Turk J Gastroenterol 2009; 20 (2): 122-128

The beneficial effect of propolis on cerulein-induced experimental acute pancreatitis in rats

Cerulein ile indüklenen deneysel akut pankreatitte propolisin faydalı etkisi

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Background/aims: *Inflammatory cytokines and oxidative stress have a central role in the pathogenesis of acute pancreatitis. Propolis is a resinous hive product collected by honeybees from various plant sources and has anti-inflammatory and anti-oxidant effects. The present work aimed to investigate the therapeutic role of ethanolic extract of propolis on a cerulein-induced acute pancreatitis model in rats.* ***Methods:*** *Seventy male Wistar albino rats were used in the study. Acute edematous pancreatitis was induced by subcutaneous cerulein injection (20 µg/kg) four times at one-hour intervals. Ethanolic extract of propolis 300 mg/kg was given subcutaneously at the beginning of the procedure (ethanolic extract of propolis-1 group) or 12 h after the last cerulein injection (ethanolic extract of propolis-2 group). Serum amylase and lipase levels, white blood cell count and serum tumor necrosis factor-α levels were measured and pancreatic tissue was evaluated histologically.* ***Results:*** *In the acute pancreatitis group, serum amylase and lipase levels were found to be elevated and the histopathological evaluation of the tissue revealed massive edema and inflammation with less fatty necrosis when compared to the sham and control groups. Serum amylase and lipase levels and edema formation were significantly decreased in the ethanolic extract of propolis-treated groups (p<0.001). In the ethanolic extract of propolis-2 group, in particular, tissue edema was improved markedly (p=0.001). Tissue inflammation and fatty necrosis were decreased with ethanolic extract of propolis treatment; however, the improvement was not statistically significant.* ***Conclusions:*** *Treatment with ethanolic extract of propolis improved the biochemical and histopathological findings in a rat model of experimental pancreatitis. Although our findings suggest that ethanolic extract of propolis might be considered an effective agent for the treatment of acute pancreatitis, this notion should be supported with further experimental and clinical investigations.*

Key words: Acute pancreatitis, cerulein, ethanolic extract of propolis

Amaç: *İnflamatuvar sitokinler ve oksidatif stres akut pankreatitin patogenezinde oldukça önemli bir role sahiptirler. Propolis bal arıları tarafından çeşitli bitkilerden toplanarak kovanda üretilen anti-inflamatuvar ve anti-oksidan etkileri olan bir reçinedir. Bu çalışmanın amacı sıçanlarda cerulein indüksiyonuyla oluşturulan akut pankreatit modelinde etanolle ekstrakte edilmiş propolisin tedavi edici etkilerini araştırmaktır.* ***Yöntem:*** *Çalışmada 70 adet erkek Wistar albino sıçanlar kullanıldı. Her saatte bir kez olmak üzere toplam dört kez 20 µg/kg cerulein subkutan enjekte edilerek akut ödematöz pankreatit oluşturuldu. Etanolle ekstrakte edilmiş propolis-1 grubundaki hayvanlara işlemin başında, etanolle ekstrakte edilmiş propolis-2 grubundakilere ise son cerulein enjeksiyonundan 12 saat sonra olmak üzere subkutan olarak 300 mg/kg etanolle ekstrakte edilmiş propolis enjeksiyonu yapıldı. Alınan kan örneklerinde serum amilaz, lipaz ve tümör nekrozis faktör-α düzeyleri ölçüldü. Pankreas dokusunda histopatolojik değerlendirme yapıldı.* ***Bulgular:*** *Kontrol grubu ve sham grubuna kıyasla akut pankreatit grubunda; serum amilaz ve lipaz düzeyleri yüksek bulunurken, histopatolojik değerlendirmede pankreas dokusunda yoğun ödem ve inflamasyon ile birlikte az oranda yağ nekrozu gözlendi. Etanolle ekstrakte edilmiş propolis tedavi gruplarında, serum amilaz, lipaz düzeylerinde ve pankreatik ödemde anlamlı azalma tespit edildi (p<0.001). Özellikle etanolle ekstrakte edilmiş propolis-2 grubunda ödem tama yakın düzelme gösterdi (p=0.001). Etanolle ekstrakte edilmiş propolis tedavi gruplarında, inflamasyon ve yağ nekrozunda azalma gözlendi; ancak bu azalma istatistiksel olarak anlamlı değildi.* ***Sonuç:*** *Cerulein ile oluşturulan akut pankreatit modelinde, etanolle ekstrakte edilmiş propolis tedavisi ile biyokimyasal ve histopatolojik bulgularda iyileşme gözlenmiştir. Etanolle ekstrakte edilmiş propolis akut pankreatit tedavisinde faydalı bir ajan olabilir; ancak bunu söyleyebilmek için etanolle ekstrakte edilmiş propolis tedavisinin faydalı olduğuna dair gözlemlerimizi destekleyecek çok sayıda klinik ve deneysel çalışmalara ihtiyaç vardır.*

Anahtar kelimeler: Akut pankreatit; cerulein, etanolle ekstrakte edilmiş propolis

INTRODUCTION

Acute pancreatitis (AP) is a process of acute inflammation of the pancreas, with variable involvement of regional tissues or organ systems (1). The pathophysiology of AP is poorly understood, but interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α as proinflammatory cytokines, oxidative

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Manuscript received: 15.06.2007 **Accepted:** 03.12.2008

stress and microvascular ischemia are important factors (2-4).

In most patients, AP is associated with remote organ failure, sepsis and high death rate despite the development of new diagnostic and therapeutic procedures (5). For this reason, in recent years, pathogenesis-oriented treatments in AP have gained importance and new experimental studies have been focused on pathophysiologic mechanisms including oxidative stress and inflammatory cytokines (5, 6).

Propolis is a resinous dark-colored natural substance collected by honeybees from the gum of various plants with bee wax and salivary secretions (7). It contains more than 180 chemical compounds such as polyphenolic compounds (i.e. flavonoids), cinnamic acid derivatives, various steroids, amino acids, glucose, fructose, vitamins (B1, B2, C and E), and essential elements such as magnesium, calcium, nickel, iron and zinc (8, 11).

Several studies have demonstrated that propolis exerts anti-inflammatory effects via inhibiting the release of arachidonic acid from cell membranes and suppressing cyclooxygenase (COX)-1 and COX-2 enzyme activities (12). It has anti-bacterial (13), anti-viral (14), antioxidant (15, 16), immunostimulatory activities (17) and anti-proliferative, anti-mutagenic, and anti-tumoral effects in several tumor cell lines (18). It has been shown that the efficacy of propolis depends mainly on the presence of flavonoids. In particular, caffeic acid phenethyl ester (CAPE) is also a phenolic antioxidant and an active anti-inflammatory component of propolis (12, 19).

The aim of this study was to evaluate the therapeutic effectiveness of propolis in cerulein-induced acute edematous pancreatitis in rats.

MATERIALS AND METHODS

Animal Groups

Seventy male Wistar albino rats (250-320 g) were used in this study. The animals, supplied from Gaziantep University Faculty of Medicine, Physiology Laboratory, were housed under a 12 h light-dark cycle at a temperature of 24°C. The experiments were performed after 12 h of fasting. All experiments were done in accordance with the recommendations of the national guidelines for the care and handling of laboratory animals, and a protocol approved by the local animal ethics committee was followed.

Experimental Design

Acute edematous pancreatitis was induced by subcutaneous cerulein (Sigma Chemical Co.) injection (20 µg/kg in saline) four times at one-hour (1-h) intervals. Rats were divided into 7 groups, each consisting of 10 animals, as demonstrated in Table 1. No treatment/procedure was applied to Group 1 (Sham group). Group 2 (Control group) received 1 ml/kg saline subcutaneously four times at 1-h intervals. Group 3 (AP group) was injected with cerulein subcutaneously (20 µg/kg in saline) four times at 1-h intervals. Animals were killed 12 h after the last cerulein injection. Group 4 (EPE-1): Ethanolic propolis extract (EPE; 300 mg/kg) was administered by subcutaneous injection at the beginning of the procedure and at the same time AP

Table 1. The experimental design of the study.

	0-h	1-h	2-h	3-h	15-h	21-h
Group 1	Sham					
Group 2	Saline 1 ml/kg	Saline 1 ml/kg	Saline 1 ml/kg	Saline 1 ml/kg	Saline 1 ml/kg	Anesthesia
Group 3	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Anesthesia
Group 4	Cerulein 20 µg/kg + EPE 300 mg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Anesthesia
Group 5	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	EPE 300 mg/kg	Anesthesia
Group 6	Cerulein 20 µg/kg + Saline (1 ml/kg)	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Anesthesia	
Group 7	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Cerulein 20 µg/kg	Saline 1 ml/kg	Anesthesia

was induced by subcutaneous cerulein injection as described before. Animals were killed 12 h after the last cerulein injection. EPE was obtained from Sigma Chemical Co. Group 5 (EPE-2): AP was induced in the same manner and EPE (300 mg/kg) was given 12 h after the last cerulein injection. Animals were killed 6 h after the EPE injection. Group 6 (Placebo-1): AP was induced by subcutaneous cerulein injection (20 µg/kg) four times at 1-h intervals and saline 1 ml was used at the begining of the studies. Animals were killed 12 h after the last injection. Group 7 (Placebo-2): AP was induced in the same manner and saline 1 ml was used at 15th h of the procedure. Animals were killed 6 h after the saline injection.

Evaluation of the Effectiveness of Treatment

Under ketamine anesthesia, a midline laparotomy was performed in rats 12 h after the last cerulein or saline injection except for those in Groups 5 and 7. Group 5 and Group 7 rats underwent laparotomy 6 h after EPE (Group 5) or saline injection (Group 7). Shortly after the blood sample was taken from the inferior vena cava, the whole pancreas was extracted and the animals were sacrificed. Blood samples were centrifuged at 3,000 rpm for 10 min and the plasma was stored at -70°C until assayed. White blood cell count, serum amylase and lipase levels and TNF-α concentrations were assessed. Serum TNF-α levels were measured by immunoassay kit (Rat TNF-α immunoassay, R&D Systems Inc.; Minneapolis, MN), while serum amylase and lipase were assessed by enzymatic photometric method. The results are expressed as U/L.

Histopathological Scoring of the Pancreatic Tissue

Pancreatic tissue was fixed in formaldehyde solution and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated under light microscope by two experienced pathologists blinded to the experimental groups. The scoring was based on the Schoenberg grading system, as shown in Table 2 (20).

Statistical Analysis

Results are given as mean ± SD. Comparisons were made using Mann-Whitney *U* test and one way ANOVA test. Data were evaluated using the SPSS (Statistical Program for Social Science, version 10.0, Chicago, IL, USA) software package. *P* values less than 0.05 were considered significant.

RESULTS

Biochemical Assays

Serum amylase, lipase and TNF-α levels and morphological examination findings are shown in Table 3. In the AP group, serum amylase and lipa-

Table 2. Pathological grading of the pancreatic tissue

Edema	0	No edema
	1	Interlobular edema
	2	Moderate interlobular edema + Intra-acinar edema
	3	Severe interlobular and intra-acinar edema
Inflammatory infiltration	0	No infiltration
	1	Intravascular margination of granulocytes
	2	Granulocytes present in the perivascular tissue
	3	Diffuse infiltration of entire pancreatic gland
Fat necrosis	0	No necrosis
	1	1-4 necrotic cells (per microscopic area)
	2	5-10 necrotic cells
	3	11-16 necrotic cells

Table 3. The biochemical and pathological parameters of the study groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
TNF-α (pg/ml)	65.1±1.7	63.3±3.8	63.8±3.8	65.8±9.9	65.3±10.0	62.4±9.5	61.7±2.5
Amylase (U/L)	665,14± 5	630.2±64.0	4752±1328[a]	2200±1791[b]	1063±339[d]	4516±749	4219±235
Lipase (U/L)	14,41±1,7	14.9±1.7	112.3±34.8[a]	26.9±5.7[b]	23.2±4.7[d]	49.2±5.3	52.5±4.8
White blood cell count (/mm^3)	9279±1867	8755±1098	8574±1437	8583±1915	9316±458	9267±927	8544±895
Edema	0±0	0±0	2.5±0.5	1.5±0.5	0.5±0.5[e]	2.3±0.6	2.2±0.6
Fat necrosis score	0±0	0±0	0.3±0.5	0±0	0±0	0.3±0.5	0.3±0.5
Leukocyte infiltration	0±0	0±0	2.9±0.4	2.5±0.5	2.5±0.5	2.7±0.5	2.7±0.5
Total pathological score	0	0	8	4[c]	3[f]	8	8

Data are given as mean ± SD.
[a] p<0.001 c; compared to Groups 1-2 [b] p<0.001, and [c] p<0.05, compared to Group 3 ; [d] p<0.001, [e] p<0.001, and [f] p<0.05 compared to Group 3.

se levels were significantly elevated compared to control and sham groups ($p<0.001$). Control and sham groups did not differ statistically in terms of serum amylase and lipase levels. On the other hand, serum amylase and lipase levels significantly decreased in the EPE-treated groups ($p<0.001$), but the levels were higher than those of the control and sham groups. In addition, serum amylase and lipase levels of the placebo groups were similar to those of the pancreatitis group (Figures 1, 2). Study groups were not statistically different in terms of serum TNF-α levels and white blood cell counts ($p>0.05$, Table 2).

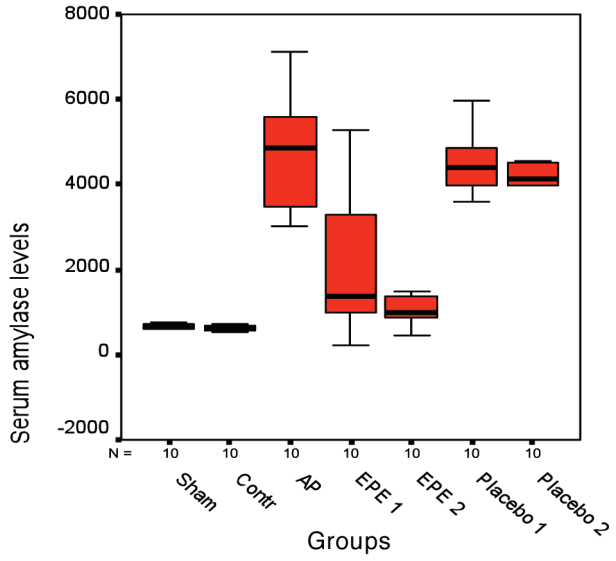

Figure 1. The serum amylase levels in study groups.

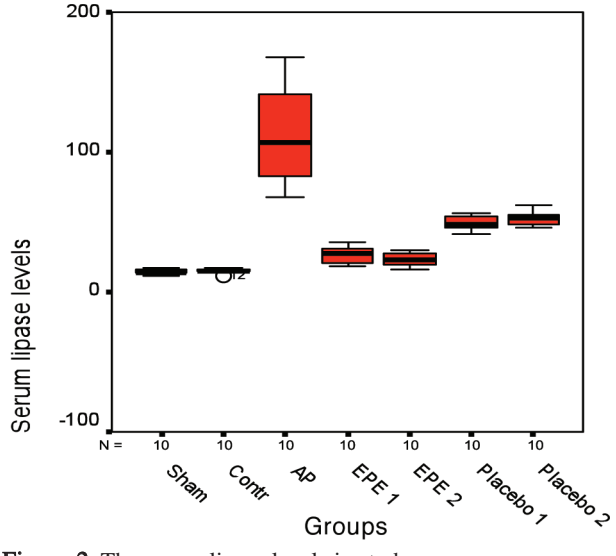

Figure 2. The serum lipase levels in study groups.

Histopathological Evaluation

In the AP group, the histopathological evaluation showed massive edema and inflammation with less fatty necrosis compared to the control and sham groups. Edema formation was significantly improved with the EPE treatment. The most striking finding was that edema was ameliorated almost completely in the EPE-2 group ($p=0.001$, Figure 3). There was marked leukocyte infiltration in the pancreatitis group (Figure 4). In the EPE-treated groups, the inflammation was resolved although the improvement was not statistically significant ($p>0.05$, Figure 5). In the AP group, the fatty necrosis score was 0.30 ± 0.48 and grade 1 fatty necrosis was observed in three rats. Fatty necrosis was decreased in the EPE-treated groups; however, this improvement was not statistically significant ($p>0.05$). The histopathologic data of the placebo groups were similar to that of the AP group. In addition, the total pathologic score showed a significant decrease in the EPE-treated groups ($p<0.05$). None of the animals died during the study.

DISCUSSION

The spectrum of AP ranges from mild to severe inflammation with systemic manifestations (21). It has been demonstrated that the pathogenesis of AP is an inappropriate activation of proteolytic enzymes causing autodigestion. On the other hand, the oxidative stress, COX-2 and proinflammatory cytokines (including IL-1, IL-6, IL-8, TNF-α, platelet activating factor) play a central role in the pat-

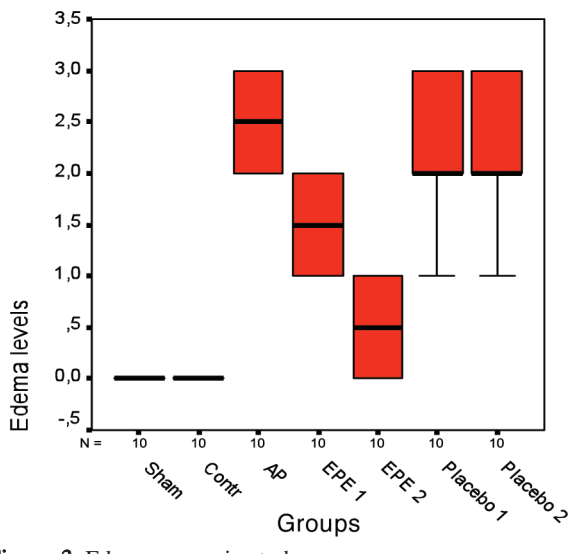

Figure 3. Edema scores in study groups.

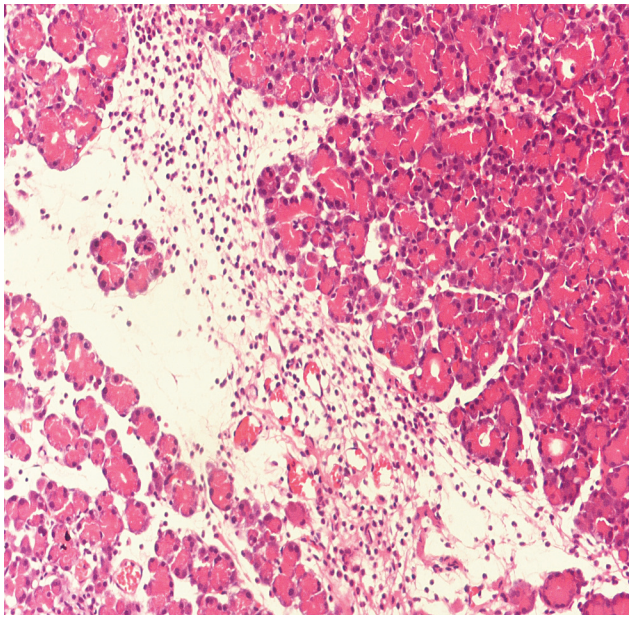

Figure 4. Severe edema and leukocyte infiltration in pancreatic tissue of the acute pancreatitis group (hematoxylin and eosin [H&E], X 200).

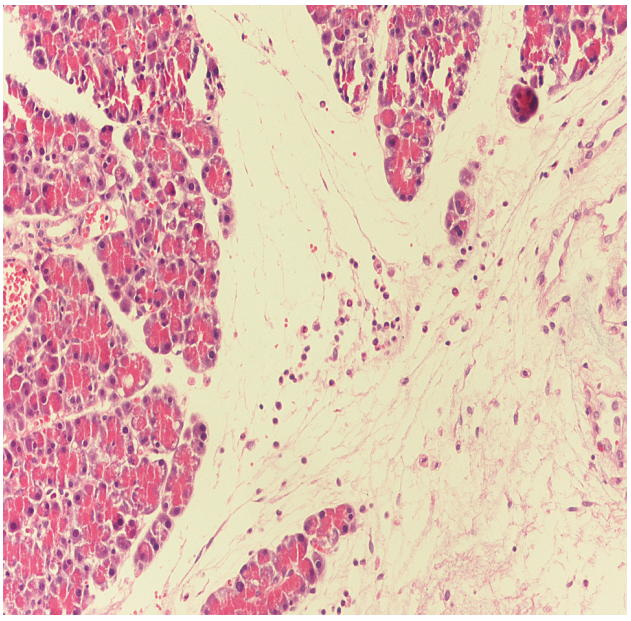

Figure 5. Decreased inflammatory cell infiltration in the pancreatic tissue after EPE treatment (H&E, X 200).

hogenesis of AP and mediate the systemic complications of the disease (4, 22, 23). Currently, there are several problems in the therapy of severe AP despite the development of new diagnostic tools and treatment options. Thus, several experimental and clinical studies have been focused on the pathogenesis of AP. In the present experimental study, we evaluated the therapeutic role of propolis as a putative new agent for the treatment of AP.

The mechanism of the anti-inflammatory activity of propolis is still unclear. Previous studies suggested that propolis has anti-inflammatory effects via inhibiting the release of prostaglandins, leukotrienes and histamine (15, 25). A recent study demonstrated that propolis exhibited anti-inflammatory effects in acute and chronic models of inflammation in rats (including carrageenan-induced paw edema, carrageenan-induced pleurisy and adjuvant-induced arthritis) and suggested that its anti-inflammatory effect is dose-dependent and mainly due to CAPE (26). However, another study suggested that EPE inhibits the COX activity in lung homogenates of saline- or lipopolysaccharide (LPS)-treated rats in a concentration-dependent manner, and that CAPE and galangin contribute to the anti-inflammatory effect of propolis, CAPE being more effective (27). CAPE is a phenolic compound and an active substrate of propolis. Several investigators showed that CAPE exerts anti-inflammatory actions via inhibition of the release of arachidonic acid from cell membranes and suppression of COX-1 and COX-2 enzyme activities (12, 27). AP is known to be associated with induction of COX-2 expression. In cerulein-induced pancreatitis, Ethridge et al (22). showed that COX-2 gene expression was increased in the pancreatic tissue and that the severity of pancreatic necrosis and leukocyte infiltration were significantly decreased by treatment with the selective COX-2 inhibitor NS-398.

Oxidative stress plays an important role in the pathophysiology of AP. A recent study on a sodium-taurocholate model of pancreatitis in rats demonstrated that serum amylase and lipase levels, edema, leukocytic infiltration, parenchymal necrosis and hemorrhage were significantly decreased with N-acetylcysteine (NAC) treatment (5). Likewise, Vaquero et al (28). showed that treatment with NAC reduced neutrophil infiltration, IL-6 mRNA expression and inducible nitric oxide synthase (iNOS) activity in pancreatic tissue via inhibition of nuclear factor (NF)-κB activity. In experimental pancreatitis, the beneficial effects of the antioxidants may be associated with the inhibition of NF-kB activity. CAPE has been shown to inhibit the production of proinflammatory cytokines by inhibiting NF-κB activity (29) Fitzpatrick et al (30). showed that CAPE (30 μg/kg) treatment inhibited NF-κB activity and colonic cytokine (TNF-α and IL-1ß) production in experimental colitis in

rats. In our study, the serum amylase and lipase levels were decreased with EPE treatment. There were no statistically significant differences between the study groups in terms of serum TNF-α levels and white blood cell counts. It is important to note that serum TNF-α levels show marked increases only in severe AP. Due to its transient elevation in AP and its high hepatic clearance, it is not always possible to determine high TNF-α levels in the serum. For this reason, TNF-α levels were not estimated in most of the previous experimental and clinical studies and not used as a follow-up criterion (6, 23).

In addition, EPE therapy significantly reduced the extent of edema and the total pathologic score. The effect of EPE on edema formation is possibly due to the anti-inflammatory action of CAPE (26). Although the tissue inflammation and fatty necrosis score were improved with EPE, the improvement was not statistically significantly. It is particularly important that there was marked improvement in the EPE-2 group in terms of biochemical and pathological parameters. This could be explained by the fact that the production of the cytokines (e.g. IL-6, IL-8) show marked increases mainly in the late phases of AP (21). Since CAPE is a specific and potent inhibitor of NF-kB, it may inhibit the production of proinflammatory cytokines (29).

There are several models of experimental pancreatitis, including the cerulein-induced and sodium-taurocholate models. Pancreatic injury was evenly distributed throughout the pancreas in the cerulein-induced model. The reasons we preferred the cerulein-induced AP model were that the characteristics of AP in this model are very similar to those of human pancreatitis and because the inflammation develops rapidly (31). We ended the study 12 h after cerulein injection since it is known as the time point for pancreatic tissue inflammation to reach a peak (32). We included the EPE-2 group to investigate the effect of the compound on the most severe stage of AP. Here, our concern was to study the efficacy of the treatment in severe pancreatitis. In clinical practice, the patients with AP often admit to the hospital at advanced stages, even with systemic complications.

In conclusion, EPE treatment improved the biochemical and histopathological parameters in cerulein-induced experimental AP in rats. EPE was particularly effective in the EPE-2 group in which AP had already occurred. The findings of this study might provide a basis for new experimental and clinical studies investigating the therapeutic role of EPE in severe necrotizing pancreatitis.

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