

The impact of pretreatment with simvastatin on kidney tissue of rats with acute sepsis

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It has been reported that changes in cytokine levels affect mitochondrial functions, levels of hypoxia-inducible factor α (HIF-1 α), and tissue damage during sepsis. We aimed to investigate the effects of simvastatin pretreatment on mitochondrial enzyme activities, and on levels of ghrelin, HIF-1 α , and thiobarbituric acid reactive substances (TBARS) in kidney tissue during sepsis. Rats were separated into four groups, namely, control, lipopolysaccharides (LPS) (20 mg/kg), simvastatin (20 mg/kg), and simvastatin + LPS. We measured the levels of mitochondrial enzyme activities and TBARS in the kidney using spectrophotometry. The histological structure of the kidney sections was examined after staining with hematoxylin and eosin. Tumor necrosis factor α (TNF- α), IL-10, HIF-1 α , and ghrelin immunoreactivity were examined using proper antibodies. In tissue, TNF- α ($p < 0.01$) and HIF-1 α ($p < 0.05$) levels were increased in the simvastatin + LPS and LPS groups. TBARS levels were higher in the LPS group than in the other groups ($p < 0.01$), but they were similar in the simvastatin + LPS and control groups ($p > 0.05$). Ghrelin immunoreactivity was lower in the LPS group ($p < 0.05$) and higher in the simvastatin + LPS group than in the LPS group ($p < 0.01$). We observed tubular damage in the sections of the LPS group. There were no differences in mitochondrial enzyme activities between the groups ($p > 0.05$). We observed that pretreatment of simvastatin caused favorable changes on ghrelin and TBARS levels in rats with sepsis.

Keywords: hypoxia-inducible factor, ghrelin, kidney, lipopolysaccharides, sepsis, simvastatin

Introduction

Sepsis is a common and often devastating condition characterized by uncontrolled infection and multisystem organ failure (40). Acute kidney injury (AKI) incidence is about 40% in patients with severe sepsis and septic shock. Within the kidney, there are multiple levels at which significantly different changes might occur. These include alterations in renal blood flow, glomerular and peritubular microcirculation, tubular cell function, and structure (6). Lipopolysaccharides (LPS) can lead to septic shock characterized by endothelial damage, loss of vascular tone, coagulopathy, and multiple system organ failure, which often result in death. Moreover, LPS induces the occurrence of inflammatory reactions with the release of cytokines, complement factors, and increments of reactive oxygen species (ROS) (41). It has been demonstrated that increased ROS affects the structure of renal tubular cells by inducing

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lipid peroxidations of the cell membrane, and oxidation of proteins, carbohydrates, and DNA molecules (3).

The role of tumor necrosis factor α (TNF- α) has been shown to be pivotal in the inflammatory response in endotoxemia-related AKI. During sepsis, TNF- α mediates the release of pro-inflammatory cytokines with anti-inflammatory mediators, such as IL-1Ra, IL4, IL10, and IL13 (4). LPS inhibits respiration by mitochondria *in vivo* and *in vitro* (15). Pathologies such as ultrastructural mitochondrial alterations and oxidative mechanisms in sepsis and septic shock have attracted attention in recent years (8).

Mitochondrial dysfunction has been suggested to contribute to the development of organ dysfunction and failure in sepsis. The mitochondrial electron transport chain consists of four complexes (CI–CIV) and its function can be assessed with different approaches (16). It has been shown that excessive production of cytokines caused alterations of oxygen consumption and mitochondrial complexes, and these changes contributed to mortality during sepsis (32). Treatment of macrophages with LPS can increase the expression of a number of hypoxic genes. Hypoxia-inducible factor α (HIF-1 α) is recognized as a mediator that allows for cellular adaptation and energy homeostasis at low oxygen concentrations (42).

Statins, such as simvastatin and atorvastatin, possess pleiotropic effects, including antioxidant and anti-inflammatory properties (22). In particular, the anti-inflammatory properties correlate with reduced sepsis-induced morbidity and mortality. However, there is no consensus in the literature for this protective effect, as some studies drew attention to the lack of randomized controlled trials in these earlier reports. Studies have also demonstrated that statins have an adverse effect on sepsis (29, 33). Ando et al. (2) reported that pretreatment with statins improved survival in a murine sepsis model. In the literature, the useful effect of statins has been shown in sepsis-induced AKI in an experimental model (30). In addition, *in vitro* and *in vivo* studies have demonstrated that simvastatin has an anti-inflammatory effect in patients with pre-dialytic chronic kidney disease, and may play an important role in counteracting the mechanisms involved in the pathogenesis of inflammation (25).

Ghrelin has anti-inflammatory properties and its receptors are widely expressed throughout the body. It has been shown that ghrelin levels decreased during sepsis and ghrelin administration protected against organ damage by preventing the release of norepinephrine and pro-inflammatory cytokines in rats with sepsis (36).

We aimed to investigate the effects of pretreatment with simvastatin on mitochondrial enzyme activities, and on levels of thiobarbituric acid reactive substances (TBARS) as end products of lipid peroxidation, HIF-1 α , and ghrelin in kidney tissue during early-phase sepsis.

Materials and Methods

Experimental groups

This study was conducted at the Istanbul University experimental research center, (resolution no: 2012/138). We used male adult *Wistar albino* rats that weighed 200–250 g as experimental models. The rats were fed a commercial diet and tap water *ad libitum*. They were housed in cages and maintained at a controlled temperature (22 ± 2 °C) and humidity (55–60%) with a 12-h light/dark cycle.

The rats were divided into four groups, each comprised eight rats: (1) control group ($n = 8$), (2) LPS group ($n = 8$), (3) simvastatin group ($n = 8$), and (4) simvastatin + LPS group ($n = 8$).

Experimental procedures

LPS from *Escherichia coli* O127:B8 (Sigma Aldrich, Product No: L5668) was intraperitoneally injected at a dosage of 20 mg/kg; and simvastatin (20 mg/kg) (Lactone form, Sigma Aldrich, Product No: S0650000) was dissolved in physiological saline and provided *through* oral gavage for 5 days (7). In the simvastatin + LPS-treated group, LPS was provided 1.5 h after the fifth dosage of simvastatin. All rats treated with LPS were sacrificed after 4 h.

Histological procedures

The kidney tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. Five-micrometer-thick sections were placed on polylysine-coated slides and stained with hematoxylin and eosin (H&E). The slides were evaluated under light microscopy (Olympus BX51; Olympus Corp., Tokyo, Japan) at 40× magnification.

Immunohistochemical procedures

A total of 8 slides for each group were then incubated with primary antibodies: TNF- α (1:100; NBP1-47581, Novus, USA), HIF-1 α (1:100; NB100-479, Novus, USA), IL-10 (1:100; ARC0102, Invitrogen, USA), and ghrelin (1:100; NBP1-51224, Novus, USA) for 1 h at 37 °C. The slides were then incubated with the secondary antibody (859043 Histostain-Plus Kit, Invitrogen, USA) at 37 °C for 30 min, washed with a phosphate-buffered saline buffer, and 3-amino-9-ethylcarbazole (AEC) (2007, AEC reagent set, Invitrogen) staining was applied. The sections were counterstained with Mayer's hematoxylin (MHS16, Sigma-Aldrich, USA) (9). All sections were photographed using an Olympus C-5050 digital camera. For each specimen, a score was assigned according to the percentage of positive cells: <5% of the cells: 1 point; 6–35% of the cells: 2 points; 36–70% of the cells: 3 points; and >71% of the cells: 4 points. The percentage indicated how many cells out of 100 had stained positive for the targeted protein per area. The intensity of staining was also quantified, with negative staining equal to 1 point; weak staining, 2 points; moderate staining, 3 points; and strong staining, 4 points. The expression score was then calculated by multiplying the two scores above (23).

TBARS

The levels of kidney TBARS were determined using a thiobarbituric acid assay (TBA) (5). Tissue homogenates were reacted with TBA reagent that contained trichloroacetic acid and HCl, boiled for 15 min, cooled, and then centrifuged. Absorbances of the supernatants were spectrophotometrically measured at 532 nm. TBARS concentrations were calculated using 1,3,3,3 tetraethoxypropane as a standard. The results were expressed as nmol/g tissue (5).

Mitochondrial enzyme activities

The absorbances of all activities were kinetically recorded using a spectrophotometer (Shimadzu, Japan) at 30 °C. Citrate synthase activity was initiated by adding of dithiobis 2-nitrobenzoic acid into a mixture containing oxaloacetate, acetyl-CoA, and tissue homogenate. The absorbance was measured at 412 nm. Complex I [nicotinamide adenine dinucleotide (NADH) dehydrogenase oxidation of NAD] activity was first monitored using potassium phosphate, NADH, and potassium ferricyanide, and then by adding tissue homogenate at 340 nm. The tissue homogenate for complex II + III (succinate–cytochrome c reductase) was added to a mixture of fresh cytochrome c containing succinate, and potassium cyanide (KCN). Complex II + III activity was observed as the increase in rate of reduction of

cytochrome c. This was measured at 550 nm (18). Complex I+III (NADH-cytochrome c reductase) activity was observed as a result of the rate of decrease in the addition of cytochrome c homogenate, which included freshly prepared cytochrome c, NADH, and KCN alterations after adding rotenone at 550 nm (31). Complex II (succinate dehydrogenase) activity was determined through the reduction of 2,6-dichlorophenolindophenol at 600 nm (18).

Statistical analysis

Data were expressed as mean \pm standard deviation. Tukey's test was used to test the differences among means for which ANOVA indicated significant for TBARS levels and mitochondrial enzyme activities ($p < 0.05$). The results of immunohistochemistry analysis were statistically analyzed using Student's *t*-test. The data were represented as mean \pm standard error of the mean. $p < 0.05$ was considered to be statistically significant.

Results

Histological findings

We observed dilatation of tubule lumens and partial damage to the epithelial cells in the LPS group. The tubule structures in the simvastatin group were found to resemble those in the controls' sections. The tubule dilatation and proximal cell damage were observed in the simvastatin + LPS group (Fig. 1A–D).

Immunohistochemistry findings

As shown in Table I, there was increased TNF- α in the LPS and simvastatin + LPS groups ($p < 0.01$ for both groups) (Fig. 2A–D) and HIF-1 α levels ($p < 0.05$ for both groups) (Fig. 3A–D) in the LPS and simvastatin + LPS groups compared with the control group ($p < 0.05$). Although there was increased IL-10 immunoreactivity in the LPS and simvastatin + LPS groups, we found no statistically significant differences ($p > 0.05$) (Fig. 4A–D). Ghrelin immunoreactivity was decreased in the LPS group compared with the controls ($p < 0.05$); however, it was increased in the simvastatin + LPS group compared with the LPS group ($p < 0.01$) (Fig. 5A–D). There were no differences in the levels of TNF- α , IL-10, and HIF-1 α in the LPS and simvastatin + LPS groups ($p > 0.05$) (Table I).

TBARS

TBARS levels were significantly increased in the LPS group compared with the other groups ($p < 0.01$). In the simvastatin + LPS group, TBARS levels were found to be the same as in the control group ($p > 0.05$) (Fig. 6).

Mitochondrial enzyme activity

There were no changes in the activities of citrate synthase, complex I, complex II, complex I + III, and complex II + III in tissue homogenate ($p > 0.05$) (Table II).

Discussion

We aimed to investigate the effect of pretreatment with simvastatin on the early phases of sepsis induced by LPS (20 mg/kg i.p., 4 h) in the kidney tissue of rats, which is known to have

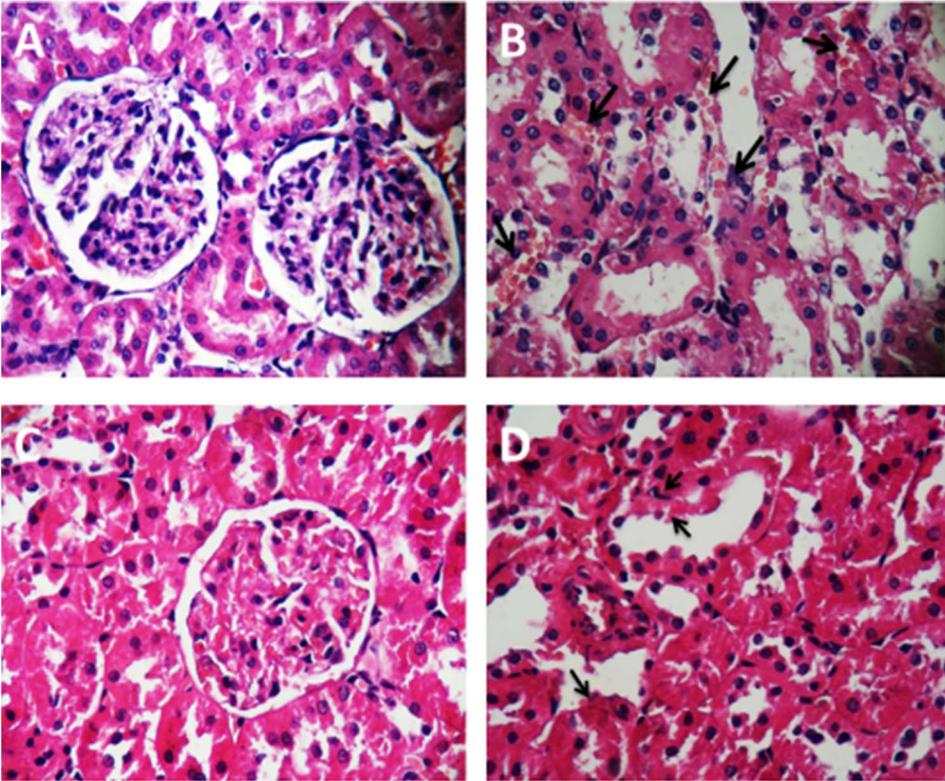


Fig. 1. Section of kidney tissue from the control group stained with H&E, 40× magnification (A). Section of kidney tissue from LPS group, 40× magnification (B). Section of kidney tissue from the simvastatin group, 40× magnification (C). Section of kidney tissue from the simvastatin + LPS group, 40× magnification (D). The arrows show tubular damage in B and D sections

an important role in drug clearance. Sepsis is a common cause of acute renal failure and accounts for up to 60% of cases in clinical studies (40).

In this study, we evaluated the changes of histological appearance, immunohistochemistries of cytokines, HIF-1 α , and ghrelin, mitochondrial enzyme activities, and TBARS levels of kidney tissue in the first hours of sepsis. Although TNF- α was found to be significantly higher, there was slightly increased IL-10 immunoreactivity in tissue sections in the LPS and simvastatin + LPS groups compared with that of the controls.

In our previous study, we showed that serum TNF- α and IL-10 levels were significantly higher in the simvastatin + LPS and LPS groups compared with controls. In addition, according to immunohistochemical findings, the levels of TNF- α and IL-10 were higher in the liver tissue of these groups (41).

The kidneys are the most important catabolic organs in the clearance of plasma cytokines. Cytokines and their degradation products are detectable in urine, and in nephrectomized rats, the half-life of cytokines is dramatically increased (27). TNF- α is an important mediator of the systemic and renal effects of sepsis. Continuous intravenous infusion of TNF- α into rabbits produced endothelial cell damage and accumulation of neutrophils in the glomerular capillary lumen, suggesting a chemotactic and/or toxic role for TNF- α *in vivo* (4).

Table 1. The expression scores of cytokines, hormone, and factor

	IL-10			TNF- α			HIF-1 α			Ghrelin		
	Percentage	Intensity	Score	Percentage	Intensity	Score	Percentage	Intensity	Score	Percentage	Intensity	Score
Control	1	1	1	1	1	1	1	1	1	1	1	1
LPS	1	2	2	3	3	9**	2	2	4*	1	0.5	0.5***
Simvastatin	1	1	1	1	1	1	1	1	1	1	1	1
Simvastatin + LPS	1	2	2	3	3	9**	1	3	3*	2	2	4****

The score results were showed as multiplication of percentage and intensity.

* $p < 0.05$, LPS and simvastatin + LPS groups vs. other groups.

** $p < 0.01$, LPS and simvastatin + LPS groups vs. other groups.

*** $p < 0.05$, LPS vs. control group.

**** $p < 0.01$, simvastatin + LPS group vs. LPS group

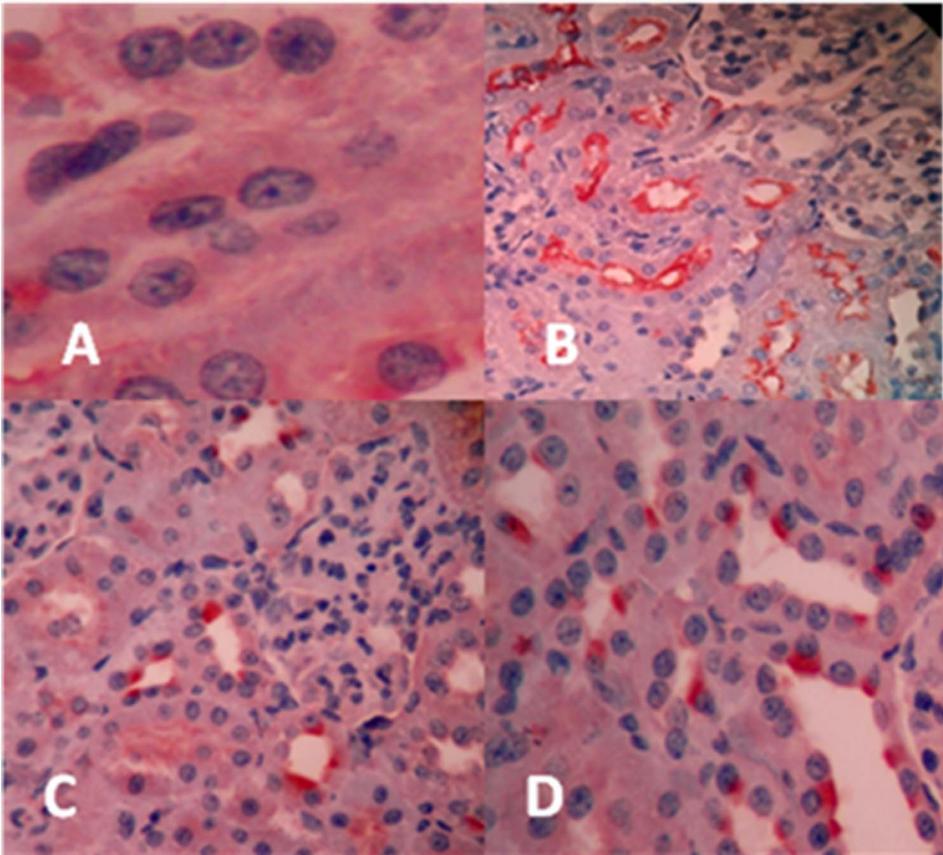


Fig. 2. Immunohistochemical staining of kidney sections for TNF- α . Section of kidney tissue from the control group (A), LPS group (B), simvastatin group (C), and simvastatin + LPS group (D)

We observed the dilatation of tubule lumens in the LPS group and partial damage to epithelial cells. In the literature, there are many studies in which histological changes in the kidney tissue of rat have been demonstrated (37).

A controlled study revealed that pretreatment with statins was associated with a reduction of severe sepsis and intensive care unit admission (2). In this study, 5 days of oral simvastatin had no effect on kidney tissue injury, tubule dilatation, or damage in proximal cells in the LPS-treated rats. The failure to prevent tissue damage in the simvastatin + LPS group may be explained by non-reduction of cytokine levels. Yasuda et al. (38) demonstrated that priorly administered simvastatin decreased the cecal ligation and puncture (CLP) induced renal dysfunction at 24 h, but not 6 h after CLP. They hypothesized that simvastatin might have influence on the late phase of CLP-induced vascular effects. In addition, there were studies which found no effect of simvastatin using either clinical (35) or experimental sepsis model (1).

In sepsis, the interaction of cellular hypoxia with microorganisms and the host immune system contributes to the formation of ROS, which results in oxidative damage of the kidney (3).

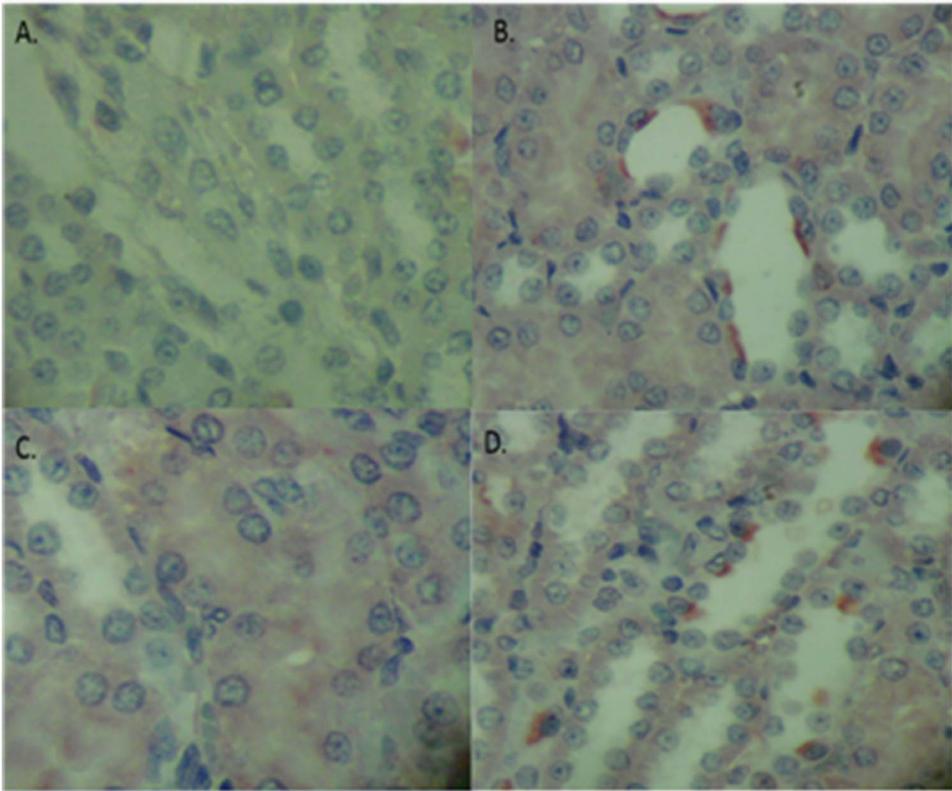


Fig. 3. Immunohistochemical staining of kidney sections for HIF-1 α . Section of kidney tissue from the control group (A), LPS group (B), simvastatin group (C), and simvastatin + LPS group (D)

Santos et al. (30) reported that the sepsis group showed increased TBARS through the formation of ROS in the cecal puncture ligation group. In this study, we found increased TBARS levels of kidney tissue in the sepsis group; TBARS levels were reduced in the simvastatin-treated sepsis group compared with those of the LPS group. Iseri et al. (14) showed that treatment with simvastatin ameliorated nephrotoxicity and reversed renal function by increasing the level of antioxidant enzymes. Teshima et al. (34) demonstrated that statin treatment revealed decreased ROS and subsequent improvement in renal function in an *in vivo* ischemic AKI model.

The anti-inflammatory effects of ghrelin have also been shown to inhibit sympathetic activation, which is known to be increased in sepsis and endotoxemia (36). Serum ghrelin levels have been reported to be decreased or increased after LPS injection depending on the timing of disease course (13, 19). Compared with controls, ghrelin immunoreactivity decreased in the LPS group; however, it increased in the simvastatin + LPS group compared with the LPS group. There were increased HIF-1 α levels in the LPS and simvastatin + LPS groups compared with the controls. We found that the results of immunoreactivities of ghrelin and HIF-1 α levels were similar to the results obtained in liver tissue in our previous study (39). LPS can trigger HIF-1 α activation in macrophages and this is crucial for the development of a LPS-triggered sepsis (12). LPS raises levels of HIF-1 α in macrophages through

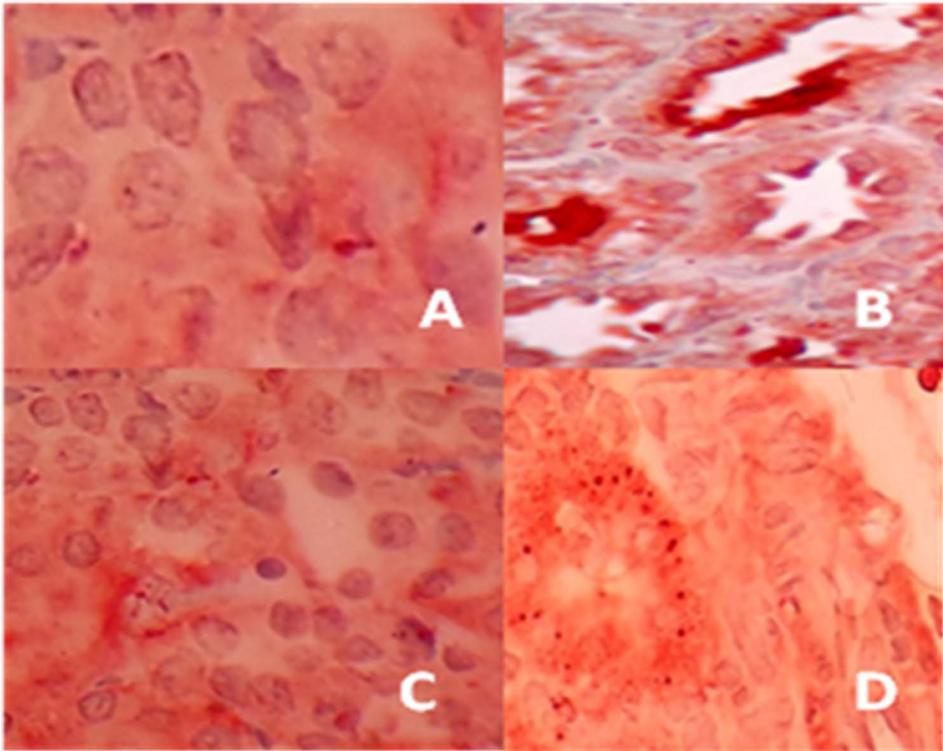


Fig. 4. Immunohistochemical staining of kidney sections for IL-10. Section of kidney tissue from the control group (A), LPS group (B), simvastatin group (C), and simvastatin + LPS group (D)

activating the p42/44 MAPK and NfκB signal transduction pathways (26). HIF-1 α induction in macrophages promotes the production of inflammatory cytokines, including TNF- α and IL-1; deletion of HIF-1 α is protective against LPS-induced sepsis in rats (17). In a previous study, although we found alterations in mitochondrial enzyme activity and morphology in muscle, no alterations in the activities of mitochondrial enzymes were observed in the kidney tissue of rats treated with LPS, similar to our previous study in the liver (24, 39).

In the literature, there are a number of studies about investigating experimental sepsis rat models with different approaches using different exposure times and dosages of LPS. Consequently, the results are conflicting. In our applied model, in consistence with literature findings (10, 11, 20, 28), the cause of mitochondrial enzyme activity results is depending on exposure time and dosage of LPS in kidney tissue. In the literature, during the early phase of exposure time of LPS; namely, the first 4 h after injection of LPS, mitochondrial oxidative phosphorylation has been supporting ATP synthesis and cellular energy. In a recent study, Porta et al. (28) have reported that mitochondrial function was not impaired by endotoxin infusion in kidney tissue. Therefore, our results showing unchanged mitochondrial enzyme activities are in accordance with other studies.

LPS has been shown by other authors to elicit alterations in mitochondrial function in rats and subcellular fractions. Decreased mitochondrial enzyme activity of the cells was dependent upon the exposure time and dosage of LPS. This decrease was correlated with

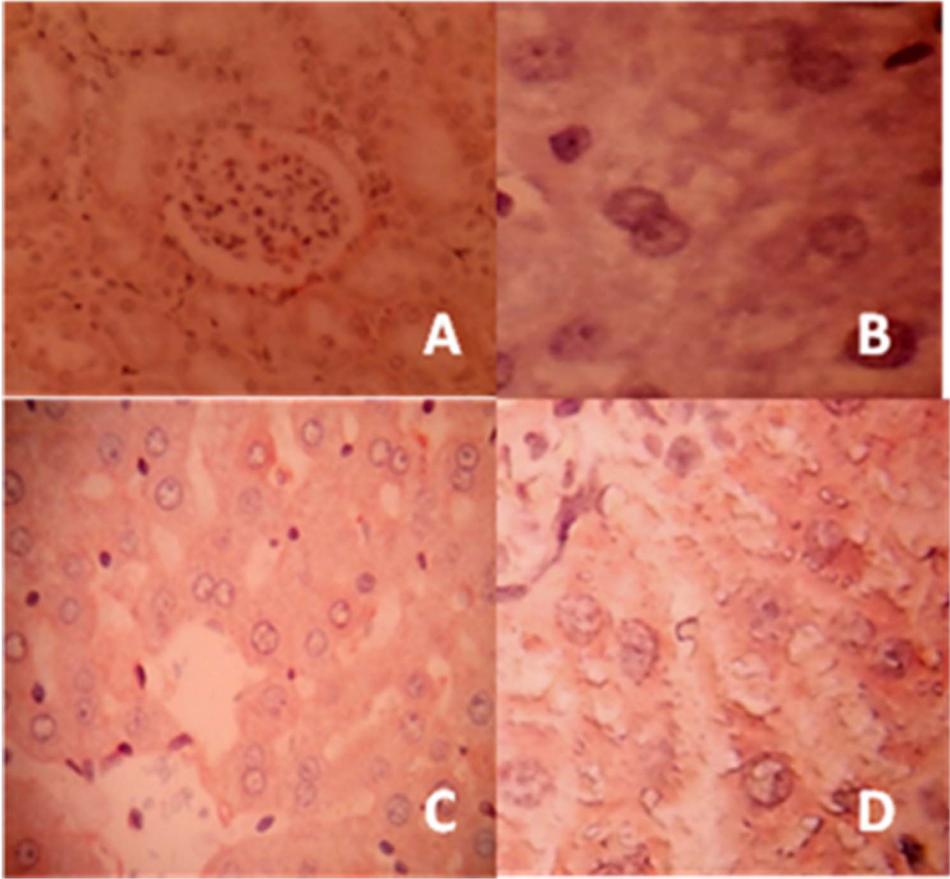


Fig. 5. Immunohistochemical staining of kidney sections for ghrelin. Section of kidney tissue from the control group (A), LPS group (B), simvastatin group (C), and simvastatin + LPS group (D)

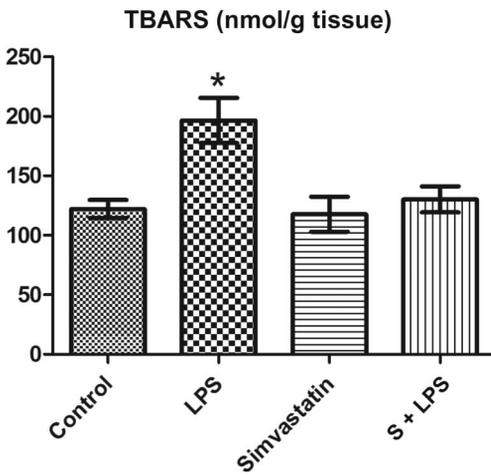


Fig. 6. TBARS in experimental groups. * $p < 0.01$, LPS group vs. control, simvastatin, simvastatin + LPS groups

Table II. Complex I, citrate synthase, I–III, II–III enzymes activities of experimental groups in kidney tissues

Mitochondrial enzyme activity ($\mu\text{mol}/\text{min}/\text{g}$ tissue)	Control	LPS	Simvastatin	Simvastatin + LPS
Complex I	26.56 \pm 6.95	30.07 \pm 4.69	29.24 \pm 12.33	23.56 \pm 5.427
Citrate synthase	2.331 \pm 0.19	2.228 \pm 0.74	1.763 \pm 0.48	1.805 \pm 0.37
Complex I–III	2.457 \pm 1.26	2.332 \pm 0.90	2.88 \pm 2.11	2.529 \pm 0.89
Complex II–III	0.19 \pm 0.04	0.24 \pm 0.09	0.18 \pm 0.04	0.17 \pm 0.04

leakage of enzyme activity from the mitochondrial fraction. It has been reported that mitochondrial oxygen consumption in the liver was either decreased or not affected (21). To our knowledge, no other study has investigated mitochondrial enzyme activities in kidney tissue in parallel with an LPS-induced sepsis model.

Although we found that, in the LPS group, there was dilatation of tubular lumens and partial damage to the epithelial cells in histological sections, there was no damage at the mitochondrial level.

In this study, simvastatin was observed to increase ghrelin levels and decrease TBARS in the kidney tissues of the simvastatin + LPS group; however, the effect of simvastatin may not be sufficient to protect against the damage of sepsis in this early-phase sepsis model.

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