05$ ) than that of the C group. The 6 and 12 h SAP subgroups were significantly higher than those of the C group ( $P < 0.01$ ). Expression levels of the UT subgroups at each time point were not statistically significant compared with those of the C group ( $P > 0.05$ ), however pancreatic tissue FKN mRNA expression levels at 3, 6, and 12 h in the UT subgroups decreased significantly compared to those in the SAP subgroups ( $P < 0.05$ ; Figure 3).



**Figure 3.** FKN mRNA levels at various time points in pancreatic tissues. Mean  $\pm$  SD. Compared with C; \* $P < 0.05$ ; \*\* $P < 0.01$ ; #Compared with SAP;  $P < 0.01$ .

Expression of fractalkine (FKN) mRNA in lung tissue

The expression level of FKN mRNA in SAP lung tissue gradually increased over time. The 1 h subgroup showed no significant differences compared with the C group, while the expression levels of the 3, 6, and 12 h subgroups were significantly higher than those of the C group ( $P < 0.01$ ). The expression levels of the UT groups at each time point showed no significant difference compared with those of the C group ( $P > 0.05$ ; Figure 4).



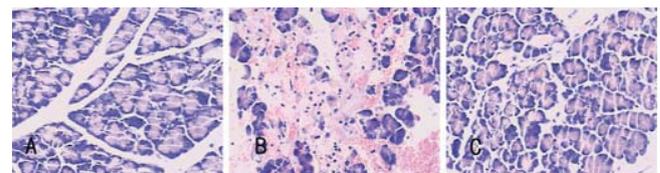
**Figure 4.** FKN mRNA levels at various time points in lung tissues. Mean  $\pm$  SD. \*Compared with C;  $P < 0.05$ ; #Compared with SAP;  $P < 0.01$ .

Histopathological features of pancreatic tissue

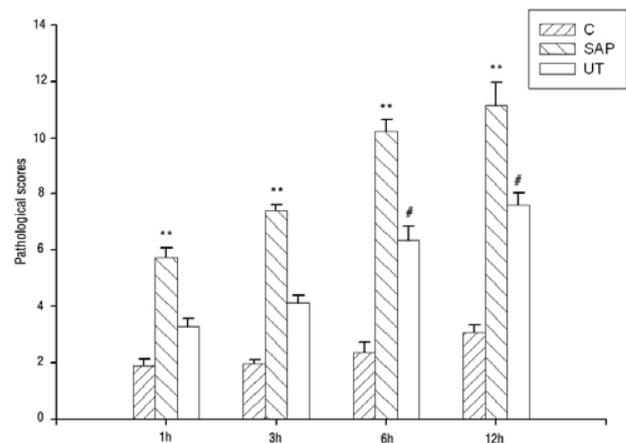
Under light microscope, rat pancreatic cells in the C group were clear, complete, well-defined in lobular, had no interstitial edema with the presence of a small amount of inflammatory cell infiltration, occasional spotting, and slight acinar cell necrosis.

SAP 1 and 3 h subgroups showed obvious interlobular edema, sheet hemorrhage, inflammatory cell infiltration, partial acinar cell degeneration, and necrosis. In the 6 h and 12 h subgroups there was infiltration by large numbers of inflammatory cells, flaky bleeding, destruction of the pancreatic lobular structure, and acinar cell necrosis. Hematoxylin and eosin (H&E) staining of the pancreatic tissue as visualized under light microscope showed large areas of necrosis, interstitial hemorrhage, a large number of inflammatory cell infiltrates, and lobular contour damage.

The UT group showed evidence of pancreatic interstitial edema in the 1 and 3 h subgroups. At 6 h after surgery infiltration by inflammatory cells and necrotic spotting in addition to interstitial edema were visualized. Further increase in inflammatory cell infiltration with focal necrosis was seen 12 h following surgery. Pancreatic pathological damage of the UT group at each time point was significantly reduced compared to the SAP group. Under light microscope there were pancreatic necrosis and interstitial hemorrhage with infiltration by numerous inflammatory cells (Figure 5). Figure 6 shows each group at different pathological time points.



**Figure 5.** H&E staining of rat pancreatic tissue (x200). **A)** Control group, **B)** SAP group, **C)** UT group.



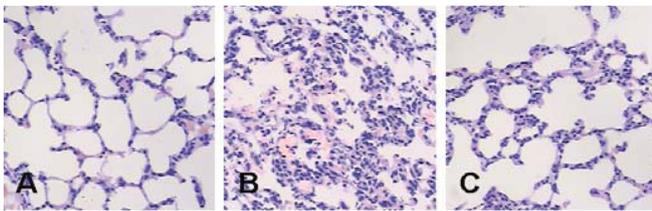
**Figure 6.** Pathologic scores of pancreatic tissues in each groups at various time points. Mean  $\pm$  SD. \*\*Compared with C;  $P < 0.05$ ;  $P < 0.01$ ; #Compared with SAP;  $P < 0.05$ .

Histopathological features of the lung tissue

No significant edema, inflammatory cell infiltration, alveolar hemorrhage, or necrosis appeared in the lung tissue at each time point in group C. Under light microscope, the alveolar structure was clear with thin walls; no apparent alveolar exudate was visualized.

The SAP group had no significant post-operative changes in the 1 h subgroup with a histopathological rating grade of 0. The 3 h subgroup showed a visible pulmonary interstitial edema, infiltration of inflammatory cells, occasional spotting, and alveolar cell necrosis with a histopathology of I. The 6 h subgroup had widened alveolar septum, small pulmonary vascular dilatation and congestion, infiltration by numerous neutrophils with a tissue pathology rating of grade II. The 12 h subgroup showed interstitial pulmonary edema, diffuse hemorrhage, alveolar structural disorder, a large number of alveolar cell necrosis, vascular dilatation and congestion, and infiltration by large numbers of inflammatory cells with a histological rating grade of III.

In comparison with the SAP subgroup pathological changes in the lung at each time point, the 3 h UT subgroup showed significant alleviation of symptoms, no patchy necrosis, and alveolar septal thinning. The histopathological class rating was grade I (Figure 7).



**Figure 7.** H&E staining of rat lung tissue (x200). **A)** Control group, **B)** SAP group, **C)** UT group.

## Discussion

SAP is the digestive symptom of acute abdomen, and the development is very fast with high mortality.<sup>10-13</sup> Its pathogenesis is not fully clear. Researchers have proposed a microcirculation theory, a network theory of inflammatory mediators, as well as a leukocyte and endothelial cell interaction theory, among others.<sup>14,15</sup> However, these could only explain one aspect of the pathogenesis of AP. At the present time, it has been accepted that inflammatory cell infiltration plays an important role in the process of its formation. In the early stages of AP, systemic inflammatory response syndrome (SIRS) may occur as well as multiple organ dysfunction syndrome (MODS), thus leading to functional and organic damage of important organs outside the pancreas.<sup>11</sup> Our results show that in the SAP 1 h subgroup and the C group, FKN mRNA expression levels of pancreatic tissue were not high. However, FKN mRNA expression levels in the 3 h, 6 h and 12 h SAP rat pancreatic tissue increased significantly compared with those of the C group. At the same time we found increasing pathological damage from the pancreatic biopsy, which suggested that FKN might play an important role in the pathogenesis of SAP. Some studies have shown<sup>16-18</sup> that when AP occurs, the damaged pancreatic tissue, as an antigen or inflammatory stimuli, activate macrophages and other inflammatory cells to release large amounts of cytokines, and then triggers an inflammatory mediator waterfall-like cascade reaction. The cytokine network and immune dysfunction could be the reason that AP was likely resulted from the local lesions due to rapid development of SIRS and multiple organ failure (MOF).

Chemokines are proteins that control directional migration of cytokines. At present, increasing evidence has shown that chemo-

kines CXC and CC may play important roles in the development of AP, evolution from AP to SAP, and local and systemic complications of SAP.<sup>16,17,19,20</sup> The application of chemokine blockers could significantly reduce the severity of SAP, reduce complications, and improve efficacy.<sup>21-23</sup> In this study, serum FKN levels in the modeling group increased continuously with time. A small amount of serum FKN expression levels were present in the 1 h SAP subgroup and the C group, whereas increasing levels appeared in the 3 h ~ 12 h SAP subgroups compared with the C group ( $P < 0.05$ ). It has been suggested that the pancreatic tissue continuously releases FKN, which entered the bloodstream because of the role of sodium taurocholate. The circulating FKN in blood has chemotaxis characteristics to neutrophils, lymphocytes, and mononuclear cells, so that a large number of inflammatory cells in the body continue to enter the inflammatory tissue. At the same time, the pathological tissue sections have shown large numbers of infiltrating inflammatory cells associated with pancreatic tissue necrosis in the 3 h ~ 12 h subgroups; pancreatic histopathology scores also increased with time. We presumed that the leakage of large numbers of FKN factors into the blood stream induced large amounts of inflammatory cells to migrate into the pancreatic tissue, which promoted the occurrence and development of the inflammatory cascade. This eventually resulted in the necrosis of a large number of pancreatic acinar cells.

Ulinastatin is a purified glycoprotein from healthy adult males out of fresh urine. Our study showed that subcutaneous injection of ulinastatin immediately after establishment of the SAP rat model led to a significant decline in serum amylase levels and a significant reduction in pathological changes in the pancreas. This has suggested that ulinastatin has a significant therapeutic effect on SAP. We also observed that FKN mRNA expression levels at 6 and 12 h in the UT subgroups in pancreatic serum and tissue showed a significant decrease compared to the SAP group ( $P < 0.05$ ). This has suggested that ulinastatin can significantly lessen the severity of SAP in rats; the mechanism may be associated with the inhibition of expression of chemokine FKN.

Acute lung injury (ALI) associated with AP resulted in the symptoms in the lungs and caused AP. In severe cases can develop acute respiratory distress syndrome (ARDS) and MODS may develop. SAP combined ALI will be dangerous and have a high mortality rate. Around 1/3 patients of SAP combined with ALI and ARDS, and about 60% of deaths are caused by complications of the latter.<sup>24-26</sup> Our study showed that FKN mRNA expression levels in the 3, 6, and 12 h SAP subgroups were significantly higher than the C group ( $P < 0.01$ ). Histopathological observation showed visible pulmonary interstitial edema, infiltration of inflammatory cells in the 3 h subgroup. A large number of infiltrating inflammatory cells accompanied by edema, hemorrhage, and necrosis were observed in the 6 and 12 h subgroups. This has suggested that FKN plays an important role in ALI associated with SAP. The results were somewhat similar with earlier studies of monocyte chemoattractant factor-1.<sup>19, 27, 28</sup>

We noted in this study that with increased experimental time, the results of the C group also increased. We believed it could be for two reasons: first, the level of chemokine expression increased due to surgical trauma to the C group. Second, due to the reverse method of bile duct injection that we used, damage to the bile duct might cause local tissue damage and possibly result in the increase in experimental results.

Abnormal expression of chemokine and its receptor will not al-

low immune cells to exercise their normal function. Chemokines and their receptors (as therapeutic targets) by activation or antagonism of chemokine receptor signaling in order to regulate the function of the chemokine system could be used for control and treatment of related diseases.

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**Ethical approval:** Not needed.

**Competing interest:** No benefits in any form have been received or will be received from a commercial party related directly or indirectly to subject of this article.

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