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The Comparison of Effect of Human Milk and Powdered Milk on the Shigella dysenteriae Invasion in Cell Culture

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Abstract

Background: *Shigella* species are the common etiologic agents of bacterial dysentery. Many epidemiological studies have shown that breastfeeding may protect infants against intestinal infections. Among the components of milk, glycosylated proteins inhibit the adhesion of enteric pathogens in the laboratory. Immunoglobulins mainly secretory immunoglobulin A, glycosylated compounds, and oligosaccharides of breast milk are associated with protection against different intestinal pathogens.

Objectives: This study aimed to evaluate the effect of different proteins of breast milk and powdered milk on the invasion of *Shigella* colonies.

Materials and Methods: To accomplish this goal, breast milk samples were provided from two donors in the first 6 months of breastfeeding and powdered milk with different brands were obtained from the market. Then the proteins were extracted by precipitation using ammonium sulfate and dialysis using dialysis bag and protein bands were separated through SDS-PAGE electrophoresis. Finally, the obtained milk proteins through Hela cells culture were tested and evaluated for the adhesion and invasion of the *Shigella*.

Results: Our results revealed that the adhesion and invasion of *Shigella* stains were more inhibited by low concentrations of breast milk proteins in comparison with powdered milk. This concentration was about 2.75 mg/mL for the proteins of breast milk and 0.5 mg/mL for the proteins of powdered milk and this inhibition in different dilutions of breast milk was 71.21% and those of powdered milk was 27.19% in average. There was a significant difference between breast milk and powdered milk ($P<0.5$) considering their inhibitory behavior.

Conclusion: The results revealed that the components of breast milk inhibit the adhesion and consequently invasion of *Shigella* and inhibit bacterial dysentery.

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Background

Diarrheal diseases are responsible for 1.3 million deaths per year. The main causes of intestinal infections and diarrhea are *Vibrio cholerae*, *Shigella*, *Escherichia coli* and so on. Shigellosis is responsible for about 600 000 to 1 000 000 deaths per year.

Diarrhea is a common cause of death in children, and it is important for determination of protection, provided with breastfeeding. Many studies have emphasized that breastfeeding is associated with an indication of reduction in morbidity and mortality.[1] In a randomized study in Belarus it was found that breastfeeding protected the children against gastroenteritis during the first year of their life.[2] It was shown that breastfeeding for 6 months reduced the cases of gastroenteritis in comparison with breastfeeding for 3 months.[3]

Protection against diarrheal bacteria and their toxins is offered by secretory IgA (sIgA) antibodies of milk along with the study of infections with the origin of enterotoxigenic *E. coli*, *Shigella*, *Vibrio cholerae*, and *Giardia lamblia*.[4] Recently, it was shown that the human anti secretory peptide factor, when induced in breast milk, had more protective activity against acute diarrhea than chronic diarrhea.[5,6] A study in Mexico has revealed that breastfeeding is associated with 5-fold lower risk of diarrhea with *G. lamblia* agent in comparison to feeding without breast milk, and 1.8-fold lower risk with incomplete breastfeeding.[7] For protection against rotaviruses by breastfeeding, no obvious protection has been recorded or a delay in diseases and asymptomatic infections been occurred.[8]

Due to the specific characteristics of the proteins of breast milk, the prevalence of infectious diseases among the infants fed with breast milk is lower than that among

those fed with powdered milk and this is because of special proteins of breast milk.

Different species of mammals produce breast milk with different amounts of antibacterial factors. For example, cow milk has the highest levels of lactoperoxidase. However, it has low levels of lysozyme and lactoferrin (Lf). While human milk has high levels of Lf and lysozyme, the amount of lactoperoxidase is very low in it.[9]

Lf is an iron binding glycoprotein (80 KDa) which was isolated from the cow milk for the first time and subsequently its presence in human milk was proved.

Lysozyme is another enzyme of the milk of some species especially humans. In human and solipeds, type C lysozyme is more important. Lysozyme represents antibacterial activity and destroys the cell wall of pathogens through the destruction of glycoside linkages between peptidoglycans (the main part of bacterial cell wall). High concentration of lysozyme (0.12 g/L) is reported in human milk. It has been reported that human colostrum has the highest levels of lysozyme.[9]

N-acetyl-β-D-glucosaminidase is used as an indicator for the detection of mastitis. So far, few studies have been done in this area and therefore further research is needed.[9]

Objectives

This study intended to isolate and identify *Shigella* dysentery strains from clinical samples, purify the proteins of breast milk and powdered milk, prepare the cell culture and evaluate the effect of purified proteins on the amount of *Shigella* strains' invasion.

Materials and Methods

Clinical strains were isolated and identified from patients' stools, referred to Taleghani children's hospital, Tehran, Iran. The strains were identified using Bridge biochemical tests.[10]

Protein Purification

One hundred milliliter of human milk samples (from donors in the first 6 months of breastfeeding) and powdered milk were centrifuged at 5000×g for 1 hour to separate the fat. In combination with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM EDTA, the samples were acidified by citric acid to pH 4.6 and incubated overnight. Then they were centrifuged at 3000 × g for 20 minutes to separate the casein.

At this stage, the proteins of human milk and powdered milk were concentrated to 70% by ammonium sulfate. For this purpose, 53 g of solid ammonium sulfate per 100 mL of human milk was added to the sample in a beaker of ice on the shaker. After precipitation, the milk sample was centrifuged at 10000 × g and 4°C for 20 minutes. The sediment produced by centrifuge was solved in 15 mL of Tris HCL buffer pH 6.7. Dialysis was done using a dialysis bag with a cut-off point of 10 KDa at 4°C for 24 hours in 0.1 M Tris HCL buffer pH 7.6.

SDS-PAGE electrophoresis (Bio-Rad Company, USA) was done for the samples with gel 10% and the voltage of 60-70 V for 3 hours. To measure the protein, Bradford method was used as follows: after reading the relevant density with a spectrophotometer at a wavelength of 590 nm, protein samples' concentrations were determined using the standard curve. Bovine serum albumin (BSA) is used as a standard protein to measure the concentration of a compound that contains protein.[11]

Cell Culture and Inoculation

Hela cell line obtained from the cell bank of Pasteur Institute of Iran was maintained and amplified in RPMI-1640 medium (Sigma, Germany). The cultured cells were incubated in the incubator (Napco, USA) at 37°C, with 5% CO_2 and 95% humidity.[12] Replacement of medium was performed according to the cell density. Samples at cell density of 80%-85% were transferred to new flasks with complete medium containing serum, antibiotics, and 10% FBS with a ratio of 1:3. To count the cells, 0.08% trypan blue solution was used. Cryo contents were transferred to falcon tubes and the volume was reached to 10 mL by medium. Then the serum and antibiotic were added to them and the cells were evaluated using inverted microscope. Again the serum was washed with phosphate buffered saline (PBS) and dead cells were removed to prevent the neutralization of trypsin.[13] After centrifugation, the supernatant was removed and the sediment was dissolved in the complete medium and transferred into the flask. The flasks were incubated in CO_2 incubator at 37°C for 2-3 minutes. To count the cells, 100 to 200 µL of cell suspension was taken and the number of cells was determined in each milliliter using the following formula: volume conversion coefficient × mean of the counted cells × dilution coefficient × 10 000. Then by multiplying the obtained number by cell suspension volume, the total number of flask's cells were determined.

Inoculation of bacteria in cell culture

The suspension of the bacterial cells (wavelength A_{600}, OD 0.8) was incubated for 30 minutes with 0.1 M Tris HCL pH 7.6 and with dilutions 1, 1.2, 1.4, 1.8, 1.16, and 1.32 of proteins of breast milk and powdered milk which were added to them. Then they were inoculated in Hela cells on 96-well microtiter plate. The plate was centrifuged at 2200 × g for 10 minutes and then incubated for 45 minutes at 37°C and in 5% CO_2 atmosphere. To remove the bacteria which were not adhered, Hela cells were washed four times with PBS and were placed in normal saline containing gentamicin and reincubated for 90 minutes. Hela cells were washed again four times with PBS and trypsinized by trypsin and EDTA to separate the cells from the bottom of the vials. Then they were poured into the microtubes and centrifuged at 5000 × g for 10 minutes. The trypsin was removed and the cells were deposited and observed by inverted microscope. To determine the amount of bacteria penetrated into the cells, Hela cells were vortexed with sterile distilled water to decompose the lysed cells and thus the bacteria could release from the cells. The remained bacterial suspension was cultured on Muller-

Hilton agar and Salmonella Shigella agar (SS) (Merck, Germany) to ensure the prevention of *Shigella* strains entry. Colony-forming unit (CFU) was determined after incubation at 37°C. All tests were performed 3 times independently.

Statistical Analysis

Results are explained as the mean ± standard deviation (SD). The statistical significance of differences between treatment groups was appraised by a Student's *t* test for unpaired observations and one-way analysis of variance (ANOVA) test using the Analysis Toolpak of Microsoft Excel. In all analyses, The *P* value of 0.05 was considered as significant parameter of the results using SPSS software version 19.0.

Results

Purification, Separation, and Extraction of Proteins by Electrophoresis and Bradford

At the stage of purification and isolation of *Shigella* species, the bacteria were detected as rod-shaped, circular, convex, gram-negative, lactose-negative, citrate negative, urease negative, mannitol negative, and glucose positive. Then, during the next stage, the proteins of human milk and powdered milk were concentrated to 70% using ammonium sulfate. The results of the protein extraction from human milk and powdered milk by the electrophoresis are shown in Figure 1. In this study, the protein markers for electrophoresis had a wide range of molecular weight in order to have all possible molecular weights. The molecular weights of markers were from 14.4 KDa to 116 KDa and after staining with Coomassie Brilliant Blue, the protein bands appeared based on their molecular weight (Table 1). In the electrophoresis evaluation, the number of protein bands before adding the sulfate to the milk was lower than that after adding the sulfate and concentrating the protein by the dialysis. In this study, Bradford method was used to determine the amount of samples' proteins, and the concentration of proteins of human milk and powdered milk before and after adding the sulfate (dialysis is shown in Table 1).

A reduction in the adhesion of bacteria with the dilutions of 1, 1.2, 1.4, 1.8, 1.16, and 1.32 was calculated to be 71.21% in average in human milk and approximately 27.19% in powdered milk. A significant difference was observed in the reduction of bacterial adhesion, with different dilutions of milk. Evaluation of Hela cells by inverted microscope revealed that the number of cells at their entry into the plate was about 10 000. By entering the *Shigella* incubated with proteins of human milk and powdered milk, the number of Hela cells reached to 4920 in average for different concentrations, while all of

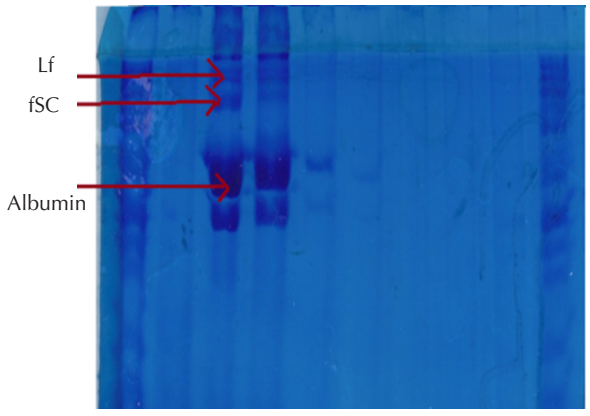

Figure 1. Electrophoresis for Purification of Proteins of Human Milk and Powdered Milk.
Line 1 and end: protein markers; Line 3 and 4: protein bands of human milk; Line 5: protein bands of powdered milk; Line 6: protein bands of milk before dialysis.
Abbreviations: fSC, free secretary component; Lf, lactoferrin.

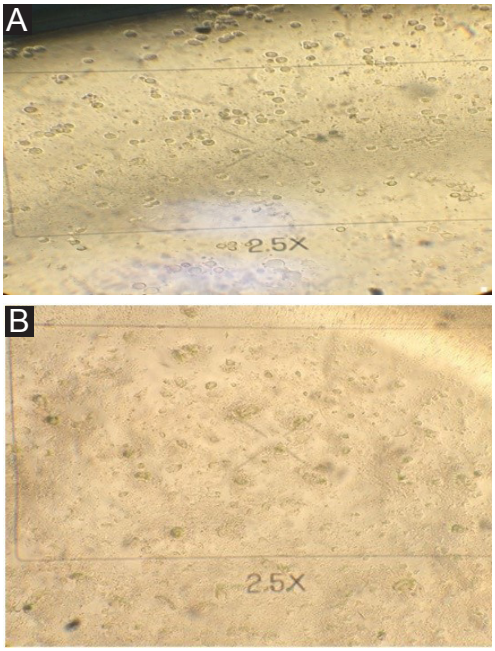

Figure 2. Hela Cells Into Cell Culture With Human milk (A) and Powdered Milk (B).
(A) Hela cells after inoculation of incubated bacteria with human milk into cell culture; (B) Hela cells after inoculation of incubated bacteria with powdered milk into cell culture.

Table 1. Values Obtained Through the Analysis of SDS-PAGE and Bradford

Samples	Concentration (mg/mL)	OD (590 nm)	Protein Components	Molecular Weight (KDa)
Powdered milk after dialysis	0.57	1.32	Albumin	66.5
Human milk after dialysis	2.75	2.01	Lf	80
Milk before dialysis	0.47	1.10	fSC	78

Abbreviations: fSC, free secretary component; Lf, lactoferrin; OD, optical density.

Table 2. The Impact of Human Milk's Proteins on Inhibition of Adhesion of Shigella in Hela Cell Culture

Human Milk	Shigella Wild Type		
Protein Concentration (mg/mL)	Before Incubation[a]	Test[b]	% of Inhibition[c]
0.7	3300	103	96.87%
0.35	3300	568	82.78%
0.17	3300	750	77.27%
0.08	3300	1110	66.36%
0.04	3300	1288	60.96%
0.02	3300	1880	43.03%

[a] The number of bacteria alive without incubation with human milk after inoculation in cell culture.
[b] The number of bacteria alive after incubation with human milk after inoculation in cell culture.
[c] The inhibitory effect of proteins on adhesion of Shigella to Hela cells.

Table 3. The Inhibitory Impact of Proteins From Powdered Milk on Adhesion of Shigella to Hela Cells

Powdered Milk	Shigella Wild Type		
Protein Concentration (mg/mL)	Before Incubation[a]	Test[b]	% of Inhibition[c]
0.7	3300	103	96.87%
0.35	3300	568	82.78%
0.17	3300	750	77.27%
0.08	3300	1110	66.36%
0.04	3300	1288	60.96%
0.02	3300	1880	43.03%

[a] The number of bacteria alive without incubation with powdered milk after inoculation in cell culture.
[b] The number of bacteria alive after incubation with powdered milk after inoculation in cell culture.
[c] The inhibitory effect of proteins on adhesion of Shigella to Hela cells.

these cells were missed by direct inoculation of *Shigella* without milk protein (Figures 2A and 2B). At this stage, the number of bacteria alive that were able to stay in the cell culture after lysis of the cells with distilled water and adding into Salmonella Shigella agar and Mueller-Hilton agar were defined by counting the number of colonies. The obtained numbers from the test in different dilutions are shown in Tables **2** and **3**.

Discussion

In this study, it was found that the various components of proteins of human milk and powdered milk inhibit the adhesion of *Shigella* dysentery strain to Hela cells. This level of inhibition was about more than 71% for human milk and approximately more than 27% for powdered milk. The difference between the result of this study and that of other studies could be due to various causes such as genetic differences among individuals in different parts of the world, differences in personal hygiene that results in different amounts of protein in their milk, using different protocols from previous researches and so on. Thus, the inhibitory activity of human milk and powdered milk can have a similar mechanism and purpose and can prevent the toxicity of *Shigella* dysentery strain. The mechanism for this is that various components of proteins of human milk and powdered milk adhere to the special mucosal receptors of Hela cells and prevent the adhesion of bacteria. In cases that the proteins are not able to adhere to the cells, a number of bacteria attaches the Hela cells. At these times, overlapping occurs between different forms of α and β proteins and consequently the receptors cannot recognize the protein and finally the proteins are washed and removed and sometimes the proteins may be used by cells as food and thus cannot prevent the adhesion of bacteria.[14]

Studies have indicated that the glyco components of human milk as homologous of cell surface prevent pathogens from binding to the receptors of host cells.[15] Newburg et al in 1992 described that the glycolipid Gb3 of human milk is a natural receptor of Shiga toxin and can eliminate the effects of this toxin on host cells.[16] Human proteins such as free secretary component (fSC) and Lf have the same role and probably work as the analogues of adhesion and invasion. On the other hand, Gomez et al showed that recombinant Lf decompose the toxicity factors such as IpaB and interfere with the formation of IpaBC complex on the surface of host cells. The findings suggest that secretory immunoglobulin A (sIgA) and Lf bind to proteins with similar masses to *Shigella* protein: VirB (35 KDa), VirA (45 KDa), IpaJ, MxiJ (26 KDa), IpaD (37 KDa), IpaA (68 KDa). These results show that Lf and sIgA prevent the interaction between bacteria and host cells and interfere with the formation of invasive complex.[17]

Several reports have mentioned the important role of immunoglobulins such as sIgA in the protective effect of human milk against diarrhea.[18] sIgA prevents further adhesion of *E. coli* pathotypes such as enteroinvasive *E. coli* (EIEC) which shows the similar toxicity aspects of these pathotypes compared to *Shigella*.[19] In addition, 2 glycol proteins, Lf and fSC, are involved in the inhibition of adhesion of enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) .[20,21] These glycol proteins also prevent the adhesion of *Shigella* dysentery in lower concentrations, found in human milk.

Previous studies have established the association of fucosylated oligosaccharides in human milk with the adhesion inhibition of enter pathogens to Hep-2 cells.[22] Gomez et al revealed that the recombinant Lf has no effect on the adhesion of *Shigella flexneri* M90T to Hela cells. Since the recombinant Lf does not have the same structure, the ability of *Shigella* in the inhibition of adhesion is missed.[17]

Gomez et al in 2002 and 2003 showed that high concentrations of recombinant Lf in *Saccharomyces cerevisiae* protect rabbits against inflammatory bowel diseases caused by *S. flexneri*[21] and interfere with the invasion of *S. flexneri* M90T but do not attach to Hela cells through inducing the separation and decomposition

of ApaB. Similarly, recent studies have found that fSC prevents *E. coli* from binding to the gut and inhibits the adhesion of ETEC.[19,23]

Research has shown that breastfeeding protects the child against many types of infections caused by the *Shigella* species which is remarkable compared to powdered milk. As a result, bacteria were not able to bind to Hela cells, however the rate of inhibition by proteins of powdered milk was considerably lower than that by proteins of breast milk, as only a small number of proteins were able to inhibit the adhesion of bacteria.

Conflict of Interest Disclosures
None.

Ethical Approval
None.

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