Biochemical Properties of Milk Reflect Evolutional Adaptations of Reproductive Strategy in Women, Cows and Mice

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ABSTRACT

We hypothesized that the biochemical properties of milk, specifically the concentrations of lactose, glucose, glucose 6-phosphate, citrate and malate reflect evolutional adaptations of distinct reproductive strategies in women, cows and mice. The concentration of lactose was highest in women's milk, lowest in mouse milk and intermediate in cow milk. Glucose concentration paralleled lactose concentrations. The ratio of glucose 6-phosphate to glucose in mouse milk was twofold greater and significantly higher than that in women's milk. Although the concentration of citrate in mouse milk was lower than in women's and cows' milk, it defied the long-held notion that mouse milk does not contain citrate. Malate concentration was highest in mouse milk. The gross composition of milk and lactation length in the studied species indicated that they represent three reproduction-strategy archetypes.

Keywords: Inter-species comparison; Lactose; Glucose; Glucose 6-phosphate; Malate; Citrate.

INTRODUCTION

The most prominent characteristic of mammals is nourishment of newborn offspring with milk produced in special glands adapted for that purpose – the mammary glands. Milk production level and composition vary greatly among mammalian species in a manner that is closely related to their overall reproduction strategy (1, 2). For instance, the following large variations in milk composition have been reported: proteins (~1% w/v in humans to >15% in rabbits), fat (<0.5% in rhinoceros to ~50% in seals), and carbohydrates (<0.5% in seals to >12% in wallabies) (1). The contribution of lactation to the total growth and development of the young can be quite small in some species, such as guinea pigs, but its proportion is very high in others, such as marsupials and primates (2). Lactation is the most energetically demanding period of a female's life and this trait can vary considerably among species. The metabolic demand for lactation is particularly high in mammals with a small body mass, such as mice, in which energy demands increase by 400% compared to the non-lactating state (2), versus typical increases of 35-150% in larger sized mammals (2, 3). In women, caloric intake in well-nourished subjects is 1.45 times greater than in non-lactating women (4). In cows, the production of each 10 liters of milk doubles their maintenance requirements (5). Thus, in cows that are not selected for milk production, the cost of this production is within the above-mentioned range. On the other hand, in modern cows, which have been intensively selected for high milk yield, the cost of milk production is 4-6 times their maintenance requirements (5). The nutritive constituents in milk differ greatly among species and vary during lactation according to the demands of their young (1, 2).

The ability to understand the evolutionary adaptations that enable close coordination between the glandular func-

tion and reproductive strategy might be improved by identifying general patterns among milk constituents in different mammals. A few examples support this notion:

i. Milk is strictly isotonic compared to blood plasma in all studied mammals (6);
ii. Lactose concentration is inversely related to that of major monovalent ions (Na^+ and K^+) (6);
iii. Casein and lactose concentrations are inversely related (7);
iv. The concentrations of Ca and Mg after ultrafiltration are positively correlated with that of citrate (8);
v. The colloidal concentrations of Ca, Mg and citrate are positively correlated to that of P (8).

These patterns of variation could result from evolutionary adaptations in the framework of a universal secretory mechanism (6-8).

Previous studies have shown that the concentration of metabolites produced and secreted by mammary epithelial cells (MECs) in humans (9,10), rats (11), pigs (12) and goats (13) is closely associated with MEC metabolic state. More recent studies have shown that the concentration of glucose and glucose-derived carbons in cow milk reflects metabolic functions in the MECs (14-19). These studies have shown the utility of modifications in milk concentrations of glucose, glucose 6-phosphate (G6-P), lactate, malate and citrate in identifying acute or chronic stresses and negative energy balance. In particular, modifications in milk concentrations of lactate, malate and citrate under acute stress (15,18), chronic stress (17), negative energy balance (19) and pro-involution (milk stasis) oxidative stress reflect the conversion of MEC metabolism to glycolysis at the expense of oxidative phosphorylation (16,18).

Very little is known about the concentrations of glucose and glucose-derived carbons in the milk of different mammals. The current knowledge on citrate concentration in mouse milk is based on findings from four and five decades ago (20,21), which in turn, were based on earlier M.Sc. thesis works that are not traceable.

The aim of the present study was to compare the concentrations of glucose and glucose-derived carbons in three mammalian species representing three major mammalian species (archetypes) of reproduction and milk composition in order to determine whether: (i) the information can help understand their evolutional adaptation to their mode of reproduction; (ii) any general patterns emerging among milk constituents. The chosen species were: humans, representing long lactation-high lactose concentration; mice, representing short lactation-low lactose concentration and cows, representing an intermediate model.

METHODS

Milk sampling

Women

Milk was sampled from 34 healthy women who delivered a singleton, term (>37 weeks gestation) infant in a major medical center in Israel between January and June 2014, and who initiated breastfeeding. In the first 5 days postpartum, mammary secretions of ~5 mL were collected by each postpartum woman with guidance from a lactation consultant, using manual expression or pumping, as has been found to be effective for early postpartum milk expression (22). The samples were stored in a -70°C freezer in the hospital until transport on dry ice for continued storage at the affiliated university laboratory; sample analysis was conducted within 3 months of commencement of sample collection.

Mice

Milk (~500 µL) was sampled in pools from the thoracic and abdominal mammary glands of 10 Swiss lactating mice at 3-4 days after parturition. Mice were separated from pups 2 hours prior to milk sampling. Milk was extracted by gentle pressure of the mammary glands and collected into 2-mL sterile tubes. The samples were stored in a -20 °C freezer and analyzed for the measures described for women within 3 weeks.

Cows

Milk samples (~20 mL) from 12 cows were taken at mid to late lactation. The samples were commingled milk of the four glands' yields, which were taken from bacterium-free glands with somatic cell counts of <100,000/mL. The samples were stored at -20 °C and analyzed for the measures described for women within 3 weeks.

Analytical procedures

Frozen milk samples were gradually defrosted without formation of clots. The concentrations of lactose, glucose, G6-P, citrate and malate were determined in whole milk samples by classical enzymatic reactions that use dehydrogenases (NAD^+-dependent oxidoreductases) coupled to conversion

of NAD^+ to NADH + H^+. The last stage in these determinations was linked to the formation of the fluorochromophore as follows:

$$NADH+H^+ \xrightarrow{\text{resazurin+diaphorase}} NAD^+ + \text{resorufin}$$

The detailed procedures for the determination of lactose (23), glucose and G6-P (17), citrate and malate (24) have been previously described. These fluorometric procedures are insensitive to the disrupting effects of fat and casein micelles and their coupling to form fluorochromophores increases their sensitivity. The limit of detection of these procedures was 10-50 μM and their sensitivity, (residual standard deviation), was ± 2-5 μM.

Statistical analysis

The UNIVARIATE procedure of the SAS (SAS Institute, Cary, NC, USA) statistical package program was used to check the normality of the data within each species. The result of this analysis showed that the data for all measures were normally distributed. Subsequently, ANOVA procedures of SAS were used to test the effects in women, mice and cows on concentrations of lactose, glucose, G6-P, citrate and malate by one-way analysis of variance (ANOVA). When statistical significance was detected ($P < 0.05$) for a particular measure, paired comparisons between means were carried out using Tukey's range test.

RESULTS

In women the pattern of changes in the concentrations of lactose, glucose, G6-P, citrate and malate during the first 5 days postpartum followed a gradual increase until stabilization on days 4 and 5 postpartum, consistent with previous reports (25). Consequently, the concentrations of those metabolites on day 5 postpartum were representative of milk composition in early lactating women.

The concentrations of lactose, glucose, G6-P, citrate and malate are summarized in Table 1. Lactose concentration was highest in women's milk (208 mM, 71.1 g/L), much lower in cow milk (147 mM, 50.2 g/L) and lowest in mouse milk (61 mM, 20.9 g/L), with all species differences being statistically significant. Glucose concentration was highest in women's milk, much lower in the cow milk and lowest in the mouse milk, with all differences between species being significant (Table 1). The concentration of G6-P was similar in women's milk and mouse milk, and significantly lower in cow milk. Consequently, the G6-P/glucose ratio was lowest in women and highest in mice (Table 1). Citrate concentration was highest in cow milk, much lower in women's milk and lowest in mouse milk. Malate concentration was highest in mouse milk, much lower in women's milk and lowest in cow's milk. Consequently, the malate/G6-P ratio was highest in mouse milk and much lower in women's and cow's milk. All the above measured species differences were statistically significant.

DISCUSSION

The three tested species represented three different types of milk composition (Table 2). Empirically, there was a close positive association between lactose and glucose concentrations in milk among the three tested species (Table 1, Fig. 1). However, to the best of our knowledge, very little information exists to support or reject the existence of a general positive relationship among species between milk lactose and glucose concentrations. In rat milk, the concentrations of lactose (111 mM) (26) and glucose (290 μM) (11) were found to be higher than in mouse milk in the present study (60 mM & 70 Mm, respectively) and consistent with such an inter-relationship (Fig. 1). Lactose is synthesized in the Golgi apparatus by condensation of one molecule of glu-

Table 1: Concentrations of lactose, glucose (G), glucose 6-phosphate (G6-P), G6-P/G ratio, malate (Ma), citrate (Cit) and Ma/G6-P ratio in milk from women, cows and mice (mean ± SE).

	N*	Lactose (mM)	G (μM)	G6-P (μM)	G6-P/G	Ma (μM)	Cit (mM)	Ma/G6-P
Women	34	$208\pm27^{c***}$	$1105\pm51^{c***}$	$140\pm19^{b**}$	0.13 ± 0.06^{a}	$1243\pm212^{b***}$	$5.71\pm0.160^{b***}$	8.88 ± 1.9^{a}
Cow	12	$147\pm17^{b***}$	$220\pm40^{b***}$	85 ± 25^{a}	$0.39\pm0.08^{b**}$	580 ± 155^{a}	$15.61\pm3.30^{c***}$	6.82 ± 0.9^{a}
Mice	10	61 ± 8^{a}	70 ± 7^{a}	$150\pm21^{b**}$	$2.1\pm0.11^{c***}$	$2405\pm230^{c***}$	0.97 ± 0.08^{a}	$16.0\pm2.1^{c***}$

* N = number of subjects.

[a,b,c] Values with different superscripts are significantly different by Tukey's pair-comparison test, where: * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Table 2: Gross milk composition: dry matter (DM), lactose, crude protein (CP), casein, and crude fat (CF) in milk of women, cows and mice, along with length of lactation (compiled from cited literature).

	DM[1] g/L	Lactose[2] g/L	CP[3] g/L	Casein[4] g/L	% Casein (casein/CP ratio)	CF g/L	Lactation length, months
Women	104	71.1	12.0	5.5	0.46	40	6-24
Cows	121	50.2	33.1	25.8	0.78	33	6-8
Mice	378	20.9	127.0	78.5	0.62	200	0.7-1

Sources of information:
[1] Women: (39); Cows: (38); Mice: (40).
[2] Present study.
[3] Women: (39); Cows: (38); Mice: (40).
[4] Women: (41); Cows: (38); Mice: (42).
[5] Women: (43); Cows: (44); Mice: (40).
[6] Women: (45); Cows: traditional in beef cows, (46); Mice: (47).

cose and one molecule of galactose (27). α-lactalbumin and galactosyltransferase make up the lactose synthase enzyme, which converts glucose and UDP-galactose into lactose with liberation of free UDP (27). UDP-galactose is derived from glucose in three enzymatic steps (27; Fig. 2). The inhibition of glucose uptake into lactose-synthesis chambers by phlorizin suggests the involvement of one or more of the members of the facilitated glucose transporter (GLUT) in this process (27). Thirteen members of this family have been identified: GLUT 1-12 and H^+/*myo*-inositol transporter, plus four pseudogenes (28). Affinities for glucose and transport kinetics of each transporter differ, which range from 0.2 to 17 mM. There is no precise information regarding the GLUT

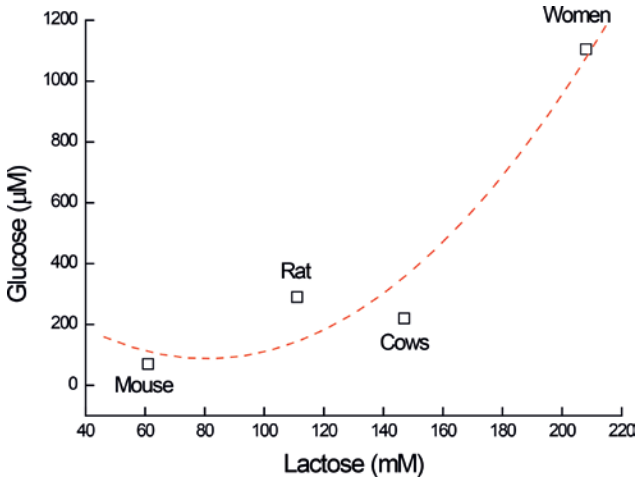

Figure 1: Inter-relationship between lactose and glucose concentration in the milk of mice, rats, cows and women*.

* The data were best fitted by a second-order polynomial. However, because of the small number of data points, the regression is not significant and as pointed out in the text. Further research is needed to establish its inter-species validity.

family members that are involved in glucose transport though Golgi apparatus membranes (27). Even less is known about how UDP-galactose is transported into the Golgi apparatus lumen, although the participation of an active transporter is assumed (27).

Based on the present results, the following assumptions can be made: (i) UDP-galactose, particularly in the case of cows' and women's milk, is taken into the Golgi apparatus by active transport, and (ii) in the case of mice, the Km of glucose transporters (an indicator of the affinity of the transporter protein for glucose molecules) is in the lowest range of GLUTs, whereas in the case of women it is in the highest range.

A central metabolite in cells is G6-P (Fig. 2): in non-MECs, glucose is rapidly phosphorylated to G6-P by G-hexokinase to be further metabolized. In MECs, free glucose is required for lactose synthesis. G6-P can then be further metabolized through the glycolytic axis or through the *pentose phosphate pathway* (PPP) (29). Because G6-P is derived from glucose, the only possible explanations for a G6-P/G ratio > 1 in milk are: (i) inhibition of G6-P's passage through the downstream stages of glycolysis in the MECs, or (ii) recycling of glucose-derived carbons, such as fructose 6-phosphate, formed in the PPP to G6-P (29). In dairy cows, a G6-P/ glucose ratio > 1 was found in early lactation, a period defined by negative energy balance and oxidative stress (19) and during acute inflammatory stress (18). It was concluded that the increase in G6-P concentration likely brings it closer to the optimal Km of G6-P dehydrogenase which shunts G6-P through the PPP (18,19). This results in the production of 2 molecules of NADP per molecule of G6-P. Conversion of oxidized glutathione to reduced glutathione by

glutathione reductase is a central reaction in the degradation of H_2O_2 (29). This reaction requires NADP as a cofactor, thereby increasing the cell's requirement for NADP during oxidative stress. Because of the tight energy budget in mice, the high G6-P/glucose ratio suggests that during lactation, most of the G6-P is metabolized through the PPP.

Although the concentration of citrate in mouse milk was the lowest among the three species tested (~1 mM), it was far from being negligible, defying the long-held notion that mouse milk does not contain citrate (20, 21). As traces or very low concentrations of citrate have also been reported in rat, elephant and viscacha rodent's milk (20, 21), it seems that there is a need to update the current state of knowledge in those and perhaps other mammals.

Milk is a challenging medium for measuring metabolite concentrations because: (i) it contains fat globules of varying sizes that scatter light in an unpredictable fashion; (ii) it is opaque owing to the casein micelles that scatter and absorb light (23,24). Taking these and the small volume that can be sampled from mouse mammary glands into consideration, it is reasonable to assume that the colorimetric method used for measuring citrate concentration in the study cited by Jenness (21) was not appropriate for the task.

Once formed in the tricarboxylic acid (TCA) cycle, citrate can be transported from the mitochondria to the cytosol by specific carriers (30). There is some evidence that citrate is accumulated from inter-cellular fluids, most likely from the cytosol, by the Golgi apparatus and along with lactose, casein and Ca, it is secreted into the milk by secretory vesicles (20, 31, 32). The Golgi apparatus fractions rapidly accumulate citrate; its transport is rapid, it exhibits saturation kinetics and it is temperature-sensitive, suggesting that it is carried by a protein transporter (33). It has been suggested that citrate transport into the Golgi apparatus may consist of either

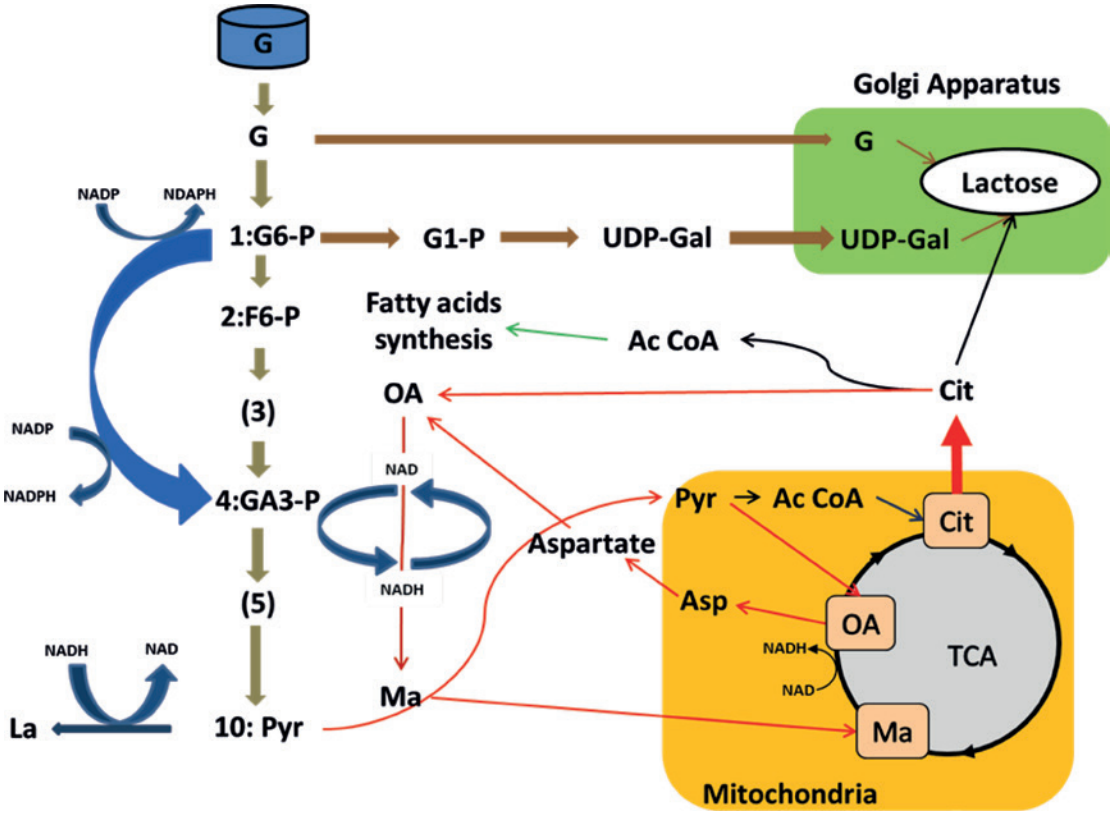

Figure 2: Schematic illustration of metabolic pathways associated with homeostatic aerobic glycolysis.

Glycolysis axis is marked in grey. Pentose phosphate pathway is marked in blue. Metabolic pathways of lactose synthesis are marked in brown. Transhydrogenation pathways between the mitochondria and the cytosol are marked in red (see text for further explanations).
Abbreviations: G – glucose; G6-P – glucose 6-phosphate; G1-P – glucose 1-phosphate; UDP-Gal – uridine diphosphate galactose; F6-P – fructose 6-phosphate; GA3-P – glyceraldehyde triphosphate; TCA – tricarboxylic acid; Cit – citrate; La – lactate; Ma – malate; Pyr – pyruvate; Asp – aspartate; Ac CoA – acetyl coenzyme A.

facilitated diffusion or active transporters (20). Considering the second option, it is possible that in mice, the gene responsible for active transport of citrate into the Golgi apparatus was evolutionarily negatively selected against, for reasons discussed further on. Considering the first option, formation of complexes with Ca and casein in the Golgi apparatus and secretory vesicles can effectively reduce the concentration gradient of free citrate (20), as demonstrated for other ions (32). In that case, the concentration of citrate in the Golgi apparatus and cytosol would be similar and large stores of citrate would be identified in the casein micelles. In cow milk, ~7% of total citrate is present in the non-aqueous phase, most likely in Ca-casein complexes (20). The much higher content of casein in mouse vs. cow milk (Table 2) may account for the binding of a larger proportion of total milk citrate within the casein micelles.

In small mammals, such as mice and rats, citrate is an important intermediate in fat synthesis within the cell, as it is involved in providing reducing power in the form of acetyl-coenzyme A (CoA) through the activity of ATP-citrate lyase (34,35). The high requirement for citrate-dependent acetyl-CoA formation might explain the sparing secretion of large amounts of citrate into mouse milk. The factors that govern the usage of cytosolic citrate between the options presented in Fig. 2 are not clear. An early hypothesis suggested that use of cytosolic citrate is controlled by the presence (e.g. in mice and rats) or absence (e.g. in cows) of ATP citrate lyase in the mammary gland (36). However, in the present study, we challenged the idea that cytosolic citrate is completely degraded by ATP citrate lyase. Secondly, Faulkner and Peaker (20) noted inter-species inconsistencies in the level of ATP citrate lyase activity in mammary glands and the expected level of citrate in their milk.

The efficiency of ATP production within the mitochondria may differ within and between species according to many factors such as growth rate, feeding rate and lactation capacity, and can vary between different tissues within the same animal (37). The efficiency of the coupling between the reducing factors formed in the TCA cycle, NADH and $FADH_2$ to ATP production by oxidative phosphorylation may explain the large proportion of the variation in the efficiency of ATP production under the above-described situations (37). Increase in the efficiency of ATP production per single cycle of the TCA can be achieved by increasing the number of NADH molecules produced per single cycle/unit of substrate supply. NAD itself cannot pass the mitochondria membrane; however, this can be achieved by replenishing the supply of metabolites that can pass through the mitochondria membrane and form NADH in the TCA cycle with 2 electrons needed to convert NAD to NADH. One such major pathway is the malate-aspartate shuttle (Fig. 2).

The much higher concentration of malate and much higher malate/G6-P ratio in mice milk in comparison to cows and human milk strongly suggest that the velocity of the malate-aspartate shuttle is more intense in mice than in cows and women. A critical regulatory support of such a cycle is the availability of NADH, which is needed to convert oxaloacetate to malate. The extra production of NADPH in the PPP at not necessarily on the expense of NADH in the glycolytic axis, because metabolites formed in the non-oxidative form of the PPP, fructose 6-phosphate and glyceraldehyde 3-phosphate can unite into the glycolytic axis and contribute to formation of NADH (Fig. 2). Thus, the high concentration of malate and high malate/glucose in mouse milk is consistent with the hypothesis on evolutionarily negative selected citrate uptake into the mitochondria and above mentioned hypothesis, because OA is a by-product of acetyl-CoA formation (Fig. 2).

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IN MEMORY OF DR. NISSIM SILANIKOVE 1952-2017

Nissim Silanikove passed away on August 13, 2017, after combating cancer for many years. Nissim fulfilled his Ph.D. program at Tel-Aviv University with Prof. A. Shkolnik on the adaptation of Bedouin desert goats to harsh conditions. After a short period that he spent at Migal (the scientific R&D program of the Upper Galilee), in 1986 Nissim joined the Institute of Animal Science at the Agricultural Research Organization (ARO, The Volcani Center), Bet Dagan, where he was employed until his last days. Nissim developed interest in physiology of lactation, including sheep and goat milk quality and the impact of mastitis on the milk quality. He developed a Casein hydrolyzate preparative for drying-off mammary glands to assist end of lactation and intramammary infection. Nissim published over 150 scientific articles and was cited > 7300 times, which places him at the top of his field.

This manuscript is the last one, which was submitted for publication as part of an ongoing study for understanding the physiology and immunity of the mammary gland and its relation to milk constituents for offspring in relation to the environment and evolution. Nissim was an open-minded scientist who always searched new avenues of interpretation of phenomena of mammalian physiology, pursuing a common evolutionary denominator.

In remembrance of Nissim Silanikove and his contribution to the scientific community, his memory will always be cherished by us.

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