

Cytoprotective and antioxidant effects of human lactoferrin against H₂O₂-induced oxidative stress in human umbilical vein endothelial cells

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Abstract

Background: Lactoferrin (LF) is an iron-binding glycoprotein with antioxidant, anti-inflammatory and nitric oxide-dependent vasodilatory properties. In the present study, we investigated the protective and antioxidant effects of LF on H₂O₂-induced oxidative stress in human umbilical vein endothelial cells (HUVECs).

Materials and Methods: HUVECs were pretreated by (6.25–100 µg/ml) LF for 24 h and then exposed to 0.5 mM H₂O₂ for 2 h. Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The intra- and extra-cellular hydroperoxides concentration and ferric reducing antioxidant power (FRAP) were determined in pretreated cells.

Results: Pretreatment of HUVECs with LF at the concentrations of 25–100 µg/ml significantly reduced the cytotoxicity of H₂O₂ in a concentration-dependent manner using MTT assay. LF pretreatment at different concentration ranges also decreased the hydroperoxides level and augmented the FRAP value in both intra- and extra-cellular assay.

Conclusion: These findings revealed antioxidant and cytoprotective effects of LF against H₂O₂-induced oxidative stress in HUVECs. With regard to the beneficial vascular activity of LF, further investigations are suggested for understanding its clinical value in human endothelial dysfunction and prevention and/or treatment of CVDs.

Key Words: Antioxidant, human umbilical vein endothelial cells, lactoferrin, oxidative stress

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INTRODUCTION

Cardiovascular diseases (CVDs) include any disease

that affects the cardiovascular system such as heart, brain, kidney and other organ's blood vessels. CVDs are responsible for significant morbidity and mortality in the world.^[1] According to the World Health Organization global report on noncommunicable diseases, 17.3 million people died from CVDs in 2008 that representing 30% of all global deaths.^[2] Furthermore, it is estimated that mortality rate from heart disease and stroke will increase to 23.3 million by 2030.^[3]

The vascular endothelium is the inner layer of blood vessels which has an important role in the

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regulation of vascular function.^[4] A growing body of evidence suggests the great role of oxidative stress in the endothelial dysfunction. Risk factors including hypertension, hypercholesterolemia, diabetes, and cigarette smoking are associated with increased oxidative stress and alteration in the endothelial function.^[5] Reactive oxygen species (ROS) including superoxide anion, lipid radicals, hydroxyl radical, peroxynitrite and hydrogen peroxide released from various sources are involved in the inactivation of nitric oxide (NO), disruption of NO function and reduction of antioxidant capacity in the vascular system.^[5,6] Oxidative stress has a causal role in the oxidation of low density lipoprotein (LDL) and in the development of atherosclerosis,^[7] and also in the induction of apoptosis of endothelial and myocardial cells following some stimulus such as hypoxia, ischemia, reperfusion and inflammation.^[8]

Recent studies have shown some beneficial effects from antioxidant supplementation in the improvement of NO availability and endothelial function.^[9,10]

Lactoferrin (LF), an iron-binding glycoprotein, was first isolated from the bovine milk by Sorensen in 1939.^[11] This glycoprotein exists in different biological fluids and in specific granules of neutrophils. In human, LF is found in milk, colostrum, and other mucosal secretions.^[12] LF is a critical component in mediation of first-line defense against infections and acts as a regulator of organ morphogenesis and promoter of wound healing and bone growth. It has various physiological and pharmacological activities including antioxidant, anti-infective, anti-inflammatory and anticancer effects.^[12-14] LF also possesses beneficial cardiovascular properties such as antihypertensive activity.^[15,16]

The present study aimed to evaluate the protective effects of human LF under oxidative stress induced by H₂O₂ in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Reagents

Human umbilical vein endothelial cells were obtained from the National Cell bank of Iran (Pasteur Institute Tehran, Iran). Human LF was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL Life Technologies (Grand Island, USA). Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assessment kit was purchased from Bioidea Company (Tehran, Iran). The kits for hydroperoxides measurement and

ferric reducing antioxidant power (FRAP) assay was obtained from Hakiman Shargh Research Co., (Isfahan, Iran). All other chemicals with analytical grade were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA).

Cell culture

The HUVECs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in a 95% humidified atmosphere of 5% CO₂ at 37°C.

Cell viability evaluation

Cell viability was assessed using MTT method based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazan product.^[17] Briefly, the cell suspension at a concentration of 1 × 10⁵ cells/ml was transferred to 96-well plates and incubated for 24 h at 37°C. At the optimum phase of proliferation, the cells were treated with different concentrations of human LF (6.25, 12.5, 25, 50, 100 µg/ml) for an additional 24 h.^[18] After that the medium of each well was removed, and the cells were washed out with phosphate buffered saline (PBS) at pH 7.4. Then, a new medium and 0.5 mM H₂O₂ were added to the wells.^[19] After 2 h incubation, medium of each well was removed and the cells were washed out with PBS, and 20 µl MTT (0.5 mg/ml) and 50 µl of new medium were added into the each well and incubated for 3 h at 37°C. Then the MTT-formazan product dissolved in 50 µl of dimethyl sulfoxide and absorbance was measured at 570 nm by microplate reader (BioTek Instruments, PowerWave XS, Wincoski, USA). The wells containing the cells without being exposed to the LF or H₂O₂ were considered as control. Cell viability was determined as a percentage of viable cells of treated samples to control samples, and each experiment was tested in triplicate.

Measurement of extra-and intra-cellular hydroperoxides concentration

The effects of LF on intra-and extra-cellular hydroperoxides level were measured based on ferrous ion oxidation by xylenol orange reagent 1 (FOX-1).^[20] The FOX-1 reagent containing ammonium ferric sulfate in an aqueous medium with sorbitol was prepared according to the manufacturer's protocol. After pretreatment of HUVECs with LF, the cells were exposed to the H₂O₂. Then, 10 µl of supernatant of the cells or the cell lysates from each well was added to 190 µl of reagent and incubated for 30 min at 40°C. Absorbance was determined at 540 nm against the blank using a microplate reader/spectrophotometer. The hydroperoxides content of samples were subsequently calculated using a standard curve of H₂O₂ concentrations (0.005–1M).

Measurement of cell-free and intra-and extra-cellular ferric reducing antioxidant power

The effect of LF on total antioxidant capacity of the samples was determined by the evaluation of FRAP.^[21] FRAP value was measured based on the reduction of ferric-tripyridyltriazine complex to the ferrous form by colorimetric assay. The FRAP reagent containing tripyridyltriazine/ferric chloride/acetate buffer was prepared based on the manufacturer's protocol. For each well, 10 μ l of sample was added to 200 μ l of FRAP reagent. Different concentrations of LF were evaluated for FRAP in cell-free assay. In cell-based assay, supernatant of the cells or the cell lysates from each well were analyzed. The mixture of sample and reagent was incubated for 40 min at 40°C. Then the absorbance was determined at 570 nm against the blank using a microplate reader/spectrophotometer. The FRAP values of samples were calculated using the standard curve, which was obtained from $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$ concentrations (0.1–10 mM) and were expressed as μM of FeII equivalents.

Statistical analysis

The results were represented as mean \pm standard error of the mean statistical analyzes were conducted using a one-way analysis of variance, followed by Tukey *post-hoc* test SPSS software version 16.0, (SPSS Ltd, Quarry Bay, Hong Kong). $P < 0.05$ were considered as significant.

RESULTS

Cytoprotective effect of lactoferrin against H_2O_2 -induced oxidative stress

Figure 1 shows the cytoprotective effect of LF on the viability of HUVECs, which were exposed to

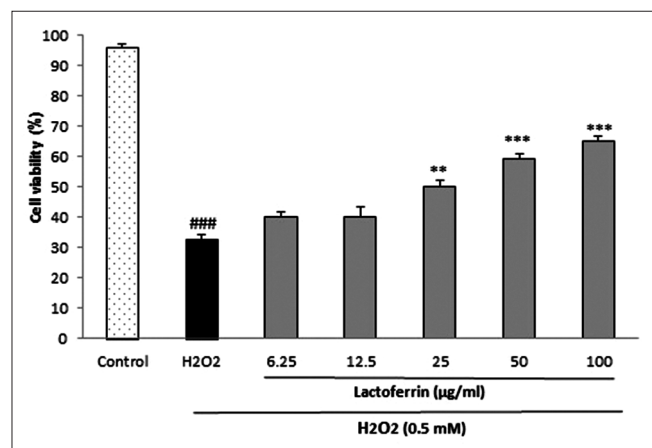


Figure 1: Cytoprotective effect of lactoferrin on H_2O_2 -induced oxidative stress in human umbilical vein endothelial cells. Cells were incubated with H_2O_2 (0.5 mM, 2 h) after pretreatment with different concentrations of lactoferrin (6.25–100 $\mu\text{g/ml}$). The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values are means \pm standard error of the mean from three independent experiments in triplicate. $###P < 0.001$ versus control (H_2O_2 -untreated cells), $**P < 0.01$ and $***P < 0.001$ versus H_2O_2 -stimulated cells

the oxidative damage induced by H_2O_2 using the MTT method. The exposure of HUVECs to H_2O_2 at 0.5 mM for 2 h caused a significant reduction in cell viability ($P < 0.001$). Pretreatment of HUVECs with LF at the concentrations of 25–100 $\mu\text{g/ml}$ reduced the cell death resulted from H_2O_2 in a concentration-dependent manner. The cytoprotective effect was not observed at the concentrations of 6.25 and 12.5 $\mu\text{g/ml}$ of LF.

Effect of lactoferrin on intra-and extra-cellular hydroperoxides concentration

Figures 2 and 3 are shown the effects of LF on intra-and extra-cellular hydroperoxides concentration in HUVECs culture, which was exposed to the oxidative stress induced by H_2O_2 . After pretreatment of HUVECs with LF (6.25–100 $\mu\text{g/ml}$), the intra-cellular hydroperoxides level were significantly declined compared to the control group. LF pretreatment also significantly reduced the extra-cellular hydroperoxides level at the concentrations of 6.25–100 $\mu\text{g/ml}$. Increasing the concentration of LF concentration-dependently prevented the increase in hydroperoxides level.

Effect of lactoferrin on cell-free and intra-and extra-cellular ferric reducing antioxidant power value

The FRAP value of LF was evaluated in cell-free and in intra-and extra-cellular fluids. In cell-free assay, our data showed increasing trend in FRAP with increasing LF concentrations [Figure 4]. In cell-based assay, LF at concentrations of 6.25–100 $\mu\text{g/ml}$ significantly increased the FRAP levels in intra-cellular fluid [Figure 5] and at the concentrations of 12.5–100 $\mu\text{g/ml}$ in extra-cellular fluid [Figure 6] concentration-dependently.

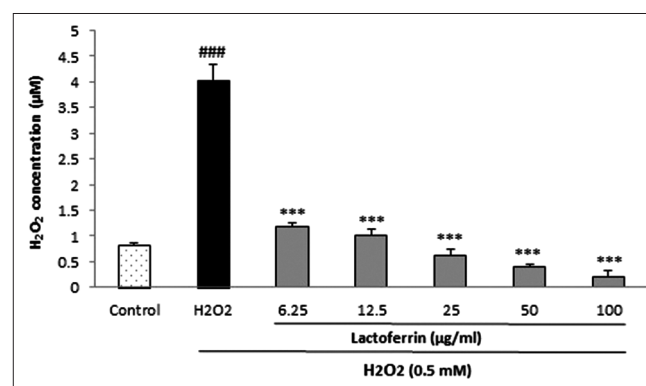


Figure 2: Effect of lactoferrin on intra-cellular hydroperoxides concentration in human umbilical vein endothelial cells. Cells were incubated with H_2O_2 (0.5 mM, 2 h) after pretreatment with different concentrations of lactoferrin (6.25–100 $\mu\text{g/ml}$). The hydroperoxides concentration was determined by ferrous ion oxidation by xylenol orange method. Values are means \pm standard error of the mean from three independent experiments in triplicate. $###P < 0.001$ versus control (H_2O_2 -untreated cells), and $***P < 0.001$ versus H_2O_2 -stimulated cells

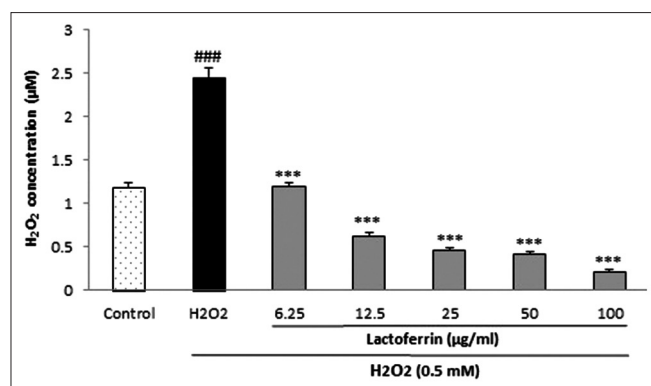


Figure 3: Effect of lactoferrin on extra-cellular hydroperoxides concentration in human umbilical vein endothelial cells. Cells were incubated with H₂O₂ (0.5 mM, 2 h) after pretreatment with different concentrations of lactoferrin (6.25–100 µg/ml). The hydroperoxides concentration was determined by ferrous ion oxidation by xylenol orange method. Values are means ± standard error of the mean from three independent experiments in triplicate. ###*P* < 0.001 versus control (H₂O₂-untreated cells), and ****P* < 0.001 versus H₂O₂-stimulated cells

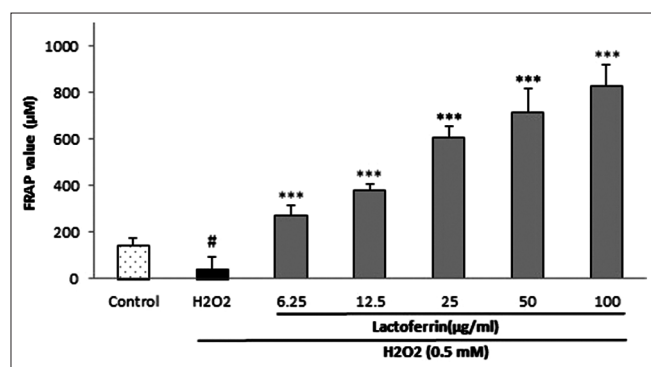


Figure 5: Effect of lactoferrin on intra-cellular ferric reducing antioxidant power value in human umbilical vein endothelial cells. Cells were incubated with H₂O₂ (0.5 mM, 2 h) after pretreatment with different concentrations of lactoferrin (6.25–100 µg/ml). Values are means ± standard error of the mean from three independent experiments in triplicate. ##*P* < 0.05 versus control (untreated cells), and ****P* < 0.001 versus H₂O₂ stimulated cells

DISCUSSION

In the present study, our findings revealed cytoprotective effect of LF at the concentration range of 25–100 µg/ml against oxidative stress induced by H₂O₂ in HUVECs. It also decreased hydroperoxides concentration and increased FRAP value in both intra-and extra-cellular fluid at different concentration ranges.

Associations between oxidative stress and endothelial dysfunction have been confirmed in numerous studies. Oxidative stress has a crucial role in the impairment of endothelium-dependent vasodilation and also in the induction of hypertrophy, apoptosis and inflammation via activation of many signaling pathways. Excessive ROS result in damage to the endothelial cells through

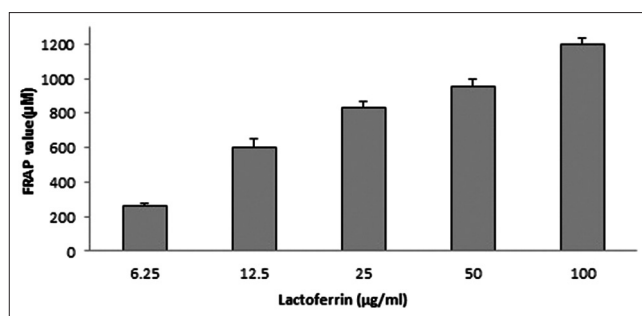


Figure 4: Concentration-dependent ferric reducing antioxidant power values of different concentrations of lactoferrin (6.25–100 µg/ml). Values are means ± standard error of the mean from three independent experiments in triplicate

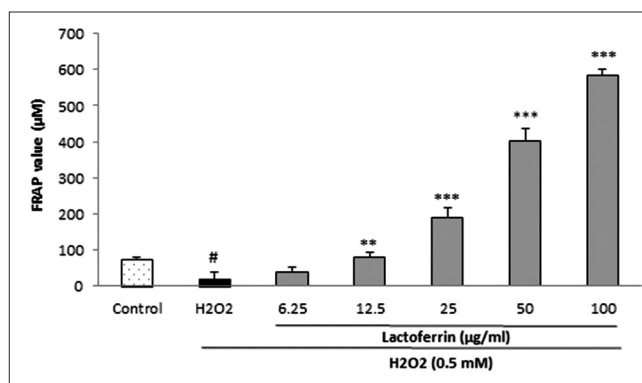


Figure 6: Effect of *Echinum amoenum* extract on extra-cellular ferric reducing antioxidant power value in human umbilical vein endothelial cells. Cells were incubated with H₂O₂ (0.5 mM, 2 h) after pretreatment with different concentrations of *E. amoenum* extract (25–1000 µg/ml). Values are means ± standard error of the mean from three independent experiments in triplicate. ##*P* < 0.05 versus control (untreated cells), ***P* < 0.01 and ****P* < 0.001 versus H₂O₂ stimulated cells

lipid peroxidation, protein oxidation, and DNA fragmentation.^[22] ROS are produced by different oxidase enzymes, however, nicotinamide-adenine dinucleotide phosphate oxidase is a main producer of ROS in the vasculature.^[23] Besides inactivation of NO, ROS are associated with down-regulation of endothelial NO synthase (eNOS) through degradation of tetrahydrobiopterin (BH₄). BH₄ is an essential cofactor for eNOS and its deficiency results in eNOS uncoupling and production of ROS rather than NO.^[24]

In this research, pretreatment of HUVECs with LF (6.25–100 µg/ml) significantly reduced the intra-and extra-cellular hydroperoxides level by FOX-1 assay. FOX method is not specific only for H₂O₂, and also is able to detect other hydroperoxides (ROOH). FOX-1 assay is a sensitive method for ROOH estimation due to the consisting of sorbitol, which is a radical scavenger and increases the yield of ferric ion.^[25]

Our results also showed significant increasing in total antioxidant capacity in intra-and extra-cellular

fluids after pretreatment of HUVECs with LF. Decreased antioxidant capacity such as superoxide dismutase (SOD), glutathione peroxidase, catalase and Vitamins C and E has been revealed in CVDs. SOD has three enzymatic types including Cu/Zn SOD, Mn SOD, and extracellular SOD. It seems that extracellular SOD has a more important role in NO bioavailability in the vasculature and so in the prevention of atherosclerosis.^[26] Administration of Vitamin C has been able to restore impaired endothelium-dependent vasodilation in patients with CVDs.^[22]

Lactoferrin is a food-derived multifunctional glycoprotein whose receptors are found in various cell types including endothelial cells. Some of its receptors are involved in LF uptake. In the cerebral endothelial cells, transportation of LF occurs through a receptor-mediated process without any intra-endothelial degradation.^[27] In some capillary endothelial cells, LF may transport via a specific LDL receptor-related protein and mediate antioxidant effect.^[27]

The potent antioxidant effect of LF and its ability to increase antioxidant capacity have been described in some researches. In healthy humans, LF supplementation has been associated with an increase in the hydrophilic antioxidant capacity.^[28] LF has also shown antioxidant effect on erythrocytes through inhibition of lipid peroxidation and hemolysis.^[29] Declining intracellular levels of ROS induced by glucose oxidase has been observed following LF treatment suggesting its ability to reduce oxidative stress-induced apoptosis.^[12]

Lactoferrin possesses metal ions-binding capacity and, therefore, can exert a protective effect against iron-catalyzed hydroxyl radicals through Fenton reaction, which is an important source of ROS.^[30] Therefore, the LF antioxidant activity is most likely related to its iron scavenging ability and inhibition of iron-catalyzed formation of ROS.^[11,31]

Furthermore, the beneficial vascular properties of LF have been reported in different studies. LF possesses vasodilator activities through eNOS-dependent pathway, inhibition of angiotensin I-converting enzyme and inhibition of endothelin-converting enzyme.^[32-34] LF has been shown anti-inflammatory and protective effects on endothelial cells through chelating lipopolysaccharide, reducing the release of pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha, inhibition of expression of endothelial adhesion molecules and prevention of NF- κ B activation.^[35] It has been suggested that plasma LF level may

be useful as a predictor to endothelial dysfunction in some diseases. LF also has a helpful effect on lipid profile by increasing high-density lipoprotein-cholesterol.^[36]

CONCLUSION

The finding of this study revealed the antioxidant and protective effect of LF against H₂O₂ induced oxidative stress in HUVECs. With regard to beneficial vascular activity and safety, LF could be suggested for clinical trial studies for understanding its clinical value.

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