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Oxidative DNA damage and total antioxidant status in rats during experimental gram-negative sepsis

C Kaymak[1], E Kadioglu[2], E Ozcagli[2], G Osmanoglu[3], S Izdes[4], C Agalar[5], H Basar[6] and S Sardas[7]

[1]Faculty of Medicine, Department of Anesthesiology and Reanimation, Kirikkale University, Kirikkale, Turkey; [2]Faculty of Pharmacy, Department of Toxicology, Gazi University, Ankara, Turkey; [3]Faculty of Medicine, Department of General Surgery, Kirikkale University, Kirikkale, Turkey; [4]Department of Anesthesiology and Reanimation, Ataturk Training and Research Hospital, Ministry of Health, Ankara, Turkey; [5]Faculty of Medicine, Department of Infection Diseases, Kirikkale University, Kirikkale, Turkey; [6]Department of Anesthesiology and Reanimation, Ankara Training and Research Hospital, Ministry of Health, Ankara, Turkey; and [7]Faculty of Pharmacy, Department of Toxicology, Marmara University, Istanbul, Turkey

Sepsis and septic shock remains as leading cause of death in adult intensive care units. It is widely accepted that gram-negative bacteria and their endotoxins cause sepsis and septic shock, predominantly. Enhanced generation of reactive oxygen species may be responsible for tissue injury in septic shock and endotoxemia. The aim of this study was to assess oxidative DNA damage and the total antioxidant status (TAS) in peripheral lymphocytes of rats during different intraperitoneal gram-negative sepsis stages. Adult male Sprague-Dawley rats were divided randomly into four groups. Control group was intraperitoneally inoculated with 2 mL of pyrogene-free saline (Group I, $n = 6$), and the other rats received an intraperitoneal inoculum with 2 mL of saline containing 2×10^8 CFU of *Escherichia coli*. The animals were killed at time zero (Group I, $n = 6$), at 6th (Group II, $n = 7$), 12th (Group III, $n = 7$), and 24th (Group IV, $n = 7$) hour after the *E. coli* inoculation. Oxidative DNA damage in peripheral lymphocytes of rats was evaluated by modified comet assay (single-cell gel electrophoresis). Formamidopyrimidine DNA glycosylase (Fpg) and Endonuclease III (Endo III) were used to detect oxidized purines and pyrimidines, respectively. Total antioxidant quantification was carried out using ABTS+ (2,2′-Azino-di-[3 ethylbenzthiazoline sulphonate]) radical formation kinetics (Randox kit) in serum samples. Significant elevations of basal levels of strand breaks (SB) in Group IV were observed as compared with Group I, II, and III. There was a significant increase in Fpg sites in Group III as compared with Group I and II. However, there was no significant difference in terms of Endo III sites in any of the groups. Although the TAS was decreased with the stages of sepsis, this moderate decrease was significant in only Group IV as compared with Group I. There was no statistically significant correlation between DNA damage and TAS for any of the groups.

Key words: modified comet assay; oxidative DNA damage; sepsis; total antioxidant status

Introduction

Sepsis and septic shock still remain as leading cause of death in adult intensive care units.[1] It is widely accepted that sepsis and septic shock are caused predominantly by gram-negative bacteria and their endotoxins.[2,3] Endotoxin or Lipopolysaccharide (LPS) plays a pivotal role in the initiation of host responses caused by gram-negative bacterial infection and can trigger the inflammatory processes. Inflammation is characterized by leukocyte recruitment and activation, a process involving the secretion of an array of inflammatory cytokines (Tumor necrosis factor-α, interleukin (IL)-1β, IL-8, and neutrophil-activating proteins).[4,5] These mediators can be responsible for ongoing interactions of different cell types and can aggravate the inflammation and cause multiple damages in organs throughout the body.[6,7]

Enhanced generation of reactive oxygen species (ROS) may be responsible for tissue injury in septic shock and endotoxemia. Production of oxygen radicals by neutrophils and macrophages such as ROS, nitric oxide (NO), and peroxynitrite promote gene expression of proinflammatory mediators.[8] The

Correspondence to: Semra Sardas (Professor), Faculty of Pharmacy, Department of Toxicology, Marmara University, Haydarpasa 34668, Istanbul, Turkey. Email: semrasardas@gmail.com

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primary production of the two free radicals O_2^- and NO by activated leukocytes initiates a free radical chain reaction leading to the formation of excited species (singlet molecular oxygen and excited carbonyl groups) as secondary by-products.[9] The activation of NO has direct effects on various enzymes that contain an iron–sulphur moiety in their catalytic center. Such enzymes include ribonucleotide reductase (necessary for DNA synthesis) and several mitochondrial enzymes that are essential for survival of the cell. NO, or more likely its reactive derivatives, are mutagenic agents, with the potential to produce nitration, nitrosation, and deamination reactions on DNA bases.[10,11] Oxidative reactions are responsible for mitochondrial and DNA damage observed during inflammation. ROS, NO, or peroxynitrite, which are produced intracellularly in different cells, can also induce apoptosis and DNA damage in surrounding cells and leukocytes.[12–14]

Single-cell gel electrophoresis (SCGE) or 'comet assay' is a rapid and very sensitive method to examine DNA damage and repair at the individual cell level.[15] Comet assay has further been modified to detect specific classes of DNA damage such as oxidative DNA damage. This method makes use of repair DNA glycosylases/endonucleases with specificity for oxidative base damage to create breaks in DNA at sites of damage, and by comparing the DNA migration in enzyme-treated and untreated slides; quantitation can easily be made.[16]

Oxidative stress is defined as an unbalance between oxidants and antioxidants. This may be due to increased free radical formation in the body and/or loss of normal antioxidant defenses.[17] Antioxidant capacity may be compromised in patients with severe infections and high levels of the metabolic products of free radical damage can be observed.[11,18] The sum of endogenous and ingested antioxidants represents the antioxidant activity of the extracellular fluid. Cooperation of all the different antioxidants provides greater protection against attack by ROS or reactive nitrogen species (RNS), than any single compound alone. Thus, the total antioxidant status (TAS) might give more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids.[19]

The aim of this study was to assess oxidative DNA damage by using modified comet assay with lesion-specific endonucleases [Endonuclease (Endo) III and Formamidopyrimidine glycosylase (Fpg)] and to evaluate the TAS in peripheral lymphocytes of rats during different stages of gram-negative sepsis.

Materials and methods

Animals

Adult male Sprague-Dawley rats (weight range, 250–300 g) were used in all experiments. All animals were housed in the same temperature-controlled (22 °C) room and humidity with 12-h light/dark cycle and had access to chow and water *ad libitum* throughout the study. The study was approved by the Animal Research Ethics Committee of Ankara Training and Research Hospital, Ministry of Health.

Chemicals

All chemicals were purchased from Sigma Chemical (Steinheim, Germany) unless otherwise stated. Lymphocyte separation medium and Dulbecco's phosphate-buffered saline, without Mg and Ca, were from ICN Flow (Thame, England). Freeze-dried Endo III and Fpg were kindly provided by Dr Andrew Collins, University of Oslo, Norway.

Organisms

Escherichia coli was obtained from a clinical case, which was grown in brain–heart infusion broth. Bacteria were stored in aliquots were stored at −70 °C. A fresh aliquot was taken and a suspension containing 0.5 Mc Forland (2×10^8 CFU/mL). *Escherichia coli* was resuspended and diluted in 2 mL pyrogene-free saline containing 2×10^8 CFU of *E. coli*, clinically isolated. Sepsis was induced by intraperitoneal inoculum of *E. coli*.

Experimental design

Twenty-seven rats were divided randomly into four groups. All animals were anesthetized by intramuscular injection of ketamine (30 mg/kg). The abdomen of each animal was shaved and prepared with iodine. Control group were intraperitoneally inoculated with 2 mL of pyrogen-free saline (Group I, $n = 6$), and the other rats received an intraperitoneal inoculum with 2 mL of saline containing 2×10^8 CFU of *E. coli*. The animals were killed at time zero (Group I, $n = 6$), at 6th (Group II, $n = 7$), 12th (Group III, $n = 7$), and 24th (Group IV, $n = 7$) hour after *E. coli* inoculation.

Evaluation of microbial assay

Blood samples were obtained by aseptic direct aspiration via percutaneous transthoracic cardiac puncture. The peritoneal cavity was opened with a midline incision and tissue samples from the mesenteric lymph nodes (MLNs), liver, kidney, and spleen were removed in aseptic conditions and quantitative

microbiological analyses were performed. The organ samples were collected under sterile conditions, washed, and weighed in pyrogen-free saline and homogenized in 2 mL brain–heart infusion broth. Homogenates were cultured in eosin–methylene blue agar and blood agar. All culture plates were incubated for 24 h in aerobic conditions at 37 °C and the bacteria were identified from the plates by using standard microbiological techniques. The number of bacteria in tissue specimens was then expressed as colony forming units per gram (CFU/g) of tissue sample. Blood samples from all killed animals were collected into standard EDTA-coated test tubes. Total counts of white blood cells (WBC) were measured. Blood smears were prepared and stained for counting polymorphonuclear leukocytes (PMNL). Rectal temperature was measured in all animals.

In this model, the presence of systemic symptoms was defined in analogy to the criteria applied for humans. Rectal temperature above 38 °C or below 36 °C, WBC more than 12,000 or less than 4000/μL, colony forming gram-negative bacteria isolated from organ cultures were the criteria for satisfaction for the animals to show sepsis.

Total antioxidant status

Randox manual commercial kit (NX 2332, Randox Laboratories Ltd., Cork, Ireland) was used to determine the TAS of serum samples. The principle of the assay is to incubate ABTS (2,2'-Azino-di-[3 ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. This has a relatively stable blue–green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree, which is proportional to their concentration. The TAS value of the samples tested is expressed as an equivalent of the millimolar concentration of Trolox solution.[20]

Oxidative DNA damage estimation with the comet assay

Oxidative DNA damage in peripheral lymphocytes of rats was evaluated in four groups in separate runs of modified comet assay (SCGE).[21] The isolated lymphocytes from heparinized blood samples were suspended (at $\sim 2 \times 10^5$ cells/mL) in 100 μL of 1% low melting point agarose at 37 °C and placed on a glass microscope slide (precoated with agarose to aid attachment of the gels). Two gels were prepared for each sample. The gels were allowed to set at 4 °C, and cells were lysed for at least 1 h in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris–HCl (pH 10), and 1% Triton X-100 at 4 °C. For analysis of base oxidation, after the lysis stage, agarose embedded nucleotides were incubated at 37 °C for 45 min with Endo III (specific for oxidized pyrimidines) or for 30 min with Fpg DNA glycosylase (Fpg; recognizes altered purines including 8-oxoGua) in 40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL bovine serum albumin (pH 8.0), or with this buffer alone. To measure DNA strand breaks, the slides were immersed in 0.3 M NaOH, 1 mM Na_2EDTA for 20 min at 4 °C before electrophoresis at 0.8 V/cm for 20 min at 4 °C. After neutralization, gels were stained with Ethidium bromide and viewed by fluorescence microscopy. Nucleotide DNA extends under electrophoresis to form 'comet tails', and the relative intensity of DNA in the tail reflects DNA break frequency. The percentage of DNA in the tail (percentage of tail DNA) was taken as a measure of DNA-break frequency. Tail intensity was assessed in 50 cells by using Comet Assay III image analysis system (Perceptive Instruments, Haverhill, Suffolk, UK). Analysis was performed blindly by one slide reader.

Statistical analysis

Data were expressed as the mean ± SD. The level of TAS, WBC, and PMNL were analyzed by using one-way ANOVA post-hoc Bonferroni test. Differences among groups for DNA damage (SB, Endo III sites, Fpg sites) were evaluated by Kruskal–Wallis variance analysis because the distribution of variances among groups were statistically different. When the P value from the Kruskal–Wallis test statistics was found to be statistically significant, Mann–Whitney U-test was used to assess the groups that differed from others. Intragroup comparisons for the differences between Fpg sites and Endo III sites were evaluated by Paired t or Wilcoxon Sign Rank tests, where applicable. Degree of association between TAS and DNA damage were calculated by Pearson's correlation coefficent. Bonferroni correction was applied for either all possible pairwise comparisons or within group comparisons. P value of less than 0.05 was considered to be significant. SPSS (SPSS Inc., Chicago, IL, USA) 10.0 was used for statistical analysis.

Results

Colony-forming bacteria were isolated from MLNs, liver, kidney, and spleen specimens in Group II, III, and IV. Bacteriological evaluation of Group II, III, and IV showed positive organ cultures. Statistically

Table 1 WBC count, the percentage of PMNL, and the evaluation of tissue invasions in control and experimental gram-negative sepsis rats

Groups	*WBC*	*PMNL (%)*	Escherichia coli *(CFU)*			
			Kidney	*Spleen*	*Liver*	*Liver*
I	5×10^3	60	—	—	—	—
II	14×10^3*	74.28*	1×10^2	2×10^2	2×10^2	2×10^3
III	16×10^3*	82.85*	6×10^2	6×10^2	1.6×10^4	3×10^3
IV	17×10^3*	88.57*	4.3×10^4	2.6×10^4	14.8×10^4	2×10^4

Group I: Inoculated with pyrogen-free saline; Group II: Killed at 6th hour after *E. coli* inoculation; Group III: Killed at 12th hour after *E. coli* inoculation; Group IV: 24th hour after *E. coli* inoculation.
*$P < 0.05$ vs Group I.
WBC, white blood cells; PMNL, polymorphonuclear leukocytes; MLN, mesenteric lymph nodes.

significant differences in the total counts of WBC were observed in Group II ($P = 0.006$), Group III ($P = 0.006$), and Group IV ($P = 0.003$) as compared with Group I. The percentage of PMNL was statistically different in Group II ($P = 0.001$), Group III ($P = 0.001$), and Group IV ($P = 0.001$) as compared with Group I (Table 1). In addition, the measurements of rectal temperature were above 38 °C in Group II, III, and IV.

Table 2 The strand breaks (SB), SB+Endo III sites, and SB+Fpg sites values for each control and experimental gram-negative sepsis rats

Groups	*Rats*	*SB (% of tail DNA)*	*SB+Endo III sites (% of tail DNA)*	*SB+Fpg sites (% of tail DNA)*
Group I	1	7.55	6.31	7.79
	2	7.88	8.34	5.73
	3	10.53	8.98	8.73
	4	8.29	11.59	9.17
	5	6.42	8.28	10.89
	6	5.13	5.54	18.27
Group II	7	7.59	7.85	10.01
	8	9.73	9.73	12.12
	9	4.53	5.57	7.96
	10	9.97	6.39	6.75
	11	6.85	5.69	9.61
	12	5.67	6.80	4.67
	13	6.76	6.98	5.74
Group III	14	9.53	19.94	40.61
	15	9.43	19.83	25.26
	16	9.88	20.36	26.54
	17	8.08	13.71	18.50
	18	7.30	9.06	20.67
	19	7.87	7.72	17.12
	20	5.75	9.30	16.55
Group IV	21	29.67	19.67	33.90
	22	20.06	20.17	28.43
	23	18.26	41.09	10.79
	24	54.60	43.88	70.54
	25	23.26	36.42	36.78
	26	14.12	32.29	21.36
	27	14.26	39.14	8.49

Group I: Inoculated with pyrogen-free saline; Group II: Killed at 6th hour after *Escherichia coli* inoculation; Group III: Killed at 12th hour after *E. coli* inoculation; Group IV: 24th hour after *E. coli* inoculation.
SB, Single breaks.

Table 2 shows the strand breaks (SB), SB+Endo III sites, and SB+Fpg sites values for each control and experimental gram-negative sepsis rats. The mean values of SB, Endo III sites, and Fpg sites are presented in Table 3. Significant elevation of basal levels of SB and alkali-labile sites were observed in Group IV as compared with Group I ($P = 0.001$), Group II ($P = 0.001$) and Group III ($P = 0.001$). There were no statistically significant differences in terms of Endo III sites in any groups. There was a significant increase in Fpg sites in Group III as compared with Group I ($P = 0.008$) and Group II ($P = 0.001$).

However, no statistically significant difference in oxidative damage to DNA purines (as detected with Fpg) as compared with damage to pyrimidines (as detected with Endo III) was observed in any of the groups.

TAS expressed as an equivalent of the millimolar concentration of Trolox solution (mmol Trolox Equiv/L) in serum samples of control and experimental gram-negative sepsis rats are shown in Table 4. Although TAS decreased with the stages of sepsis, this decrease was significant in only Group IV as compared with Group I ($P = 0.013$). There was no statistically significant correlation between DNA damage and TAS for any of the groups.

Discussion

Systemic inflammatory response to infection and microbial products is known as a major component in the pathogenesis of sepsis and septic shock. Several studies have been focused in modulating the activation of this complex cascade of mediators.[3–5] LPS is a complex endotoxin macromolecule, which is a main component of the gram-negative bacterial cell wall and contains lipid A. LPS, as a known endotoxin, activates the host effector cells by stimulating receptors on their surfaces.[4,5,8]

Table 3 Mean values of the strand breaks (SB), Endo III sites, and Fpg sites for groups

	Group I (n = *6*)	*Group II* (n = *7*)	*Group III* (n = *7*)	*Group IV* (n = *7*)	P *value*
SB (% of tail DNA)	7.63 ± 1.82	7.3 ± 1.99	8.26 ± 1.47	24.89 ± 14.16[a]	0.001
Endo III sites (% of tail DNA)	0.54 ± 1.83	−0.29 ± 1.63	6.01 ± 4.48	8.34 ± 15.11	0.145
Fpg sites (% of tail DNA)	2.46 ± 5.74	0.82 ± 2.53	15.34 ± 7.47[b]	5.15 ± 8.94	0.008

Percentage of tail DNA (Fpg and Endo III sites, the difference in score between slides treated with and without Fpg and Endo III enzymes). Values are mean ± SD.
SB, single breaks.
[a]$P < 0.0083$ vs Group I, II and III ($P = 0.001$).
[b]$P < 0.0083$ vs Group I ($P = 0.008$); $P < 0.0083$ vs Group II ($P = 0.001$).

A single intraperitoneal inoculum of gram-negative species has been one of the experimental models used in sepsis.[22–24] Many animal sepsis models have been described, but none has proved to be superior.[25] In our experimental rat model of sepsis, expression of *E. coli* is detected in the liver, kidney, spleen, and MLNs. Group II, Group III, and Group IV showed models of gram-negative sepsis when compared with pyrogen-free saline-treated rats (Group I) with systemic symptoms in analogy to the criteria applied for humans. However, the use of currently, widely accepted sensitive and specific methods to screen markers of infection with gram-negative bacteria for the detection of endotoxin content such as limulus amebocyte lysate reagent are also available.

LPS induces expression of cytokine genes through stimulation of specific receptors on macrophages/monocytes, neutrophils and on the surface of other target cells. These target cells secrete large quantities of proinflammatory cytokines, which play a critical role in the induction of inducible NO synthesis in the endothelium, macrophages, and vascular smooth muscle cells.[6,9] NO possessing properties of a free radical is an important molecule involved in the inflammatory response. Furthermore, the reaction of NO with superoxide anion results in the formation of cytotoxic oxygen radicals, such as peroxynitrite, which decomposes to an intermediate with the biological activity of the hydroxyl radical. Peroxynitrite is a strong and stable oxidant that is thought to be responsible for many cytotoxic actions of NO via a number of independent mechanisms.[8,10] NO may act indirectly by inhibition of antioxidative enzymes and respiratory chain or by modulation of inflammation-related transcription factor.[12]

Damage to DNA by ROS or RNS seems to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues. DNA damage by ROS/RNS can cause multiple lesions including single and double strand breaks, apurinic/apyrimidinic sites and modified pyrimidines and purines.[26] The chemistry of DNA damage by several ROS/RNS has been well characterized *in vitro*, and 8-oxoGua, which is abundant in DNA, is often measured as an index of oxidative DNA damage.[27] Here we used the method of modified comet assay combined with repair enzymes to measure oxidative damage to DNA. Fpg removes 8-oxoGua and the FaPy products, leaving AP sites that are converted to strand breaks by the associated AP endonuclease activity. The extra breaks represent sites of endonucleolytic attack at 8-oxoGua, which is the principal substrate for Fpg,

Table 4 Total antioxidant status (TAS) expressed as an equivalent of the millimolar concentration of Trolox solution (mmol Trolox Equiv/L) in serum samples of control and experimental gram-negative sepsis rats

Rats	*Group I* (n = *6*)	*Group II* (n = *7*)	*Group III* (n = *7*)	*Group IV* (n = *7*)
1	2.34	1.96	1.29	0.85
2	1.81	1.75	2.81	1.55
3	1.93	1.7	2.05	0.91
4	1.99	1.64	0.94	1.26
5	2.16	1.08	1.43	0.94
6	2.13	0.53	1.30	1.81
7		1.61	1.50	0.99
Mean values ± SD	2.06 ± 0.18*	1.46 ± 0.49	1.50 ± 0.4	1.18 ± 0.36

Group I: Inoculated with pyrogen-free saline; Group II: Killed at 6th hour after *Escherichia coli* inoculation; Group III: Killed at 12th hour after *E. coli* inoculation; Group IV: 24th hour after *E. coli* inoculation.
*$P < 0{,}05$ vs Group IV ($P = 0.013$).
Values are expressed as an equivalent of the millimolar concentration of Trolox solution (mmol Trolox Equiv/L).

and it also detects various kinds of ring-opened bases such as FapyGua and FapyAde. However, an analogous enzyme, endonuclease III, creates strand breaks at sites of oxidized pyrimidines.[28]

In our study, the increase in SB in Group IV and Fpg sites in Group III and IV indicates that septic shock may result in elevated levels of DNA damage. Inclusion of Fpg in the comet assay showed extra sites of damage in 12 h after *E. coli* inoculation (Group III), which indicates the oxidized purine bases. However, no oxidative damage to pyrimidine bases has been shown in any stages of gram-negative sepsis. Furthermore, different patterns are observed in the level of SB and Endo III sites as compared with Fpg sites. We observed the highest basal DNA damage and Endo III sites in 24 h after *E. coli* inoculation (Group IV). However, the highest effect of Fpg sites were reached after 12 h (Group III) and the effect decreased after 24 h (Group IV). This data suggests some kind of recovery in terms of purine bases, whereas the basal DNA damage and pyrimidine base damage seem to have an increasing trend in our sepsis model. One limitation of our study was not to use a matched vehicle-treated control group on every time point, to avoid the experimental or diurnal variations.

In accordance with our results, Bohlinger, *et al.*[29] reported a significant increase in the amount of low–molecular weight DNA in cytosolic fractions from liver, lung, and cecum by an ELISA specific for histone-bound DNA at 12 h after injection of LPS and suggested that oligonucleosomal DNA fragmentation is an indicator of apoptotic cell death and an early and sensitive marker of endotoxic shock-induced tissue injury. DNA injury can initiate apoptosis by a powerful, early-activated mechanism dependent on the nuclear phosphoprotein activated by both transcriptional and post-translational means and is a critical element in the cellular response to double-strand DNA breaks.[30] Morphologically, apoptosis is characterized by cell and organelle shrinkage (pyknosis), condensation of chromatin, DNA fragmentation, and membrane blebbing.[31] Grasl, *et al.*[32] showed that the number of positive hepatocyte nuclei exceeded the incidence of apoptosis observed in hematoxylin and eosin-stained liver tissue by the TUNEL assay, 24 h after endotoxic shock. Apoptosis was evaluated in DNA gel electrophoresis by Hotchkiss, *et al.*[33] and reported that demonstration of apoptosis in septic tissues by DNA agarose gel electrophoresis was highly specific. In our present study, single strand DNA damage and alkalilabile sites have been evaluated with the alkaline comet technique, and the observed damage is important in sepsis.

Antioxidants form an important line of endogenous defense against free radical damage in both human and animal lymphocytes.[34,35] Antioxidant capacity may be compromised in patients with sepsis through redistribution and increased utilization of plasma-binding proteins as part of the acute inflammatory response, poor nutritional state, and inadequate provision of optimal nutrition.[11,36] In our experimental rat model of gram-negative sepsis, the TAS was significantly decreased 6 h after *E. coli* inoculation and this decrease was continued for 12 and 24 h. Previous studies have also reported that the circulating concentrations of components of the plasma antioxidant defense system decrease in sepsis.[18,37] Goode, *et al.*[18] showed that patients with sepsis and secondary organ dysfunction had extremely low plasma concentrations of vitamins C, E, and β-carotene. Cowley, *et al.*[37] found that the plasma antioxidant potential of patients with sepsis was reduced when compared with the plasma antioxidant potential of healthy subjects.

In conclusion, the data indicate elevated levels of oxidized pyrimidine bases, which were correlated with the different stages in sepsis, whereas purine base damage seems to have a different trend as evaluated by modified comet assay. Although TAS was decreased slightly with the stages of our sepsis model, statistically significant difference was only seen in late stages, and no correlation with DNA damage and TAS were shown. The role of ROS and oxidative DNA damage in sepsis is still unclear; more studies are needed to determine this correlation and the protective effects of antioxidants against this damage.

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