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Effect of suboptimal nutrition during lactation on milk protein gene expression in the rat☆

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Abstract

Human milk provides infants with proteins that aid in the prevention of infections and facilitate the digestion and absorption of other nutrients. Maternal diet is not believed to affect the protein concentration of breast milk. However, the maternal factors that regulate the expression of genes for specific milk proteins are not well characterized. We hypothesized that nutrition could be one of the factors. We fed Sprague–Dawley rats five diets representing common nutrient deficiencies and energy deficiency during pregnancy and lactation: low-zinc (Zn; 7 μg/g), low-iron (Fe; 6 μg/g), low-protein (12.5% albumin), pair-fed control diet (lactation only, 20% less kcal) and control diet (Zn, 25 μg/g; Fe, 100 μg/g; protein, 21%) ad libitum. At day 10 of lactation, the mammary gland was removed for RNA extraction. Northern blots of mRNA from the different groups were performed by hybridization with β-casein and whey acidic protein (WAP) cDNA probes. The expression of β-casein mRNA in rat mammary gland was significantly ($P<.005$) increased in the pair-fed group when compared to the control group. The expression of WAP mRNA was also significantly ($P<.005$) increased in the pair-fed group as well as in the low-Fe group when compared to the control group. The concentration of β-casein in milk was significantly higher for the low-zinc and the pair-fed groups only. The concentration of WAP in milk was not different among groups. These results suggest that compromised maternal nutrition can affect the expression of two individual milk proteins and may have functional implications with regard to bioactive proteins in milk.

Keywords: Lactation; Suboptimal nutrition; β-Casein; Whey acidic protein; Gene expression

1. Introduction

The positive effects of breast-feeding on the health of infants are well recognized. However, our knowledge of the effect of maternal nutrition on lactation performance is still incomplete. Milk proteins not only provide the infant with a source of amino acids, thereby playing a nutritional role, but they also have specific physiological functions that protect the infant against infections and that aid in the absorption of other nutrients [1]. The individual proteins in milk show distinct patterns of distribution during lactation [2]. For example, in human milk, casein is present at very low concentrations early in lactation, but it rapidly increases through the first month when it peaks and gradually decreases afterward [3]. The opposite effect is seen for whey proteins, which start being high in colostrum, but early in lactation, a decreasing pattern is seen, which continues throughout lactation. In addition, there is significant variation among women at the same stage of lactation [3]. The factors that determine the patterns that individual women will follow throughout lactation are not clear. It has been speculated that ethnicity, genetic background and age may account for part of this individual variation [4–6]. External insults such as nutrient deficiencies may also affect this variation [7].

A number of studies have shown that the protein concentration of milk does not seem to be significantly affected in poorly nourished women who confront an inadequate intake of energy and nutrients in general [8–11]. Coward et al. [9] analyzed the impact of poor nutrition during human lactation and concluded that it appears that suboptimal nutrition has little, if any, effect on the protein concentration of human milk. Additional studies

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performed in The Gambia [10] and Kenya [11] did not find lower milk protein concentrations in poorly nourished women compared with well-nourished women. However, two short-term and small-scale studies have showed that reduced protein intake can lead to changes in milk composition [12,13]. These studies found a decrease in total nitrogen, true protein, nonprotein nitrogen and total free amino acids when women were fed a low-protein diet. Because both of these previous studies were of short duration, it is uncertain whether these effects are maintained long term with protein-deficient diets. Our group has previously analyzed concentrations of lactoferrin, α-lactalbumin, IgG and IgM in milk from privileged and nonprivileged Ethiopian and Swedish mothers during lactation [8]. No differences were found between these groups of women for several of these proteins, but the Ethiopian mothers showed higher concentrations of lactoferrin compared to the Swedish mothers. It is, however, difficult to detect statistically significant differences in concentrations due to the large interindividual variation coupled with normal variations in milk proteins occurring during lactation. It should be emphasized that nutrition may have specific effects on individual milk proteins even if total protein concentration is not affected. Therefore, a possible scenario is to have a nutritional insult affecting a physiological important protein such as secretory IgA by decreasing its expression, and at the same time, the expression of another protein that may not have a physiological role in the infant may increase; however, there may be no net effect on total protein concentration.

Unfortunately, there is limited information available about the direct effects of single nutrients on milk protein gene expression. It is known that transferrin is a major iron-binding protein in rat milk, and that its expression does not seem to be affected by maternal iron status [14]. In addition, no correlation was found between milk iron and milk transferrin levels. We speculate that differences in transferrin gene expression in the mammary gland may be masked by regulation of transferrin receptor expression in the gland. This speculation is based on the findings that transferrin receptor levels in the rat mammary gland are affected by maternal iron status [14,15]. Although there is evidence that other nutrients like zinc have a direct effect on gene expression [16], effects on milk protein expression have not yet been studied.

The extent to which maternal nutrition can affect milk protein gene expression in lactating animals and humans is not known. It is clear that hormones are involved but studies are needed to understand the mechanisms involved in the regulation of gene expression of milk proteins during lactation. In this study, we examined the effects of nutrient deficiencies during pregnancy and lactation in the rat on the expression of a major casein subunit, β-casein, and a major whey protein, whey acidic protein (WAP). β-Casein and WAP were selected as proteins being representative of the two major classes of milk proteins and, therefore, expected to be under different mechanisms of regulation. They consequently serve as specific molecular markers for studying the effects of maternal malnutrition on mammary gland milk protein gene expression.

2. Methods and materials

2.1. Animals

Virgin female Sprague–Dawley rats weighing between 220 and 250 g were purchased from a commercial source (Simonsen Laboratory, Gilroy, CA). The animals were acclimatized to the new environment and fed the purified diet (control diet) (Table 1) for at least 1 week prior to mating with stock-fed males (Stock chow #5012 from PMI Purina Mills, San Carlos, CA). All females were housed in individual stainless steel cages in a temperature (23°C) and light-controlled room (12-h reverse light cycles, starting at 0600 h). This study was approved by the Animal Research Services at the University of California, Davis, CA, which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

2.2. Experimental design

Pregnancy was confirmed by the presence of vaginal plugs, and pregnant animals ($n=35$) were randomly assigned to one of five dietary groups: (1) low-zinc (7 mg/kg), (2) low-iron (6 mg/kg), (3) low-protein (12.5% albumin) and (4) pair-fed groups (20% less kcal), and (5) control (Zn, 25 mg/kg; Fe, 100 mg/kg; protein, 21%; energy, 3.8 kcal/g). Due to death by unknown causes, the pair-fed group had only five animals. The purpose of the pair-fed group was to imitate the food intake of the zinc-deficient animals (anorexic effect) by feeding them a control diet. Direct analysis of the diets by atomic absorption spectrophotometry and micro-Kjeldahl confirmed these values. These levels of nutrients were carefully selected after an initial pilot study to accomplish marginal deficiencies in these animals while still allowing successful pregnancy and lactation. Demineralized water and control and experimental diets, except the energy-restricted diet, were fed ad libitum. Pregnant animals were transferred to a plastic cage with bedding where they could comfortably deliver their pups. By day 5 of lactation, litters were adjusted to eight pups to standardize the number of animals

Table 1
Composition of the albumin-based control diet (AIN-93) [17]

Egg white[a]	210
Corn starch	200
Glucose (cerelose)	395
Corn oil	80
Mineral mix[b]	60
Cellulose (alphacel)	40
Vitamin mix	15

Values are presented as grams per kilogram.
[a] Egg white (125 g/kg) was used for the low-protein diet.
[b] Adjusted for the low iron ($FeSO_4$) and low zinc ($ZnCO_3$) diets.

Table 2
Effects of dietary treatment on maternal outcome (means±S.D.)

Measurements	Diets				
	Control	Low iron	Low zinc	Low protein	Pair-fed
Weight gain, pregnancy (g)	97±68	123±27	102±15	146±13	140±19
Weight gain, lactation (g)	9.9±5.7[a]	9.0±11.7[a]	7.7±23.2[a]	8.3±10.9[a]	–18±8.9[b]
Weight, day 10 of lactation (g)	313±23	313±13	294±22	306±23	284±13
Food intake, pregnancy (g/day)	399±38	400±26	396±21	403±26	390±2
Food intake, lactation (g/day)	260±22[c]	258±23[c]	195±19[d]	245±29[c]	203±15[d]

[a,b] $P<.05$ (pair-fed group vs. control group) by ANOVA.
[c,d] $P<.005$ (low zinc and low pair-fed groups vs. control group) by ANOVA.

nursing each dam (adjustment for pup sex was not done). At day 10 of lactation, dams were separated from their litters 2 to 4 h prior to milking; longer periods of separation were not used as they can affect milk composition [18]. Dams were always milked between 1200 and 1500 h in order to minimize possible diurnal variations in milk composition [19]. Dietary intake of the dams was registered every other day throughout pregnancy and lactation. The dams were anesthetized with a ketamine/xylazine cocktail (4.2 ml ketamine, 1.0 ml xylazine and 5.2 ml saline) administered intraperitoneally at 0.25 ml per 300 g rat. Oxytocin (20 IU, Sigma, St. Louis, MO) was injected intraperitoneally (0.3 ml per 300 g rat) to stimulate milk flow. Milk was obtained by gentle hand stripping of the teats, and the free flowing milk was collected in plastic 1.5-ml Eppendorf tubes. Collected milk was stored frozen until protein analysis was performed.

From the dam, milk was collected for protein and mineral determinations (efforts were made to empty the gland) and blood was collected by heart puncture for hematocrit, hemoglobin (HemoCue, Mission Viejo, CA) and mineral analysis. In addition, a piece of the mammary gland was removed for RNA extraction (about 1 g), which was rinsed and placed immediately in cold TRIzol solution, homogenized and stored at −70°C to prevent RNA degradation. The liver was removed for mineral analysis. Birth weight of the pups, number of pups at birth as well as weight of the pups at day 10 of lactation were also recorded to evaluate the effect of the nutritional insults on growth. Tissues and milk samples were stored at −20°C.

2.3. Mineral analysis

Diet, tissue and milk samples (0.3 g) were wet-ashed in concentrated nitric acid, and mineral concentrations were determined by atomic absorption spectrophotometry (Thermo Jarrell Ash SH4000, Franklin, MA) as previously described [20].

Table 3
Characteristics of the pups (means±S.D.)

Diet	No. of pups at birth	Birth weight (g)	Weight at day 10 (g)
Control	10.0±2.8	6.04±0.60	19.02±3.2[a]
Low iron	11.4±4.4	5.80±0.49	15.30±2.3[a,b]
Low zinc	10.0±4.2	5.30±0.63	11.40±2.5[b]
Low protein	12.3±1.3	5.53±0.69	15.3±2.9[a,b]
Pair-fed	12.8±3.7	5.47±0.40	15.4±1.8[a,b]

[a,b] $P<.005$ (low zinc group vs. control group) by ANOVA.

2.4. Analysis of milk

Total milk protein was determined (10 μl of a 1:100 dilution) by a dye-binding method (Bio-Rad Protein Assay, Richmond, CA) [21], and analysis of individual milk proteins was done using 12% polyacrylamide gel electrophoresis (Bio-Rad). Individual proteins were confirmed by N-terminal sequencing using in-gel digestion of the bands, microbore HPLC purification of the fragments and sequence analysis of purified peptides [22,23]. Quantification of individual proteins was performed by the Storm system (Molecular Dynamics, Sunnyvale, CA) after staining gels with SYPRO Orange protein gel stain (Molecular Probes, Eugene, OR) and measuring the signal as chemifluorescence. The Image QuaNT program (Molecular Dynamics) was used to quantify variations in the signal. This procedure was selected because antibodies to these rat milk proteins are not commercially available.

2.5. Analysis of transcript levels

Total RNA extraction was done with the TRIzol Reagent (Gibco-BRL, Grand Island, NY). Transcript levels were measured in the mammary gland tissues by Northern blot analysis using nylon membranes (Boehringer Mannheim, Indianapolis, IN). Rat β-casein and WAP cDNAs (obtained from Dr. Jeffrey Rosen at the Department of Cell Biology, Baylor College of Medicine) were purified with the Plasmid Midi Kit from Qiagen (Qiagen, Valencia, CA). The cDNAs

Table 4
Hematological values of dams and Cu and Zn concentrations in dam plasma (means±S.D.)

Diet	Hematocrit (%)	Hemoglobin (g/L)	Cu (mg/L)	Zn (mg/L)
Control	45.2±5.2[a,b]	129±12.5[c]	0.80±0.3	1.42±0.3[e]
Low iron	40.8±3.5[a]	113±9.5[d]	0.78±0.1	1.41±0.2[e]
Low zinc	46.2±2.5[b]	129±8.2[e]	0.68±0.1	0.90±0.4[f]
Low protein	44.0±1.7[a,b]	128±7.3[c,d]	0.75±0.1	1.27±0.2[e,f]
Pair-fed	44.3±2.9[a,b]	130±7.1[c]	0.71±0.1	1.11±0.2[e,f]

[a,b] $P<.05$ (low Fe group vs. low Zn group) by ANOVA.
[c,d] $P\leq.05$: low Fe group vs. control group and low Zn group by ANOVA.
[e,f] $P\leq.05$: low Zn group vs. control group and low Fe group by ANOVA.

Table 5
Cu, Zn and Fe concentrations in dam liver and mammary gland (means±S.D.)

Diet	Liver (çg/g)			Mammary gland (çg/g)		
	Cu	Zn	Fe	Cu	Zn	Fe
Control	1.73±0.3	10.5±1.5	163.9±36.6[a]	2.9±0.3	5.5±1.0	12.3±5.8
Low Fe	1.85±0.2	10.1±1.3	44.4±13.6[b]	3.1±0.5	5.2±1.0	9.4±5.7
Low Zn	1.71±0.2	9.4±1.0	152.6±53.4[a]	2.8±0.3	5.0±0.8	20.3±11.7
Low protein	1.70±0.1	9.5±0.9	137.4±30.9[a]	2.9±0.5	5.2±0.4	14.2±12.3
Pair-fed	1.81±0.2	10.4±0.9	174.2±20.8[a]	2.6±0.7	5.5±0.6	15.9±6.7

[a,b] $P<.00001$ (low Fe vs. all other groups) by ANOVA.

were labeled with α^{32}P-dCTP (Amersham, Piscataway, NJ) utilizing the Random Primed DNA Labelling Kit (Boehringer Mannheim) to be used for hybridization of the membranes. Hybridization was performed for 20 h at 45°C and followed by four washes with different concentrations of SSC with 0.1% SDS for 15 min each. Membranes were exposed to a phospho-screen (Molecular Dynamics) for 24 h, and the bands were captured using a Storm system (Molecular Dynamics) and quantified by the Image QuaNT program (Molecular Dynamics). Measurements of β-casein and WAP expression by Northern analysis are given as pixels per 23 μg RNA as determined by the density of the band in the phospho-imager. GAPDH was analyzed as a control as described previously [24].

2.6. Statistical methods

Data were hand-entered and sorted using Microsoft Excel (Redmond, WA) and rechecked for accuracy to minimize entry errors. Statistical analyses were run using STATA (Stata, College Station, TX). ANOVA with Bonferroni adjustment was used to compare the means and set up P value cutoffs between the control group and the experimental groups, and the pair-fed group with the low-zinc group.

3. Results

Weight gain and food intake of dams during gestation and dam weight at day 10 of lactation were not significantly different between groups (Table 2). However, food intake during lactation was lower for the dams in the low-zinc and the pair-fed groups compared to the control, low-protein and low-iron groups (Table 2). Weight gain for dams during lactation was not significantly different between groups,

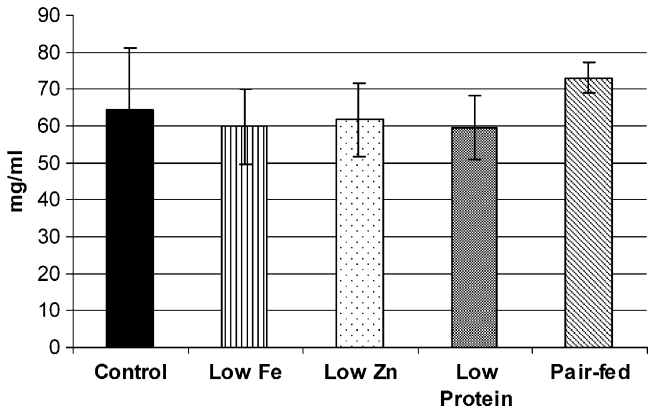

Fig. 1. Total protein concentration in milk ($n=5$–7) determined by a dye-binding method (Bio-Rad Protein Assay). ANOVA with Bonferroni adjustments was used to compare the means among the five groups. Total milk protein concentration was not significantly different between the groups.

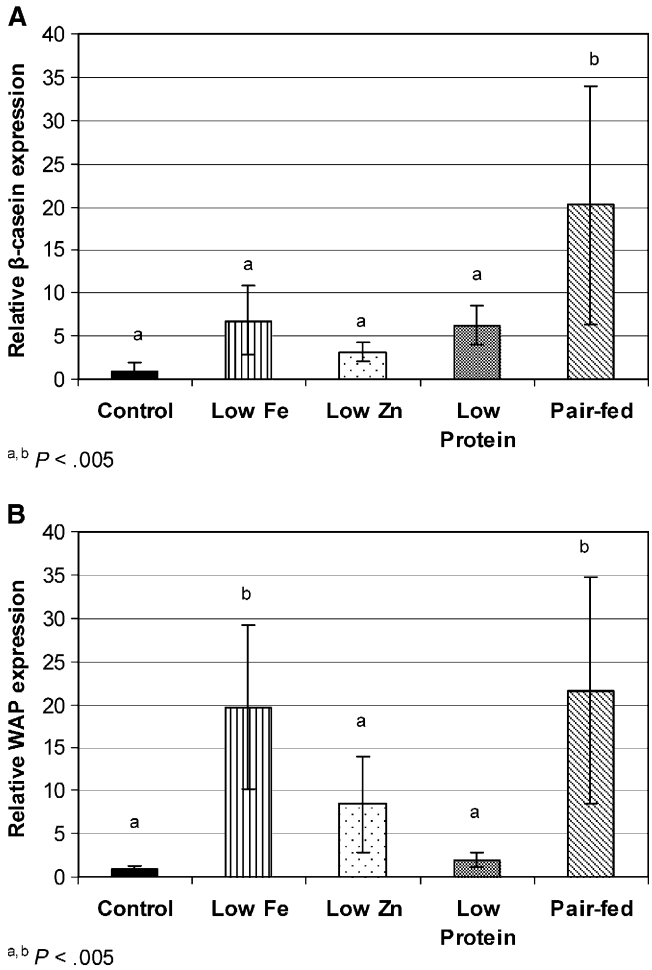

Fig. 2. β-Casein and WAP expression in the mammary gland ($n=5$–8). Transcript levels were measured in the mammary gland tissues by Northern blot analysis. Nylon membranes were hybridized with rat β-casein and WAP cDNAs labeled with α^{32}P-dCTP. Measurements of β-casein and WAP expression were determined by band density using a phospho-imager. ANOVA with Bonferroni adjustments was used to compare the means among the five groups. β-Casein mRNA expression in the rat mammary gland was significantly higher ($P<.005$) for the pair-fed group when compared with the control group (A). Whey acidic protein mRNA expression was also significantly higher ($P<.005$) in the low-iron and pair-fed groups when compared to controls (B).

with the exception of the pair-fed group that lost weight compared to the control and the low-zinc, low-protein and low-iron groups (Table 2). Litter sizes of the dams fed the five different diets, and birth weight of the pups were not significantly different (Table 3). Weight of pups at day 10 of lactation, however, was significantly lower ($P<.005$) for pups from dams fed the low-zinc diet (Table 3). As expected, hemoglobin concentrations were lower ($P\leq.05$) for dams fed the low-iron diet as compared to the control and the low-zinc and pair-fed groups (Table 4). Dams consuming the low-iron diet had significantly lower hematocrit values ($P\leq.05$) than the low-zinc group but not the control group (Table 4). Concentrations of copper (Cu) and Zn in the liver of dams were not significantly different between groups, but concentrations of iron in the liver was significantly lower in dams fed the low-iron diet than all the other groups ($P<.00001$) (Table 5). There was no difference in mammary gland iron, Zn and Cu concentrations among groups (Table 5). As expected, plasma Zn concentrations were significantly lower ($P<.05$) in dams fed the low-zinc diet in comparison to the control and the low-iron diet (Table 4), whereas plasma Cu concentrations did not differ between groups.

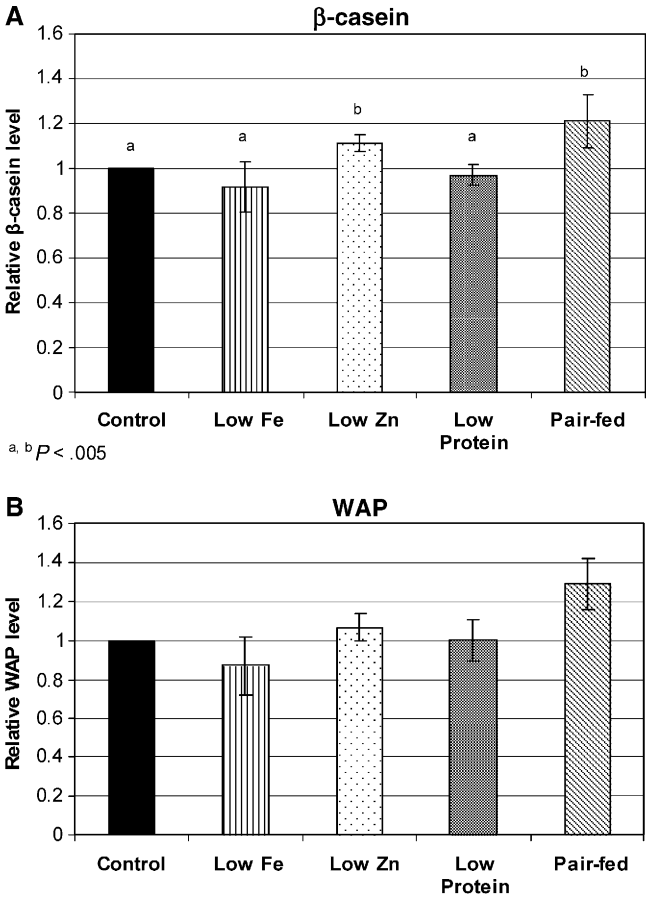

Fig. 3. β-Casein and WAP concentration in milk ($n=5$–7). Individual milk proteins were assessed using a 12% polyacrylamide gel electrophoresis. Gels were stained and quantified by measuring the chemifluorescence signal. ANOVA with Bonferroni adjustments was used to compare the means among the five groups. The concentration of β-casein in milk analyzed as the difference between each experimental group to its control was significantly higher for the low-zinc and the pair-fed groups only (A). The concentration of WAP in milk analyzed as the differences between each experimental group to its control was not significantly different between the four groups (B).

Total milk protein concentration was not significantly different between groups (Fig. 1). β-Casein mRNA expression in the rat mammary gland was significantly higher ($P<.005$) for the pair-fed group when compared with the control group (Fig. 2A). Whey acidic protein mRNA expression was also significantly higher ($P<.005$) in the low-iron and pair-fed groups when compared to the control group (Fig. 2B). The concentration of β-casein in milk, analyzed as the difference between each experimental group to its control, was significantly higher for the low-zinc and the pair-fed groups as compared to the control group (Fig. 3A). The concentration of WAP in milk analyzed as the differences between each experimental group to its control was not significantly different between the five groups (Fig. 3B).

4. Discussion

The imposed nutritional insults clearly had an impact on the lactating dams. Iron status was impaired in the low-iron group of dams as demonstrated by hemoglobin values (Table 4) as well as by dam liver Fe concentrations (Table 5). Plasma Zn concentrations (Table 4) in the low-zinc group confirm that this nutritional insult affected the dams. Food intake during pregnancy was not significantly different between the groups, but food intake during lactation was clearly lower in the low-zinc and the pair-fed groups as compared to the control group (Table 2). As mentioned earlier, the purpose of the pair-fed group was to mimic the food intake of the low-zinc animals while feeding a control diet, thereby, controlling for the anorexic effect in the zinc-deficient animals. It is puzzling that despite similar food intake by the low-zinc and the pair-fed groups during lactation, only the pair-fed group lost weight during this period. There is no clear explanation to this observation, but it is obvious that the low-zinc and the pair-fed groups responded very different to the nutritional insults imposed on them. We hypothesize that there are different reactions to the two treatments that can be explained by differences in response to the two types of deficiencies that they produced as discussed hereinafter. Dams in the low-protein group responded similarly in weight gain and food intake during pregnancy and lactation to the control group, which was probably due to the protein level in the low-protein diet (12.5%), which was higher than the levels frequently used (6–9%) [25–28]. Our level was chosen after a pilot study and was intended to achieve only a marginal protein deficiency. Birth weight of the pups was not significantly different among the groups (Table 3). At day 10 of lactation, pups nursing dams in all experimental groups weighed less than pups from dams fed

the control diet, although only pups nursing zinc-deficient dams had weights that were significantly lower than all the other groups.

Nutrient deficiencies can affect the body very differently. Golden [29] has postulated that a response to a deficiency of an essential nutrient can be perceived as one of two signs. The first one is when the animal continues to grow, consumes the body stores and then has a reduction in the bodily functions that depend upon the deficient nutrient. The second one is when the animal stops growing and avidly conserves the nutrient in the body to make it more available internally and maintain tissue levels. The nutrient deficiencies that give rise to the first or the second response, respectively, are referred to as Types I and II nutrients. It is important to distinguish between Types I (e.g., iron) and II (e.g., zinc and protein) nutrients when discussing how nutrient deficiencies can affect protein expression. Although energy could be classified as comparable to a Type II nutrient, it really falls under a third category of nutrient deficiencies. Energy is not a nutrient, per se; it is sufficient food being available (hence, pair-fed group). However, low energy intake, as a result of the anorexia produced by a Type II nutrient deficiency, is different from low energy intake caused by a lack of food because it is not the primary cause of malnutrition but rather secondary to anorexia. By clarifying these differences and understanding these definitions, the validity of the pair-fed group to control for the anorexia produced in animals deficient in Type II nutrients (e.g., zinc) can be questioned. Our data on weight of pups on day 10 clearly show that pups nursing pair-fed dams were heavier than pups nursing zinc-deficient dams, which could explain why dams fed the pair-fed diet lost weight while the animals in the zinc-deficient diet still gained some weight. Relating these definitions to our findings in this paper could lead us to a better understanding of the differences between the dietary groups.

Suboptimal nutrition did not affect total milk protein concentration (Fig. 1), which is in agreement with previous studies [9–11]. The pair-fed group showed the highest transcript levels for β-casein and WAP, but the low-zinc group showed no effect on the expression of these proteins (Fig. 2). The low-iron group showed higher transcript levels for WAP compared to the control group (Fig. 2B). The low-protein group did not show a significant difference in transcript levels for β-casein and WAP.

Prolactin, a major regulatory hormone during lactation, is involved in protein synthesis. Plasma levels of this hormone are increased during restricted energy intake [30]. This may be a possible explanation for the pair-fed group showing increased expression of two major proteins in rat milk. The mechanisms by which this hormone increases expression of these proteins are not yet understood, but it can be speculated that increased levels of prolactin may increase the transcription of β-casein and WAP as well as the half-life of these proteins [31]. Furthermore, the stability of β-casein mRNA may also be enhanced by high prolactin levels [32], which could possibly explain the increased concentration of this protein in the milk of the pair-fed group. Therefore, dietary insults (such as energy restriction) seem to affect β-casein expression in the rat mammary gland, which also results in increased concentrations of β-casein in milk. Although zinc deficiency causes increased prolactin levels in lactating rats [33], zinc deficiency can have independent effects on milk protein gene expression, and also on prolactin receptor [24], possibly explaining the difference in results between the low-zinc and the pair-fed groups. Unfortunately, we did not have sufficient sample volumes to analyze prolactin in the present study.

The concentration of β-casein in milk was significantly higher for the low-zinc and the pair-fed groups as compared to the other groups (Fig. 3A), whereas concentrations of WAP in milk did not show significant differences between groups (Fig. 3B). This illustrates how nutritional insults can have different effects on the concentration of different milk proteins although not affecting total milk protein concentration.

Transcript levels for β-casein were significantly higher for the pair-fed group, and this higher expression was clearly translated to a higher concentration of β-casein in the milk of pair-fed dams. We also found a higher concentration of β-casein in milk from dams in the zinc-deficient group even though no higher transcript levels were seen. Transcript levels for WAP were significantly higher for the low-iron and the pair-fed groups, but mRNA expression did not seem to be translated to a higher concentration of WAP in the milk, possibly due to degradation of WAP mRNA before it is translated to protein. These results indicate that diets deficient in iron or energy during pregnancy and lactation affect β-casein and WAP mRNA expression, but this does not necessarily translate into higher protein concentrations in the milk.

It is important to understand the underlying mechanisms for the effect of maternal nutrition on concentrations of specific proteins in milk. Previous studies [9–11,34] and the results of this study show that total milk protein concentration does not seem to be affected by suboptimal maternal nutrition. However, there are probably situations when some specific proteins increase and others decrease in concentration, although not significantly affecting total protein concentration in the milk [35,36]. The implications of such events could be considerable in situations when the concentration of proteins in milk that have specific physiological roles decreases, whereas other proteins not having bioactivity increase. The results of this study suggest that compromised maternal nutrition, such as energy restriction, affects the expression of individual milk proteins such as β-casein and WAP, which in turn could affect infant nutrition and health.

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