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LACTOFERRIN MODULATES INCREASED LIVER DNA DAMAGE BY REDUCING SERUM CYTOKINE, HEPCIDIN AND IRON LEVELS IN LIPOPOLYSACCHARIDE-INDUCED ENDOTOXEMIA IN RATS

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ABSTRACT. Endotoxemia, known as the presence of Gram (-) bacterial endotoxin in the circulation, is associated with high mortality. Here, it was aimed to investigate the effects of lactoferrin on inflammation and iron homeostasis and cytokine production in lipopolysaccharide (LPS)-induced experimental endotoxemia in rats. Tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ) levels, which are important cytokines, and iron and hepcidin levels, which play a role in iron homeostasis, DNA damage marker 8-hydroxy-2'-deoxyguanosine levels were evaluated in endotoxemia model. Enzyme-linked Immunosorbent Assay (ELISA) method is a technique based on antigen-antibody complex and using enzymes as marking. Serum TNF-α, IFN-γ and hepcidin levels and the liver 8-OHdG levels were measured by ELISA methods, serum iron levels were determined using iron assay kit. The results indicated that increased serum TNF, hepcidin and iron levels decreased at 1st, 3rd and 6th hours after LPS injection with lactoferrin supplementation, and increased IFN-γ level at 3rd and 6th hours returned to normal range. 8-OHdG increased immediately after LPS injection and this damage returned to normal within 6 hours by lactoferrin. The findings of the study revealed that lactoferrin might be beneficial in the prognosis and treatment of endotoxemia.

Keywords: *lactoferrin, endotoxin, cytokine, 8-hydroxy-2'-deoxyguanosine, iron transport*

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INTRODUCTION

Lipopolysaccharide (LPS), also known as endotoxin, is the component of gram (-) bacterial cell wall. LPS is the best-defined antigen in immunology, being major factor in formation of endotoxemia and septic shock. In experimental studies, LPS has a wide array of uses from modeling local inflammations to modeling septic shock but being dependent on the dose, frequency and way of administration and exposure time. Bacteria, viruses, fungi and parasites can cause septic shock and result in a mortality rate of around 20-80%. The presence of bacteria in the systemic circulation, the bacteremia, the systemic state of the infection is called sepsis whilst the presence of Gram (-) bacterial endotoxin in the circulation is called endotoxemia [1,2].

Lactoferrin is naturally present in colostrum and breast milk and is produced from milk proteins. It is a multifunctional protein that prevents the growth and reproduction of various infectious agents including gram-positive and negative bacteria, viruses, protozoa, or fungi. Lactoferrin is a single-chain glycoprotein of the transferrin gene family that binds iron at 80-kDa. Lactoferrin plays a role in many physiological processes such as regulation of iron metabolism, defense against a wide range of microbial infections, anti-inflammatory activity, regulation of cell growth and differentiation, and cancer prevention and anti-metastasis [3].

In any case of infection, cytokine release develops as a normal immune response. When LPS passes into the circulation, an inflammatory response is triggered by defense cells. Lactoferrin shows a prophylactic effect by reducing the release of interleukin-1, interleukin-6, tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) from monocytes in *in-vitro* and *in-vivo* conditions [3-6]. One of the most important antimicrobial functions of lactoferrin is to bind free iron to prevent infections. Iron is an important catalyst in the formation of reactive oxygen species. It shows important functional properties due to its characteristics such as high iron binding ability even at very low pH, resistance to proteolysis, net positive charge, and presence in many tissues. For this reason, it has been reported that lactoferrin reduces the harmful effects of reactive oxygen species produced by leukocytes in the inflammation region [7,8]. The hepcidin plays a central role in the regulation of systemic iron homeostasis by coordinating iron absorption, mobilization, and storage to meet the iron requirements of erythropoiesis and other iron-dependent processes [9]. It has also been reported that hepcidin, an acute phase 2 protein, has a positive correlation with inflammation indicators [10]. Lactoferrin has a great potential due to its roles in carrying iron and exhibiting antimicrobial, immune-modulatory, anti-inflammatory, and antineoplastic activities. Despite many new available drugs,

antibiotics and treatment protocols for immunomodulation, septic shock is still an important health problem with a 30-90% mortality rate [11]. Revealing the safe uses of LF for clinic and veterinary purposes with respect to the potent protective effects of LF against endotoxemia is important. Herewith the target, in our previous study, we aimed to determine the effect of lactoferrin on adenosine deaminase, nitric oxide and liver enzyme levels in endotoxemic rats. The results showed that LPS increased adenosine deaminase activity synthesis and NO release and LF acted as an anti-inflammatory and immunosuppressor in stimulating immune response [12]. In this study, we investigated the effect of lactoferrin on some important biochemical parameters such as serum TNF-α, IFNγ, hepcidin, iron and liver 8-OHdG levels at different hours (1, 3 and 6 h) following LPS administration.

RESULTS AND DISCUSSION

The experimental model can be designed according to the differences in the dose and frequencies of lipopolysaccharide administration in rat [5,12,13]. In this study, after lactoferrin supplementation (20 mg/kg/day) for 1 week, rats were given LPS (20 µg/kg) at 3 different hours (1, 3 and 6 h post-injections of LPS) and blood was collected at the 1st, 3rd and 6th hours. Herewith the blood samples, the changes in serum TNF-α, IFN-γ, hepcidin, iron and liver 8-OHdG levels in an experimental endotoxemia model in Swiss Sprague rats were recorded after LF supplementation.

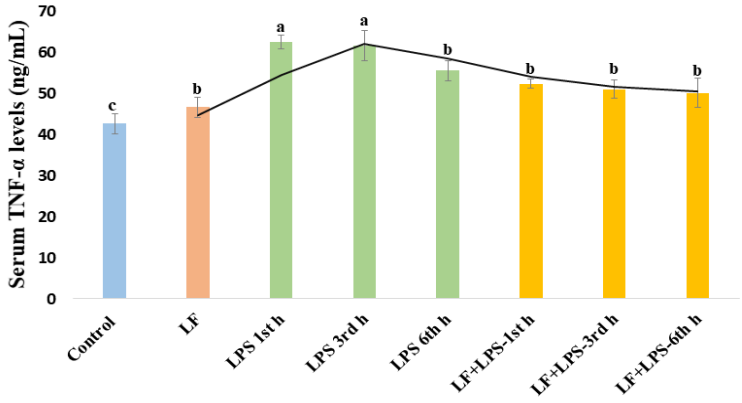

Figure 1. The effect of lactoferrin on serum TNF-α levels in LPS-induced endotoxemia model. The difference among the groups in different columns (a,b,c) was statistically significant ($P<0.05$ for the 1st, 3rd and 6th hours).

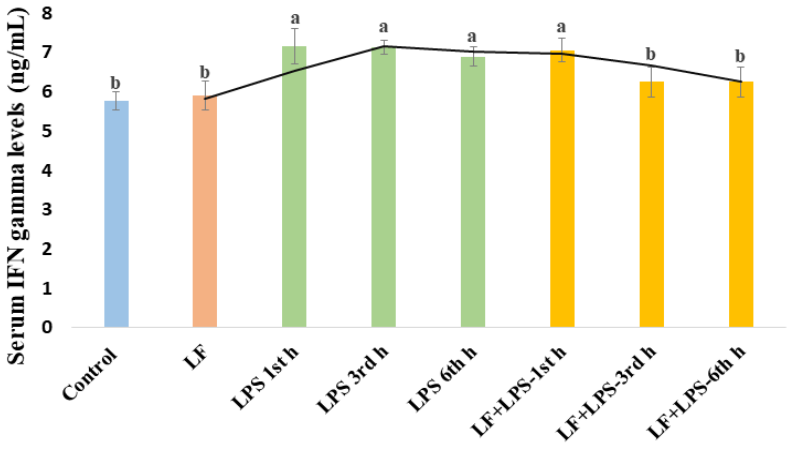

Figure 2. The effect of lactoferrin on serum IFN-γ levels in LPS-induced endotoxemia model. The difference among the groups in different columns (a,b) was statistically significant (P<0.05 for the 1st, 3rd and 6th hours).

The findings of the study revealed that at serum TNF-α levels were higher in the LPS group compared to other groups (P<0.05) (Figure 1). When the TNF-α levels in the groups were analyzed according to hours, no statistical difference was observed between the 1st, 3rd and 6th hours of the LF+LPS group. The IFN-γ levels were higher in the LPS group at the 1st, 3rd and 6th hours in comparison to the other groups (P<0.05) (Figure 2). At 3rd and 6th hours IFN-γ levels in the LF + LPS group were lower than the LPS group and no statistical difference was observed between the control, LF and LF + LPS groups at the same hours.

The proinflammatory cytokines are endogenous polypeptides generated by immune system cells and mediate many kinds of immune response. The serum cytokine levels increase after injection of gram-negative bacteria, which is a component of the cell wall [14, 15]. In a previous study, it was reported that endotoxin remained in the blood for 6 hours and its level was higher than 1584.9 EU/mL in LPS-induced endotoxemia model in mice [16]. Hasegawa-Ishii et al. found that in sepsis-associated encephalopathy mouse model, LPS exposure (30 mg/kg, i.p.) responded to the production of multiple cytokines in the acute phase (4-24 hours) after LPS injection in all brain regions [17]. In endotoxemia, proinflammatory cytokines (TNF-α, IF-Ɣ), which are associated with the severity of the disease, are secreted and anti-inflammatory cytokines are secreted in response to the continuation of the infection [18-20]. TNF-α and IFN-γ is generally considered to be the critical

mediator for septic shock-related lethality induced by LPS [5, 21]. In this study, the serum TNF-α and IFN-ɣ levels after LPS injection was increased at all hours (P<0.05). Lactoferrin administration significantly normalized TNF-α levels at the 1st, 3rd and 6th hours. It has been found that TNF-α, whose synthesis is stimulated by LPS, causes longer circulation (from 3 hours to 6 hours) in endotoxemia model. LF may play a protective role in endotoxemia by affecting the transcriptional levels of proinflammatory cytokines by affecting various signal pathways that control cytokine production involved in the immune response. Moreover, the inhibition effect of LF can be explained by the disruption of the membrane structure as a result of binding iron in the gram-negative bacterial membrane and thus showing prophylactic properties against septic shock [6,19,20].

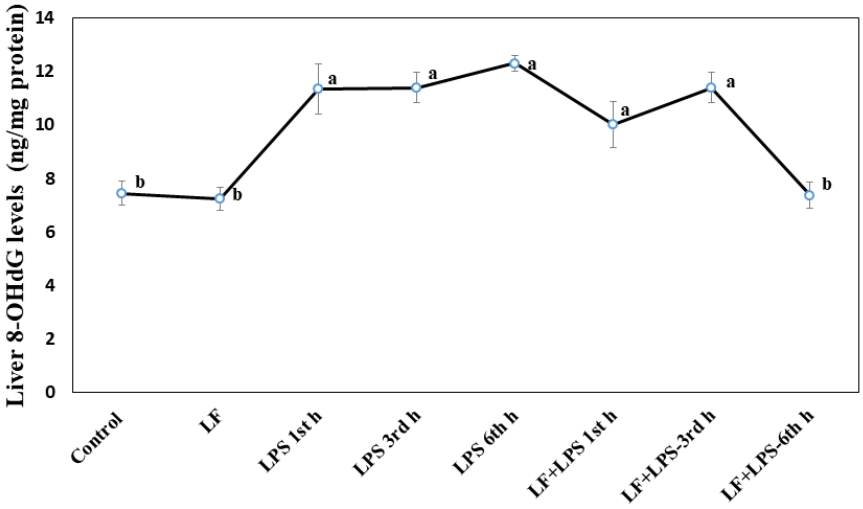

Figure 3. The effect of lactoferrin on liver 8-OH-2-Deoxyguanosine levels in LPS-induced endotoxemia model. The difference among the groups in different columns (a,b) was statistically significant (P<0.05 for the 1st hour, P<0.001 for the 3rd hour).

At the 1st and 3rd hours, the liver 8-OHdG levels were higher in the LPS and LF + LPS group than the other groups (P<0.05 for the 1st hour, P<0.001 for the 3rd hour) (Figure 3). At the 6th hour, liver 8-OHdG levels were significantly higher in the LPS group than in the other groups. While there was no difference between the 1st and 3rd hours, there was a difference between the 1st and the 6th and the 3rd and 6th hours (P<0.05).

The 8-OHdG has been used commonly in many studies as a biomarker for the measurement of endogenous oxidative DNA damage and as a risk factor for many diseases. After LPS treatment, the liver is more susceptible to oxidative damage than other organs and causes mutagenic lesions such as 8-OHdG with DNA breakage [22]. In our previous study, LPS significantly was increased liver enzymes aspartate aminotransferase and gamma glutamyltranspeptidase activities, which are the precursors of disease risk. Also, liver NO level significantly with LPS injection. LF attenuated increased NO and liver enzyme levels during inflammation [12]. Liver 8-OHdG level reduced by LF supplementation was positively correlated with liver enzymes and NO level. In the LPS-induced murine neuroinflammation model, a significant increase in both peripheral and brain IL-1β, TNF-α levels were shown to be accompanied by an increase in 8-OHdG levels [23]. In another study, it was stated that urothelium 8-OHdG expression increased in the LPS-induced cystitis model [24]. In this study, the liver 8-OHdG levels at the 6th hour were higher in the LPS group than in the other groups ($P<0.001$). Lactoferrin supplementation did not reduce DNA damage in a short time, but normalized it by reducing DNA damage to the 6th hour in experimental endotoxemia model. Ogasawara et al. [25] showed that LF acts not only as a transient metal chelator, but also as a sacrificial scavenger for reactive oxygen species (ROS), and that it protects through direct interaction with hydrogen radicals. In another study, LF regulated the endotoxemic effect of LPS by reducing DNA damage especially after the 3rd hour. Some of the inflammation cascade mediators such as TNF-α, IFNγ, interleukins 1 and 6 and nuclear factor kappa B related to the action mechanism of 8-OHdG have been proposed [26].

The iron levels were higher in the LPS group at the 1st, 3rd and 6th hours compared to the other groups ($P<0.05$) (Figure 4). At all hours, iron levels in the LF + LPS group were lower than the LPS group, and higher than the control and LF ($P<0.05$). There was a difference between the 1st hour and the 6th hour in the LF + LPS group ($P<0.05$). The hepcidin hormone levels were higher in the LPS group at the 1st, 3rd and 6th hours compared to the other groups ($P<0.001$) (Figure 5). At all hours, hepcidin levels in the LF + LPS group were lower than the LPS group, and higher than the control ($P<0.001$). Hepcidin levels increased at 1st hour compared to 6th hours in the both LPS and LF+LPS groups ($P<0.05$). The hepcidin levels of LPS group were lower at 6th hours than both 1st and 3rd hours.

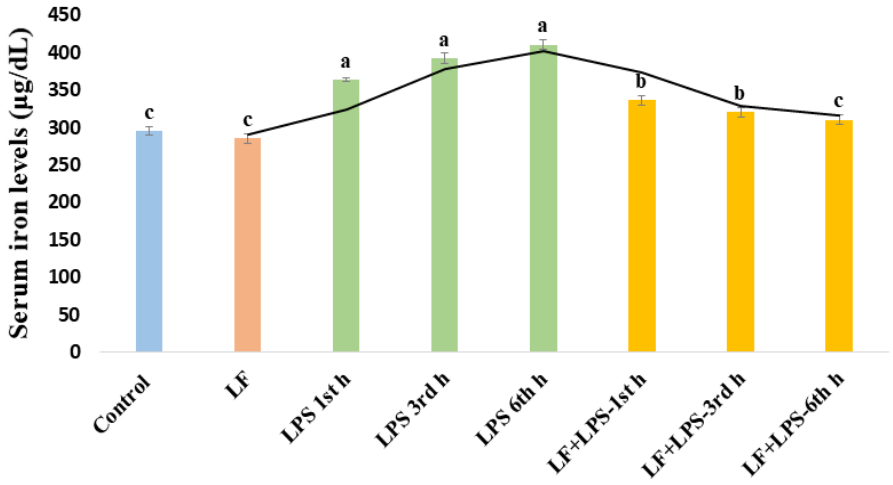

Figure 4. The effect of lactoferrin on serum iron levels in LPS-induced endotoxemia model. Serum iron levels were determined spectrophotometrically by drawing a standard curve at 100-1000 µg/dL Fe^{2+} concentrations at 590nm. The difference among the groups in different columns (a,b,c) was statistically significant ($P<0.05$ for the 1st, 3rd and 6th hours).

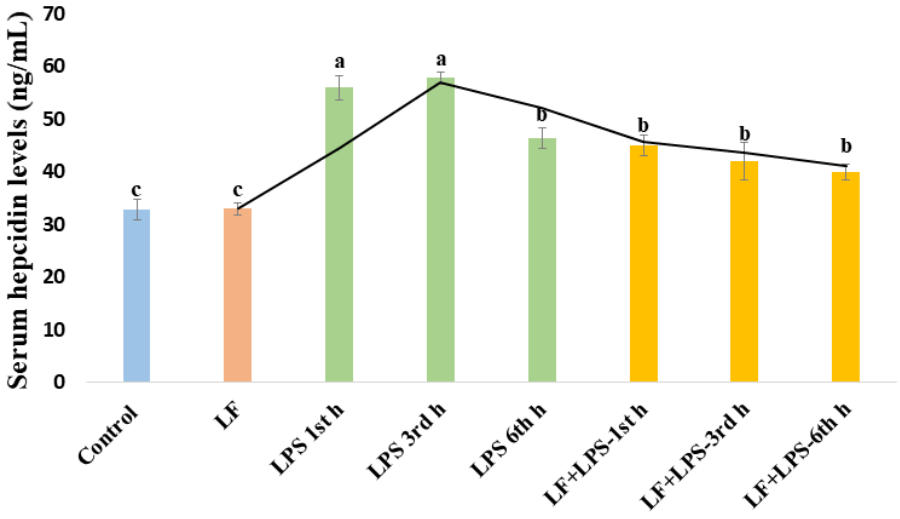

Figure 5. The effect of lactoferrin on serum hepcidin levels in LPS-induced endotoxemia model. The difference among the groups in different columns (a,b,c) was statistically significant ($P<0.05$ for the 1st, 3rd and 6th hours).

The iron is necessary for pivotal metabolic processes including energy production, synthesis, repair and transcription of DNA, oxygen transport/ storage and drug detoxification. The free available not bound-Fe^{3+} causes the ROS production through the Fenton and Haber-Weiss reactions, ROS causes oxidative stress and acute and chronic inflammation processes associated with various diseases. The high hepcidin levels, hindering iron export by ferroportin (Fpn) causes intracellular iron overload and the increased hepcidin level correlates with an increase in ferritin level in chronic inflammatory process [27]. It has been reported that serum hepcidin levels increase significantly after 4^{th} and 8^{th} hours compared to start values in acute kidney injury [28]. In LPS-induced inflammatory model mice, LPS has been reported to reduce the iron level and increase hepcidin, TNF-α and hepcidin signal pathway molecule STAT3 expressions [29]. Hepcidin is expressed to negatively regulate iron in circulation by inhibiting iron absorption from the duodenum. Therefore, hepcidin expression increases significantly during infection. This leads to a significant reduction in serum iron, thus bacteria are deprived of iron and reduce their growth rate [30]. Iron binding capacity of lactoferrin (bi) carbonate ion (CO_3^{2-}) depends on its existence and thus balances its positive charge. It shows this effect by interacting with the N-terminal lipid A portion and thus causing the lipopolysaccharide to separate, destabilize the outer membrane and increase its permeability [31].

Intracellular iron retention can be an inducer of infection, so the cell has to maintain the anti-inflammatory state in order to balance the iron level between tissues and blood. Previous studies found that the cyclic peptides formed by a bridge of disulfide bond tend to change structures and antimicrobial activity of some peptides. Despite the limited information evaluating the changes in the molecular structure of LF, there are studies regarding the effect of disulfide bonds on its antibacterial and anti-inflammatory activities [32,33].

CONCLUSIONS

The study emphasizes that lactoferrin is vital in the reduction of TNF-α and IFN-γ cytokines production and the complex relationship between iron and inflammation homeostasis during the endotoxemia process. It was observed that 8-OHdG levels increased immediately after LPS injection and this level returned to normal within 6 hours by lactoferrin. In addition to serum cytokines, changes in iron, hepcidin and 8-OHdG levels are important in the treatment and prognosis of endotoxemia. It has been concluded that increased serum cytokine, hepcidin, iron levels during endotoxemia contribute to 8-OHdG increase in the liver. It is worthy to note herein that lactoferrin might be beneficial in the prognosis and treatment of endotoxemia because it significantly reduces this damage.

EXPERIMENTAL SECTION

Animals

Swiss Sprague female rats, aged 6 months and weighing 280-310 g were used in this study. Before starting the study, a consent was obtained from Kafkas University, Animal Experiments Local Ethics Committee (Decision no: KAÜ-HADYEK: 2015/018). Animals were kept at constant conditions as follows: temperature (23 ± 2 °C), humidity (50 ± 10 %), and light (12 h light/dark cycles). All animals were allowed to access free standard chow and *ad libitum* freshwater. Same conditions were applied to all groups during the experiment.

Experimental design

A total of 80 Swiss Sprague rats were divided equally into 4 groups: Group I (control, n=10), Group II (20 mg/kg/day LF for 7 days (n=10)), Group III (20 µg/kg LPS single dose, n=30)) and Group IV (20 mg/kg/day LF for 7 days + the end of 7 days 20 µg/kg LPS single dose (n = 30). In order to determine the effects of LPS administration at 3 different hours (1, 3 and 6 h post-injections of LPS), 30 rats were preferred in Groups III and IV. Lipopolysaccharides from *Escherichia coli* 0111:B4 (Sigma-Aldrich: L4391) were used as the LPS source**.** All injections were done as intraperitoneal injection. Blood samples and liver tissues were taken under anesthesia from the groups at 1, 3 and 6 h post-injections of LPS. Serum samples were obtained by centrifuging the blood samples for 15 minutes at 3000 rpm. All the samples were stored at -45°C until analyses. Serum samples taken were used to measure TNF-α and IFN-γ cytokines, hepcidin and iron levels. The liver samples were used to measure 8-OHdG levels.

Biochemical Analysis

Serum TNF-α, IFN-γ and hepcidin levels and the liver *8-OHdG levels* were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Sunred Biological Technology Co. Ltd., Shanghai, CHINA). Serum iron levels were measured using commercial kit (BioAssay Systems, Hayward, USA). All spectrophotometric analyzes were performed on the microplate reader (Bio-Tek Eon, USA).

Statistical Analysis

The statistical significance was evaluated using the SPSS 20.0 software package (SPSS ver. 20.0 for windows professional edition). Mean and standard error were used in data analyses. Kruskall-Wallis H analysis was conducted to determine the differences between the groups. Mann Whitney U-test was determined as the source of significant differences among groups. Level of significance was accepted as $P< 0.05$.

AUTHOR CONTRIBUTION

C.G. and K.Y.D performed experiments. All authors wrote the manuscript. O.A and E.A. conceived the original idea, designed and supervised the project.

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CONFLICT OF INTEREST

All authors declare that there is no potential conflict of interest.

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