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Camel milk lactoferrin reduces the proliferation of colorectal cancer cells and exerts antioxidant and DNA damage inhibitory activities

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ABSTRACT

Lactoferrin (Lf), the main iron-binding protein of milk, has biological activities. We have evaluated the potential of camel milk lactoferrin for its ability to inhibit the proliferation of the colon cancer cell line, HCT-116, *in vitro*, DNA damage and its antioxidant activities for the first time. The antioxidant capacity of Lf was evaluated by different assays, including ferric-reducing/antioxidant power assay (FRAP), free radical-scavenging activity (DPPH), nitric oxide (NO) radical-scavenging assay, total antioxidant activity and DNA damage, compared with vitamin C and rutin.

1. Introduction

Lactoferrin is a mammalian cationic iron-binding glycoprotein belonging to the transferrin family, which was discovered 70 years ago, and isolated simultaneously from human and bovine milks in 1960. It is widely distributed in all biological fluids and is also expressed by immune cells, which release it under stimulation by pathogens. Lactoferrin is a multi-functional protein with many beneficial properties, which makes it a functional food for a number of product, commercial and clinical applications (Adlerova, Bartoskova, & Faldyna, 2008).

Lactoferrin is a glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron. The molecular structure and amino acid sequence of human lactoferrin were discovered in 1984. Lactoferrin was then classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin (Metz-Boutique et al., 1984).

The protective character of lactoferrin has been demonstrated, on numerous occasions, on chemically induced tumors in laboratory rodents. Lactoferrin has even been reported to inhibit the development of experimental metastases in mice (Bezault, Bhimani, Wiprovnick, & Furmanski, 1994; Wang, Iigo, Sato, Sekine, Adachi, & Tsuda, 2000; Wolf, Li, Taylor, & O'malley, 2003). Lactoferrin-mediated inhibition of tumor growth might be related to apoptosis of these cells, induced by the activation of the Fas signalling pathway. Nevertheless, the exact mechanism of this function has not been discovered so far (Fujita, Matsuda, Sekine, Iigo, & Tsuda, 2004).

Lactoferrin was thought to support cell proliferation due to its ability to transport iron into cells. However, lactoferrin has later been proven to act as a growth factor activator. The effect of lactoferrin alone on small intestine epithelial cells is more potent than that of the epidermal growth factor. Lactoferrin alone (without the presence of any other cytokines and factors) is able to stimulate the proliferation of endometrium stroma cells. Lactoferrin has also been identified as a transcription factor. It can penetrate a cell and activate the transcription of specific DNA sequences (Adlerova et al., 2008).

In this study, to evaluate camel milk lactoferrin as a novel antioxidant material for a pharmaceutical agent or a food additive, its stable radical-scavenging activity, inhibitory effect on the major inflammation-related reactive oxygen species (ROS) and reactive nitrogen species (RNS) including hydrogen peroxide (H_2O_2), superoxide (O_2^{-}), and nitric oxide ($NO^{\cdot}$), were elaborated. Furthermore, the inhibitory effect of camel milk lactoferrin on protein degradation, DNA strand cleavage and cellular damage from ROS attack, were determined to verify the specific ROS/RNS scavenging

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function. In addition, the ability of camel milk lactoferrin to inhibit cell proliferation of colon cancer cell line HCT-116 was evaluated.

2. Materials and methods

2.1. Materials

Camel milk lactoferrin was kindly donated by the Department of Food Science, College of Food and Agriculture, UAE University, Al Ain. It was isolated from commercially available camel milk in the market. Rutin, vitamin C, TPTZ, $FeCl_3$, acetate buffer, ferrous sulfate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside, sulfanilic acid, glacial acetic acid, naphthyl ethylene diamine dihydrochloride, sulphuric acid, sodium nitroprusside, sodium phosphate, ammonium molybdate, $FeSO_4$, H_2O_2, agarose, and pRK5 DNA were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Cell proliferation

Colon cancer cells HCT-116 were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Gibco, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2, 95% air, supplemented with 1% Penicillin–Streptomycin (100 U/ml) (Gibco, Germany) and 10% foetal bovine serum (Gibco, Germany). Cells were seeded at 105 cells/ml and exposed to lactoferrin (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml).

2.2.2. Crystal violet assay

The cultivation medium was removed gently from the test wells, and cells were fixed with 1 ml of 96% ethanol for 10 min. A volume of 1 ml of 0.05% crystal violet (CV) solution in 20% ethanol was added, and cells were allowed to stain for 30 min after staining. The extracellular dye was removed by rinsing the cell monolayers with tap water thoroughly. The remaining cell-attached dye was dissolved in 2 ml of 0.1% acetic acid solution in 50% ethanol, and the OD at 585 nm was recorded. The mean OD_{585} of the control cells exposed to test-compound-free culture medium was set to represent 100% of viability, and the results were expressed as the percentage of these controls (Mickuviene, Kirveliene, & Juodka, 2004).

2.2.3. Ferric-reducing/antioxidant power assay

The ferric-reducing/antioxidant power (FRAP) assay was conducted for rutin, vitamin C, and lactoferrin at concentrations of 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml according to the method of Benzie and Strain (1996) with slight modifications. The FRAP reagent included 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.3 M acetate buffer (pH 3.6) in proportions of 1:1:10 (v/v/v). 1 ml of each diluted solution from the tested sample was mixed with 2 ml of freshly prepared FRAP reagent, and the reaction mixtures were incubated at 37 °C for 30 min. Absorbance at 593 nm was determined against distilled water as a blank. Aqueous solutions of ferrous sulfate (0–100 μM) were used for calibration. Triplicate measurements were taken and the FRAP values were expressed as μmol of Fe(II).

2.2.4. DPPH-free radical-scavenging assay

Free radical-scavenging capacity for rutin, vitamin C and lactoferrin (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml) was also studied through the evaluation of the free radical-scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The determination was conducted based on the method proposed by De Ancos, Sgroppo, Plaza, and Cano (2002), with slight modifications. An aliquot (200 μl) of tested sample was mixed with 3.8 ml of 0.25 mM methanolic DPPH solution. The mixture was thoroughly vortex-mixed and kept in the dark for 30 min. After this, the absorbance was measured at 515 nm against methanol without DPPH as blank. Results were expressed as a percentage of inhibition of DPPH radical. Percentage of inhibition of the DPPH radical was calculated according to the following equation:

$$\%\ \text{inhibition of DPPH} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \qquad (1)$$

where Abs control is the absorbance of DPPH solution without the tested sample.

2.2.5. Nitric oxide radical scavenging assay

Nitric oxide radical inhibition was estimated using the Griess reaction. A 3 ml reaction mixture containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline, rutin (0.5 ml), vitamin C, or lactoferrin (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml) was incubated at 25 °C for 150 min. After this, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completion. Then, 1 ml of 0.1% naphthyl ethylene diamine dihydrochoride was added, mixed and allowed to stand for 30 min at 25 °C. The concentration of nitrite was assayed at 540 nm. The determination was conducted based on the method proposed by Sreejayan and Rao (1997). Percentage of inhibition of nitric oxide inhibition was calculated according to Eq. (1) as given above.

2.2.6. Total antioxidant activity

The total antioxidant activity for rutin, vitamin C and lactoferrin (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml) was investigated according to the method of Prieto, Pineda, and Aguilar (1999). Briefly, 0.1 ml samples having different concentrations were mixed with 0.3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solutions). The tubes were capped and the reaction mixtures were incubated for 90 min at 95 °C. The absorbance of the cooled mixture was measured at 695 nm against a blank sample. The blank contained the reagent solution and the solvent. The total antioxidant activity was expressed as the absorbance of the sample. A higher absorbance value indicated higher antioxidant activity.

2.2.7. DNA damage by free radical

The assay reaction was conducted in an Eppendorf tube at a volume of 12 μl containing 0.5 μg of pRK5 DNA in 3 μl of 50 mM PBS (pH 7.4), 3 μl of 2 mM $FeSO_4$ and 10 μl of lactoferrin (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml). Subsequently, 4 μl of 30% H_2O_2 was added, and the mixture was incubated at 37 °C for 30 min. At the end of the incubation, the sample was exposed to UV light for 5 min (Young, Je, Park, Kim, & Ahn, 2009). Samples were run on 0.8% agarose. Gel was stained with ethidium bromide and photographed and analysed using Doc-It software (Kumar & Chattopadhyay, 2007).

2.2.8. Statistical analysis

All analytical determinations were performed in triplicate. Statistical analysis was performed using SPSS for windows (version 19; SPSS Inc., Chicago, IL, USA). The data obtained were analysed using analysis of variances to determine the significance ($P < 0.05$) of the main effects. Values of different parameters are expressed as the mean ± standard deviation.

3. Results and discussion

Camel milk Lactoferrin is a multifunctional glycoprotein. Due to its anti-inflammatory and anti-cancer properties and the resulting therapeutic potential, lactoferrin is presently a focus for a variety of research areas. The regulation of cell growth represents one of the prominent performances of lactoferrin.

3.1. Proliferation of HCT-116 colon cancer cells

Lf was found to inhibit the proliferation of HCT-116 cells in a dose and time dependent manner at higher concentration (3 mg/ml and 5 mg/ml), among all concentrations studied. To grade the impact of this inhibitory effect, we enumerated it by Crystal violet assay (Fig. 1). Cells were incubated with Lf (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml and 5 mg/ml) for 24 and 48 h. The lower concentrations of Lf (0.1 mg/ml, 0.5 mg/ml and 1 mg/ml) failed to inhibit the proliferation rate of HCT-116 cells at any of the time points investigated. However, Lf induced growth inhibition at concentrations of 3 mg/ml and 5 mg/ml. The inhibition was evident after 2 days, and a highly significant ($P < 0.001$) 56% decline of growth was observed after 48 h at a concentration of 5 mg/ml. Lf recruits a multitude of mechanisms to exert its multiple effects. It binds to several types of receptors that carry out cargo transport as well as signal transduction. Additionally, Lf can chelate and bind (Fe^{3+}), (Cu^{2+}), (Mn^{3+}) and (Zn^{2+}) which are necessary for cell proliferation. Pinpointing the pathways and mechanisms of the action of Lf is thus important to gather insight into its role in cellular metabolism, growth and differentiation (Zemann, Klein, Wetzel, Huettinger, & Huettinger, 2010).

The antioxidant activity of Lf cannot be fully described using a single method because antioxidant activity is influenced by many factors. Therefore, various assays were performed for the determination of antioxidant activity for Lf.

3.2. Ferric-reducing/antioxidant power assay

The FRAP assay depends on reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) to ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant (antioxidants or other reducing agents) at low pH (Xu & Chang, 2007). Fe(II)-TPTZ has an intensive blue colour and can be monitored at 593 nm. In contrast to other tests for assessing total antioxidant power, the FRAP assay is simple, fast, inexpensive and highly reproducible. The FRAP values for Lf, rutin and vitamin C are presented in Fig. 2. Rutin exhibited the highest FRAP value (1705–10,644 μmol FE), while Lf exhibited the lowest FRAP value (317.63–1057.97 μmol FE).

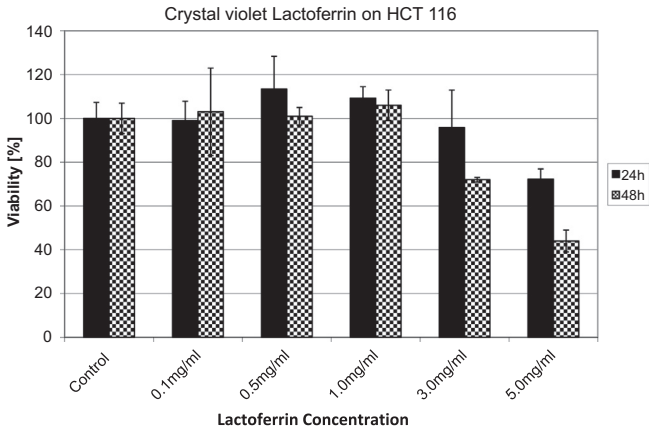

Fig. 1. Proliferation rate of HCT-116 colon cancer cells treated with the Lf.

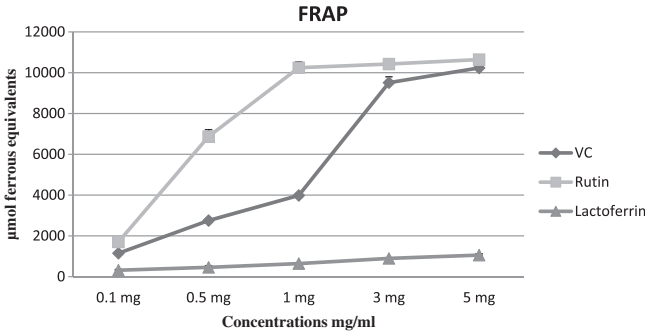

Fig. 2. FRAP assay for Lf, vitamin C and rutin at different concentrations.

3.3. DPPH free radical-scavenging assay

The DPPH free radical-scavenging capacities of Lf, vitamin C and rutin are presented in Fig. 3. Rutin showed the highest inhibition effect on DPPH (11.49–40.72%) followed by vitamin C (4.25–67.9%), while Lf showed the lowest effect of inhibition on DPPH radical (2.77–5.54%).

3.4. Nitric oxide radical scavenging assay

Oxidative stress caused by the production of excess nitric oxide during infection or inflammation, has been implicated in the pathogenesis of several diseases, including cancer, diabetes and renal disease. Accordingly, the scavenging of NO· may be a promising indicator in healthy foods. The NO· scavenging capacities of Lf, vitamin C and rutin are shown in Fig. 4. The NO· scavenging capacity increased significantly in a concentration dependent manner for Lf and rutin. Lf NO· scavenging capacity value was (5.09–38.24%) and rutin NO· scavenging capacity value was (59.42–79.01%). Surprisingly, vitamin C NO· scavenging capacity started with a value of 59.10%, reduced to 40.92% and 29.57% at 0.5 mg/ml and 1 mg/ml respectively, then increased to 55.12% and 56.55% at 3 mg/ml and 5 mg/ml, respectively.

3.5. Total antioxidant activity

The total antioxidant activity method is based on the reduction of Mo(VI)–Mo(V) by the antioxidant compounds, with the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The assay is simple and independent of other antioxidant measurements commonly employed. It should be recalled that higher absorbance indicates a higher antioxidative activity for the assay. Fig. 5 indicates that rutin exhibits the highest phosphomolybdenum reduction effect (0.13–1.57 nm), followed by Lf (0.05–0.09 nm). On the other hand, vitamin C was seen

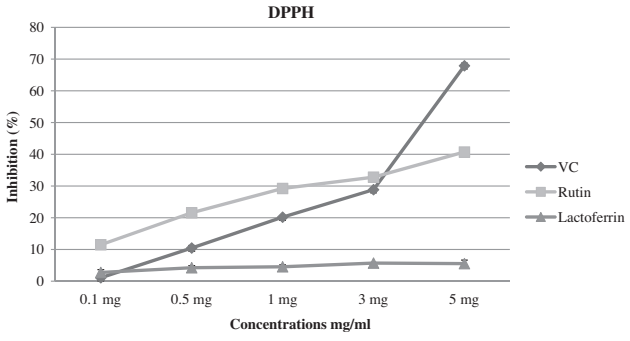

Fig. 3. DPPH assay for Lf, vitamin C and rutin at different concentrations.

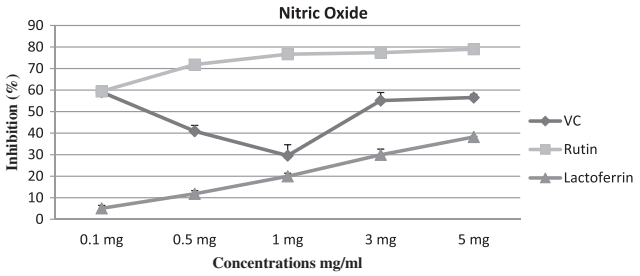

Fig. 4. Nitric oxide assay for Lf, vitamin C and rutin at different concentrations.

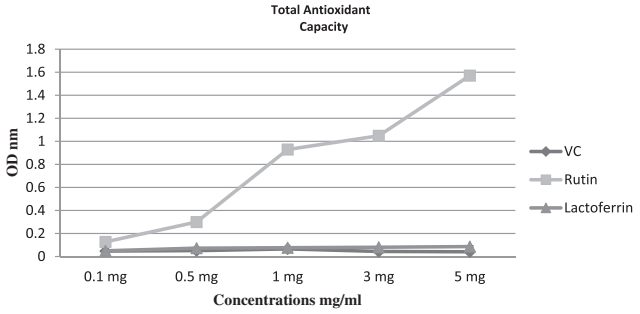

Fig. 5. Total antioxidant capacity for Lf, vitamin C and rutin at different concentrations.

to exhibit the lowest reduction of phosphomolybdenum (0.041–0.066 nm).

3.6. DNA damage by free radical

The protective effect of Lf on H_2O_2 + UV-induced DNA damage was studied on pRK5 plasmid DNA. Fig. 6(a) and (b) shows the electrophoretic pattern of pRK5 plasmid DNA after UV-photolysis of H_2O_2 and exposure to $FeSO_4$ in the presence or absence of Lf. The plasmid DNA showed two bands on agarose gel electrophoresis. The faster moving band (lane OC) corresponded to the open circular DNA and the slower moving band (lane SC) corresponded to the native supercoiled circular DNA form. The hydroxyl radical ($OH^{\cdot}$) generated from UV-photolysis of H_2O_2 and $FeSO_4$ produced DNA strand scission activity. Most of the oxidative damage in biological systems is caused by the $OH^{\cdot}$, which is generated by the reaction between $O_2^{\cdot-}$ and H_2O_2 in the presence of metal ions (Guitteridge, 1984). The results showed complete degradation of plasmid DNA treated with UV, H_2O_2 and $FeSO_4$. This damage was reduced in DNA treated with UV, H_2O_2 and $FeSO_4$ in the presence of Lf. Lactoferrin may involve in an antioxidant defense mechanism by binding any catalytic iron, and inhibiting the Haber–Weiss reaction:

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

Densitometric analysis strongly suggests that Lf inhibits the Haber–Weiss reaction thus protecting the pRK5 plasmid DNA from damage. The plasmid alone showed 89.75% SC and 10.25 % OC, while the plasmid exposed to H_2O_2, $FeSO_4$ and UV showed 44.08% SC and 55.92% OC. On the other hand, when Lf was present at different concentrations, the damaged decreased. The SC and OC values ranged from 85.51% to 14.49% and from 80.92% to 25.31%, respectively.

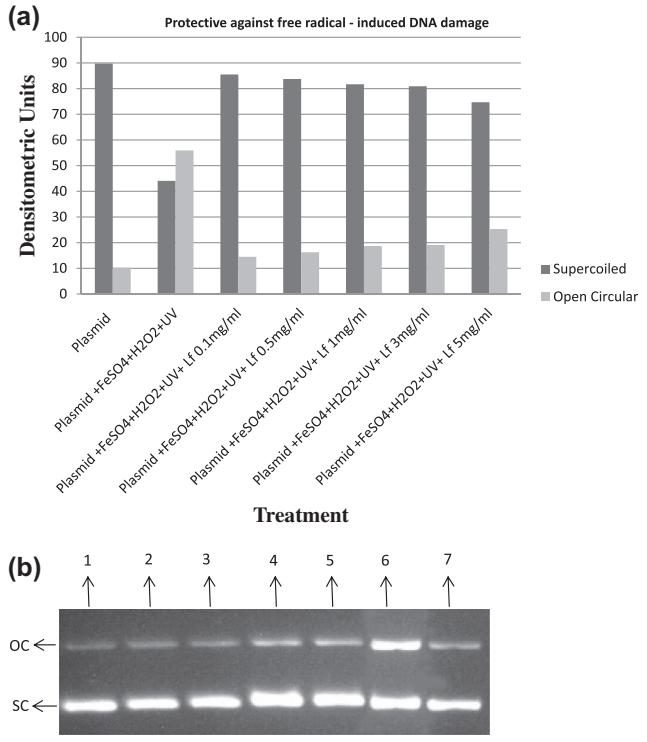

Fig. 6. (a) Densitometric analysis. (b) 1-Plasmid + $FeSO_4$ + H_2O_2 + UV + Lf 0.1 mg/ml, 2-plasmid + $FeSO_4$ + H_2O_2 + UV + Lf 0.5 mg/ml, 3-plasmid + $FeSO_4$ + H_2O_2 + UV + Lf 1 mg/ml, 4-plasmid + $FeSO_4$ + H_2O_2 + UV + Lf 3 mg/ml, 5-plasmid + $FeSO_4$ + H_2O_2 + UV + Lf 5 mg/ml, 6-plasmid + $FeSO_4$ + H_2O_2 + UV, 7-plasmid, OC (open circular), SC (supercoiled).

4. Conclusions

Results obtained showed that camel milk Lf exerted antioxidant activity through scavenging $NO^{\cdot}$ and the DPPH free radical, as well as the capability to furnish reducing power as evident by the FRAP and the total antioxidant assays. Lf also inhibited DNA damage most likely through binding catalytic iron. Moreover, Lf inhibited the growth of colon cancer cells. Studies are underway to determine the mechanisms underlying the antiproliferative effects of camel milk lactoferritin. Lactoferrin seems to have great potential in practical medicine. Nevertheless, much research and many experiments still need to be carried out in order to obtain a better understanding of its activity and interactions. In addition, the use of Lf in combination with other milk components or drugs may be an increasing consideration.

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