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Mastitis Is Associated with Increased Free Fatty Acids, Somatic Cell Count, and Interleukin-8 Concentrations in Human Milk

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Abstract

Background: Research in bovine lactation has demonstrated that milk produced by a mammary gland displaying inflammation-based symptoms of mastitis has increased levels of free fatty acids (FFAs) compared with milk produced by a contralateral asymptomatic gland. However, the effects of mastitis on lipid classes in milk have not been investigated in humans.

Methods: The study described here compared milk collected from the symptomatic breast of women with mastitis ($n = 14$) with that collected from the contralateral asymptomatic breast to determine if mastitis caused alterations in the quantity of total lipids, FFAs, and phospholipids (PLs), as well as the fatty acid profiles of these lipid classes. To assess their efficacy as biomarkers of mastitis, samples were also analyzed for selected markers of local inflammation: sodium, somatic cell count (SCC), and interleukin-8 (IL-8).

Results: FFAs were higher in milk from the mastitic breast compared with that from the healthy breast (1.31 vs. 1.07 ± 0.10 g/100 g of lipid, $p < 0.05$). Similarly, SCC and IL-8 were elevated roughly 10-fold in milk from mastitic breasts, compared with milk from healthy breasts, and sodium tended to be higher in milk from mastitic breasts ($p < 0.10$). However, there were no differences in total lipid, PLs, or fatty acid profiles within each lipid class.

Conclusions: In summary, mastitis is associated with increased lipolysis in the human breast but not alterations in milk fat synthesis, as evidenced by a lack of alteration in total milk lipids. Additionally, these results indicate that SCC and IL-8 may be better indicators of mammary inflammation than sodium content.

Introduction

Decades of research have established that breastfeeding is not only the optimal mode of infant nutrition, but that it confers several health benefits upon the lactating mother and her infant not related to nutrition per se. For example, women who breastfeed for a minimum duration of 6 months are less likely to develop postmenopausal breast cancer,[1] and breastfed infants have a reduced incidence of respiratory[2] and diarrheal[3] illnesses and childhood obesity[4] compared with formula-fed counterparts. Nonetheless, in the United States only 43% of women breastfeed their children for at least 6 months.[5] One of the primary causes of precocious weaning is mastitis (breast inflammation)[6]; up to 30% of lactating women suffer at least one episode over the course of lactation.[7] Consequently, it is important to understand the physiological changes that accompany mastitis in order to seek potential alternative forms of prevention and treatment.

Research in the dairy industry has demonstrated that, compared with milk produced by a healthy mammary gland, fresh milk produced by a mastitic gland has elevated levels of free fatty acids (FFAs)[8,9] and when stored at 4°C exhibits greater rates if lipolysis.[10] These composition-related changes are intriguing, as FFAs and monoacylglycerols common to bovine and human milk have demonstrated potent antibacterial properties when added to cultures at concentrations similar to what naturally occurs in milk.[11–13] Indeed, various FFAs have long been investigated for their antibacterial properties against infections of staphylococcal species in skin abscesses.[14] It is therefore intriguing to consider that increased

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lipolysis in the mammary gland, and the subsequent increase in milk FFA concentration, may be an important element of the host's inflammatory response to bacterial infection. However, the potential for this type of alteration in human milk has not been investigated.

Our study used a matched-pair design to examine the differences between milk collected from symptomatic and asymptomatic breasts of women with unilateral mastitis. The primary objective was to test the hypothesis that milk produced by an inflamed mastitic breast has an increased FFA concentration compared with milk produced by an asymptomatic breast. Additionally, we sought to determine the relationship between mastitis and milk lipids by testing for differences in triglycerides (TGs), phospholipids (PLs), and lipid class fatty acid profiles. Lastly, we examined the associations between mastitis and three commonly used indices of mammary gland inflammation: milk sodium concentration, somatic cell count (SCC), and interleukin-8 (IL-8), a chemoattractant that plays a major role in orchestrating immunity.

Materials and Methods

Subjects

The Institutional Review Board at the University of Idaho (Moscow, ID) approved this study. Lactating women ($n = 14$) with a current case of mastitis were diagnosed and referred by physicians or certified lactation consultants in the Moscow, ID area to participate in the study. Mastitis was defined as exhibiting soreness/redness of the breast or pain related to breastmilk expression with or without fever or flu-like symptoms. To participate in the study, women had to be otherwise healthy and breastfeeding at least four times daily. Prior to milk sample collection, written informed consent was obtained, and a questionnaire was completed to ascertain anthropometric data, current antibiotic use, and background health information.

Sample collection and storage

Milk was collected by trained personnel simultaneously from the inflamed, symptomatic breast and the asymptomatic "control" breast using sterile, dual-breastmilk collection kits and an electric breast pump (Ameda®, Lincolnshire, IL). Participants were instructed to continue pumping until the flow of milk had ceased—ensuring the collection of a complete milk expression. Samples were placed on ice and transported to the laboratory as quickly as possible for immediate storage at −80°C within an hour of milk expression.

Quantification of inflammatory markers

SCC was determined for each fresh sample using a commercial somatic cell counter (DeLaval® cell counter, DeLaval International AB, Tumba, Sweden). A 2-mL aliquot of each frozen sample was rapidly thawed and centrifuged at 10,000 *g* for 30 minutes before removal of the cream layer. The aqueous portion was then used to measure the IL-8 concentration using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Sodium concentrations were determined by analyzing the cream-free samples with a sodium-specific electrode (model 8611BNWP, ThermoOrion, Beverly, MA) as previously described.[15] In order to verify that our IL-8 measurements were not altered as a result of the lysis of somatic cells upon freezing samples for storage, a small test was performed. Fresh milk samples collected from the symptomatic and asymptomatic breasts of a woman with mastitis were split in half. Half of each sample was processed by immediately removing cells before freezing, whereas the other half of each sample was processed without removing the cells before freezing. IL-8 measurements of both sets of samples revealed similar results, indicating that the lysis of milk cells during frozen storage did not alter the observed IL-8 concentrations.

Lipid analysis

Lipid was extracted from 2 mL of milk by a modified Folch procedure using 2:1 chloroform:methanol,[16] and lipid content was determined gravimetrically. Lipid classes were fractionated using a modification of the method of Kaluzny et al.[17] In brief, 3 mg of lipid was dissolved in 0.5 mL of chloroform and applied to a 500-mg aminopropyl cartridge (Waters, Milford, MA) attached to a vacuum manifold. Because approximately 98% milk lipid is TG,[18] the volume of chloroform:2-propanol applied to the cartridge to elute the neutral lipid fraction was increased to 32 mL to assure that the TG fraction was completely removed, thereby preventing carryover of TG into the FFA and PL fractions. Elution of FFA was then performed with 4 mL of 2% acetic acid in diethyl ether, followed by elution of PL with 4 mL of methanol. To verify that the fractionation method successfully isolated TG-free FFA and PL fractions, a test sample of each fraction was dissolved in 30 μL of chloroform and applied to a silica gel thin-layer chromatography plate (Alltech Corp., Deerfield, IL). The plate was eluted with 80:20:2 (by volume) hexane:diethyl ether:acetic acid and developed with iodine to visualize the lipid classes and confirm the lack of TG in the FFA and PL fractions. Fatty acid methyl esters (FAMEs) were synthesized from TGs and PLs using a base-catalyzed *trans*-esterification procedure,[19] whereas FFAs were converted into FAME using a two-step methylation procedure.[20] The FAMEs were analyzed on a gas chromatograph (Hewlett-Packard® 6890 Series fitted with an auto injector; Agilent Technologies, Palo Alto, CA) fitted with a flame ionization detector and a 100-m × 0.25-mm (0.2-μm film thickness) capillary column coated with CP-Sil 88 (Varian Inc., Lake Forest, CA) with previously described settings.[21] The fatty acid 13:1 *cis* 12 (Nu-Chek-Prep, Inc., Elysian, MN) was used as an internal standard for the quantification of the FFA and PL fractions.

Statistical analysis

R statistical software (R Foundation for Statistical Computing, Vienna, Austria) was used to employ paired *t* tests to examine differences in lipid fraction concentrations, fatty acid profiles, and inflammatory markers by matching the sample produced by the symptomatic breast from each subject to the sample produced by her asymptomatic breast. Correlations between inflammatory markers were investigated using Pearson's correlation coefficients. Statistical significance was declared at $p < 0.05$, and data are presented as means and the SE of the difference between the paired symptomatic versus asymptomatic samples.

Results

Participant description

Subjects were 29.1 ± 1.0 (mean ± SEM) years of age and of parity 1.9 ± 0.2 children and had an average body mass index of 28.1 ± 2.2 kg/m^2. Time postpartum ranged from 5 to 280 days with a mean of 100 ± 26 days. At the time of sample collection, 10 subjects reported fever or flu-like symptoms, and all subjects described pain associated with lactation. Nine of the subjects had started antibiotic treatment at the time of sample collection; however, they were included in the study because they still displayed clear signs of breast inflammation.

Lipid classes and fatty acids

Mean FFA concentration was higher ($p < 0.05$) in samples produced by the symptomatic breasts compared with those collected from the contralateral asymptomatic breasts (1.31 vs. 1.07 ± 0.10 g/100 g of lipid; Table 1). Whereas there was an elevation in overall concentration, the fatty acid profiles of the FFA fractions showed little difference between the inflamed and control samples (Table 2). Five fatty acids constituted 5% or greater of the relative abundance in the FFA lipid profiles: 18:0 (21.2 ± 0.9), 18:1 *cis* 9 (19.4 ± 1.6), 16:0 (17.2 ± 1.1), 18:2 *cis* 9, *cis* 12 (9.8 ± 0.9), and 12:0 (5.6 ± 0.6).

The overall lipid content was not different between milk collected from symptomatic versus asymptomatic glands (3.58 vs. 3.39 ± 0.25 g/100 g of milk, respectively); likewise, TG fatty acid profiles were similar (Table 3). Much like the TG fraction, the overall quantity of PLs did not differ between milk produced by symptomatic and asymptomatic breasts (1.59 vs. 1.93 ± 0.34 g/100 g of lipid, respectively). Fatty acid profiles of PL fractions (Table 4) were also similar between breasts, with the exception of docosanoic acid (22:0), the level of which was elevated in milk collected from symptomatic compared with asymptomatic breasts (5.7 vs. 4.8 ± 0.23 g/100 g of PL, respectively; $p < 0.05$).

Inflammatory markers

The sodium concentration of milk from symptomatic breasts tended to be greater than that of milk from asymptomatic breasts (7.3 vs. 5.0 ± 1.1 mmol/L; $p < 0.10$). Mean milk SCC from symptomatic breasts was 10-fold greater than that of milk from asymptomatic breasts (1,564 vs. 120 ± 322 cells/μL, $p < 0.05$). Likewise, the concentration of IL-8 was greater in symptomatic versus asymptomatic samples (2,960 vs. 302 ± 647 pg/mL, $p < 0.05$).

Table 1. Comparison of Concentrations of Total Lipids, Free Fatty Acids, Phospholipids, Interleukin-8, Somatic Cell Count, and Sodium in Milk Produced by Breasts with Symptoms of Mastitis Versus Milk Produced by Contralateral Asymptomatic Breasts

	Symptomatic	*Asymptomatic*	*SED*
Total lipids (g/100 g)	3.58	3.39	0.25
FFA (g/100 g of lipid)[a]	1.31	1.07	0.10
PL (g/100 g of lipid)[b]	1.59	1.94	0.34
IL-8 (pg/mL)[a]	2960	302	647
SCC (cells/μL)[a]	1564	120	322
Sodium (m*M*)	7.3	5.0	1.1

Data are mean and SE of the difference (SED) values ($n = 14$).
[a]Different between symptomatic and control samples, $p < 0.05$.
[b]Means calculated with $n = 13$ due to the loss of a sample.
FFA, free fatty acid; IL-8, interleukin-8; PL, phospholipid; SSC, somatic cell count.

Table 2. Comparison of Fatty Acid Profiles of the Free Fatty Acid Fraction of Milk Produced by Breasts with Symptoms of Mastitis Versus Milk Produced by Contralateral Asymptomatic Breasts

Fatty acid	*Symptomatic (g/100 g)*	*Asymptomatic (g/100 g)*	*Difference (symptomatic – asymptomatic)*	*SED*
12:0	5.1	6.0	−0.9	0.5
14:0	4.7	4.6	0.1	0.3
16:0	16.8	17.6	−0.8	0.9
16:1 *cis*-9	1.3	1.1	0.2	0.2
17:0	0.6	0.6	0.0	0.1
18:0	20.9	21.5	−0.6	1.3
18:1 *trans* isomers	2.0	2.2	−0.2	0.3
18:1 *cis*-9	20.4	18.5	1.9	1.8
18:2 *cis*-9, *cis*-12	10.1	9.4	0.7	0.8
18:3 (n-6)	0.3	0.3	0.0	0.0
18:3 (n-3)	0.8	0.7	0.1	0.1
20:0	0.3	0.2	0.1	0.1
18:2 *cis*-9, *trans*-11	0.2	0.3	−0.1	0.1
22:0	0.5	0.6	−0.1	0.1

Data are mean and SED values ($n = 14$).

Discussion

The results of this study support the conclusion that milk produced by a breast displaying symptoms of mastitis has an increased FFA concentration compared with milk produced by the contralateral asymptomatic breast, similar to what has been observed in dairy cattle.[8–10] This elevation of FFA level is interesting because even trace amounts of FFAs have exhibited inhibitory effects against *Staphylococcus aureus*,[11–13] a pathogen commonly associated with lactational mastitis. It is possible that the elevation of FFA is an element of the nonspecific immune response to pathogen-associated lactational mastitis. It is interesting that studies have demonstrated that the potency of these compounds varies among bacterial strains.[11] Therefore the effects of elevated FFA levels on mastitis pathogens would likely vary depending upon the bacterial strain associated with the infection.

The "within-subject" matched-pair element of this study was ideal because it added statistical power by eliminating the effects of inter-individual variation. However, it is important to consider that systemic inflammation may have contributed to increased inflammation/lipolysis not only in milk from the symptomatic breast, but also in milk from the asymptomatic breast. This is a possibility because nine of 14 subjects reported symptoms of fever at the time of sample donation and may help explain why the quantity of FFAs and PLs in the asymptomatic samples was somewhat elevated compared with previously reported values in breastmilk.[18,22]

Table 3. Comparison of Fatty Acid Profiles from Triglycerides of Milk Produced by Breasts with Symptoms of Mastitis Versus Milk Produced by Contralateral Asymptomatic Breasts

Fatty acid	Symptomatic (g/100 g)	Asymptomatic (g/100 g)	Difference (symptomatic – asymptomatic)	SED
6:0	0.1	0.1	0.0	0.0
8:0	0.2	0.2	0.0	0.0
10:0	1.3	1.3	0.0	0.1
12:0	4.8	4.8	0.0	0.2
14:0	5.4	5.4	0.0	0.2
i15	0.1	0.1	0.0	0.0
a15	0.1	0.1	0.0	0.0
14:1 *cis*-9	0.2	0.2	0.0	0.0
15:0	0.3	0.3	0.0	0.0
i16	0.1	0.1	0.0	0.0
16:0	20.5	20.4	0.1	0.2
16:1 *cis*-9	2.1	2.0	0.1	0.1
17:0	0.3	0.3	0.0	0.0
i18	0.1	0.1	0.0	0.0
18:0	6.5	6.6	−0.1	0.1
18:1 *trans* isomers	1.8	1.7	0.1	0.2
18:1 *cis*-9	31.2	31.2	0.0	0.2
18:2 *cis*-9, *cis*-12	16.4	16.5	−0.1	0.2
20:0	0.2	0.2	0.0	0.0
18:3 (*n*-6)	0.2	0.2	0.0	0.0
18:3 (*n*-3)	1.0	0.9	0.1	0.1
18:4 (*n*-3)	0.5	0.6	−0.1	0.1
18:2 *cis*-9, *trans*-11	0.3	0.3	0.0	0.0
22:0	0.3	0.3	0.0	0.0

Data are mean and SED values ($n = 14$).

Table 4. Comparison of Fatty Acid Profiles of the Phospholipid Fraction of Milk Produced by Breasts with Symptoms of Mastitis Versus Milk Produced by Contralateral Asymptomatic Breasts

Fatty acid	Symptomatic (g/100 g)	Asymptomatic (g/100 g)	Difference (symptomatic – asymptomatic)	SED
14:0	0.8	0.7	0.1	0.2
16:0	11.3	11.4	−0.1	1.3
16: 1 *cis*-9	1.0	1.4	−0.4	0.7
17:0	0.4	0.4	0.0	0.1
18:0	21.2	20.3	0.9	1.1
18:1 *trans* isomers	2.1	1.8	0.3	0.2
18:1 *cis*-9	13.1	12.6	0.5	1.2
18:2 *cis*-9, *cis*-12	21.0	20.0	1.0	1.0
18:3 (*n*-6)	1.8	1.0	0.8	0.5
20:0	0.3	0.5	−0.2	0.3
18:3 (*n*-3)	0.6	0.7	−0.1	0.1
18:2 *cis*-9, *trans*-11	0.9	0.9	0.0	0.1
20:3 (*n*-6)	1.3	1.2	0.1	0.1
22:0[a]	5.7	4.8	0.9	0.3
24:0	0.6	0.6	0.0	0.2

Data are mean and SED values ($n = 14$).
[a]Different between symptomatic and asymptomatic samples, $p < 0.05$.

However, subjects indicated that the pain associated with lactation was exclusive to the symptomatic breast. Similarly, IL-8 concentration and SCC were elevated by roughly 10-fold in the symptomatic versus asymptomatic samples. This suggests that, although milk produced from the asymptomatic breast may not have been completely inflammation-free, the comparison that was made captured a large difference in inflammatory status between the glands.

It is interesting that whereas the overall quantity of FFA increased in the mastitic breast, the fatty acid profile of the FFA fraction did not change. This finding may aid in identifying the source of lipolysis that generated the increase in FFAs. In human milk, the placement of several major fatty acids is specific to a particular location on the glycerol backbone.[23] For example, 16:0 is most often located at the *sn*-2 position of the glycerol molecule, whereas 18:0 is most often found at the *sn*-1 position.[24] Consequently, the lack of observed alteration to the quantity of any single fatty acid would indicate that the increase in lipolysis that occurred was not position specific.

Bile salt–stimulated lipase is a prevalent lipase in human milk that does not show specificity for any location when cleaving fatty acids from the glycerol molecule.[25] In contrast, human milk lipoprotein lipase, the other major lipase in milk, displays specificity for fatty acids at the *sn*-1 position.[26] Previous work from our laboratory used a bovine model of mastitis to examine differential gene expression in mammary tissue collected from symptomatic versus asymptomatic quarters in animals with experimentally induced staphylococcal mastitis.[27] In this model, expression of the gene for bile salt–stimulated lipase increased 1.2-fold ($p < 0.03$) in mastitic quarters compared with asymptomatic quarters. These data and the non-stereospecific increase in fatty acids in the FFA fraction demonstrated in the present study suggest that one potential mechanism for the increased lipolysis observed during lactational mastitis may be the activation of bile salt–stimulated lipase as a result of inflammation. Additionally, there are several bacterial lipases that do not display specificity for any location on the glycerol molecule[28]; these lipases also may have contributed to increased liberation of FFAs from the glycerol backbone during infection of the mammary gland.

In contrast with the changes observed in the FFA fractions, the effect of breast inflammation on total lipid and TG fatty acid profiles was negligible; furthermore, the observed values were within the range of what is commonly reported.[29] This is contradictory to a previously published report that found a marked decrease in total lipid of milk produced by women with mastitis.[30] However, that study did not use a within-subject design to compare the lipid content of milk produced by a symptomatic breast and a contralateral asymptomatic breast. Rather, milk samples donated from healthy women served as controls. Furthermore, the aforementioned study did not match the subjects with mastitis to their respective controls by stage of lactation, which is important because total lipid content of milk increases over the course of lactation[18]; stage of lactation, therefore, may have introduced an important confounding variable. Nonetheless, the lack of alteration to total milk lipid and the fatty acid profiles observed in our

study supports the conclusion that mammary gland lipid biosynthesis in humans is not altered by inflammation associated with lactational mastitis.

The majority of lipids in milk are TG contained in small globules encased in a membrane composed primarily of PLs.[31] Therefore, the PL fraction makes up only 0.2–1% of total milk lipid, yet it performs an important structural role. In contrast to work performed in the dairy industry demonstrating a modest decrease in the PL content of mastitic milk,[8] no alteration to the PL content or PL fatty acid profiles was observed this study. This suggests that mammary inflammation does not affect formation of the milk fat globule in women.

At the onset of an inflammatory episode, one of the primary responses of the innate immune system is to activate an influx of immune cells (mostly neutrophils) into the affected area—in mastitis, the mammary gland.[32] The three inflammatory markers tested in this study are all related to this element of the immune response. The measurement of somatic (immune) cells (SCC) in the milk is commonly used in the dairy industry to evaluate severity of mammary inflammation.[33] The concentration of IL-8, a cytokine produced to elicit the infiltration of immune cells, has been shown to increase during inflammatory episodes in bovine[34] and human[35] milks. As the immune cells proceed from the bloodstream into mammary tissue, passing through the paracellular junctions of the mammary alveolar cells, there is an accompanying movement of ions down their respective concentration gradients such that the concentration of sodium in milk increases.[36] Therefore, evaluating milk sodium concentration is common when testing samples for mastitis.[15,37] SCC and IL-8 concentration were both elevated in the milk produced by symptomatic breasts compared with asymptomatic breasts, supporting the presence/existence of mastitis. Sodium concentration only tended to be greater in the symptomatic samples, with concentrations exceeding 12 m*M*—a previously utilized cutoff to indicate mastitis[15]—in only five of the 14 symptomatic samples. Therefore, our data suggest that milk SCC or IL-8 concentrations may be more informative in this regard. Furthermore, the data displayed excellent agreement between these two metrics, further supporting their validity. Considering that IL-8 is a potent chemoattractant that recruits immune cells (particularly neutrophils) to the site of inflammation, it is not surprising that a strong positive correlation ($r = 0.843$) was observed between SCC and IL-8 concentration.[34,35]

In summary, results of this study support the conclusion that in milk produced by breasts displaying signs of mastitis, there is increased lipolysis as evidenced by increased FFA concentration. Furthermore, the lack of alteration to the concentrations and fatty acid profiles of total milk lipids and PLs indicates that the effects of mastitis do not influence lipid biosynthesis or lipid fat globule formation in the human mammary gland. Lastly, human milk IL-8 and SCC may be more effective indicators of mastitis than sodium concentration.

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Disclosure Statement

No competing financial interests exist.

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