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Deleterious effects of inflammatory cytokines induced by endotoxin on hepato-renal functions and attenuating role of estrogen in vivo

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Abstract

Objective

One of the potent innate immune system stimulators is endotoxin or lipopolysaccharide (LPS). This stimulator can lead to systemic inflammatory response syndrome (SIRS). It may also ultimately lead to septic shock. Estrogen is a steroid hormone that plays an important role in female reproduction. This hormone also has multiple roles in other body systems, including the immune system. The aim of this study was to investigate the expression of TNF-α, IL-6, CCL2 and CCL3 genes in response to LPS. Other aims of this study were to evaluate histological and functional changes in the kidney and liver, measure serum levels of inflammatory cytokines such as TNF-α, IL-6 and MCP-1, and investigate the effects of ovariectomy (removal of the ovaries and consequently estrogen deficiency) on the extent of organ damage and inflammatory response induced by LPS in three-month-old adult female Wistar rats.

Materials and methods

Several groups would typically be included in an experiment to examine the effects of estrogen, endotoxins, and their combination. All laboratory tests for assessing inflammatory cytokines, kidney and liver function, and gene expression were carried out in the laboratories of the College of Veterinary Medicine, Al-Qadisiyah University, Iraq. The study included 138 adult female Wistar rats (3 months old) distributed across six main groups; each set contains 23 adult female Wistar rats including G1-Sham, G2-OVX, G3-LPS, G4-E2+LPS, G5-LPS+E2, and G6-E2).

Results

The results of this study reported a statistically significant difference ($p<0.05$) in serum levels of inflammatory cytokines TNF-α, IL-6 and MCP-1. In addition, creatinine and urea (kidney function), ALT and AST (liver function) and TNF-α, IL-6, CCL2 and CCL3 gene expression were observed between 3-month-old female Wistar rats.

Conclusions

The study concluded that estrogen reduces the harmful effects of inflammatory cytokines that increase due to endotoxins, and therefore estrogen activates liver and kidney functions in adult female Wistar rats. Estrogen signaling may represent a promising therapeutic target for mitigating sepsis-induced multi-organ injury in estrogen-deficient states. Estrogen pre-treatment reduced TNF-α expression by ~40% relative to LPS-only group.

Keywords: endotoxin, estrogen, IL-6, inflammatory cytokines, TNF-α

Paper Type: Research Paper.

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Introduction

A basic biological reaction to infection and tissue damage is inflammation, but excessive or dysregulated inflammation may cause serious organ malfunction (Fang et al., 2024; Ghareeb et al., 2024). One of the important components of the outer membrane of Gram-negative bacteria is lipopolysaccharide (LPS), which is commonly used in experimental models to model endotoxemia and simulate the systemic inflammatory response (Wójcik et al., 2024). LPS stimulates immune cells through Toll-like receptor 4 (TLR4) and leads to the release of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and chemokines such as CCL2 and CCL3 (Fan et al., 2020). Cells that play a key role in immune and inflammatory responses produce proteins called cytokines. These cytokines participate in these processes by interfering with the regulation of other cells (Mohammadabadi et al., 2025b). Because they can activate the immune system to provide effective protection, they can be used in vaccine development. Different cells can produce cytokines. For example, epithelial cells produce the cytokines IL-6 or IL-8 (Khezri et al., 2025). Macrophages produce cytokines involved in the activation and regulation of T helper (Th) lymphocytes in inducing an adaptive immune response.

The mode of action of all cytokines is similar. They bind to receptors on the surface of target cells and, through these receptors, affect the activity of the target cells (Mohammadabadi et al., 2025a). There are three criteria for classifying cytokines. These criteria include the cells that produce the cytokines, the cells that the cytokines affect, and the type of activity of the cytokines themselves. Cytokines include several groups, although there is considerable overlap between them. These groups include interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), transforming growth factors (TGF), migration inhibitory factors, and smaller chemokines (Mohammadabadi et al., 2025b). They have a role in the pathophysiology of renal and hepatic damage, where they are believed to promote oxidative stress, microvascular dysfunction, and apoptosis of the affected cells. If these cytokines are produced in excess, they cause reactive oxygen species (ROS) and apoptosis. Therefore, mitochondrial function is impaired and tissue damage in the liver and kidneys is aggravated (Yousif et al., 2023; Ma et al., 2025). The cellular stress caused by adipocyte hypertrophy causes inflammatory pathways to be activated. When macrophages infiltrate adipose tissue, they increase the production of inflammatory mediators such as macrophage chemotactic protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) (Ma et al., 2021). These proinflammatory mediators have important effects on the heart, liver, kidneys, etc. The translation is as follows: In the absence of estrogen's protective effects, especially those of 17β-estradiol (E2), which are mediated by estrogen receptor-α (ERα), and the expression of estrogen receptor-β (ERβ) in hepatic and renal organs. ERα and ERβ activation regulates inflammatory signaling via inhibition of NF-κB nuclear localization which lowers cytokine gene expression (Darvishzadeh Mahani et al., 2021; Al-hashemi et al., 2023). When the levels of glutathione peroxidase, catalase, and superoxide dismutase (SOD) enzymes are increased, estrogen can enhance antioxidant defense mechanisms. Meanwhile, it can reduce the levels of lipid peroxidation markers such as malondialdehyde (MDA). Estrogen is one of the main female sex hormones and plays an important role in reproduction. In addition, estrogen has anti-inflammatory, antioxidant, and cytoprotective biological effects (Yuan et al., 2022). Because estrogen can reduce the excessive production of cytokines and inhibit inflammatory signaling pathways such as NF-κB and MAPK, it is possible that it can affect immune responses. By these combined mechanisms—inhibition of NF-κB-mediated inflammation, receptor-dependent signaling via ERα/ERβ, reafirmation of antioxidant pathways—estrogen alleviates the harmful hepato-renal consequences of endotoxin exposure, facilitating cellular homeostasis and organ preservation in endotoxemia (Ma et al., 2021). In addition, estrogen deficiency during the postmenopausal state has been associated with enhanced risk of diseases and organ damage due to inflammation (Xu et al., 2020). The function of estrogen in LPS-induced inflammation offers important implications for treatment of inflammatory and septic conditions (Durmaz et al., 2020). The aim of this investigation was to assess for protective effects of supplementation with different doses of estrogen replacement therapy (17β-estradiol (E2)) on LPS-induced endotoxemia-induced injurious effects on kidney and liver function in the rat. Accordingly, this research concentrated on the gene expression of inflammatory cytokines (TNF-α, IL-6, CCL2, and CCL3) in certain experimental groups as well as the blood levels of biochemical indicators of organ function (ALT, AST, creatinine, and urea). Through side-by-side comparisons of estrogen-treated to untreated animals, this work aims to clarify the mechanism and fashion in which estrogen attenuates inflammatory responses and protects against the end-organ damage often accompanying the injurious effects of endotoxins. Endotoxin (lipopolysaccharide; LPS)-mediated systemic inflammation, as evidenced by excessive generation of cytokines that promote inflammation, such as TNF-α and IL-6, causes hepatic and renal dysfunction, yet the precise mechanisms responsible for the protection against these deleterious effects by estrogen (mostly 17β-estradiol) have not been fully elucidated. Though data about this pathophysiologic effect of estrogen is available, it is primarily in reference to estrogen's general anti-inflammatory and antioxidant actions, offering scant specific insight on estrogen's modulatory capacity to cytokine-mediated hepatotoxicity and nephrotoxicity in endotoxemic settings. Based on the research conducted so far in experimental rat endotoxemia models, it is not clear what the different roles of estrogen receptor subtypes (ERα and ERβ) are in regulating these inflammatory pathways in vivo. Therefore, it is hypothesized that the use of estrogen in rats prevents the excessive production of proinflammatory cytokines (TNF-α, IL-6, and MCP-1) and reduces endotoxin (LPS)-induced liver and kidney injury. It is likely to do this by modulating the expression of NF-κB-dependent cytokine genes. It is likely to use estrogen receptor-dependent pathways to carry out this protective pathway. Because this pathway inhibits inflammatory signaling cascades and oxidative stress and maintains liver-renal integrity during endotoxemia. Thus, the aim of this study was to investigate the expression of TNF-α, IL-6, CCL2 and CCL3 genes in response to LPS. Other aims of this study were to evaluate histological and functional changes in the kidney and liver, measure serum levels of inflammatory cytokines such as TNF-α, IL-6 and MCP-1, and investigate the effects of ovariectomy (removal of the ovaries and consequently estrogen deficiency) on the extent of organ damage and inflammatory response induced by LPS in three-month-old adult female Wistar rats.

Materials and methods

Experimental design and grouping: Endotoxin, estrogen, and their combination treatments were provided by the Laboratory Animal Care Center, Faculty of Veterinary Medicine, Al-Qadisiyah University, Iraq. A standard experimental design would include many groups to compare the effects of these substances. All animals were maintained under standard laboratory conditions. The light/dark cycle was 12 h. The temperature and humidity were 22 ± 2 °C and 50-60%, respectively. Standard food and water were available to the animals ad libitum. The experimental period was from September 3, 2025 to June 17, 2025. 138 adults female Wistar rats, 3 months old, were used in this study to investigate the effects of endotoxin, estrogen, and their combination on these animals. The selection of rats was completely randomized into four main experimental groups (23 animals per group). Randomization was performed by computer. The first group (Sham) included rats that underwent sham surgery (laparotomy without ovariectomy) and received vehicle solution (normal saline). The second group (OVX) included rats that were ovariectomized and received vehicle solution. The third group (LPS) underwent sham surgery, i.e., received an intraperitoneal (IP) injection of lipopolysaccharide (LPS, 7 mg/kg), an endotoxin from Gram-negative bacteria known to induce systemic inflammation. The dose was chosen to cause organ dysfunction without causing immediate death. Group 4 (E2 + LPS) included ovariectomized rats that had been pretreated with estrogen (17β-estradiol, 0.5 mg/kg/day, subcutaneously in sesame oil, for seven consecutive days prior to LPS injection (7 mg/kg, intraperitoneally). Group 5 included rats that had received concurrent treatment with LPS (7 mg/kg, IP) and estrogen (17β-estradiol, 0.5 mg/kg/day, subcutaneously) on the same day. Group 6 (E2) contained ovariectomized rats received estrogen replacement therapy only (17β-estradiol, 0.5 mg/kg/day, subcutaneously) without LPS administration. All sample collection and biochemical analyses were carried out by researchers who were not aware of the treatment groups. All laboratory tests for assessing inflammatory cytokines, kidney and liver function, and gene expression were conducted in the laboratories of the College of Veterinary Medicine, Al-Qadisiyah University. Coded labels were used during the ELISA assays and statistical analysis to ensure unbiased data interpretation. ELISA kits were used to measure inflammatory cytokine levels. These kits included TNF-α: Rat TNF-alpha ELISA Kit (Category #ab208348), IL-6: Rat IL-6 ELISA Kit (Category #ab234570), and MCP-1: Rat macrophage chemotactic protein-1 ELISA Kit (Category #ab100777).

Analysis of gene expression: TNF-α, IL-6, CCL2, and CCL3 are examples of inflammatory cytokines whose gene expression may be measured in kidney and liver tissues using quantitative polymerase chain reaction (qPCR). 25 mg of liver and kidney tissue is taken and rapidly frozen in liquid at a temperature of approximately -20°C, where it is used to obtain RNA yield.

RNA extraction: 25 mg of tissue was placed in a chilled tube. 500 µl of TRIzol was added. A rotor-stator or Bead Mill was used to completely homogenize the tissue (2 times, 30 s each time at high speed). Homogenization was performed so that no visible fragments remained. The tube was kept on ice between each homogenization. The homogenized mixture was incubated for 5 min at room temperature to completely separate the nucleoprotein complexes. For every 0.5 ml of TRIzol, 100 µl of chloroform was added. The tube was then closed and shaken by hand for 15 s. After this step, it was left at room temperature for 2-3 min. The tube was centrifuged at 12,000 × g for 10 min at 4°C. Three phases were obtained: the upper aqueous phase (RNA), the intermediate phase (DNA/proteins), and the lower organic phase. The upper aqueous phase was gently transferred to a fresh RNase-free tube without contact with the middle phase. Then, for every 1 ml of TRIzol used, 0.5 ml of isopropanol was added. For samples with low RNA, 1 µl of linear polyacrylamide was added as a carrier. After mixing by inversion, the mixture was left at room temperature for 10 min. It was then centrifuged at 12,000 × g for 10 min at 4°C. An RNA pellet was formed, although it was not very visible. To wash the RNA pellet, the supernatant was gently discarded, 1 mL of 75% ethanol (with RNase-free water) was used to wash the pellet, the sample was vortexed briefly, centrifuged for 5 min at 7500 × g at 4°C, the ethanol was slowly removed, and the pellet was air-dried for 5-10 min. The pellet was then dissolved in 40 µL of RNase-free water. To aid dissolution, the sample was placed at 55°C for 10 min and gently pipetted up and down.

RNA concentration and quality control measurement: A Qubit analyzer was used to measure RNA concentration, yielding a concentration of 40 µg/mL. A NanoDrop analyzer (A260/A230 ratio) was used to assess RNA purity, yielding a value of 1.8. To check the integrity of the RNA, samples were run on a Bioanalyzer/TapeStation to determine the RIN number for qPCR (RIN ≥ 7). The presence of intact rRNA bands including 28S and 18S on the gel indicated good RNA quality. Finally, RNA samples were stored at -20°C.

cDNA synthesis: The assay is performed on a Promega (USA) Quantus™ Fluorometer. RNA was turned into cDNA using a cDNA synthesis kit (ADDBio/Korea). Aliquot a volume of each RNA sample into a PCR tube containing 2X reverse transcriptase, cDNA, dNTPs, and random hexamer oligonucleotides. Able to convert RNA to cDNA by glass of cDNA synthesis kit (ADDBio/Korea) The cDNA that was isolated is amplified with specific primers. Housekeeping: GAPDH: F; 5′-TGCACCACCAACTGCTTAGC-3′ , R; 5′-GGCATGGACTGTGGTCATGAG-3′ , TNF-α gene: F; 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, R; 5′-TGGGAGTAGACAAGGTACAACCC-3′ (Khatri et al., 2020), IL-6 gene: F; 5′-GAGGATACCACTCCCAACAGACC-3′, and R; 5′-AAGTGCATCATCGTTGTTCATACA-3′ (Xu et al., 2007), CCL2 (MCP-1) gene: F; 5′-TTAAAAACCTGGATCGGAACCAA-3′, and R; 5′-GCATTAGCTTCAGATTTACGGGT-3′ (Wu et al., 2023), CCL3 (MIP-1α) gene: F; 5′-GCTGCTCAGAGACAGGAAGTCTT-3′, and 5′-ACAGGAACTGCGGAGAGGAGT -3′ (Osawa et al., 2006), using a Real Time qPCR machine (BioRad, USA) device. BioRAD (USA) was responsible for the thermal conditioning process.

RT-qPCR amplification: Genomic RNA from tissues was isolated using an RNA Isolation kit (ADDBio/Korea) at 40 µg/ml or less. This test is performed using a Promega (USA) Quantus™ Fluorometer. CDNA from total RNA can be converted from RNA by cDNA synthesis kit (ADDBio/Korea).

Quantification methods: The Ct values obtained from the genomic DNA sample were used to calculate the copy counts for the genes GADPH, TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α). These values were then compared with the slopes and intercepts of the previously constructed standard curves. The formulas and ratios used in the two quantification processes (relative quantification) were thoroughly compared using the relevant mathematical techniques. Either the actual results or 100% efficiency for all tests were taken into consideration for relative quantification. For example, other methods may be used to compare the target gene's expression level in the test sample to that in the calibrator sample after the analysis of Ct values. The most popular and simple approach is used to estimate the probability that both target and reference genes are amplified with similar ~100% efficiency (E%). One of the assumptions that should be confirmed before to using the Livak method is that the amplification efficiencies of the target and reference genes should be equal. The following formula was used to normalize the transcription level using GADPH, TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α) data based on the delta-delta Ct method:

$\Delta Ct(test) = Ct\ (target, test) - Ct\ (ref, test)$, $\Delta Ct(control) = Ct\ (target, control) - Ct\ (ref, control)$, $\Delta\Delta Ct = \Delta Ct(test) - \Delta Ct(control)$, $2^{-\Delta\Delta Ct}$ = fold change, and %Efficiency (E%) = $(10^{(-1/slope)}-1)\times 100$.

Animal model and ethical considerations: Adult female Wistar rats weighing 250 g were used in this study. The acclimation phase of the animals to the environment began one week before the start of the experiment. Exposure was performed on a 12-hour light/12-hour dark cycle. Temperature and humidity were fully controlled and regulated. This study was conducted in accordance with the ARRIVE 2.0 guidelines and the International Ethics Standards for Animal Care and Use. It was also approved by the Al-Qadisiyah University Animal Care and Use Committee (IACUC). Humane endpoints were also determined. Animals that showed signs of severe distress such as loss of appetite, weight loss of more than 20%, or the appearance of symptomatic behaviors including unconsciousness were immediately euthanized with a high dose of sodium pentobarbital (200 mg/kg, intraperitoneally). Animals were examined daily for signs of pain or illness. In addition, every effort was made to reduce the number of animals used and to minimize their distress.

Ethical approval: It is important to note that this research was approved by the Ethical Approval Committee with reference number 459 dated 9/3/2025. This study was also approved by Al-Qadisiyah University, Faculty of Veterinary Medicine.

Statistical analysis: RT-qPCR amplification and data normalization for TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α), and the housekeeping gene (GAPDH) in the two groups were statistically associated, according to descriptive statistics and in all groups by one-way ANOVA and Tukey's multiple comparison, and provide 95% confidence intervals where applicable, with a P-value less than 0.05. The findings of the present investigation are shown as mean ± SD or as percentages. The T-test was used to assess mean differences between two groups for quantitative variables using SPSS version 27 (SPSS Inc., Chicago, IL, USA). A P-value was deemed statistically significant if it was below 0.05 at a 95% confidence interval. Clinical biological indicators were subjected to statistical analysis using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). The findings were shown as $P \leq 0.05$ and mean ± SD. The analysis of the mean ± SD was made easier using the student's t-test. Statistical significance is shown when the p-value is less than 0.05.

Results

Evaluation of the effect of lipopolysaccharide (LPS, 7 mg/kg) and estrogen replacement therapy (E2-17β-estradiol, 0.5 mg/kg/day) on liver and kidney function and inflammatory cytokines: For inducing an inflammatory response, LPS was injected intravenously at a dose of 7 mg/kg. Injections were performed seven days after ovariectomy (or a specified time). For evaluating liver function, serum ALT and AST levels was examined. Moreover, for evaluating kidney function, urea and creatinine levels were measured. The expression of TNF-α, IL-6 and MCP-1 in kidney and liver tissues were determined using immunohistochemistry (Table 1).

Evaluation of the effect of ovariectomy on estrogen deficiency, liver and kidney function and inflammatory cytokines: This evaluation was performed by comparing the G1-Sham group with the G2-OVX group. These results are shown in Table 1.

The effect of lipopolysaccharide (LPS, 7 mg/kg) on liver and kidney function and inflammatory cytokines: The effect of LPS was studied by comparing the endotoxin group (G3) with the sham group (G1) and the results are shown in Table 2.

The effect of estrogen replacement therapy (17β-estradiol, 0.5 mg/kg/day) on liver and kidney function and inflammatory cytokines: This effect was studied by comparing the estrogen-only group (G6-E2) with the ovariectomized control group (G2-OVX). The results are shown in Table 3.

Effect of estrogen prophylactic replacement therapy treatment (17β-estradiol, 0.5 mg/kg/day) with lipopolysaccharide (LPS, 7 mg/kg) on liver and kidney function and inflammatory cytokines: This effect was done through a comparative study between the estrogen pre-treatment group (G4-E2+LPS) and the endotoxin group (G3-LPS). The results are shown in Table 4.

Table 1. The clinical and biochemical features of (G1 and G2) groups in this study

Parameters	Groups		T-test	P-value
	G1-Sham Mean ± SD	G2-OVX Mean ± SD		
TNF-α (pg/mL)	17.26± 4.70	43.04± 7.31	14.23	0.023*
IL-6 (pg/mL)	25.91± 6.28	56.43± 8.30	14.07	0.008*
MCP-1 (pg/mL)	69.43± 13.92	124.04± 18.84	11.18	0.015*
ALT (U/L)	34.74± 6.06	53.70± 7.80	9.224	0.012*
AST (U/L)	74.04± 11.31	103.3± 15.98	7.157	0.016*
Creatinine (mg/dL)	0.951± 0.15	0.769± 0.14	4.164	0.043*
Urea (mg/dL)	19.83± 3.20	28.61± 4.87	7.994	0. 01*

Table 2. The clinical and biochemical features of (G1 and G3) groups in this study

Parameters	Groups		T-test	P-value
	G1-Sham Mean ± SD	G3- LPS Mean ± SD		
TNF-α (pg/mL)	17.26± 4.70	480.1± 87.83	25.24	0.001*
IL-6 (pg/mL)	25.91± 6.28	1319± 304	20.40	<0.0001*
MCP-1 (pg/mL)	69.43± 13.92	604± 126	20.23	<0.0001*
ALT (U/L)	34.74± 6.06	153± 18.52	29.11	0.001*
AST (U/L)	74.04± 11.31	292.1± 38.15	26.29	0.004*
Creatinine (mg/dL)	0.951± 0.15	1.52± 0.20	18.10	0.002*
Urea (mg/dL)	19.83± 3.20	53.39± 7.86	18.97	0.037*

Table 3. The clinical and biochemical features of (G2 and G6) groups in this study

Parameters	Groups		T-test	P-value
	G2-OVX Mean ± SD	G6-E2 Mean ± SD		
TNF-α (pg/mL)	43.04± 7.31	13.65± 2.71	18.09	0.041*
IL-6 (pg/mL)	56.43± 8.30	21.48± 4.36	17.89	0.01*
MCP-1 (pg/mL)	124.04± 18.84	51.52± 9.40	16.52	0.002*
ALT (U/L)	53.70± 7.80	40.04± 2.84	7.19	0.046*
AST (U/L)	103.3± 15.98	76.74± 7.33	7.23	0.003*
Creatinine (mg/dL)	0.769± 0.14	0.64± 0.054	4.251	0.01*
Urea (mg/dL)	28.61± 4.87	25.87± 2.24	2.766	0.038*

Effect of concurrent estrogen replacement therapy (17β-estradiol, 0.5 mg/kg/day) with lipopolysaccharide (LPS, 7 mg/kg) on liver and kidney function and inflammatory cytokines: This effect was done through a comparative study between the estrogen co-treatment group (G5-LPS+E2) and the endotoxin group (G3-LPS) to demonstrate the effect of administering oral estrogen replacement therapy (17β-estradiol, 0.5 mg/kg/day) simultaneously with giving LPS (7 mg/kg) administration. The results are shown in Table 5.

Table 4. The clinical and biochemical features of (G3 and G4) groups in this study

Parameters	Groups		T-test	P-value
	G3-LPS Mean ± SD	G4-E2+LPS Mean ± SD		
TNF-α (pg/mL)	480.1± 87.83	321.4± 50.58	9.205	0.013*
IL-6 (pg/mL)	1319± 304	785.6± 139.6	9.782	0.0001*
MCP-1 (pg/mL)	604± 126	376.3± 73.78	7.223	0.0026*
ALT (U/L)	153± 18.52	93.7± 7.33	16.13	0.034*
AST (U/L)	292.1± 38.15	187.5± 10.54	13.15	0.019*
Creatinine (mg/dL)	1.52± 0.20	1.030± 0.133	11.29	0.028*
Urea (mg/dL)	53.39± 7.86	39.83± 1.43	7.620	0.001*

Table 5. The clinical and biochemical features of (G3 and G5) groups in this study

Parameters	Groups		T-test	P-value
	G3-LPS Mean ± SD	G5-LPS+E2 Mean ± SD		
TNF-α (pg/mL)	480.1± 87.83	232.2± 51.25	16.74	0.011*
IL-6 (pg/mL)	1319± 304	528.6± 122.1	13.07	0.001*
MCP-1 (pg/mL)	604± 126	264.8± 58.10	12.26	0.0023*
ALT (U/L)	153± 18.52	90.52± 12.22	14.99	0.026*
AST (U/L)	292.1± 38.15	171.1± 25.67	12.50	0.001*
Creatinine (mg/dL)	1.52± 0.20	0.978± 0.157	8.924	0.036*
Urea (mg/dL)	53.39± 7.86	32± 4.543	11.54	0.01*

Evaluating the expression of TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α) genes comparing with housekeeping (GAPDH) gene on liver and kidney function and inflammatory cytokines in studied groups: TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α) gene expression were examined in the studied groups (G1, G2, G3, G4, G5, and G6). The results are shown in Table 6. The results of relative gene expression are shown in Table 7. Amplification curves of genes are shown in Figures 1, 3, 5, and 7 and the melting curves of genes are shown in Figures 2, 4, 6, and 8.

Discussion

The results showed that there are significant differences ($p < 0.05$) between the G1-Sham and G2-OVX groups in terms of inflammatory cytokines (TNF-α, IL-6, and MCP-1), renal function (creatinine and urea), and liver function (ALT and AST). In addition, these differences were also observed between the G1-Sham and G3-LPS groups (Table 2), between G2-OVX and

G6-E2 (Table 3), between G3-LPS and G4-E2+LPS (Table 4), and between G3-LPS and G5-LPS+E2 (Table 5).

Table 6. The comparison between the gene expression of TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α) genes comparing with the housekeeping gene (GAPDH) in the studied groups

Genes	Groups Mean ± SD						T-test	P-value
	G1-sham	G2-OVX	G3-LPS	G4-E2+LPS	G5-LPS+E2	G6-E2		
TNF-α	8.5± 0.5	8± 0.3	5.5± 0.6	6.5± 0.4	6.8± 0.3	8.3± 0.4	5.66	0.001*
IL-6	9.1± 0.6	8.5± 0.8	5± 0.7	6± 0.6	6.3± 0.5	8.8± 0.4	7.02	0.01*
CCL2	8.7± 0.3	8.3± 0.2	5.2± 0.3	6.2± 0.4	6.4± 0.7	8.5± 0.4	6.77	0.03*
CCL3	8.8± 0.5	8.4± 0.4	5.4± 0.6	6.3± 0.3	6.5± 0.2	8.6± 0.4	6.48	0.04*

Table 7. Relative quantification of TNF-α, IL-6, CCL2 (MCP-1), and CCL3 (MIP-1α) genes comparing with the housekeeping gene (GAPDH) in the studied groups

Groups	Gene											
	TNF-α (E = 98%)			IL-6 (E = 95%)			CCL2 (E = 92%)			CCL3 (E = 96%)		
	Δct	ΔΔct	2-ΔΔct	Δct	ΔΔct	2-ΔΔct	Δct	ΔΔct	2-ΔΔct	Δct	ΔΔct	2-ΔΔct
G1-sham	8.5	0.01	1	9	0.02	0.9	8.7	0.23	0.8	8.8	0.01	0.97
G2-OVX	8	0.5	1.41	8.5	0.52	1.48	8.3	0.4	1.32	8.4	0.45	1.36
G3-LPS	5.5	0.3	8	5	0.46	16	5.2	0.35	8.31	5.4	0.34	8.56
G4-E2+LPS	6.5	0.24	4	6	0.37	8	6.3	0.25	5.66	6.8	0.27	5.56
G5-LPS+E2	6.8	0.17	3.25	6.3	0.27	6.9	6.4	0.29	4.93	6.3	0.23	4.42
G6-E2	8.3	0.2	1.7	8.8	0.25	1.26	8.7	0.21	1.27	8.6	0.21	1.28

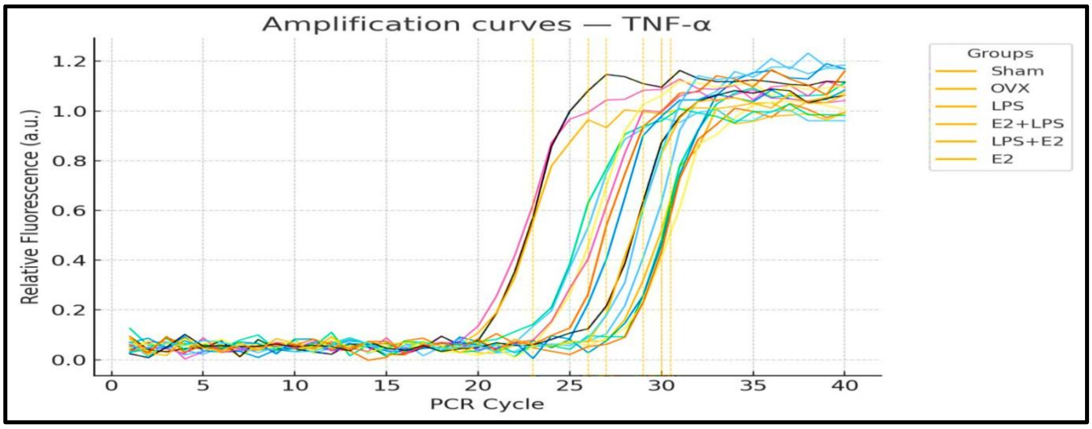

Figure 1. Amplification curve of TNF-α gene expression in study groups

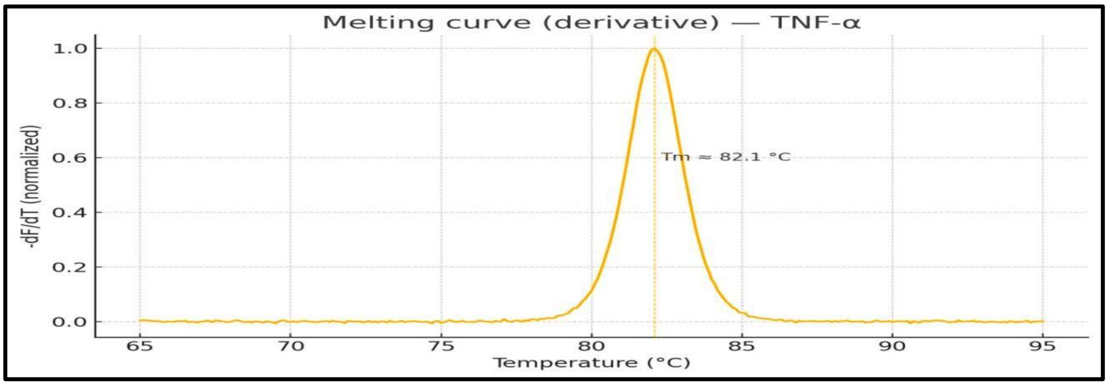

Figure 2. Melting curve analysis of TNF-α gene expression in study groups

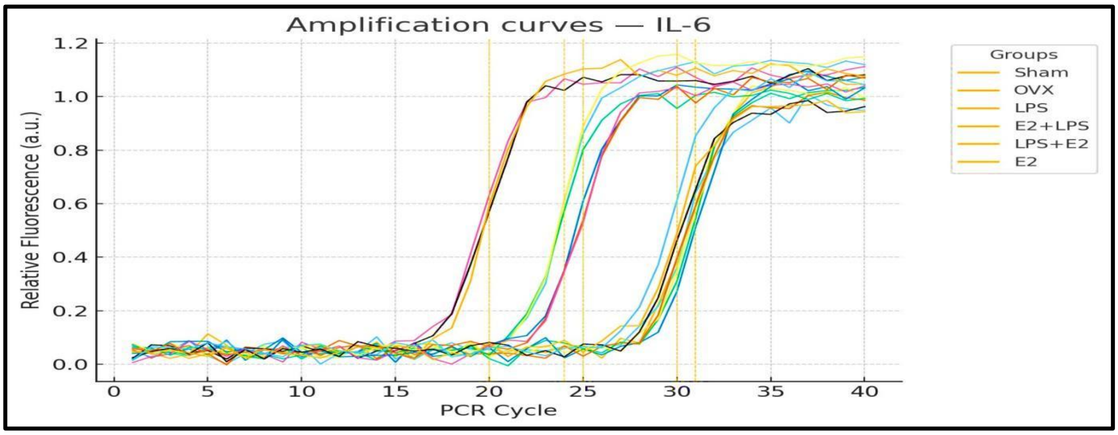

Figure 3. Amplification curve of IL-6 gene expression in study groups

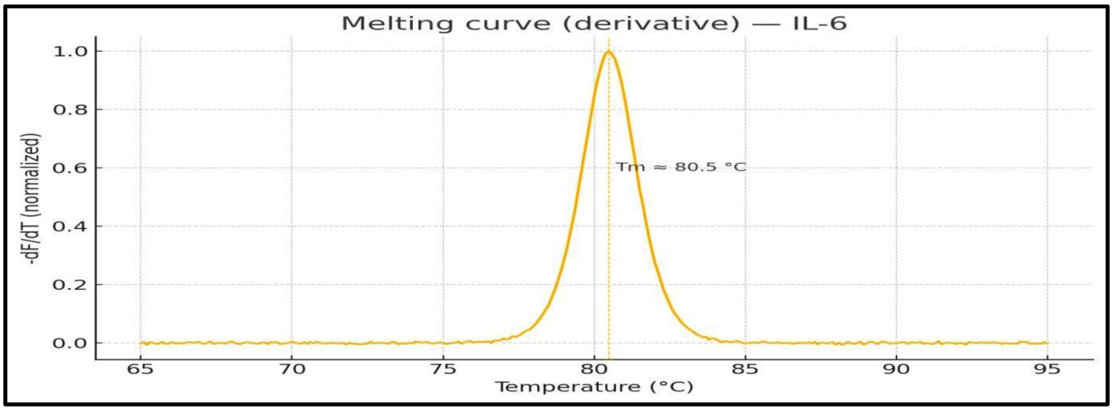

Figure 4. Melting curve analysis of IL-6 gene expression in study groups

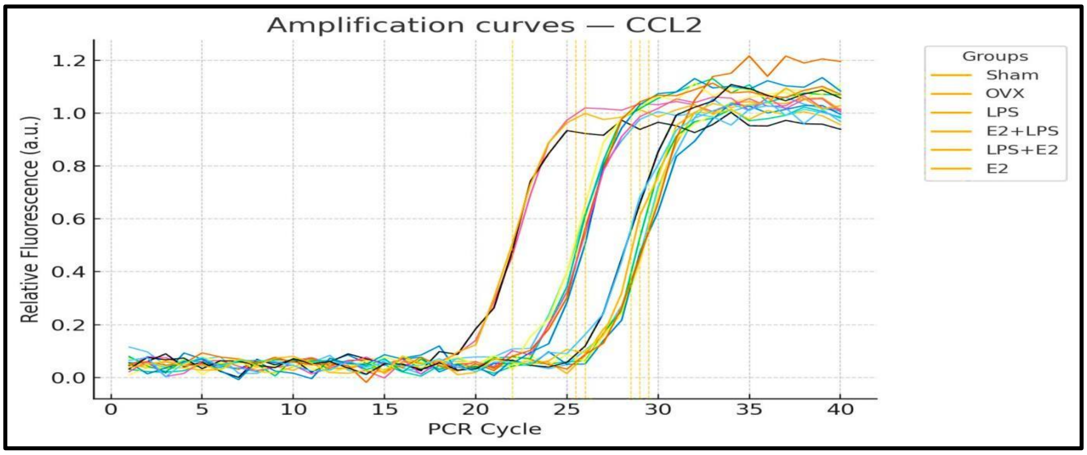

Figure 5. Amplification curve of CCL2 gene expression in study groups

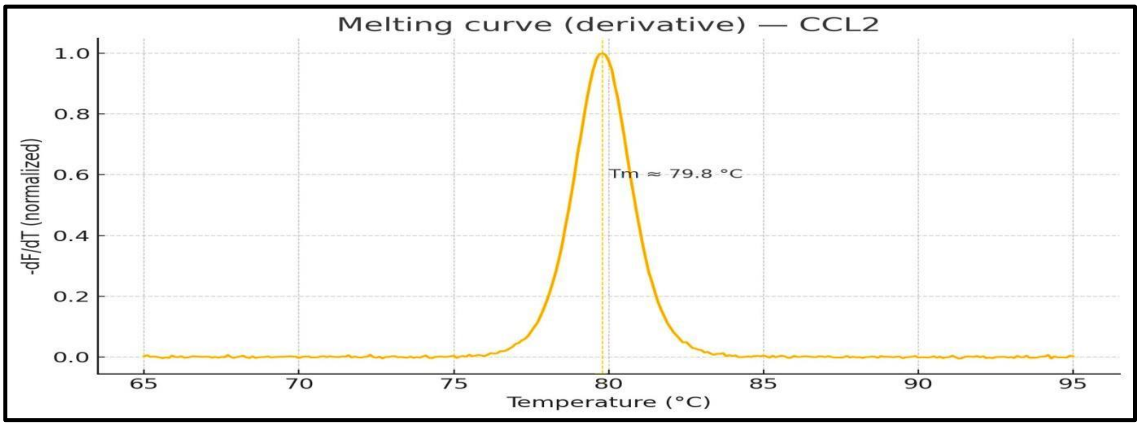

Figure 6. Melting curve analysis of CCL2 gene expression in study groups

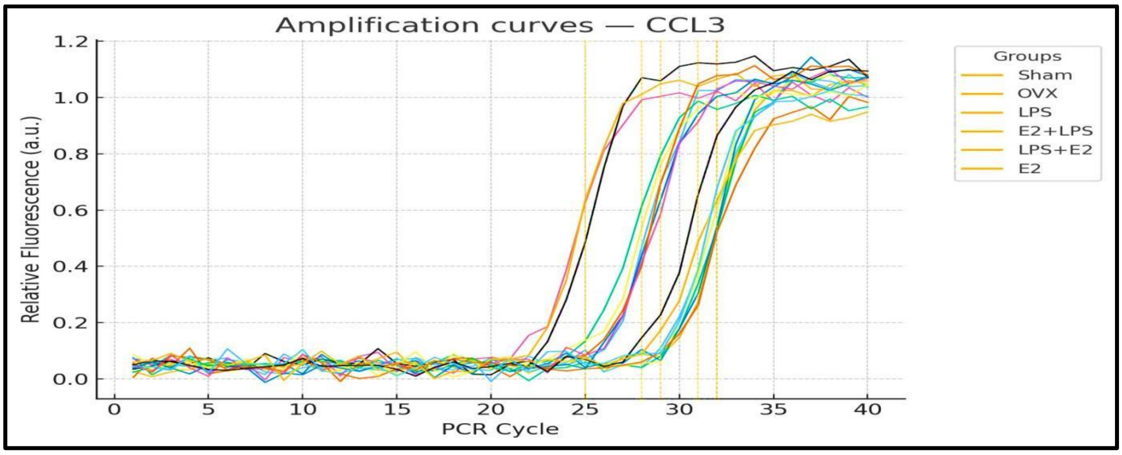

Figure 7. Amplification curve of CCL3 gene expression in study groups.

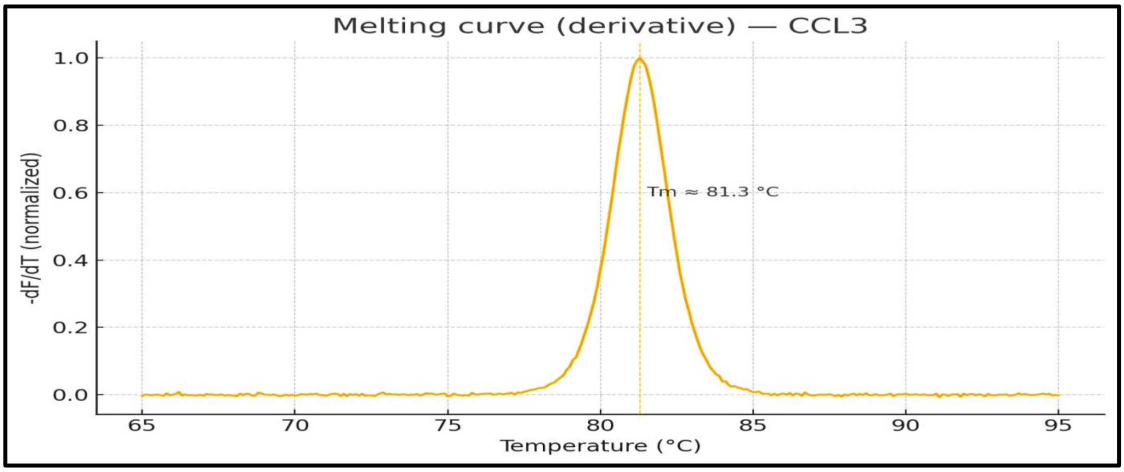

Figure 8. Melting curve analysis of CCL3 gene expression in study groups

As can be seen in Table 1, the comparison between the sham-operated control group (G1) and the ovariectomy group (G2) showed a significant increase in the levels of inflammatory cytokines and biochemical indices of liver and kidney injury after estrogen deprivation. Serum concentrations of TNF-α, IL-6, and MCP-1 were significantly increased in the G2-OVX group compared to the G1-Sham group ($p < 0.05$), indicating increased systemic inflammatory activity. Similarly, serum levels of liver function enzymes (ALT and AST) were also significantly higher ($p < 0.05$). This could indicate liver cell damage. In addition, in the G2-OVX group, urea showed a significant increase and serum creatinine levels were slightly but significantly elevated ($p < 0.05$), suggesting an initial renal dysfunction. Together, these results demonstrate that the absence of endogenous estrogen enhances proinflammatory cytokine production and further increases hepatic and renal stress, reinforcing a critical function of estrogen in the maintenance of organ homeostasis and the inhibition of endotoxin-induced inflammatory responses (Eisa et al., 2023). As listed in Table 2, LPS exposure significantly increased the levels of pro-inflammatory cytokines and biochemical markers of hepatic and renal injury compared to sham-operated controls. Serum TNF-α, IL-6, and MCP-1 concentrations were markedly raised in the G3-LPS group (p-value 0.001), showing a powerful systemic inflammatory response in response to endotoxin challenge. Concurrently, the liver enzymes (ALT and AST) activities were also significantly increased (p value of $\leq$ 0.004), showing severe hepatocellular lesions. Moreover, there was a significant increase in the levels of creatinine and urea in the G3-LPS-treated rats when compared with G1-sham (p-values $\leq$ 0.05), indicating deteriorating renal function. Together, these findings confirm that LPS administration indeed produced a strong inflammatory response with hepatic and renal dysfunction, suggesting this model can be used to study opposing actions of estrogen in endotoxin-induced organ injury (Gao et al., 2021). As indicated in Table 3, estrogen treatment in ovariectomized rats led to systemic anti-inflammatory effects accompanied by ameliorated hepatic and renal biochemical values compared with the untreated G2-OVX group. Conclusion: Estrogen replacement has a strong anti-inflammatory effect, as reflected by a significant reduction of TNF-α, IL-6, and MCP-1 serum levels in G6-estrogen-treated mice ($p<0.05$). Likewise, liver enzyme activities ALT and AST were significantly ($p < 0.05$) depressed, indicating that hepatic injury was also ameliorated. Moreover, treated rats showed lower concentrations of creatinine and urea (p-value <0.05), suggesting that estrogen has a beneficial effect on renal function. Importantly, these results indicate that protection from the inflammatory and organ-damage effects of estrogen deficiency is countered by estrogen supplementation, emphasizing a protective role for estrogen in maintaining liver and kidney integrity (Sucedaram et al., 2021). Effect of lipopolysaccharide (LPS) administration on inflammatory and biochemical disturbances in female rats treated with estrogen. When estrogen pretreatment was used, the inflammatory and biochemical abnormalities induced by LPS administration were significantly reduced (Table 4). Comparison of TNF-α, IL-6, and MCP-1 in the G4-E2+LPS group compared to the G3-LPS group ($p < 0.05$) showed that E2 pretreatment effectively prevented the excessive production of LPS-dependent cytokines. In addition, liver damage was also reduced. As the liver enzymes ALT and AST were also significantly reduced ($p < 0.05$). It was found that estrogen has a protective role against LPS-induced nephrotoxicity. As the renal function indices (creatinine and urea) in the G4-E2+LPS group were significantly reduced compared to the LPS-treated mice (G3 group) ($p < 0.05$). Taken together, these findings suggest that prior exposure to estrogen provides significant protection against endotoxin-induced systemic inflammation and organ damage, likely mediated through estrogen's anti-inflammatory and antioxidant effects (Jiang et al., 2021). As shown in Table 5, co-administration of estrogen with lipopolysaccharide (LPS) significantly diminished the inflammatory and biochemical disturbances produced by endotoxin exposure. This result indicated that estrogen co-treatment effectively inhibited the increase of the serum concentrations of TNF-α, IL-6, and MCP-1 in response to LPS ($p < 0.05$, vs G3-LPS-only group) in the G5-LPS+E2 group. Hepatic enzyme activities (ALT and AST) were also significantly reduced (p-value < 0.05) in co-treated rats, consistent with decreased hepatocellular injury. Additionally, the concentrations of serum creatinine and urea were significantly lower compared with LPS-treated rats ($p < 0.05$) and suggest an estrogen protective effect on renal function. The estrogen treatment is effective in providing potent hepatic and renal organ protection against endotoxin-induced dysfunction by modulating cytokine signaling and oxidative stress pathways (Tan et al., 2025). As shown in Table 6, significant differences ($p < 0.05$) were observed in the mean ± standard deviation values of TNF-α, IL-6, CCL2 (MCP-1), CCL3 (MIP-1α) and gene expression of G1-Sham, G2-OVX, G3-LPS, G4-E2+LPS, G5-LPS+E2 and G6-E2 groups. The expression of proinflammatory genes was decreased in LPS-treated mice on which quantitative RT-PCR analysis was performed. In LPS-treated rats (G3), mRNA expression levels for all four genes were markedly down-regulated (p-value<0.05) relative to the Sham and OVX controls, and clearly indicative of a strong LPS-induced inflammatory response (without upregulation of mRNA) resulting in depletion of mRNA, presumably due to overproduction of cytokines and feedback suppression. In contrast, both estrogen pretreatment (G4:E2+LPS) and co-treatment (G5:LPS+E2) meaningfully restored gene expression levels to those of the control groups, indicating that estrogen can both act to modulate transcription regulation to counteract and mediate the suppression of inflammatory cytokine genes associated with endotoxin exposure. The E2-only group (G6) preserved gene expression patterns similar to the Sham group, further confirming that E2 on its own does not affect basal cytokine transcription. Taken together, these results show that in the sphere of inflammatory gene expression in hepatic and renal tissues, estrogen treatment either before or simultaneous to LPS exposure has a stabilizing influence, consistent with its known anti-inflammatory and protective properties (Küçükler et al., 2021). Quantitative PCR analysis showed distinct changes in gene expression patterns between the experimental groups based on ΔCt, ΔΔCt, and $2^{-\Delta\Delta Ct}$ values (Table 7). Compared to levels in the Sham (G1) and Estrogen-only (G6) groups, ΔCt values for TNF-α, IL-6, CCL2, and CCL3 were highly maintained, resulting in minimal ΔΔCt shifts and $2^{-\Delta\Delta Ct}$ values next to 1 (stable basal expression of inflammatory cytokines at normal physiological state). In contrast, the LPS group (G3) showed significantly lower ΔCt levels and higher $2^{-\Delta\Delta Ct}$ values (8-16-fold increases) corresponding to a net upregulation of inflammatory genes triggered by endotoxins. Notably, estrogen pre-treatment (G4: E2+LPS) and co-treatment (G5: LPS+E2) substantially attenuated these increases, with intermediate ΔCt and moderate $2^{-\Delta\Delta Ct}$ values (approximately 4-8-fold), indicating partial suppression of LPS-induced inflammation. Meanwhile, ovariectomized rats (G2) displayed modest elevations in relative expression, confirming that estrogen deficiency enhances inflammatory sensitivity even without

LPS challenge. The calculated amplification efficiencies TNF-α (98%), IL-6 (95%), CCL2 (92%), and CCL3 (96%) validate the reliability of qPCR performance. Overall, these results demonstrate that estrogen replacement significantly downregulates the expression of inflammatory genes, returns cytokine levels towards baseline values, and confirms its protective regulatory role in liver and kidney tissues against endotoxin-induced inflammation (Tran et al., 2021).

Conclusion: The results of this study showed that the use of estrogen can significantly improve endotoxin-induced liver and kidney disorders. Because estrogen can inhibit the damaging effects of inflammatory cytokines by reducing the expression of TNF-α, IL-6, and CCL2/CCL3 that are increased by endotoxins. Therefore, because estrogen can activate liver and kidney function and reduce elevated levels of inflammatory cytokines, it can be used to reduce inflammation-induced damage in adult female Wistar rats. These findings suggest that the estrogen signaling pathway may be a promising therapeutic target to reduce the long-term damage caused by sepsis in estrogen-deficient conditions. Estrogen pre-treatment reduced TNF-α expression by ~40% relative to LPS-only group.

Author Contributions

Ozdan Akram Ghareeb: Conceptualization and methodology, software and validation, formal analysis and investigation, resources, data curation, writing-original draft preparation, writing-review, visualization, and funding acquisition.

Data Availability Statement

Data are available from the authors upon reasonable request.

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Ethical Considerations

This research was approved by the Ethical Approval Committee with reference number 459 dated 9/3/2025. This study was also approved by Al-Qadisiyah University, Faculty of Veterinary Medicine. The study was carried out with integrity, with no fabrication, falsification, plagiarism, or scientific misconduct.

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Conflict of Interest

The authors declare no conflict of interest.

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مجله بیوتکنولوژی کشاورزی
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شاپا چاپی: ۶۷۰۵-۲۲۲۸ شاپا الکترونیکی: ۶۵۰۰-۲۲۲۸

اثرات مخرب سیتوکین‌های التهابی ناشی از اندوتوکسین بر عملکرد کبد و کلیه و نقش تعدیل‌کننده استروژن در شرایط زنده

اوزدان اکرام غریب

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چکیده

هدف: یکی از محرک‌های قوی سیستم ایمنی ذاتی، اندوتوکسین یا لیپوپلی‌ساکارید (LPS) است. این محرک می‌تواند منجر به سندرم پاسخ التهابی سیستمیک (SIRS) شود و در نهایت ممکن است به شوک سپتیک منجر گردد. استروژن یک هورمون استروئیدی است که نقش مهمی در تولیدمثل ماده‌ها دارد و همچنین عملکردهای متعددی در سایر سیستم‌های بدن از جمله سیستم ایمنی ایفا می‌کند. هدف این مطالعه بررسی بیان ژن‌های TNF-α، IL-6، CCL2 و CCL3 در پاسخ به LPS بود. اهداف دیگر این مطالعه شامل ارزیابی تغییرات هیستولوژیکی و عملکردی در کلیه و کبد، اندازه‌گیری سطح سرمی سیتوکین‌های التهابی مانند TNF-α، IL-6 و MCP-1 و بررسی اثرات اواریکتومی (برداشتن تخمدان‌ها و در نتیجه کمبود استروژن) بر میزان آسیب اندام‌ها و پاسخ التهابی ناشی از LPS در موش‌های بالغ ماده ویستار سه ماهه بود.

مواد و روش‌ها: برای بررسی اثرات استروژن، اندوتوکسین‌ها و ترکیب آن‌ها، گروه‌های متعددی در آزمایش در نظر گرفته شدند. تمام آزمایش‌های آزمایشگاهی برای ارزیابی سیتوکین‌های التهابی، عملکرد کبد و کلیه و بیان ژن‌ها در آزمایشگاه‌های دانشکده دامپزشکی، دانشگاه القادسیه، عراق انجام شد. مطالعه شامل ۱۳۸ موش بالغ ماده ویستار (۳ ماهه) بود که در شش گروه اصلی تقسیم شدند (G1-Sham, G2-OVX, G3-LPS, G4-E2+LPS, G5-LPS+E2, and G6-E2)؛ هر گروه شامل ۲۳ موش بالغ ماده ویستار بود.

نتایج: نتایج این مطالعه تفاوت آماری معناداری ($p<0.05$) در سطح سرمی سیتوکین‌های التهابی TNF-α، IL-6 و MCP-1 نشان داد. علاوه بر این، کراتینین و اوره (عملکرد کلیه)، ALT و AST (عملکرد کبد) و بیان ژن‌های TNF-α، IL-6، CCL2 و CCL3 نیز بین موش‌های بالغ ماده ویستار سه ماهه مشاهده شد.

نتیجه‌گیری: مطالعه نشان داد که استروژن اثرات مضر سیتوکین‌های التهابی افزایش‌یافته به‌واسطه اندوتوکسین‌ها را کاهش می‌دهد و بنابراین عملکرد کبد و کلیه را در موش‌های بالغ ماده ویستار فعال می‌کند. مسیر سیگنالینگ استروژن می‌تواند یک هدف درمانی

امیدوارکننده برای کاهش آسیب چنداندامی ناشی از سپسیس در شرایط کمبود استروژن باشد. پیش‌درمانی با استروژن بیان TNF-α را تقریباً ۴۰٪ نسبت به گروه دریافت‌کننده تنها LPS کاهش داد.

کلمات کلیدی: استروژن، اندوتوکسین، سیتوکین‌های التهابی، IL-6، TNF-α

نوع مقاله: پژوهشی

استناد: اوزدان اکرام غریب (۱۴۰۵) اثرات مخرب سیتوکین‌های التهابی ناشی از اندوتوکسین بر عملکرد کبد و کلیه و نقش تعدیل‌کننده استروژن در شرایط زنده. *مجله بیوتکنولوژی کشاورزی*، ۱۸(۱)، ۲۵۹-۲۸۰.

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