

Concentración de grelina y leptina en suero y leche humana en madres lactantes según el tipo de alimentación

Concentration of ghrelin and leptin in serum and human milk in nursing mothers according to the type of feeding

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ABSTRACT

Objective: we assessed the relationship between serum and human foremilk and hindmilk concentrations of ghrelin and leptin in nursing mothers according to the type of feeding.

Methods: this cohort design was carried out on 131 mother-newborn dyads admitted to a physiological puerperium ward. The independent variables were the type of feeding, full breastfeeding (FBF, 56.5%) and

partial breastfeeding (PBF, 43.5%). The dependent variables were the concentration of total ghrelin (pg/ml) and leptin (ng/ml) in serum, foremilk and hindmilk at eight and 16 weeks. Fasting blood samples were obtained from the nursing mothers at four months for serum assays. Unpaired Student's t-test, Mann-Whitney U test, Pearson's correlation tests, coefficient of determination and linear regression were used.

Results: the concentration of ghrelin and leptin in hindmilk was higher than that of foremilk in both groups at eight and 16 weeks. The concentration of ghrelin and leptin was higher in serum than in foremilk in both groups. These values showed a direct and significant linear correlation with the exception of ghrelin in the FBF group. The serum concentration of leptin in mothers explained 32% of the variance of its concentration in foremilk in the FBF and 13% in the PBF groups.

Conclusion: the hindmilk/foremilk gradient suggests an intake regulating mechanism during the fed. The concentration of ghrelin and leptin was higher in the serum than in foremilk and its correlation and determination coefficients could suggest plasma-milk transfer in addition to synthesis regulation by the mammary gland, adipose tissue or other organs.

Key words: Human milk. Leptin. Ghrelin. Nursing mothers. Foremilk. Hindmilk.

RESUMEN

Objetivo: evaluar la relación entre la concentración de suero y la leche materna y la concentración de grelina y leptina en leche materna en madres lactantes según el tipo de alimentación.

Métodos: diseño de cohorte realizado en 131 diadas madre-lactante que ingresaron en una sala de puerperio fisiológico. Variables

independientes: tipo de alimentación, lactancia materna completa (LMC, 56,5%) y lactancia materna parcial (LMP, 43,5%). Variables dependientes: concentración sérica de grelina total (pg/ml) y leptina (ng/ml), leche humana pre-tetada y pos-tetada a las ocho y 16 semanas. Se utilizaron pruebas no pareadas t de Student, U de Mann-Whitney, correlación de Pearson, coeficiente de determinación y regresión lineal.

Resultados: la concentración de grelina y leptina en leche humana pre-tetada fue mayor que en leche humana pos-tetada en ambos grupos a las ocho y 16 semanas. La concentración de grelina y leptina fue mayor en suero que en leche humana en ambos grupos; estos valores mostraron una correlación lineal directa y significativa con la excepción de la grelina en el grupo de LMC. La concentración sérica de leptina en las madres explicó el 32% de la varianza de su concentración en leche humana en LMC y el 13% en madres en LMP.

Conclusión: el gradiente de leche humana pre-tetada/pos-tetada sugiere un mecanismo de regulación e ingestión de leche. La concentración de grelina y leptina fue mayor en suero que en leche humana y los coeficientes de determinación sugieren una transferencia de suero-leche, además de una regulación de la síntesis por la glándula mamaria, el tejido adiposo u otros órganos.

Palabras clave: Leche humana. Leptina. Grelina. Madres lactantes. Leche humana pre-tetada. Leche humana pos-tetada.

INTRODUCTION

The World Health Organization (WHO) (1) recommends offering “exclusive breastfeeding for the first six months, the time when safe and nutritious foods are introduced while breastfeeding continues and can be extended to the second year of life”. The creation of the WHO growth standard of healthy breastfed infants supports the perception that

growth and cognitive development are optimal when this standard is adhered to, and that formula-fed infants deviate from this reference (2,3). Theoretically, exclusive breastfeeding (EBF) for six months propitiates the ability to self-regulate energy intake by the infants according to their needs, unlike formula-fed infants (4,5). It has also been noted that the protective role of breastfeeding against the development of obesity could be partially explained by the composition of human milk and also by the presence of appetite-regulating hormones (ARHs) (6-8).

Breast milk contains many bioactive factors, including growth factors and hormones. It has been hypothesized that breast milk may act as a metabolic messenger between mother and infant, whose composition influence infant appetite and growth (9). ARHs have been identified in breast milk, and it has been suggested that they may also influence infant appetite and appetite-regulatory pathways (10). Andreas et al. have pointed out that if breastfeeding is capable of protecting infants against obesity in later life, this may potentially be partially explained by the presence of these ARHs (9). ARHs concentration has been investigated in foremilk and hindmilk to establish whether they changed over the course of a feeding, potentially acting as hunger or satiety factors. Apparently, leptin was not observed to alter its concentration between foremilk and hindmilk. Other investigators have shown lower concentration of ghrelin in hindmilk in comparison with foremilk, and have observed higher concentration of leptin in hindmilk (11,12).

It is accepted that satiety and ARHs play a role in the regulation of food intake and body composition by signaling satiety and energy reserves through hypothalamic receptors, during and after the lactation stage (13,14). Appetite-stimulating hormones, such as ghrelin, are important in the initiation, cessation and frequency of eating (15). Satiety-regulating hormones, such as leptin, decrease food intake, promote satiety,

decrease the desire to eat, and increase the metabolic rate (11). Therefore, the purpose of this report is to show the relationship and differences between serum and human milk concentration of ghrelin and leptin in nursing mothers, between human milk and serum concentration in their infants and before and after breastfeeding in FBF and partially breastfed (PBF) infants.

METHODS

This non-random cohort design was carried out on mother-newborn dyads who were admitted to a physiological puerperium ward in a shared room at the Nuevo Hospital Civil de Guadalajara Dr. Juan I Menchaca (NHCG DJIM). The independent variables were the type of feeding and the dependent variables were the human milk and serum ARHs total ghrelin (pg/ml) and leptin (ng/ml). Subjects were included and evaluated at two and four months if they met the inclusion criteria: healthy postpartum women living in the metropolitan area of Guadalajara who had signed an informed consent form and had a healthy full-term single infant of either sex, with an adequate weight for his/her gestational age. Dyads were not included when mothers had a history of chronic, genetic, or congenital diseases, addiction to alcohol, tobacco, or drugs, or if their newborns had congenital malformations and/or genetic diseases; dyads were also excluded by maternal causes such as loss of follow-up, presence of intercurrent subacute or chronic disease, occurrence of serious accident and/or infant causes such as subacute or chronic disease, occurrence of an accident, serious illness, or incomplete data regarding the mother or infant. With averages and variances of serum leptin from nursing and non-lactating mothers (16), an alpha of 0.05 and a power of 0.80. The sampling system was non-probabilistic at the site of birth concentration.

Measuring instruments and techniques

Human milk

Human milk was collected from the mothers at eight and 16 weeks before and after breastfeeding. It was obtained with a breast pump and collected in previously labeled plastic containers. Immediately after collection, all of the samples were cold centrifuged at 4 °C at 3,000 rpm for ten minutes. The top layer of fat was removed using a Pasteur pipette and the remaining liquid phase was divided into eight aliquots. All of the aliquots were placed in a 0.6 ml tube and stored at -80 °C, until the day of the assay. The quantification determination of the leptin and ghrelin levels in the breast milk samples was performed using commercial ELISA kits (Leptin Ultrasensitive, ALPCO® USA and Human Ghrelin Total, EMD Millipore, Billerica, MA, USA, respectively) and processed according to the manufacturer's instructions. All of the samples were duplicated and 1:5 dilutions were made for the leptin assay. The sensitivity of the leptin assay was 0.01 ng/ml while for ghrelin it was 50 pg/ml. Dilutions were made with the standards provided in the trials, and were analyzed in duplicate to generate standard curves of the hormones and subsequently adjusted to a four-parameter logistic regression model (4-PL). Both curves showed a correlation coefficient (R^2) above 0.95. The ghrelin and leptin sample levels were interpolated from the standard curve.

Collection of blood assays

At 16 weeks of age, postpartum blood samples were obtained from the mother (3 ml) while fasting. To stabilize the ARHs, the samples were placed in Vacutainer® EDTA anticoagulant tubes treated with aprotinin protease inhibitor (500 KIU/ml, EMD Millipore, Billerica, MA, USA) and inhibitor of dipeptidyl peptidase-4 (DPP4, 10 µl/ml, EMD Millipore, Billerica, MA, USA). After collection, the plasma was separated by

centrifugation at 3,000 g for 15 minutes at 4 °C, and aliquoted in 0.6 ml tubes; then, they were stored at -80 °C. The leptin hormones (R&D Systems, Minneapolis, MN, USA) and ghrelin (EMD Millipore, Billerica, MA, USA) were analyzed with commercial ELISA kits. For the determination of leptin the samples were diluted 1:20. The sensitivity of the assay for leptin and ghrelin was 7.8 pg/ml and 30 pg/ml, respectively. The concentrations of the hormones were determined from curves generated from the standards of each kit, using a logistic regression model of four parameters. Both curves showed a correlation coefficient (R^2) above 0.95. The levels of leptin and ghrelin in the samples were interpolated from the standard curve.

Field work criteria and strategies

The recruitment of the dyads was performed at a physiological puerperium ward of the Gyneco-Obstetrics Division of the NHCG DJIM. Mothers were invited to participate after researchers (EGM and NME) promoted full breastfeeding for at least six months. We clarified that we were interested in including all of the mothers who wanted to participate regardless of the mode of feeding that they chose for their infants. At eight and 16 weeks, samples of human milk were collected from the mothers before and after feeding their infants; at 16 weeks a blood sample was also obtained from both.

Collection of information, databases and computer programs

Once the information was obtained, the database was elaborated, the data were captured, and the statistical analysis was performed using the Statistical Package for Social Sciences version 24.

Statistical analysis

Levene's test was used to assess equality of the variances and for two or more groups, and Shapiro-Wilk and Kolmogorov-Smirnov tests were used to explore the normality of the distributions. The unpaired Student's t tests were used to show the contrast between two independent samples with normal distribution. In variables with non-normal distribution, the Mann-Whitney U test on samples was used. Linear regressions and Pearson's correlation coefficient between parametric variables and Spearman's correlation between non-parametric variables with very wide variances were also obtained. The level of significance was a p value ≤ 0.05 .

Biosecurity

The handling of the biological samples was carried out according to the specifications of the Mexican Official Standard NOM-087-ECOL-SSA1-2002. The chemical substances were handled and stored in accordance with the Mexican Official Standards NOM-052-SEMARNAT-2005 and NOM-054-SEMARNAT-1993, in addition to information indicated in the biosafety sheets for each chemical substance used in each experiment.

Ethical considerations

The recommendations of the Declaration of Helsinki were followed in its last amendment during the 64th Annual Assembly organized by the World Medical Association, 2013. The protocol was applied to each of the participating dyads that met the inclusion criteria once the mother had given her authorization by signing the informed consent form. The protocol was approved by the Committees of Bioethics and Research of the HCG DJIM and the Committees of Biosecurity, Bioethics and Research at the University of Guadalajara, Center of Health Sciences (CI-01314).

RESULTS

A total of 131 mothers of infants who were cared for at the NHCG DJIM from the birth of their children were studied. Seventy-four (56.5%) fed their children with FBF and 57 (43.5%) with PBF. The age of the mothers in the FBF group was 23.7 (\pm 4.6) years and that of the PBF group 23.5 (\pm 4.5) years, without difference of means ($p = 0.819$).

HRAs in human milk, comparison between groups

The results of the comparison of HRAs in human milk are presented in table I. The concentrations of ghrelin and leptin on milk samples taken at eight and 16 weeks, before and after the feeding to the breast between the FBF and PBF groups are presented in table I. In some cases, it was not possible to obtain enough breast milk for the ARHs assay, particularly in the postprandial sample. The comparison of the ghrelin concentration between the FBF and PBF groups at eight and 16 weeks and both in the foremilk and hindmilk did not show statistical differences. In the PBF group, the concentration of leptin in breast milk was significantly higher in the hindmilk than in foremilk at eight but not at 16 weeks.

HRAs in human milk, comparison within groups

In the FBF group the concentration of ghrelin in the hindmilk was higher than in the foremilk at eight and 16 weeks. In the PBF group, no differences were observed in the concentration of ghrelin in foremilk when compared with hindmilk at eight and 16 weeks.

In the FBF group, the comparison of leptin in foremilk and hindmilk was greater at 16 weeks compared to eight weeks. In the PBF group no differences were observed in foremilk or hindmilk when comparing their concentration between eight and 16 weeks.

HRAs in the serum of mothers and in human milk

The comparison, correlations and linear regressions between the serum of the mothers and breast milk are presented in table II. The concentration of ghrelin and leptin was higher in serum than in foremilk in both groups. The correlations and linear regressions between the concentrations of ARHs in maternal serum (assigned as independent variable) *versus* the concentration in milk (assigned as dependent variable) are shown in table II. The correlation of the serum ghrelin values with those of human milk showed a nonsignificant trend in the FBF group. However, in the PBF group they presented a weak significant linear correlation with a prediction value less than 1. The values of the leptin showed a direct and significant linear correlation. The leptin serum concentration in the mothers explained 32% of the variance of concentration in milk in the FBF and 13% in the PBF groups.

DISCUSSION

Almost no differences were found in the comparison of FBF *vs* PBF regarding foremilk and hindmilk ghrelin concentration and in foremilk and hindmilk leptin concentration at eight and 16 weeks postpartum except for hindmilk leptin at eight weeks, which was higher in the PBF group. Our results were very similar to those reported by Karatas et al. (12). These findings are likely to coincide with the greater amount of fat in the hindmilk at two months of postnatal infant age, which is a critical time of lactation. It is known that hindmilk is a high-fat, high-calorie human milk (17); in addition, it has also been described that leptin is produced and secreted by mammary epithelial cells in milk fat globules, although other amounts of leptin may be transferred from the blood to milk by secretory epithelial cells (18,19).

The concentration of ghrelin in human milk in the FBF group was significantly higher at eight and 16 weeks postpartum in the foremilk *vs* hindmilk sample ($p < 0.001$). Andreas et al. (9) also found higher

concentration of ghrelin and leptin in foremilk vs hindmilk at three months of postnatal age. The powerful orexigenic action of ghrelin is well known and one might think that the reason for this is the body's natural pretension to stimulate the infant's appetite so that he obtains a greater quantity of human milk in a critical period of growth and mass increase. It has been suggested that ghrelin in human milk could be absorbed by the infant's gut and thus influences metabolic pathways and growth based on infant's needs (20). Savino et al. have pointed out that considering the orexigenic function of ghrelin, its presence in human milk may directly influence milk intake in breastfed infants, acting on feeding behavior, and control infants growth in the early period of life (21). Ghrelin concentration in human milk has been found to increase during lactation and it correlates with serum ghrelin levels in BF infants (22).

It was also noted that in the PBF group the concentration of ghrelin in the foremilk at eight weeks postpartum was significantly higher than the concentration of this hormone in the foremilk at 16 weeks postpartum. It is likely that it is due to the same reason. At four months the infant has practically doubled his weight and has less need of a higher amount of milk than the mother already produces at that infant's age.

A slightly more contradictory effect is observed in infants receiving PBF because the concentration of ghrelin is slightly lower in the foremilk phase than in the hindmilk phase at eight weeks postpartum ($p = 0.026$). However, at 16 weeks postpartum the foremilk concentration of ghrelin is significantly lower than the hindmilk ghrelin concentration ($p < 0.001$). These results do not coincide with others (12). It is likely that a gradually smaller amount of human milk and gradually higher amount of human milk substitutes is the reason for a lower production of ghrelin in the first moment of milk extraction by the infant.

In the FBF group the concentration of leptin was higher at eight weeks postpartum in the foremilk phase than at 16 weeks postpartum in the same phase ($p = 0.046$). It is likely that this effect is related to the greater adiposity that characterizes the infant in the fourth month of life. If this effect is valid, the adiposity of the infant would influence more than the adiposity of the mother in the concentration of leptin in human milk. In fact, some investigators (9) have suggested that the concentrations of ghrelin and leptin in human milk are regulated independently of maternal body mass index, with other factors determining their concentrations in this milk.

The estimation of the correlation and the coefficient of determination between the serum concentration of ghrelin and its concentration in human milk was observed to be discrete in both the FBF ($r = 0.222$, $p = 0.065$) and PBF ($r = 0.208$, $p = 0.044$) groups. This finding would indicate that the concentration of ghrelin in human milk would reflect the needs of the infant more than the concentration of this hormonal biomarker in the mother's serum, which would be more related to her own physiological needs. Our results partially agree with those in other studies in which ghrelin levels in human milk correlated positively with maternal serum concentrations and that ghrelin has a direct passage from serum to milk; its production by the mammary glands is more likely (23).

In contrast, the concentration of leptin in maternal serum correlated more with its concentration in human milk in both the FBF group ($r = 0.570$, $p < 0.001$) and in the PBF group ($r = 0.366$, $p = 0.013$). A particularly noticeable effect was observed in the FBF group. The characteristics of the mother, such as her body composition and adiposity, would have a greater influence on the concentration of anorexigenic hormones in human milk. This finding could be interpreted as an effect of prevention of an excessive consumption of food of the

infant, trying to avoid an excess of adiposity as it has been pointed out in other studies (21,24,25). Leptin is known to play an important role in the central regulation of energy balance, acting both at the level of the hypothalamus, inhibiting the hunger center with an anorexigenic effect, and at the peripheral level, inhibiting the synthesis of fatty acids and triglycerides and increasing the oxidation of fatty acids (21).

One strength of this study is the determination of ghrelin and leptin in human milk in FBF and PBF at eight and 16 months of postnatal age, two crucial moments of infant's growth. One limitation is that we determined total ghrelin, which is the sum of the acylated and deacylated ghrelin instead of the acylated form (known as active ghrelin) because it is thought to be essential for binding to the growth hormone secretagogue receptor 1a. However, the deacylated form is not totally inactive; it has influence on both cell proliferation and adipogenesis and counteracts the metabolic effects of active ghrelin (12).

In conclusion, the comparison of the concentration of ghrelin and leptin in human milk was made under different approaches: foremilk vs hindmilk in FBF and in PBF; two different moments of breastfeeding, eight and 16 weeks postpartum and FBF vs PBF. Our results showed significant differences with ghrelin and leptin that might have different interpretations. Ghrelin concentration in human milk showed a mild positive correlation with maternal serum concentration; therefore, it is likely that the main concentration in human milk comes from the mammary glands. In contrast, the concentration of leptin in maternal serum highly correlated with the concentration in human milk. It is likely that the characteristics of the mother, probably her body composition and adiposity, would have a greater influence on the concentration of this hormone in human milk.

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Table I. Ghrelin (pg/ml) and leptin (ng/ml) concentration in milk of lactating mothers according to the type of feeding at eight and 16 weeks postpartum and at the time of lactation (foremilk and hindmilk)* (the p-values < 0.05 of the intragroup comparisons are noted at the bottom of the table)

ARH	Age (weeks)	Feeding time	Full breastfeeding			Partial breastfeeding			p [†]
			n	Median	IQR	n	Median	IQR	
Ghrelin	8	Foremilk	74	154.1	75.1	53	192.2	113.1	0.510
		Hindmilk	74	178.8	88.1	47	185.4	87.3	0.832
	16	Foremilk	74	162.4	102.1	51	168.2	96.0	0.800
		Hindmilk	69	210.6	153.1	47	211.2	115	0.948
Leptin	8	Foremilk	69	0.316	0.50	54	0.359	0.63	0.298
		Hindmilk	68	0.317	0.57	46	0.468	0.6	0.032
	16	Foremilk	67	0.447	0.48	46	0.574	0.12	0.407
		Hindmilk	62	0.411	0.64	43	0.492	0.01	0.393

ARH: appetite regulatory hormones; FBF: exclusive breastfeeding; PBF: partial breastfeeding; IQR: interquartile range (percentile 75-percentile 25). *Some “n” values are smaller because they were excluded as outliers. †Mann-Whitney test. Ghrelin, FBF, eight weeks, foremilk *versus* hindmilk, Wilcoxon test: $p < 0.001$. Ghrelin, FBF, 16 weeks, foremilk *versus* hindmilk, Wilcoxon test: $p < 0.001$. Ghrelin, FBF, foremilk eight weeks *versus* foremilk 16 weeks, Wilcoxon test: $p = 0.038$. Ghrelin, FBF, hindmilk eight weeks *versus* hindmilk 16 weeks, Wilcoxon test: $p = 0.020$. Leptin, FBF, foremilk eight weeks *versus* foremilk 16 weeks, $p = 0.024$. PBF, foremilk eight *versus* 16 weeks, $p = 0.454$.

Table II. Comparison, correlation and determination coefficients between the concentration of ghrelin (pg/ml) and leptin (ng/ml) measured in serum (independent variable) and in foremilk (dependent variable) of four-month-old infant's mothers

ARH	Feeding type	n	Mother's serum		Mother's milk		p*	r	R†	p†
			Median	IQR	Media	IQR				
Ghrelin	FBF	70	336.3	234.9	162.4	100.4	< 0.001	0.222	0.048	0.065
	PBF	55	313.2	229.2	168.2	139	< 0.001	0.208	0.084	0.044
Leptin	FBF	67	9.3	11.9	0.55	0.49	< 0.001	0.570	0.325	< 0.001
	PBF	46	14.9	26	0.63	0,75	< 0.001	0.366	0.134	0.013

ARH: appetite regulatory hormones; IQR: interquartile range (IR = centile 75-centile 15); FBF: full breastfeeding; PBF: partial breastfeeding. *Comparison between mother's serum and mother's milk, Mann-Whitney U t-test. †p value of correlations and linear regressions.