

Original article

Triiodothyronine (T_3), insulin and characteristics of 5'-monodeiodinase (5'-MD) in mare's milk from parturition to 21 days post-partum

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Abstract – It is generally accepted that hormones and tissue growth factors are supplied from mother to neonate via mammary secretion. Among the protein hormones, insulin and prolactin are considered as the most important milk components for neonates. The significance of the thyroid hormones, namely triiodothyronine (T_3) generated locally by 5'-monodeiodinase (5'-MD) in the mammary tissues, for the mammary gland itself and for suckling neonates is still under consideration. In the present study the activity of the 5'-MD and the concentrations of T_3 and insulin in mare's colostrum and milk during the first 21 days of lactation were measured. Post partum, T_3 increased to its highest concentration around day 4 (1.14 ± 0.08 nmol/L), then progressively decreased until day 7, reaching a relatively stable concentration of 0.71 ± 0.06 nmol/L (overall mean for days 7–21). The colostrum insulin concentration, highest on the day of parturition (401.0 ± 24.9 μ U/mL), decreased to a nadir value (25.0 ± 3.4 μ U/mL) on day 5, after which it tended to increase. The mare's milk showed the presence of PTU-sensitive (type I) and PTU-insensitive (type II) 5'-monodeiodinases (5'-MD). Contrary to the classical type II 5'-MD, the mare's milk isoenzyme was inhibited non-competitively by aurothioglucose. A significant relationship ($r = 0.962$, $P < 0.01$) between T_3 concentration and 5'-MD activity, from the 1st to the 6th lactational day was found, which may indicate a dependence of T_3 concentration on the milk 5'-MD activity. The presence of 5'-MD of type II suggests that intra-mammary T_3 generation may play a paracrine role supporting lactogenesis. Estimating that 1.8 μ g of colostrum T_3 (0.456 μ g/L) is consumed daily by a suckling foal, the T_3 hormone action within the intestinal tract cannot be ruled out. This is the first paper to provide evidence of T_3 and insulin concentrations, and of T_4 -5'-monodeiodinases activity in colostrum and milk of the mare. © Inra/Elsevier, Paris

triiodothyronine in milk / insulin in milk / 5'-deiodinase in milk / mare's milk / hormones in milk

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Résumé – Concentrations de T₃, d'insuline et caractéristiques de la 5' monodéiodinase dans le lait de jument au cours des 3 semaines post-partum. Le lait permet le transfert d'hormones maternelles (en particulier la prolactine et l'insuline) de la mère vers le nouveau-né. De plus, un rôle local de la triiodothyronine (T₃) sur la mamelle comme un rôle sur le jeune via l'allaitement sont possibles. Dans ce travail, l'évolution, pendant les 21 jours post-partum des concentrations d'insuline et de T₃ dans le colostrum, et de l'activité de la 5' monodéiodinase a été examinée. Les concentrations de T₃ dans le colostrum présentent un pic à 4 j post-partum ($1,14 \pm 0,08$ nmol/L) avant de redescendre jusqu'à 7 j et se stabiliser à des niveaux de $0,71 \pm 0,06$ nmol/L. Les concentrations d'insuline dans le colostrum sont maximales le jour de la parturition ($401,0 \pm 24,9$ μ U/mL) puis diminuent jusqu'à j 5 post-partum ($25,0 \pm 4$ μ U/mL). Des monodéiodinases de type I (PTU sensible) et de type II (PTU insensible) sont présents par l'aurothioglucose de façon non compétitive. Une corrélation très significative ($r = 0,96$, $P < 0,01$) existe entre les concentrations de T₃ et l'activité monodéiodinase lors de la première semaine post-partum. Cette corrélation suggère un rôle de la monodéiodinase dans la production mammaire de T₃. De plus, compte tenu des quantités de T₃, ingérées quotidiennement par le poulain (1,8 μ g), une action de la T₃, sur l'ingestion est possible. © Inra/Elsevier, Paris

jument / lait / insuline / triiodothyronine / 5' déiodinase

1. INTRODUCTION

Hormones and growth factors are generally in high concentrations in colostrum and milk. This finding has generated discussion on their role in the neonate [3, 33]. Pearlman [34] inferred from comparative studies across a wide range of species that the timing of birth and the postnatal growth rate are both reflected in the supply of hormones and tissue growth factors from mother to neonate via mammary secretion. Among the protein hormones present in colostrum and milk, only two – insulin and prolactin, have been considered as milk factors influencing newborns, offering selective advantages for reproductive success [34]. This view is supported by results of other studies [14, 28, 35].

The presence of thyroid hormones (TH) in milk has been reported many times but their role for neonates is so far less documented. From thyroxine (T₄) and triiodothyronine (T₃) concentrations in sow and cow milk, it has been calculated that the daily consumption of the hormones in piglets and calves, relative to

the normal secretion rate, is about 2 % for T₄ and 4 % for T₃ in piglets [42], and 4 % for T₄ and 7 % for T₃ in calves [1]. The quantity consumed may suggest little systemic influence but it does not rule out the possibility of a local action in the alimentary tract. In fact, a significant role of thyroid hormones (TH) in the process of intestinal enzyme metabolism has been documented in rodents [20, 21], and in macromolecule transfer in newborn calves [43].

In contrast to insulin, for which the pancreatic islets are the only natural source, T₃ (but not T₄) is locally generated within the mammary gland of the cow [19, 40] and pig [40], by an enzymic mechanism deiodinating T₄ to T₃. Two thyroxine 5'-monodeiodinases (5'-MD deiodinating T₄ to T₃) have been found in the cow and the pig mammary gland [40]. One is inhibited by prophyllthiouracil (PTU), type I 5'-MD, and the other is PTU resistant (type II 5'-MD). Deiodination in the milk or mammary gland tissue in the cow and in the pig is catalysed predominantly by type II 5'-monodeiodinase. The importance of this lies in the fact that the type II 5'-MD

has so far been found only in those tissues where T_3 appears to act as a paracrine factor [38]. A local (paracrine) role for T_3 generated in the mammary gland has already been postulated [17].

In spite of the recognized importance of insulin and thyroid hormones for mammary gland function and their benefit to offspring in various species, similar studies in horses are lacking. The aim of the present study was to describe the presence and sequential changes in T_3 and insulin concentrations, to prove the existence of a monodeiodinating process (the factor responsible for local T_3 generation) in colostrum and milk and to describe the characteristics of the deiodinating enzyme.

2. MATERIAL AND METHODS

Ten mares (Wielkopolska Breed; saddle-carriage type) from a single-breeding stud were used. The history of the horses indicated that the mares were from 5 to 17 years old and in the 2nd to 13th lactation period. Colostrum or milk samples were taken sequentially from the day of parturition (day 0) and on days 2–7, 14 and 21 post-partum. After cooling in an ice-water bath, the milk samples were transported cold to the laboratory.

2.1. Extraction of iodothyronines from milk

The extraction was performed with alkaline ethanol at low temperature, as described previously [42]. In brief, to 1 mL of whole mare's colostrum or milk, 2 mL of cold (-20°C) alkaline ethanol, pH 9.0 (7 mmol of NH_4OH commercial solution in 100 mL of 96% ethanol) were added, mixed thoroughly with a glass rod, vortexed and left overnight in a freezer (-20°C). After 24 h the milk-ethanol mixture was vortexed, left in a freezer for a further 24 h and then centrifuged at 3 500 g for 30 min. Supernatant was stored at -20°C until analysed. This procedure was validated for the mare's milk. The recovery of added T_4 and T_3 was 90 and 98%, respectively.

2.2. T_3 radioimmunoassay

One day before analysis, the milk extracts were diluted (1:1) with buffer used for RIA. This procedure was aimed at removing excess ammonia which might interfere with the assay. The T_3 concentration was measured by an RIA which is validated for colostrum or milk extracts from human, cow, pig and rabbit [42]. Charcoal methyl-cellulose was used for the separation of free and bound fraction. Intra-assay and inter-assay coefficient of variation for a quality control extract was 4.14 and 8.0%, respectively, and the detection limit was 0.02 nmol T_3/L . Cross reactivity with other iodothyronines was negligible. The antibodies were the same as those used in previous work [41].

2.3. RT_3 -5'-monodeiodinase determination

3,3',5'-triiodothyronine (reverse T_3 , rT_3) was used as a substrate for the 5'-MD activity determination. The measurements of 5'-MD activity was based on release of $^{125}\text{I}^-$ from (^{125}I) rT_3 according to the method of Leonard and Rosenberg [24] and modifications of Jack et al. [17]. The (^{125}I) rT_3 (DuPont-NEN, Belgium), labelled in the outer ring only (specific activity 976–1 260 $\mu\text{Ci}/\mu\text{g}$), was purified by Sephadex LH-20 chromatography immediately before assay [27]. The (^{125}I) rT_3 fraction was dried under nitrogen and dissolved in 0.1 M potassium phosphate buffer (pH 7.0).

Colostrum or milk samples were centrifuged at 2 500 g for 20 min at 4°C and the infranatant below the fat layer was stored at -20°C until analysed. The assay mixture consisted of 10 mM dithiothreitol (DTT), 5 μg of milk protein and between 60 000 and 80 000 c.p.m. (^{125}I) rT_3 , mixed with unlabelled rT_3 to a final concentration of about 400 nmol/L. For kinetic studies rT_3 concentrations were used as indicated in figure 1.

The assay mixture was incubated at 37°C for 2 min (blanks) and 12 min (samples), each in triplicate. The enzymic reaction, which was linear for more than 30 min, was terminated by the addition of 0.5 mL ice-cold horse serum followed by 0.5 mL of 10% (w/v) trichloroacetic acid. After centrifugation for 10 min at 1 500 g, radioactivity of free $^{125}\text{I}^-$ in 0.5 mL of supernatant was measured. Radioactivity in blanks was subtracted from that in samples, and the results expressed in pmol I^- liberated/mg

protein/min. Protein concentration in milk was determined by the method of Hartree [15].

2.4. Insulin radioimmunoassay

After acidification of colostrum or milk samples with concentrated acetic acid to pH 4.6 (isoelectric point of casein), and centrifugation at 2 500 g for 15 min at 0 °C, the fat layer and casein pellets were discarded and the infranatant was stored at -20 °C until analysis.

Insulin concentration was measured in the whey samples (0.2 mL each) using the method of Kulski and Hartmann [22] and the modifications of Nowak and Nowak [29], with the

use of RIA-INS test kits (Swierk, Poland). For low and high quality control samples, the intra-assay coefficient of variation was 7.5 and 5.2 % ($n = 18$) and the inter-assay coefficient (13 assays) was 10.7 and 8.3 %, respectively. Recovery of added insulin was between 89 and 105 %, mean 96.1 %.

2.5. Statistical analysis

Data were analysed by the paired Student's *t*-test, and curvilinear regression analysis with F-test of Snedecor [44]. Differences between means were considered significant when $P < 0.05$. Values are given as means \pm S.E.M.

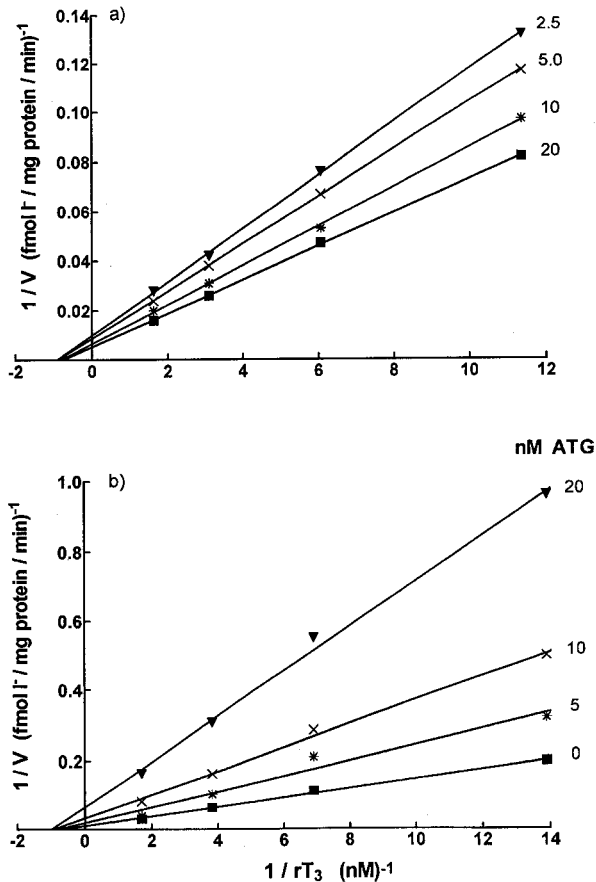


Figure 1. Lineweaver-Burk plots of the rT_3 -5'-deiodination by mare milk: a) at 1 mM PTU in the presence of 2.5 (\blacktriangledown), 5.0 (\times), 10.0 ($*$) or 20.0 (\blacksquare) mM DTT; and b) at 10 mM DTT and 1 mM PTU in the absence (\blacksquare) or presence of 5.0 ($*$), 10.0 (\times) or 20.0 (\blacktriangledown) nM ATG. Points are the mean of four determinations.

3. RESULTS

Colostrals T_3 concentration increased post partum from 0.76 ± 0.04 nmol/L to the maximal value (1.14 ± 0.08 nmol/L) on day 4, then decreased rapidly (between days 5 and 7). The mean T_3 concentration between days 7 and 21 was 0.71 ± 0.06 nmol/L (figure 2a).

Insulin concentration (figure 2b), was high on the day of parturition (401.0 ± 24.9 μ U/mL), and decreased rapidly to the level of 35.0 ± 2.3 μ U/mL on day 2 (figure 2b). From day 5 (25.5 ± 3.71 μ U/mL), the insulin concentration gradually increased to the latest recorded value of 40.1 ± 4.15 μ U/mL ($P < 0.02$), on day 21 post partum.

Colostrals 5'-MD activity, expressed as pmol I^- released /mg protein/min, was highest on the day 0 (7.3 ± 0.20), and decreased rapidly to a nadir (5.5 ± 0.10) on day 1. Between days 1 and 4, a statistically significant increase ($P < 0.05$) in 5'-MD activity to the value of 6.0 ± 0.15 pmol I^- /mg protein/min was observed. The activity of 5'-MD from days 1 to 6 post partum ($n = 6$) displayed a curvilinear relationship with time, described by a second-degree polynomial equation (figure 3a). A similar curvilinear relationship was observed for T_3 concentration in milk during the first 7 consecutive days post partum (figure 3b). The relationship between 5'-MD activity and T_3 concentration was linear and highly correlated (correlation coefficient, $r = 0.962$; $P < 0.01$; figure 3c).

As summarized in table 1 there was a 46 % inhibition of rT_3 -5'-MD activity in the presence of 1 mM propylthiouracil (PTU) and 61 % inhibition in the presence of 10 nM aurothioglucose (ATG). The addition of non-radioactive thyronines (T_4 and T_3) to the incubation mixture showed that the 5'-MD present in the mare's milk was more sensitive to T_3 than to T_4 inhibition.

In the presence of 50–450 nM rT_3 and 10 mM DTT, the apparent K_m value and maximum velocity (V_{max}) of the milk 5'-MD were 0.2 ± 0.06 μ M and 8.2 ± 0.36 pmol I^- /mg protein/min, respectively. The apparent K_m value in the presence of 1 mM PTU at 10 mM DTT (the activity of type II 5'-MD) was 0.8 ± 0.02 nM and V_{max} 104.9 ± 3.22 fmol/mg protein/min.

Because rT_3 5'-deiodination in mare's milk was only partially inhibited by PTU, and substantial activity remained in the presence of PTU at concentrations as high as 1 mM, Lineweaver-Burk plots were calculated, at various rT_3 concentrations, in the presence of 2.5–20 mM DTT and 1 mM PTU (figure 1a). The sequential-type reaction kinetics of PTU-insensitive deiodination was obtained. The effect of gold (aurothioglucose) on the PTU-insensitive pathway of rT_3 5'-deiodination (in the presence of 1 mM PTU) showed that ATG inhibited the reaction non-competitively with rT_3 . The calculated apparent K_i value was 11.5 nM (figure 1b).

4. DISCUSSION

T_3 concentration in mare's milk during the early stage of lactation (days 1–7), was linearly correlated with 5'-MD activity (figure 3c). This agrees well with our previous finding in lactating rabbits, indicating that the ultimate T_3 level in milk is in part a result of enzymic generation of T_3 from T_4 within the mammary gland tissue [40]. In milk, 5'-MD may originate from plasma membrane components and/or from cellular components, namely epithelial cells. In fact, relative to the 5'-MD in the whole milk, the deiodinating activity exerted by macrophages, lymphocytes and granulocytes was found to be low [40]. The high or increasing T_3 concentrations observed in milk at the beginning of lactation was described previously in pigs [42]. In rats, changes in

5'-MD activity proportional to lactational intensity were observed by Kahl et al. [18]. It has been postulated that T_3 generated in the mammary gland during the early lactation period, serves as a paracrine factor to support lactogenesis and at the same time to supply the hormone to the neonate through milk intake [32].

Two distinct types of enzyme (type I and type II) are known to be responsible for outer ring deiodination of iodothyronines in the 5'-position [25]. Type I 5'-MD, inhibited by PTU is present in the

liver, kidney, skeletal muscle, heart and lung, while type II 5'-MD, insensitive to PTU, is present in the pituitary, the central nervous system and brown adipose tissue. The current results show that in mare's milk, both types of 5'-MD exist. The 5'-MD type I (table I) is affected by 1 mM PTU (about 50 % inhibition) and sensitive to ATG (an inhibitor of selenocysteine-containing enzymes [4, 5]), while the other 5'-MD, shows some characteristics of the type II enzyme [46]. The latter is characterized by sequential type reac-

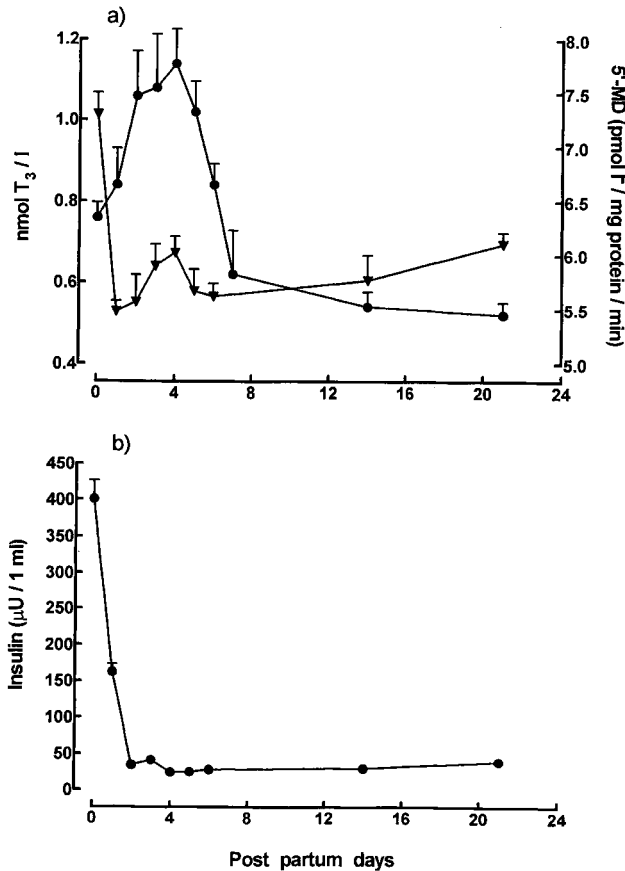


Figure 2. Sequential observation of changes in: a) triiodothyronine (T_3) concentrations (●), rT3-5'-deiodinase activity (▼); and in b) insulin levels in colostrum and milk of mares from parturition (day 0) until day 21 of lactation. Means \pm S.E.M.; $n = 6$ for T_3 and 5'-MD and $n = 10$ for insulin.

tion kinetics in the presence of 1 mM PTU (with various rT_3 and DTT concentrations; *figure 1a*), and by the low K_m value (in nanomolar range) for rT_3 . The type II 5'-MD activity of the mare's milk was sensitive to the gold-containing compound (ATG) which suggests the presence of the selenocysteine in this enzyme molecule and agrees with recent evidence that type II 5'-MD [11] and other deiodinases are selenoenzymes [45]. However, the inhibition of the mare milk type II 5'-MD by ATG was non-competitive with rT_3 as a

substrate (*figure 1b*). The amount of ATG required for a 50% reduction of the enzymatic activity (7.7 nM) was similar to that found by Santini et al. [36] for inhibition of the rat hepatic type I 5'-MD (8.2 nM ATG); however, the inhibition in the rat liver was competitive. The K_i value obtained for ATG inhibition of type II 5'-MD (11.5 nM) in mare's milk was close to the K_i value of the rat liver type I 5'-MD [6]. The existence of the both 5'-deiodinases (types I and II) in one tissue has already been observed in the rat pituitary and brain

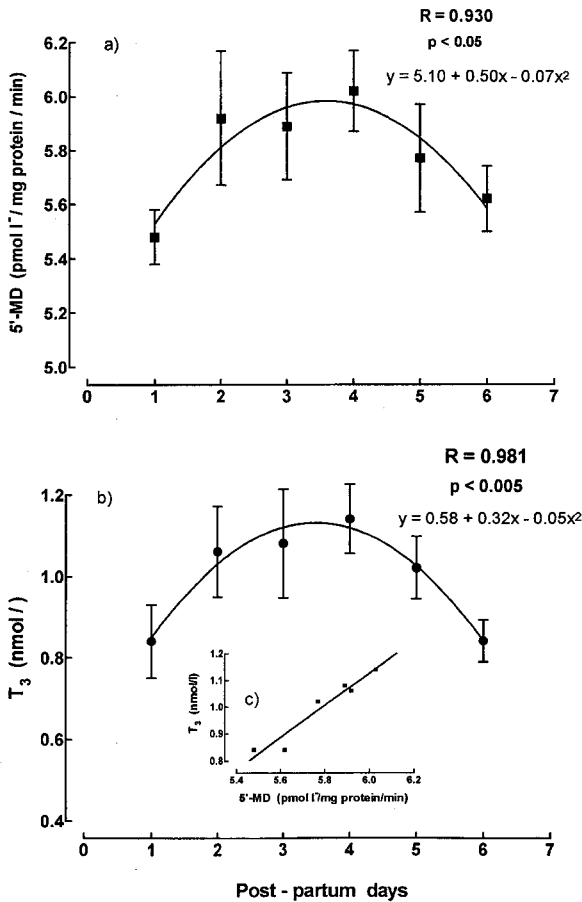


Figure 3. Curvilinear relationship of milk 5'-MD (a) and milk T₃ (b) concentrations during the early lactation period in mares. Statistical significance ($P < 0.05$) of multiple correlation coefficient, R , was verified using F-test, with 2 and 5 d.f. Note the linear relationship between the T₃ concentration and 5'-MD activity (c).

Table I. Properties of the mare's milk 5'-MD ($n = 5$).

Inhibitor	pmol I ⁻ / mg × min	Percent of the enzyme activity
–	5.45 ± 0.34	100
T ₄ (10 μM)	4.10 ± 0.23*	75.2 ± 3.73
T ₃ (10 μM)	3.18 ± 0.19***	58.3 ± 4.35
PTU (0.5 mM)	3.80 ± 0.31*	69.7 ± 5.67
PTU (1.0 mM)	2.95 ± 0.18***	54.1 ± 3.21
PTU (2.5 mM)	2.40 ± 0.10***	44.0 ± 1.93
PTU (5.0 mM)	1.95 ± 0.08***	35.8 ± 1.45
ATG (5 nM)	3.60 ± 0.10**	65.9 ± 1.73
ATG (10 nM)	2.10 ± 0.20***	38.5 ± 3.63
ATG(20 nM)	1.02 ± 0.08***	18.7 ± 1.45

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with normal value (Student's t -test).

[46], as well as in the same tissue of different species: type I 5'-MD in the bovine brown adipose tissue (BAT; [12]), type II in BAT of rodents [25]; the type I 5'-MD as the only type in the rat mammary gland [2], and the type II as the predominant type in the cow and sow mammary gland [40].

The two types of 5'-MD present in the mare's milk may play different physiological roles: 1) as a source of the systemic T₃ (product of T₄ deiodination by type I 5'-MD; [23]), and 2) as a source of T₃ (product of T₄ to T₃ conversion by type II 5'-MD) involved in a local or paracrine regulation of galactopoiesis. Studies on somatotropin, which is not galactopoietic itself, show an increase in lactation intensity relating to insulin-like growth factor and the mammary tissue 5'-MD in cows [7, 13]. Moreover, recent investigations in mice suggest that neither somatotropin nor prolactin are galactopoietic when thyroid hormones are absent [8].

Apart from the postulated paracrine function of locally generated T₃, the T₃ taken with colostrum or milk may play a significant role in the process of intraluminal digestion, absorption and the maturation of enzyme systems [37]. In the neonatal calf, peroral administration of T₃ can exert a positive effect on macromolecule

transfer in the intestines [43]. The high T₃ level in foal's serum [16], and in colostrum (figure 2) may be temporally associated with the responsiveness to gastrin and gastric secretion in the neonates [26].

The ratio of T₃ concentration in colostrum (0.74 ng/mL on day 4 after birth) to the mean T₃ concentration in mare's serum (0.90 ng/mL [10]) gives an approximate colostrum/serum ratio of 0.82. At a mean colostrum T₃ concentration of 0.456 μg/L and a daily intake of colostrum by a foal of about 4 L, the daily T₃ consumed by a suckling foal is approximately 1.8 μg, which may have some stimulatory effect within the intestinal tract [43].

Unlike thyroid hormones, there is very little information concerning the insulin concentrations in colostrum and milk across species. Until now, insulin concentration has been determined in the colostrum and milk of human [9, 35], pig, cow and sheep [29, 30].

The profiles of 5'-MD activity and insulin in the mare's mammary secretion showed some similarities in that the both show a rapid decrease after parturition (figure 2). The observed high colostrum insulin levels relative to that in full milk agree well with the findings described already in human and animals [29].

Colostrum and milk insulin can cross the intestinal wall to regulate the development of variety of tissues [35]. Studies on insulin and its binding to receptors in the alimentary tract suggest the hormone is important in alimentary tract development [3, 31]. On the basis of available data it has been concluded that, together with prolactin and milk growth factors, insulin is one of the candidates for selective advantages for reproductive success [34]. The presence of insulin in the mare's mammary secretion may suggest a similar importance for colostrum insulin in the horse, namely for alimentary tract development and maturation.

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