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Effects of Oleuropein on Serum Inflammatory Cytokines and Histopathological Changes in Rats with Pancreatitis

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Objectives. Acute pancreatitis is a severe debilitating disease with high morbidity and mortality. The severity of the disease is largely due to the actions of various inflammatory mediators. No specific therapy is indicated for AP and its treatment remains largely supportive. This study investigated the effect of oleuropein on serum inflammatory cytokines and distant organ injury in rats with acute pancreatitis induced by bile-pancreatic duct obstruction. **Material and Methods.** Thirty adult male Wistar rats were randomly assigned to 3 groups of 10 rats each: group I: sham, group II: pancreatitis and group III: pancreatitis + oleuropein. Rats in Group III received oleuropein 20 mg/kg intragastrically during 10 days before surgery. The rats were sacrificed at 24 h after surgery and venous blood, pancreatic and lung tissue samples were obtained for analysis.

Results. A statistically significant difference was found between groups regarding serum amylase, LDH, lipase, pancreatic amylase, IL-6, IL-10 and TNF- α values with a Kruskal-Wallis test ($p < 0.05$). No statistically significant difference was found between Group II and III regarding pairwise comparison of amylase, LDH, lipase, pancreatic amylase, IL-6, IL-10 and TNF- α value by using pairwise comparisons with a Mann-Whitney U test ($p > 0.01$). A statistically significant difference between groups was found regarding edema, inflammation, hemorrhage and acinar cell necrosis in histopathological examination of the pancreas ($p < 0.05$). No difference was found between group II and III ($p > 0.01$). A difference was found between the groups in terms of lung tissue edema ($p < 0.05$) but no difference was found regarding hemorrhage and inflammation ($p > 0.05$). No statistically significant difference was found between group II and III with pairwise comparison ($p > 0.01$).

Conclusions. The experimental model used in this study showed to be appropriate for creation of acute pancreatitis. It was concluded that oleuropein as a prophylactic treatment has no protective effect on serum proinflammatory and anti-inflammatory cytokines as well as pancreatic tissue (*Adv Clin Exp Med* 2015, 24, 2, 213–218).

Key words: acute pancreatitis, oleuropein, cytokines, pancreatic damage.

Mortality in acute pancreatitis (AP) is associated with systemic inflammatory response syndrome in the early period and superinfection of pancreatic necrosis in the late period. Although the mechanisms that trigger the inflammatory and necrotizing process are not fully understood, in general, activated leukocytes are considered to play an important role in the pathogenesis. Proinflammatory cytokines like serum tumor necrosis factor α (TNF- α),

interleukin-1 beta (IL-1 β), interleukin-6 (IL-6) and interleukin-8 (IL-8) levels were found significantly higher in severe pancreatitis compared to mild pancreatitis [1–4]. Therefore, treatment methods for the prevention of pancreatic superinfection may play an important role in the prevention of lung injury. The antimicrobial activity of probiotics can reduce secondary infections in acute pancreatitis and thus reduce activated leukocytes and the production

of proinflammatory mediators [5]. Although in the literature probiotic use in *in vitro* studies was found to decrease IL-10 production by monocytes and lymphocytes and decrease post-operative IL-6 production in clinical trials [6], Besselink et al. [7] expressed in their study that probiotic prophylaxis was not effective in reducing infectious complications and associated mortality in acute pancreatitis. Despite the large accumulation of information about the pancreas and associated diseases, treatment of acute pancreatitis remains a serious problem and there is no specific treatment [3]. Nowadays, new treatment strategies against the pathophysiology of the disease are still experimental [8]. Oleuropein is found in olives, olive leaves and olive oil and is thought to be a substance with different pharmacological properties such as antioxidant, antitumoral, anti-viral, and proinflammatory cytokine inhibitor. The anti-inflammatory effect of oleuropein acts *via* the lipooxygenase enzyme and inhibition of leukotriene B₄ production. As a result of *in vitro* studies, oleuropein is considered to have strong antimicrobial activity against both gram-positive and gram-negative bacteria [9–11]. In an experimental model of sepsis by Bourbollus et al. [12], levels of pro-inflammatory cytokines such as TNF- α and IL-6 significantly decreased. Therefore, oleuropein may be considered to be useful in reducing morbidity and mortality in acute pancreatitis due to the preventive effect on proinflammatory cytokines and the bactericidal effect. The aim of this study is to evaluate the effects of oleuropein on acute pancreatitis in rats, emphasizing the changes of serum levels in proinflammatory and anti-inflammatory cytokines and histopathological changes in the pancreas and lungs.

Material and Methods

The study was approved by the Ondokuz Mayıs University Local Ethics Committee for Animal Experiments (date: 08/10/2010, no: 2010/43). Thirty Wistar albino rats, female, weight ranging 250–300 g, were used in the study. All rats were fed *ad libitum* with standard rat chow and tap water. All subjects were kept in 12 h darkness and 12 h light before and after study (at standard temperature -22°C).

Animal Groups

The rats were randomly divided into 3 groups, each containing 10 rats. Group I: sham-operated rats; a distal common bile duct exploration was performed without ligation of the bile-pancreatic duct. Group II (Control): rats with AP induced by bile-pancreatic duct obstruction (BPDO) as described.

Group III (Oleuropein): animals in the pancreatitis group were administered 20 mg/kg oleuropein *via* gavage during 10 days before surgery (Kale Naturel Limited Company, Balıkesir, Turkey).

Surgical Procedure

50 mg/kg ketamine (Ketalar, Pfizer, Turkey) with 10 mg/kg xylazine HCl (Rompun, Bayer, Turkey) were given to all animals for anesthesia. The abdominal skin of all subjects was shaved and painted with iodine povidone under general anesthesia. All surgical procedures were performed under sterile conditions. Laparotomy was performed with a mid-line incision of approximately 2 cm. Acute pancreatitis was induced by bile-pancreatic duct obstruction as previously described [13]. After recovery from anesthesia, the rats were allowed free access to water only. All rats were sacrificed at 24 h after induction of pancreatitis. Blood samples were taken by cardiac puncture for measuring total amylase, pancreatic amylase, lactate dehydrogenase, lipase (LDH), IL-6, IL-10 and TNF- α . Tissue samples of the pancreas and lung were removed for histological examination *via* laparotomy and sternotomy.

Biochemical Analysis

The blood samples were centrifuged for 10 min at $3500 \times g$. Serum was separated and stored frozen at -80°C in individual aliquots for each assay. Four analyses including amylase, pancreatic amylase, lipase, and lactate dehydrogenase (LDH) were assayed using the spectrophotometric method by Roche Modular P800 autoanalyzers. The levels were expressed as IU/L.

The concentrations of IL-6, IL-10 and TNF- α in the serum were measured using commercially available enzyme amplified sensitivity immunoassay (EASIA) kits (BioSource Europa SA, Nivelles, Belgium). The enzymatic reactions were quantified in an automatic microplate photometer. The levels were expressed as pg/mL. The lower detection limits were 2.0 pg/mL, 1.6 pg/mL, and 0.7 pg/mL for IL-6, IL-10 and TNF- α respectively. The mean interassay coefficient of variation (CV) % and intraassay CV % for IL-6, IL-10 and TNF- α were 4.4%, 2.8% and 4.5% and 4.2%, 2.8% and 6.6%, respectively. All assays were conducted according to the manufacturer's instructions. The samples which showed higher concentrations were diluted and measured in duplicate.

Histopathological Analysis

Samples were taken from the pancreas and lung for histopathological examination. Tissue samples were fixed for 12 h in a 10% buffered

neutral formaline solution. After a routine follow-up, samples were embedded in paraffin blocks and sections of 5 μm thickness were stained with hematoxylin and eosin (H & E) and finally examined under light microscope. Section was conducted by an experienced histologist who was blinded to the experimental groups. For evaluation of pancreatitis, Schmidt's standards were modified as follows [5]: grading for edema was scaled as 0 – absent or rare; 1 – edema in the interlobular space; 2 – edema in the intralobular space; 3 – isolated island shape of pancreatic sinus. Inflammation was scored as 0 – absent; 1 – mild; 2 – moderate; 3 – severe. Acinar cell degeneration was scaled as 0 – absent; 1 – focal (< 5%); 2 – and/or sublobular (< 20%); 3 – and/or lobular (> 20%). Parenchyma hemorrhage was scored as 0 – absent; 1 – mild; 2 – moderate; 3 – severe. The lung edema was assessed as follows: 0 – no; 1 – mild; 2 – moderate; 3 – severe edema with hemorrhagic lesions. For inflammatory and red blood cell infiltration (CI), the grading was similar to edema formation. Degrees 0, 1, 2, and 3 were scored for absent, mild, moderate, and severe CI.

Statistical Analysis

Computer analysis was performed using SPSS 15.0 after data obtained from the research program

was encoded. The Kruskal-Wallis test was used for comparisons between groups and the Bonferroni-corrected Mann-Whitney *U* test was used for pairwise comparison of significant values. Statistical significance level was $p < 0.01$ for the Mann-Whitney *U* test and $p < 0.05$ for other tests.

Results

Evaluating the Results of Biochemistry

A statistically significant difference was found between two groups regarding multiple comparison of serum amylase, LDH, lipase, pancreatic amylase, IL-6, IL-10 and TNF- α values with the Kruskal-Wallis test ($p < 0.05$). The results of the statistical biochemical evaluation of the groups are shown in Table 1. The difference between the groups was found to be caused by the sham group. According to these results, acute pancreatitis occurred in group II and III, and it was concluded that the model used is appropriate. Amylase, LDH, lipase, pancreatic amylase, IL-6, IL-10 and TNF- α values were evaluated and found statistically significantly higher in group II and III compared to group I ($p < 0.01$). No statistically significant difference between Group II and III was detected ($p > 0.01$).

Table 1. Evaluation of biochemistry results and histopathological examination

		Group I M (range)	Group II M (range)	Group III M (range)	P-value*
Amylase (IU/L)		318.00 (190–665)	3643.50 (2115–21690)	3935.50 (1458–12360)	0.000
LDH (IU/L)		379.00 (217–821)	2155.00 (1458–2409)	2220.50 (1521–2418)	0.000
Lipase (IU/L)		8.00 (7–478)	75.50 (27–702)	67.50 (17–713)	0.002
Pancreatic amylase (IU/L)		309.00 (188–567)	2921.00 (1547–15980)	3054.50 (978–9012)	0.000
IL-6 (pg/mL)		7.190 (2.18–11.50)	28.970 (10.36–45.98)	16.316 (10.39–28.35)	0.000
IL-10 (pg/mL)		17.581 (13.29–25.84)	45.080 (22.83–60.48)	36.900 (19.42–59.15)	0.000
TNF- α (pg/mL)		6.280 (2.88–7.48)	8.584 (5.60–14.25)	7.221 (5.10–17.14)	0.025
Pancreas	edema	1 (0–2)	3 (1–3)	3 (3–3)	0.000
	inflammation	1 (0–1)	2 (1–2)	2 (1–3)	0.000
	acinar necrosis	0 (0–1)	2 (0–2)	2 (1–3)	0.000
	hemorrhage	1 (0–3)	2 (1–3)	2 (1–3)	0.016
Lung	edema	1 (1–2)	1 (1–2)	2 (1–2)	0.040
	hemorrhage	1.5 (0–2)	2 (1–3)	1 (0–2)	0.148
	inflammation	2 (1–2)	2 (0–3)	2 (1–3)	0.516

M – median, range – min–max, * – Kruskal-Wallis test, $p < 0.05$.

Table 2. Mann-Whitney *U* test results of biochemistry values and histopathological examination findings ($p < 0.01$)

		Group II/Group III	Group II/Group I	Group III/Group I
Amylase (IU/L)		0.241	0.000	0.000
LDH (IU/L)		0.326	0.000	0.000
Lipase (IU/L)		0.762	0.002	0.002
Pancreatic amylase (IU/L)		0.545	0.000	0.000
IL-6 (pg/mL)		0.005	0.000	0.000
IL-10 (pg/mL)		0.326	0.001	0.002
TNF- α (pg/mL)		0.449	0.013	0.041
Pancreas	edema	0.067	0.003	0.000
	inflammation	0.925	0.000	0.001
	acinar necrosis	0.094	0.001	0.000
	hemorrhage	0.776	0.013	0.012
Lung	edema	0.022	0.542	0.075

In terms of the severity of pancreatitis, when IL-6 and TNF- α levels were evaluated in Group II and III compared to Group I, a statistically significant elevation was found ($p < 0.01$). These results refer to the rise of proinflammatory cytokines IL-6 and TNF- α in pancreatitis. In terms of the effectiveness of the treatment, no significant difference was found between group II and III for IL-6 and TNF- α level ($p > 0.01$). It was concluded that secretion of cytokines in pancreatitis is not affected by the use of oleuropein. When the levels of anti-inflammatory cytokine IL-10 were evaluated, a statistically significant elevation was found in Group II and III compared to Group I ($p < 0.01$). However, no statistically significant difference was found between Group II and III ($p > 0.01$). It was found that oleuropein treatment has no anti-inflammatory cytokine-stimulating effect. The results of pairwise comparisons of the groups described above are summarized in Table 2.

Evaluation of the Results of Histopathological Examination

As shown in Table 1, a statistical difference was found with multiple comparisons between the groups regarding pancreatic edema, inflammation, acinar cell necrosis and hemorrhage ($p < 0.05$). The difference between the groups was found to be caused by the sham group. When the results of the examination of pancreatic tissue are interpreted, it

was concluded that the model used is appropriate. Table 2 shows the results of pairwise comparisons of groups. The significantly higher levels of pancreas edema, inflammation and acinar cell necrosis in group II and group III compared to group I shows that acute pancreatitis has occurred ($p < 0.01$). However, in terms of these parameters between group II and group III, the lack of statistical difference suggests that oleuropein is not effective ($p > 0.01$). When the histopathology results of lung tissue edema are analyzed, a statistically significant elevation was detected in group II and group III compared to group I ($p < 0.05$). There was no significant difference in pairwise comparison of group II and group III ($p > 0.01$). However, no statistical difference was found between the groups in terms of hemorrhage and inflammation ($p > 0.05$). Absence of a significant difference between the groups regarding interpretation of the results of the histopathological examination of lung tissue was thought to be due to premature termination of the experiment and non-formation of severe pancreatitis.

Discussion

Amylase and LDH are released from acinar cells and used to confirm the diagnosis of acute pancreatitis [5]. Meyerholz and Samuel [13] expressed that the BPDO model is a suitable method for creating an experimental model of acute pancreatitis. In this study, a statistically significant elevation

was detected regarding amylase, LDH and lipase levels in group II and III compared to group I in BPDO-model pancreatitis. As the histopathological examination showed significantly higher pancreatic acinar cell necrosis in both control and treatment groups compared to the sham group, it was concluded that the experimental model used is appropriate. In clinical and experimental studies, regardless of the etiology of pancreatitis, increased serum proinflammatory cytokines and their important role in the pathogenesis of pancreatitis were seen. Cytokines are not only related to the severity of pancreatic injury, but also related to systemic inflammatory response syndrome and mortality. It has been stated that serum TNF- α , IL-6 and IL-1b levels increase in BPDO and cerulein-induced acute pancreatitis and IL-6 indicate the severity of pancreatitis in the early stage of pancreatitis and that these cytokines are higher in severe pancreatitis [3, 5, 14, 15]. IL-10 has been defined as an anti-inflammatory cytokine which strongly suppresses the production of proinflammatory cytokines. IL-10 production may happen *via* negative feedback of pro-inflammatory stimuli for controlling inflammation. Serum IL-10 levels were found to be higher in patients with severe pancreatitis compared to patients with moderately severe pancreatitis [3, 14]. The severity of pancreatitis-induced lung injury depends on the balance between pro-and anti-inflammatory factors [16]. The effectiveness of the substance used in experimental studies of acute pancreatitis is evaluated with its effect on cytokines such as serum IL-6, IL-10 and TNF- α [4, 17]. In this study, the IL-6 and TNF- α levels of both the control and treatment groups are statistically significantly higher than in the sham group, showing an increase of proinflammatory cytokines in acute pancreatitis. No statistically significant difference was found between the oleuropein and the control group ($p > 0.01$). When these are interpreted, it was concluded that oleuropein as a prophylactic treatment has no inhibitory effect on cytokine release in acute pancreatitis. Detection of significantly higher IL-10 serum levels in the control and treatment groups than in the sham group shows that IL-10, an anti-inflammatory cytokine, increases in acute pancreatitis ($p < 0.01$). However, the absence of a difference between group II and III showed that oleuropein treatment was not effective ($p > 0.01$). It was concluded that oleuropein therapy is not effective in terms of results of both pro-inflammatory and anti-inflammatory cytokine levels.

However, different publications are available in the literature about the anti-inflammatory efficacy of oleuropein. For example, Miles et al. [18] noted that oleuropein inhibited the production of

IL-1 in blood cultures but had no effect on IL-6 and TNF- α levels, and their results are similar to this presented study. In another study, Motilva V. et al. [19] stated that oleuropein usage in indomethacin-induced gastritis decreased TNF- α levels and were beneficial in reducing the inflammatory response.

In recent studies, it has been expressed that acinar cell damage determines the severity of acute pancreatitis [8]. Horst et al. [1] stated that the substance used in the experiment was effective due to a significant difference between the control group and the treatment groups in terms of pancreatic damage in an experimentally-induced acute pancreatitis model. Similarly, Turkyilmaz et al. [20] stated that the material used in their study could be useful, as a decrease in pancreatic damage was observed. Similar to this, in this presented study, as a result of histopathological examination, higher acinar cell necrosis in the control and oleuropein groups than in the sham group ($p < 0.05$) showed that acute pancreatitis had occurred. However, no significant difference between the control and oleuropein groups suggests that the treatment was not effective. In the same way, no statistically significant difference was found between the control and oleuropein groups in terms of changes observed in the case of pancreatitis such as edema, inflammation and hemorrhage.

Lung injury in acute pancreatitis is the most important extra-abdominal organ dysfunction. The mechanism of lung injury caused by acute pancreatitis is not fully understood, but a variety of proinflammatory mediators and activated leukocytes in pancreatitis are known to contribute to this injury [5]. Histopathologically diffuse alveolar blood stasis, edema, and inflammation are seen in lung damage [21]. In this presented study, a histopathological examination of lung tissue revealed a statistical difference between the groups regarding edema, but no difference was detected in terms of hemorrhage and inflammation. No statistically significant difference was found between the control and oleuropein groups when lung tissue edema is compared. Absence of a statistical difference between all groups except in lung tissue edema was attributed to early termination of the experiment and being less affected of lung tissue because of this.

The use of oleuropein in acute pancreatitis was found to be inadequate on regulating serum proinflammatory and anti-inflammatory cytokines and preventing acinar cell necrosis in pancreatic tissue. However, results of further studies on this issue might clarify the effects of oleuropein usage in reducing the severity of acute pancreatitis and thus reducing mortality.

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