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Original Article

Serum M30 and M65 levels and effects of Ankaferd blood stopper in cerulein induced experimental acute pancreatitis model in rats

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Abstract: Background and Aim: The incidence of acute pancreatitis is increasing recently. The aim of this study is to investigate the effect of Ankaferd Blood Stopper (ABS) on experimental model of cerulein induced acute pancreatitis in rats. Materials and Methods: Forty Wistar Albino rats were divided into five groups. Group 1: Sham (n = 8), Group 2: Control group (n = 8), Group 3: Treatment group (n = 8), Group 4: Prophylaxis group (n = 8), Group 5: Prophylaxis treatment group (n = 8). Any practice was not administered to Group 1. Rats were treated with either 1 ml ABS or 1 ml saline via intraperitoneal route before and after inducing acute pancreatitis. Pancreatic tissues were examined histopathologically. Amylase, cytokines (tumor necrosis factor- α and interleukin-1 β), and markers of apoptosis (M30 and M65) were also measured in blood samples. Immunohistochemical staining was performed with caspase 3 antibody. Results: We found a statistically significant improvement in histopathological scores in treatment group and prophylaxis group compared with controls. In treatment group, M30 and M65 levels were lower when compared with controls. In prophylaxis group, there was not a statistically significant difference in M30 levels, but M65 levels were lower when compared with controls. Conclusion: In this experimental acute pancreatitis model, we found high histopathological healing effects of ABS treatment and also prophylaxis. ABS treatment and prophylaxis reduced apoptosis.

Keywords: Ankaferd blood stopper, experimental acute pancreatitis, animal models, apoptosis

Introduction

Acute pancreatitis is the acute inflammation of pancreas in which pancreatic tissues and remote organs are also involved in varying degrees. While a complete recovery is obtained through a non-complicated progress in the majority of patients with acute pancreatitis, in 15% of them severe pancreatitis occurs. Necrotizing pancreatitis is a serious clinical problem with a high mortality rate up to 30% [1]. After pancreatitis develops, the disease becomes limited through pancreatic acinar cell apoptosis; Apoptosis is related with mild pancreatitis, unlike necrotic cell death. Acute pancreatitis, where necrosis is dominant, is associ-

ated with the intense inflammatory reaction and apoptosis inhibition increases [2].

Experimental studies suggest that pancreatic enzymes are the most significant factors in the pathogenesis of intra-pancreatic activation of pancreatic enzymes. Some cytokines such as TNF- α , IL-1 and PAF secreted by damaged cells and systemic immune cells during inflammation, increase the capillary permeability, leukocyte adherence, leukocyte extravasation and the release of proteases and cause the severity of acute pancreatitis and systemic complications [3, 4]. Upon the connection of TNF to TNF receptors (TNFR) in alpha pancreatic acinar cells, the part of receptors within cells, the

Table 1. Histological scoring (Spearman *et al.*)

	Evaluation	Score
Edema	None-mild-medium-severe	0-1-2-3
Fat inflammation	None-mild-medium-severe	0-1-2-3
Parenchymal inflammation	None-mild-medium-severe	0-1-2-3
Peripheral necrosis	None-medium-severe	0-1-2
Fat necrosis	Focal (< 5%)-Sublobular (< 20%)-Lobular (> 20%)	3-5-7
Parenchymal necrosis	Focal (< 5%)-Sublobular (< 20%)-Lobular (> 20%)	3-5-7

TNFR interacts with the adapter protein (TRADD). Then the TRADD causes apoptosis by combining with Phase adapter protein (FADD), which is the part of Phase receptor of cell surface receptor within the cell, and by activating caspase 8 and caspase 3 cause apoptosis [5, 6]. A lot of studies found that apoptosis have significant pathophysiological role especially in the beginning of acute pancreatitis. Apoptosis and necrosis are two different death formulas of the damaged cells. Acute pancreatitis, where necrosis is dominant, is associated with the intense inflammatory reaction and apoptosis inhibition increases [2].

The main component of filament proteins, which exist in the glandular epithelial cells, is Cytokeratin-18 (CK-18). CK-18 is a protein fragment including M65 and M30. While M30 fragment reflects apoptotic cell death, M65 reflects necrosed cell death. During apoptosis, M30 fragment is separated from CK 18 through caspase 3, 6, and 7. While the high rate of M30/M65 suggests the fact that the inflammation proceeds mainly with apoptosis, the low rates suggest the development of necrosis [7]. Recently, there has been a growing interest in determination of the role of apoptosis and necrosis in pathogenesis, and in measuring cytokeratin-18 (CK 18) forms in serum, which indicates the harshness of the disease and the prognostic factor, in many acute and chronic diseases [8].

ABS is an herbal medicine used for haemostatic purposes for many years in the traditional Turkish medicine. It is composed of a standard mixture of *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*. Besides the haemostatic effects of ABS there are its effects on endothelial cells via angiogenesis, cellular proliferation, and vascular mediators. In the proteomic analysis that Demiralp *et al* made to clarify the biological

activity of ABS, it was found that ABS contains many anti-inflammatory proteins [9]. We investigated the effectiveness of ABS in the prevention of the development of acute pancreatitis and treatment of acute

pancreatitis because of its anti-inflammatory and antioxidant effects.

Materials and methods

For this experimental study, an approval from the ethical committee of the Yıldırım Beyazıt Training and Research Hospital was obtained and Etlik clinics' animal laboratory was used. The study was carried out by using Wistar Albino rats with at least 3 months of age whose weight varied between 200-250 grams. The subjects, placed in the room temperature with 5 rats in each cage, were fed by being given free water and food before one week from the study. In the night before the experiment, the rats were allowed free water but no food for 12 hours. 40 rats were included in the study and they were randomly divided into 5 groups ($n = 8$). For achieving acute pancreatitis, intraperitoneal cerulein was applied in a 50 mcg/kg dose of a total 4 times in one hour intervals. At the end of the experiment, the rats were scarified by being applied cervical dislocation under ketamine/xylazine anesthesia in the 12th hour after the last cerulein application. In all groups, blood samples were taken via 5 cc syringe from the truncal main vascular access just before the scarification. Blood samples were immediately centrifuged and the serums were separated. After the rats were sacrificed, their abdomens were opened with a longitudinal incision, and their pancreas gland was taken out. The serums were stored in the deep freezer in -80°C to be studied for TNF-alpha, IL-1 beta, amylase, M30, and M65. Some part of the tissue was sent within 10% formalin to the Pathology laboratory in order to be studied for immunohistochemical analysis for the histopathological examination and apoptotic index.

Animals and grouping

Group 1 (Sham Group) ($n = 8$), Group 2 (Control Group) ($n = 8$) were intraperitoneally injected 1

Table 2. Comparison of histopathological features and *P* values between all groups

	Edema	Fat inflammation	Pancreatic inflammation	Fat necrosis	Pancreatic necrosis	Total score
Group 1: Sham Group*	0	0.2 ± 0.7	0	0	0	0.2 ± 0.7
Group 2: Control Group*	2.1 ± 0.6	2.7 ± 0.4	2.3 ± 0.9	3.8 ± 2.1	3.0 ± 2.0	14.1 ± 4.0
Group 3: ABS treatment group*	1.6 ± 0.5	2.1 ± 0.9	2.0 ± 0.9	2.5 ± 2.8	0.7 ± 1.3	9.0 ± 5.7
Group 4: Prophylaxis Control Group*	2.2 ± 0.7	2.8 ± 0.3	2.2 ± 0.4	4.2 ± 1.0	2.7 ± 1.9	14.3 ± 2.7
Group 5: ABS Prophylaxis Group*	2.0	2.5 ± 0.5	2.0	2.3 ± 2.1	0	8.8 ± 2.5
Comparisons						
Group 1 & 2	< 0.001	< 0.001	< 0.001	0.002	0.010	< 0.001
Group 1 & 4	< 0.001	< 0.001	< 0.001	< 0.001	0.010	< 0.001
Group 2 & 3	0.195	0.279	0.442	0.382	0.050	0.083
Group 4 & 5	0.442	0.234	0.442	0.083	0.010	0.002

*All values are given as mean ± sd. **Comparisons was done by Mann Whitney U test.

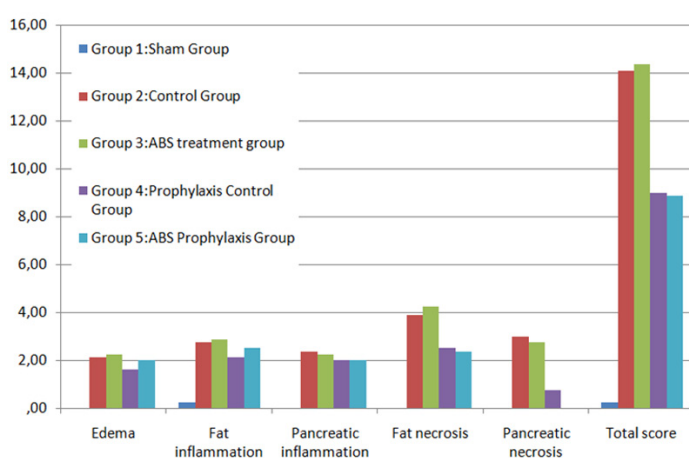


Figure 1. Improvement of pancreatic necrosis and total score are apparent in the group 3 and group 5 (Group 2 & 3; *P* = 0.05 and *P* = 0.083, group 4 & 5; *P* = 0.01 and *P* = 0.002, respectively).

cc of normal saline in total after 6 hours following the last dose injection of cerulein to the group of acute pancreatitis. Group 3 (ABS Treatment Group) (*n* = 8) 1 ml Ankaferd was injected intraperitoneally after 6 hours from the last dose of cerulein to the acute pancreatitis generated group. Group 4 (Prophylaxis Control Group) (*n* = 8) 1 cc normal saline was injected intraperitoneally before the acute pancreatitis was generated. Group 5 (ABS Prophylaxis Group) (*n* = 8) 1 ml was injected before the acute pancreatitis was generated.

Histopathological and immunohistochemical evaluation

Pancreatic tissue samples were detected within the 10% formaldehyde solution. 4 micron thick sections were prepared in the paraffin block and stained with H & E. Pancreatitis grad-

ing was performed according to the modified criteria of Spormann's and his colleagues' working criteria (Table 1) [10]. For the immunohistochemical analysis of the pancreatic tissue, Caspase-3 antibody (1:100; Thermo Scientific, USA) was used. The cells were counted by being nuclear stained with caspase-3 through selecting 10 random area with ×400 magnification in light microscope.

Evaluation of biochemical parameters

For the measurement of TNF-alpha, IL-1β levels, (eBioscience, AUSTRIA) brand ELISA kits, and for the measurement of amylase, M30, M65 levels (Cusabio biotech Co) ELISA kits were used. The measuring ranges for TNF-alpha, IL-1β, amylase, M30, and M65 were 39.1-2500 pg/ml; 31.3-2000 pg/ml; 0-200 mIU/ml; 0.156-10 mIU/ml and 0.9-60 ng/ml, respectively.

Statistical analysis

SPSS (Statistical Package for Social Science) 17.0 for Windows was used to analyze the data. The continuous variables are presented as mean and standard deviations, and box plot graphs were done for better expression. Histograms and "One-Sample Kolmogorov-Smirnov Test" were used to check whether the continuous variables were distributed normally. The meaning of the difference between independent variables that are normally distributed was evaluated with "Independent samples t-test". The meaning of the difference between

Ankaferd blood stopper and apoptotic markers on rat pancreatitis

Table 3. Results of mean values, standard deviations and *p* values of biochemical and apoptotic parameters among all groups

	Amylase (mIU/ml)	TNF alpha (pg/ml)	IL-1beta (pg/ml)	M30 (mIU/ml)	M65 (ng/ml)	M30/M65 ratio	Caspase-3 index (%)
Group 1: Sham Group*	21.9 ± 4.4	46.9 ± 10.0	41.1 ± 7.1	0.160 ± 0.002	1.31 ± 0.75	0.146 ± 0.055	1.03 ± 0.18
Group 2: Control Group*	36.1 ± 14.2	62.5 ± 8.9	173.0 ± 74.1	2.990 ± 2.467	11.72 ± 1.91	0.255 ± 0.238	3.73 ± 1.21
Group 3: ABS treatment group*	24.4 ± 4.3	57.6 ± 2.1	92.2 ± 33.0	0.178 ± 0.059	8.58 ± 3.24	0.021 ± 0.005	1.36 ± 0.39
Group 4: Prophylaxis Control Group*	35.3 ± 7.9	58.3 ± 3.1	179.9 ± 63.1	1.011 ± 1.658	12.33 ± 5.04	0.045 ± 0.057	2.17 ± 0.44
Group 5: ABS Prophylaxis Group*	21.1 ± 6.7	59.4 ± 4.4	101.8 ± 40.2	0.765 ± 1.149	3.44 ± 1.49	0.043 ± 0.022	1.71 ± 0.62
Comparisons Group 1 & 2	0.006	0.003	0.001	0.006	< 0.001	0.229	< 0.001
(<i>P</i> values)** Group 1 & 4	0.002	0.003	0.001	0.059	< 0.001	0.003	< 0.001
Group 2 & 3	0.027	0.430	0.014	0.006	0.034	0.015	< 0.001
Group 4 & 5	0.003	0.713	0.011	0.735	< 0.001	0.955	0.110

*Values are given as mean ± sd. **Comparisons was done by Independent Samples t-test.

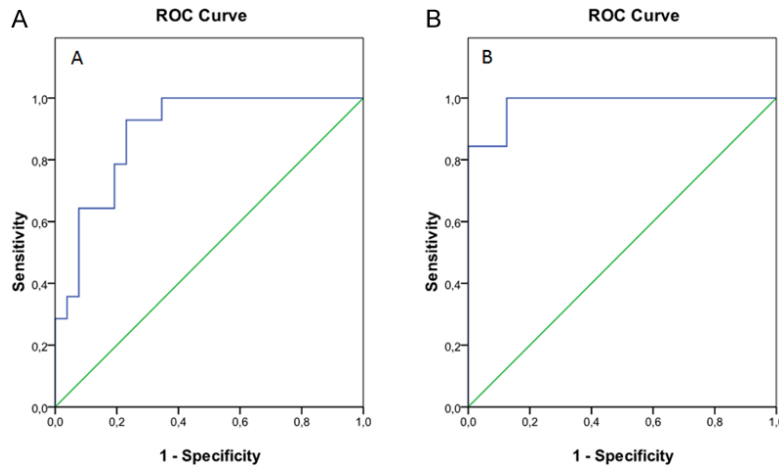


Figure 2. A: For M65, AUC is 0.980 ($P < 0.001$; 95% CI: 0.000-1.000). Cut-off point of M65 for the presence of edema or pancreatic inflammation is 1,839 ng/ml (sensitivity: 100.0% specificity: 87.5%), and 3,575 ng/ml (sensitivity: 84.4%, specificity: 100.0%). B: For M65, AUC is 0.890 ($P < 0.001$; 95% CI: 0.792-0.988). Cut-off point of M65 for the presence of pancreatic necrosis is 7,346 ng/ml (sensitivity: 92.9% specificity: 76.9%).

independent variables that are not normally distributed was evaluated with “Mann Whitney U test”. ROC analysis was used to calculate the cut-off point of M30 and M65 in predicting the edema, pancreatic inflammation and pancreatic necrosis; AUC values by 95% confidence intervals (CI) was indicated; the highest value of the sum of sensitivity and specificity indicated as cut-off point; also at that points the sensitivity and specificity were calculated. For statistical significance testing, two-tailed test were used; < 0.05 was considered significant.

Results

When comparing sham group and control groups, the results of histopathological evaluation, apoptotic markers and biochemical parameters were found high in statistically significant in the control groups. When comparing group 2 with group 3, only parenchymal necrosis score was better and statistically significant ($P = 0.003$). Although the total histopathological score was better in group 3, no statistically significant difference was detected ($P = 0.082$). When we compare group 4 and 5, pancreatic necrosis score and total score was better and statistically significant in group 5 ($P = 0.003$, $P = 0.003$; respectively) (Table 2, Figure 1). Amylase, IL-1 beta levels were significantly lower in group 3 compared to the group 2 ($P = 0.027$, $P = 0.021$, respectively). TNF alpha lev-

els were lower in group 3 compared to the group 2 but it was not statistically significant difference ($P = 0.430$). M30, M65 levels, M30/M65 ratio and apoptotic index were significantly lower in group 3 compared to the group 2 ($P = 0.05$, $P = 0.046$, $P = 0.05$, $P = 0.001$; respectively). Amylase, IL-1 beta levels in ABS prophylaxis group (group 5) was statistically significantly lower compared to the control prophylaxis group (group 4) ($P = 0.003$, $P = 0.014$, respectively). When comparing group 5 and group 4, TNF alpha and M30 levels was no statistically significant difference ($P = 0.713$, $P = 0.834$, respectively). M65 levels were statistically significantly lower ratio in group 5 ($P = 0.001$) (Table 3).

In all cases, edema and pancreatic inflammation presence were similar; showing a similar ROC curve analyses. For M30, AUC was 0.629 ($P = 0.265$; 95% CI: 0.466-0.791). Cut-off point of M30 for the presence of edema or pancreatic inflammation was 0.1625 mIU/ml (sensitivity: 53.1%, specificity: 100.0%). For M65, AUC was 0.980 ($P < 0.001$; 95% CI: 0.000-1.000). Cut-off point of M65 for the presence of edema or pancreatic inflammation was 1.839 ng/ml (sensitivity: 100.0% specificity: 87.5%), and 3,575 ng/ml (sensitivity: 84.4%, specificity: 100.0%) (Figure 2A). For M30, AUC was 0.629 ($P = 0.674$; 95% CI: 0.482-0.867). Cut-off point of M30 for the presence of pancreatic necrosis was 0.2425 mIU/ml (sensitivity: 57.1%, specificity: 88.5%). For M65, AUC was 0.890 ($P < 0.001$; 95% CI: 0.792-0.988). Cut-off point of M65 for the presence of pancreatic necrosis was 7,346 ng/ml (sensitivity: 92.9% specificity: 76.9%) (Figure 2B).

Figure 3 is a sample of histopathological view of serious pancreatitis, characterized by necrosis, edema and intense inflammatory cell infiltration at control group. Figure 4 is the histopathological view of this regressed pancreatitis after treatment with Ankaferd blood stopper, characterized by mild edema, inflammatory cell infiltration.

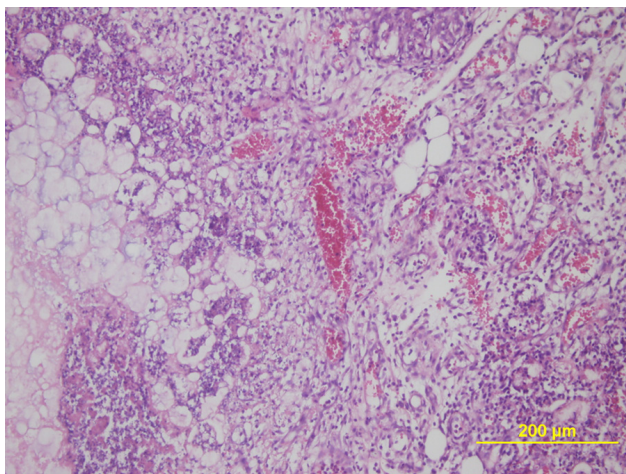


Figure 3. Histopathological view of serious pancreatitis, characterized by necrosis, edema and intense inflammatory cell infiltration at group 2 ($\times 200$).

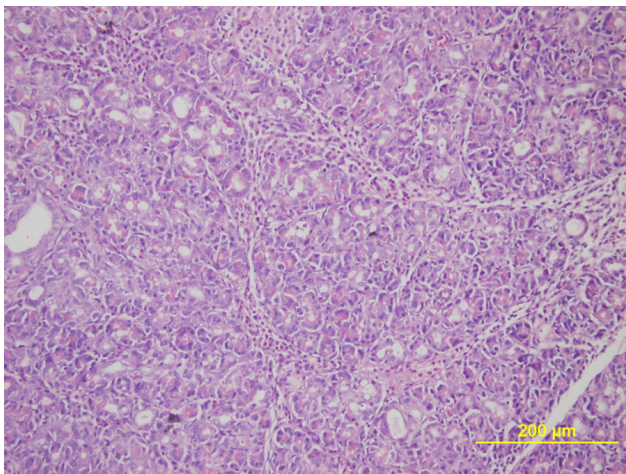


Figure 4. Histological view of regressed pancreatitis after treatment with ABS, characterized by mild edema, inflammatory cell infiltration ($\times 200$).

Discussion

In this experimental model of acute pancreatitis, the histopathologically positive effect of ABS usage in acute pancreatitis prophylaxis and treatment is observed. In our study, M30 and M65 levels were high in acute pancreatitis generated rats. Especially, M65 levels were significantly correlated with severe acute pancreatitis. We detected low levels of apoptotic markers which were correlated with histopathological improvement in groups that were ABS administrated.

In acute pancreatitis, local inflammatory reaction becomes the major cause of mortality by

leading to systemic inflammatory response syndrome. While biochemical and morphological studies in experimental models, where acute pancreatitis was generated, depict that severe pancreatitis were related primarily with necrosis and minimally with apoptosis, apoptosis was primarily on the forefront in mild pancreatitis. TNF alpha is the key regulator in the activation of other pro-inflammatory cytokines and leukocyte adhesion molecule. It is thought that TNF alpha produced and secreted by acinar cells have a role in apoptosis. High TNF alpha increases NF-KB expression and inflammatory response [1, 11]. TNF alpha indicates activity in the early period of the disease and is quickly removed from the serum [12]. IL-1 beta is the major pro-inflammatory cytokine in the development of SIRS response. It was found that high levels of IL-1 beta in serum are related with severe pancreatitis and that there is a positive correlation between mortality and serum levels [13]. In our study, TNF alpha and IL-1 beta levels were high in pancreatitis generated rats. IL-1 beta levels in both the treatment group and the prophylaxis group was low when comparing to control groups. These results was parallel with other studies in the literature [13, 14]. The reason for TNF alpha's quick removal from the serum may be that we could not detected the higher values of TNF alpha.

The anti-inflammatory mechanism of ABS is not fully known, but it might be indicating an anti-inflammatory activity through the variety of proteins [9]. The herbal ingredients consisting ABS have characteristic anti-inflammatory and antioxidant activities. G. Glabra's anti-inflammatory, antioxidant activities have been shown [15]. T. vulgaris shows antioxidant activity, it assumes the protective role against oxidative damage caused by lipid peroxidation [16]. In experimental studies using animal models, ABS was found to increase new bone formation with a reduction in inflammation and activating the antioxidant, and reducing the development of necrosis [17]. It was depicted that ABS prevented stricture development in rats, on which caustic injury of the esophagus was created, and accelerated healing [18]. In another study, in the partial nephrectomy model in rats, renal tubular apoptosis down-regulation was determined through endothelial nitric oxide synthase (eNOS) expres-

sion, inducible nitric oxide synthase (iNOS), and apoptosis protease-activating factor-1 (Apaf-1). As a result, ABS reduced the expression of apoptotic molecules by modifying the topical biological response [19]. Anti-inflammatory and antioxidant activity as well as haemostatic effect of ABS might have a role in tissue damage reduction. ABS haemostatic mechanism is different from other haemostatic agents. ABS stimulates the formation of an encapsulated protein network, which includes fibrinogen, total protein, albumin and globulin, and the erythrocytes and thrombocytes are aggregated across this network [9]. The formed protein network might be preventing the migration of inflammatory cells to the damaged area, and the arrival of inflammatory mediators. In the experimental animal model, which Isler et al made and received a result parallel to this view, inflammation was not observed histopathologically in the bone defects treated with ABS [17]. In a recent study, the effect of ABS on the pancreatic fluid was evaluated, and protein aggregates in pancreatic secretion and a frozen gel-like solid layer within the pancreatic fluid were observed [20]. Haznedaroglu et al reported that ABS-induced protein network formation functions with anti-infectivity, anti-neoplastic, anti-inflammatory activity as well as the haemostatic affectivity [21].

In the literature, there is no study evaluating the M30/M65 ratio to predict the severe necrotizing pancreatitis development where necrosis is dominant, or the mild acute pancreatitis where apoptosis is dominant. In our study, in the group in which ABS was applied to the acute pancreatitis generated rats with treatment aims, levels of apoptotic index, M30 and M65 were found lower along with the meaningful development in pancreatic necrosis. These results suggest that ABS treatment suppresses both apoptosis and necrosis. The rate of M30/M65 was found to be lower in the treatment group compared with the control group; suggesting that the necrosis is suppressed more than the apoptosis. In the group where ABS was applied with prophylaxis aims, low levels of pancreatic necrosis, fat necrosis, and betterment in the total score were found along with the levels of M65 compared to the control group.

Our findings suggest that total cell death plays a significant role in pancreatitis pathogenesis.

Evidently, there are changes in CK 18 forms after cell death and that they are detectable in the serum. As an indirect indication of cell death, we detected that M65 was a more valuable predictive factor than M30 in indicating severe pancreatitis, inflammation and necrosis. In predicting edema, pancreatic inflammation and necrosis presence, M30 is not a good marker because of its low p and AUC values. However, M65 is an excellent marker with its high AUC, sensitivity and specificity values. The effectiveness of M65 in determining acute pancreatitis prognosis should be supported by further studies.

In conclusion, we found the total histopathological score was better and necrosis development was less in ABS treatment and prophylaxis groups in experimental model of acute pancreatitis. The necrosis marker M65 levels were found lower, which was also supported by histopathological findings. Our findings suggest that ABS suppresses both the apoptotic process and the development of necrosis. ABS might be inhibiting the cascade of apoptosis and necrosis development through an unknown mechanism.

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Disclosure of conflict of interest

None.

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