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FACTORS AFFECTING THE HYPOCALCAEMIC RESPONSE TO PROTAMINE

By

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PERSSON, J. and J. LUTHMAN: *Factors affecting the hypocalcaemic response to protamine.* Acta vet. scand. 1975, *16*, 51—62. — The hypocalcaemic response to protamine, as a measure of bone resorption rate, was studied in cattle and sheep. Three groups of calves were studied (1—3 days, 2—3 weeks and 4 months old). The oldest calves showed the greatest response, indicating a more rapid skeletal turnover in these animals, probably related to a higher growing rate. A group of cows was tested at 2 occasions, near parturition and 2—3 weeks after parturition. The decrease in plasma calcium was small near parturition, while a profound drop was obtained 2—3 weeks post partum, which supports earlier findings that bone resorption is more or less blocked in parturient cows. Young pregnant ewes showed the same degree of hypocalcaemia as adult pregnant ewes but the recovery was more rapid in the young animals. Young rams were given protamine before and after diethylstilboestrol treatment. No differences in calcium response were obtained. Varying plasma phosphate responses were obtained. A pronounced hyperphosphataemia was found in the adult pregnant ewes. Diethylstilboestrol caused hypermagnesaemia in the rams.

protamine; hypocalcaemia; bone resorption; cattle; sheep.

The concentration of calcium in blood plasma is regulated in a very precise manner by parathyroid hormone and calcitonin. One of the 2 main sources of calcium in this homeostatic mechanism is bone. The rate of bone resorption is known to vary during different periods of life, it is high in young growing animals and decreases in adult animals. The need of calcium is greatly increased during pregnancy and lactation, and this normally leads to accelerated resorption of bone.

It was reported by *Johnston et al.* (1970) that protamine produces hypocalcaemia in rats by direct inhibition of bone resorp-

tion. The drug was found to be effective also in ruminants (*Luthman et al.* 1973 a) and was later suggested as a useful tool in the study of bone resorption rate during different physiological conditions (*Luthman et al.* 1973 b).

The primary aim of the present investigation was to study the hypocalcaemic response to protamine, as an indicator of bone resorption rate, during various conditions in cattle and sheep. The study includes in most cases also the changes in inorganic phosphorus and magnesium.

MATERIAL AND METHODS

In the first series of experiments protamine was tested in 3 groups of calves, 1—3 days old (group 1), 2—3 weeks old (group 2) and 4 months old (group 3). The animals in group 1 had received at least 2 meals of whole-milk before the experiment was performed. The calves in group 2 were fed whole-milk, 1 tenth of the body weight in liter, divided in a morning and an evening meal. In these 2 groups the milk ration was withheld on the morning of the experimental day since, otherwise, protamine produced a lipaemic plasma. Group 3 was fed hay and grain and was non-fasted when used. Protamine chloride (Protamin, 10 mg/ml, Vitrum, Stockholm, Sweden) was injected into the jugular vein at the dose of 10 mg/kg. Blood was drawn in heparinized tubes (Heparinrör, Vitrum, Stockholm, Sweden) at 1, 2, 4 and 6 hrs. post injection, and plasma was analysed for calcium and inorganic phosphorus.

Four dairy cows (5—8 years old) were tested at 2 occasions, 1—3 days post partum and 2—3 weeks post partum. All cows had an uncomplicated parturition. Protamine sulphate (Protamine sulfate, No. P-4380, Sigma Chemical Company, St. Louis, USA) was dissolved in saline to a concentration of 10 mg/ml and was infused into the jugular vein at the dose of 10 mg/kg. The time of infusion was about 5 min. Blood was sampled as above and plasma was analysed for calcium, inorganic phosphorus and magnesium.

A comparison was made between the responses to protamine in young (10 months) and adult (3—5 years) ewes. All animals were in the last month of pregnancy. All young ewes bore single foetuses, while all adult ewes except 1 bore twins. The animals were fed 1.0 kg hay and 0.6 kg of a mineralized pelleted concen-

trate a day, providing about 9 g calcium a day. Protamine as the chloride was used (10 mg/kg) and the experimental and analytical procedures were performed as in the cows.

Two series of experiments were designed to investigate a possible inhibitory effect of oestrogen on the calcium response to protamine. In a preliminary study protamine chloride (10 mg/kg) was given intravenously to 6 young rams (8—10 months old). After 3 daily intramuscular injections of diethylstilboestrol (Stilbol, ACO, Stockholm, Sweden) at the dose of 10 mg/animal/day the protamine injection was repeated. The rams in the second study were of the same age and the experiment was performed in the same way with the exception that diethylstilboestrol was given for 12 days before the second protamine injection. Only calcium was analysed in the preliminary study, while in the second experiment determinations were made on calcium, inorganic phosphorus and magnesium. The weight of the animals varied between 30 and 35 kg. All animals were fed 1.0 kg hay and 0.5 kg mineralized pelleted concentrate a day.

All cattle were of the Swedish Red and White Breed and the sheep were all of the Swedish landrace. In all groups except the 2 youngest calf groups the animals had free access to tap water.

The preinjection levels of the measured parameters given in the text and in the Tables are the mean of 2 determinations, at —30 min. and immediately before administration of protamine.

Calcium was analysed by EDTA titration according to *Skerry* (1965), and commercial reagent kits were used for the determinations of inorganic phosphorus (Sigma Kit 670, Sigma Chemical Company, St. Louis, USA) and magnesium (Merckotest Magnesium, E. Merck, Darmstadt, Germany).

Conventional statistical methods were used (Student's t-test for paired and unpaired data).

RESULTS

The responses to protamine in calves of various ages are given in Table 1. No significant difference in calcium response was obtained between groups 1 and 2. Comparison between groups 1 and 3 showed a difference in calcium decrease, which was highly significant ($P < 0.001$) at 1 hr. and significant ($0.01 > P > 0.001$) at 2 hrs. The difference between groups 2 and 3 was almost significant ($0.05 > P > 0.01$) at 1 and 2 hrs. For inorganic phosphorus

Table 1. Changes in the plasma levels of calcium and inorganic phosphorus in calves of various ages after intravenous injection of protamine (10 mg/kg). Mean±s.

Group no.	Age	Pretreatment level (mg/100 ml)	Hours 1	2	4	6
			Δ Ca (mg/100 ml)			
1	1—3 days (n=6)	10.80±0.54	—0.17±0.33	—0.45±0.44	—0.27±0.33	+0.12±0.48
2	2—3 weeks (n=6)	10.55±0.70	—0.45±0.53	—0.60±0.51	—0.43±0.33	—0.13±0.29
3	4 months (n=5)	10.52±0.36	—1.22±0.26	—1.36±0.41	—0.74±0.52	—0.10±0.26
			Δ P (mg/100 ml)			
1	1—3 days (n=6)	6.72±0.90	—0.27±0.30	—0.25±0.38	+0.25±0.36	+0.45±0.45
2	2—3 weeks (n=6)	9.20±0.76	—0.33±0.37	—0.27±0.37	+0.18±0.30	+0.55±0.50
3	4 months (n=5)	7.56±0.98	—0.44±0.57	—0.80±0.66	—0.36±0.70	+0.16±0.49

the difference in pretreatment level was highly significant between groups 1 and 2 and almost significant between groups 2 and 3. The changes after protamine injection were small and the differences between the groups were not significant.

As shown in Table 2, the plasma calcium decrease in the cows was small when protamine was given near parturition, while a profound drop was obtained when the drug was administered 2—3 weeks after parturition, in 1 cow plasma calcium decreased from 9.4 to 6.3 mg/100 ml. No statistical calculations were made

Table 2. Changes in the plasma levels of calcium, inorganic phosphorus and magnesium in cows (n=4) after intravenous infusion of protamine (10 mg/kg). Mean±s.

Time after parturition	Pretreatment level (mg/100 ml)	Hours 1	2	4	6
			Δ Ca mg/100 ml		
1—3 days	8.33±0.70	—0.25±0.19	—0.38±0.30	—0.50±0.58	—0.40±0.29
2—3 weeks	9.68±0.32	—1.18±0.36	—1.55±0.13	—2.30±0.54	—1.13±1.32
			Δ P mg/100 ml		
1—3 days	5.73±1.54	+0.60±0.34	+0.45±0.81	+0.40±0.65	+0.33±0.41
2—3 weeks	5.23±1.30	+0.15±0.56	+0.03±0.61	—0.90±1.10	—1.18±1.28
			Δ Mg mg/100 ml		
1—3 days	2.20±0.53	—0.13±0.06	—0.13±0.15	—0.33±0.23	—0.28±0.12
2—3 weeks	2.23±0.22	—0.05±0.10	—0.18±0.05	—0.25±0.21	—0.08±0.17

since only 4 cows were available. The changes in plasma inorganic phosphorus were small and seemed to be negligible, while the maximum decrease in magnesium was about 15 % at both occasions.

Table 3 shows the responses to protamine in young and adult pregnant ewes. The maximum calcium decrease was of about the same degree in both groups and occurred 2 hrs. after protamine injection. The young ewes, however, showed a more rapid recovery and, in this group, plasma calcium exceeded the preinjection level at 6 hrs. In the adult ewes, plasma calcium never returned to the pretreatment level and the difference in calcium response between the groups was significant at 6 hrs. There was a striking difference in phosphate response between young and adult ewes. An initial rise followed by a decrease was obtained in both groups. The rise was, however, much more pronounced in the adult ewes and was also more sustained. The difference in phosphate response between the groups was highly significant. Plasma magnesium remained unchanged in both groups throughout the observation period.

Table 3. Changes in the plasma levels of calcium, inorganic phosphorus and magnesium in young and adult pregnant ewes after intravenous injection of protamine (10 mg/kg). Mean±s.

	Pretreatment level (mg/100 ml)	Hours			
		1	2	4	6
			Δ Ca (mg/100 ml)		
Young ewes (n=6)	10.62±0.22	—0.82±0.35	—1.27±0.39	—0.18±0.39	+0.25±0.18
Adult ewes (n=6)	10.23±0.66	—0.80±0.13	—1.10±0.20	—0.60±0.26	—0.35±0.39**
			Δ P (mg/100 ml)		
Young ewes (n=6)	5.65±0.46	+0.45±0.83	+0.25±0.37	—0.08±0.28	—0.73±0.38
Adult ewes (n=6)	5.07±1.86	+1.77±0.40**	+1.70±0.64***	+1.48±0.47***	+0.05±0.62*
			Δ Mg (mg/100 ml)		
Young ewes (n=6)	2.17±0.16	+0.10±0.14	+0.02±0.12	+0.07±0.15	+0.08±0.07
Adult ewes (n=6)	2.18±0.23	+0.05±0.19	+0.05±0.14	+0.05±0.14	+0.07±0.15

Three daily injections of diethylstilboestrol in young rams did not affect the plasma calcium concentration. The responses to protamine before and after hormone treatment were also almost identical. Pretreatment levels were 9.86±0.35 and 9.94± 0.15 mg/100 ml. The maximum response to protamine occurred at both occasions 2 hrs. after injection and were 0.98±0.11 and 1.00±0.35 mg/100 ml, respectively. Neither did treatment with diethylstilboestrol for 12 days change the calcium response to protamine (Table 4). As seen from this Table, also plasma phosphate response was unaffected. The hormone treatment caused an increase in plasma magnesium of almost 25 %. The rise was highly significant. A small, but highly significant, difference in magnesium response was obtained at 4 hrs.

DISCUSSION

The oldest calves showed the greatest hypocalcaemic response to protamine (Table 1). The effect of calcitonin is known to depend on rate of growing and thereby also on the rate of skeletal

Table 4. Changes in the plasma levels of calcium, inorganic phosphorus and magnesium in young rams (n=6) after intravenous injection of protamine (10 mg/kg) before and after treatment with diethylstilboestrol (10 mg/day for 12 days). Mean±s.

	Pretreatment level (mg/100 ml)	Hours			
		1	2	4	6
			Δ Ca (mg/100 ml)		
Before diethylstilboestrol	10.27±0.57	—1.15±0.89	—1.28±0.39	—0.78±0.65	—0.63±0.69
After diethylstilboestrol	10.07±0.66	—0.88±0.28	—0.90±0.40	—0.70±0.20	—0.43±0.24
			Δ P (mg/100 ml)		
Before diethylstilboestrol	7.20±0.78	+0.63±0.57	+0.63±0.66	—0.42±0.95	—0.15±0.39
After diethylstilboestrol	7.82±1.64	+0.05±0.62	—0.18±1.00	—0.72±0.76	—0.27±1.10
			Δ Mg (mg/100 ml)		
Before diethylstilboestrol	1.95±0.19	—0.12±0.15	—0.13±0.10	+0.02±0.19***	—0.12±0.20
After diethylstilboestrol	2.40±0.13***	—0.15±0.14	—0.22±0.12	—0.13±0.15	—0.10±0.21

turnover. The differences in response between the groups thus probably reflect differences in growing and bone resorption rates. It can, however, not be excluded that protamine was metabolized in a different way in the milk-fed calves.

There has earlier been presented support to the current concept of the aetiology to parturient paresis that bone resorption is decreased at parturition. *Ramberg et al.* (1970) reported from kinetic calcium studies in cows during late pregnancy, parturition, and early lactation that gastrointestinal absorption was the major inflow to plasma during the prepartal period, while the rate of calcium removal from bone was low before and for the first 2 weeks after the onset of lactation. This is consistent with the results of *Mayer et al.* (1969) that infusions of calcitonin were ineffective in lowering plasma calcium in cows during the period from 2 days before to 5 days after parturition. In the present study protamine caused only a slight reduction of plasma calcium when infused near parturition, while a marked hypocalcaemia was obtained 2—3 weeks after parturition. These results give further support to the earlier findings that bone resorption is more or less blocked in parturient cows and also that the mechanism of adaptation to the increased need of calcium for milk production has caused an increased rate of bone resorption 2—3 weeks post partum. The endocrinological and cellular nature of this phenomenon is not yet known, although it has been proposed that high calcium feeding during the dry period causes a high level of calcitonin in plasma, thereby making the skeleton more or less unresponsive to parathyroid hormone. Diminished plasma calcium response in prepartum cows to parathyroid hormone has been reported by *Martig & Mayer* (1973).

There was no difference in maximum hypocalcaemic response to protamine between young and adult pregnant ewes (Table 3), which indicates that the losses of calcium from bone were of the same degree. The daily movement of calcium across the placenta depends on the number of foetuses (*Braithwaite et al.* 1970). Assuming that the effect of protamine on bone is gradually lost, this may explain the more rapid calcium recovery in the young ewes, who all bore single foetuses. One might, however, not exclude a role of parathyroid hormone in this respect. It is true that *Johnston et al.* (1970) found protamine to inhibit the hypercalcaemic effect of parathyroid extract in rats, but no experiments were performed to study if this effect was related to age.

We have earlier found that the rise in plasma calcium after administration of parathyroid hormone is more pronounced in young ewes than in adult ewes (*Persson & Luthman* 1974). The difference in calcium normalization rate may thus also be a result of different skeletal sensitivity to parathyroid hormone.

The protamine injections in young rams before and after treatment with diethylstilboestrol (Table 4) were performed in view of the controversy concerning the role of oestrogens in calcium homeostasis. An interaction between oestrogens and bone calcium has long been considered in humans, where senile osteoporosis is most prevalent in elderly women, a fact which sometimes has been attributed to oestrogen deficiency. Oestrogen has been the most widely used therapeutic agent of the disease (*Riggs et al.* 1973). It is, however, still an unsolved problem whether the loss of bone mass is a result of decreased bone formation rate, accelerated resorption or a combination of the two (*Urist* 1973). The effect of oestrogens on calcium metabolism evidently varies between species. *Magargal et al.* (1969) reported from studies in female rats that oestradiol enhanced the calcium response to low doses of parathyroid hormone and inhibited the response to high doses. *Aitken et al.* (1972) observed osteoporosis in rats 11 months after oophorectomy, and *Orimo et al.* (1972) found ovariectomy to increase the sensitivity of rat bone to parathyroid hormone. *Currie & Black* (1972) reported that oestradiol treatment of rabbits blocked the hypocalcaemic effect of exogenous calcitonin and raised the serum calcium concentration up to 80 % in animals not given calcitonin. Oestrogens have by some authors been thought to play an aetiological role in parturient paresis in dairy cows. For example, *Stott* (1968) suggested that high plasma levels of oestrogen at parturition could interfere with the action of parathyroid hormone on bone. *Bach & Messervy* (1969) reported a decrease in total and ultrafiltrable calcium in both lactating and non-lactating cows during oestrus. They also found that stilboestrol treatment of an ovariectomized cow produced a drop in the level of ultrafiltrable calcium without a decrease in total calcium. Contrary to this, *McLennan & Willoughby* (1973) obtained no changes of total and ionized calcium in cows during oestrus. *Braithwaite et al.* (1972) found hexoestrol implantation in wether lambs not to change the rate of bone resorption. The skeletal retention of calcium obtained in their study was caused by increased accretion of calcium into

bone. *Muir et al.* (1972) studied the effects of sex hormones on bone resorption in cows by measuring the urinary excretion of hydroxyproline. Neither oestrogen nor progesterone were found to change hydroxyproline excretion. Since oestrogen caused reduced appetite they suggested that an effect of oestrogen on calcium metabolism would be mediated by decreased intestinal absorption rather than by inhibition of bone resorption. In the present study neither 3 days nor 12 days of diethylstilboestrol treatment inhibited the hypocalcaemic effect of protamine in young rams. The failure to demonstrate such an effect is most easily interpreted as an unchanged rate of calcium resorption from bone, which is in agreement with reports of *Braithwaite et al.* (1970, 1972) and *Muir et al.*

Calcitonin is known to produce hypocalcaemia and hypophosphataemia by inhibition of bone resorption. However, *Talmage & Anderson* (1972) found the hypophosphataemia to be a result of increased phosphate movement out of plasma instead of inhibition of its entry. Thus, the 2 effects of calcitonin do not seem to be absolutely coupled. *Johnston et al.* characterized protamine as a hypocalcaemic and hypophosphataemic agent. On the other hand, we have found protamine to cause varying effects on plasma phosphate in sheep, in most cases a rise has been obtained (*Luthman et al.* 1973 a, b). Varying responses were found also in the present study. The most pronounced effect was obtained in adult pregnant ewes (Table 3), where the rise persisted throughout the observation period and, when compared with the young pregnant ewes, the difference in response was highly significant. It is obvious that protamine must affect phosphate metabolism in other ways than by reduced removal of phosphate from bone, but no reasonable explanation can be given from the present data. The high pretreatment level of phosphate in the second group of calves is in agreement with the results of *Long et al.* (1965), who found a maximum serum phosphate level in lambs at about 14 days of age.

Nothing is known about the influence of protamine on magnesium metabolism. In the present study the greatest response was found in the cows, a decrease of almost 15 %, and one can only speculate about the nature of this hypomagnesaemia. About 70 % of the total body magnesium is in the skeleton, and an inhibition of calcium resorption from bone would be expected to cause a concomitant reduced outflow of magnesium. Since the

milk is high in magnesium this might explain why the cows were most responsive. The rise in plasma magnesium after diethylstilboestrol treatment in the rams is in agreement with the report of *Addis et al.* (1969) who obtained hypermagnesaemia in cattle after treatment with a synthetic oestrogen for 3 days.

The results obtained in the present study confirm our earlier findings that the role of bone resorption in calcium homeostasis may be studied by measuring the hypocalcaemic response to protamine.

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SAMMANFATTNING

Olika faktorers inverkan på det hypokalcemiska svaret av protamin.

Det hypokalcemiska svaret av protamin, som ett mått på benresorptionen, studerades hos nötkreatur och får. Tre grupper av kalvar undersöktes (1—3 dagar, 2—3 veckor samt 4 månader gamla). De

äldsta kalvarna visade den kraftigaste kalciumsänkningen, vilket tolkades som en snabbare „turnover" i dessa djurs skelett, troligen relaterad till en högre tillväxttakt. En grupp av kor testades vid två tillfällen, nära förlossningen samt 2—3 veckor efter förlossningen. Kalciumsänkningen var liten vid första tillfället, medan en uttalad hypokalcemi erhölls vid andra infusionen. Resultaten stöder tidigare uppgifter om att benresorptionen är mer eller mindre blockerad hos kor vid tiden för förlossning. Unga dräktiga tackor uppvisade samma grad av hypokalcemi som gamla dräktiga tackor. Hos de unga djuren skedde emellertid normaliseringen av plasmakalcium snabbare. Unga baggar erhöll injektioner med protamin före och efter behandling med dietylstilboestrol. Kalciumsvaret var detsamma vid båda tillfällena. Varierande svar i halten av oorganiskt fosfor erhölls. De gamla dräktiga tackorna uppvisade en påtaglig hyperfosfatemi. Behandling med dietylstilboestrol medförde hypermagnesemi hos ungbaggarna.

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