Turk J Gastroenterol *2016; 27: 68-72*

Oxidative status and lymphocyte DNA damage in patients with acute pancreatitis and its relationship with severity of acute pancreatitis

PANCREAS

Ali Dur[1], Orhan Kocaman[2], Abdurrahim Koçyiğit[3], Kenan Ahmet Türkdoğan[4], Ertan Sönmez[1], Sıddıka Keskin[3], Mehmet Yiğit[1], Bedia Gülen[1], Elif Kılıç[3], Ömer Uysal[5]

[1]Department of Emergency Medicine, Bezmialem Vakıf University Faculty of Medicine, İstanbul, Turkey
[2]Department of Gastroenterology, Bezmialem Vakıf University Faculty of Medicine, İstanbul, Turkey
[3]Department of Biochemistry, Bezmialem Vakıf University Faculty of Medicine, İstanbul, Turkey
[4]Department of Emergency Medicine, Adnan Menderes University Faculty of Medicine, Aydın, Turkey
[5]Department of Biostatistics, Bezmialem Vakıf University, Faculty of Medicine, İstanbul, Turkey

Original Article

ABSTRACT

Background/Aims: Acute pancreatitis (AP) is a life-threatening disease with a rising incidence. The aim of this study was to investigate the association between oxidative status, lymphocyte deoxyribonucleic acid (DNA) damage, and acute pancreatitis.

Materials and Methods: A total of 45 patients with AP and 35 healthy controls were included in the study. We assessed pancreatic enzymes, oxidative stress, and lymphocyte DNA damage. The severity of AP disease was determined by the Harmless Acute Pancreatitis Score (HAPS) and Balthazar scoring systems.

Results: In AP patients, lymphocyte DNA damage was significantly higher than in controls [49.84±25.48 arbitrary units (AU) vs. 28.80±13.98 AU, p<0.001]. The plasma total oxidative status (TOS) and oxidative stress index (OSI) were higher in patients than in healthy controls (10.36±5.54 vs. 8.47±2.66, p<0.05; 0.64±0.35 vs. 0.45±0.13 AU, p<0.001, respectively). The plasma total antioxidant status level in patients was lower than in healthy controls (1.66±0.19 vs. 1.86±0.18, p<0.001). Lymphocyte DNA damage was correlated with TOS, OSI, and HAPS and Balthazar scores.

Conclusion: This study shows that patients with AP have higher lymphocyte DNA damage and more deteriorated oxidative status than healthy controls.

Keywords: Acute pancreatitis, plasma oxidative status, lymphocyte DNA damage

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease that is prevalent worldwide and causes local and systemic complications. Although the majority of cases with AP present with mild disease, AP causes inability to work, decrease in productivity, and work loss. In the severe form of AP, systemic complication and multiorgan failure may develop and result in mortality. In patients with severe AP (SAP), the range of mortality changes between 10% and 30%, and selecting patients with SAP as early as possible after the onset of symptoms is crucial for effective treatment (1,2). Acute Physiology and Chronic Health Evaluation (APACHE), Balthazar, and Harmless Acute Pancreatitis Score (HAPS) are the conventional scoring systems used in clinical practice (3). However, the optimal scoring system for the initial phase of AP is still lacking. Recently, high-sensitivity C-reactive protein (HsCRP); interleukin (IL)-2, 6, and 8, and B-type natriuretic peptide (BNP) are the investigational parameters for determining the severity of AP.

The relation of oxidative stress-induced free oxygen radicals with pancreatitis, chronic diseases, cancer, and neurodegenerative and cardiovascular diseases is one of the most studied areas in the last decade. The excessive production of free oxygen radicals may damage macromolecules such as protein, fat, and DNA (4). The experimental studies in rats showed that increased free

Address for Correspondence: Orhan Kocaman E-mail: drokocaman@hotmail.com
Received: August 23, 2015 **Accepted:** December 14, 2015

oxygen radicals cause damage to cells and induce apoptosis; in contrast, antioxidant substances decrease the severity of AP (4,5). Lymphocyte oxidative DNA damage is considered as a reference marker for oxidative stress in the cell and apoptosis (6). Hitherto, there has been no study conducted on the topic of relation between lymphocyte DNA damage in the early phase of AP and plasma total oxidative status (TOS), plasma total antioxidant status (TAS), and the severity of AP.

In this study, we aimed to compare lymphocyte DNA damage and oxidative stress values in patients with AP scored according to the HAPS and Balthazar scoring system. To the best of our knowledge, the current study is the first in the literature which evaluates the association between lymphocyte DNA damage and AP and its severity.

MATERIALS AND METHODS

Patients and controls

This prospective study included 45 consecutive patients with AP and 35 controls and was conducted between November 2013 and December 2014. The control group consisted of sex-matched and age-matched healthy individuals. The diagnosis of AP was based on the clinical manifestation of acute upper abdominal pain with or without guarding and/or rebound tenderness associated with an increased serum amylase level higher than three times the normal value or elevated serum lipase levels and radiological evidences compatible with AP (7). Patients with pain lasting longer than 24 h, usage of supplemental vitamins, intravenous drug abuse, pregnancy, ischemic and inflammatory disease, and chronic renal failure were excluded from the study. Patients with AP with alcoholic and biliary origin were excluded from the study because these etiologies would interfere with the oxidative status of the patients. All the AP cases were extensively studied for the etiology of AP and classified as idiopathic AP. All patients were hospitalized and administered palliative treatment, including intravenous fluid replacement and opioids, as needed. Abdominal computed tomography with contrast enhancement was performed for all patients, and the Balthazar scoring system (8) was used for calculating the severity of AP. In addition, the HAPS system (9) was used to determine the severity of AP for all patients on presentation to the emergency unit.

According to the ethical guidelines of the Helsinki declaration, as revised in 1989, informed consent was obtained from all participants, and the study was monitored by the local ethical committee of the Bezmialem Vakif University.

Measurement of total oxidant status

Plasma TOS was measured using a novel automated method developed by Erel et al. (10). Oxidants present in a sample oxidize the ferrous ion of an o-dianisidine complex to ferric ion. Oxidation is enhanced by glycerol, which is abundant in the reaction medium, and the ferric ion forms a colored complex with xylenol orange under acidic conditions. The color intensity (which can be measured spectrophotometrically) is associated with the total level of oxidants present. Hydrogen peroxide (Sigma-Aldrich®; Munich, Germany) is used to calibrate the assay, and the results are expressed in terms of micromoles of hydrogen peroxide equivalent per liter (μmol H_2O_2 equiv./l).

Measurement of total antioxidant status

Plasma TAS was measured using another novel automated method developed by Erel et al. (11). This involves production of the hydroxyl radical, which is a potent biological reactant. A ferrous ion solution (Reagent 1) is mixed with hydrogen peroxide (Reagent 2). Radicals produced by the hydroxyl radical, including the brown dianisidinyl radical cation, are also potent in biological terms. Thus, it is possible to measure the antioxidative capacity of a sample in terms of the inhibition of free radical reactions initiated by the production of the hydroxyl radical. Variation in assay data is very low (less than 3%), and the results are expressed as mmol Trolox equiv./L.

Determination of the oxidative stress index

The percent ratio of TOS to TAS level was accepted as the oxidative stress index (OSI), an indicator of the degree of oxidative stress (10). To perform the calculation, the result unit of TAS, mmol Trolox equivalent/L, was converted to μmol equivalent/L, and the OSI value was calculated as follows: OSI=[(TOS, μmol/L)/(TAS, mmol Trolox equivalent/L)×100].

Lymphocyte DNA damage determination by the alkaline comet assay

Human blood samples were collected by venipuncture from the antecubital vein (~5 mL) and immediately transferred into tubes containing sterile EDTA solution (EDTA Titriplex® III, SCM Corp.; Burlingame, CA, USA). In total, 250 μL of blood samples were transferred by a pipette into an Eppendorf tube. Blood was kept in fridge at -80°C and studied in 4 weeks.

Endogenous DNA damage was analyzed by the alkaline comet assay in accordance with Singh et al. (12) with little modifications. Microscope slides were coated with 1.0% hot (60°C) normal melting point agarose (NMA) prepared in phosphate buffered saline (PBS) and then covered with a coverslip at 4°C for at least 5 min to allow the agarose to solidify. After the removal of coverslips, 5 μL of whole blood was embedded in 0.7% low-melting point agarose (LMA) at 37°C. In addition, 85 μL of this cellular suspension was then spread onto slides that had previously been coated with NMA and was covered with a coverslip. The slides were allowed to solidify for 10 min at 4°C in a moist box. After the removal of coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH 10–10.5; 1% Triton X-100 was added just before use) for at least 1 h. The slides were then placed on the horizontal electrophoresis unit. The DNA was allowed to unwind for 40 min in the electrophoresis running buffer solution (0.3 mol/L NaOH and 1 mmol/L Na_2EDTA, pH >13). Electrophore-

Original Article

sis was set at 25 V, 300 mA for 20 min at an ambient temperature of 4°C. After electrophoresis, the slides were placed in a horizontal position and washed two times for 5 min each to remove alkali and detergent in a phosphate buffer saline and once with distilled water. Finally, 85 µl of ethidium bromide (2 µg/mL) was added to each slide, which was then covered with a coverslip and analyzed using a fluorescence microscope. Hundred images were randomly selected from each sample and were classified according to their tails (from undamaged class 0 to maximally damaged class 4). The total score of samples were between 0 and 400 arbitrary unit.

Determination of severity the disease

We used the Balthazar scoring system and HAPS to assess the severity of AP. These scoring systems have been developed for the early prediction of disease severity and to further enable an earlier risk stratification of SAP patients and assist physicians to start appropriate therapy (8,9).

The Balthazar scoring system is widely accepted, fast and easy to perform, and does not require the intravenous administration of contrast material. This system can be used to identify a subgroup of individuals (with grade D or E) at risk of death or a high morbidity rate. In this study, HAPS-positive and Balthazar grade D and E patients were considered as patients with SAP.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 15 (SPSS Inc.; Chicago IL, USA). Continuous variables were expressed as means±standard deviation or medians and 25^{th}–75^{th} percentile values (Inter quartile range: IQR) (normally and not normally distributed, respectively). Categorical variables were also expressed as percentages. Statistical differences among groups were tested by one-way analysis of variance with post hoc Scheffé correction or Kruskal–Wallis test for parametric or nonparametric variables, respectively.

RESULTS

Forty-five patients with AP (15 males, 33.3%) and 35 age-matched healthy controls (8 males, 29.6%) were enrolled in this study. The demographic, clinical, and laboratory features of the patients and controls are presented in Table 1. There were no differences in age and sex, but differences were observed in diabetes mellitus and hypertension. Between the patients with AP and controls, diabetes, hypertension, and smoking were more prevalent in patients with AP than in controls ($p<0.05$, for each variable). White blood cell, glucose, amylase, lipase, total bilirubin, and lactic dehydrogenase (LDH) levels were significantly higher in patients with AP.

The value of lymphocyte DNA damage and TOS were significantly higher in AP subjects than that in healthy controls. The OSI levels were also significantly higher in AP subjects than in healthy controls, while the TAS levels were significantly lower in the AP group (Figure 1). HAPS-positive patients had significantly higher lymphocyte DNA damage and OSI levels and significantly lower TAS levels than HAPS-negative patients (Table 2). We also evaluated serum TAS, TOS, OSI, and lymphocyte DNA

Table 1. Demographic and clinical parameters in AP patients and controls

	Control (n=35)	AP (n=45)	p
Baseline characteristics			
Age (years)	49.62±9.06	52.73±15.38	0.264
Male/female	8/27	15/30	0.304
Hypertension	0	11	
Diabetes mellitus	0	7	
Smoking	0	5	
Biochemical tests			
Glucose (mg/dL)	105.6 (68–165)	154.95 (63–769)	0.008
Creatinine (mg/dL)	0.74±0.11	0.79±0.22	0.277
Total bilirubin (mg/dL)	0.72 (0.3–1.6)	1.48 (0.3–5.7)	0.002
AST (U/L)	39.22 (15–98)	149.26 (15–935)	0.001
ALT (U/IL)	30.20 (10–78)	171.51 (10–894)	0.001
Amylase (U/L)	66.94 (28–110)	1834 (66–9422)	0.001
Lipase (U/L)	48.91 (20–130)	2962 (25–14000)	0.001
Calcium (mg/dL)	9.49±0.59	9.43±0.74	0.696
Hs-CRP (mg/dL)	0.73 (0.2–2.2)	2.5 (0.1–33.30)	0.070
WBC ($\times10^9$ cells/L)	6.48±1.83	11.47±4.45	0.001
Hematocrit (%)	40.83±2.30	39.75±4.74	0.188
Thrombocyte count ($\times10^9$ cells/L)	241.05±90.57	263.37±68.50	0.230

Results are n, mean±SD or median (interquartile range).
AP: acute pancreatitis; BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; hs-CRP: high-sensitivity C-reactive protein; WBC: white blood cell

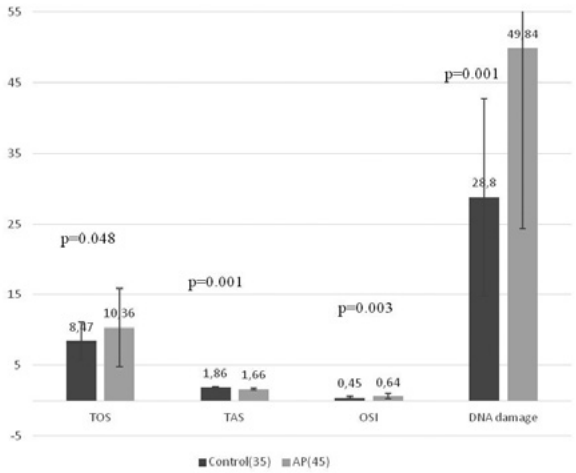

Figure 1. DNA damage values and oxidative stress parameters in AP patients and controls.
Values are mean±SD. Significance was defined as p<0.05.
AP: acute pancreatitis; TOS: total oxidative status; TAS: total antioxidant status; OSI: oxidative stress index

damage levels according to the Balthazar score by logistic regression analysis and found that lymphocyte DNA damage and TAS levels were significantly correlated with the severity of pancreatitis (Table 3).

DISCUSSION

Oxidative damage to DNA was shown to be increased in a number of pathologies, including neoplastic, neurodegenerative, and cardiovascular autoimmune diseases, and very little data regarding oxidative status and DNA damage were present in case of inflammatory conditions (13-16).

In this study, lymphocyte DNA damage was found to be significantly higher in patients with AP than healthy controls. One of the most striking aspects of our results was a very high level of lymphocyte DNA damage in the SAP patients. We also found decreased TAS levels and increased TOS and OSI levels in patients with AP. According to the Balthazar scoring system, the TAS levels and lymphocyte DNA damage were also correlated with the severity of AP. Similar studies in the literature used the same sensitive, easy, and fast-applicable Comet assay technique to reveal DNA breaks and damage induced by oxidative stress (17). However, in our study, the TOS levels compared to other parameters were less correlated with AP and had no correlation with the severity of AP staged according to the Balthazar scoring system. Therefore, it is thought that DNA damage may be more related with insufficient antioxidant capacity and less excessive ROS generation, which contributed to the pathogenesis of the disease in AP patients. Methemalbumin, IL-6, IL-8, and C-reactive protein (CRP) were used to determine the severity of AP in the literature (18). Although CRP is the most commonly used inflammatory marker in daily clinical practice in patients with AP, the yield of CRP for the evaluation of the severity of AP within 12 h of AP is very low (19). The findings of increased DNA damage in concordance with the severity of AP even in the first 12 h of AP in all patients may be complementary to CRP evaluation and help triage of the patients with AP in the very early stages of disease.

Table 2. DNA damage values and oxidative stress parameters according to the HAPS score

Parameters	HAPS positive (n=24)	HAPS negative (n=56)	p
TOS (μmol H_2O_2 equiv./L)	10.85±5.74	8.97±3.92	0.153
TAS (mmol Trolox equiv./L)	1.65±0.17	1.79±0.21	0.006
OSI (AU)	0.66±0.35	0.51±0.26	0.046
DNA damage (AU)	60.16±22.06	32.27±18.91	0.001

Values are mean±SD. Significance was defined as p<0.05.
HAPS: harmless acute pancreatitis score; TOS: total oxidative status; TAS: total antioxidant status; OSI: oxidative stress index; AU: arbitrary units

Although there are many scoring systems developed for evaluating the severity of AP, HAPS with its easy-applicability and cost-effectivity is better than the other scoring systems and had more use in daily clinical practice (9). In our study, we found more lymphocyte DNA damage and lower TAS levels in HAPS-positive patients than HAPS-negative patients. Similar to the Balthazar scoring system, the TOS levels was not a statistically significant parameter for the determination of SAP according to the HAPS score. Thus, in patients with SAP, the evaluation of the TAS levels in conjunction with lymphocyte DNA damage may better delineate the protective status of the patients against oxidative stress. The absence of significant TOS levels in correlation with TAS and lymphocyte DNA damage levels support the finding in the literature that different oxidative stress factors with different anti-oxidant capacity may function in distinct inflammatory conditions (20,21). The reflection of oxidative status in AP with the findings of reduced TAS levels and severity of lymphocyte DNA damage and severity of AP in the early stages are the two main important finding in our study. The determination of the clinical scenario from the onset of AP by the measurement of DNA damage and the TAS levels may give the opportunity for establishing innovative treatments to reduce the risk of severe AP.

This study has some limitations which have to be highlighted. Because of the technical difficulty regarding the necessity of the rapid measurement of lymphocyte DNA damage, limited number of patients was recruited for the study. The cost of measuring the TAS, OSI, TOS, and lymphocyte DNA damage is high, and the evaluation of these parameters requires sophisticated laboratory equipment. The cost-effectivity, easy-applicability, and reproducibility of these laboratory parameters remain as an important problem in daily clinical practice.

Table 3. DNA damage values and oxidative stress parameters according to the Balthazar scores

Parameters	Grade A (n=7)	Grade B (n=6)	Grade C (n=16)	Grade D (n=7)	Grade E (n=9)	p
TOS (μmol H_2O_2 equiv./L)	8.72±3.57	8.41±5.5	11.21±6.36	11.88±5.66	10.25±5.60	>0.05
TAS (mmol Trolox equiv./L)	1.74±0.14	1.64±0.1	1.68±0.24	1.70±0.14	1.56±0.14*	<0.05*
OSI (AU)	0.50±0.20	0.52±0.3	0.68±0.40	0.72±0.36	0.67±0.40	>0.05
DNA damage (AU)	28.85±13.8	44.33±3.	46.56±22.6	55.43±16.5	71.33±25.6	<0.05$^{\Omega}$

TOS: total oxidative status; TAS: total antioxidant status; OSI: oxidative stress index; AU: arbitrary units
*TAS levels are significantly higher in grade A than in grade E
$^{\Omega}$DNA damage is significantly more in grade E than in grades A, B, and C
Values are mean±SD. Significance was defined as p<0.05.

When contrast-enhanced tomography-based Balthazar scoring system is correlated with lymphocyte DNA damage for the evaluation of AP, the data of lymphocyte DNA damage can differentiate stage A from stage E and stage C from stage E. Thus, to avoid contrast and radiation exposure at the onset of the staging process, the measurement of lymphocyte DNA damage may form a reference for following computed tomography (CT) evaluation. The AP patients with high lymphocyte DNA damage undergo CT evaluation and may be triaged to the intensive care unit because of the high risk of developing complications. To make a joint consensus statement about this innovative approach, we need a higher sample size to clarify the accuracy of this new triage method.

In conclusion, this study revealed that the patients with AP have higher lymphocyte DNA damage and more deteriorated oxidative status than healthy controls. The finding of higher DNA damage in concordance with the severity of AP even in the first 12 h of AP may help the clinician predict the severity and prognosis of AP within the early onset of AP. The development of a new triage system for AP cases based on the measurement of oxidative status and lymphocyte DNA damage may provide a more predictable and cost-effective approach for the treatment of AP.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Bezmialem Vakıf University.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - A.D., O.K.; Design - A.D., O.K., A.K., K.A.T.; Supervision - O.K., A.K.; Funding - S.K., M.Y., B.G., E.K.; Materials - A.D., E.S.; Data Collection and/or Processing - A.D., O.K.; Analysis and/or Interpretation - Ö. U., O.K., A.D.; Literature Review - O.K., E.S., A.D.; Writer - O.K., A.D.; Critical Review - O.K., E.S., A.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

REFERENCES

1. Talukdar R, Swaroop Vege S. Early management of severe acute-pancreatitis. Curr Gastroenterol Rep 2011; 13: 123-30. [CrossRef]
2. Servín-Torres E, Velázquez-García JA, Delgadillo-Teyer G, Galindo-Mendoza L, Bevia-Pérez F, Rivera-Bennet F. Severe acute pancreatitis: surgical management in a third-level hospital. Cir Cir 2009; 77: 407- 10.
3. Lempinen M, Puolakkainen P, Kemppainen E. Clinical value of severity markers in acute pancreatitis. Scand J Surg 2005; 94: 118-23.
4. Esrefoglu M. Experimental and clinical evidence of antioxidant therapy in acute pancreatitis. World J Gastroenterol 2012; 18: 5533-41. [CrossRef]
5. Booth DM, Murphy JA, Mukherjee R, et al. Reactive oxygen species induced by bile acid induce apoptosis and protect against necrosis in pancreatic acinar cells. Gastroenterology 2011; 140: 2116-25. [CrossRef]
6. Collins AR. Oxidative DNA damage, antioxidants, and cancer. Bioessays 1999; 21: 238-46. [CrossRef]
7. Rau BM, Kemppainen EA, Gumbs AA, et al. Early assessment of pancreatic infections and overall prognosis in severe acute pancreatitis by procalcitonin (PCT): a prospective international multicenter study. Ann Surg 2007; 245: 745-54. [CrossRef]
8. Balthazar EJ. Acute pancreatitis: assessment of severity with clinical and CT evaluation. Radiology 2002; 223: 603-13. [CrossRef]
9. Lankisch PG, Weber-Dany B, Hebel K, Maisonneuve P, Lowenfels AB. The harmless acute pancreatitis score: a clinical algorithm for rapid initial stratification of nonsevere disease. Clin Gastroenterol Hepatol 2009; 7: 702-5. [CrossRef]
10. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005; 38: 1103-11. [CrossRef]
11. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004; 37: 277-85. [CrossRef]
12. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175: 184-91. [CrossRef]
13. Banks PA, Freeman ML. Practice Guidelines in Acute Pancreatitis. Am J Gastroenterol 2006; 101: 2379-400. [CrossRef]
14. Rahman SH, Menon KV, Holmfield JH, McMahon MJ, Guillou JP. Serum macrophage migration inhibitory factor is an early marker of pancreatic necrosis in acute pancreatitis. Ann Surg 2007; 245: 282-9. [CrossRef]
15. Dong M, Wang C, Deen WM, Dedon PC. Absence of 2-deoxyoxanosine and presence of abasic sites in DNA exposed to nitric oxide at controlled physiological concentrations, Chem Res Toxicol 2003; 16: 1044-55. [CrossRef]
16. Cooke MS, Olinski R, Evans MD. Does measurement of oxidative damage to DNA have clinical significance? Clin Chim Acta 2006; 365: 30-49. [CrossRef]
17. Petrovic S, Bogavac-Stanojevic N, Kotur-Stevuljevic J, et al. Oxidative status parameters in children with urinary tract infection. Biochem Med (Zagreb) 2014; 24: 266-72. [CrossRef]
18. Aslan M, Nazligul Y, C. Bolukbas, et al. Peripheral lymphocyte DNA damage and oxidative stress in patients with ulcerative colitis. Pol Arch Med Wewn 2011; 121: 223-9.
19. Moller P, Knudsen LE, Loft S, Wallin H. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. Cancer Epidemiol Biomarkers Prev 2000; 9: 1005-15.
20. Demirbag R, Yilmaz R, Gur M, et al. Lymphocyte DNA damage in patients with acute coronary syndrome and its relationship with severity of acute coronary syndrome. Mutat Res 2005; 578: 298-307. [CrossRef]
21. Ji LL. Antioxidants and oxidative stress in exercise. Proc Soc Exp Biol Med 1999; 222: 283-92. [CrossRef]