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BRIEF ARTICLE

Assay of ghrelin concentration in infant formulas and breast milk

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Abstract

AIM: To test if total ghrelin is present in infant formulas.

METHODS: Using a radioimmunoassay, we measured total ghrelin concentrations in 19 samples of commercial infant formulas and in 20 samples of human milk. We also determined ghrelin concentration in the serum of infants and lactating mothers.

RESULTS: Ghrelin concentrations were significantly higher in artificial milk (2007.1 ± 1725.36 pg/mL) than in human milk (828.17 ± 323.32 pg/mL) (P = 0.005). The mean ghrelin concentration in infant serum (n = 56) was 1115.86 ± 42.89 pg/mL, and was significantly higher (P = 0.023) in formula-fed infants (1247.93 ± 328.07 pg/mL) than in breast-fed infants (1045.7 ± 263.38 pg/mL). The mean serum ghrelin concentration (mean ± SD) in lactating mothers (n = 20) was 1319.18 ± 140.18 pg/mL.

CONCLUSION: This study provides evidence that total ghrelin is present in infant formulas. This finding raises diverse questions regarding the uptake, absorption and metabolic effects of this hormone.

Key words: Breast feeding; Ghrelin; Human milk; Infants; Infant formulas

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INTRODUCTION

It is now well established that early life nutrition plays an important role in long-term appetite control[1]. Indeed, neonatal nutrition is involved in the programming of feeding regulatory mechanisms in the central nervous system, and in those mediated by factors secreted from peripheral tissues. Besides peripheral circulating factors that regulate energy balance and adiposity, gastrointestinal peptides have been demonstrated to act as hunger signals. Among the known orexigenic peptides, ghrelin has been found to be the most powerful[2].

Ghrelin is involved in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity[3]. It is released in a pulsatile manner, with a nocturnal peak. Ghrelin responds to meals, increasing 1-2 h before eating and returning to trough levels 1-2 h after a meal[4,5]. Ghrelin secretion increases under negative energy-balance

conditions, and decreases under positive energy-balance conditions, such as food intake and obesity[6,7]. It is one of the most powerful orexigenic and lipogenic hormones and represents an interface between energy balance regulation, glucose homeostasis and hypothalamic neuropeptides[8]. The amino acid sequence of ghrelin is highly conserved throughout mammalian species[9]. Ghrelin has been found in human milk, but the source of the hormone is unclear. Aydin *et al*[10] reported that its levels in colostrum, transitional and mature milk were lower than those found in plasma and they assumed that ghrelin present in milk probably comes from the plasma of lactating mothers. In contrast, Kierson *et al*[11] showed that ghrelin levels in breast milk are higher than plasma levels and identified ghrelin mRNA from human mammary epithelial cells and mammary gland. Based on these findings these authors suggested that ghrelin in breast milk is probably synthesized and secreted from the breast. The identification of ghrelin in breast milk suggests that breast milk is a source of compounds critical for the metabolic development of infants[12,13].

Early feeding mode affects growth and body composition[14]. Breast-fed (BF) and formula-fed (FF) infants have similar weight gains in the first three months of life, however, BF infants gain weight less rapidly during the following months of the first year[15]. BF and FF infants have different feeding behaviors: FF infants eat less frequently and consume higher amounts of food than BF infants[16,17]. We previously reported that serum ghrelin concentrations were higher in infants exclusively FF than in infants exclusively BF[18]. Recently, serum ghrelin values were positively correlated with fasting times only in FF infants[19].

The aim of this study was to investigate whether infant formulas contain ghrelin.

MATERIALS AND METHODS

In this study, we enrolled 56 infants aged from 11 d to 5 mo (mean ± SD: 81 ± 46 d) born from a normal spontaneous vaginal delivery, consecutively referred to the Department of Paediatrics of the University of Turin, Regina Margherita Children's Hospital. The inclusion criteria for infants were gestational age between 37 and 42 wk, birth weight appropriate for gestational age (between 2500 and 4000 g), Apgar score higher than 7 at 5 min, no fetal anomaly, absence of acute or chronic gastroenteric diseases or other growth-affecting pathologies.

We collected milk samples from 20 lactating mothers of the infants enrolled. Eligibility criteria for mothers were: no maternal medical complications, non-smoking mothers, normal response to a glucose tolerance test, no mastitis, no prescribed medication, no digestive disorders.

Appropriate Ethics Committee permission was obtained and each parent signed a written informed consent. Milk samples (2 mL) were collected from the lactating women (n = 20) before breakfast at around 09:00 h. Samples of 19 infant formulas were collected: 9 starting formulas, 6 follow-on formulas and 4 special formulas (anti-regurgitation and hydrolyzed casein protein formulas). We also obtained unpasteurized milk samples (2 mL) from 5 dairy cows. Breast, cow and artificial milk were separated by centrifuging the samples twice at 2000 r/min and 4℃ for 20 min. After the first centrifugation, the thick fat layer at the top of the tube, which could interfere with detection of the hormone, was removed with a sterile toothpick. We collected a venous blood sample from 56 infants (37 BF infants and 19 FF infants) after a fast of 3 h. We also collected venous blood samples from 20 lactating mothers. Blood samples were immediately centrifuged at 4000 r/min and 4℃ for 10 min and each sample of the resulting serum was divided in 3 tubes and these were stored at -30℃ until analysed.

Hormone assays

Serum and milk total ghrelin were assayed by radioimmunoassay using a commercial kit (Ghrelin (total) RIA 3967, DRG Diagnostic, New Jersey, USA) according to the manufacturer's instructions (using a polyclonal antibody that recognizes octanoylated and non-octanoylated ghrelin with I^{125} ghrelin as a tracer molecule). Measurements of total ghrelin in mature milk and serum samples have been validated as reported elsewhere[10]. The intra-assay and inter-assay coefficients of variation of ghrelin were 5% and 7.6%, respectively. The lowest level of ghrelin that can be detected by this assay is 93 pg/mL with a 100-μL sample size. The specificity for human ghrelin is 100%. The limit of linearity for the ghrelin assay is 6000 pg/mL (any result greater than 6000 pg/mL was repeated on dilution using Assay Buffer as a diluent). Spike and Recovery of ghrelin in human plasma are shown in Table 1. As the kit is designed for human samples, a validation process was undertaken for use with bovine milk. We obtained 3 measurements of the hormone for each blood sample and milk sample. The manufacturer's protocol was followed and standard validations, including parallelism and recovery, conducted.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

A normal distribution was verified with the Shapiro-Wilk test ($P > 0.05$). The data are expressed as arithmetic means ± SD; $P < 0.05$ was considered statistically significant. Differences in ghrelin concentrations between human milk and formula milk were determined by the Student's t test.

RESULTS

As shown in Table 2, the mean ± SD of ghrelin concentration in human milk was 828 ± 323 pg/mL. Ghrelin levels varied widely in infant formulas (from 300 to 6110 pg/mL; mean: 2007 ± 1725 pg/mL). The mean ghrelin concentration in starting formulas and in follow-on formulas was 2699 ± 233.5 pg/mL and 2561 ± 215.2 pg/mL, respectively, whereas the mean ghrelin concentration was 1160 ± 340 pg/mL in special formulas (Table 3). Ghrelin

Table 1 Spike and recovery of ghrelin in human plasma (mean of the observed levels from 3 duplicate determinations in 3 separate assays)

Sample No.	Ghrelin added[1] (pg/mL)	Recovery (%)
1	500	96
2	1000	90
3	2000	91

[1]Different concentrations of human ghrelin were added to 3 different human plasma samples and the ghrelin content was determined by RIA. Percent recovery was calculated on the observed *vs* expected.

Table 2 Ghrelin concentrations in infant formulas, in mother's milk, in cow's milk and in serum of lactating mothers, breast-fed infants and formula-fed infants

	Ghrelin concentration (pg/mL ± SD)
Milk	
Different kinds of artificial infant formula (n = 19)	2007.1 ± 1725.36[a]
Breast milk (n = 20)	828.17 ± 323.32[a]
Non pasteurized cow milk (n = 5)	2816.00 ± 219.00
Serum	
Lactating mothers (n = 20)	1319.18 ± 140.18
Breast-fed infants (n = 37)	1045.7 ± 263.38[b]
Formula-fed infants (n = 19)	1247.93 ± 328.07[b]

[a]P = 0.005, infant formulas *vs* human milk, student's t test; [b]P = 0.023, breast-fed infants *vs* formula-fed infants, student's t test.

levels were significantly higher in infant formulas than in human milk (P = 0.005). The mean ghrelin concentration in unpasteurized cow milk was 2816 ± 219 pg/mL. The mean ghrelin concentration in infant serum was 1115.86 ± 42.89 pg/mL, and was significantly higher (P = 0.023) in FF infants (1247.93 ± 328.07 pg/mL) than in BF infants (1045.7 ± 263.38 pg/mL). The mean serum ghrelin concentration (mean ± SD) in lactating mothers was 1319.18 ± 140.18 pg/mL.

DISCUSSION

In this study, we showed that artificial milk contains the orexigenic hormone, ghrelin, and that its concentration was higher in infant formulas than in human milk. This finding might explain the higher serum levels we previously observed in FF infants *vs* BF infants, which was also confirmed in the present research[18,19].

If FF infants receive a higher amount of ghrelin, it is conceivable that they have a greater feeding stimulus than BF infants, and a consequent increase in weight and growth rate. Our observations could explain the more appropriate growth curves of BF infants, who physiologically receive less ghrelin[20]. Therefore, breast-feeding may protect against the development of obesity in childhood and adulthood, not only because of its nutrient composition, but also because of the presence of bioactive factors such as ghrelin, leptin and adiponectin[12,21].

Table 3 Ghrelin concentrations in three different types of artificial infant formula

Type of infant formula	Ghrelin concentration (pg/mL ± SD)
Starting infant formulas (n = 9)	2699 ± 233.5
Follow-on infant formulas (n = 6)	2561 ± 215.2
Special infant formulas (anti-regurgitation and hydrolyzed casein protein formulas) (n = 4)	1160 ± 340

Several assays are available to measure human serum ghrelin, whereas there is no commercial milk ghrelin assay kit. Consequently, we carried out a validation process using a basic clinical chemistry method (linearity)[10]. Similarly, there is no assay kit specific for bovine ghrelin; however, given the high structural homology between human ghrelin and mammalian ghrelin, the kit used in our study to detect ghrelin in infant formulas can be considered reliable[22].

The ghrelin concentrations reported in our study refer to the final volume after centrifugation, because the RIA kit instructions indicate that samples with high lipidemia be avoided. After centrifugation, we removed the supernatant and the fat layer that could interfere with detection of the hormone. This method has been used in previous studies[10]. Kierson *et al*[11] reported higher ghrelin levels in whole milk than skimmed milk, with a direct relationship between estimated milk fat content and ghrelin levels. Therefore, it is possible that ghrelin levels detected in milk after centrifugation are lower than those present in whole milk and consumed by the infants.

The mechanism by which ghrelin influences growth in early infancy is not yet completely known. Ghrelin levels are higher in small-for-gestational-age (SGA) newborns than in adequate-for-gestational age (AGA) newborns[23]. Reduced ghrelin suppression and higher postprandial ghrelin concentrations in SGA infants could cause a sustained orexigenic drive and may contribute to catch-up growth in these infants. Kitamura *et al*[24]. reported high ghrelin levels during the early neonatal period. Onal *et al*[25] observed that plasma ghrelin concentration was inversely associated with birth weight and body length in term newborns. We previously observed a negative correlation between ghrelin concentration and weight gain in BF infants in the first months of life, which suggests that ghrelin may play a role in body weight regulation in healthy infants[26,27]. More recently, we found a positive correlation between circulating ghrelin concentration and fasting time in FF infants; these infants have a higher serum ghrelin concentration, longer fasting time and fewer meals than BF infants[19]. Clearly, we need to learn more about early feeding and the mechanisms regulating satiety and feeding behavior.

Finally, it should be noted that, similar to previous studies[11,28], we measured total milk ghrelin level. Deacyl ghrelin influences food intake, gut motility, insulin secretion and resistance and adipogenesis, whereas the acylated form of ghrelin, known as active ghrelin, is essential for binding to the growth hormone secretagogue receptor 1a[29]. Additional studies are needed to evaluate active ghre-

lin in infant formulas.

There have been some studies conducted on the effects of ghrelin administration (i.e. in anorexia, cachexia), however, the hormone was administered intravenously and not orally[30]. More recently, animal studies have investigated the effects of oral administration of ghrelin receptor agonist, and found that it survives the acid environment of the stomach and could exert some of its biological functions[31,32].

In conclusion, specific research is needed to understand the origin of ghrelin found in infant formulas. Furthermore, it would be interesting to determine whether ghrelin present in infant formulas survives the acid environment of the stomach in humans, exerts biological activity through receptors present in the gastrointestinal tract of newborns and whether the higher ghrelin concentration in infant formulas result in a higher serum concentration in FF infants. Lastly, investigations are required to determine whether these higher levels of ghrelin affect feeding habits and thus obesity in later life.

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COMMENTS

Background

Current research highlights the importance of early life nutrition in long-term appetite control, with consequent programming of regulatory mechanisms. Ghrelin is a recently discovered hormone involved both in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Research frontiers

Ghrelin has recently been detected in breast milk, but data on ghrelin in infant formula are lacking. Using a radioimmunoassay, the authors measured ghrelin concentrations in commercial infant formulas *versus* concentrations in human milk. Surprisingly, ghrelin was significantly higher in artificial formulas. This finding raises diverse questions.

Innovations and breakthroughs

Little is known about ghrelin regulation, especially in early infancy. Breast milk contains ghrelin, but it was not known whether infant formulas contain ghrelin. The finding that infant formulas do indeed contain ghrelin, and at levels higher than those found in breast milk, raises questions about the uptake, absorption and metabolic effects of this feeding stimulus and growth rate of artificially fed infants. Further research is needed to determine whether the higher levels of ghrelin in formulas could affect infant feeding habits and thus obesity in later life.

Applications

The higher ghrelin levels found in artificial milk in the present study might explain the higher serum values the authors recently observed in formula-fed infants compared with breast-fed infants. Thus, if artificially fed infants receive a higher amount of ghrelin together with a higher intake of protein, it is conceivable that formula-fed infants have a greater feeding stimulus with a consequent increase in weight and growth rate.

Terminology

Ghrelin is involved in the short-term regulation of food intake, with an orexigenic action, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Peer review

The manuscript by Savino *et al* provides original data on the presence of the orexigenic hormone ghrelin cow's milk formulas. The authors suggest that the high levels of hormone present in formulas can influence the feeding habits of formula feed subjects in infancy and also later and can be responsible for the high risk of obesity observed in formula fed infants as compared to breast fed ones. The article could be of some general interest, both for pediatricians and for nutritionists.

REFERENCES

1 **Bouret SG**. Early life origins of obesity: role of hypothalamic programming. *J Pediatr Gastroenterol Nutr* 2009; **48** Suppl 1: S31-S38
2 **Cummings DE**. Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiol Behav* 2006; **89**: 71-84
3 **Kojima M**, Kangawa K. Ghrelin: more than endogenous growth hormone secretagogue. *Ann N Y Acad Sci* 2010; **1200**: 140-148
4 **Korbonits M**, Goldstone AP, Gueorguiev M, Grossman AB. Ghrelin--a hormone with multiple functions. *Front Neuroendocrinol* 2004; **25**: 27-68
5 **Hosoda H**, Kojima M, Kangawa K. Biological, physiological, and pharmacological aspects of ghrelin. *J Pharmacol Sci* 2006; **100**: 398-410
6 **Zou CC**, Liang L, Zhao ZY. Factors associated with fasting plasma ghrelin levels in children and adolescents. *World J Gastroenterol* 2008; **14**: 790-794
7 **Morash MG**, Gagnon J, Nelson S, Anini Y. Tissue distribution and effects of fasting and obesity on the ghrelin axis in mice. *Regul Pept* 2010; **163**: 62-73
8 **Schwartz MW**, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000; **404**: 661-671
9 **Kojima M**, Ida T, Sato T. Structure of mammalian and non-mammalian ghrelins. *Vitam Horm* 2008; **77**: 31-46
10 **Aydin S**, Aydin S, Ozkan Y, Kumru S Ghrelin is present in human colostrum, transitional and mature milk. *Peptides* 2006; **27**: 878-882
11 **Kierson JA**, Dimatteo DM, Locke RG, Mackley AB, Spear ML. Ghrelin and cholecystokinin in term and preterm human breast milk. *Acta Paediatr* 2006; **95**: 991-995
12 **Savino F**, Fissore MF, Liguori SA, Oggero R. Can hormones contained in mothers' milk account for the beneficial effect of breast-feeding on obesity in children? *Clin Endocrinol* (Oxf) 2009; **71**: 757-765
13 **Savino F**, Liguori SA, Fissore MF, Oggero R Breast milk hormones and their protective effect on obesity. *Int J Pediatr Endocrinol* 2009; **2009**: 327505
14 **Lamb MM**, Dabelea D, Yin X, Ogden LG, Klingensmith GJ, Rewers M, Norris JM. Early-life predictors of higher body mass index in healthy children. *Ann Nutr Metab* 2010; **56**: 16-22
15 WHO Child Growth Standards based on length/height, weight and age. *Acta Paediatr Suppl* 2006; **450**: 76-85
16 **Sievers E**, Oldigs HD, Santer R, Schaub J. Feeding patterns in breast-fed and formula-fed infants. *Ann Nutr Metab* 2002; **46**: 243-248
17 **Li R**, Fein SB, Grummer-Strawn LM. Do infants fed from bottles lack self-regulation of milk intake compared with directly breastfed infants? *Pediatrics* 2010; **125**: e1386-e1393
18 **Savino F**, Fissore MF, Grassino EC, Nanni GE, Oggero R, Silvestro L. Ghrelin, leptin and IGF-I levels in breast-fed and formula-fed infants in the first years of life. Acta Paediatr 2005; **94**: 531-537
19 **Savino F**, Fissore MF, Liguori SA, Grassino EC, Guidi C, Oggero R, Silvestro L, Miniero R. Serum ghrelin concentration, fasting time and feeding in infants. *J Pediatr Endocrinol Metab* 2007; **20**: 1027-1033
20 **Dewey KG**, Heinig MJ, Nommsen LA, Peerson JM, Lönnerdal B. Growth of breast-fed and formula-fed infants from 0 to 18 months: the DARLING Study. *Pediatrics* 1992; **89**: 1035-1041
21 **Koletzko B**, von Kries R, Closa R, Escribano J, Scaglioni S,

Giovannini M, Beyer J, Demmelmair H, Anton B, Gruszfeld D, Dobrzanska A, Sengier A, Langhendries JP, Rolland Cachera MF, Grote V. Can infant feeding choices modulate later obesity risk? *Am J Clin Nutr* 2009; **89**: 1502S-1508S

22 **Kojima M**, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005; **85**: 495-522

23 **Chiesa C**, Osborn JF, Haass C, Natale F, Spinelli M, Scapillati E, Spinelli A, Pacifico L. Ghrelin, leptin, IGF-1, IGFBP-3, and insulin concentrations at birth: is there a relationship with fetal growth and neonatal anthropometry? *Clin Chem* 2008; **54**: 550-558

24 **Kitamura S**, Yokota I, Hosoda H, Kotani Y, Matsuda J, Naito E, Ito M, Kangawa K, Kuroda Y. Ghrelin concentration in cord and neonatal blood: relation to fetal growth and energy balance. *J Clin Endocrinol Metab* 2003; **88**: 5473-5477

25 **Onal EE**, Cinaz P, Atalay Y, Türkyilmaz C, Bideci A, Aktürk A, Okumuş N, Unal S, Koç E, Ergenekon E. Umbilical cord ghrelin concentrations in small- and appropriate-for-gestational age newborn infants: relationship to anthropometric markers. *J Endocrinol* 2004; **180**: 267-271

26 **Savino F**, Liguori SA, Fissore MF, Oggero R, Silvestro L, Miniero R. Serum ghrelin concentration and weight gain in healthy term infants in the first year of life. *J Pediatr Gastroenterol Nutr* 2005; **41**: 653-659

27 **Savino F**, Grassino EC, Fissore MF, Guidi C, Liguori SA, Silvestro L, Oggero R, Miniero R. Ghrelin, motilin, insulin concentration in healthy infants in the first months of life: relation to fasting time and anthropometry. *Clin Endocrinol* (Oxf) 2006; **65**: 158-162

28 **Ilcol YO**, Hizli B. Active and total ghrelin concentrations increase in breast milk during lactation. *Acta Paediatr* 2007; **96**: 1632-1639

29 **Chen CY**, Asakawa A, Fujimiya M, Lee SD, Inui A. Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacol Rev* 2009; **61**: 430-481

30 **Cheung WW**, Mak RH. Ghrelin and its analogues as therapeutic agents for anorexia and cachexia in end-stage renal disease. *Kidney Int* 2009; **76**: 135-137

31 **Smith RG**, Sun Y, Jiang H, Albarran-Zeckler R, Timchenko N. Ghrelin receptor (GHS-R1A) agonists show potential as interventive agents during aging. *Ann N Y Acad Sci* 2007; **1119**: 147-164

32 **Shafton AD**, Sanger GJ, Witherington J, Brown JD, Muir A, Butler S, Abberley L, Shimizu Y, Furness JB. Oral administration of a centrally acting ghrelin receptor agonist to conscious rats triggers defecation. *Neurogastroenterol Motil* 2009; **21**: 71-77

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