EXPERIMENTAL STUDY

Effect of N-acetylcysteine on neutrophil functions during experimental acute pancreatitis

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ABSTRACT

BACKGROUND: Systemic inflammatory responses and extrapancreatic vital organ impairment are mediated by activated neutrophil functions and products, such as oxygen-derived free radicals, in patients with acute pancreatitis (AP). The present study is an examination of effects of an antioxidant, N-acetylcysteine (NAC), on local and systemic histopathological changes and neutrophil functions during AP.

METHODS: This experimental study was performed on 24 Wistar albino rats equally divided into 3 groups: Group 1 comprised sham laparotomy, Group 2 had AP induced with taurocholate infusion, and Group 3 consisted of AP with NAC treatment. Histopathological features in pancreas, kidney, and lung tissues were examined for local and systemic changes during AP. Neutrophil functions were evaluated using flow cytometry.

RESULTS: Serum levels of pancreatic enzymes were elevated, and histopathological parameters showed acinar cell damage and pancreatic tissue necrosis in the 2 groups with AP. Severe histopathological changes were found in pulmonary and renal tissues, and flow cytometry results indicated defective neutrophil functions in the group with AP alone. NAC treatment significantly ameliorated phagocytosis, chemotaxis, and opsonization of neutrophils ($p<0.05$). NAC treatment also ameliorated systemic changes in pulmonary and renal tissue damage in all microscopic parameters ($p<0.05$).

CONCLUSION: Uncontrolled and defective neutrophil functions could provoke severe systemic inflammatory responses. In addition to local inflammation and necrosis, severe systemic responses and histopathological changes in extrapancreatic vital organs occur during AP. Treatment with antioxidant NAC significantly reverses detrimental systemic responses in extrapancreatic vital organs by significantly ameliorating neutrophil functions despite ongoing AP.

Keywords: Flow cytometry; leucocytes; pancreatitis; systemic inflammatory response.

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory condition that is thought to be due to activation of enzymes in pancreatic acinar cells, with inflammation spreading into surrounding tissues. It is mediated by proinflammatory factors released from acinar cells and leucocytes infiltrating in glandular tissue.

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Previous reports have suggested that pathogenesis of AP is associated with oxidative stress.[1–3] Activation of circulating leucocytes and their subsequent interaction with endothelial vascular cells results in local, initial oxidative stress and production of inflammatory mediators within the pancreas, leading to cell damage.[4] Sanfey et al.[5] designed an experimental model in which capillary injury developing due to increased permeability was reported to be commonly observed phase in pathogenesis of AP. Oxygen-derived free radicals (OFR) were shown to have been predominating determinants of this stage. Many studies have been conducted to investigate role of oxidative stress in different experimental models of AP. OFR production overwhelms cellular antioxidant defense systems, and thus, oxidative stress develops. This leads to disturbances in cellular homeostasis because these OFR can cause biochemical and functional alterations at different cellular levels.[4,6] Local pancreatic inflammation and necrosis triggers activation of neutrophils that produce and secrete cytokines, and OFRs create systemic inflammatory responses.

An end result of this systemic response may be vital organ dysfunction and failure.

N-acetylcysteine (NAC) is well-known free radical scavenger capable of stimulating glutathione synthesis. It has been investigated as an antioxidant compound in various experimental studies.[7,8] Various antioxidants have no useful effects in taurocholate-induced pancreatitis, but NAC was found to be beneficial. Therefore, OFR formation may be an important factor in taurocholate-induced pancreatitis.[9]

First aim of the present study was to investigate effects of NAC in the course of experimental AP. In addition, pathway for pathogenesis via neutrophil functions was examined based on histopathological changes in the pancreas.

MATERIALS AND METHODS

All experiments were performed in accordance with the National Institutes of Health guidelines for the care and handling of animals. This study was approved by the animal ethics commitee of Haydarpaşa Numune Training and Research Hospital.

Twenty-four female Wistar albino rats (250–300 g) were used in this study. The animals were housed in metabolic cages with free access to standard food and water at controlled temperature with 12-hour light-dark cycles during entire study, beginning 1 week prior to experimental stage.

Experimental Groups

A total of 24 rats were randomly divided into 3 groups.

Group 1 [(G1); n=8; sham group]: Only laparotomy was performed. One mL 0.9% saline was intraperitoneally injected at postoperative days 1 and 2.

Group 2 [(G2); n=8; pancreatitis group]: Pancreatitis was induced by sodium taurocholate infusion. One mL 0.9% saline was intraperitoneally injected at postoperative days 1 and 2.

Group 3 [(G3); n=8; pancreatitis and NAC treatment group]: Pancreatitis was induced by sodium taurocholate infusion, and 200 mg/kg NAC (Asist 300 mg, 10%; Bilim Pharmaceuticals, Istanbul, Turkey) in 1 mL 0.9% saline was intraperitoneally injected at postoperative days 1 and 2.

Operative Procedure

According to previous experimental studies,[10] AP was induced by retrograde infusion of sodium taurocholate into the common bile duct with surgical techniques. Anesthesia was induced via intraperitoneal injection of 50 mg/kg of ketamine hydrochloride (Ketalar; Pfizer, Inc., NY, NY, USA) and 10 mg xylazine hydrochloride (2%, 20 mg/mL; Alfasan International B.V., Woerden, The Netherlands). All groups underwent median laparotomy to manipulate the duodenum and common biliopancreatic duct (BPD). In Groups 2 and 3, the duodenal wall was punctured at anti-mesenteric side with 24-G catheter (Introcan-W; B. Braun Medical, Inc., Bethlehem, PA, USA), and catheter was advanced into the common BPD. Leakage of sodium taurocholate was prevented by temporary ligature of the proximal bile duct with 4/0 silk (Mersilk; Ethicon, Inc., Somerville, NJ, USA), while the distal bile duct was occluded with microvessel clip. Sodium taurocholate 5% (0.15 mg/kg; Sigma-Aldrich, Corp., St. Louis, MO, USA) was infused into the common BPD via catheter. After infusion, microvessel clip, injection needle, and silk ligature were removed, reconstituting physiological flow of bile. Puncture site at the duodenal wall was closed with 5/0 Prolene suture (Ethicon, Inc., Somerville, NJ, USA), and the abdomen was closed with 3/0 silk.

Sample Collection and Laboratory Analyses

After 72 hours, rats were anesthetized with sodium pentobarbital. Blood samples were collected by cardiac puncture. All animals were sacrificed with excessive anesthesia, and the pancreas, lungs and kidneys were excised for histopathological examination.

Biochemical Analyses

Serum amylase and lipase levels were measured using automated analyzer.

Histopathological Evaluation

Samples of pancreatic, pulmonary, and renal tissue were collected for histopathological examination. Relevant specimens were fixed in 10% formalin solution. Formalin-fixed tissues were embedded in paraffin, and 3 to 5 μm sections were cut and stained with hematoxylin and eosin to be evaluated by light microscopy. Injury in the pancreas was scored by recording number of lobes affected according to several criteria, including edema, hemorrhage, leucocyte infiltration, acinar necrosis, peripancreatic fat necrosis, fibrosis, and acinar vacuolization. Injury in the lungs was scored with respect to intraalveolar hemorrhage, parenchymatous congestion, edema, and inflammation. Injury in the kidneys was scored for interstitial hemorrhage, vacuolization in tubular epithelium, and epithelial necrosis. Each criterion was graded on scale of 0 to 3.

Neutrophil Function Evaluation

In assessment of neutrophil functions, phagocytosis, oxidative burst, opsonization, and chemotaxis tests were evaluated using flow cytometry. Dihydrorhodamine 123 (DHR 123) dye (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA) was used to measure mitochondrial responses to different stimulants: Escherichia coli for phagocytosis, C3b for opsonization, N-Formylmethionyl-leucyl-phenylalanine for chemotaxis, and phorbol 12-myristate 13-acetate for oxidative burst. Neutrophils were obtained by layering 1:1 saline-diluted whole blood onto Ficoll (Sigma-Aldrich Corp.,

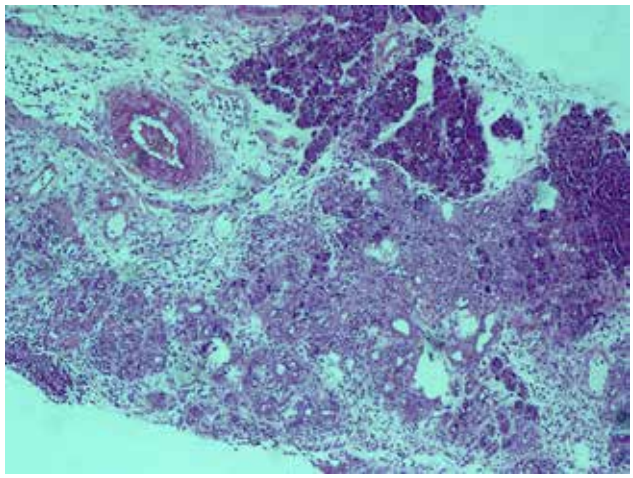

Figure 1. Microscopic image. Histopathology of the pancreas from Group 2 (pancreatitis) revealing inflammatory cell infiltration creating significant acinar and fat necrosis (hematoxylin and eosin stain x40).

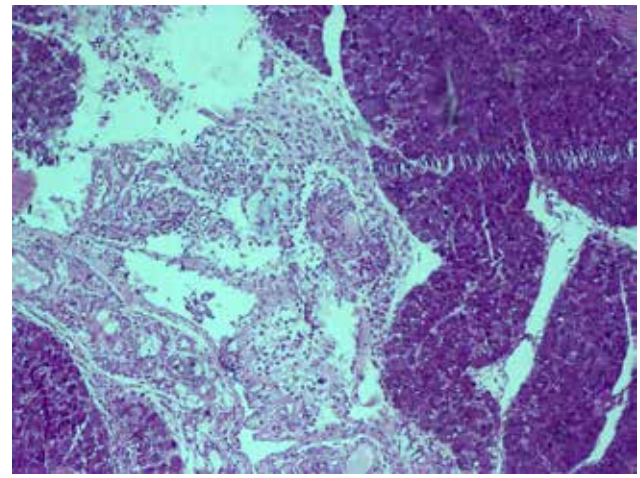

Figure 2. Microscopic image. Histopathology of the pancreas from Group 3 (pancreatitis and N-acetylcysteine treatment) revealing peripancreatic area of fat necrosis (hematoxylin and eosin stain x100).

St. Louis, MO, USA) and left at room temperature for 40 minutes for density separation by gravity. Upper 500 µL was collected, and each group of samples was run in parallel with healthy rat sample as control. After stimulants were added to granulocytes, measurements of fluorescence were completed at 0 and 20 minutes using BD FACSCalibur flow cytometer (BD BioSciences, Inc., San Jose, CA, USA). Daily calibration and quality control of the system was performed with BD CaliBrite beads (BD BioSciences, Inc., San Jose, CA, USA). Forward scatter/side scatter gram was used for identification and gating of granulocytes and FL1 (green fluorescence) histogram was used to observe fluorescence changes. For each tube, 2500 granulocytes were counted. Index value for each test was obtained by dividing test sample mean fluorescence channel value by control sample.

Statistical Methods

All statistical measurements were performed using SPSS software, version 15.0 (IBM, Corp., Armonk, NY, USA). Comparison of mean values of groups for statistical analysis as well as histopathological scores was performed using Kruskal-Wallis test, whereas dual comparisons were performed using Mann-Whitney U test. Value of $p<0.05$ was considered statistically significant.

RESULTS

Biochemical Findings

Serum amylase and lipase levels were significantly higher in both AP groups (G2 and G3) compared with sham group (G1). These levels were significantly lower in NAC-treated group (G3) than group with AP alone (G2).

Local (Pancreatic) Histopathological Changes

Polymorphonuclear leucocyte (PNL) infiltration was elevated in both pancreatitis groups. NAC treatment slightly, but not

Table 1. Results of histopathological analysis of pancreatic tissue in study groups

	Group 1 (G1)				Group 2 (G2)				Group 3 (G3)				Means[1]		
Pathology	**Control**				**Acute pancreatitis**				**Acute pancreatitis+NAC**				**G1**	**G2**	**G3**
Grade	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**			
Edema	7	1	–	–	–	3	2	3	–	5	3	–	0.13	2*	1.38*
Hemorrhage	4	4	–	–	–	4	1	3	–	3	4	1	0.50	1.88	1.5
Leukocyte infiltration	8	–	–	–	–	4	4	–	–	5	3	–	0	1.5	1.38
Acinar necrosis	8	–	–	–	–	–	4	4	–	2	3	3	0	2.5	2.13
Peripancreatic fat necrosis	8	–	–	–	–	2	4	2	–	2	4	2	0	2	2
Fibrosis	8	–	–	–	–	7	1	–	1	4	3	–	0	1.13	1.25
Acinar vacuolization	8	–	–	–	–	–	6	2	–	3	2	3	0	2.25	2

[1]Comparison between G2 and G3 results: No changes was significant ($p>0.05$) except edema (*$p<0.05$). NAC: N-acetylcysteine.

significantly, reduced leucocyte infiltration. Significant acinar and fat necrosis was histopathologically observed in AP groups (Figure 1). Fat necrosis was not reduced with NAC treatment (Figure 2). All microscopic findings were slightly, but not significantly, reduced by NAC treatment ($p>0.05$). Edema was the only microscopic finding for which NAC treatment provided significant reduction (Table 1).

Systemic Histopathological Changes

Significant detrimental histological changes were found in pulmonary tissue of animals with AP compared with sham group. NAC treatment significantly decreased damage, such as congestion, edema, inflammation, and intra-alveolar hemorrhage, in the pulmonary tissue, compared with group with AP alone (Table 2).

Significant detrimental changes were also observed in renal tissue of animals with AP compared with sham group. NAC treatment significantly ameliorated renal histology compared with group with AP alone (Table 3).

Table 2. Results of histopathological analysis of pulmonary tissue in study groups

	Group I (G1)				Group 2 (G2)				Group 3 (G3)				Means[1]		
Pathology	Control				Acute pancreatitis				Acute pancreatitis+NAC				G1	G2*	G3*
Grade	0	I	2	3	0	I	2	3	0	I	2	3			
Congestion	3	4	I	–	–	2	4	2	–	6	2	–	0.75	2	1.25
Edema	7	I	–	–	–	3	4	I	2	6	–	–	0.13	1.75	0.75
Inflammation	4	4	–	–	–	2	4	2	–	5	3	–	0.50	2	1.38
Intra-alveolar hemorrhage	5	3	–	–	–	I	3	4	–	4	4	–	0.38	2.38	1.50

[1]Comparison between G2 and G3 results: All changes were significant (*$p<0.05$). NAC: N-acetylcysteine.

Table 3. Results of histopathological analysis of renal tissue in study groups

	Group I (G1)				Group 2 (G2)				Group 3 (G3)				Means[1]		
Pathology	Control				Acute pancreatitis				Acute pancreatitis+NAC				G1	G2*	G3*
Grade	0	I	2	3	0	I	2	3	0	I	2	3			
Interstitial hemorrhage	6	2	–	–	–	3	4	I	3	4	I	–	0.25	1.75	0.75
Vacuolization in tubular epithelium	4	4	–	–	–	–	7	I	2	4	2	–	0.50	2.13	1.0
Necrosis in tubular epithelium	6	2	–	–	–	I	6	I	I	4	3	–	0.25	2	1.25

[1]Comparison between G2 and G3 results: All changes were significant (*$p<0.05$). NAC: N-acetylcysteine.

Table 4. Mean values of neutrophil function tests in acute pancreatitis

	Group I (G1)	Group 2 (G2)	Group 3 (G3)	Statistical analysis
Function	Sham	Acute pancreatitis	Acute pancreatitis + NAC	P (G2 vs G3)
	Mean±SD	Mean±SD	Mean±SD	
Phagocytosis	2.0±0.28	1.39±0.18	2.06±0.18	0.001
Chemotaxis	2.13±0.40	1.33±0.22	1.84±0.15	0.002
Opsonization	2.1±0.28	1.47±0.17	1.94±0.35	0.003
Oxidative burst	1.78±0.51	1.38±0.15	1.47±0.09	0.313

SD: Standard deviation; NAC: N-acetylcysteine.

Flow Cytometry of Neutrophil Activity

Flow cytometry results revealed that all tested neutrophil functions were significantly affected in group with AP alone (G2) compared with sham group (G1). In AP group with NAC treatment (G3), phagocytosis, chemotaxis, and opsonization of PNL were significantly improved with NAC treatment ($p<0.05$). After NAC treatment, only oxidative burst was not changed significantly among neutrophil functions (Table 4).

DISCUSSION

Severity of AP depends on extent of systemic inflammatory response, as well as cytokines and chemokines, which play critical roles in the activation and migration of inflammatory cells. It is generally believed that pancreatitis begins with intrapancreatic activation of digestive enzyme zymogens, acinar cell injury, and activation of transcription factors.[1,2] Active enzymes in pancreatic acinar cells create inflammation, spreading into the pancreatic tissues. Our results of serum level of pancreatic amylase and lipase indicated that AP was induced by taurocholate infusion. Our histopathological findings confirmed cellular damage due to detrimental enzymatic activity in the pancreatic tissue. In addition, we found that NAC treatment mildly ameliorated edema and hemorrhage in tissues. Histopathological examination confirmed slight reduction of acinar necrosis with NAC treatment. NAC provides effective protection against pancreatic and vital organ damage and extrapancreatic complications during severe AP.[11,12] NAC improves pancreatic microvascular perfusion and alleviates severity of AP.[13] Histopathological results of vital organs, the lungs and kidneys, indicated systemic deteriorating effects of AP. Although AP is local inflammatory process in pancreatic tissue, systemic responses increase severity of the pathological condition. Results in NAC-treated group revealed that NAC ameliorated systemic effects of the disease. Seo et al.[3] recently reported that antioxidant NAC attenuates cellular responses and reduces cell death.

Flow cytometry indicated that PNL activity was ameliorated by NAC treatment in rats with experimental AP. Neutrophils play central role in producing cellular immune response. They are the first phagocytic cells to appear at site of inflammation. Neutrophil activation, subsequent phagocytosis, and release of oxygen radicals play crucial role in host defense. Defects in neutrophil function cause increased incidence of sepsis and multiorgan failure.[14] Experimental and clinical studies have shown that oxidative stress and leucocyte infiltration play important roles in AP. Reactive oxygen species (ROS) produced within the pancreas by acinar cells and infiltrating leucocytes trigger activation of signaling pathways regulating the gene expression of inflammatory mediators and increased production of cytokines and chemokines, which induce the progression of local pancreatic inflammation to systemic inflammatory reaction and remote organ damage.[3–6] In our study, ameliorating the action of NAC on PNL activity was confirmed with chemotaxis, phagocytosis, and opsonization of PNL measured with flow cytometry. Therefore, we can conclude that NAC reduced systemic responses via pathways for neutrophil action during AP process. In addition to pancreatic cellular damage and necrosis, systemic inflammatory responses significantly affect clinical outcomes of AP cases. We found that main neutrophil functions, such as phagocytosis, chemotaxis, and opsonization, were impaired by AP. These effects could increase production of ROS and provoke severe inflammatory responses. We can hypothesize that some antioxidants reverse effects of OFR and palliate local and systemic complications during AP. Santos et al.[15] recently reported that NAC prevents inflammatory responses induced by various detrimental biochemical materials. NAC reduces pancreatic oxidative stress and inhibits signaling pathways involved in generation of inflammation.[16] The outcome of a patient is mostly dependent on systemic complications of AP. NAC-induced antioxidant effects could be helpful to lessen local and systemic alterations of AP.[3,11–13] In our study, results of flow cytometry indicated detrimental neutrophil functions during AP. These functions were recovered with NAC treatment despite ongoing pancreatitis. Yagci et al.[8] found that leucocyte infiltration and production of lipid peroxidation were lower in NAC-treated group. Due to direct scavenging effects of NAC on ROS, higher remaining nitric oxide may have been beneficial to regulation of microcirculation in the pancreatic tissue in NAC-treated group. Improvement in pancreatic microvascular perfusion with NAC treatment alleviates severity of AP.[13] In some clinical studies, NAC has been used for AP, post-endoscopic retrograde cholangiopancreatogram pancreatitis, and chronic pancreatitis. They have reported that NAC decreases severity of AP.[9,17] Severity of systemic responses and vital organs impairment are main determinants of prognosis during AP. Neutrophil functions, such as phagocytosis, chemotaxis and opsonization, could increase production of ROS and cytokines and provoke severe changes. Recent publications have studied effects of some chemicals on systemic responses to experimental pancreatitis by way of neutrophil function. They reported that reduction of neutrophil accumulation, depletion of neutrophils, and inhibited infiltrations of macrophages and neutrophils improved systemic events during experimental AP.[18–20] Therefore, chemicals that improve neutrophil functions may reduce detrimental systemic effects of AP.

Gender of animals may affect systemic and local inflammatory response. In the literature, majority of studies used male rats,[3,4,6,7,13,14,19] some both male and female rats without sex discrimination,[12] and finally some studies used only female rats.[21,22] In this study, we used female rats. We believe that using animals of same gender provided homogeneity of the study in terms of inflammatory response. Based on results of present and previous studies, we can comment that whatever the gender of experimental animals, NAC treatment alleviates systemic response to experimental AP.

Based on results of current study, we concluded that NAC

treatment cannot prevent spreading active enzymes into pancreatic tissue. Although mild improvement was observed in NAC-treated group, cellular damage and tissue necrosis occurred despite NAC treatment. In addition to local inflammation and necrosis, severity of systemic inflammatory responses and extrapancreatic vital organ impairment are main determinants of prognosis of patients with AP. Cytokines, PNL products, and OFRs are effective against severity of systemic events. Uncontrolled and defective neutrophil functions, such as phagocytosis, chemotaxis, and opsonization, could increase production of ROS and cytokines and provoke severe inflammatory responses. Therefore, effects of antioxidants that reverse (or ameliorate) PNL functions may have beneficial effects on systemic changes during AP. Based on our findings, we observed that neutrophil functions were recovered by NAC treatment despite ongoing AP. This effect may prevent systemic responses and extrapancreatic damages. We concluded that NAC treatment strengthens functions of neutrophils, which play important role in enhancement of host immune responses during AP, and reduces extrapancreatic tissue damage.

Conflict of interest: None declared.

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DENEYSEL ÇALIŞMA - *ÖZET*

Deneysel akut pankreatitte nötrofil fonksiyonları üzerine N-asetilsistein'in etkisi

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AMAÇ: Akut pankreatitli (AP) olgulardaki sistemik enflamatuvar cevap ve pankreas dışı vital organ bozuklukları aktive olmuş nötrofillerin fonksiyonları ve ürünleri olan serbest oksijen radikalleri aracılığıyla oluşur. Çalışmamızda, deneysel AP süresince sistemik histopatolojik değişiklikler ve nötrofil fonksiyonları üzerinde antioksidan N-asetilsistein'in (NAC) etkilerinin araştırılması amaçlandı.

GEREÇ VE YÖNTEM: Bu deneysel çalışma eşit olarak üç gruba ayrılmış 24 Wistar-albino sıçan üzerinde gerçekleştirildi. Grup 1: Yalnız laparotomi grubu, grup 2: Taurokolat infüzyonu ile oluşturulan AP grubu, grup 3: NAC tedavisi yapılan AP grubu. Lokal ve sistemik etkiler için böbrek, akciğer ve pankreas dokusundaki histopatolojik değişiklikler araştırıldı. Nötrofil fonksiyonları flow sitometri yöntemiyle belirlendi.

BULGULAR: Her iki AP grubunda pankreas enzimlerinin serum seviyeleri yüksek bulundu. Histopatolojik bulgularda asiner hücre hasarı ve pankreas doku nekrozu vardı. Eş zamanlı olarak renal ve pulmoner dokuda ciddi histopatolojik değişiklikler gözlendi. Flow sitometri bulguları, tedavisiz AP grubunda bozulan nötrofil fonksiyonlarını göstermekteydi. Akut pankreatitli deneklerde NAC tedavisi nötrofillerin fagositoz, kemotaksis ve opsonizasyon işlevlerini anlamlı olarak iyileştirdi ($p<0.05$). Aynı zamanda NAC tedavisiyle pulmoner ve renal dokudaki hasarların, bütün mikroskobik parametreler için anlamlı olarak düzeldiği gözlendi ($p<0.05$).

TARTIŞMA: Deneysel AP süresince, bozulan nötrofil fonksiyonları ciddi sistemik enflamatuvar cevapları uyarabilir. Lokal enflamasyon ve nekroza ek olarak pankreas dışı vital organlarda ciddi sistemik cevap ve histopatolojik değişiklikler oluşur. Antioksidan NAC tedavisi, pankreas dışı vital organlardaki zararlı sistemik cevapları, nötrofil fonksiyonlarını anlamlı ölçüde iyileştirerek geri döndürmektedir.

Anahtar sözcükler: Flow sitometri; lökosit; pankreatit; sistemik enflamatuvar cevap.

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