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DETERMINATION OF INSULIN-LIKE GROWTH FACTORS IN BOVINE MILK AND COLOSTRUM BY RADIOIMMUNOASSAY

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Bovine milk is an important ingredient in human nutrition as well as in the diet of the young calf. In view of the role of insulin-like growth factors (IGFs) in growth and development attempts were made to determine the concentrations of both IGF-I and IGF-II in colostrum and milk. Colostrum was collected on the day of calving from six dairy cows on a large co-operative farm. Milk samples were taken directly from nine cows at different stages of lactation on two private farms. Pasteurized milk and various milk powders available commercially were also examined. The mean concentrations of IGF-I and IGF-II in colostrum were found to be 345 ± 200 ng/mL and 406 ± 46 ng/mL, respectively. When milk samples were centrifuged to remove the fat before radioimmunoassay, IGF-I levels were found to be lower than in non-centrifuged samples, whereas no differences were detected concerning IGF-II concentrations. This indicates that these growth factors are distributed differently in the milk emulsion. Thus, concentrations of IGF-I decreased from 38.7 ± 3.5 ng/mL and 24.0 ± 2.8. ng/mL at four days postpartum to 7.2 ± 6. ng/mL and 4.1 ± 3.7 ng/mL in whole and centrifuged mature milk samples, respectively. Mean IGF-II concentrations were 14.1 ± 6.0 ng/mL and 13.7 ± 6.1 ng/mL in whole and centrifuged milk samples, respectively. Pasteurized milk contained lower concentrations of both factors (IGF-I - 1.1 ± 0.9 ng/mL; IGF-II - 6.5 ± 3.2 ng/mL). Even lower amounts, near or below the limit of the sensitivity of the assay systems (1 ng/mL) were found for humanised milk powders intended for human infants.

Thus, while consumption of colostrum leads to considerable intake of IGF-I and IGF-II, commercially available milk and milk products contain negligible amounts.

Key words: IGF-I, IGF-II, radioimmunoassay, milk, colostrum, cows

INTRODUCTION

In addition to the provision of macronutrients, immunological protection and antibacterial agents, milk is a rich source of biologically active compounds including hormones and growth factors (Belford et al. 1997).

Insulin-like growth factors are multifunctional mitogenic polypeptides (7.5 kD) interacting with specific receptors and binding proteins (IGFBPs), which modify their activity. Several studies have demonstrated the presence of IGF-I and IGF-II in bovine prepartum mammary secretions (Vega et al.1991), colostrum (Francis et al. 1988) and milk (Campbell & Baumrucker, 1989).The bovine mammary gland accumulates large quantities of IGFs during late gestation which are secreted at parturition (Skaar et al. 1991). Both IGF-I and IGF-II levels are considerably higher in colostrum than in mature milk (Zumkeller, 1992). The large quantities of IGF-I and IGF-II present in colostrum might play a role as maternal mediators for growth and development of the gastrointestinal tract or have an overall systemic effect on the neonate (Vega et al. 1991). Since bovine IGF-I has an identical amino acid sequence to human IGF-I (Honegger & Humbel, 1986) it would therefore be active in humans.

Since bovine milk is an important ingredient in human nutrition as well as in the diet of the young calf, both IGF-I and IGF-II were determined in colostrum and milk collected on the farm and in commercially available liquid and powdered milk. The aim of the present investigation was to detect possible changes in the concentrations of these factors during lactation and to determine the extent to which they survive technological processing.

MATERIAL AND METHODS

Sample collection and treatment. Milk was obtained directly from nine healthy, black and white or red and white cows in different stages of lactation, on two private dairy farms. Colostrum samples were collected from six East Friesian cows on a large co operative dairy farm before suckling on the day of parturition. The samples were stored at -20^{0}C until analysed. Colostrum was centrifuged at 20000xg at 4^{0}C, for 25 min. The top fat layer was discarded and the infranatant was used for assay (Francis et al. 1988). Fresh milk samples were treated under the same conditions. Powdered milk products were reconstituted as directed.

Total nitrogen concentration in some samples of milk and in colostrum was determined by the Kjeldahl method. Protein concentration was calculated using 6.37 as the conversion factor.

IGF extraction protocol. IGF-I and IGF-II in bovine milk are principally associated with binding proteins (Campbell & Baumrucker, 1989) which must be dissociated and removed prior to radioimmunoassay (RIA). Whole milk and centrifuged samples of milk and colostrum were extracted in acid-ethanol to remove binding proteins and then neutralised as described by Daughaday et al., 1982, with the subsequent modification involving an additional cryoprecipitation step (Breier et al. 1991).

Briefly, to 0.2 mL milk or colostrum was added 0.8 mL of an acid-ethanol solution containing 87.5% ethanol and 12.5% 2M HCl. After thorough mixing and standing for 30 min at room temperature, the tubes were centrifuged at 3000xg for 30 min. Neutralization of 0.5 mL of the resulting supernatant was accomplished by the addition of 0.2 mL of 0.855 M Tris base (pH 10.5). The resulting solution was incubated at -20^{0}C for a minimum of 2 h and then centrifuged at 3000xg for 30 min. The supernatant obtained was used for determination of IGF-I and IGF-II by liquid phase RIA.

A blank extract was prepared from 0.2 mL of 0.05 M phosphate buffer, containing the same concentrations of ethanol, HCl and Tris as those present in the milk extract. This blank was introduced to standards and to the reference preparation of IGF-I in the same amount as the milk extracts.

IGF-I RIA. IGF-1 concentrations were measured by homologous liquid-phase RIA as described by Nikolić et al. (1996) using polyclonal rabbit antibodies to human IGF-I (A 677/R1H Biogenesis, England) as the reagent. Cross-reactivity for human IGF-II was 0.05% at 50% inhibition of tracer binding in our assay conditions. Recombinant human IGF-1 (ICN, USA), which has the same structure as bovine IGF-I was used for preparing the tracer (^{125}I-IGF-I), which had a specific activity of approximately 150 mg, and standards. The assay system was calibrated using a recognized reference preparation of human IGF-I (WHO 87/518). All samples were analyzed in duplicate and the intra-assay coefficient of variation (CV) was 9.2%.

IGF-II RIA. Milk and colostrum extracts were assayed for IGF-II as described by Nikolić et al. (1988). A monoclonal antibody (subclass IgG-1) raised in the mouse against rat IGF-II (A681/M1H, Biogenesis, England) was used as the reagent (crossreactivity with human IGF-1 was 0.4% at 50% inhibition). Rat IGF-II differs from bovine IGF-II by one amino acid only (Sara & Hall, 1990). In the absence of a recognised international reference preparation it was impossible to standardise the measurements completely. Recombinant human IGF-II (ICN, USA) was labelled with ^{125}I to a specific activity of about 150 mCi/mg. For preparing standards recombinant human IGF-II was dissolved in PBS (pH 7.5) containing 0.1% BSA and appropriate dilutions prepared. Ovine anti-mouse immunoglobulin-G antibodies (INEP, Zemun) suspended in 12.8% polyethylene glycol solution were used for separation of antibody-bound from free ligand. The immunocomplexes formed were separated from other components by centrifugation. Samples were assayed in duplicate with an intra-assay CV of 6.1%.

The assay was validated for these extracts by dilution and recovery tests. Recovery of human IGF-II added to colostrum was incomplete, probably due to the effect of residual binding proteins in the assay mixture. Sequestration of these binding proteins by including IGF-I (3 ng/tube)in the test at a concentration too low to react with the specific anti-IGF-I antibody had a small effect. Namely, apparent IGF-II concentrations were increased by 14-22%. However, since the recovery of added IGF-II averaged 46% only, the results were corrected using this factor and should be regarded as relative rather than absolute values.

Statistical analysis. The effect of centrifugation of the milk samples on the results was assessed by the paired t-test, while the significance of duration of lactation was tested by factorial ANOVA using missing values to equalise the number of cows on both farms.

RESULTS

IGF-I and IGF-II concentrations in bovine colostrum. The IGF-I and IGF-II concentrations found in colostrum are presented in Table 1. Both IGF-I and IGF-II levels considerably varied from animal to animal with average values of 345 ± 200 ng/mL and 406 ± 46 ng/mL for IGF-I and IGF-II, respectively. However, these growth factors represented a very small partof the total colostral protein.

Table 1. Concentrations of IGF-I, IGF-II and total protein in colostrum

Cow	IGF-I (ng/mL)	IGF-II (ng/mL)	Total protein (g/100g)	IGF-I (μg/g protein)	IGF-II (μg/g protein)
1	545.5	382	15.4	3.5	2.5
2	394.0	444	17.5	2.3	2.5
3	351.5	377	17.8	2.0	2.1
4	300.0	360	14.5	2.1	2.5
5	272.0	418	16.3	1.7	2.6
6	210.5	454	13.0	1.6	3.5
X̄(SD)	345(200)	406(46)	15.8(1.8)	2.2(0.7)	2.6(0.5)

IGF-I and IGF-II concentrations in fresh milk. IGF-I concentrations were consistently higher in whole milk than in centrifuged samples on both farms (Tables 2 and 3; t =3.44 P = 0.015), indicating that some IGF-I was associated with the protein emulsifying the fat droplets. The mean concentration of IGF-I was more than 5-fold higher 4 days postpartum than later (F=30.1; P=0.001). Namely, the average values of 38.7 ± 3.5 and 24.0 ± 2.8 ng/mL for IGF-I in whole and centrifuged milk at 4 days decreased to 7.2 ± 6.0 and 4.1 ± 3.7 ng/mL respectively.

Table 2. Concentrations of IGF-I and IGF-II in milk obtained from dairy cows at different stages of lactation on Farm 1.

Cow	Time after calving	Milk sample	IGF-I (ng/mL)	IGF-II (ng/mL)
1	4 days	Centrifuged	21.2	12.1
		Whole	35.2	11.0
2	14 days	Centrifuged	1.0	7.6
		Whole	2.3	8.1
3	2 months	Centrifuged	1.7	10.3
		Whole	2.2	10.6
4	5 months	Centrifuged	7.8	16.5
		Whole	9.4	16.7
5	8 months	Centrifuged	5.7	18.8
		Whole	13.1	19.9
	X̄(SD)	Centrifuged	7.5 (8.2)	13.1 (4.6)
		Whole	12.4(13.6)	13.3 (4.9)

On Farm 1 (Table 2) IGF-I levels tended to be lower in early lactation (14 days to 2 months) than in later lactation, possibly reflecting the known change from negative to positive energy balance during lactation in dairy cows. However, this trend was not apparent on Farm 2 (Table 3), possibly due to differences in husbandry and characteristics of the cows. Crude protein was also determined in the samples from Farm 2. It can be seen that the relative contribution of IGF-I to total milk protein was about 8-fold lower that the relative amount in colostral protein (Tables 1 and 3).

Table 3. Concentrations of IGF-I and IGF-II in fresh milk taken from dairy cows at different stages of lactation on Farm 2.

Cow	Time after calving	Milk sample	IGF-I (ng/mL)	IGF-II (ng/mL)	Total protein (g/100g)	IGF-I (ng/g protein)	IGF-II (ng/g protein)
1	4 days	Centrifuged	26.7	15.9	3.7	722	430
		Whole	42.2	19.8	4.5	938	440
2	18 days	Centrifuged	2.7	15.5	2.8	96	554
		Whole	7.4	13.9	3.6	206	386
3	4 months	Centrifuged	7.0	17.9	2.7	261	663
		Whole	9.1	14.3	4.8	191	298
4	8 months	Centrifuged	2.5	8.7	3.2	80	272
		Whole	6.7	12.4	3.9	172	318
	$\bar{X}$ (SD)	Centrifuged	9.7(11.5)	14.5(4.0)	3.1 (0.5)	290(299)	479(168)
		Whole	16.4(17.3)	15.1(3.2)	4.2 (0.5)	376(374)	360 (65)

Since IGF-II concentrations were found to be very similar in whole and centrifuged milk (13.7 ± 4.1 ng/mL and 14.1 ± 4.1 ng/mL, respectively), it appears that IGF-II is present mainly in the soluble phase and is not associated with the protein fraction surrounding the fat droplets. Moreover, no differences were detected between IGF-II concentrations in early milk samples 4 days after calving and mature milk samples on either farm. Namely, IGF-II concentrations decreased rapidly from the high levels found in colostrum (Table 1) to values below those of IGF-I on day 4 of lactation. Thereafter, levels were maintained relatively constantly and were similar for all cows on both farms throughout lactation.

IGF-I and IGF-II concentrations in milk products. The mean concentration of these growth factors in commercially packed liquid pasteurized milk was found to be several times lower than the mean levels found in centrifuged mature milk taken directly from the farm (Tables 2, 3 and 4). Namely, partially defatted pasteurized milk contained 1.1 ± 0.9 ng/mL IGF-I and 6.5 ± 3.2 ng/mL IGF-II (Table 4). IGF-I could not be detected in long-life homogenised milk and many products intended for human infant nutrition. The mean value found was around the limit of valid measurement of the assay system (1 ng/mL). Levels of IGF-II were somewhat higher indicating that this growth factor survives possible enzyme activity and heat treatment better than IGF-I. Values were lower for noncentrifuged samples suggesting the presence of substances interfering with the assay procedure.

Table 4. IGF-I and IGF-II concentrations in some commercially available milk products

Product	Treatment	IGF-I (ng/mL)	IGF-II (ng/mL)
Pasteurized milk (2.8% fat)	Sample 1	0.5	4.2
	Sample 2	1.8	8.8
Long-life homogenised milk (2.8% fat)	Centrifuged	ND*	3.8
	Whole	ND	1.8
Infant formula A**	Centrifuged	3.7	9.6
	Whole	1.1	5.8
Infant formula A1	Centrifuged	3.5	9.2
	Whole	0.9	3.8
Infant formula B1**	Centrifuged	ND	3.8
	Whole	3.3	1.8
Infant formula B2	Centrifuged	ND	7.6
	Whole	ND	0.6
Infant formula B3	Centrifuged	0.9	7.2
	Whole	ND	1.6
Infant formula B4	Centrifuged	ND	0.8
	Whole	ND	0.6
Infant formula B5	Centrifuged	0.5	1.2
	Whole	0.4	0.8
$\bar{X}$ (SD) $N \leq 8$	Centrifuged	1.1 (1.6)	5.4 (3.5)
	Whole	0.7 (1.1)	2.1 (1.8)

*ND-Not detected. Zero value included in calculation of mean.
**A-Proprietary products from manufactures A and B.

DISCUSSION

IGFs in bovine milk are closely associated with specific binding proteins (IGFBPs). However, Campbell & Baumrucker (1989) validated acid-ethanol extraction as a reliable procedure for the removal of these binding proteins prior to RIA and this was confirmed in later investigations (Vega et al. 1991; Zhao et al. 1991) for both IGF-I and IGF-II. In our case, dilution curves for colostrum were parallel to the standard curve for human IGF-I giving 99.6% of the expected results, which validates our assay. Moreover, the mean value found for colostral IGF-I (Table 1) was within the limits reported by other authors, which vatied from 179.8 ± 16.8 ng/mL (Skaar et al. 1991) to 768 ng/mL (Hadsell et al. 1993) for Holstein dairy cows on the day of parturition. Zumkeller (1992) concluded that colostral IGF-I may vary from 100 to 2000 ng/mL, while IGF-II occurs at somewhat higher concentrations (230-2300 ng/mL).

Even though it was not possible to standardise the heterologous IGF-II assay properly in the absence of a recognised reference preparation, our results confirm this conclusion. Namely, mean colostral IGF-II tended to be slightly, but

not significantly higher than IGF-I. Other studies in which human IGF-II was also used as the standard letd to similar findings (Skaar et al. 1991; Vega et al. 1991). Thus, IGF-I is present in colostrum at about 5-fold higher concentrations than those prevailing in maternal serum around the time of parturition (Vega et al. 1991; Nikolić 1996). Hadsell and co-workers (1991) found that alteration of blood IGF-I through chronic administration of somatotropin did not alter the concentration of IGF-I in mammary secretions but caused a dramatic increase in total mass of IGF-I secreted by the gland with only a marginal increase in IGF-I concentrations in colostrum. They concluded that IGF-I does not enter mammary secretions by passive diffusion from blood. It is possible that IGF-I is autonomously produced in the mammary gland in response to locally synthesized somatotropin induced by the influence of high progestin concentrations during gestation, as has been found in bitches, cats and women (Rijnberk & Mol, 1997). Certainly, this synthesis must decrease rapidly after parturition because, within a few days, the concentrations of both growth factors in milk are much lower than those in blood serum. The mean concentrations of 7.2 ± 6.0 ng/mL,observed here for IGF-I and IGF-II respectively, in mature milk, are similar to those previously reported. Namely, other investigators found IGF-I levels from 3.3 ng/ml (Zhao et al. 1991) to 34 ng/mL (Vega et al. 1991; Skaar et al. 1991). These small amounts of IGFs in fresh milk do not appear to survive the technological procedures used to prepare commercial products. Thus, IGF-I was below the level of detection of our assay system in many products, while the IGF-II concentration was more than halved (Table 4).

Campbell & Baumrucker (1989) found that IGF-I was primarily associated with 45 kD binding proteins in bovine colostrum. The binding activities of the 42-40 kD IGFBP declined postpartum, while the activities of 30-34 kD IGFBPs increased (Skaar et al. 1991). Our investigations indicate that the IGFs are differently distributed between the IGFBPs present in milk, IGF-II being primarily bound to the soluble fraction while IGF-I was associated significantly with the lipid fraction removed by centrifugation. On the other hand, Zhao et al. (1991) reported that centrifugation for 3 min at 10.000 xg had no effect on the values obtained for IGF-I in late lactation milk. The different findings may be due to differences in methodology.

Zumkeller (1992) raised the question of the effect of increased levels of IGF-I in the milk of somatotropin treated cattle affecting infant health. However, since the eventual increases appear to be small and immunoreactive IGF-I and IGF-II decline markedly during processing, it seems that daily consumption of these growth factors in dairy products is likely to be negligible.

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ODREĐIVANJE INSULINU-SLIČNIH FAKTORA RASTA U KRAVLJEM MLEKU I KOLOSTRUMU RADIOIMUNOESEJOM

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SADRŽAJ

Kravlje mleko je važan sastojak humane ishrane, kao i ishrane teladi. Imajući u vidu ulogu insulinu-sličnih faktora rasta IIGF-ova) u rastu i razvoju pokušali smo da odredimo koncentracije IGF-I i IGF-II u kravljem kolostrumu i mleku. Kolostrum je uzet od šest krava, na sam dan teljenja, sa jedne velike kooperativne farme. Sakupljeni su uzorci mleka devet krava, koje su bile u različitim fazama laktacije, sa dve privatne farme. Takođe je ispitivano pasterizovano mleko, kao i različita komercijalno dostupna mleka u prahu, koja se koriste u prehrani odojčadi. Srednje vrednosti koncentracija IGF-I i IGF-II izmerenih u kolostrumu iznose 345 ± 200 ng/mL i 406 ± 46 ng/mL, respektivno. Kada su uzorci mleka centrifugovani pre radioimunoeseja, da bi se uklonile masti, nađene su niže koncentracije IGF-I u odnosu na one izmerene u punom (necentrifugovanom) mleku, što nije bio slučaj sa IGF-II. Smatramo da to može biti posledica različite distribucije ovih faktora rasta u mleku. Koncentracije IGF-I u punom i centrifugovanom mleku su smanjene sa 38.7 ± 3.5 ng/mL i 24.0 ± 2.8 ng/mL, koliko su iznosile 4 dana nakon partusa, na 7.2 ± 6.6. ng/mL i 4.1 ± 3.7 ng/mL u uzorcima zrelog mleka. Što se tiče IGF-II, srednje vrednosti merenih koncentracija su iznosile 14.1 ± 6.0 ng/mL i 13.7 ± 6.1 ng/mL, u punom i centrifugovanom mleku, respektivno. Pasterizovano mleko je imalo niže koncentracije oba faktora rasta (IGF-I: 1.1 ± 0.9 ng/mL; IGF-II: 6.5 ± 3.2 ng/mL). Još niže koncentracije, ispod ili blizu granice osetljivosti testa (1 ng/mL), su izmerene u uzorcima mleka u prahu, namenjenih ishrani odojčadi.

Dok konzumiranje kolostruma dovodi do značajnog unosa IGF-I i IGF-II u organizam, komercijalno dostupno mleko sadrža zanemarljive količine ovih faktora rasta.