Original Communication

Bovine lactoferrin ameliorates antioxidant esterase activity and 8-isoprostane levels in high-cholesterol-diet fed rats

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Abstract: The main aim of the present study was to show the effect of bovineLactoferrin (bLF), an 80 kD iron-binding glycoprotein, its application on antioxidant esterase activities and 8-isoprostane changes in high-cholesterol-diet fed (HCD-Fed) rats. The 44 adult Sprague-Dawley male rats were randomly assigned into four experimental groups. They were randomly assigned into four equivalent groups (n = 11). The groups included the control group which was fed with normal diet, bLF group, the third group which were made hypercholesterolemia by being fed with high cholesterol diet, and the last group which consisted of hypercholesterolemia rats treated with bLF (HCD + bLF) for 4 weeks (200 $mg.kg^{-1}$ per day wt. dissolved in 0.9% normal saline).After 4 weeks, the serum Paraoxonase1 (PON1), Arylesterase (ARE) activity and 8-isoprostane with lipid profile were measured. Upon treatment with the bLF, the decrease in LDL-Cholesterol (LDL-C), Glucoses, Triglyceride (TG) and Total-Cholesterol (TC) levels and an increase in HDL-Cholesterol (HDL-C) level were observed. The co-administration of bLf for 4 weeks had decreased the 8-isoprostane levels significantly ($P < 0.001$) (86.36 ± 7.1 vs 117.18 ± 8.62) when compared to hypercholesterolemia-induced rats. Also, the Atherogenic Index (AI) in HCD + bLF group showed a significant decrease as compared to the HCD group ($P < 0.001$) (0.37 ± 0.07 vs 0.57 ± 0.09). The results indicated that bLF was effective against oxidative stress by its ability to increase PON1 activity and reduce the lipid peroxidation in high-cholesterol-fed rats.

Keywords: Bovine lactoferrin, Hyperlipidemia, Antioxidant enzymes, Lipid peroxidation

Introduction

Cardiovascular disease is the most common cause of mortality around the world and especially in developing countries [1]. Atherosclerosis is characterized by the accumulation of lipids in the wall of arteries. According to the epidemiological studies, the Hyperlipidemia is characterized by hypercholesterolemia and high concentration of LDL-cholesterol (LDL-C) which are important denominators and major factors in atherosclerosis occurrence and development [2]. Atherosclerosis is a chronic inflammatory disease associated with lipid metabolism disorder. Excessive production of inflammatory marker and oxidative stress could mediate chronic disease which is correlated with artery walls damage and the development of atherosclerotic lesions [3]. Hyperlipidemia increases monocyte chemotactic movement into the intima. In the case of this process, chronic inflammation is created which

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results in atherosclerotic plaque. The formation of these plaques is an important prognosis for the occurrence of cardiovascular diseases [4].

Excessive production of reactive oxygen species (ROS) or imbalance between oxidative and anti-oxidative are known as oxidative damage and are involved in disease progression. Excessive ROS in vivo causes oxidation of lipoproteins and cell membrane phospholipids and is in turn associated with pro-inflammatory state and cellular damage [5]. Change in the cell membrane fluidity by lipid peroxidation reduces the capacity to maintain an equilibrated gradient of concentration, resulting in increase in membrane permeability and inflammation [6]. Increased production of ROS during Hypercholesterolemia causes endothelial cell injury and contributes with up-regulation of some inflammatory factors such as Nuclear factor-κB (NF-κB), interleukin-6, and Tumor Necrosis Factor alpha (TNF-α). Studies have shown that Oxidized LDL-Chas a critical role in the pathogenesis of atherosclerosis by inducing the endothelial dysfunction. So, inhibition of oxidative stress or increase in antioxidant capacity as well as reduction in cholesterol levels might be an important step by affecting atherosclerosis progression and development [7].

Now days the investigations have mainly focused on vitamin- and mineral-binding proteins, on antimicrobial, immunosuppressing/-modulatory proteins, and on proteins with enzyme inhibitory activity as well as on hormones and growth factors from different food proteins; most research has been performed on milk proteins [8]. Lactoferrin (bLF), an immunological 80 KD iron-binding glycoprotein is a member of transferrin family present in "Whey" protein fraction of milk. bLF has immunological activity which is secreted by the epithelial cells and neutrophils [8, 9]. bLF receptors on the surface of activated lymphocytes have an inhibitory role in the production of pro-inflammatory cytokines such as NF-κB, TNF-α, and interleukin-10. In fact, LF is a link between the innate, and acquired immune system [10]. Studies have shown important biological activity of LF including anti-inflammatory, antibacterial, antifungal, anti-cancer and antioxidant activities in many biological and chemical environments [11]. High affinity and reversible binding of bLF to the ferric ion could prevent the production of free radicals such as OH° radicals during the Fenton reaction, and thereby prevents the oxidation of lipoproteins [12]. Studies have revealed the increased activity of catalase, glutathione peroxidase, superoxide dismutase and total antioxidant capacity following bLF administration [11]. Serum Paraoxonase 1 (PON1), lipophilic enzyme is a calcium-dependent esterase which was identified as a component of HDL during electrophoresis in 1961. PON1 improves the antioxidant activity of HDL which is attributed with prevention of lipid peroxidation [13]. The studies have shown a significant reduction in PON1 activity in diabetics and cardiovascular diseases [14].

The main aim of this study is to evaluate the effect of bLF on 8-isoprostane levels as an exclusive lipid peroxidation biomarker in an experimental hypercholesterolemia condition in male rats. We also measured the antioxidant activity of PON1 and Arylesterase (ARE) with 8-isoprostane for better assessment of antioxidant/oxidative stress status in male rats.

Material and Methods

Animal treatment

This study was carried out on 44 adult male Sprague-Dawley rats weighing average 180-250 g. Rats were kept in laboratory condition at room temperature of 25 °C, 48 ± 5% humidity rate, and 12-h light-dark cycle in special cages which have plastic bottoms and wire tops. They were fed with the same type of baits without nutritional limitations. All experimental procedures were approved by the ethical committee of Tabriz University of Medical Sciences (Department of Medicine). Also, all procedures and techniques were conducted according to the CPCSEA guidelines for the use and care of experimental animals.

Experimental design

Rats were randomly assigned into foure quivalent groups (n = 11) including the control group which was fed with normal diet and bLF group.Third group rats were made hypercholesterolemia by feeding them with high cholesterol diet containing 1% cholesterol, 0.05% cholic acid and 5% lard for four weeks. The last group included hypercholesterolemia rats treated with bLF (HCD + bLF); (Morinage Milk Industry, Tokyo, Japan) for four weeks (200 $mg.kg^{-1}$ per day wt. dissolved in 0.9% normal saline). Dosage of lactoferrin was selected based on published reports of the absence of side effects of the lactoferrin values between 0 and 200 $mg.kg^{-1}$ per day [15]. At the end of the experimental period, all of the rats were weighed, their blood was drawn, and serums were stored at −70 °C for further studies.

Assessment of PON1 and ARE activity

The serum PON1 activity was measured using paraoxon substrate (diethyl- p-nitrophenyl phosphate; Sigma Chemical Co) and measured by increases in the absorbance at 412 nm due to the formation of 4-nitrophenol. Briefly, the

activity was measured at 25 °C by adding 30 µl serum to Tris/HCl (100 mmol, pH: 8.0) buffer containing 2 mmol paraoxon and 2 mmol calcium chloride. The rate of 4-nitrophenol generating was determined at 412 nm with a spectrophotometer (Techcomp 8500 II UV/VIS, China). PON1 activity was defined as 1 µmole of p-nitrophenol formed per minute and was calculated using the molar extinction coefficient 17,100 M^{-1} cm^{-1} and the enzyme activity was expressed in U/L. The Arylesterase activity was also evaluated using phenyl acetate (Sigma Co, London, UK) as substrate in 3 ml assay mixture (100 mMTris-HCl, 2.0 mM CaCl2, 4.0 mM phenylacetate, pH 8.0). The hydrolysis rate of the substrate was determined using the photospectrometry method at wavelength of 270 nm. The results were reported as U/l. A blank sample containing the assay buffer without sample was run [16].

Determination of lipid profile and 8-isoprostane levels

8-isoprostane levels were also determined using the ELISA kit (Cayman Chemical Ann Arbor, MI) as instructed by the manufacturer. Determination of 8-isoprostane levels in each sample was conducted according to the kit protocol, using the mouse anti-rabbit IgG coated plate provided in the kit and standard curve of 8-isoprostane. The serum glucose, HDL-Cholesterol (HDL-C), LDL-Cholesterol (LDL-C), Triglyceride (TG) and Total Cholesterol (TC) levels were measured directly with standard laboratory techniques using commercially available assay kits (Biosystem, Barcelona, Spain). The AtherogenicIndex (AI) was calculated as follows: AI = log (TG / HDL-cholesterol)

Statistical analysis

The results were expressed as means ± SD. Variance analysis was performed by one way ANOVA. Student's unpaired T-test followed by Tukey test were also applied to assess the significance of differences between groups (if homogeneity of variables was assumed). A value of $P < 0.05$ was considered as statistically significant. All statistical analyses were conducted using SPSS v.18.0software for Windows (SPSS, Chicago, IL, USA).

Results

Effect of bLF on serum lipid profile and AtherogenicIndex

Table I shows the beneficial effects of bLF treatment on PON 1 and ARE enzyme activity, serum lipid profile, and glucose levels in experimental animals. The TC, glucose, TG and LDL-C with final weight were markedly increased in HCD group as compared with the control group ($P < 0.001$). However, the treatment with bLF significantly alleviated TC,Glucose, TG and LDL-C levels as compared with the HCD rats ($p < 0.001$).Also, rats' body weight was decreased at the end of experiment in the treatment group as compared with the HCD rats. HDL-C levels showed increase in bLF group as compared with the control group; although it was not statistically significant. Lower level of LDL-C concentration was observed by bLF supplementation in HCD group as compared with HCD rats ($p < 0.001$). Also, the same results were observed for TG and glucose levels ($p < 0.001$). Significantly elevated level in

Table I. The Bovine lactoferrin effects on PON 1 and ARE activities, lipid profile and AI in male rats*(N = 11).

Parameter	Control	bLF	HCD	HCD + bLF
Final Weight (g)	346.36 ± 26.93	356.72 ± 13.10	547.81 ± 25.19[a]	461.09 ± 25.57[ac]
PON (U/l)	126.9 ± 10.59	136.63 ± 7.91	86.36 ± 7.1[a]	117.18 ± 8.62[bc]
ARE (U/l)	97.45 ± 6.69	112.9 ± 5.12[b]	69.36 ± 7.47[a]	90.36 ± 6.05[bc]
Glucose (mg/dl)	82.36 ± 11.42	83.90 ± 7.48	123.45 ± 9.18[a]	105.54 ± 9.71[ac]
Triglyceride (mg/dl)	72.72 ± 8.69	68.72 ± 8.56	114 ± 9.08[a]	91.90 ± 9.87[ac]
Total-Cholesterol (mg/dl)	61.45 ± 8.69	53.9 ± 6.96	141.36 ± 9.03[a]	103.27 ± 10.29[ac]
LDL-Cholesterol (mg/dl)	19.55 ± 2.94	16.91 ± 2.11	89.73 ± 10.27[a]	50.09 ± 6.51[ac]
HDL-Cholesterol (mg/dl)	28.09 ± 4.57	34.55 ± 3.5	30.73 ± 6.63	39 ± 8.03[ad]
VLDL-C	14.4 ± 1.73	13.6 ± 1.7	22.8 ± 1.8	18.2 ± 2
AI**	0.41 ± 0.09	0.29 ± 0.07[b]	0.57 ± 0.09[a]	0.37 ± 0.07[c]

PON: Paraoxonase; ARE: Arylesterase; AI: Atherogenic Index.
Statistical significance: a, $p < 0.001$; b, $p < 0.05$; c, $p < 0.001$; d, $p < 0.05$ (Mean ± sd). (a& b compare groups with control rats; c &d compare groups with High-Cholesterol-Diet (HCD) group)
* Experimental groups were fed diets supplemented with 0 mg (control), 1% cholesterol (HCD) and hypercholesterolemia rats treated with 200 $mg.kg^{-1}$/day Bovine lactoferrin (HCD + bLF)
** AI = log (triglyceride / HDL-cholesterol).

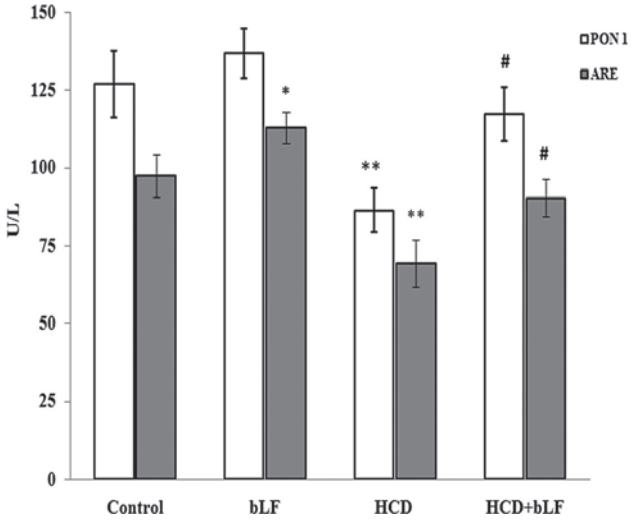

Figure 1. The effect of bLF (Bovine lactoferrin) on levels of Paraoxonase1 (PON1) and Arylesterase (ARE)activity affected by high-cholesterol-diet (HCD) and/or bovine lactoferrin (bLF) in the study groups. Data are expressed as mean ± sd. *P < 0.05, **P < 0.001 vs. control; #P < 0.001 vs. HCD (N = 11 in all groups of study)

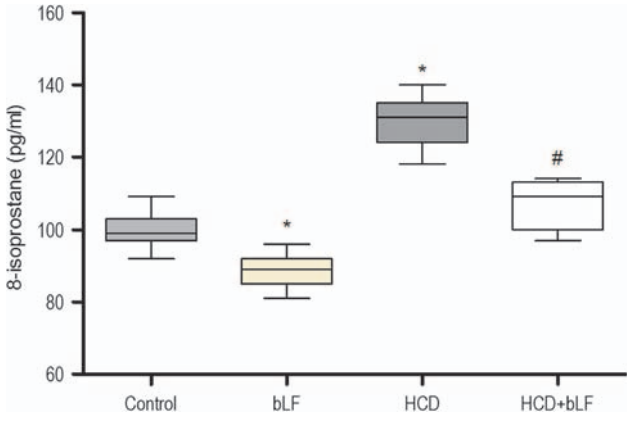

Figure 2. 8-isoprostane levels in experimental rats affected by high-cholesterol-diet (HCD) and/or bovine lactoferrin (bLF) in the study groups. Levels of 8-isoprostane in HCD group (129.54 ± 6.9 pg/ml) were significantly higher than those in the other study groups (control, 99.81 ± 4.7; HCD + bLF, 106.81 ± 6.6; bLF, 86.81 ± 4.55; pg/ml). Data are expressed as mean ± sd. *P < 0.001 vs. control; #P < 0.001 vs. HCD (N = 11 in all groups of study)

the Atherogenic Index in rats fed with high-cholesterol diet ($p < 0.001$) was observed compared with the control group. Co-administration of bLF along with HCD significantly improved the Atherogenic Index as compared with HCD rats ($p < 0.001$).Interestingly, bLF treatment shows a significant decrease in Atherogenic Index as compared with control rats ($p < 0.05$).

Effect of bLF on activities of PON1 and ARE enzymes with8-isoprostane levels

Comparing with the control group, the PON1 and ARE activity was significantly decreased in the HCD group ($P < 0.001$).The treatment of high-cholesterol diet fed rats with bLF increased the PON1 and ARE activity as compared with the HCD group ($P < 0.001$); (Figure 1).Also, a significant increase in ARE activity in bLF rats was observed compared with the control group ($P < 0.05$); (Table I). Figure 2 shows the bLF effect on lipid peroxidation in terms of 8-isoprostanein HCD rats. Upon bLF, feeding significantly decreased the 8-isoprostane levels compared to HCD group ($P < 0.001$); (Figure 2).

Discussion

Atherosclerosis resulting from hyperlipidemia and lipid abnormalities has increased the epidemic of overweight and metabolic syndrome in the worldwide. The elevation of serum TC and alteration of lipid parameters with an accumulation of free radicals have been implicated as the main causes of cardiovascular diseases [7, 17]. Oxidative stress plays an important role in the pathogenesis of hypercholesterolemia atherogenesis [18]. PON1 as a lipophilic antioxidant enzyme has a protective role in preventing the increase of ROS and specially lipid peroxidation [19]. In recent years, drug treatment or diet therapies have been more focused due to their important role in preventing diseases associated with cardiovascular diseases and oxidative stress [20, 21]. In this study, we studied the cardiovascular protective effect of bLF by reduction in the level of TC, glucose, TG, LDL-C and increase in HDL-cholesterol in hypercholesteremia male rats. Both antioxidant activity of PON1 and ARE were shown increased in the present study by bLF treatment. Also, we observed the lower level of 8-isoprostane levels in bLF rats.

Studies have demonstrated that elevated TC, TG and LDL-C have been implicated as a primary risk factor for cardiovascular diseases. AtherogenicIndex (AI) is a powerful indicator of cardiovascular diseases risk. However, administration of bLF, reduced the AI, which is commonly used as an index of risk for Coronary Heart Disease (CHD); mainly by increasing the HDL-C level rather than reducing the TC level. Researchers have shown that HDL promotes the reverse cholesterol transport pathway, in which, HDL induces efflux of excess accumulated cellular cholesterol and prevents the generation of an oxidative modified LDL [22]. Badimon et al, stated that HDL administration not only inhibits the progression of atherosclerosis, but also reduces atherosclerotic lesions effectively [23]. Nakamura et al. showed that lactoferrin interrelates with bile acids and enhance fecal cholesterol excretion in rats. They previously

reported that enteric-LF prevented hypercholesterolemia and atherosclerosis in a diet-induced atherosclerosis model using Microminipig. In accordance of our study, the results of studies showed benefit effect of lactoferrin in protection of atherosclerosis [24]

Thus, our results suggest that bLF may have a beneficial effect by promoting efflux of cholesterol accumulated in cells due to increase in the serum concentrations of HDL.

Changes in lipid composition and oxidative stress cause lipid peroxidation in hypercholestermia condition [25]. PON1 enzyme has the ability to protect HDL and LDL from oxidation. It is believed that the enzymes related to HDL prevent lipoproteins from oxidative modifications [26]. In the present study, we found that co-administration of bLF along with HCD significantly increases the PON1 and ARE enzymes activity which was associated with a reduction in 8-isoprostane concentration. Increase in enzyme activity of PON1 could be related to the reduction of lipid peroxidation. Lipid peroxidation has been reported to inhibition of PON1 activity as an index of ROS metabolite [27].

Studies have demonstrated that human LF has an ability to inhibit lipid peroxidation by iron-binding capacity and has preventative mechanism toward hydroxyl radical formation via the fenton reaction [28–30]. However, the study with bLF monotherapy in patients with hepatitis C showed that bLF decreases the 8-isoprostane without influencing on iron level [31]. Wang and co-workers examined the effect of dietary bLF on antioxidant and performance status in piglet. They found that treatment with bLF has improved activity and mRNA levels of antioxidant and indicated the exogenous antioxidant activity of bLF [32]. Mulder et al, reported the potential immune modulating properties and antioxidant activity of an oral supplementation of bLF in humans [33]. Similarly, Konishi et al, showed that bLF administration in patients with chronic hepatitis C was associated with improvement in lipid peroxidation and ALT levels. They demonstrated that therapy with bLF is a promising therapeutic approach for suppressing oxidative stress in non-responders to antiviral therapy [31]. Takeuchi et al, demonstrated that bLF has a beneficial effect on plasma total cholesterol and Triacylglycerol concentrations. They showed that adding bLF to the standard commercial diet of animals retardsthe hepatic lipid accumulation andsuppresses the intestinal absorption of lipids in a chronic treatment with bLF [34]. Also, Li et al, indicated that bLF protects against metabolic syndrome which is induced by high-fructose corn syrup in male mice. They reported the reduction in interleukin-1b, interleukin-6, Tumor Necrosis Factor-α (TNF-α), monocyte chemotactic protein-1, Adiponectinand a reduction in serum alanine aminotransferase. Furthermore, they demonstrated that bLF decreases lipid peroxidation by reducing serum and hepatic TG, and prevents the lipid accumulation in the liver [35]. The enhanced activity of PON1 is attributed to the free radical scavenging that induced lipid peroxidation such as 8-isoprostane and LDL oxidation [36, 37].

In conclusion, our results proposed the beneficial effects in attenuating the atherosclerotic process through increase in PON1 and ARE antioxidative activity, serum HDL-C and decrease in serum TC, LDL-C, TG levels in hypocholesterolemic male rats. Therefore, using supplements that prevent lipid peroxidation and enhance PON1 activity should be considered.

Our team attempted to conduct a well-designed study by considering all the parameters which are the strength of the study; however, some limitations of the present study are lack of studying about gene expressions which are associated with lipid profile alteration and reverse cholesterol transport pathway. Another limitation of this study is the use of single doses of bLF in current protocol. Further studies are warranted to investigate the exact mechanism action of bLF effect in hypercholesterolemia condition in male rats.

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