Original Article
Effect of simvastatin on mitochondrial enzyme activities, ghrelin, hypoxia-inducible factor 1α in hepatic tissue during early phase of sepsis

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Abstract: We aimed to investigate the effects of prior treatment of simvastatin on mitochondrial enzyme, ghrelin, and hypoxia-inducible factor 1 α (HIF-1 α) on hepatic tissue in rats treated with Lipopolysaccharides (LPS) during the early phase of sepsis. Rats were divided into four groups: control, LPS (20 mg/kg, i.p.), Simvastatin (20 mg/kg, p.o.), and LPS + Simvastatin group. We measured citrate synthase, complex I, II, I-III, II-III enzymes activities, serum and tissue levels of TNF-α, IL-10 using ELISA. Liver sections underwent histopathologic examination and TNF-α, IL-10, HIF-1α and ghrelin immunoreactivity were examined using immunohistochemistry methods. There were no differences in all groups for mitochondrial enzyme activities. In terms of both ELISA and immunohistochemistry findings; the levels of serum and tissue TNF-α and IL-10 were higher in the experimental groups than controls (P < 0.05). In the LPS group, the hepatocyte cell membrane and sinusoid structure were damaged. In the Simvastatin +LPS group, hepatocytes and sinusoidal cord structure were partially improved. For HIF-1α, in all experimental groups immunoreactivity was increased (P < 0.05). In the Simvastatin group, Ghrelin levels were increased in comparison with the other groups (P < 0.01). Ghrelin levels were greatly decreased in LPS (P < 0.05). We observed that the degree of hepatocellular degeneration was partially reduced depending on the dosage and duration of prior simvastatin treatment with LPS, probably due to alterations of Ghrelin and HIF-1α levels.

Keywords: Lipopolysaccharide, mitochondrial enzyme activites, ghrelin, hypoxia-inducible factor 1-α, simvastatin, liver

Introductıon

Sepsis, defined as a systemic inflammatory response, leads to shock and multiorgan system failure with high mortality [1]. Lipopoly-saccharide (LPS) is an endotoxin in the cell membrane of gram negative bacteria [2]. At low doses, LPS causes an inflammatory response, and at higher doses, it leads to septic shock. LPS cause the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin1 (IL-1), IL-6, IL-8 [3]. IL-1 and TNF-α lead to an increase in expression of other proin-flammatory cytokines (IL-12, IL-18) during sepsis [4]. Proinflammatory cytokine release is regulated by antiinflammatory cytokine such as

IL-1Ra, IL4, IL-10, IL-13. Imbalance of cytokine levels trigger a systemic proinflammatory reaction (SIRS) or compensatory anti-inflammatory reaction (CARS), which leads to organ damage [5]. Morever, LPS caused secretion of hypoxia inducible factor (HIF-1) from macrophages by increasing cytokines such as TNF, IL-1, IL-12, IL-4. HIF-1α is recognized as a major regulator of cellular adaptation and energy homeostasis under conditions of low oxygen concentrations and low pH [6].

It has been reported that alterations of oxygen consumption and mitochondrial complex were affected by excessive production of cytokines and these changes caused cell death during sepsis [7].

Statins are agents that inhibit cholesterol synthesis by HMG-CoA reductase enzyme and have antioxidant, anti-inflammatory, and pro-apoptotic effects such as regulating macrophages, monocytes, and lymphocyte functions [8]. Ando et al. demonstrated that pretreatment with a statin prevented excessive cytokine production and improved survival in mice with sepsis [9].

Recent studies reported that ghrelin levels were decreased during sepsis and that ghrelin administration protected against organ damage by preventing the release of norepinephrine and proinflammatory cytokines in animals with sepsis [10, 11]. The liver plays important roles in a host's defense mechanisms during sepsis, by clearing inactivate bacterial products, cytokines, and bioactive lipids. It was reported that hepatic dysfunction occured as a result of hepatosplanic hypoperfusion of in the early phases of sepsis [12].

In this study, we aimed to investigate the effects of simvastatin on mitochondrial enzyme activities, HIF-1α, and ghrelin in the liver tissue of rats with sepsis.

Materials and methods

Experimental groups

This study was conducted at the experimental research center, University of Istanbul (Resolution No: 2012/138). Male adult *Wistar albino* rats weighing 200-250 g were used in the experiments. The rats were divided into four groups, each composed of eight rats: (1) control group, (2) LPS group, (3) Simvastatin group, and (4) LPS + Simvastatin group

Experimental procedures

Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (Sigma Aldrich, Product No: L5668) injected intraperitoneally at a dosage of 20 mg/kg, Simvastatin (20 mg/kg) (Sigma Aldrich, Product No:S0650000) were given p.o. *via* oral gavage for 5 days [13].

Biochemical procedures

Serum concentrations of creatinine kinase (CK), aspartate aminotransferase (GOT), alanine aminotransferase (GPT), lactate dehydrogenase (LDH) were measured using the corresponding kit in an autoanalyzer (Cobas auto-

analyzer, DPC, Diagnostic products corporation, USA).

Cytokine levels

The cytokine levels were measured using the ELISA method for TNF-α (BioSource, Invitrogen, USA, Cat No, KRC3012) and IL-10 (BioSource, Invitrogen, USA, Cat No, KRC0101). The absorbance values were measured at 450 nm using a micro-ELISA automatic analyzer.

Histological procedures

Five-micrometer-thick liver 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 × and × 100 magnification.

Immunohistochemical procedures

Eight slides for each group were then incubated with primary antibodies: TNF-α (1:100; NBP1-47581, Novus, USA), HIF-1α (1:100; NB100-479, Novus, USA), IL-10 (1:100; ARC0102, Invitrogen, USA) and Ghrelin (1:100; NBP1-51224, Novus, USA) for 1 hour at 37°C. The slides incubated with the secondary antibody (859043 Histostain-Plus Kit, Invitrogen, USA) at 37°C for 30 minutes, washed with PBS 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) [14]. All the sections were examined by two blinded experts and photographed using an Olympus C-5050 digital camera. For each specimen, one 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. Another score was assigned according to the intensity of staining, with negative staining equal to 1 point; weak staining, 2 points; moderate staining, 3 points; and strong staining, 4 points [15].

Mitochondrial enzyme activities

Enzyme measurements were taken kinetically using a spectrophotometer (Schimadzu, Japan) at 30 °C. Citrate synthase was measured after the addition dithio-bis 2-nitrobenzoic acid using the method of Srere [16]. Oxidation of NADH in Complex I (NADH dehydrogenase) activity was

Table 1. Values of serum CK, GOT, GPT, LDH for groups

Experimental Groups	CK (U/L)	GOT (U/L)	GPT (U/L)	LDH (U/L)
Control	892.3 ± 216.8	122.7 ± 14.7	51.0 ± 10.0	1176.75 ± 310.36
LPS	1565.2 ± 635.8*	220.5 ± 66.6*	68.5 ± 14.7	1267.20 ± 630.65
Simvastatin	1908.8 ± 412.7*	218.8 ± 32.2*	58.2 ± 16.96	2803.20 ± 986.10*
Simvastatin +LPS	1843 ± 512.49*	306.33 ± 70.57*	56.8 ± 12.21	1913.4 ± 252.95

*$P < 0.05$.
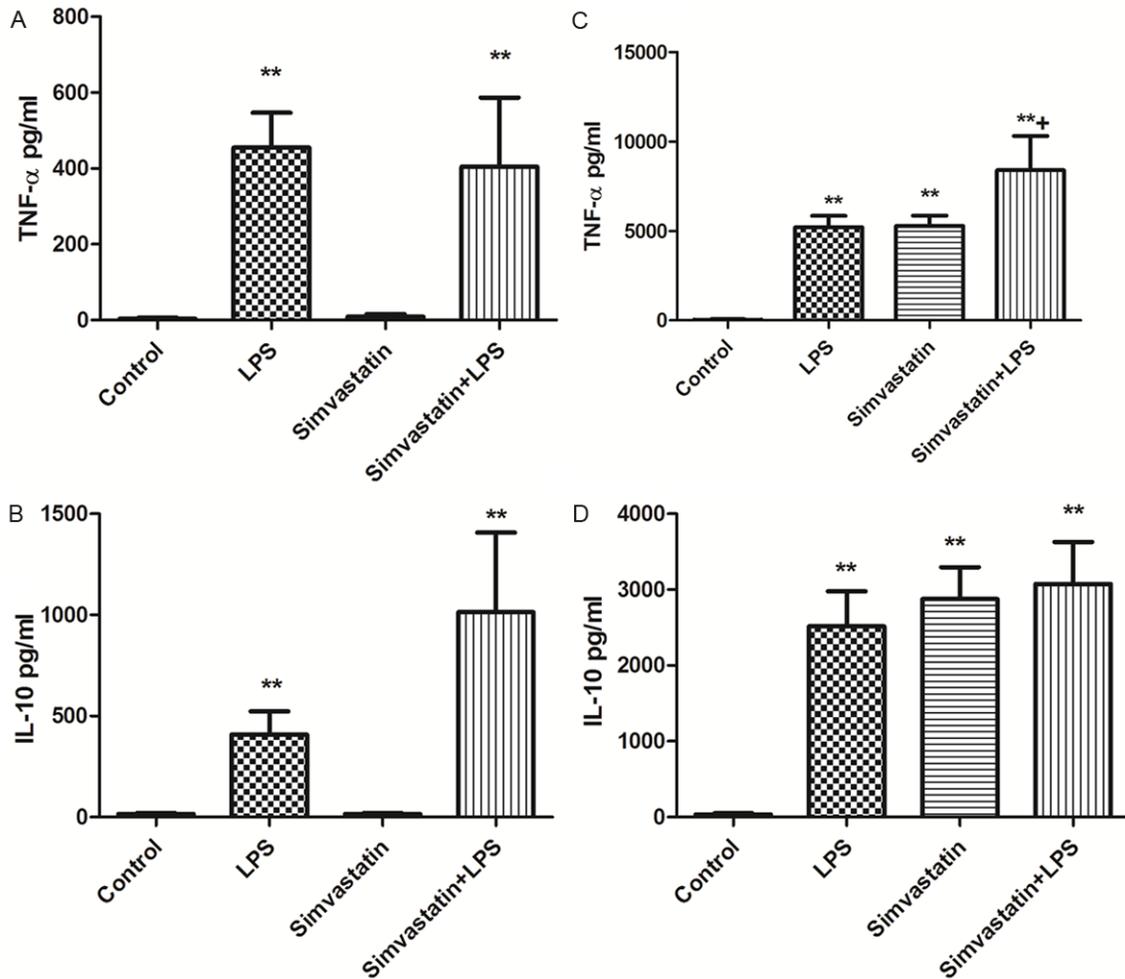
Figure 1. Cytokine values of experimental groups. Serum TNF-α values in experimental groups.**Significant differences at $P < 0.01$, LPS and Simvastatin +LPS vs. other groups (A). Serum IL-10 values in experimental groups. **Significant differences at $P < 0.01$, LPS and Simvastatin +LPS vs. other groups (B) Liver TNF-α values in experimental groups.**Significant differences at $P < 0.01$, experimental groups vs. control group, +Significant differences at $P < 0.05$, Simvastatin +LPS group vs. LPS and Simvastatin groups (C) Liver IL-10 values in experimental groups. **Significant differences at $P < 0.01$, experimental groups vs. control group (D).

monitored with potassium ferricyanide adding tissue homogenate using the King and Howard method [17]. The increase rate of the reduction of cytochrome c was measured in Complex II + III (Succinate Cytochrome c Reductase) by King's method. Complex I + III (NADH Cyto-chrome c Reductase) was measured according to Sottocosa's method [18]. Complex II (Succinate dehydrogenase) activity was determined by the reduction of 2,6-dichlorophenolindophenol (DCIP) according to King's method [19].
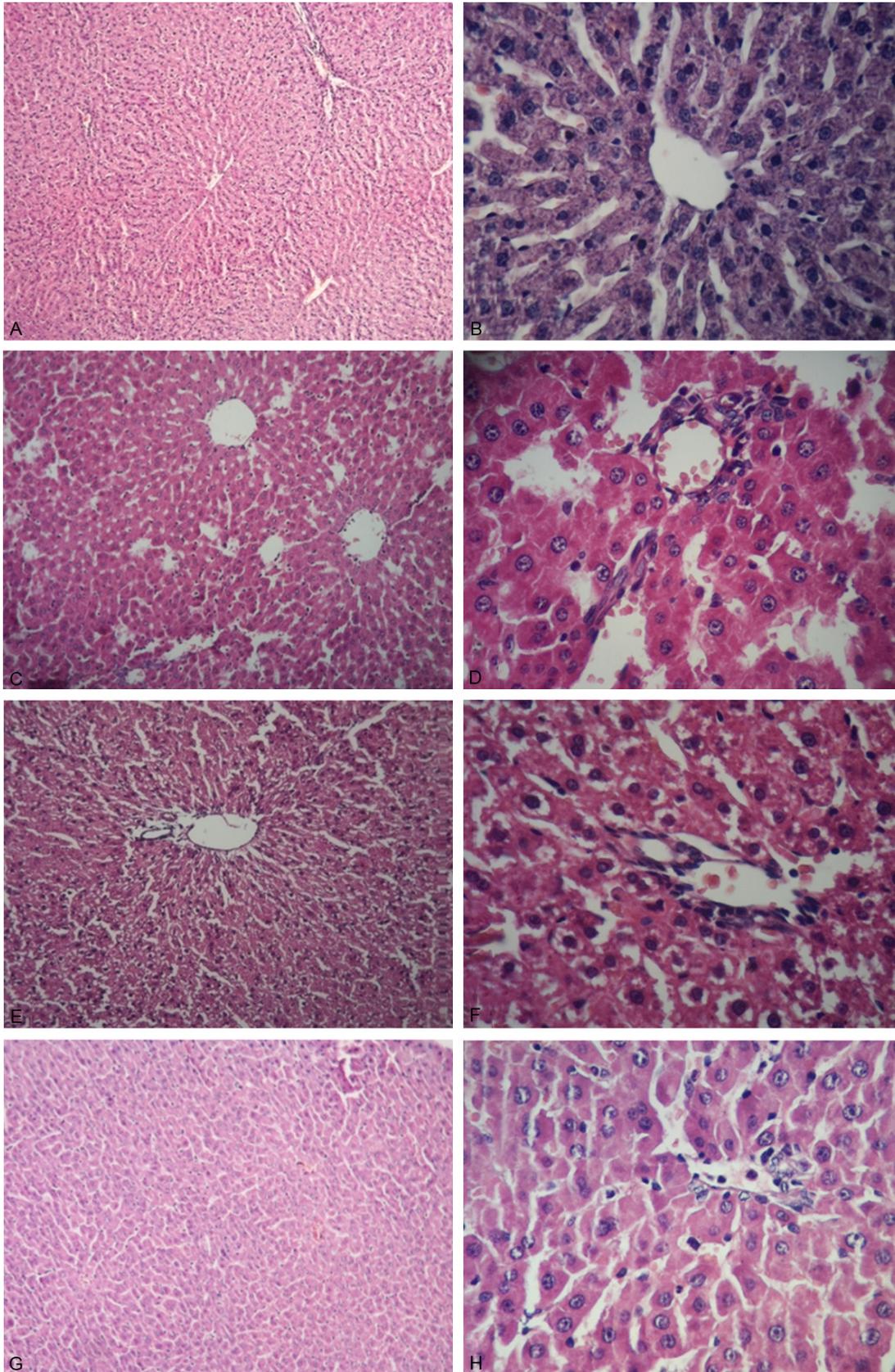
Figure 2. Section of liver tissue stained with H&E. Section of liver tissue from control group stained with H&E, × 40 magnification (A). Section of liver tissue from control group, × 100 magnification (B). Section of liver tissue from LPS group, × 40 magnification (C). Section of liver tissue from LPS group, × 100 magnification (D). Section of liver tissue from Simvastatin group, × 40 magnification (E). Section of liver tissue from Simvastatin group, × 100 magnification (F). S ection of liver tissue from Simvastatin +LPS group, ×40 magnification (G). Section of liver tissue from Simvastatin +LPS group, × 100 magnification (H).
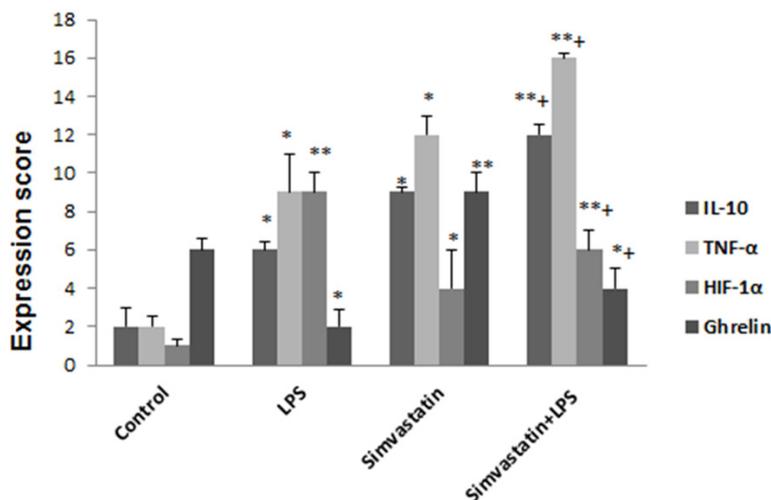
Figure 3. Alterations at cytokines, HIF-1α and Ghrelin hormone levels in liver after LPS, simvastatin and Simvastatin +LPS applications. **Significant differences at P < 0.01, *Significant differences at P < 0.05, +Significant differences at P < 0.05.

Statistical analysis

SPPS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. The results were statistically analyzed using the ANOVA and Tukey test. Data were presented as the mean ± standart deviation (SD). P < 0.05 was considered to be statistically significant.

Results

Biochemical findings

Serum CK and GOT values were found to be increased in experimental groups as compared with those of controls (P < 0.05).

There were no significant changes in GPT levels in any groups (P > 0.05). However, levels of GPT were lower in the control than the other groups'. In the simvastatin group, LDH levels were observed to be higher in the Simvastatin group than in the other groups (P < 0.05) (**Table 1**).

Cytokine findings

Serum TNF-α and IL-10 levels were found higher in Simvastatin +LPS and LPS groups com-

pared with the other groups. Liver TNF-α and IL-10 levels were higher in the experimental groups than the control group. (P < 0.01), However, levels of liver TNF-α were higher in the Simvastatin +LPS group than the LPS and Simvastatin groups (P < 0.05) (**Figure 1A-D**).

Histological findings

In the microscopic examination of the H&E stained sections. In the LPS group, hepatocyte cell membrane and the sinusoid structure were damaged. The Simvastatin group was the same as the control sections. In the Simvastatin +LPS group, hepatocytes and sinusoidal cord structure in periportal areas were partially improved, in some areas it was even close to the classic liver lobule structure (**Figure 2A-H**)

Immunhistochemistry findings

As shown in **Figure 3**, in all experimental groups, there was increased IL-10 and TNF-α levels in comparison with control group (P < 0.05); however, IL-10 and TNF-α levels were at the highest level in the Simvastatin+LPS group (P < 0.01). IL-10 and TNF-α levels of Simvastatin +LPS group had increased compared to LPS group (P < 0.05). For HIF-1α evaluation, its clear that in all groups the levels increased in comparison with the control group: the rank from highest to lowest was LPS (P < 0.01), Simvastatin+LPS group (P < 0.05) and Simvastatin (P < 0.01). HIF-1α levels of Simvastatin +LPS group had decreased compared to LPS group (P < 0.05). Only the Simvastatin group had increased ghrelin levels in comparison with the control group and other groups (P < 0.01). Ghrelin level was highly decreased in the LPS group, the Simvastatin +LPS group had a mild decrease (P < 0.05). Ghrelin levels of
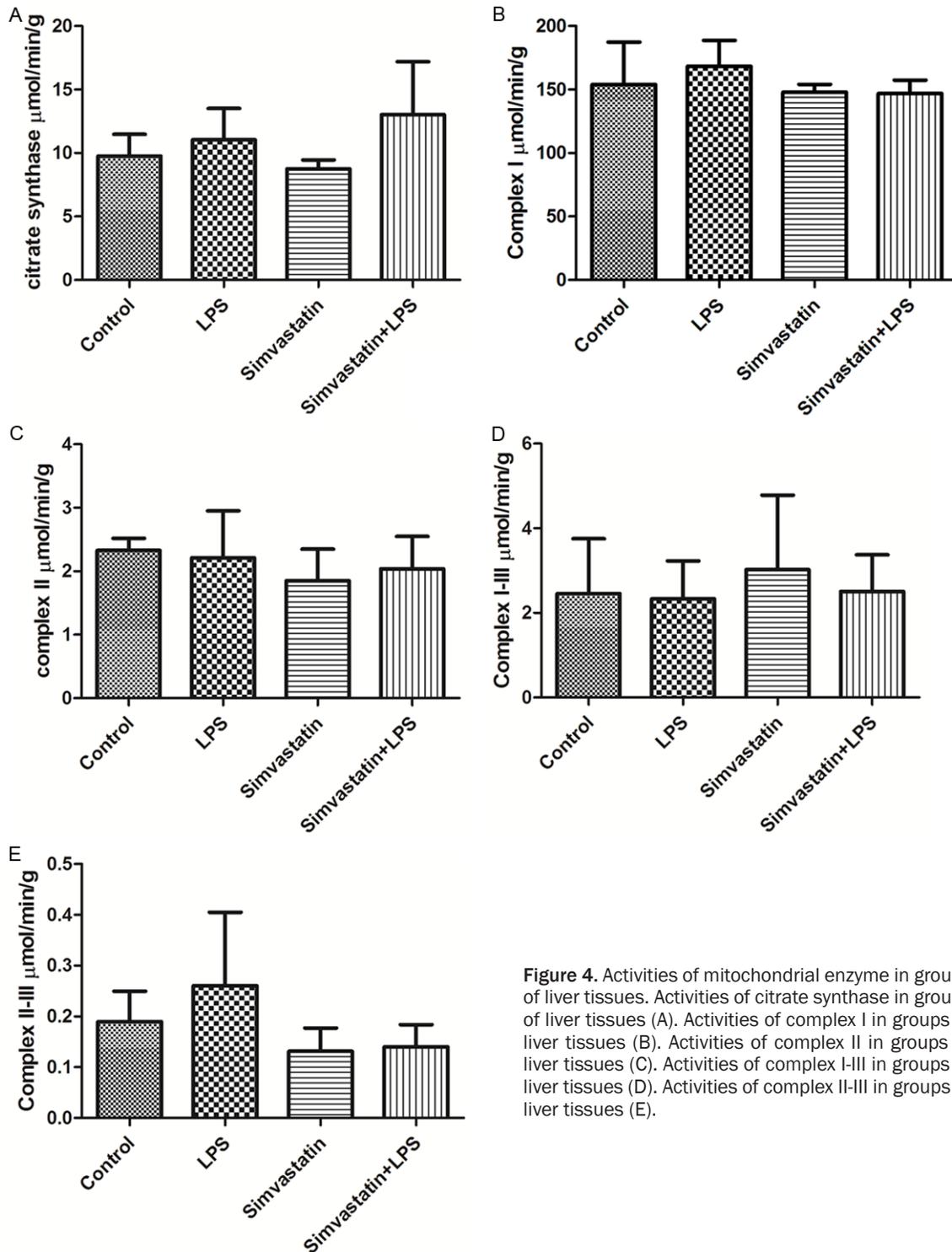
Figure 4. Activities of mitochondrial enzyme in groups of liver tissues. Activities of citrate synthase in groups of liver tissues (A). Activities of complex I in groups of liver tissues (B). Activities of complex II in groups of liver tissues (C). Activities of complex I-III in groups of liver tissues (D). Activities of complex II-III in groups of liver tissues (E).

Simvastatin +LPS group had increased compared to LPS group (P < 0.05).

Mitochondiral enzyme activities findings

We found citrate synthase, complex I, II, I-III, II-III enzymes activities in liver tissues, there were no significant differences in all groups (P > 0.05) (**Figure 4A-E**).

Discussion

In our study, we aimed to research the effects of prior treatment of simvastatin on the early

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phase of sepsis in liver tissue. Sepsis is a clinically important disease, causing organ dysfunction as a systemic inflammatory response to infections [20]. In experimental studies LPS has been shown to cause an inflammatory response at low doses and septic shock at high doses [21, 22]. It is known that LPS stimulates release of proinflammatory cytokines such as TNF-α and IL-1β, and antiinflammatory cytokines from monocytes, macrophages, and Kuppfer cells [23]. We observed significant increments of serum proinflammatory and antiinflammatory cytokines released in the LPS group at the end of 4th hours. In this study, while we determined significantly increased levels of tissue TNF-α and IL-10 in all experimental groups compared with controls, serum TNF-α and IL-10 levels were higher in the Simvastatin +LPS and LPS groups. In addition, according to immunohistochemical findings, levels of liver TNF-α and IL-10 were higher in the experimental groups than the control group.

In the literature, some studies suggest that statins potentiate LPS signaling, which is in agreement with our results, whereas some other studies show that statins inhibit LPS-induced increases in cytokines. It is known that after inducing sepsis with LPS, statins potentiate the reduction of proinflammatory cytokines [24-26]. On the other hand, statins have been demonstrated to lower C-reactive protein (CRP), and proinflammatory cytokines such as TNF-α, IL-1β. In agreement with our results, Eristrup et al. showed that [27] short-term treatment of simvastatin (20 mg/kg) did not influence cytokine levels during endotoxemia in an in vivo study. Moreover, Stolf et al. demonstrated that [28] both simvastatin and atorvastatin, both at low and high doses had no affect on expressions of antioxidant superoxide dismutase (SOD) and proinflammatory TNF-α genes in protecting aginst cecal-ligation-puncture induced liver injury. Matsumoto et al. reported that simvastatin increased the production of proinflammatory cytokines by inhibiting c-Fos with AP-1 binding site of IL-12p40 promoter, and stimulating JNK-dependent phosphorylation of c-Jun inducing to LPS activation [29].

In the histologic findings, we defined the structure of sinusoids and damaged cell membrane as injured in the hepatocytes of the LPS induced group. Many studies have reported histological alterations in liver tissue when using LPS [30, 31]. Abel-Salam et al. demonstrated that [32] LPS induction caused lymphocyte infiltration in the portal area in which caused vessel blockage, and injury to hepatocytes. Stolf et al. demonstrated that [28] there were narrowing of sinusoid lumens, capsular infiltration in inflammatory cells and these lesions could be prevented by statin treatment in cecal ligation perforation induced sepsis.

In our study, histologic alterations were observed to have partially recovered with using Simvastatin in animals treated with LPS. The production of many acute phase reactants are reported to be influenced by statins, such as IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 (MCP-1), and CRP [33].

Sepsis-associated hepatocellular dysfunction is mediated by antiinflammatory cytokines [34]. In our study in paralel with findings in the literature, raised levels of serum IL-10 contributed to reducing tissue damage in Simvastatin +LPS groups.

Yasuda et al. indicated that [35] short period application of simvastatin may prevent tissue damage and mortality by improving tissue oxygenation and microvasculer perfusion.

Serum CK and GOT values increased in all groups compared with controls; however, there were no significant changes in GPT levels, all groups were higher than controls. Stolf et al. [32] consistent with our study, found that GOT activity was higher while GPT activity was not changed in the group with sepsis, and this increment in GOT could not be reversed with statin treatment. Wu et al. demonstrated that [36] endotoxin shock significantly increased blood GOT, GPT, BUN, Creatinine, LDH, CPK, glucose, TNF-α, and IL-6 levels. Treatment with statins has been reported to elevate CK levels. In our study, we found increased serum LDH levels in the simvastatin group. It has been reported that lovastatin may induce myopathy and elevate LDH levels [37].

Although we observed damage to the hepatocyte membrane in histologic sections, mitochondrial enzyme activities were not affected in tissue homogenates in the LPS induced group. These findings may be attributed to the liver's kupffer cells affect in removing LPS from circulation [38].

There are controversial results of studies investigating sepsis where liver mitochondrial oxygen consumption was not affected or decreased. There are studies in the literature about mitochondrial function that are consistent with our findings [39-41]. Geller et al. showed [42] a high dose of LPS (40 mg/kg i.p.) with faecal peritonitis for 18 h had no effect on muscle or hepatic mitochondrial respiration. Brealey et al. demonstrated that [43] activity of complex IV muscle or liver mitochondria did not alter regardless of the duration and severity of sepsis. Taylor et al. observed [44] no changes in hepatic mitochondrial complex I- and complex II-dependent respiration in rats for 16 h after cecal ligation perforation.

In simvastatin our group, we observed a significantly higher level of ghrelin immmunoreactivity compared with the other groups, while it decreased in the LPS and LPS+Simvastatin groups. There are studies about ghrelin levels being altered during endotoxic shock but its mechanism remains unclear [45]. Ghrelin exerts anti-inflammatory effects in sepsis. Serum ghrelin levels are reduced during sepsis, and administration of exogenous ghrelin improves survival in sepsis. Another study has shown that Ghrelin suppresses inflammation in sepsis by activating the vagus nerve and sympathetic nervous system activity. In the literature, the source of elevated tissue ghrelin in the simvastatin group is unclear [46, 47]. In the simvastatin +LPS group, ghrelin levels were higher than the LPS group. In histologic sections, we propose that the improvement of liver tissue was a result of increased ghrelin levels in the Simvastatin +LPS group. However, we did not find any study in the literature which addresses the effects of exogenously given statins on endogenous ghrelin. We think that this is necessary to understand the relationship between ghrelin levels and statins during the early phase of sepsis.

Hypoxia-inducible factor (HIF)-1 is a transcription factor which is essential for regulating oxygen homeostasis [48]. HIF-1α participates in the transcriptional activation of hypoxia-responsive genes by binding to the hypoxia-response element in the promoter or enhancer region of these genes, and promotes the production of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-12, which reach harm-ful levels during sepsis [49]. HIF-1α is also activated by LPS and contributes to the cytokine activation and affect on lethality of LPS-induced sepsis. Therefore, inhibiting HIF-1α activity may be a novel therapeutic strategy for LPS-induced sepsis [6].

We observed that HIF-1α immunoreactivity in the liver sections was highest in the LPS group, and was reduced in rats with sepsis that had prior treatment with simvastatin. Jung et al demonstrated that [50] stimulation of HIF-1 alpha was needed for inducing nuclear factor kappa B (NF-kB).

Hisada et al [51] investigated the effect of fluvastatin, their study showed increments of degradation of HIF-1α by down-regulation of isoprenoid intermediates in vascular smooth muscle cells. On the other hand, Loboda et al. demonstrated that [52] atorvastatin initiated the activation of HIF-1α by inducing hypoxia in endothelial cells. We found the increased immunoreactivity of HIF-1α in the LPS group compared with those of other groups. Frede et al. showed that NF-kB increased expression of HIF-1α gene response to LPS secreted in human monocytes. Studies in the literature have suggested the increment of HIF1α translation is caused by proinflammatory mediators such as TNF-α and IL-1β [53]. Hazuku et al. showed that [54] simvastatin increased protein levels but not mRNA levels of HIF-1α in normoxic human endothelial cells. In contrast, Huang et al. showed that [55] nuclear HIF-1α protein, a subunit of HIF-1, and HIF-1 mRNA expression were coincidently reduced in late septic liver of rats.

Limitations of our study include not measuring the levels of serum ghrelin and HIF-1α. Also, to comply with ethics rules in experimental studies we should use the fewest number of animals possible, thus limiting our sample size. In conclusion, we found that Simvastatin reduced LPS induced tissue damage by increasing ghrelin and decreasing HIF-1α during early phase of sepsis.

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Disclosure of conflict of interest

None.

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