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Hematological and Biochemical Status in Norm and with LPS-Induced General Inflammation

A. G. Sysa
Belarusian State University, ISEI BSU, Minsk, Belarus

E. I. Kvasyuk
Belarusian State University, ISEI BSU, Minsk, Belarus

A. Shihad
Belarusian State University, ISEI BSU, Minsk, Belarus
University of Al-Qhadisayh, College of Veterinary Medicine, Al Diwaniyah, Iraq

M. S. Hassan
University of Al-Qhadisayh, College of Veterinary Medicine, Al Diwaniyah, Iraq

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ABSTRACT

The present study investigated the effects of E. coli lipopolysaccharide (LPS) induced mastitis model in rat on the activity of antioxidant enzyme systems under modified nucleosides/nucleotides administration. To achieve this purpose, E. coli LPS was injected intraperitoneally. The antioxidant activities of the enzymes, superoxide dismutase, glutathione peroxidase, and catalase together with malondialdehyde (MDA) level were assayed in blood serum. Results obtained showed that, the antioxidant enzyme activities were significantly decreased while the level of MDA, the indicator of lipid peroxidation were significantly increased following intramammary inoculation of LPS compared to the control animals. The results indicated that E. coli LPS-induced mastitis could alter antioxidant enzymes and increase lipid peroxidation.

Introduction

Although oxidative reactions are essential for the body, an excess of oxidative reactions of the anti-bacterial processes may cause tissue damage. An excess of ROS and the absence of optimal amounts of antioxidants are leading to oxidative stress. Many cells are susceptible to this oxidative stress, which can cause necrosis or apoptosis [1]. Different enzymes can prevent the formation of radicals or scavenge radicals or hydrogen peroxide and other peroxides. Among antioxidant enzymes, superoxide dismutase and catalase have been demonstrated in blood serum. Superoxide dismutase (SOD) is considered the most important enzyme and the presence of this enzyme is important in maintaining the antioxidant stability. Studies have shown that exogenous addition of SOD causes a reduction in lipid peroxidation processes [2]. Catalase (CAT) is an enzyme that catalyzes the decomposition of hydrogen peroxide. The enzyme can be bacterial origin, is heat labile and are inactivated within minutes at 65℃. Present in large quantities, catalase is an indication that the udder has held an inflammatory process [3].

Another enzyme with antioxidant functions is the glutathione peroxidase. The enzyme glutathione peroxidase (GPx) is a family of enzymes play important roles in the protection of organisms from oxidative damage that converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing lipid hydro- peroxides to their corresponding alcohols or free hydrogen peroxide to water [4].

Coliform bacteria (including Escherichia coli, Klebsiella species, and Enterobacter species) are the most common causative agents in severe clinical cases. Therefore, the search for new compounds with potential antibacterial activity in a series of modified nucleosides and nucleotides is of fundamental and practical importance.

Objective – to characterize changes in diagnostically significant antioxidant defence blood parameters during systemic inflammation caused by *Escherichia coli* lipopolysaccharide administration to rats and its modulation by modified nucleosides/nucleotides in a model experiment on laboratory animals.

Material and Methods. The modified nucleosides and nucleotides of the purine and pyrimidine series studied in the work were synthetized as described [8]. Purine nucleoside analogs are represented by compounds halogenated at the nitrogenous base, 2-fluoro-arabinofuranosyladenine (fludarabine) and 2-amino-6-chloro-arabinofuranosylpurine (2-NH_2-6-Cl-araPur). Pyrimidine nucleosides are represented by sugar-modified arabinofuranosylcytosine (cytarabine, ara-C), which contains arabinose instead of ribose, and [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil (TTU), which is modified at the carbohydrate fragment (three acetate groups) and the nitrogenous base (triazole in the 4th position) (Figure 1).

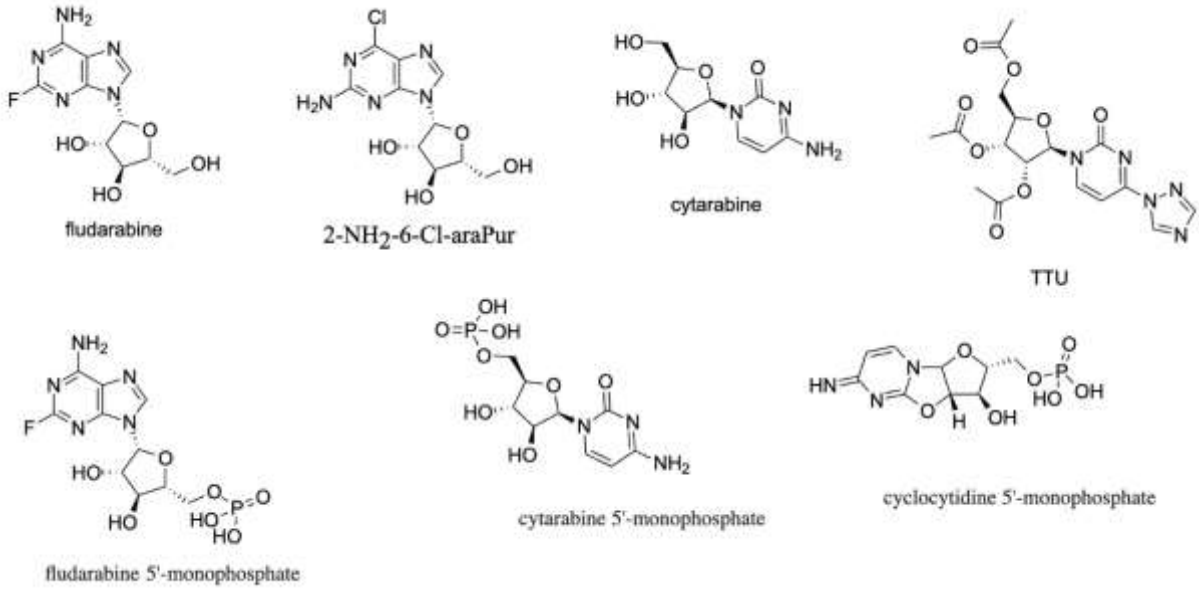

Figure 1 – The structure of the modified nucleosides and nucleotides used in the work

To study the changes in blood biochemical marks caused by of *E. coli*, the isolates were tested for their activity to grow in a biofilm [5], briefly in this method, brain heart infusion broth (BHIB) containing tubes were inoculated with the isolated bacteria, incubated at 37 °C for 24 h, then the content of the tubes was poured and drops of crystal violet was added to the tubes with gentle rotation, left for two minutes, then the tubes placed upside down on a filter paper after rinsing with distilled water to remove excess stain.

Hemolytic activity of *E. coli* was tested by cultivation of bacteria on blood agar plates then incubated at 37 °C for 24 h, a clear zone around the colonies, indicating a positive result [6].

Urine isolate with the highest biofilm formation activity and hemolytic activity were selected for LPS extraction as a pathogenic isolate. In brief, 250 ml of the 24h bacterial growth in brain heart infusion broth (BHIB) were centrifuged at 6000 rpm for 20 minutes, the sediment washed with phosphate buffered saline (PBS) twice then subjected to 15 ml of lysis buffer containing Tris HCl, MgCl2, SDS and β-mercaptoethanol, then placed in water bath at 65 °C for 1 hour to solubilize the sediment, then, 1 ml of proteinase K was added to the preparation to remove contaminants proteins, preparation was incubated at 37 °C for 24 h, then LPS was precipitated at -20 °C using 3 M sodium acetate and cold absolute ethanol, after final centrifugation, the pellet was re-suspended in 9 ml of 10 mM Tris-HCl followed by extraction using hot phenol, where 9 ml of phenol at 65 °C was added and mixed vigorously and then was immediately placed in an ice bath, preparation was centrifuged at 6000 rpm for 15 min and the top aqueous layer was taken, and the extraction was repeated again, then the aqueous layers of the extracted LPS were subjected to dialysis for 48 h against distilled water, and the LPS was lyophilized and stored at -20 °C until use [7].

The experiment was performed at the College of Veterinary Medicine of University of Al-Qhadisayh in strict compliance with The European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Di-rective2010/63/EU) [8], Institutional animal care and use committee (IACUC) approval № 1047/2 from 20.06.2023.

Eighty-five Swiss adult male Wistar rats of 8-10 weeks age and weights between 160-210 grams were obtained from the Animals House, College of Veterinary Medicine, University of

Al-Qhadisayh were kept under standard laboratory condition, they were given standard locally prepared diets and were placed in cages and were acclimatized for a week. Next rats were divided into 17 groups (5 rats each), one group was the control group (was given standard saline solution) and the other 8 groups were the experimental groups. The first experimental group was injected intraperitoneally with only LPS (5 mg/kg BW). Eight others experimental groups were injected with LPS and the synthesized nucleosides/nucleotides. All concentrations of used materials were injected with 3 repeated doses 48 h between them, after 7 days of immunization with LPS, the blood was collected using retro orbital blood collection technique and placed in EDTA tubes.

The portion of blood put into anticoagulant tubes was centrifuged at 90g for 10 min at room temperature for assay of oxidative stress status. Total protein levels were analysed to calculate final values of oxidative stress biomarkers superoxide dismutase (SOD), total glutathione (GSH), glutathione peroxidase (GPx) and thiobarbituric acid reactive species (TBARS) (Ferreira et al., 1999). TBARS was evaluated as an index of lipid peroxidation. Briefly, 1.0 ml of blood serum was added to the test tube containing 1.0 ml of 3.0% sulphosalicilic acid, agitated for 10 s, centrifuged at 18,000g for 3 min and rested for 15 min. The sample was diluted to 500 µl of 0.67% TBA solution. The mixture was heated to 80 °C for 30 min and the absorbance measured at a wavelength of 535 nm. The results were expressed as µmol of TBARS per gram of total protein (µmol\mg PTN) (Ferreira et al., 1999).

SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogallol. The reaction mixture (1.0 ml) consisted of 5.0 mmol/l Tris (hydroxymethyl) aminomethane (pH 8.0), 1.0 mmol/l EDTA, bidistilled water and 20 µl of the sample. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mmol/l), and the absorbance was measured by a spectrophotometer with a wavelength of 420 nm (25 °C) for 5 min. Enzymatic activity unit was defined as SOD units able to produce 50% of pyrogallol oxidation inhibition. All data were expressed in units of SOD per mg of total protein (Ferreira et al., 1999).

GSH, which consists of reduced and oxidized glutathiones, was enzymatically determined using 5,50-dithiobis-2-nitrobenzoic acid (DTNB; Sigma) and glutathione reductase in the presence of NADPH, forming 2-nitro-5-thiobenzoic acid. A mixture consisting of 1290 µl of distilled water, 200 µl of Tris/HCl buffer (1 mol/l, pH 8.0, 5 mmol/l EDTA), 200 µl of 10 IU/ml glutathione reductase (Sigma), 200 µl of 2 mmol/l NADPH (Sigma) and 100 µl of 12 mmol/l of DTNB was added to 10 µl of the sample. Activity was measured at 412 nm on a spectrophotometer. One unit of activity was equal to µmol of substrate reduced per gram of total protein (Ferreira et al., 1999).

GPx content was performed by monitoring NADPH oxidation. The mixture consisted in the addition of 1300 µl of distilled water, 200 µl of Tris/HCl buffer (EDTA 1 mol/l; pH 8,0; 5 mmol/l), 200 µl of 10 IU/ml of glutathione reductase, 200 µl of NADPH (2 mmol/l), 40 µl of GSH (0.1 mol/l) to 40 µl of hemolysate. The mixture was agitated in a vortex mixer for 10 s. Next, 20 µl of t-Butyl hydroperoxide (7 mmol/l) were added and maintained at 37 °C for 10 min.

Absorbance was determined by a spectrophotometer with a wavelength of 340 nm. GPx activity was expressed in enzymatic activity units per gram of total protein (U\mg PTN) (Ferreira et al., 1999).

H_2O_2 is the usual oxidizing substrate of horseradish peroxidase (HRP). The reaction medium (20µl) contained H_2O_2 at different concentrations in different buffers: 50 mM sodium hydroxide (pH 3.0- 5.8), 50 mM Sodium phosphate monobasic monohydrate (pH 6.0-7.5) at 25

°C. The reactions were started by the addition of 10–50 µl of HRP in oxygen-free (nitrogen-bubbled) water. Enzyme concentrations were estimated by measuring the absorbance of solutions at 620 nm at 37 for 10min (Aebi et al. 1983).

Catalase activity was determined spectrophotometrically by the method of Aebi et al. 1983. Briefly, 10 μL of sample was incubated with 100 μmol/mL of H_2O_2 in 0.05 mmol/L Sodium phosphate monobasic monohydrate for 10 min. Changes in absorbance at 240 nm at a span of 15 s for 2 min at 25°C were noted.

Four clinical and morphological blood parameters were measured on Gen5 (Bio Tek, UV Probe (Shimadzu), Statistica (StatSoft) and Microsoft Office 365 (Microsoft): TBARS (µmol\mg PTN); SOD (U\mg PTN); GSH (Um\mg PTN; CAT (U\mg PTN; H_2O_2 (nmol\mg PTN); and GPx (U\mg PTN).

The data was summarized, analyzed, and presented using the SPSS version 23 software program and the difference in mean of quantitative variables between groups was investigated using a one-way ANOVA, which was accompanied by a post hoc Duncan multiple range test.

Results. We used the reference values for Wistar rats in a comparative analysis of hematological study data, but we used the outcomes of control group investigations as the reference. Results obtained from analysis of antioxidant enzyme activity in blood samples (mean ± SD) are presented in Table 1. The level of SOD, CAT, GSH, H_2O_2, and GPx in experimental LPS-treated rat compared to control rat showed significant decrease ($p < 0.05$) and the level of TBARS showed marked elevation ($p < 0.05$) in comparison with control rats.

Table 1 - Oxidative stress parameters of laboratory rats with experimental systemic inflammation induced by bacterial endotoxin

Groups	**TBARS (µmol\mg PTN)**	**SOD (U\mg PTN)**	**GSH (Um\mg PTN**	**CAT (U\mg PTN**	**H_2O_2 (nmol\mg PTN)**	**GPx (U\mg PTN)**
Control	0.42±0.63	7.8±0.54	1.66±0.42	110.5±0.58	2.34±0.75	22.3±0.62
LPS-treated	0.91±0.71	6.3±0.42	1.21±0.52	81.9±0.33	0.82±0.54	15.6±0.77
LPS + Fludarabine	1.3±0.54	5.7±0.74	0.93±0.63	74.3±0.74	0.84±0.57	13.2±0.76
LPS + 2-NH_2-6-Cl-araPur	1.0±0.64	5.6±0.74	1.14±0.32	78.2±0.37	0.81±0.57	12.84±0.66
LPS + ara-CMP	0.98±0.87	4.98±0.34	1.19±0.47	79.9±0.45	0.79±0.84	14.1±0.63
LPS + TTU	1.1±0.44	5.5±0.62	1.10±0.87	76.6±0.34	0.87±0.46	14.9±0.53
LPS + Fludarabine phosphate	1.4±0.78	5.9±0.45	1.17±0.43	72.8±0.42	0.78±0.63	13.2±.0.34
LPS + araC	1.5±0.76	4.5±0.25	1.03±0.56	69.5±0.68	0.74±0.66	10.1±0.49
LPS + cCMP	1.5±0.54	4.9±0.66	1.09±0.76	71.4±0.82	0.72±0.27	11.2±0.35

The administration of modified nucleosides or nucleotides to animals resulted in an increase in symptoms of an acute inflammatory process on the part of leukocytes, as indicated by the results provided in Table 1. The most prominent oxidative stress was found with the cCMP and ara-C nucleotides (a decrease in H_2O_2 of 69.23 and 68.47%) respectively, followed by Fludarabine phosphate, araCMP both 66.66%, as well as Fludarabine 64.102%, NH_2-6-Cl-araPur 65.3%, and TTU 62.82% compared to the LPS-immunized group).

We used the reference values for Wistar rats in a comparative analysis of biochemical blood test data, but we used the findings of investigations of the control groups as the reference. Results obtained from analysis of antioxidant enzyme activity in blood samples (mean ± SD) of rats treated with combination of nucleoside/nucleotide with quercetin are presented in Table 2.

Table 2 - Oxidative stress parameters of laboratory rats in experimental systemic inflammation induced by bacterial endotoxin with antioxidant administration

Groups	**TBARS (µmol\mg PTN)**	**SOD (U\mg PTN)**	**GSH (Um\mg PTN**	**CAT (U\mg PTN**	**H_2O_2 (nmol\mg PTN)**	**GPx (U\mg PTN**
Control	0.42±0.63	7.8±0.54	1.66±0.42	110.5±0.58	2.34±0.75	22.3±0.62
LPS-treated + Quercetin	0.56±0.51	7.2±0.62	1.55±0.61	102.4±0.74	1.20±0.65	21.1±0.43
LPS + Fludarabine + Quercetin	0.72±0.64	6.7±0.43	1.40±0.54	88.6±0.78	0.97±0.44	17.4±0.82
LPS + 2-NH_2-6-Cl-araPur + Quercetin	0.61±0.41	6.8±0.53	1.38±0.42	95.9±0.65	0.98±0.67	18.7±0.57
LPS + ara-CMP + Quercetin	0.86±0.54	6.9±0.86	1.43±0.74	87.4±0.56	0.88±0.73	19.4±0.75
LPS + TTU + Quercetin	0.73±0.77	6.9±0.65	1.39±0.78	91.4±0.76	1.1±0.55	16.8±0.65
LPS + Fludarabine phosphate + Quercetin	0.88±0.57	7.0±0.34	1.47±0.64	94.2±0.32	0.93±0.76	16.6±0.49
LPS + araC+ Quercetin	0.66±0.65	6.2±0.43	1.49±0.65	89.9±0.42	0.92±0.78	17.2±0.58
LPS + cCMP + Quercetin	0.87±0.47	6.8±0.87	1.46±0.41	85.7±0.46	0.90±0.56	17.7±0.95

According to the data in Table 2, TBARS has been significantly increased and the most effective compound was cCMP. Reducing of SOD has been notice with all compounds. The other oxidative stress GSH, H_2O_2, CAT, and GPx all significantly havw decreased after exposed to antioxidant quercetin.

Discussion. Although laboratory changes are well examined in many diseases in rats,

oxidative stress parameters induced by LPS are poorly studied. Also, oxidative statuses of rat inflammation induced by LPS are weak investigated. Many potentially toxic ROS are generated through normal oxidative metabolism and the body has adapted by developing a complex system of protective antioxidants [9]. Oxidative stress may be defined as an alteration in the steady-state balance between oxidant and antioxidant agents in the cells. With increase of intracellular sources of ROS, several physiological processes may be disturbed [10]. Under more stressful conditions such as inflammation, hydroxy radicals released by infiltrated neutrophils caused cell injury. Antioxidant enzymes and the determination of TBARS concentrations are among the most widely used methods for determination of oxidative stress [11]. Increased plasma TBARS concentration is considered as references a marker of lipid peroxidation [12].

Reactive Oxygen Species (ROS) are produced by cellular metabolism. However, the overproduction of ROS and its derivatives may poorly affect health. Among ROS, the superoxide anion plays a vital role in inflammation. The enzyme SOD neutralizes superoxide anion by converting it into hydrogen peroxide so that it prevents the formation of aggressive compounds like peroxynitrite and hydroxyl radical [13].

In the present study, values of GPx, SOD and CAT significantly were decreased in serum, however, TBARS levels were increased significantly. The mean SOD level at each measurement was lower in the intervention group than that of in control group. A study by Mittal at al reported that SOD is one of antioxidant in the body which can be imbalanced when ROS is excessive due to continuous inflammation. Hence, SOD could not neutralize ROS, leading to cell damage [14].

In contrast to SOD, we found that TBARS levels in the control and the intervention groups demonstrated an increasing trend over time. However, the intervention group had a higher TBARS level than that of the control group. Our finding is line with the study by Tasdemir et al., which investigated the effect of *E. Coli* induction in a dose of 0,5 ml with the concentration 2×10^8 an animal model revealing *E. Coli* induction resulted in increased MDA level [15]. Another study by Chen et al. and other studies found that the MDA level was elevated significantly while SOD activity and Glutathione (GSH) content were decreased remarkably in LPS-induced acute kidney injury model [16].

The higher TBARS levels in mastitic rats reported in this study demonstrated that the auto-oxidative activity of mastitic case is higher than normal. Because during inflammation, oxidation of long chain fatty acids in cell membranes lead to lipid peroxidation [17] which may inhibit the activity of some antioxidant molecules as GPx leading to oxidative stress [18]. The present results indicated that increasing levels of oxidative stress markers in rats might have an essential role in the process of inflammation and tissue damage and TBARS is the final product of lipid peroxidation and therefore is used as index of this process.

These changes in enzyme activities in blood could be consequence of cell structural damage and may indicate that GPx, SOD and CAT levels were depressed when lipid peroxidation was increased and Low GSH-Px activity with high SCCs and high level of prostaglandin formation has been recorded [19] which may be attributed to the excessive release of free radicals that may result in inhibiting enzymes activity and lead to an exacerbation of the oxidative stress. On the other hand, decrease in enzymatic antioxidant activities might be attributed either to the increase in consumption or to the counteraction with ROS produced from inflamed gland, suggesting a compromise in antioxidant defence of the body.

Conflict of interest. The authors declare no conflict of interest.

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