ABSTRACT. Objective. Leptin, a hormone present in breast milk, is involved in energy regulation and metabolism. The purpose of this investigation was to determine whether leptin is present in either preterm breast milk (PBM) or preterm formula (PF). The effects of delivery methods and pasteurization on leptin levels also were evaluated.

Methods. PBM samples were obtained from 29 mothers who delivered infants at between 23 and 34 weeks’ gestation. Leptin levels were measured in PBM and PF with the use of a radioimmunoassay specific for human and bovine leptin, respectively. Milk samples were pasteurized by fast- and slow-heating methods. PBM and PF spiked with human leptin were delivered through catheters by bolus and continuous administration to determine the effects of delivery method on recoverable leptin levels.

Results. Median PBM leptin concentration was 5.28 ng/mL (intraquartile range: 24.79). Birth gestational age, birth weight, and gender of the infant did not significantly influence PBM leptin levels. Neither bolus nor continuous feeding practices affected leptin levels in PBM or spiked PF. However, pasteurization significantly reduced the amount of detectable leptin in PBM.

Conclusions. PBM leptin levels were highly variable and similar to levels reported for term breast milk. There was no effect of postnatal age on PBM leptin concentrations. Sterilization decreased detectable leptin levels, whereas feeding practices had no adverse effect on the quantity of leptin delivered. Although no infant formula contained leptin, leptin could be added to formula and delivered through various feeding methods without loss. Pediatrics 2001;108(1). URL: http://www.pediatrics.org/cgi/content/full/108/1/e15; delivery method, infant formula, leptin, pasteurization, preterm breast milk.

ABBREVIATIONS. PBM, preterm breast milk; PF, preterm formula; RIA, radioimmunoassay.

Leptin can no longer be viewed as solely an antiobesity hormone. Although leptin plays an important role in modulating adaptation to energy regulation and utilization in the fasting state, it also affects angiogenesis, wound healing, hematopoiesis, bone metabolism systems, and the neuroendocrine and immune systems. In utero, the fetus is exposed to leptin derived primarily from the placenta and from its own tissues. Premature delivery separates the infant from its principal source of leptin before the late-gestation rise in leptin levels. Premature infants have significantly lower serum leptin levels than full-term infants. This has significant implications for the premature infant, who is in a catabolic state.

Breast milk and formula are the only sources of nutrition and growth factors for the infant in the postnatal environment. Mammary epithelial cells produce leptin, and leptin is secreted into term breast milk. A previous study showed that leptin can pass from mother’s milk into the circulation of rat pups, suggesting that term breast milk is an exogenous source of leptin. Whether preterm breast milk (PBM) and preterm formula (PF) also are a potential source of leptin has not been previously established.

Because human breast milk contains nutrients, growth factors, and other factors that benefit infants, mothers of premature infants are strongly encouraged to provide breast milk for their infants. In the absence of an adequate maternal supply of breast milk, mothers of preterm infants are offered the option of donated banked term breast milk or PFs from the Christiana Care Hospital Milk Bank. Breast milk samples, including all donated and those that test positive for high levels of pathogenic bacteria, are pasteurized to reduce the risk of infectious contamination. Preterm infants are fed through a tube before they are capable of oral feedings. Leptin in term human breast milk is associated with milk fat globules. Although Stocks et al found that the fat in pasteurized, human breast milk adheres to the lining of the feeding tube, Mehta et al showed that the fat in fresh milk does not. Thus, pasteurization and tube-feeding practices may affect the delivery of leptin to the infant.

The first purpose of this study was to determine whether PBM and PF contain leptin. Second, because it is unknown how pasteurization and delivery method affect PBM leptin levels, we also evaluated the effect of these processes on leptin content.

METHODS

Participants and PBM Study Design

A total of 29 mothers consented to the study and donated a total of 42 samples. Five mothers declined because of low milk supply. Criteria for enrollment were as follows: mothers who delivered infants at 23 to 34 weeks’ gestation, mothers who were planning to
lipid hydrolysis. Lipase-treated samples were stained with Sudan black to confirm in all PF samples (Linco Research, St Charles, MO). Aliquots of or a multispecies RIA that can detect the presence of bovine leptin. Leptin levels were determined with the use of commercial RIAs. Samples were incubated at 37°C for 1 hour and then placed on ice. Leptin levels were determined with the use of commercial RIAs, which measure the presence of leptin: premixed Similac Special Care (24 calories), Neosure liquid, and Neosure powder (Ross Laboratories, Columbus, OH); and premixed Premature Enfamil (24 calories) and Enfamil liquid, and Neosure powder (Ross Laboratories, Columbus, OH); and premixed Premature Enfamil (24 calories) and Enfamil (22 calories) liquid and powder (Mead Johnson and Company, Evansville, IN). We spiked formulas, Similac Special Care and Premature Enfamil, with known quantities of leptin to give final concentrations of 10 or 20 ng/mL human leptin to determine whether any is lost by delivery method. The formulas, both unmodified and spiked, were separated into 3 aliquots to be administered as described in Delivery Methods.

Delivery Methods

To determine the effect of feeding practices on leptin levels, we compared baseline levels with postfeeding levels. Mock feedings were performed through bolus or continuous routes of administration. For the bolus delivery method, PBM or PF was placed in a 5-mL syringe with the plunger removed. One end of 50 cm of #5 French enteral feeding tube (Écouen, France) was attached to the needle end of the syringe, and the other was placed in a collection tube. The syringe was held ~30 cm above the collection tube so that the PBM or PF dripped for ~15 minutes into the tube by force of gravity. The same procedure was done for the continuous feeding example except that the nasogastric tubing was attached to an IVAC 710 syringe pump (IVAC Corporation, San Diego, CA) and delivered at a continuous rate over 3 hours. The syringe containing the PBM was held at an angle to prevent the milk from separating during the feed. The collected samples were frozen immediately at ~70°C and then thawed at room temperature at the time of processing.

PBM Sterilization Methods

We evaluated 2 methods of sterilizing breast milk currently used in the United States—fast- and slow-heat pasteurization. Before pasteurization, an aliquot of unpasteurized PBM was removed and stored for baseline comparison. For the fast-heat method, PBM was autoclaved at a constant temperature of 100°C for 5 minutes. For sterilization with the use of the slow-heat method, a glass jar containing PBM was submerged for 30 minutes in a 57°C agitating water bath.

PF and Study Design

The following commonly used PFs were assayed to determine the presence of leptin: premixed Similac Special Care (24 calories), Neosure liquid, and Neosure powder (Ross Laboratories, Columbus, OH); and premixed Premature Enfamil (24 calories) and Enfamil (22 calories) liquid and powder (Mead Johnson and Company, Evansville, IN). We spiked formulas, Similac Special Care and Premature Enfamil, with known quantities of leptin to give final concentrations of 10 or 20 ng/mL human leptin to determine whether any is lost by delivery method. The formulas, both unmodified and spiked, were separated into 3 aliquots to be administered as described in Delivery Methods.

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Leptin Level Measurement

All samples were thawed at room temperature before analysis. Pancreatic lipase (3 μL) and 6 μL of 1 M sodium bicarbonate were added to 600 μL of either breast milk or formula to degrade triglycerides thought to interfere with the radioimmunoassays (RIAs). Samples were incubated at 37°C for 1 hour and then placed on ice. Leptin levels were determined with the use of commercially available RIAs specific to human leptin for all PBM samples or a multispecies RIA that can detect the presence of bovine leptin in human PBM samples (Linco Research, St Charles, MO). Aliquots of lipase-treated samples were stained with Sudan black to confirm lipid hydrolysis.

Western Blot Assay

PBM and PF samples were diluted to protein concentrations of 3 and 5 mg/mL in sample buffer containing 0.1 M dithiothreitol as a reducing agent. The samples were electrophoresed on a 4% to 15% polyacrylamide gradient gel, and the separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as described by Fawcett et al.9 The PF protein blots were probed with a polyclonal rabbit leptin antibody that recognized multispecies leptin (Linco Research). PBM protein blots were probed with a monoclonal goat antihuman leptin (R&D Systems, Minneapolis, MN).

Creamatocrit and Protein Content

The percentage of fat was measured for all PBM samples with the use of the creamatocrit method described by Lucas et al.20 Each sample was run in duplicate, and the average of the duplicates was reported. Protein concentrations were determined with the use of the Bradford microassay (BioRad, Richmond, CA).

Statistical Analysis

Demographic data are described as mean and standard deviation. Nonparametric testing was used because leptin was significantly positively skewed (K-S Lilliefors tests) and secondary outcome variables used small n values. The influence of postnatal age (2- and 4-week samples) was determined by Wilcoxon signed rank test. The effects of feeding practice, pasteurization method, and gender on leptin were performed with the use of Mann-Whitney and Kruskal-Wallis analysis of variance when appropriate. The relationship between gestational age and leptin was analyzed through regression analysis. Demographic data are described through parametric distributions. The primary outcome being studied, whether leptin is present in PBM, had not been previously determined. Power determination for secondary outcomes, such as the effect of feeding methodology and pasteurization, were based on previous data from our laboratory on term breast milk. Significance was set at α = 0.05 (2-tailed), β = 0.2. All analyses were conducted with the use of Statistica 5.1 (StatSoft, Inc, Tulsa, OK).

RESULTS

Leptin Levels in PBM and PF

The median leptin level for the PBM samples was 5.28 ng/mL, with an intraquartile range of 24.79. The mean postmenstrual age at the time of donation was 30 ± 2 weeks (range: 25–35 weeks). Gestational age, birth weight, and gender of infant did not significantly influence leptin levels with P values of 0.4, 0.6, and 0.9, respectively. Freezing and thawing fresh PBM 3 times had no effect on leptin levels. All samples were run in triplicate. The leptin levels were averaged, and the means were used for all comparisons. Mean breast milk leptin level at 2 weeks’ postnatal age was 6.02 ± 8.97 ng/mL and did not differ from that of 5.18 ± 4.96 ng/mL at 4 weeks’ postnatal age for 11 case-matched samples (P = .37).

Bovine leptin initially was detected in the PFs as determined by RIA. After treatment with pancreatic lipase, leptin levels detected in powder or concentrated formulas with the RIA were reduced to 2.8 ng/mL. We found that the supplemental iron in PFs also produced interference (2.46 ng/mL), which explained the low levels that we detected even after enzyme treatment. Emulsifiers are added to formulas to maintain homogeneity in the ready-to-use solutions. The formula manufacturers maintain emulsifier composition and concentrations as proprietary information. Therefore, we could not develop an RIA-emulsifier interference standard curve. To overcome this problem, we assayed ready-to-use and powder formulations for Neosure and Enfamil and found interference with the ready-to-use formula but not from the powdered form. This suggests that the
leptin levels that were detected initially most likely were attributable to interference from the added emulsifiers and iron. The European formula of Similac has a different emulsifier preparation than that made in the United States and demonstrated no detectable leptin levels. Western blot analysis confirmed that there was no detectable leptin protein in PFs (data not shown).

Effect of Sterilization on Leptin Levels, Protein Content, and Fat Concentration in PBM

Sterilization by the slow-heat (9.15 ± 9.72 ng/mL) or fast-heat method (9.17 ± 15.46 ng/mL) significantly decreased detectable leptin levels in PBM compared with unpasteurized PBM (25.37 ± 22.59 ng/mL; $P = .013$ and $P = .009$, respectively). Similar to the effect on leptin, fast-heat pasteurization significantly decreased the percentage of fat ($P = .001$). However, this effect was not seen with slow-heat sterilization ($P = .39$). Total protein content was not significantly decreased by either sterilization method ($P = .36$). There was no difference between the 2 pasteurization methods on total protein content ($P = .14$).

Effect of Delivery Method on Leptin Levels in PBM and PF

There were no differences in either delivery method on leptin levels in PBM. Human leptin supplemented to PFs was recovered in full after mock feedings (Table 1).

**TABLE 1.** Leptin Levels (ng/mL) in Spiked Preterm Formulas Before and After a Mock Feeding*

<table>
<thead>
<tr>
<th>Delivery Method</th>
<th>SSC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>23.22 ± 0.17</td>
<td>23.92 ± 0.82</td>
</tr>
<tr>
<td>Bolus</td>
<td>20.49 ± 5.15</td>
<td>19.53 ± 2.6</td>
</tr>
<tr>
<td>Continuous</td>
<td>21.16 ± 1.5</td>
<td>28.98 ± 12.6</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviation. Spiking and delivery methodologies for Similac Special Care (SSC) and Premature Enfamil (PE) are described in “Methods”.

**DISCUSSION**

We are the first to show that leptin is present in PBM. The leptin levels in human PBM were highly variable but similar to levels noted in term breast milk.13–15 We did not measure leptin in premature fore- and hindmilk because leptin levels did not differ in these.18 Maternal factors21–26 and neonatal factors21,27 in part influence infant serum leptin variability. In our study sample, there was no effect of infant gender on breast milk leptin concentrations. The risk of a type II error may exist for gender, because the study sample size was not designed to answer this question. Whether the same factors that influence serum leptin levels also influence breast milk leptin concentrations warrants additional investigation.

Breast milk leptin levels did not vary between 2 and 4 weeks of postnatal age. This period may not be long enough to detect a change in PBM leptin levels. A previous study that investigated changes in breast milk leptin levels over time did not demonstrate an increase until after 4 weeks of lactation (D. O’Connor, et al, unpublished data). We suspect that leptin concentrations in breast milk are not dependent on gestational age at time of birth. A change in the hormonal balance after pregnancy, such as a decrease in estrogen, progesterone, and possibly leptin, permits prolactin to initiate lactation.

In addition to determining the presence of leptin in PBM, we investigated whether milk bank processing and feeding practices affected the quantity of leptin delivered to the infant. Pasteurization adversely affected PBM leptin concentrations, whereas method of delivery had no effect. Pasteurization did not lower total protein levels but did lower leptin levels. We speculate that this difference is because of irreversible denaturation of leptin by heat. Although others16 showed that fat sticks to the nasogastric tubing, the more recent work of Mehta et al17 showed that milk fat does not stick. One possible explanation for this difference is that Stocks et al16 used banked, pasteurized breast milk, whereas Mehta et al17 used unpasteurized PBM. Pasteurizing the milk most likely disrupted the milk fat globules, allowing the fats to adhere to the tubing. In support of this hypothesis, we showed that leptin associated with milk fat globules does not stick to nasogastric tubing. This is important because very premature infants are fed by this method until they are able to coordinate their feeding reflexes.

We hypothesized that premature formulas do not contain leptin because whey proteins added to formula are isolated from skim, bovine milk, and leptin associated with milk fat globules would be removed during the skimming process. On the basis of our study results, there was no detectable leptin in the formulas we analyzed. Although leptin can be added to formula and delivered through standard feeding methodologies, additional studies are necessary to determine how well leptin is absorbed by the premature infant. Leptin may have a protective role for the premature infant who exists in a high-stress environment. Premature infants often are catabolic and nutritionally deprived, which alters their immunologic status. Leptin affects immunologic potential by stimulating proliferation and differentiation of hematopoietic precursors and increasing the number of macrophages and granulocyte colonies.28,29 This argues that administration of leptin to premature infants may be beneficial. Additional investigation into the role of exogenous leptin in PBM or supplemented formula preparations is warranted.

**CONCLUSION**

Our study is the first to show that leptin is present in PBM but not in PF. We showed that 2 methods of pasteurization significantly reduced the amount of detectable leptin in PBM. Common methods for delivering food to premature infants did not affect leptin concentrations in either PBM or spiked PF. Additional studies are needed to determine the factors that influence PBM leptin levels. This may have particular importance to the premature infant.
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