A Pilot Study on the Protein Composition of Induced Nonpuerperal Human Milk. Journal of Human Lactation

By: Maryanne Tigchelaar Perrin, Erica Wilson, Ellen Chetwynd, and April Fogleman

Abstract:

Background: Our understanding of the components of human puerperal milk is extensive and increasing, yet the literature on nonpuerperal human milk has been limited to studies that measure the success of induced lactation. **Objective:** This study aimed to describe the composition of total protein and key bioactive proteins when lactation is induced in nonpuerperal women. **Methods:** Two women who induced lactation in the absence of pregnancy provided weekly milk samples over a 2-month period for analysis of total protein, secretory immunoglobulin A (sIgA), lysozyme, and lactoferrin. Composition was compared to the mature milk of 3 puerperal control subjects who were 11 months postpartum. **Results:** Median total protein for subject A was 2.30 g/dL (interquartile range [IQR] = 0.46) and showed a significant downward trend over time ($P < .0001$), whereas the median total protein for subject B was 2.21 g/dL (IQR = 0.18) and showed a nonsignificant decline ($P = .232$). Total protein in both nonpuerperal subjects was elevated compared to control subjects. Secretory IgA activity declined for both nonpuerperal subjects over time, whereas lysozyme concentrations increased over time. Both sIgA and lysozyme approached the levels seen in the puerperal controls. Lactoferrin levels remained stable for both nonpuerperal subjects and were elevated compared to puerperal milk samples. **Conclusion:** This pilot study suggests that nonpuerperal milk has similar or higher levels of total protein, sIgA, lactoferrin, and lysozyme compared to puerperal, mature milk at 11 months postpartum, which warrants more attention as adoptive mothers increasingly choose to induce lactation.

Keywords: adopted children | breastfeeding | human milk | milk composition

Article:

Well Established

Lactation can be induced without pregnancy, and many women are choosing this option. Puerperal human milk has been analyzed under many conditions. We have a growing understanding of the composition and function of many elements of human puerperal milk.

Newly Expressed

A pilot study of sequential, nonpuerperal human milk samples from induced lactation describes comparable or greater concentrations of total protein, secretory immunoglobulin A, lactoferrin, and lysozyme compared to mature milk samples from puerperal subjects.

Background

It is well established that human milk is not only a source of essential nutrients for the infant but also a dynamic substance that reacts to the mother's gestation at the time of birth, the infant's age, whether breastfeeding has been stopped and then resumed, and the immunologic environment of both the mother and the infant.[1-4] Not only does the protein, fat, and carbohydrate content of milk change with both the gestational age at birth and the stage of lactation, the milk also contains important bioactive compounds, which help prevent a variety of infections and promote the health and development of the infant.[5-7]

Given the nutritive benefits of human milk, it is not surprising that women in a variety of settings choose to induce lactation to feed a baby in their care but to whom they did not give birth. Methods of induction of human milk vary but encompass several different types of regimens. The most complex regimens are those that include use of hormones and galactogogues for a period of time and then elimination of the hormones, leaving the galactogogues and adding breast or nipple stimulation.[8-12] In contrast, simpler protocols use breast or nipple stimulation alone or with the addition of herbal or dietary supplements.[13,14] Sometimes, women who induce lactation are biologically related to the babies they are inducing for, such as grandmothers who induce lactation for grandchildren, or biological mothers of surrogate pregnancies, and sometimes lactation is induced in the absence of any genetic connection.[15-17]

Although relatively few studies have looked at induced lactation in humans, the dairy industry has been studying induced lactation in ruminants since World War II, when the majority of an edition of the *Journal of Endocrinology* was devoted to studies of induction protocols, milk production, and milk composition.[18-20] Although these studies focus on macronutrients and not on other bioactive compounds, they show that the composition of induced nonpuerperal milk is similar to that of puerperal milk, and 1 study noted that the transitional period from colostrum to mature milk lasted longer in the induced animals.[18,21]

Our understanding of the components of human puerperal milk is extensive and increasing, yet the literature on nonpuerperal human milk has been limited to studies that measure the success of induced lactation[8,13,14,22,23] and studies of the milk composition of relactation.[4] Despite a growing number of women choosing to induce lactation without pregnancy, we do not yet have an understanding of the composition of the nonpuerperal human milk (induced without pregnancy) that they are producing. The objective of this pilot study was to describe the composition of nonpuerperal human milk as it relates to total protein and select bioactive proteins. Specifically, we looked at lactoferrin, lysozyme, and secretory immunoglobulin A (sIgA), as these proteins are known to be abundant in human milk and provide immunological benefits to the infant, including the lysing and binding of potential pathogens.[24-26]

Methods

Two women interested in inducing lactation in the absence of pregnancy were recruited from a lactation clinic. Subjects were ages 38 (subject A) and 46 (subject B) with no serious medical conditions. Subject A had a body mass index (BMI) of 22.5 and subject B had a BMI of 20.5. Neither participant had been pregnant or lactated previously. Both used a combination of domperidone, birth control pills, pumping, and herbal galactogogues to induce lactation. Each woman recorded a detailed log of hormone and galactagogue use, pumping duration, and milk production volumes. Details of the induction process and milk volumes are described elsewhere.[27] A weekly milk sample was collected with an electric pump for approximately 2 months from the commencement of lactation. All the milk pumped on a given collection day was pooled, and study samples were drawn from the pooled milk and stored in sterile containers in a home freezer until they were picked up by a researcher and transported on ice to the lab at North Carolina State University. Upon delivery to the lab, they were aliquoted and stored at −20°C until assaying. All assays were performed in triplicate by the same researcher using the methods described below. Milk samples from 3 puerperal women that were collected by our lab at 11 months postpartum for another study were used as a control to represent mature, puerperal milk.

Total Protein

Total protein was analyzed using a bicinchoninic acid kit (Pierce BCA protein assay kit; Thermo Scientific, Rockford, Illinois, USA) and spectrophotometry. This technique has been suggested as the best suited method for human milk analysis[28] and is based on the Biuret reaction, where protein in an alkaline medium reduces copper from the cupric state (Cu^{+2}) to the cuprous state (Cu^{+1}). Two molecules of bicinchoninic acid chelate the cuprous ion and change the color of the solution to a deep purple. The degree of color change is proportional to the protein content of the sample. Briefly, 100 µL of human milk diluted 1:10 with deionized water were added to a test tube. Two milliliters of working reagent were added and the samples were incubated at 37°C for 30 minutes in a shaking water bath. Samples were immediately cooled in an ice bath and absorbance was measured at 562 nm using a Spectronic Genesys 2 spectrophotometer (Spectronic Instruments, Leeds, United Kingdom). Known quantities of protein from bovine serum albumin were used as a standard to compute protein quantity in the milk samples. The average intra-sample coefficient of variation (CV) was 2.5%.

Secretory IgA Activity

Secretory IgA activity was quantified based on the milk samples' ability to bind *Escherichia coli* antigens using an enzyme-linked immunosorbent assay (ELISA) previously described by Viazis et al[29] and Chen.[30] A 96 well plate was coated with *E coli* antigens obtained from the National Food Safety and Toxicology Center at Michigan State University and prepared according to Viazis et al.[29] One hundred microliters of human milk samples diluted 1:100 with a 0.01M phosphate buffered saline and 0.05% Tween 20 (PBST) were added to each well, incubated for 3 hours, and washed in triplicate with PBST. One hundred microliters of horseradish peroxidase labeled anti-human IgA from goat (A0295; Sigma-Aldrich, St Louis, Missouri, USA) diluted 1:2000 with PBST were added to each well, incubated for 1 hour, and washed in triplicate with PBST. One hundred microliters of a substrate containing 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (A1888; Sigma-Aldrich) in a .05M citrate buffer

were added to each well and absorbance was measured at 405 nm every 2 minutes for 20 minutes. The rate of absorbance change of known quantities of human IgA from colostrum (I2636; Sigma-Aldrich) was used as a standard to compute sIgA concentrations in milk samples. The average intra-sample CV was 4.5%.

Lysozyme

Lysozyme was analyzed by a bacterial-turbidimetric assay using methods described in detail by Viazis et al.[29] Briefly, lysozyme quantities were determined by the rate of lysis of a *Micrococcus lysodeikticus* (M3770; Sigma-Aldrich) bacterial suspension. Changes in the turbidity of the bacterial suspension were measured over time at 450 nm using a Spectronic Genesys 2 spectrophotometer (Spectronic Instruments), and lysozyme concentrations were computed based on 1 unit of lysozyme producing a .001 change in absorption per minute at a pH of 7.0 and a temperature of 25°C.[31] The average intra-sample CV was 7.0%.

Lactoferrin

Lactoferrin was quantified using a sandwich ELISA kit for lactoferrin analysis in human milk (ab108882; Abcam, Cambridge, Massachusetts, USA). Briefly, 50 µL of human milk diluted 1:100 000 with a diluent were added to a 96 well plate that was precoated with lactoferrin specific antibodies. The plate was incubated for 2 hours and then washed in triplicate. Fifty microliters of biotinylated anti-human lactoferrin antibodies were added to each well, incubated for 1 hour, and washed in triplicate to remove any unbound biotin. Fifty microliters of streptavidin-peroxidase conjugate were added to each well to detect the bound biotin, incubated for 30 minutes, and washed in triplicate. Fifty microliters of chromogen were added to each well, which was catalyzed by streptavidin-peroxidase conjugate to produce a blue color product. An acidic stop solution was added to stop the reaction and change the color to yellow. The density of the yellow color was proportional to the amount of lactoferrin in the sample. Absorbance was read on a Thermo Scientific Multiskan EX spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 450 nm. The average intra-sample CV was 4.2%.

Statistical Analysis

Summary statistics for each assay were calculated by subject. Linear regression was performed on the time series of measurements by subject to look for significant changes in composition of total protein, lactoferrin, lysozyme, and sIgA over time. SAS software version 9.3 (SAS Institute Inc, Cary, North Carolina, USA) was used to perform the analysis. This study received institutional review board approval from the University of Alabama at Birmingham.

Results

The first visible milk appeared after 12 days of pumping for subject A and 2 days for subject B. Both participants were able to induce lactation, with total production levels peaking at 64 mL and 26 mL daily, respectively. Milk appeared white without a period of thick yellow colostrum as would be expected immediately after birth. A total of 19 samples of milk were collected over a period of 2 months.

Total Protein

Table 1 provides summary statistics for subject A and subject B, as well as reference values for mature milk samples provided by 3 puerperal mothers at 11 months postpartum. Mean total protein in the milk of subject A showed a significant downward trend over time ($P < .0001$), whereas the total protein for subject B showed a nonsignificant decline ($P = .232$) over the course of the study (Figure 1A). Total protein in both nonpuerperal subjects was higher than the mean total protein in puerperal mature milk samples throughout the study period.

Table 1. Summary Statistics, Slope from Regression Analysis, and Significance of Change over Time.

Assay	Subject A (n = 10)			Subject B (n = 9)			Mature Milk (n = 3)
	Median (IQR)	Slope	Slope Change	Median (IQR)	Slope	Slope Change	Mean (SD) (Min, Max)
Total protein, d/dL	2.30 (0.46)	–0.0872	$P < .0001$[a]	2.21 (0.18)	–0.0312	$P = .232$	1.38 (0.08) (1.28, 1.43)
Active sIgA, g/dL	0.105 (0.018)	–0.0245	$P = .041$[a]	0.091 (0.025)	–0.0254	$P = .072$	0.089 (0.032) (0.064, 0.125)
Lysozyme, units/mL	32 444 (10 489)	2760	$P = .0002$[a]	9667 (3556)	1014	$P = .403$	42 933 (31 814) (19 733, 79 200)
Lactoferrin, g/dL	0.411 (0.025)	–0.0003	$P = .549$	0.432 (0.050)	–0.0002	$P = .720$	0.178 (0.042) (0.130, 0.206)

Abbreviations: IQR, interquartile range; SD, standard deviation; sIgA, secretory immunoglobulin A.
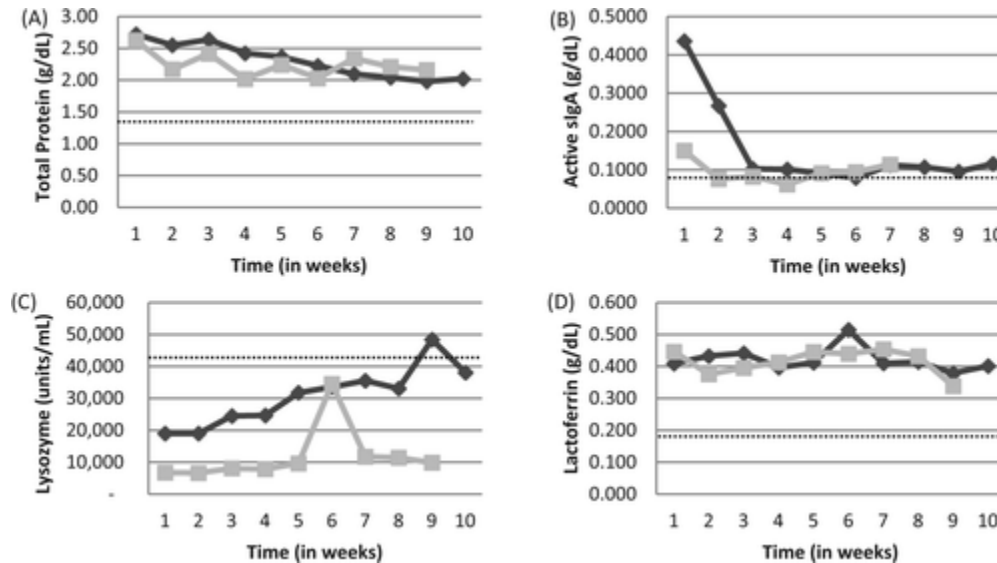[a] $P < .05$ indicates significant change.

Figure 1. Changes in Non-Puerperal Milk Composition over Time.
Changes in composition over time for subject A (-♦-) and subject B (-□-) for the following assays: (A) total protein for subject A ($P < .0001$) and subject B ($P = .232$); (B) secretory immunoglobulin A activity for subject A ($P = .041$) and subject B ($P = .072$); (C) lysozyme for subject A ($P = .0002$) and subject B ($P = .403$); and (D) lactoferrin for subject A ($P = .549$) and subject B ($P = .720$). Levels observed in mature, puerperal milk are denoted by ---------.

Secretory IgA Activity

Secretory IgA levels were variable by subject in early lactation and showed a decline over the course of lactation (Figure 1B), with subject A showing a significant decline ($P = .041$) and subject B approaching significance ($P = .072$). Both subjects showed a steep drop in the first 2 weeks of milk production, followed by levels that stabilized at a value similar to the puerperal control samples from week 3 until the end of the study (Figure 1B).

Lysozyme

Lysozyme levels increased over the course of lactation for both subjects (Figure 1C), with subject A showing a pronounced and significant increase in lysozyme ($P = .0002$), eventually reaching levels similar to the control samples. Increases for subject B were not significant over time ($P = .403$) and only temporarily approached the levels seen in the control samples, before falling again at the end of the study.

Lactoferrin

Lactoferrin levels were similar for both nonpuerperal subjects (Figure 1D), and there was no significant change over time for either subject A or subject B ($P = .544$ and $P = .720$, respectively). Both nonpuerperal subjects had higher lactoferrin levels than was observed in the puerperal mature milk samples over the course of the study.

Discussion

This is the first study that we are aware of to report on the protein composition of human milk when lactation is induced in nonpuerperal women. Longitudinal studies of total protein composition in puerperal human milk have described significant changes in early lactation, with levels falling over the first 2 weeks and then stabilizing to around 1.2 g/dL throughout the course of lactation.[32-34] Our understanding of this process is grounded in a mother's timeline from birth. Narang et al[35] described higher levels of total protein in puerperal milk at day 3 postnatal for mothers who delivered less than 33 weeks' gestation compared to the milk of mothers who delivered between 37 and 41 weeks gestation (mean ± standard deviation = 4.1 ± 0.52 g/dL and 1.9 ± 0.69 g/dL, respectively). Changes in protein composition have also been described during gradual weaning, with protein levels rising to approximately 2.0 g/dL as milk production fell below 300 mL per day.[36] We observed gradually declining total protein levels in weekly samples of nonpuerperal human milk over a 2-month period (Figure 1A), with protein levels at the end of our observation period approaching 2.0 g/dL. These levels were elevated compared to the levels we observed in mature milk and those reported in the literature for term milk at 2 months postpartum.[32-35] A possible explanation for the elevated protein levels in nonpuerperal milk in our study is that neither woman reached levels of milk production greater than 64 mL per day and may not have achieved the physiological changes in the mammary gland associated with stage II lactogenesis.[37] Future research is needed on nonpuerperal milk composition in women who reach higher milk production volumes.

We observed steep declines in sIgA levels in nonpuerperal milk samples (Figure 1B). Secretory IgA levels in puerperal milk have been shown to decline rapidly in the first days of lactation, followed by stable levels during established lactation.[38-40] Mehta and Petrova[41] reported that

there was no change in sIgA levels based on stage of lactation, but they first sampled subjects between day 6 and day 8 postpartum and, therefore, they may not have observed changes that occurred in the first days after the initiation of milk production. Secretory IgA levels stabilized in our samples to levels that were similar to average values assessed by our lab in mature milk samples, suggesting that nonpuerperal milk behaves like puerperal milk in terms of sIgA compositional changes and concentrations.

Lysozyme levels in our study showed increasing trends over time (Figure 1C). Lewis-Jones et al[38] and Mehta and Petrova[41] both reported declines in lysozyme levels over the first 14 days postpartum; however, Lewis-Jones et al found that lysozyme levels began to increase between 43 and 56 days postpartum. An increase in lysozyme levels during established lactation has been reported in other studies.[39,40] Lysozyme levels for subject A and subject B both reached levels near those observed in puerperal mature milk; however, lysozyme levels for subject B subsequently declined, which may be related to a drop in subject B's production volume.

Lactoferrin levels in our study showed no significant change over time for either subject (Figure 1D); however, they were higher than the levels of lactoferrin observed in puerperal mature milk, suggesting that lactoferrin is one of the proteins contributing to a greater total protein concentration in our induced lactation samples. In puerperal milk samples, lactoferrin has been reported to decline over the first weeks of lactation,[38,41] with stable levels reported in mature milk.[38-40]

The components in the milk from subject A showed significant trends in milk composition over time that were consistent with the scientific literature, whereas the milk of subject B showed similar trends, although they were not statistically significant. Subject A consistently achieved higher milk volumes than subject B, which may explain the difference in achieving significance. A limitation of this study is the small sample size of nonpuerperal and puerperal subjects; therefore, future studies with greater sample sizes are warranted. An additional limitation is that longitudinal nonpuerperal milk samples were compared to cross-sectional control samples of mothers at 11 months postpartum. Finally, this study did not look at the composition of lactose, oligosaccharides, fats, hormones, and other compounds due to the small sample volumes.

Conclusion

In this small pilot study, we observed that nonpuerperal human milk had similar or higher levels of total protein, secretory IgA, lactoferrin, and lysozyme compared to puerperal, mature milk at 11 months postpartum, suggesting that the milk produced through induced lactation in nonpuerperal women is a good source of total and bioactive proteins. This pilot study describes a potential similarity in protein composition between puerperal and nonpuerperal human milk that warrants more attention as adoptive mothers increasingly choose to induce lactation. More research is needed on the composition of nonpuerperal milk when higher total milk production is achieved. In addition, future research should look at changes to the composition of induced lactation milk once breastfeeding is initiated to examine whether there is a different response to a suckling child compared to a pump. Other areas of future research include comparing nonpuerperal milk to longitudinal milk samples at the time of parturition, differences in milk

composition and production based on the age and BMI of the mothers who are inducing lactation, and measuring other nutritive and bioactive compounds in nonpuerperal human milk.

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