Original Article

Effects of Powdered Human Milk Fortifiers on the Antibacterial Actions of Human Milk

Gary M. Chan, MD

OBJECTIVES:

To evaluate the effects of powdered fortifiers and the addition of iron and medium-chain triglycerides on preterm human milk antibacterial activity.

STUDY DESIGN:

Human milk samples were obtained from 42 preterm lactating mothers after the first week of postnatal life. Enfamil® (EHMF) and Similac® (SHMF) Human Milk Fortifiers were evaluated. All mothers were healthy and were on no medications except for vitamins during lactation. The effects of each fortifier against *E. coli* (*E. coli*), *Staphylococcus* (Staph), *Enterobacter sakazakii* (ES), and *Group B Streptococcus* (GBS) were measured by the filter paper method and growth of the bacteria with human milk alone as control. The addition of iron and medium-chain triglycerides (MCT) to human milk was also tested.

RESULTS:

Human milk inhibited the growth of *E. coli*, *Staph*, *ES*, and *GBS*. Only the SHMF and the addition of MCT had similar antibacterial action as human milk alone. EHMF and the addition of iron to human milk removed the milk's antibacterial action against these four organisms.

CONCLUSIONS:

Preterm human milk has antimicrobial activity against *E. coli, Staph*, *ES*, and GBS. This activity can be affected by the addition of iron and fortifiers that contain iron.

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INTRODUCTION

Human milk contains cellular and hormonal factors that serve to protect infants against numerous bacteria, viruses, and fungi.[1] These immunologic factors are important for all infants but especially for the preterm infant who has an immature immunologic state. However, the nutritional content of human milk alone is not sufficient to meet the nutritional needs of preterm infants. Nutrient fortification of human milk is an acceptable and necessary method for feeding the preterm infant. There are two commercially available powdered human milk fortifiers, Enfamil® (EHMF, Mead Johnson Nutritionals, Evansville, IN) and Similac® (SHMF, Ross Products, Columbus, OH) Human Milk Fortifier. The nutrient content of these new fortifiers differs in the amounts of medium-chain triglycerides (MCT) and iron that may affect the antibacterial activity of human milk. The addition of medium-chain monoglycerides to human milk has been reported to inactivate *Haemophilus influenza* and *Group B Streptococcus* (GBS)[2] and iron may affect the milk's lactoferrin activity.[3] However, the effects of these new fortifiers on the anti-infective properties of human milk have not been reported. Therefore, the present study was designed to evaluate the effects of the addition of these powdered fortifiers, iron, and MCT on the anti-infective properties in human milk.

METHODS

Milk Samples

Human milk samples were obtained after complete breast expression from 42 preterm lactating mothers between the 7th and 112th postpartum day, using a sterile breast pump. Samples collected ranged from 30 to 100 ml. All mothers were healthy and were on no medications except for vitamins during lactation. The mean length of gestation was 32 weeks and mean length of lactation was 4 weeks. Samples were frozen immediately at −18°C and used within 4 weeks. Informed consent from all mothers was obtained to participate in this study.

Milk Processing

Each frozen sample was allowed to thaw under cool tap water. To avoid contamination, the water level was not allowed to contact the lid of the container. All thawed milk was kept refrigerated and discarded after 48 hours. All experiments were run with aliquots of the same mother's milk as control.

Human Milk Fortifiers

Enfamil® and Similac® Human Milk Fortifiers were evaluated. Both fortifiers were mixed according to the manufacturer's directions. A volume of 25 ml of mother's milk was mixed with one packet (0.81 g for EHMF and 0.9 g for SHMF) of the fortifier. The

Pediatrics (G.M.C.), University of Utah, UT, USA.

Address correspondence and reprint requests to Gary M. Chan, MD, Pediatrics, University of Utah, 30 North 1900 East, Salt Lake City, UT 84132, USA.

Table 1 Composition of Powdered Fortifiers in four packets per 100 ml

Nutrients	Enfamil® human milk fortifier	Similac® human milk fortifier
Energy, kcal	14	14
Protein, g	1.1	1.0
Fat, g	1.0	0.36
% MCT	70	100
Carbohydrate, g	<0.4	1.8
Vitamin A, IU	950	620
Vitamin D, IU	150	120
Vitamin E, IU	4.6	3.2
Thiamin, μg	150	233
Riboflavin, μg	220	417
Vitamin C, mg	12	25
Calcium, mg	90	117
Phosphorus, mg	50	67
Iron, mg	1.44	0.35

composition of each fortifier is shown in Table 1. The iron in the fortifiers is ferrous sulfate.

Bacterial Inhibition

Bacterial inhibition of the milk samples was evaluated using the filter paper method.[4] A disk of filter paper was soaked with the milk sample and placed on the surface of 5% sheep blood agar plate previously inoculated with either *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (Staph), *Enterobacter sakazakii* (ES), or *Group B Streptococcus* (GBS). Bacterial strains were from clinical isolates from our microbiology lab. The ES was from the American Type Culture Collection 29544. The addition of iron at 1.09 mg ferrous sulfate (0.36 mg iron) per 25 ml and medium-chain triglycerides (MCT) at 0.20 g per 25 ml to human milk was also tested. Both iron and MCT amounts were similar to that in EHMF. After 24 to 36 hours of incubation, the diameter of the clear zone representing inhibition of growth around the disk was measured if present.

Bacterial Growth

Bacterial growth of *E. coli*, Staph, ES, and GBS was analyzed using the method previously described.[5] Human milk samples with the fortifiers were tested. A volume of 1 ml of bacterial suspension in normal saline of 10^5 to 10^7 colony forming units per ml was added to 1 ml of the milk samples. Triplicate aliquots from each milk mixture were plated on 5% sheep blood agar plates after 3.5 hours of incubation at 37°C. The plates were incubated at 37°C for 24 hours and the number of colonies formed was counted.

Statistical Analyses

All statistical tests were two-tailed and the level of significance was set at 0.05. All analysis were performed using GraphPad InStat®. To compare means among the milk groups, ANOVA with post hoc testing using Tukey–Kramer was used. Unless indicated otherwise, the data were expressed as mean ± SD.

Results

Human milk inhibited the growth of *E. coli*, Staph, ES, and GBS using the filter paper method (Table 2). Using this method, SHMF and the addition of MCT to human milk had similar inhibitory effects to human milk alone. However, EHMF caused the milk to have no zone of inhibition, a finding similar to that when iron was added to human milk (Table 2).

After $3\frac{1}{2}$ hours of incubation, *E. coli*, Staph, ES, and GBS grew faster in the human milk with EHMF compared to human milk alone and with SHMF. The growth of these bacteria was similar for SHMF and human milk alone (Table 3).

DISCUSSION

From our preliminary study, we found that human milk from mothers delivering prematurely inhibits the growth of *E. coli*, *Staph*, *ES*, and *GBS*, major organisms causing neonatal sepsis. ES has been associated with powdered infant formula contamination and neonatal infections and death.[6] Besides these organisms, human milk has been reported to be effective against a variety of bacteria, viruses, and protozoas.[1,7] The clinical importance of these findings is illustrated by several epidemiologic studies that have demonstrated fewer infections, especially gastrointestinal and respiratory infection, in breast-fed infants than formula-fed infants.[8] Also, preterm infants fed their own mother's milk are reported to have less necrotizing enterocolitis, respiratory infections, sepsis, and meningitis compared with formula-fed infants.[9,10] However, preterm infants fed fortified human milk have a significant higher infection rate compared to controls.[11]

Table 2 Diameter of the Zone of Inhibition, mm

	n	*E. coli*	Staph	GBS	ES
Human milk	48	25.4±0.5	25.4±0.5	25.5±0.5	24.5±1.0
Human milk+SHMF	39	25.0±0.5	25.5±0.8	25.5±1.0	23.7±1.0
Human milk+EHMF	42	0*	0*	0*	0*
Human milk+iron	39	0*	0*	2.2±6.9*	0*
Human milk+MCT	39	25.6±0.7	26.0±0.8	25.6±0.7	25.5±1.0

*$p<0.001$, vs human milk or SHMF.

Table 3 Bacterial Growth × 10^7 at $3\frac{1}{2}$ Hours

	n	*E. coli*	Staph	GBS	ES
Human milk	32	13.9±6.8	21.3±3.2	3.1±2.5	17.4±4.0
Human milk+SHMF	20	15.4±3.5	19.8±3.6	2.0±3.2	18.1±4.6
Human milk+EHMF	20	28.5±5.8*	30.8±3.3*	6.9±3.8*	36.4±7.4*

*$p<0.004$, vs human milk or SHMF.

The effects of nutrient fortification on bacterial growth have been studied using a previous EHMF.[12,13] EHMF was formulated in 1983 and reformulated three times with the latest reformulation in 2002. Human milk fortification using the older EHMF product did not affect the milk total IgA concentration but did decrease the specific IgA levels to *E. coli*. Fortified human milk had 19% decrease in lysozymal activity, a measure of bacterial lysis. Similar to our study, bacterial growth was also greater in fortified human milk than unfortified milk.[12] In our study, we evaluated a reformulated EHMF, which had higher amounts of MCT and iron than its predecessor.

In the present study, we found that the fortification of human milk can affect its antimicrobial activity. Of the two commercially available powdered fortifiers, EHMF inhibited human milk antimicrobial activity against all four tested organisms *E. coli*, *Staph*, *ES*, and *GBS*. EHMF has more iron and MCT compared to the SHMF which has minimal amounts of iron. Therefore, we questioned if the difference in the amounts of iron and MCT between the two fortifiers may have affected the milk's antimicrobial activity.[2,3] In testing each of these two nutrients, we found that addition of iron but not MCT appears to abolish human milk antimicrobial activity.

The addition of iron has a direct effect on lactoferrrin. Lactoferrin is the major iron-binding glycoprotein in mother's milk. Preterm milk has higher levels of lactoferrin than in term milk from the second to the 12th week postpartum.[14] One of lactoferrin's important antibacterial properties is related to its high iron binding affinity for iron. Lactoferrin can deprive the bacteria or fungus of this essential nutrient for growth.[15–18] The action of lactoferrin depends on an iron-poor environment. Saturation of lactoferrin with endogenous iron abolishes this activity.[3,19] Lactoferrin in human milk is only 5% to 8% saturated with iron.[20] When we added iron directly or the fortifier containing iron to human milk, we may have saturated lactoferrin, thereby decreasing its antibacterial action. Lactoferrin and its peptide fragments may also act directly against the organism cell wall, but this action is probably not dependent on its iron state.[21,22]

It is well known that lactoferrin only binds iron in the ferric state. The iron used in the fortifiers is in the ferrous state. However, the oxidation of the ferrous ion to the ferric state occurs readily upon mixing with human milk. The ferrous iron is oxidized actively in solution.[23–25] Also the presence of low amounts of antioxidants in the milk and fortifier encourages the oxidation of the ferrous ion.

The clinical significance of this effect of the fortification on human milk antimicrobial activity has yet to be determined. Nevertheless, previous studies have suggested that preterm infants may benefit from the antimicrobial activity of human milk.[26–28] We are concerned that mitigating this antimicrobial activity may be detrimental to the health of preterm infants. Iron needs of the preterm infant are important, but the addition of iron directly into human milk may be ill-advised.

In summary, preterm human milk has antimicrobial activity against of *E. coli*, *Staph*, *ES*, and *GBS*. This activity can be affected by the addition of iron or fortifiers that contain a significant amount of iron.

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