Therapeutic effects of ellagic acid on L-arginin ınduced acute pancreatitis[1]

Edip Erdal Yılmaz[I], Zübeyir Bozdağ[II], Ibrahim Ibiloğlu[III], Zülfü Arıkanoğlu[IV], Ümit Can Yazgan[V], Ibrahim Kaplan[VI], Metehan Gümüş[VII], Sabri Selçuk Atamanalp[VIII]

DOI: http://dx.doi.org/10.1590/S0102-865020160060000006

[I]MD, Department of General Surgery, Diyarbakır Gazi Yaşargil Education and Research Hospital, Turkey. Intellectual and scientific content of the study, design the protocol, surgical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[II]Assistant Professor, Department of General Surgery, Faculty of Medicine, Dicle University, Turkey. Design the protocol, surgical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.
[III]Assistant Professor, Department of Pathology, Faculty of Medicine, Dicle University, Turkey. Surgical procedures, acquisition of data.
[IV]Assosciate Professor, Department of General Surgery, Faculty of Medicine, Dicle University, Turkey. Surgical procedures, acquisition and interpretation of data, statistical analysis.
[V]Assistant Professor, Department of Physiology, Faculty of Medicine, Zirve University, Turkey. Surgical procedures, statistical analysis.
[VI]Assistant Professor, Department of Biochemistry, Faculty of Medicine, Dicle University, Turkey. Surgical procedures, acquisition and interpretation of data, statistical analysis.
[VII]Assosciate Professor, Department of General Surgery, Faculty of Medicine, Dicle University, Turkey. Statistical analysis, acquisition and interpretation of data, manuscript writing, critical revision.
[VIII]Professor, Head, Department of General Surgery, Faculty of Medicine, Ataturk University, Turkey. Manuscript writing, critical revision.

ABSTRACT

PURPOSE: To investigate the therapeutic effects of ellagic acid on L-arginin ınduced acute pancreatitis in rats.

METHODS: Thirty-two were split into four groups. Group 1 (control) rats were performed only laparotomy, no drugs were administered. Group 2 (control+EA) rats were administered 85mg/kg EA orally. Rats were sacrificed by cardiac puncture 24 hours after the administration. Group3 (AP) 24 hours after intraperitoneal L-arginine administration, rats were sacrificed by cardiac puncture. Group 4 (EA)-(AP): 85mg/kg EA was administered orally after the L-arginine administration. 24 hours later, rats were sacrificed by cardiac puncture. Serum TNF-α, IL-1β, IL-6, total oxidative status (TOS), total antioxidant capacity (TAC), amylase levels were determined in all groups.

RESULTS: Group 3 (AP) rats showed significantly raised TOS level as compared to Group1 (control) rats ($p<0.001$). Following the EA therapy, a decrease in TOS was observed in Group 4 (AP+EA). TAC levels were significantly raised in the Group 4 (AP+EA) compared to the Group 3 (AP) ($p=0.003$). Group 3 (AP) showed significantly increased TNF-α, IL-1β and IL-6 serum levels as compared to Group 4 (AP+EA). Histopathological changes were supported our result.

CONCLUSION: The healing effects of ellagic acid on inflammatory and oxidative stress were confirmed by histopathological and biochemical evaluations of the pancreatic tissue.

Key words: Ellagic Acid. Pancreatitis. Oxidative Stress. Rats.

Introduction

Acute pancreatitis (AP) is an inflammatory disease caused by stimulation of inflammatory macrophages, neutrophil penetration, together with development of necrosis in the pancreatic tissue[1]. The mean incidence around the world is 40/100[2,3]. Many studies have shown that cytokines such as IL-1β, IL-6, and TNF-α[4,5], which are secreted due to ductal obstruction and ductal injury as well as oxidative stress, are involved in the pathogenesis of AP[6,8]. Oxidative stress, through reactive oxygen species, can damage the membrane resulting in lipid peroxidation, and alter the cytosol dynamics leading to early activation of pancreatic digestive enzymes that initiate pancreatic damage. The inflammatory cytokines along with oxidative stress are important in the development of AP and cause local damage in the pancreas and systemic diseases such as ARDS, shock and multi-organ failure[8,9]. Many preventative agents have been researched; however, an ideal treatment has yet to be found.

Many studies on rats have proven that high dose essential amino acids can damage the pancreas and cause acute pancreatitis. A semi-essential amino acid, L-arginine, was proven to induce the development of acute pancreatitis and cause IL-6 and TNF-α levels to significantly increase. The increase in these cytokines was hypothesized to occur due to the stimulation of peritoneal macrophages following an intraperitoneal injection of L-arginine or the activation of monocytes/macrophages in severe pancreatic damage[10]. Mizunuma *et al.*[11] have used this property of L-arginine and administered high dose L-arginine intraperitoneally to develop an experimental model for acute pancreatitis. This model is ideal due to its non-invasive and non-invasive nature and the fact that it causes dose-dependent acinar cell necrosis, allowing studies on the pathogenesis of acute pancreatitis. Szaboles *et al.*[12] have also used L-arginine to develop pancreatitis from which they have earned good results.

EA (ellagic acid) is a known antioxidant that is derivatized from phenolic acid. The chemical components of EA result in its antioxidant activity. The four hydroxyl groups are known to enhance oxidant protection and reduce lipid peroxidation as well as protect cells from acute and chronic oxidative damage. Besides its role in antioxidation, EA has been suggested to have anti-inflammatory, antifibrotic, antiangiogenic, antiproliferative, antibacterial, antiviral, antimutagenic, and anticarcinogenic effects[13]. Walnuts, tomatoes, grape juice, blackberries, carrots, grape wine, pomegranate, strawberries and blueberries have been shown to comprise EA[13,14].

EA functions acts directly on oxidative stress through the induction of cellular antioxidant signaling systems[15]. Despite its proven antiinflammatory and antioxidant effects, it wasn't shown to have any effect on acute pancreatitis. In this study, we aimed to evaluate the effects of EA that has an antioxidant and antiinflammatory effect on acute pancreatitis.

Methods

This study was conducted with the permission of Dicle University's Local Ethics Committee for Animal Experiments (Dicle -HADYEK No:29 Date: June/10/2015).

Thirty-two adult male Sprague-Dawley rats (250-350g) had free access to water and standart food, were keeped to standard animal care conditions (22±2°C) with a 12:12-h light: dark cycle. They were randomly allocated to four groups as follows: sham, EA, AP and EA-AP groups. Each group consists of 8 rats. Acute pancreatitis was induced by 2 doses of 250 mg/100 g of L-arginine (Sigma Chemical) prepared with 20% 0.15 M NaCl and administered intraperitoneally at a 1 hour interval.

Group 1: Sham Group: Intraperitoneal saline was injected at a dose of 1ml per rat. 24 hours after saline administration, rats were sacrificed by cardiac puncture. No drugs were administered.

Group 2: Ellagic acid (EA) group: 85mg/kg Ellagic acid was administered orally. Rats were sacrificed by cardiac puncture 24 hours after the administration.

Group 3: Acute Pancreatitis (AP) group: 24 hours after intraperitoneal L-arginine administration, rats were sacrificed by cardiac puncture.

Group 4: Ellagic acid (EA)- Acute Pancreatitis (AP) group: 85mg/kg Ellagic acid was administered orally after the L-arginine administration. 24 hours later, rats were sacrificed by cardiac puncture.

All surgical procedures were performed using 10 mg/mL xylazine (Rompun; Bayer) and 50 mg/mL ketamine (Ketalar; JHP), given intramuscularly in the right rear leg of the rats at a dose of 0.25 mL/100 g of body weight before the procedures. 24 hours later, experiment was terminated. Rats were sacrificed by cardiac puncture. Biochemical and histopathological tissue samples were obtained from all the groups of rats by laparotomy. Blood samples were separated immediately by centrifugation at 4000 rpm for 5 minutes and stored at -20°C for biochemical analysis. Specimens from the pancreas were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin.

Biochemical assays

In all groups, amylase levels, TNF-α, IL-1β, IL-6, serum total antioxidant capacity (TAC), total oxidative status (TOS) were determined. Total antioxidant capacity (TAC) was performed for each blood sample. Total oxidant status (TOS) and TAC analyses were performed for each tissue sample. TOS Analysis is a fully automatic colorimetric method developed by Erel. In this analysis, color intensity was measured spectrophotometrically. The TOS values of the tissues were calculated as nmol H_2O_2 equiv./mg protein. TAC analysis method is a fully automatic method developed by Erel[16] and is capable of measuring TAC of the body against strong free radicals[17]. The TAC values of the blood were calculated as μmol Trolox equiv./L, and the TAC values of the tissues were calculated as nmol Trolox equiv./mg protein.

The serum levels of amylase were determined using the Olympus Autoanalyser (Olympus Instruments, Tokyo, Japan). The IL-β, IL-6 and TNF-a levels of blood samples were using enzyme-linked immunosorbent assay (ELISA) kit specific for the previously mentioned rat cytokines (Biosource International, Belgium).

Histopathological evaluation

Pathological findings were assessed by one of the author blinded to group allocations. Acute pancreatitis was evaluated and documented in each tissue sections. We histopathologically evaluated edema, acinar cell necrosis, hemorrhage and the degree of inflammation in the pancreas. The scoring system used by Schmidt *et al*.[18] was used for histopathological evaluation.

Statistical analysis

SPSS 16 package program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were presented as mean ± SD. Comparisons were made using Mann-Whitney U test and one way ANOVA test. $p<0.05$ values were considered significant.

Results

Biochemical parameters are compared in Table 1. AP and AP+EA groups were high amylase level. Serum levels of amylase, TNF-α, IL-1β and IL-6 were meaningful lower in the AP-EA group than the AP group (p=0.009, p=0.002, p=0.004, respectively) (Table 1). Serum TAC level increased significantly in AP-EA group than AP group. AP group was the lowest level of serum TAC in all groups (Table 2). Following the EA therapy, a decrease in TOS was observed in AP+EA group (Table 2). AP group was the highest level of serum TOS in all groups (Table 2). The histopathological evaluation of the AP-EA group showed that edema, necrosis, hemorrhage and inflammation was significantly lower than AP group (Table 3 and Figure 1).

TABLE 1 - Serum biochemical parametres in all the experimental groups.

Parametres	Groups				
	Control (I)	Control+EA (II)	AP (III)	AP+EA(IV)	p-value*
Serum Amylase(U/L)	390.38±50.59	380.00±60.97	2054.50±554.68	1745.69±511.42	*p*=0.00
Serum TNF-α(pg/ml)	46.78±3.04	48.19±3.70	105.87±31.15	47.50±3.23	*p*=0.009
Serum IL-1β (pg/ml)	442.65±41.24	451.88±44.79	738.88±295.96	464.63±44.74	*p*=0.002
Serum IL-6 (pg/ml)	25.87±3.38	26.20±2.14	49.81±17.18	26.60±2.89	*p*=0.004

*Compare groups III to the others goups
AP: Acute pancreatitis; EA: Ellagic acid; TNF: tumor necrosis factor-alpha; IL: interleukin
All data were expressed as median(min-max)

TABLE 2 - The Parametres of serum oxidative status in all the experimental groups.

Parametres	Groups			
	Control (I)	Control+EA(II)	AP (III)	AP+EA(IV)
Serum TAC	0.84±3.38	0.99±0.04	0.69±0.10	0.83±0.07
Serum TOS	580.91±211.82	565.48±68.96	1478.29±446.32	804.14±140.54

AP: acute pancreatitis; EA: ellagic acid; TAC: total antioxidant capacity; TOS: total oxidant status
All data were expressed as median(min-max)

TABLE 3 - Histological injury criteria in pancreatic tissues in all experimental groups.

	Groups				
	Control (I)	**Control+EA(II)**	**AP (III)**	**AP+EA(IV)**	**p (overall)**
Edema	0	0	3	2	<0.001[a]
Necrosis	0	0	3	2	<0.001[a]
Hemorrhage	0	0	3	2	<0.001[a]
Inflammatory	1	0	3	2	<0.001[a]

AP: acute pancreatitis; EA: ellagic acid
All data were expressed as median(min-max). [a] =X^2 test

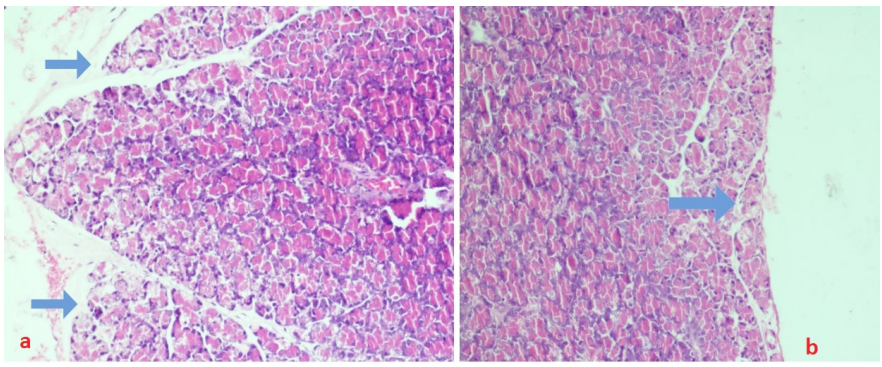

FIGURE 1 - Hematoxylin-eosin (H&E) staining (x200). Microscopic appearance. **a**) Leukocyte infiltration, edema, necrosis, hemorrhage and congestion is increased in pancreatitis group (AP). **b**) Leukocyte infiltration, edema, necrosis, hemorrhae and congestion is decreased in treatment group (AP-EA) (H&E, x200).

Discussion

In this study, we determined that ellagic acid is an effective biochemical and histological therapeutic agent for the treatment of inflammation and oxidative stress in AP.

Despite current therapies, AP still causes serious mortality and morbidity. Approaches that aim to decrease the systemic inflammatory process and oxidative stress have gained momentum, but a consensus has not yet been formed for a drug that administers effective treatment[6,7,18].

Pancreatitis starts by the cessation of secretion from acinar cells, which causes zymogene granules to accumulate intracellularly. This results in cell damage due to the combination of intracellular lyzozomal enzymes and zymogens. Increased amylase is indicative of acinar cell damage[19] and an important parameter for diagnosis. In our study, amylase was increased in all rats with induced AP (Table 1).

Cathepsin B, a lyzozomal enzyme, activates the precursor, trypsinogen, in the zymogene granules into trypsin, which is followed by the proteolysis of other digestive enzyme precursors (chymotrypsinogen, proleastase, prophospholipase, procolipase, callicreinogen) into active enzymes, resulting in pancreatic tissue damage. Many studies have shown the importance of various pro-inflammatory cytokines in this pathway, especially the secretion of *TNF-α*, *IL-1β*, and *IL-6*[20]. With the mediators it induces (proteolytic enzymes, Phospholipase A2, free oxygen radicals), TNF-α is responsible for the systemic effects. When TNF-α is neutralized, these mediators and amylase levels are observed to decrease and acinar cells begin the healing process[2,3,19]. When studies on *TNF*-α are examined, it is observed that *anti-TNF-α* treatment is effective on pancreatic cell healing[19]. In addition, many conducted studies have shown that an increase in *IL-1β and IL-6* levels may be precursor markers of pancreatic damage and these levels were seen to decrease after treatment[5]. In this study, EA was seen to correct increased *IL-1β,* IL-6, and TNF-α levels seen during AP in the AP-EA group, and these results were confirmed by histopathological evaluations as well. Many studies have shown that free radicals were important in the pathogenesis of AP[21]. Due to the high chemical reactivity of the radicals, they interact with many important molecules like lipids, proteins, deoxyribonucleic acid (DNA) and carbohydrates, causing lipid and protein oxidation, DNA breakage, and a dysfunction in the immune system by killing immunity cells and loss of cell function[22]. Free radicals are usually either neutralized or prevented from forming by antioxidants. Depending on the increase of oxidant levels and/or the decrease of antioxidants, the oxidative/antioxidative balance

shifts in favor of the oxidative system, causing oxidative stress, which is linked to the pathogenesis of many diseases[23]. From aging to cancer, anti-oxidants have drawn attention in the treatment of many diseases[22,23]. The measurement of total antioxidant capacity (TAC) or total oxidant stress (TOS) is much easier, practical, and economically convenient than many other analytical methods[16,17]. In this study, we observed that TOS levels, an indicator of lipid peroxidation, were increased and TAC levels were decreased in the AP group when compared to the other groups (Table 2). In addition, we determined a marked decrease in TOS levels and a marked increase in TAC levels in the AP-EA group, which was related to the anti-oxidant and free-radical-eliminating effects of EA. Being a strong antioxidant, EA was observed to have a marked beneficial effect on TAC and TOS levels. In many studies, despite proving the antioxidant effects of EA. To our knowledge, this is the first study to shown that EA can reduce lipid peroxidation and increase protection against AP[15].

In this study, our results show that edema, hemorrhage, severe acinar necrosis, and fibrosis were augmented in the pancreatic tissues of experimental models of rat AP. We also were able to find inflammatory cell infiltration in the inflammed pancreatic sections. Interestingly, EA treatment resulted in a reduction of inflamed pancreas and lower levels of infiltrating neutrophils in the AP-EA rats pancreas sections. This data represents an important additional mechanism whereby EA protects the pancreas from oxidative and inflammatory damage.

The lack of treatment of pancreatitis leads to mortality and morbidity in acute pancreatitis. Along with many inflammatory cytokines, IL-1β, IL-6, TNF-α, and free oxygen radicals cause increased permeability in vessels and disruption of microcirculation. These functional disorders, unless corrected appropriately, damage intestine barriers which allow the transition of endotoxins and intestinal bacteria into the blood stream, resulting in endotoxemia and multi organ disorder and failure caused by the infection[24]. IL-6 is an early biomarker, which has been determined to cause serious organ failure and mortality in acute pancreatitis. While many cytokines were reported to play a role in ARDS, IL-6 and TNF-α were demonstrated to increase phospholipase A2, cause lung damage by affecting phospholipids and surfactant in the lung, increase vessel permeability in the lung, and result in acute lung damage and ARDS[25,26]. In our study, we determined that EA was significantly beneficial in the treatment of AP through the reduction of serum levels of IL6, IL-1β, and TNF-α in the AP-EA group compared to the AP group.

Conclusions

Ellagic acid was shown to reduce inflammatory and oxidative stress in pancreatitis and cause marked improvements in the fatal damage by these two important mechanisms. The healing effects of EA on inflammatory and oxidative stress were confirmed by histopathological and biochemical evaluations of the pancreatic tissue. Ellagic acid is a drug that decreases the damage following acute pancreatitis in both the systemic response and the pancreatic tissue.

References

1. Minkov GA, Halacheva KS, Yovtchev YP, Gulubova MV. Pathophysiological mechanisms of acute pancreatitis define inflammatory markers of clinical prognosis. Pancreas. 2015;44(5):713-7. PMID: 26061557.
2. Granger J, Remick D. Acute pancreatitis: models, markers, and mediators. Shock. 2005;24(1):45-51. PMID: 16374372.
3. Banks PA. Epidemiology, natural history, and predictors of disease outcome in acute and chronic pancreatitis. Gastrointest Endosc. 2002;56(6):226-30. PMID: 26794464.
4. García-Hernández V, Sarmiento N, Sánchez-Bernal C, Coveñas R, Hernández-Hernández A, Calvo JJ, Sánchez-Yagüe J. Changes in the expression of LIMP-2 during cerulein-induced pancreatitis in rats: effect of inhibition of leukocyte infiltration, cAMP and MAPKs early on in its development. Int J Biochem Cell Biol. 2016;72(1):109-17. doi: 10.1016/j.biocel.2016.01.010.
5. Pérez S, Pereda J, Sabater L, Sastre J. Redox signaling in acute pancreatitis. Redox Biol. 2015;5(1):1-14. doi: 10.1016/j.redox.2015.01.014.
6. Kim MJ, Bae Gs, Choi SB, Jo IJ, Kim DG, Shin JY, Lee SK, Kim MJ, Song HJ, Park SJ. Lupoel protects aganist cerulein- induced acute pancreatitis in mice. Phytother Res. 2015;29(10):1634-9. doi: 10.1002/ptr.5423.
7. Gukovskaya AS, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. J Clin Invest. 1997;100(7):1853-62. PMID: 9312187.
8. Yamanel L, Mas MR, Comert B, Isik AT, Aydin S, Mas N, Deveci S, Ozyurt M, Tasci I, Unal T. The effect of activated protein C on experimental acute necrotizing pancreatitis. Crit Care. 2005;9(3):184-90. PMID: 15987389.
9. Bülbüller N, Doğru O, Umaç H, Gürsu F, Akpolat N. The effects of melatonin and pentoxiphylline on L-arginine induced acute pancreatitis. Ulus Travma Acil Cerrahi Derg. 2005;11(2):108-14. PMID: 15877240.
10. Guo F, Zheng S, Gao X, Zhang Q, Liu J. A novel acute necrotizing pancreatitis model induced by L-arginine in rats. Pancreas. 2015;44(2):279-86. PMID: 2541600.
11. Mizunuma T, Kawara S, Kishino Y. Effects of injecting excess arginine on rat pancreas. J Nutr. 1984;114(3):467-71. PMID: 6199486.
12. Szabolcs A, Reiter RJ, Letoha T, Hegyi P Papai G, Varga I, Jarmay K, Kaszaki J, Sari R, Rakonczay Z Jr, Lonovics J, Takacs T. Effect of melatonin on the severityof L-arginine- induced experimental acute pancreatitis in rats. World J Gastroenterol. 2006;12(2):251-8. PMID: 16482626.

13. Gümüs M, Tekin R, Firat U, Onder A, Kapan M, Böyük A, Aldemir M, Kilinç C. The effects of pomegranate on bacterial translocation in rats with obstructive jaundice. Eur Rev Med Pharmacol Sci. 2013;17(11):1488-94. PMID: 23771537.
14. Gümüş M, Yüksel H, Evliyaoğlu O, Kapan M, Böyük A, Önder A, Aldemir M. Effects of ellagic acid on copper, zinc, and biochemical values in serum and liver of experimental cholestatic rats. Biol Trace Elem Res. 2011;143(1):386-93. doi: 10.1007/s12011-010-8863-2.
15. Böyük A, Onder A, Kapan M, Gümüş M, Fırat U, Başaralı MK, Alp H. Ellagic acid ameliorates lung injury after intestinal ischemia-reperfusion. Harmacogn Mag. 2011;7(27):224-8. doi: 10.4103/0973-1296.84236.
16. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem. 2004;37(2):112-9. PMID: 14725941.
17. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38(12):1103-11. PMID: 16214125.
18. Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL. A better model of acute pancreatitis for evaluating therapy. Ann Surg. 1992;215(1):44-56. PMID: 1731649.
19. Grewal HP, Mohey el Din A, Gaber L, Kotb M, Gaber AO. Amelioration of the physiologic and biochemical changes of acute pancreatitis using an anti-TNF-alpha polyclonal antibody. Am J Surg. 1994;167(1): 214-8. PMID: 8311136.
20. Yang J, Zhang J, Liu K, Wang Z, Liu L. Involvement of polyamines in the drought resistance of rice. J Exp Bot. 2007;58(6):1545-55. PMID: 17332417.
21. Wereszczynska-Siemiatkowska U, Dabrowski A, Siemiatkowski A, Mroczko B, Laszewicz W, Gabryelewicz A. Serum profiles of E-selectin, interleukin-10, and interleukin-6 and oxidative stress parameters in patients with acute pancreatitis and nonpancreatic acute abdominal pain. Pancreas. 2003;26(2):144-52. PMID: 12604912.
22. Shalini M. Production of reactive oxygen species and ıts implication in human disease. Free radicals in human health and disease. Springer India, 2015:3-15. doi: 10.1007/978-81-322-2035-0_1.
23. Serdar Z, Yeşilbursa D, Dirican M, Sarandöl E, Serdar A. Sialic acid and oxidizability of lipid and proteins and antioxidant status in patients with coronary artery disease. Cell Biochem Funct. 2007;25(6):655-64. PMID: 17006879.
24. De Campos T, Deree J, Coimbra R. From acute pancreatitis to end-organ injury: mechanisms of acute lung injury. Surg Infect. 2007;8(1):107-20. PMID: 17381402.
25. Sathyanarayan G, Garg PK, Prasad H, Tandon RK. Elevated level of interleukin-6 predicts organ failure and severe disease in patients with acute pancreatitis. J Gastroenterol Hepatol. 2007;22(4):550-4. PMID: 17376050.
26. Frossard JL, Hadengue A, Pastor CM. New serum markers for the detection of severe acute pancreatitis in humans. Am J Respir Crit Care Med. 2001;164(1):162-70. PMID: 11435255.

Correspondence:
Edip Erdal Yılmaz
Diyarbakır Gazi Yasargil Education and Research Hospital, Department of General Surgery
21400, Diyarbakır Turkey
Phone: +90-412 2580061
Fax: +90 412 2580060
drediperdalyilmaz@gmail.com

Received: Feb 15, 2016
Review: Apr 18, 2016
Accepted: May 19, 2016
Conflict of interest: none
Financial source: none

[1]Researched performed at Expermental Animal Laboratory, Department of Pathology, Department of Biochemistry, and Department of General Surgery, Faculty of Medicine, Dicle University, and Depatment of General Surgery, Diyarbakır Gazi Yasargil Education and Research Hospital, Turkey.