

## Anticancer effect of lactoferrin on Gastric Cancer Cell Line AGS

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### Abstract

**Background:** Lactoferrin is a glycoprotein with a molecular weight of 80 kDa, as a member of the transferrin family. In recent investigations some important biological properties such as anti microbial, antiviral and anticancer activity of lactoferrin were studied. In the present study, the effect of antitumor induced by lactoferrin was evaluated on stomach cancer cell AGS.

**Materials and Methods:** Bovine milk was collected after parturition. After isolation of fat and casein, the milk's other proteins were separated by ammonium sulfate precipitation. Then, lactoferrin was purified using CM-Sephadex-C50 cation exchange chromatography by FPLC system. The purified protein was identified using SDS-PAGE electrophoresis. The concentration of lactoferrin from milk and colostrum by Bradford assay obtained 0.6 and 2 mg/ml, respectively. Stomach cancer cell line, AGS and normal cell lines, HEK-293 were cultured in 37 °C and 5 percent CO<sub>2</sub>. After appropriate cell growth, different concentrations of purified lactoferrin were added to cells and incubated for 20, 36 and 48 hours. The cell viability and inhibition of cell growth were determined by MTT assay, also apoptosis assay was evaluated using propidium iodide staining through flow cytometry.

**Results:** Lactoferrin (500µg/ml) inhibited the AGS growth by 55, 71 and 80% during 20, 36 and 48 h, respectively but did not have notable inhibitory effect on normal cell lines. The apoptosis level of AGS in flow cytometry was 55 and 70% in 20 and 48 h, respectively and was 5% in normal cells.

**Conclusion:** The isolated lactoferrin from bovine milk showed inhibitory effect on stomach cancer cell line whereas; it did not have any significant effect on normal cells.

**Keywords:** Flow cytometry; Lctoferrin; MTT assay; Gastric cancer cell

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### Introduction

Lf is an 80-kDa member of the transferrin family of iron-binding glycoproteins (1, 2). It is a member of the transferrin family that secretions, such as colostrum and tears, and, is mainly found in exocrine granules of neutrophils (3, 4). Lf is produced by mucosal epithelial cells in various mammalian species including humans, cows, goats, horses, dogs, and rodents, and it is also produced by fish (5). Several biological functions have now been described for Lf, including iron homeostasis, cellular growth and differentiation, host defense against microbial

infection, anti-inflammatory activity and cancer protection (6, 7). Lf is a multifunctional iron-binding glycoprotein which is particularly abundant in colostrum and is also present in mammalian epithelial cell secretions such as tears, saliva and seminal fluid in various amounts (8). A promising field of research is clinical studies with cancer preventive proteins existing in milk (9). Lactoferrin (Lf) known for its inhibitory action on cell proliferation, as well as for its anti-inflammatory and antioxidant abilities, has been described to have anti-carcinogenic properties in several in vivo and in vitro studies (10, 11).

It has been demonstrated that more than 60% of administrated bovine Lf survived passage through the adult human stomach and entered the small intestine in an intact form (12). Intact and partly intact bovine Lf is likely to exert various physiological effects in the digestive tract. Moreover, subcutaneously administration of Lf inhibited the growth of implanted solid tumors and exerted preventive effects on metastasis (13). Recently it was found that dietary supplementation with bovine Lf (bLf), derived from bovine milk (14), can inhibit the development of azoxymethane (AOM) - induced aberrant crypt foci (ACF) as precursor lesions of tumor development, as well as carcinomas in the rat colon, without any toxic effects in major organs (15,16). Also, Lf reduced the growth and metastasis of solid tumors (13) and bLF exerts chemopreventive effects in the esophagus and lung in addition to the colon (17). Therefore, bLf is considered a good candidate for a chemopreventive agent of human cancer development. In the present study, we treated human stomach cancer cell line (AGS) with different concentration of bLf and studied the anticancer inducing and apoptosis effects of bLf by MTT test and flow cytometry.

### Materials and Methods

#### *Isolation and purification of Lactoferrin from cow's milk*

Bovine colostrum was collected in sterile containers within 72 hours after delivery of ten cows. Then centrifuged in 1000 ×g for 20 min at 4 °C until the cream was isolated. In order to isolate the caseins, the pH of skim milk was reached 4.6 using HCl 2N for 30 min at 40 °C. Casein precipitates was separated by centrifugation in 12000×g for 20 min at 4 °C. The acid whey was neutralized by NaOH and its pH reached 6.8. For isolation of globulins from whey, ammonium sulfate (267 g/l) was added slowly to the acid whey then centrifuged in 1000 ×g for 20 min at 4° C. The precipitates of globulins were discarded, and 250 g/l of ammonium sulfate was added to remaining supernatant. Lf was separated by centrifugation under previous conditions. Dialysis was done in order to separate salt from the protein using dialysis bags with Cut-off 10 kDa in 20 mM phosphate buffer with 200 rpm/min speed for 24 h at 4 °C. The protein was powdered in freeze dryer (CHRIST) for 24 h. For purification of Lf, FPLC (LP models Biorad America) was used. For this purpose, the sample of powdered protein was solved in 20 mM phosphate buffer and then was taken on CM-Sephadex-C50 column. Lf protein eluted in gradient of 0.4 to 0.5 M NaCl in speed of 0.75 ml per minute under pressure 5 MPa. The Lf purity was checked by SDS-PAGE electrophoresis (BioRad) and the

concentration of protein was determined using the Bradford Assay.

#### *Cell culture*

AGS human stomach carcinoma cells and HEK human epithelial like normal cell were purchased from Pasteur institute (Iran) and fibroblast normal cell was a gift from Molecular and Cell Biology Research Center in Mazandaran University of Medical Sciences. AGS was cultured in 90% RPMI1640 plus 10% FBS. HEK normal cells were cultured in 90% DMEM plus 10% FBS. Incubation was done in 5% CO<sub>2</sub> incubator (Memert, Germany) at 37 °C.

#### *MTT Assay*

In order to assess the toxicity of Lf, MTT assay was used. MTT Reagent (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) is a yellow tetrazolium salt that is absorbed by active and live cells mitochondria and with activity of dehydrogenase enzymes, produce purple formazan crystals. The amount of purple color produced is measured with a spectrophotometer at a wavelength 490 nm. Almost 15×10<sup>3</sup> cells in complete medium were cultured in each well of 96 well plates. Cells incubated at 37 ° C and 5% CO<sub>2</sub> for 24 h. Different concentrations of Lf (0, 100, 200, 300, 400 and 500 µg/ml) were added to each well in triplicates. Incubation continued for 20 hours. MTT dye solution (50 ml, Sigma) was added to each well and, the cells were considered by microscope every one hour until the formation of purple crystals (maximum 3-4 hours). After the formation of crystals, the supernatant was carefully removed and 150 ml of acidic isopropanol was added to each well. The cells were kept in a dark place for 30 min. Absorption of purple color formation was read at 490 nm using Eliza reader (ELX800).

#### *Assessment of cell death by propidium iodide (PI) staining and flow cytometry*

The cells were cultured in 6 wells plate in complete medium containing 10% FBS and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. Lactoferrin was added in concentrations of 0 and 500 µg/ml in triplicate to each well. Incubation was continued for 20 hours. The cells were separated using 2 ml EDTA and trypsin and centrifuged at 2000 g for 5 min and washed one more time with 2 ml of PBS then centrifuged again. The cells were fixed with 2 ml of cold ethanol at 4°C overnight then washed twice with PBS and centrifuged at 2000 rpm for 5 min. PI dye solution (1 mL, Sigma) was added and treated with 50 µl of 100 µg/ml RNase A (Fermentas, Germany) to ensure that only DNA is stained then

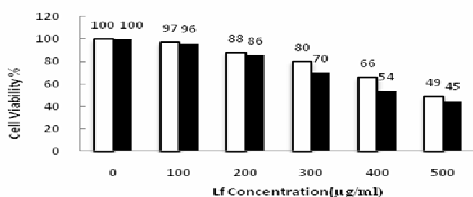
incubated in the dark at room temperature for 30 minutes. Finally, dead cells were analyzed using flow cytometry (Partec, Germany).

## Results

Lactoferrin eluted in 40-50% salt concentration. To confirm the purity of the proteins, 10% SDS-PAGE gel electrophoresis was used. Lf concentration was 2 mg/ml for colostrum and 0.6 mg/ml for milk determined by Bradford Assay in previous study (18).

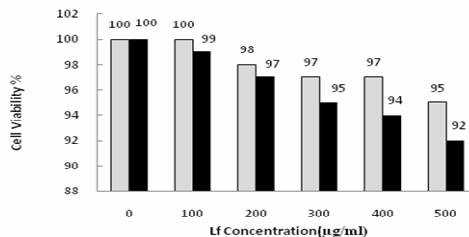
### Cell viability using MTT Assay

MTT assay was used To evaluate the toxicity effect of Lf on AGS cells, HEK and HEF normal cells, After growth of the cells up to 70% confluence, different concentrations of Lf including: 0, 100, 200, 300, 400 and 500  $\mu\text{g/ml}$  were considered for treatment of the cells. During 20 hours of treatment with lactoferrin, the lowest cell viability was observed 45 and 49% equivalent to 55 and 51% of the inhibitory effect of colostrum and milk's lactoferrins (500  $\mu\text{g/ml}$ ), respectively (Figure1).



**Figure 1.** Lf effect (concentrations of 0, 100, 200, 300, 400, 500  $\mu\text{g/ml}$ ) on AGS cells. Percentage of cell viability at 20 hours. The white column shows colostrum's lactoferrin and black column shows milk's lactoferrin.

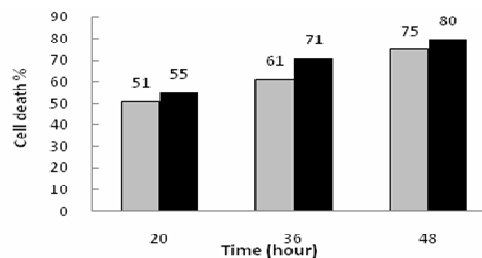
Lactoferrin effects on normal HEK cells were also studied. The inhibitory effect was not observed at different concentrations and survival rate was almost 92 to 95% for colostrums and milk, respectively (Figure2).



**Figure 2.** Lf effect (concentrations of 0, 100, 200, 300, 400, 500  $\mu\text{g/ml}$ ) on HEK normal cells. Percentage of cell viability at 20 hour. The gray column shows colostrum's lactoferrin and black column shows milk's lactoferrin.

The experiment was performed with concentration of 500  $\mu\text{g/ml}$  Lf at different intervals (20, 36 and 48

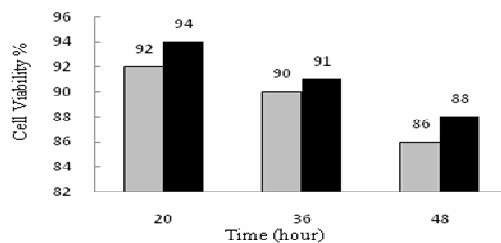
hours). In AGS cells after 20, 36 and 48 hours, cell death was 51, 61 and 75% for milk and 55, 71 and 80% for colostrum, respectively (Figure3). Cell viability present after treatment of Lf 500  $\mu\text{g/ml}$  at different intervals 20, 36 and 48 hours on HEK normal cells were found 93, 92 and 89% for colostrum, respectively and 95, 93 and 91% for milk, respectively. (Figure 4).



**Figure 3.** Lactoferrin effect (500  $\mu\text{g/ml}$ ) on survival of AGS cell line. Percentage of colostrums (the black column) and milk's lactoferrin (the gray column) inhibition in intervals 20, 36, 48 hours.

### Flow cytometry analysis using propidium iodide staining

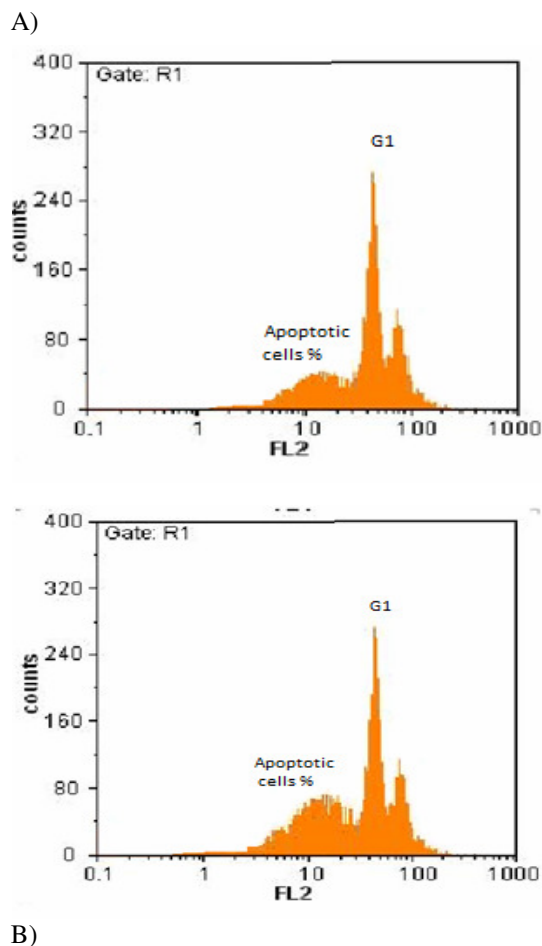
The results of the propidium iodide staining for detecting death of cells by flow cytometry are shown in figure5. The AGS and HEK cells treated with 500  $\mu\text{g/ml}$  lactoferrin for 20 and 48 hours. The apoptotic cells, among other typical features, are characterized by DNA fragmentation and consequently loss of nuclear DNA content. Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells.



**Figure 4.** Lactoferrin effect (500  $\mu\text{g/ml}$ ) on survival of HEK normal cell. Percentage of cell viability of colostrums (the gray column) and milk's lactoferrin (the black column) in intervals 20, 36, 48 hours.

Analysis of DNA stained by PI and flow cytometry showed the presence of a broad peak of hypodiploid particles characteristic of apoptotic cells and the sharp diploid DNA peak of normal cells. Debris and residuals of necrotic cells can be easily gated-off. A precise calibration of the flow cytometer is

necessary to properly gate out debris. The apoptosis in the treated AGS cells with lactoferrin was 55 and 70% in 20 and 48 hours, respectively and in HEK cells indicated 4 stages of normal cell cycle including; sub-G1, G1, S and G2 after 48 hour (Figure6).

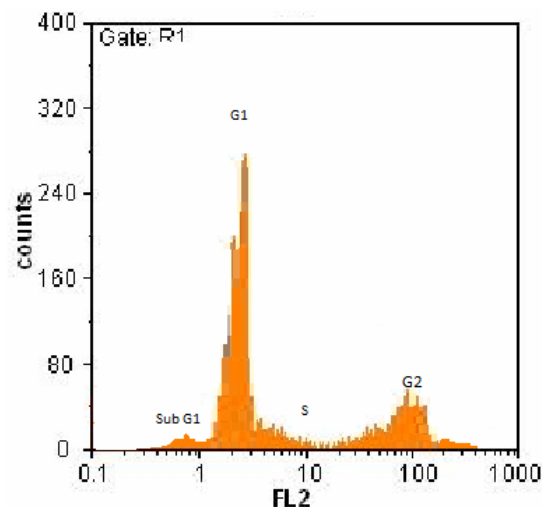


**Figure 5.** Flow cytometry diagram of percentage of AGS cells apoptosis after treated with lactoferrin at concentration of 500  $\mu\text{g/ml}$ . A) After 20 hour treatment B) After 48 hour treatment.

### Discussion

The intake of dairy products has been reported to play a role in cancer prevention (19). Several clinical or in vitro studies have shown inverse association between cancer risk and higher consumption of some dairy products, especially those containing whey proteins (20). Some research even concluded that the iron-binding capacity of whey may also contribute to anticancer potential (21). Whey lactoferrin comprises a single polypeptide chain with 2 binding sites for ferric ions, and its functionality is related to the strong iron-bind properties, such as antimicrobial, antiviral, and anticancer. However, the cancer prevention mechanism is still not clearly

known. Gastric cancer is a serious public health problem. According to the recent assessment of global cancer incidence, gastric cancer is the fourth most common cancer worldwide (22). Therefore, development of new therapeutic agents for stomach cancer is a top priority. Chemotherapy drugs are widely used in cancer treatment, but have the disadvantage of nonspecific toxicity because these factors, all rapidly dividing cells, target without any discrimination between normal and malignant cells. In this study, the amount of Lf concentration derived from one liter of bovine colostrum was about 2 mg/ml and for milk was 0.6 mg/ml. However, in previous studies the concentration of Lf derived from bovine colostrum have been reported 0.1 to 0.4 mg/ml (23). The purified lactoferrin, showed cytotoxicity on human stomach cancer cells, which had no adverse effect on viability of human normal epithelial like cells. In our research, different concentrations of lactoferrin on cancerous AGS cell line were studied for the first time. Cell viability levels decreased with increase in concentration, and at 500  $\mu\text{g/ml}$  Lf the inhibition of growth was the greatest. Duarte et al. studied the effect of 12.5  $\mu\text{g/ml}$  bLf on human breast cancer cell lines like T47D and HS578T, and showed 54% and 47% cell viability reduction after 48 hours, respectively (24).



**Figure 6.** Flow cytometry diagram of HEK normal cells after treatment of lactoferrin in 48hour indicated 4 stages of normal cell cycle including; sub-G1, G1, S and G2.

The antitumor effect of 50  $\mu\text{g/ml}$  lactoferrin studied in gastric cancer SGC-7901 cells for 24 and 48 h and inhibition of proliferation was observed (25). Zemann et al. found that lactoferrin, inhibits proliferation of human HeLa epithelial cancer cell line (26). Lactoferrin-induced growth inhibition at

concentrations of 50 and 500 µg/ml, after three days and resulted in a very substantial reduction of growth, about 75% after six days of treatment.

Propidium iodide flow cytometric method (PI) has been widely used to assess apoptosis in various experimental models. PI is a red fluorescent dye and is ready to use, which binds to the DNA of dead cells. In this case, normal cells are not stained. With some modifications to the basic method, this method is applied for different types of cells, including tumor adherent cell lines. PI is a fluorogenic compound that binds stoichiometrically to nucleic acids (27) so that fluorescence emission is proportional to the DNA content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels. This method has some advantages. It allows a fast, reliable and reproducible estimate of apoptosis, simultaneous analysis of cell cycle parameters of surviving cells (28). In 1991 a fast and simple method was published for measuring apoptosis of mouse thymocytes stained by flow cytometric with PI (29). Xiang and Liu investigated the effect of deguelin on cell cycle of Raji cells by PI flow cytometry (30). Ormored et al. examined apoptosis in IL-3-dependent haemopoietic cells by PI flow cytometry (31). In our results the apoptosis (sub-G1) peak was observed in AGS cells after Lf treatment and in normal cells four normal stages of cell cycle existed. Through this experiment we found that lactoferrin had the function of apoptosis of stomach cancer cell line AGS. According to previous study and PI staining benefits, we determined apoptosis after treatment of Lf using this method.

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### Authors' contributions

Moradian and Rafiei contributed in the study conception, design, critical revision and finalizing the manuscript.

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### Conflict of interest

The authors declare that they have no conflict of interest in this work.

### Role of sponsor

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