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Evaluation of Liver Antioxidants in an induced Oxidative Stress in Albino Rat Model

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Abstract

Background: Oxidative stress is a state of persistent imbalance between reactive oxygen species generation and ability of cellular antioxidant system to deactivate them.

Objectives: The present case-control study aimed to evaluate antioxidants of liver in an induced oxidative stress by lipopolysaccharide (LPS) in an albino rat model at different time intervals (6 and 72 hours) and the correlations between the studied antioxidant indicators.

Materials and methods: Ninety albino rats (male and female) were divided into two groups: 30 rats (control group) and 60 rats (test group)) were injected intraperitoneally (i. p) with saline and 60 rats (test group) injected i. p with 100 µg/kg of LPS. Rats were sacrificed at intervals of 6 and 72 hours for evaluatingTumor necrosis factor α) TNF-α) , Myeloperoxidase (MPO), caspase-3, Cyclooxygenase-2 (COX-2), Interleukin-6 (IL-6), Malondialdehyde (MDA), Catalase, Glutathione peroxidase (GPx), Glutathione reductase (GR) and superoxide dismutase (SOD)in liver tissues using ELISA technique.

Results: Our results revealed significant elevation in TNFα, MPO, Caspase-3, COX-2, IL-6 and MDA levels, a significant reduction in catalase level and insignificant decrease in GPx, GR and SOD levels in both time intervals compared to the controls. There was significant difference in the indicator levels after 6 hours compared to 72 hour intervals, but it was insignificant for MPO, Caspase-3, GPx, GR and SOD. There were positively significant correlations for all indicators at 6 and 72-hours intervals except for MDA with GPx, GR and SOD, for MDA with catalase, GPx, GR and SOD. They were negatively significant at 6 and 72-hours intervals respectively; also, correlations were insignificant between catalase and GR after 6-hours interval and between TNFα and catalase, MDA and MPO, IL-6 and MPO, GPx and SOD after 72-hours interval.

Conclusion: The levels of the studied indicators were increased in LPS-induced oxidative stress up to 72 hours except for GPx, GR and SOD; catalase was decreased significantly at both time intervals. Upon comparing 6-hours to 72-hours intervals, significant difference was detected in all indicators except for MPO, Caspase-3, GPx, GR and SOD. Correlations between indicators were positively and negatively significant except at 6-hours interval for catalase and GR, at 72-hours interval for TNFα and catalase, MDA and MPO, IL-6 and MPO, GPx and SOD after 72-hours interval, correlations were insignificant.

Keywords: Oxidative stress, TNF-α, Lipopolysaccharide, COX-2, Caspase-3, Glutathione peroxidase.

1. Introduction

Lipopolysaccharide (LPS) is a thermostable endotoxin,glycolipid component found in the outer membrane of Gram-negative bacteriasuch as *Salmonella* and *Escherichia* species (Kabanov and Prokhorenko 2010). The toxic proinflammatory properties of LPS are due to its lipid content (Milicaj et al. 2021), and the serological specificity is attributed to its distal polysaccharide and core oligosaccharide. The toxic effect of LPS is mediated by signaling pathway employing Toll-like receptors that stimulate NF-kβ translocation to nucleus (Di Gioia and Zanoni 2015)and enhance cytokines transcription such as TNFα and IL-6, which leads to inflammatory responses causing oxidative stress (Gutsmann et al. 2007).

Tumor necrosis factor α (TNFα) is a cytokine that has different effects on various types of cells secreted by macrophages in response to inflammatory agents and septic shock; it is involved in the pathogenesis of inflammatory and autoimmune diseases (Chen and

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** **Abbreviations**: Cyclooxygenase-2 (COX-2), Glutathione peroxidase (GPx), Glutathione reductase (GR), Lipopolysaccharide (LPS), Malondialdehyde (MDA), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ), Phosphate buffered saline (PBS), Superoxide dismutase (SOD), Tumor necrosis factor α (TNFα)

Oppenheim 2016). The expression of cyclooxygenase-2 (COX-2) is especially enhanced in damaged hepatocytes and Kupffer cells mainly during antigen induced inflammation by inflammatory cytokines under effect of stimuli such as pro-inflammatory IL-1b and TNFα resulting in increased production of prostanoids at the inflammation site (Tunctan 2020). Interlukin-6 (IL-6) is induced by infection and damage of tissues. Physiologically, serum concentration of IL-6 is very low, but it is rapidly elevated in cases of trauma, infection and injury, and it is considered as an early indicator of inflammation and a predictor of disease activity (Yang et al. 2016).

Myeloperoxidase (MPO) is a heme peroxidase enzyme; it catalyzes a reaction to produce hypochlorous acid that reacts with nucleic acids, proteins and lipids causing dangerous cellular effects and leads to inflammatory conditions such as the atherosclerotic changes (Aratani 2018). Caspase 3 is one of the pro-apoptotic caspases, contributed to signal transduction for programmed cell death. It induces apoptosis by activating caspase-activated DNase and DNA fragmentation (Kroemer 2016).Malondialdehyde (MDA) is produced endogenously from polyunsaturated fatty acids oxidation. Free MDA can be scavenged by protein and nucleic acids because it reacts with their functional groups. The level of oxidative stress cannot be reflected correctly by measuring the amount of free MDA; its toxicity is contributed to its ability to do cross linkage leading to mutagenesis(Ho et al. 2013).

Reactive oxygen species (ROS), the main oxidants are partially reduced oxygen metabolites that have strong oxidizing abilities. They are deleterious to cells at high concentrations because they can oxidize lipids and protein cellular components. Besides the damage of DNA, the prolonged production of ROS is considered a risk leading to the progression of the inflammatory processes. Their role in inducing inflammation has been excessively studied in experimental models (Pizzino et al. 2017). To avoid the harmful effects of oxidants, there are groups of antioxidants that can scavenge for removing ROS, including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Handy and Loscalzo 2012).

Superoxide dismutase (SOD) is a metalloenzyme that catalyzes superoxide radical dismutation into hydrogen peroxide and molecular oxygen to protect cells from damage that results from accumulation of ROS (Singh et al. 2017). Catalase is a cytoplasmic antioxidant enzyme that can detoxify H_2O_2 to oxygen and water to maintain the homeostasis of cellular redox. It was reported to be an important enzyme associated with mutagenesis and inflammatorydisorders; also, it is related to oxidative stress states(Zhang et al. 2015).

Glutathione peroxidase (GPx) is an antioxidant selenoenzyme; it catalyzes the reduction of H_2O_2 to water and destroy fatty acid hydroperoxidesfor mitigating their toxicity. It acts in association with SOD and catalase as an antioxidant enzyme system to minimize ROS and their toxicity(Zhao et al. 2019).Glutathione reductase (GR), homodimeric flavoprotein cytosolic and mitochondrial enzyme that catalyzes glutathione disulfide (GSSG) reduction utilizing NADPH as the reducing cofactor to sustaincellular redox homeostasis(Iozef et al. 2000).

The aim of the present study is to assess the antioxidants of hepatic tissues in an induced oxidative stress by LPSin an experimental rat model at different time intervals (6 and 72 hours), and the correlation between the studied antioxidant indicators. This study is considered as an extension of a previous one titled "Time-dependent expression patterns of inflammatory markers in rat model of lipopolysaccharide-induced acute systemic inflammation" *Medical Science*, 2021, 25(118), 3336-3344 (Albataineh et al. 2021).

2. Materials and Methodology

2.1. Design of the study

Ninety albino rats, age-matched with weight range of 200-250 gm were divided into 30and 60 rats as control and test sets, respectively; then, each set was subdivided into two subgroups each including 15 and 30 rats, respectively. A standard diet was fed to the animals; they were housed in the animal house. The rats were given a light/dark cycle of 12:12 hours and free access to get water and food during the full time of the experiment. Each rat of the two subgroups of the controls were injected once with normal saline intraperitoneally. Teeling et al. [2020] was followed for suspending LPS (*Salmonella abortusequi* derivative) (L5886, Sigma, Poole, UK) in normal saline (Teeling et al. 2010). Each rat of the two test subgroups was injected once with LPS (100 µg/kg body weight) intraperitoneally.Later, one subgroup of both the controls and the test were sacrificed at 6 hours and the second control and test subgroups were sacrificed at 72 hours of the challenge to obtain their livers for the biochemical analyses of the required parameters in the tissues. The procedure of Aksoy et al. [2014] was used to prepare tissues for biochemical analyses(Aksoy et al. 2014).

2.2. Ethical considerations

The Ethics Committee of Faculty of Medicine, Mutah University had approved the study (reference no. 1302023).

2.3. Biochemical analyses

Engvall et al. [1971] method was used to analyze all studied oxidative indicators(Aksoy et al. 2014). The used kits for the biochemical analyses were purchased from ELK Biotechnology CO.,Ltd, China. About 100 mg of liver tissues were rinsed with 1X PBS (Phosphate buffered saline); then, homogenizedin 1 ml of 1XPBS and stored at -20ºC overnight. After performing two freeze-thaw cycles to break the cell membranes, the homogenates were centrifuged at 5000 x g for 5 min at 4°C. The supernatant was withdrawn, divided into aliquots, and stored at -80°C till the assay time.

2.4. Statistical analyses

SPSS Software version 25 was used to analyze the data statistically, and expressed as mean±SD. *t*-test was applied for comparing between groups. Spearman's correlation coefficient (*r*) was used to study the correlation between the numerical data. *P*value <0.05 was considered statistically significant.

3. Results

Table 1 shows mean±SD of all studied indicators after 6 hours of LPS challenge, and the results revealed statistically significant increase in TNFα, MPO, Caspase-3, COX-2, IL-6 and MDA levels. A statistically significant decrease in the level of catalase, on the other hand, there was statistically insignificant decrease in the levels of GPx, GR and SOD when the test group was compared to the controls.

Table 1. Comparison between the levels of the studied oxidative stress and antioxidant indicators expressed as mean±SDin the test group versus the controls after 6 hours of the challenge with LPS

Parameters	Control group (no. 15)	Test group (no. 30)	*P* value
TNF-α (ng/ml)	28.6 ±1.1	82.8±7.61	0.006
MPO (ng/ml)	3.64±0.70	7.82±0.90	0.025
Caspase-3 (U/g)	19.88±.1.72	37.20±2.10	0.001
COX-2 (ng/ml)	25.46±1.65	51.43±4.21	0.001
IL-6 (pg/ml)	89.40±2.95	156±6.44	0.033
MDA (nmol/g)	8.28±0.52	14.31±0.83	0.011
Catalase (U/g)	0.307±0.073	0.179±0.042	0.005
GPx (U/g)	45.58±1.66	39.27±1.34	0.420
GR (U/g)	67.39±7.35	59.23±6.92	0.322
SOD (U/g)	196.63±10.72	187±13.79	0.215

t-test was applied, *P* value <0.05 (significant).

By comparing mean±SD of all antioxidant indicators in the test group compared to the controls after 72 hours of the LPS administration, our findings showed statistically significant increase in the levels of TNFα, MPO, Caspase-3, COX-2, IL-6 and MDA. In addition, there was a statistically significant decrease on the level of catalase, and a statistically insignificant decrease in the levels of GPx, GR and SOD when the test group was compared to the controls. (Table 2)

Table 2. Comparison between the levels of the studied oxidative stress and antioxidant indicatorsas mean±SD in the test group versus the controls after 72 hours of the challenge with LPS

Parameters	Control group (no. 15)	Test group (no. 30)	*P* value
TNF-α (ng/ml)	26.3 ±1.12	94.6±7.73	0.012
MPO (ng/ml)	4.02±0.72	9.11±1.22	0.001
Caspase-3 (U/g)	18.96±.0.81	41.38±1.02	0.017
COX-2 (ng/ml)	28.03±1.52	61.98±4.35	0.005
IL-6 (pg/ml)	86.52±6.88	198.26±10.21	0.005
MDA (nmol/g)	9.215±0.66	17.47±0.92	0.001
Catalase (U/g)	0.295±0.079	0.133±0.091	0.000
GPx(U/g)	45.82±6.35	34.31±1.03	0.339
GR (U/g)	67.31±4.41	64.27±5.34	0.441
SOD (U/ml)	197.63±10.81	176.71±13.95	0.266

t-test was applied, *P* value <0.05 (significant).

In table 3, the comparison was between the antioxidant indicators in both test subgroups after 6 and 72 hours of LPS injection. Also, the results in the present study elucidate statistically significant increase in the levels of TNF-α, COX-2, IL-6, MDA, and there is low catalase activity, which could contribute to more oxidative stress. There was, however, insignificant difference in the levels of MPO, Caspase-3, GPx, GR and SOD in the test subgroup after 72 hours of the challenge when compared to the test subgroup after 6 hours.

Table 3. Comparison between the levels of the studied oxidative stress and antioxidant indicators expressed as mean±SD in the two test subgroups after 6 and 72 hours of the challenge with LPS

Parameters	6 hours test subgroup (no. 30)	72 hours test subgroup (no. 30)	*P* value
TNF-α (ng/ml)	82.8±7.61	94.6±7.73	0.037
MPO (ng/ml)	7.82±0.90	9.11±1.22	0.147
Caspase-3 (U/g)	37.20±2.10	41.38±1.02	0.332
COX-2 (ng/ml)	51.43±4.21	61.98±4.35	0.001
IL-6 (pg/ml)	156±6.44	198.26±10.21	0.005
MDA (nmol/g)	14.31±0.83	17.47±0.92	0.042
Catalase (U/g)	0.179±0.042	0.133±0.091	0.005
GPx (U/g)	39.27±1.34	34.31±1.03	0.115
GR (U/g)	59.23±6.92	55.27±5.34	0.247
SOD (U/ml)	187±13.79	176.71±13.95	0.062

t-test was applied, *P* value <0.05 (significant).

The correlation analysis was performed for all the studied mediators at a 6-hours interval. The values of Pearson's coefficient (r^2) were positive and statistically significant among the indicators, except the correlations between MDA on one hand and catalase, GPx, Gr and SOD on the other hand, they were negative, however, these correlations were statistically significant. The highest negative correlation was between MDA and SOD (r^2=-0.822), while the lowest positive one was between GPx and GR (r^2=0.322); on the other hand, the only insignificant correlation was between catalase and GR (r^2=0.282) (table 4).

Pearson's coefficient values (r^2) for the correlations between the studied indicators at a 72-hours interval were positive and statistically significant in the range of 0.376-0.774 except the correlations between MDA on the one hand and catalase, GPx, Gr and SOD on the other hand, which were negative and statistically significant. The highest correlation was between MPO and catalase (r^2=0.774), while the lowest one was between MDA and IL-6 (r^2=0.376); the insignificant correlations were between TNFα and Catalase, MDA and MPO, IL-6 and MPO, GPx and SOD (r^2=0.301, 0.288, 0.337 and 0.341, respectively) (table 5).

Table 4. The correlations between the studied oxidative stress and antioxidant indicators after 6 hours of the challenge with LPS [Pearson Correlation (r^2)]

	TNFα	MPO	Caspase	COX 2	IL-6	MDA	Catalase	GPx	GR	SOD
TNFα	1	0.544**	0.612**	0.733**	0.512**	0.549**	0.522*	0.612**	0.533*	0.667**
MPO	0.544**	1	0.679**	0.529*	0.702**	0.423*	0.549**	0.606*	0.552**	0.693**
Caspase	0.612**	0.679**	1	0.452*	0.647**	0.463*	0.395**	0.537**	0.507**	0.445**
COX-2	0.733**	0.529*	0.452*	1	0.558**	0.557**	0.628**	0.537*	0.665**	0.455**
IL-6	0.512**	0.702**	0.647**	0.558**	1	0.560**	0.680*	0.379*	0.669**	0.477*
MDA	0.549**	0.423*	0.463*	0.557**	0.560**	1	- 0.643**	- 0.559**	- 0.359*	- 0.822**
Catalase	0.522*	0.549**	0.395**	0.628**	0.680*	- 0.643**	1	0.469**	0.282***	0.441*
GPx	0.612**	0.606*	0.537**	0.537*	0.379*	- 0.559**	0.469**	1	0.322*	0.433**
GR	0.533*	0.552**	0.507**	0.665**	0.669**	- 0.359*	0.282***	0.322*	1	0.429**
SOD	0.667**	0.693**	0.445**	0.455**	0.477*	-0.822**	0.441*	0.433**	0.429**	1

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed); ***insignificant correlation

Table 5. The correlations between the studied oxidative stress and antioxidant indicators after72 hours of the challenge with LPS [Pearson Correlation (r^2)]

	TNFα	MPO	Caspase	COX 2	IL-6	MDA	Catalase	GPx	GR	SOD
TNFα	1	0.616**	0.414*	0.403**	0.469**	0.482**	0.301***	0.726**	0.592**	0.417**
MPO	0.616**	1	0.455**	0.556*	0.337***	0.288***	0.774**	0.552**	0.479*	0.653**
Caspase	0.414*	0.455**	1	0.524**	0.643**	0.538**	0.558**	0.395**	0.686**	0.534**
COX-2	0.403**	0.556*	0.524**	1	0.567**	0.605**	0.723**	0.495**	0.510**	0.539**
IL-6	0.469**	0.337***	0.643**	0.567**	1	0.376**	0.666**	0.564**	0.644**	0.670**
MDA	0.482**	0.288***	0.538**	0.605**	0.376**	1	- 0.532**	- 0.561**	- 0.597**	- 0.652**
Catalase	0.301***	0.774**	0.558**	0.723**	0.666**	- 0.532**	1	0.545**	0.602**	0.602**
GPx	0.726**	0.552**	0.395**	0.495**	0.564**	- 0.561**	0.545**	1	0.453**	0.341***
GR	0.592**	0.479*	0.686**	0.510**	0.644**	- 0.597**	0.602**	0.453**	1	0.753**
SOD	0.417**	0.653**	0.534**	0.539**	0.670**	- 0.652**	0.602**	0.341***	0.753**	1

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed); ***insignificant correlation

4. Discussion

Oxidative stress is attributed to the imbalance between excessive accumulation of ROS intracellularly and the inability of the antioxidants to detoxify them. Physiologically, ROS can play several roles such as cell signaling. When the production of ROS is exceeding the normal physiological limit, it can cause harmful effects on cellular components such as nucleic acids, proteins and lipids of the cells (Pizzino et al. 2017).Antioxidants are stable molecules having scavenging properties to terminate the chain reaction of free radicals before damaging the cellular vital molecules (Khallouki et al. 2022).

In the present study, LPS was used for the induction of oxidative stress in the experimental rat model and the changes in antioxidant indicators were studied after 6 and 72 hours-interval of the challenge. Upon comparing the levels of the indicators in the test group versus the controls, our results after 6 hours of the challenge revealed significant elevation in the levels of TNFα, MPO, Caspase-3, COX-2, IL-6 and MDA; the level of catalase showed a significant reduction, and the decrease in the levels of GPx, GR and SOD was insignificant. In a study conducted to explore the link between oxidative stress mediated by thyroid dysfunctioninduction and TNFα, the estimated oxidative stress indicators showed significant increase in TNFα and MDA levels in the rats under effect of oxidative stress status (Hazzaa et al. 2013). Another study revealed significant increase in the levels of TNFα and caspase-3 in the test group compared to the controls. Their obtained results were based on the proven link between the deficiency of TNFα and the prevention of oxidative stress in mice (Wang et al. 2017). In addition, Awooda et al. [2014] stated that TNFα and MDA levels were higher in brain tissues of rats due to oxidative stress induced by transient cerebral ischemia which showed an association between TNF-α and oxidative stress biomarkers (Awooda et al. 2014). Francéset al, demonstrated the contribution of TNF-α and the resulting oxidative stress in hepatic tissues in streptozotocin-induced diabetes in experimental animal models (Frances et al. 2013). Additionally, both TNF-α and MDA levels are significantly elevated in an induced sepsis and its associated oxidative stress (Erbaş and Taşkıran 2014). In a Turkish study, the animal model was under ischemic-induced oxidative stress status mediated through TNF-α;

the results showed significant higher levels of TNF-α. On the other hand, there was significant reduction in the activities of catalase and SOD (Akbulut et al. 2005).

When metabolic syndrome and ulcerative colitis were induced chemically in experimental rat model, the oxidative status in both situations was associated with rise of MPO, SOD, catalase and GPx levels in the test group when compared to the control group (Garagiola et al. 2016, Geetha et al. 2017). In addition, in rheumatoid arthritis patients, there was elevation of MPO level that was contributed to the inflammatory disorder and its associated oxidative stress status (Stamp et al. 2012). In another animal model study, liver and kidney were subjected to toxicity by carbon tetrachloride; the results of studying the oxidative stress status and antioxidants levels showed significant higher SOD and MDA, lower GPx and catalase in the test group versus the controls (Balahoroğlu et al. 2008). Chlorpyrifos, the pesticide, was used for inducing oxidative stress in rat model; then, the oxidative indicators were studied. The findings showed significant rise in TNF-α and caspase-3 levels, decrease in SOD, catalase and GPx in chlorpyrifos-treated rats after 14 consecutive days of exposure compared to the control group of rats (Owumi and Dim 2019).

Sulindac sulfide, the chemo-preventive agent, its effect was studies in several dying cell lines; it can generate an oxidative stress status leading to the initiation of an increase in the synthesis and activity of COX-2 (Sun et al. 2009). In a streptozotocin-induced diabetic retinopathy, the increase of oxidative stress is apparent through elevating the levels of MDA and IL-6, but catalase is reduced in the test group when compared to the controls. Additionally, IL-6 can reduce the oxidative stress in β cells of islets of Langerhans (Robinson et al. 2020). Furthermore, the generated and accumulated ROS in the diaphragm muscle in mice is associated with over-expression of circulating IL-6. The use of antioxidants for protecting mitochondria is capable of reducing IL-6 and oxidative stress in sepsis-induced state in a rat model (Lowes et al. 2013). Mostly, our obtained results were in accordance with those reported in some of the above- mentioned studies and yet disagree with others. These could be attributed to the difference in the type of animal model, the average of their weights, the different conditions in the animal house where the animals were kept, and the sample size.

In the present study, all antioxidant indicators in the test group were compared to the control group after 72 hours of the LPS injection intraperitoneally. Our results showed elevation in the levels of TNFα, MPO, Caspase-3, COX-2, IL-6 and MDA, a decrease in catalase level, and both situations were statistically significant, while there was insignificant reduction in the levels of GPx, GR and SOD. Halawa et al. [2018] studied the enhancement of oxidative stress and apoptosis in the testicular tissues of rats by LPS intraperitoneal administration (Halawa et al. 2018).The indicators of oxidative stress, antioxidants and caspase-3were investigated after 6 and 72 hours of the challenge; their results identified a slight insignificant and significant reduction in SOD levels after 6 hours and 72 hours of the LPS-treated group, respectively, compared to the controls. In both time intervals, there was significant reduction in catalase, and the level of MDA was increased significantly but without significant difference between 6 and 72 hours. In addition, the increase in the concentration of caspase-3 was higher after 6 hours than the controls with more increase after 72 hours; the difference in both comparisons was statistically significant, proving that it was time-dependent. Those results are in agreement with the obtained ones in the present study, especially the designation of the study to be time-dependent in investigating the oxidative status and the antioxidants after 6 and 72 hours-time intervals of LPS challenge.

Concerning the correlation analysis for all oxidative mediators at a 6 and 72-hours interval of LPS injection, Spearman's correlation coefficient was significant positively among all indicators. Moreover, there was a negative and statistically significant correlation between MDA and four of the indicators (catalase, GPx, GR and SOD). On the other hand, there were insignificant correlations between catalase and GR after 6-hours of the challenge, while the insignificant correlations after 72-hours of LPS injection were between TNFα and Catalase, MDA and MPO, IL-6 and MPO, GPx and SOD. Noeman et al. [2011] found negative significant and negative insignificant correlations between MDA and GPx on one hand and between MDA and catalase on the other hand, respectively, in hepatic tissues (Noeman et al. 2011).

5. Conclusion

In the present study, LPS was used to induce oxidative stress in rat model; the oxidative status results were in significant differences in the levels of TNFα, MPO, Caspase-3, COX-2, IL-6 MDA and catalase, and insignificant differences for GPx, GR and SOD after both intervals (6 and 72-hours). Levels of indicators showed significant differences when 6-hours interval were compared to 72-hours interval except for MPO, Caspase-3, GPx, GR and SOD. There were positive and negative significant correlations between the studied indicators except for catalase and GR, on the one hand, and for TNFα and catalase, MDA and MPO, IL-6 and MPO, GPx and SOD, on the other hand, after 6 and 72 hours of the challenge.

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Conflict of Interest

The authors declare that there are no conflicts of interests.

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