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Original article

Major constituents, leptin, and non-protein nitrogen compounds in mares' colostrum and milk

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Abstract — Five Haflinger mares were hand-milked at 0 h (pre-suckle) and 6 h (postsuckle), 12, 24, 48, 72 and 96 h after parturition. Total solids, protein, fat, lactose, calculated gross energy content, leptin and non-protein nitrogen components (urea, α-amino nitrogen, creatinine and allantoin) were determined. The levels of the major constituents differed significantly in pre-suckle colostrum from subsequent samples. Leptin levels were the highest in whole (9 ng·mL^{-1} of immunoreactive human equivalent HE ± 0.48 ng·mL^{-1}, SEM) and skimmed (7.8 ng HE·mL^{-1} ± 0.52 ng·mL^{-1}, SEM) pre-suckle colostrum, declined sharply at 6 hours postpartum, and more slowly subsequently. Mean urea concentration was constant at around 5.0 mM, while α-amino N increased over the observation period and creatinine and allantoin decreased. These findings provide a further indication that mares' milk can be regarded as a functional food.

mares' milk / colostrum / major constituents / leptin / non-protein nitrogen

Résumé — Principaux constituants, leptine et métabolites d'azote non protéique dans le colostrum et le lait de jument. Cinq juments Haflinger ont été traitées à la main aux temps 0 (avant tétée) et 6 (après tétée), 12, 24, 48, 72 et 96 heures après le poulinage. Les paramètres évalués sur ces échantillons ont été les principaux constituants de la sécrétion mammaire (matière sèche, protéines, lipides, lactose), le contenu énergétique brut et la leptine ; en plus, on a considéré les teneurs en urée, α-aminoacides, créatinine et allantoïne. Une différence significative a été observée entre les valeurs enregistrées dans le colostrum prélevé avant la tétée et dans les autres échantillons. De même, des variations dynamiques concernant la leptine immunoréactive ont été relevées au cours de l'essai : les valeurs étaient les plus fortes dans le lait entier (9 ng·mL^{-1} équivalent de leptine humaine HE ± 0,48 ng·mL^{-1}, SEM) et dans le colostrum écrémé (7,8 ng HE·mL^{-1} ± 0,52 ng·mL^{-1}, SEM) prélevés avant la tétée. La concentration en urée était en moyenne de 5,0 mM et la concentration en azote des α-aminoacides dénote des bilans différents par rapport à celui mis en évidence pour la créatinine et pour l'allantoïne. Ces résultats contribuent à faire considérer le lait de jument comme un aliment fonctionnel.

lait de jument / colostrum / constituants du lait / leptine / non-protein nitrogen

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1. INTRODUCTION

Mammalian milk contains nutrients, cells, enzymes, hormones and protective and trophic factors. Milk helps to prevent metabolic disorders and chronic diseases in newborn mammals [17] and has been called a "functional food" due to its other roles in addition to that of simple nutrition [14]. Leptin, a 16 kDa protein, is the most recently discovered factor in milk [2, 11, 26].

As reported in extensive reviews on the biology of the hormone and its implications in animal production, plasma leptin, mainly secreted by mature adipocytes [31], is a metabolism modifier that plays a role in coordinating food intake, energy expenditure and nutrient utilization [12, 24]. However it is becoming clear that the peptide has other functions [10, 26].

Leptin is also synthesized by human mammary epithelial cells and secreted into milk, but whether or not this is the main source of milk leptin is unclear [11, 26]. Emerging evidence has indicated that leptin in milk may be involved in the regulation of growth, and in particular in the development and maturation of the neonatal gut, the immune system and the neuroendocrine system [12, 24]. Leptin in milk may also exert a more general regulatory activity until organ systems begin to function autonomously [19].

Serum leptin has been studied in mares in relation to reproductive activity [18], however no data are available on the leptin content of mares' milk. Since leptin may stimulate the functional development of the neonate and because newborn horses have a rapid growth rate, we decided to investigate leptin in mares' colostrum and milk.

Similarly, although α-amino acids and urea are known to play a role in the nutrition of newborn humans, other non-protein nitrogen (NPN) components of milk may have particular roles as growth modulators [14] but also as taste factors and substrates for milk microbes, as Tiemeyer et al. [28] have suggested for metabolites of nucleic acids in bovine milk. In order to contribute to the knowledge on mares' milk composition, a starting point in understanding its functional significance for the newborn foal, we decided to extend the present study to the levels of urea, α-amino nitrogen, creatinine and allantoin in mares' colostrum and milk.

2. MATERIALS AND METHODS

2.1. General

Colostrum and milk samples were collected from five multiparous Haflinger mares, housed in a sand paddock during the day and box stalls at night. The animals foaled at term from the end of March to the beginning of July with a body condition score ranging between 3 and 3.5 [15]. The average bodyweight was 416.7 kg (±3.9 kg, SEM). From about 320 d of gestation, the animals were adapted to a lactation diet composed of 8 kg meadow hay (9% CP, 6.8 MJ DE·kg^{-1}), 4 kg commercial mixed feed (15% CP, 11.5 MJ DE·kg^{-1}) and 100 g corn oil (data expressed as fed). The foals were clinically normal at birth and suckled colostrum without assistance within two hours of birth. The mares were hand-milked, without oxytocin, immediately after foaling but before the foal nursed (0 h, presuckle) and at 6, 12, 24, 48, 72 and 96 hours later.

The samples (100–150 mL) were stored at –80 °C. They were left for 24 h at –20 °C then thawed overnight at 4 °C prior to analysis.

Skimmed milk was prepared by centrifugation of whole milk (1500 × *g*, 20 min, 4 °C). For leptin determination, whole and skimmed milk samples were sonicated on ice for 2 min using an ultrasonic-homogenizer (Labsonic, Braun Biotech, Diessel, D), as described by Smith-Kirwin et al. [26].

2.2. Milk constituents and hormone determinations

Total solids (TS) were determined following drying of weighed samples at 110 °C overnight. Fat, crude protein (as N% × 6.38) and lactose were determined by IR spectroscopy (Milkoscan 605, Foss Electric, Hilleroed, DK) calibrated according to FIL-IDF [9]. Gross energy was calculated using Perrin's equation [23] and the results were converted to Joules and expressed as $kJ \cdot L^{-1}$.

Leptin was assayed with a commercial multi-species leptin RIA kit (Cat. No. XL-85K, Linco Research Inc., St. Charles, MO, USA). According to McManus and Fitzgerald [18], the results are reported as human equivalents of immunoreactive leptin (ir-leptin), since purified equine leptin was not available.

Serial dilutions (25 to 100 μL) of whole and skimmed milk in 100 μL buffer gave leptin values parallel to the standard curve. The detection limit was 0.9 ng ir-leptin$\cdot mL^{-1}$. Intra-assay coefficients of variation were 4.2% and 3.8%, and inter-assay coefficients of variation were 9.2% and 7.8 %, for whole and skimmed milk samples respectively.

The creatinine content of sonicated whole milk was determined by an enzymatic spectrophotometric assay (Cat. No. 883263**,** Boehringer Mannheim, Mannheim**,** Germany). Before determination of the other NPN constituents, samples were deproteinated. Whole milk urea was determined spectrophotometrically (Cat. No. 535A**,** Sigma Diagnostics, St. Louis, MO, USA) after deproteinisation with trichloroacetic acid 0.3 N; the α-amino nitrogen of deproteinated (sodium tungstate 1.3% and HCl 0.1 N) samples was determined by the Goodwin method [13]; allantoin was determined by the Rimini-Schryver reaction [30] on samples pretreated with perchloric acid 1.2 mM and neutralized with KOH 3 M.

2.3. Statistical analysis

Data were analyzed by analysis of variance (GLM procedure; SAS Inc., Cary, NC USA), considering mares and sampling-time effects. The differences between animals were not significant, thus the post hoc Scheffè test was applied to test the significance of differences among sampling times. A two sided *f*-test was used to test the significance of linear correlations (SAS Inc.) between leptin and milk fat and nitrogen metabolites.

3. RESULTS AND DISCUSSION

The levels of the investigated major components of colostrum and transitional milk (Tab. I) showed daily differences over the

Table I. Changes in total solids, fat, protein, lactose contents and estimated energy of mammary secretions from pre-suckle to 96 hours post-partum.

	Hours from foaling							
Parameter	0	6	12	24	48	72	96	SEM
Total solids, $g \cdot L^{-1}$	202.5 a	124.0 b	123.0 b	119.2 b	119.0 b	118.2 b	113.4 b	0.66
Protein, $g \cdot L^{-1}$	160.1 a	48.9 b	35.1 b	33.9 b	32.0 b	30.9 b	30.8 b	0.46
Fat, $g \cdot L^{-1}$	7.2 a	24.9 b	30.3 b	24.9 b	24.9 b	25.3 b	18.9 b	0.23
Lactose, $g \cdot L^{-1}$	34.0 a	49.5 b	56.3 bc	60.4 c	60.9 c	62.6 c	62.7 c	0.16
Energy, $MJ \cdot L^{-1}$	4.8 a	3.0 b	2.9 b	2.8 b	2.7 b	2.7 b	2.5 b	0.13

Within rows, means without common letter differ ($P < 0.05$).

experimental period: in general, total solids and total protein declined, while lactose and fat content increased. The average concentrations of the major components of mammary secretions in our study were similar to those reported by Csapò-Kiss et al. [4] and by Martuzzi et al. [16], while the energy content was similar to that reported by Oftedal and Jenness [22] on mares' milk samples at various stages of lactation.

In addition, the peculiar trend in fat content (Tab. I) was consistent with the findings of Doreau and Boulot [7] and Dell'Orto et al. [5].

Ir-leptin was only recently measured for the first time in mares' plasma [18] and the average value found was 3.5 ng·mL^{-1} ir-leptin. Our study provides the first evidence that mares' milk and colostrum also contain ir-leptin, whose levels are shown in Table II. Leptin coefficients of variation among mares were comprised between 7.8% and 18%,in post-suckle and pre-suckle samples respectively.

Ir-leptin levels were the highest ($P < 0.05$) in both whole and skimmed pre-suckle colostrum and declined subsequently as shown in Table II; it is interesting to note that ir-leptin levels, as well as allantoin concentrations, did not significantly vary when expressed on a total solid basis (range 43–46 ng ir-leptin·g^{-1} TS), suggesting that these constituents in mares' milk/colostrum mainly depend on a dilution effect.

The high levels of ir-leptin that we found in mares' colostrum and transitional milk are consistent with the suggestion of Mistry et al. [19] that the peptide's thermoregulatory role in the neonate differs from the anorectic role in adults, acquired most likely at the time of weaning.

Smith-Kirwin et al. [26] reported leptin levels in human milk that were 10 to 15 times higher than what we found in mares' milk, however Houseknecht et al. [11], and Rosi et al. [25] reported ir-leptin levels respectively in human breast milk and bovine milk that are similar to what we observed. By contrast, ir-leptin levels in human skimmed milk [2, 26] were about 50% lower than the level we found in skimmed mares' milk. This variation could be due to differences in the assay methods but could also reflect species-specific variations, since there are interspecies

Table II. Mean ir-leptin concentrations in whole and skimmed mammary secretions and trends in urea, α-amino nitrogen, creatinine and allantoin from pre-suckle to 96 hours post-partum.

	Hours from foaling							
Parameter	0	6	12	24	48	72	96	SEM
Ir- leptin								
In whole milk, ng·mL^{-1}	9.05 a	5.60 b	5.47 b	5.39 b	5.34 b	5.32 b	5.11 b	0.48
In skim milk, ng·mL^{-1}	7.83 a	3.81 b	3.11 b	3.06 b	3.07 b	2.81 b	2.74 b	0.52
Skim: whole secretions leptin (%)	85.9 a	70.4 ab	55.6 ab	56.6 ab	57.1 ab	53.0 b	53.0 b	5.93
NPN components								
Urea, mM	5.11	5.34	5.16	5.13	5.02	4.68	4.31	0.21
α-amino N, mM	3.61 a	3.24 a	3.78 ab	4.60 abc	5.65 bc	5.87 c	5.52 bc	0.34
Creatinine, μM	90.5 a	59.8 b	45.3 bc	40.2 bc	33.8 c	28.3 c	25.3 c	4.0
Allantoin, μM	349.4 a	267.1 ab	223.6 b	231.1 b	222.3 b	227.0 b	181.0 b	19.3

Within rows, means without common superscript differ ($P < 0.05$).

differences in milk composition [27] and growth factor contents [21].

The higher ir-leptin concentrations in whole than in skimmed mares' mammary secretions (Tab. II), suggest that a fraction of the hormone is associated with milk fat globules, as also hypothesised for human milk [26]. Ir-leptin concentrations in the milk fat layer (data not shown) ranged between 86 and 98 $ng \cdot g^{-1}$ fat and were on average 30 times higher than in skimmed milk, according to other findings [11]. However, a significant negative correlation was noted (Fig. 1a) between ir-leptin and lipid concentration in mammary secretions when pre-suckle samples were considered, but no correlation was found when these were removed (Fig. 1b), thus the correlation was due exclusively to the low fat content of the pre-suckle samples. No significant correlation between leptin levels and fat concentration of whole milk was reported by other authors [11, 25].

The majority of plasma leptin in rodents and humans is specifically and saturably bound to proteins [11]; this may also be the case in mares' milk, since the bound forms of leptin could be present both in solution in the skim phase and associated with fat globules. If a portion of the hormone is sequestered in fat globules or bound to precipitable hormone receptors associated with fat globule membranes, adequate sonication is crucial prior to leptin radioimmunoassay in order to avoid falsely high values in the fat fraction due to incomplete disruption of the fat globules (F. Rosi, unpublished data).

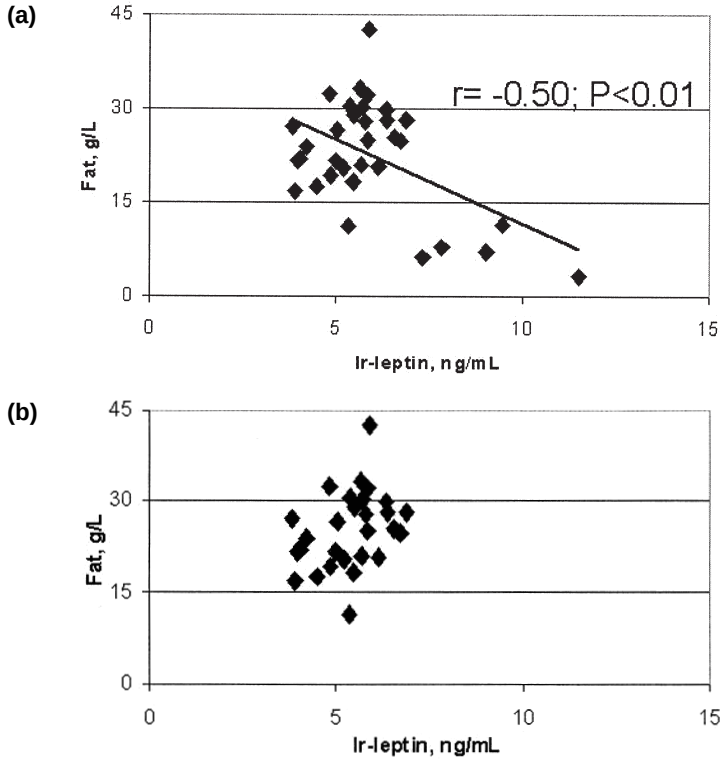

Figure 1. Relationship between leptin levels and fat content of mares' milk and colostrum. (**a**) All samples; (**b**) pre-suckle samples excluded.

Although a link between maternal adiposity and leptin content of milk has been suggested by others [3, 12], the present study does not provide evidence on this regard, since all the mares foaled in similar body condition and their feed consumption was always complete.

Urea, α-amino N, creatinine and allantoin concentrations in milk are also shown in Table II. As in human milk, a considerable proportion (30–50%) of the NPN content of mares' colostrum and transitional milk consists of urea [7]. The urea levels in mares' milk, which were stable throughout the experimental period, were close to those reported in bovine milk but slightly lower than those in human milk [1]. However, compared to herbivores, only a small proportion of the urea in human milk is available for the synthesis of amino acids in the human newborn [14], because of the relatively low efficiency of urea hydrolysis by gut micro-organisms [8].

Among the NPN metabolites we investigated, only α-amino nitrogen levels increased over the 96 hours of the study; the free amino-acid composition of mares' milk was determined by Kulisa, as reviewed by Doreau and Boulot [7]. The observed α-amino nitrogen levels were similar to those observed in human colostrum and transitional milk [1], but slightly higher than in cow's milk [6].

By contrast, creatinine and allantoin levels decreased steadily in parallel with the decline in ir-leptin levels. On this regard, the observed correlations between creatinine and allantoin levels with leptin levels (Tab. III) could be partly derived from the same time pattern. Creatinine and allantoin levels were significantly ($P < 0.001$) associated with each other over the 96 hours of the study, notwithstanding their different metabolic origins [20, 29]. Allantoin and urea levels were also directly correlated ($P < 0.01$) probably due to their roles in nitrogen disposal. Conversely, creatinine and α-amino N were inversely correlated ($P < 0.001$): this could be related to the fact that the former (high in colostrum) is derived from the maternal muscle mass [20] and the latter from maternal protein intake [1].

To conclude, we found that leptin is present in mares' colostrum and transitional milk up to four days of lactation, suggesting that the peptide may play a role in neonatal growth regulation as it seems to do in rodents and humans. Further studies of the biological role in foal metabolism and gastrointestinal development are needed. In addition, the presence of non-protein nitrogen compounds in mares' colostrum and milk also raises questions about their functional significance to the foal, as a possible further functional dimension of milk.

Table III. Correlation coefficients between leptin and NPN in colostrum and milk.

	Urea	α-amino N	Creatinine	Allantoin
Ir-leptin, ng·mL^{-1}	NS	NS	0.47 $P < 0.01$	0.48 $P < 0.01$
Urea, mM	–	NS	NS	0.52 $P < 0.01$
α-amino N, mM		–	–0.55 $P < 0.001$	NS
Creatinine, μM			–	0.69 $P < 0.001$

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