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Effect of Lactoferrin on Paraoxonase Activity, Some Acute Phase Proteins and Oxidant/Antioxidant System

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RESEARCH ARTICLE

Abstract

Lactoferrin is a member of the high affinity to glycoprotein family of transferrin for iron and plays a role in natural immunity as well as antibacterial, antifungal, antiviral, antiparasitic, antioxidant, anticancer, antiinflammatory, bone health and immunomodulation. The aim this study was to investigate of the acute effect of lactoferrin on paraoxonase activity, oxidant/antioxidant system and total protein, albumin and globulin. This study was carried out on 6-month Sprague Dawley rats in a total number of 20 subjects. Paraoxonase activity, total antioxidant capacity (TAC), total oxidant capacity (TOC), total protein, albumin and globulin levels were measured through the spectrophotometer. When the lactoferrin group was compared with the control group, it was found that TAC levels were statistically higher ($P<0.05$), TOC levels were statistically lower ($P<0.01$) and paraoxonase activity was statistically higher ($P<0.05$). There was no difference in total protein and globulin levels in the lactoferrin group compared to the control group. As a result, acute lactoferrin administration may play an important role in the regulation of antioxidant balance by increasing paraoxonase activity and decreasing the level of reactive oxygen species.

Keywords: Lactoferrin; reactive oxygen species; paraoxonase activity; rat.

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INTRODUCTION

Lactoferrin is a single-chain glycoprotein of 692 amino acids with a molecular weight of approximately 80 kDa, a positive charge and a high affinity for iron, (Steijns and van Hooijdonk, 2000). In 1961, it was first named "Lactoferrin" by Blanc and Iskiler because it was obtained from milk (Blanc and Islıker, 1961). Moreover, it is also secreted by mammary gland epithelial cells, bronchial gland epithelial cells, endometrial mucosal cells, renal tubular cells and seminal vesicles. It is also found in many exocrine fluids such as saliva, bile, pancreatic fluid, tears and in heterophilic leukocyte granules (Lonnerdal and Iyer, 1995; Masson and Heremans, 1971). Lactoferrin occurs after receptor-mediated endocytosis of phagocytic cells from the organism by iron transfer to ferritin or is directly taken up by the liver (Levay and Viljoen, 1995). Because of its similarity to transferrin and as an iron-bearing molecule, lactoferrin is thought to play an important role in iron metabolism. Initial research into the function of lactoferrin began with high iron binding ability and active activity in a wide pH range within the transferrin family. It is found in many tissues, being highly resistant to proteolysis and is positively charged, allowing lactoferrin to exhibit different properties. Lactoferrin plays a role in innate immunity and exhibits antibacterial, antifungal, antiviral, antiparasitic, antioxidant, anticancer, anti-inflammatory, and immunomodulation, which has been suggested in previous structured studies (Pan *et al.,* 2007; Ward *et al.,* 2005). Paraoxonase [aryldialkylphosphatase, EC 3.1.8.1 (PON)], is firstly synthesized in liver with arylesterase. PON is a glycoprotein structured serum ester hydrolase capable of hydrolyzing paraoxone

(o,o-diethyl-opnitrophenyl phosphate), the active metabolite of paration, an organic phosphorus insecticide. The PON gene (HUMPONA) has three members, the paraoxonase multigen family (PON1-PON2-PON3), located in the region between chromosome q21.3 and q22.1 in humans (Primo-Parmo*et al.,* 1996). During cell metabolism, free radicals are produced continuously and cause oxidative stress. PON1 is characterized as an antioxidant with its ability to hydrolyze organophosphate compounds and neutralize free radicals (Rosenblat*et al.,* 2006).

Free radicals are high-energy atoms or molecules that have one or more unpaired electrons in their outer orbital. Due to the presence of unpaired electrons, they can easily react with substances. Free radicals can originate from oxygen or nitrogen. The increase in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or the decrease in antioxidant mechanisms in living organisms creates a condition called oxidative and nitrosative stress, respectively. Enzymatic and non-enzymatic antioxidants scavenge ROS and protect tissues from oxidative damage (Bast *et al.,* 1991; Halliwell and Gutteridge, 1999; Taysi*et al.,* 2019). The studies investigating the effects of lactoferrin on the antioxidant system, such as anticancer, antiviral, antimicrobial and immune system effects, are limited. Therefore, we aimed to investigate the acute effects of lactoferrin on biochemical parameters related to oxidant/antioxidant system, paraoxonase activity and antioxidant system.

MATERIALS AND METHODS

Subjects

This study was carried out on 20 mature six-month-old Sprague Dawley rats. Prior to the experimental procedures, the permission was obtained from the Animal Experiments Local Ethics Committee at Kafkas University (KAU-HADYEK/2015-018). Housing, care procedures and experimental studies of the animals were carried out in the Experimental Animals Unit at Kafkas University. All animal groups were fed ad libitum-balanced diet. They were kept at constant room temperature (19-21°C) on a 12-hour light and dark cycles.

Experimental Design

Animals were divided into two groups: the control group (saline group) and the lactoferrin group (single dose intraperitoneal 20 mg/kg lactoferrin). During the experiment, normal feeding conditions were provided for the groups. Blood samples were taken 6 hours after the experiment. At the end of the experiment, after intraperitoneal administration of xylazine-ketamine combination (15mg/kg-50mg/kg), blood samples were taken from the hearts of rats into heparin tubes. Serum samples were obtained by centrifuging the blood samples for 15 minutes at 3000 rpm and +4°C. All the samples were stored at -45°C until analyses. PON activity, TAC and TOC levels, total protein, albumin and globulin levels were measured through spectrophotometer (Bio-Tek Eon, WINOOSKI, VT, USA).

Biochemical Analysis

Serum samples were stored at -45 °C. Paraoxonase activity in serum samples was determined according to the method specified by Furlong et al. (Furlong *et al.,*1988). TAC and TOC levels, total protein, albumin and globulin levels were measured colorimetricallythrough commercial kit spectrophotmeter.

Determination of Paraoxonase Activity

Paraoxonase activity in serum samples was analyzed according to the method specified by Furlong et al. [Furlong *et al.,* 1988]. Substrate for paraoxonase activity measurement paraoxone (o,o-diethyl-p-nitrophenyl phosphate; Supelco, USA) was used. The p-nitrophenyl released through hydrolysis by PONwas based on spectrophotometric measurement of its absorbance at 412 nm (at 25 °C). PON activity was calculated as U/L according to the molar absorption coefficient determined for p-nitrophenol and dilutions made in the experiment. Paraoxonase activity is the unit of enzyme activity (U/L) that converted 1 μmol of paraoxon to p-nitrophenol in 1 minute.

Determination of Total Antioxidant and Total Oxidant Capacity Levels

Total antioxidant status (TAS) was determined colorimetrically using commercial kit (Rel Assay®, Gaziantep, Turkey) in plasma samples. The antioxidant substances in the sample react with the dark blue-green colored 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulfonate (ABTS) radical, causing a decrease in the color of the compound or a loss of color.The change of absorbance at 660 nm is related with total antioxidant level of the sample [Erel, 2004]. Plasma total oxidant status (TOS) was determined through commercial kit (Rel Assay®, Gaziantep, Turkey). Oxidants present in the sample oxidized the ferrous ion-chelator complex to ferric ion. The oxidation reaction was prolonged by enhancer molecules, which were present in reaction medium. The ferric ion made a colored complex with chromogen in an acidic medium. The color intensity is related to the total oxidant molecules present in the sample at 530 nm. Trolox and hydrogen peroxide standards were used for total antioxidant and total oxidant capacities **[**Erel, 2005].

Determination of Oxidative Stress Index (OSI)

The OSI, which is an indicator of the degree of oxidative stress, was calculated according to the formula below, the TAS and TOS kit protocol of Rel Assay.

OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/L)/TAS (mmolTrolox Eq/L).

Determination of Total Protein, Albumin and Globulin Levels

Determination of total protein (mg/dL), albumin (mg/dL) and globulin (mg/dL) levels from the serum samples were determined by commercial kits through colourimetric assay (Erba Lachema S.R.O, Brno, Czech Republic). Serum globulin concentrations were calculated by subtracting the albumin values from total protein values.

Statistical Analysis

Statistical analyzes of the data were performed in triplicate, and the standard deviation (mean±SD) and mean values were determined. The significance of the statistical data was evaluated using the SPSS 16.0 software package. A one-way analysis of variance was performed, and Duncan's test was used to estimate significance at the 5% probability level.

RESULTS AND DISCUSSIONS

Paraoxonase activity, total antioxidant and oxidant capacity, total protein, albumin and globulin levels in serum samples were investigated. The results showed that both PON activity and TAC levels (both P <0.001) of the lactoferrin group were higher and the TOC level (P<0.01) was lower compared to the control groups. There were no differences in total protein, albumin and globulin levels in the lactoferrin group compared to the control group (Table 1).

Table 1. PON activity, TAC, TOC, Albumin, Total Protein and Globulin Levels

Groups	PON activity (U/L)	TAC (mmolTrolox equiv./L)	TOC (μmol H_2O_2 Equiv./L)	OSI (arbitrary unit)	Albumin (mg/dL)	Total Protein (mg/dL)	Globulin (mg/dL)
Control	10.628±0.914	1.427±0.079	48.515±2.788	26.57±2.45	3.09±0.262	5.32±0.197	2.23±0.206
Lactoferrin	18.91±2.78*	1.81±0.124*	35.546±1.639*	25.42±2.1	2.97±0.25	5.45±0.38	2.48±0.17
P Values	<0.05	<0.05	<0.01	Ns	Ns	Ns	Ns

*: Differences between groups are statistically significant.
Ns: There was no statistical difference between the groups. (Ns: Non-significant)

The antimicrobial effect of lactoferrin and its preventive role in oxidative reactions are due to its iron binding capacity, glutathione synthesis and being rich in cysteine amino acid structures (Al-Hashem *et al.,* 20090. This study aimed to investigate the effect of lactoferrin on some antioxidant system parameters and paraoxonase activity, known as an antioxidant enzyme in rats. It was determined that a single dose (20 mg/kg live weight) of lactoferrin application to rats significantly increased PON activity (P<0.05). Faridvand et al. investigated the effect of lactoferrin on antioxidant esterase activities. In their study, a dose of 200 mg/kg live weight lactoferrin and the normal diet increased serum PON activity in the lactoferrin. They also reported that lactoferrin is effective against oxidative stress due to its ability to reduce lipid peroxidation (Faridvand *et al*., 2017). In their study where they administered lactoferrin to rats for 3 weeks (300 mg / kg body weight), Farid et al. determined that there was no significant change in PON activity in liver tissues compared to the control groups. However, they observed that by lowering the level of malondialdehyde (MDA), it contributed to protection against reactive oxygen species-mediated cell and tissue damage and increased superoxide dismutase (SOD) activity [Farid *et al.,* 2019].

In a study in pigs, they reported that lactoferrin administration significantly increased total antioxidant capacity levels in serum and muscle tissue (P<0.01 and P<0.05, respectively) and showed this effect by affecting reactive oxygen species (ROS) levels. It was reported that lactoferrin administration significantly increased (P<0.01) activities of glutathione peroxidase and catalase enzymes, and MDA level which is an indicator of lipid peroxidation in serum and muscle tissue decreased (Wang *et al.,* 2008). In this study, it was determined that lactoferrin administration significantly increased serum total antioxidant capacity levels (P<0.05) and decreased total oxidant capacity levels (P<0.01) in rats. With these findings obtained from the study, it was concluded that lactoferrin administration decreases ROS level and accordingly increases PON activity and plays an important role in regulating antioxidant balance.

A study in mice suggested that lactoferrin significantly increases glutathione and 1,1-diphenyl-2-picryl-hydrazyl antioxidant molecules in serum and liver tissue, and significantly reduces the formation of MDA in the liver (Chen *et al.,* 2016). Ginet et al. reported in their study in rats that daily administration of 300 mg/kg body weight lactoferrin against lipopolysaccharide-induced oxidative stress provided a significant increase in glutathione (GSH) level, which is an important antioxidant parameter, and that lactoferrin reduced brain damage and increased antioxidant capacity (Ginet *et al.,* 2016). In a study, protein concentrate (WPC) was administered orally to rats daily (0.5 g / kg body weight) for 6 weeks. They reported that WPC application significantly decreased liver MDA level and serum nitric oxide (NO) levels, and there was a statistically significant increase in PON1 activity. It was suggested that this effect of WPC is related to its antioxidant activity (Abdel-Wahhab *et al.,* 2016).

In the organism there are seen changes in cases of tissue damage, infection, trauma, inflammatory diseases and similar conditions. The response to these changes is called the acute phase. The changes occur in the concentrations of some proteins during the acute phase response. Total protein, albumin and globulin synthesized from the liver are also some of the acute phase proteins (Gruys *et al.,* 1995; Petersen *et al.,* 2004). In a study, lactoferrin was administered to rats (0.5 %) through drinking water for 21 days. In the blood samples taken at the end of the first and third weeks, no significant difference was observed in the levels of total protein, albumin, alpha and beta globulin in the lactoferrin group compared with the control group (Abdalla and El-Boshy, 2013). This study shows similar results compared to the data we have obtained in our study. In another study, it was observed that there was no significant difference in total protein, α-and β-globulin levels in oral drinking water administration of lactoferrin (0.5%), but a significant increase ($P<0.001$) in albumin levels in rats (Mohamed, 2014). Similarly, in this study, it was found that serum albumin, total protein and globulin levels in rats did not change through lactoferrin administration compared to control. The data obtained are similar to the results of the above studies. In a study conducted on, over 65 years of age, 28 male and 28 female patients diagnosed with Alzheimer's disease, 250 mg capsule daily lactoferrin administration for three months significantly decreased MDA and NO levels, and increased GSH and TAC levels compared to the control. It is concluded that lactoferrin has a protective effect against free radical damage in Alzheimer's disease [Mohamed *et al.,* 2019]. In the same way, it was found that there was a significant increase in the total antioxidant capacity level and a significant decrease in the total oxidant capacity level. In a study conducted in patients with iron deficiency anemia, changes in PON activity were investigated in patients treated with elemental iron therapy of 5-6 mg / kg orally three times a day for 3 months. It was reported that there was a significant decrease ($P<0.05$) in PON activity in the pre-treatment group compared to the control group in blood samples taken before starting the treatment. It was reported that there was a significant increase in PON activity in blood samples taken after 7 days and 3 months after treatment [Koc *et al.,* 2012]. Similarly, in another study, patients received oral ferrous sulfate (Fe^{2+}) treatment. Iron sulfate tablet (195 mg elemental iron daily) was given to the patients three times a day. In patients who could not tolerate oral iron therapy, iron sucrose (200 mg/day for two consecutive days) was administered intravenously after the iron deficiency was calculated. Treatment was continued until the ferritin was >20 µg/L level, hemoglobin level was 12 g/dL in women and 13 g/dL in men. PON activities were investigated in blood samples taken before and after treatment. It was reported that PON activity was significantly lower ($P<0.05$) in pre-treatment patients compared to the control group, and there was a significant increase ($P<0.05$) in patients after treatment compared to pre-treatment patients. It was suggested that the hemoglobin and ferritin levels of the patients after the treatment were at the same level as the control group, but the increase in the PON activity of the patients did not reach the level of the control group and this situation may be related to the treatment process. They concluded that the PON enzyme is sensitive to iron deficiency, with positive and linear correlations between hematologic and iron status parameters by increasing PON activity of iron therapy (Okuturlar *et al.,* 2016). The increase in PON activity levels in these studies on iron deficiency anemia shows similar results to the data we obtained in our study. It was reported that iron as a cofactor affected the activity of some antioxidant enzymes such as SOD, glutathione S-transferases, and reduced GSH [Naithani *et al.,* 2006].

Safaeian and Zabolian investigated the effect of chronic lactoferrin administration on oxidative stress parameters and hypertension in dexamethasone application. They administered dexamethasone (30 µg/kg/day i.p.) and lactoferrin at different concentrations (30, 100, 300 mg/kg orally) to male Wistar rats for 14 days. It was determined that ROS production decreased and antioxidant capacity increasedthrough lactoferrin treatment (Safaeian and Zabolian, 2014). Thus, data presented showed that lactoferrin may play a role in its antihypertensive effect. Thus, it was concluded that lactoferrin is an antioxidant protein capable of increasing antioxidant capacity and decreasing ROS formation. The protective effects of LF were investigated in rats with sepsis-induced acute lung injury. LF treatment reduced the wet/dry ratio of lung tissue by 30.7% and 61.3%, and lipid peroxidation by 22.3% and 67%, respectively, at concentrations of 100 and 200 mg/kg. SOD, GSH, glutathione peroxidase and catalase were reported to increase by more than 50% under treatment with 200 mg/kg LF (Han *et al.,* 2019). Gulmez et al. applied lactoferrin in the experimental endotoxemia model induced by lipopolysaccharide (LPS) in rats. In this study, it was stated that liver NO levels increased significantly in the LPS group compared to other groups ($P<0.001$) and control, LF and LF+LPS groups showed statistical similarity. It was reported that liver ADA activity levels significantly

increased in the LPS group compared to other groups (P<0.001) and the ADA activity levels of the control, LF and LF+LPS groups were statistically similar. As a result, it was reported that LPS increased the ADA synthesis and NO release, and LF has an anti-inflammatory and immunosuppressive function in stimulating the immune response (Gulmez *et al.,* 2020).

CONCLUSIONS

It has been reported that interventions involving the use of powerful antioxidants from different sources can also increase PON activity. The acute lactoferrin administration increased PON activity and decreased reactive oxygen species. Although the antioxidant system-inducing property of PON is known, more studies are needed to explain how it does this and by which mechanism lactoferrin exerts its antioxidant effect.

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Conflicts of Interest:

The authors declare that they do not have any conflict of interest

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