

BIOCHEMICAL MARKERS OF BONE METABOLISM IN DAIRY COWS WITH MILK FEVER

STARIČ J and ZADNIK T

University of Ljubljana, Veterinary Faculty, Clinic for ruminants, Slovenia

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Bone metabolism was investigated in 8 healthy and 12 dairy cows suffering from milk fever (MF) in intensive dairy production. Blood samples were taken within 48 hours after calving in healthy cows and before treatment in cows with MF. Bone and mineral metabolism were evaluated by measuring blood serum bone resorption biomarker C-terminal telopeptide of type I collagen (CTx) and bone formation biomarker bone-specific alkaline phosphatase (bALP) beside the classical panel: total calcium (Ca), inorganic phosphate (iP), magnesium (Mg) and alkaline phosphatase (ALP). The results were statistically analyzed and compared between the two groups.

Mean Ca value in cows with milk fever was 1.01 ± 0.29 mmol/L and in healthy cows it was significantly ($p < 0.05$) higher (1.94 ± 0.04 mmol/L). All the cows were also hypophosphatemic with lower phosphorus values ($p < 0.05$) in MF cows. Some cows with MF were also hypermagnesemic, but the difference in Mg concentrations between the two groups was not significant. Mean total ALP and bALP activity were higher in cows with MF (65 ± 17.7 U/L and 21.3 ± 8.5 U/L, respectively) than in healthy cows (55.9 ± 7.0 U/L and 20.5 ± 8.9 U/L, respectively). The mean concentration of blood serum CTx was lower in cows with MF (0.212 ± 0.091 ng/L) than in healthy cows (0.417 ± 0.252 ng/L), but as for bALP not significantly.

Key words: biomarkers, blood, bone metabolism, cattle, milk fever

INTRODUCTION

Recent reports from Europe (Roche, 2003) and Slovenia (Gašperlin, 2002) estimate that up to 10% of all dairy cows each year develop the acute clinical form of hypocalcemia, called also milk fever (MF), where animals are unable to stand and can die if not promptly medically treated with Ca supplements. Similar findings were established also in the USA and Australian dairy herds with an incidence range from 0 to 7% (DeGaris and Lean, 2008). The incidence of subclinical hypocalcemia was estimated to be up to 50% of all mature dairy cows close to the time of parurition (Horst *et al.*, 2003). Periparturient hypocalcaemia is a metabolic disease of females i.e. cows, caused by the inability of homeostatic

mechanisms to maintain normal blood Ca level. Ca demand after calving rises to multiple values of that during the dry period due to intensive colostrum and milk production, which puts cows at risk of developing milk fever if all homeostatic mechanism for Ca balance are not functioning properly (Goff, 2000). Inadequate blood Ca concentration can cause the inability to stand, which is just the tip of the iceberg. Many more cows in the herd with a less severe form will present reduced food intake, poor rumen and intestinal motility, poor productivity, and increased susceptibility to other metabolic and infectious diseases (Goff, 2008). Milk fever in dairy cows is one of the most economically important metabolic diseases. It has a big economic impact on the dairy industry due to direct and indirect financial losses. Average costs of a single case of periparturient paresis are estimated to more than 300 EUR, taking into account loss of production, treatment costs and culling (Kossaibati and Esselmont, 1997).

Ca homeostasis around parturition can not be maintained solely by intestinal absorption of Ca, but also from resorption of mineralized bone tissue (Goff, 2008). Dairy cows are programmed to go into a state of lactational osteoporosis to maintain normal blood Ca level in early lactation. As much as 9 – 13% of skeletal Ca can be lost for milk production in the first month of lactation (Goff, 2004). Ca is obtained from bone tissue also by osteoclastic osteolysis (Christenson, 1997).

Biochemical markers of bone metabolism are byproducts of bone tissue formation and resorption that escape into blood. They can be measured in the blood or urine. Biochemical markers of bone tissue formation (for instance bone alkaline phosphatase (bALP)) are byproducts of osteoblastic activity and biochemical markers of bone tissue resorption (for instance C terminal telopeptide crosslinks (CTX)) are byproducts of osteoclastic activity. Currently, they are extensively being used for osteoporosis evaluation and monitoring in humans (Christenson, 1997). Some reports of their use in different animal species are published in scientific and professional literature (Allen, 2003). They are already implemented in diagnostic procedures also in dairy cattle (Liesegang *et al.*, 1998; Iwama *et al.*, 2004; Holtenius and Ekelund, 2005; Filipović *et al.* 2008; Starič *et al.*, 2008a,b).

In the present paper, bone metabolism in healthy mature dairy cows and dairy cows with MF was studied by using biochemical markers of bone metabolism.

MATERIAL AND METHODS

Animals and herd management

Healthy Slovenian black and white (BW) cows (n=8) and 12 BW cows with milk fever and no signs of other diseases were included in the study at a dairy farm with intensive milk production. Average yearly milk production per cow was 8.753 kg. The study was performed during the wintertime, when all the cows were housed in tie-stall type system on short stalls. All the cows were at least in the fourth lactation and were in the dry period from 45 to 60 days. Cows included in the study were fed twice a day with usual winter total mix ration (based on home produced forages: grass silage, maize silage, hay and straw; concentrates and

vitamin-mineral supplement) adapted for dry cows and fresh cows, according to NRC (2001) recommendations. Anion salts were not added to the feed in our study.

Blood sampling and laboratory analyses

Samples of venous blood were obtained using vacutainers with no additives (Venoject, plain silicone coated, Terumo Europe N.V., Belgium) by puncture of *v. caudalis mediana* before i.v. therapy for milk fever was instituted or within 48 hours after calving in healthy cows. After blood clotting, samples were centrifuged at 3000 rpm for 10 minutes and the supernatants were centrifuged again at 3000 rpm for 10 minutes at room temperature. Harvested blood serum was stored at -20° C until analyses.

Biochemical analyses of blood serum samples was performed by automatic systems.

Blood serum total Ca, iP and Mg concentrations and the activity of total ALP were measured with the biochemical analyzer RX Daytona (Randox, Ireland) according to manufacturer's instructions. Blood serum bALP activity was measured with Alkphase-B kit (Metra, Biosystems, USA) by enzyme immunoanalysis according to manufacturer's instructions. The absorbance at the end of the reaction was measured with the optical reader Humareader (Human, USA) at 405 nm wavelength. CTx concentration in blood serum was measured by electrochemiluminiscent immunoanalysis ECLIA. The test was conducted using Elecysis 3 – CrossLaps kit on Elecys analyzer 1010 (Roche Diagnostics, USA) according to manufacturers' instructions.

Statistical analysis

Data were statistically analyzed using SPSS version 15.0. software (SPSS Inc., USA). Basic descriptive statistics (mean and standard deviation (SD)) were calculated. Influence of MF on blood serum parameters was analyzed with independent samples T test. All obtained values were previously normalized according to Box-Cox. Person's correlations were also calculated for all parameters. Statistical significance was set at $p < 0.05$.

RESULTS

Mean \pm SD for Ca, iP, Mg, ALP, bALP, CTx and statistical differences between the 2 groups are presented in Table 1. All the cows affected by MF showed characteristic clinical signs of the disease, the diagnosis was further confirmed by blood analyses and favourable treatment results. Cows with MF fully recovered after i.v. treatment with preparations containing Ca, P and Mg.

Statistically significant Pearson correlation (Table 2) was established between Ca and iP ($r = 0.748$, $p < 0.001$) and Ca and CTx ($r = 0.497$, $p < 0.05$). iP also correlated with CTx ($r = 0.549$, $p < 0.05$) and a strong correlation was noticed between CTx and bALP ($r = 0.715$, $p < 0.001$). Other variables did not correlate in our study. Graphical illustration of statistically significant correlations is presented in Figures 1 to 5.

Table 1. Mean \pm SD for Ca, iP, Mg, ALP, bALP, CTx and statistical differences between the 2 groups

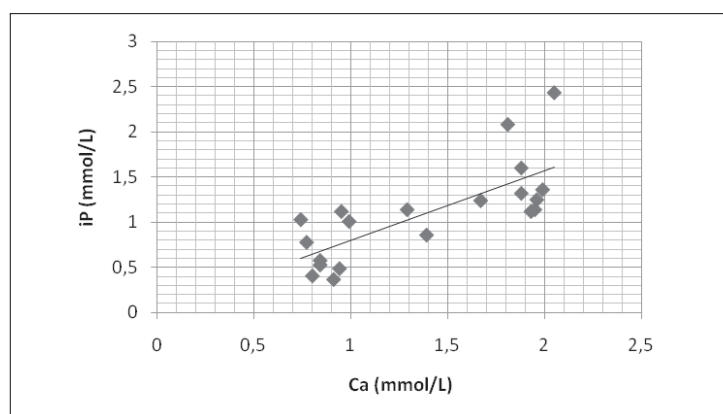
Biochemical parameter	Healthy cows	Cows with milk fever	p value
Ca (mmol/L)	1.94 \pm 0.04	1.01 \pm 0.29	**
iP (mmol/L)	1.54 \pm 0.48	0.80 \pm 0.31	*
Mg (mmol/L)	1.17 \pm 0.13	1.19 \pm 0.28	NS
ALP (U/L)	55.90 \pm 7.00	65.20 \pm 17.70	NS
bALP (U/L)	20.50 \pm 8.90	21.30 \pm 8.50	NS
CTx (ng/L)	0.417 \pm 0.252	0.212 \pm 0.091	NS

**significance at <0.01 level; *significance at <0.05 level; NS – non significant

Table 2. Pearson correlation between investigated biochemical parameters in 20 examined dairy cows

Biochemical parameter		iP (mmol/L)	Mg (mmol/L)	ALP (U/L)	bALP (U/L)	CTx (ng/L)
Ca (mmol/L)	r	0.748**	-0.108	-0.161	0.367	0.497*
	p	0.000	0.650	0.497	0.112	0.026
iP (mmol/L)	r		-0.153	-0.133	0.493*	0.549*
	p		0.519	0.577	0.027	0.012
Mg (mmol/L)	r			0.132	-0.117	0.028
	p			0.580	0.622	0.906
ALP (U/L)	r				0.260	0.021
	p				0.269	0.931
bALP (U/L)	r					0.715**
	p					0.000

**Correlation is significant at <0.01 level; *Correlation is significant at <0.05 level

Figure 1. Correlation between Ca and iP ($r=0.748$) with trend line

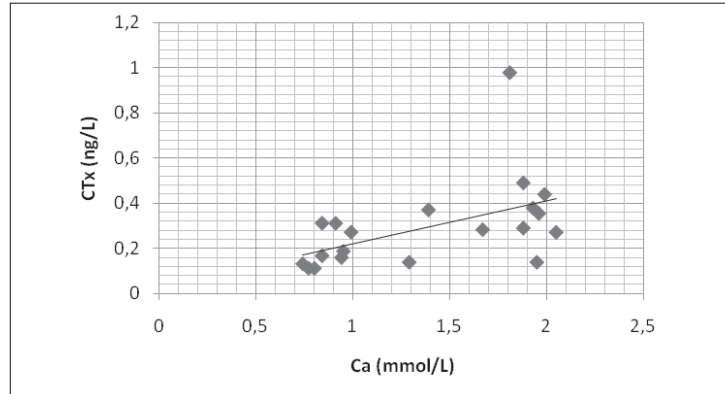


Figure 2. Correlation between Ca and CTx ($r=0.497$) with trend line

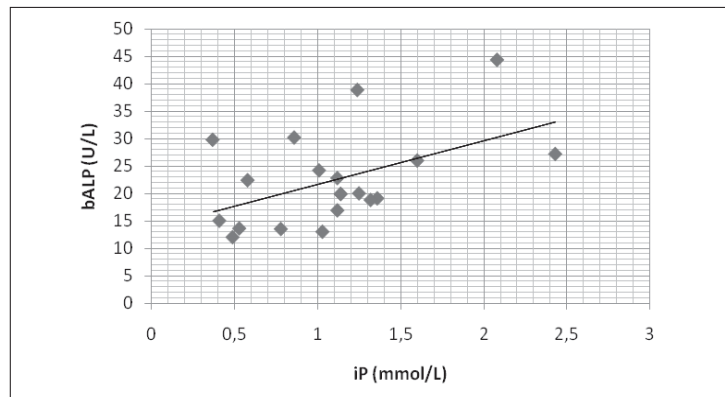


Figure 3. Correlation between iP and bALP ($r=0.493$) with trend line

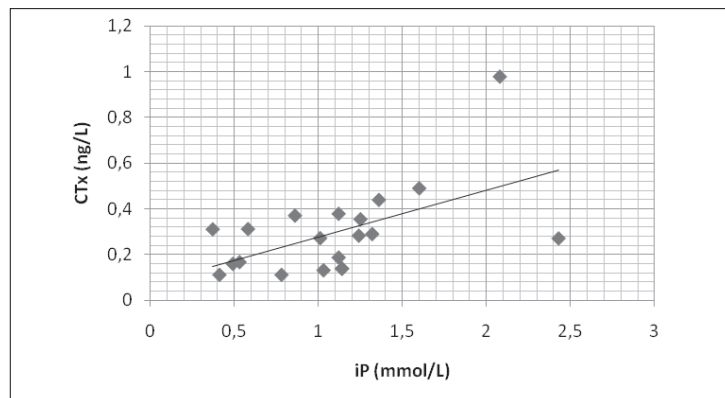


Figure 4. Correlation between iP and CTx ($r=0.549$) with trend line

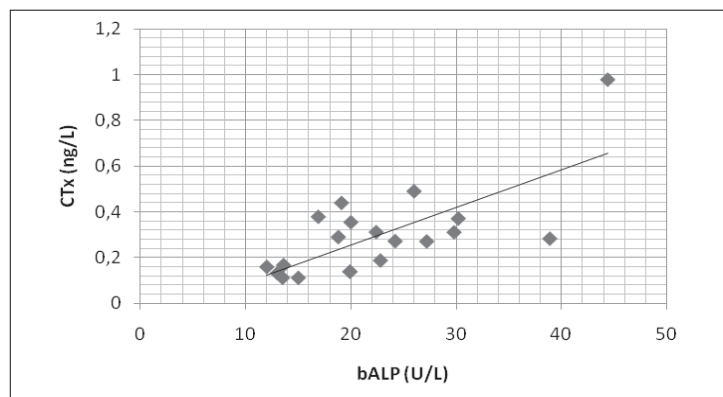


Figure 5. Correlation between bALP and CTx ($r=0.715$) with trend line

DISCUSSION

Clinical diagnosis of MF was confirmed by measurements of total blood serum Ca and complete recovery of affected cows after i.v. treatment with Ca borogluconate. Cows with MF were markedly hypocalcemic. Their Ca values were far below the suggested normal total serum Ca level ($\text{Ca} \geq 2.00 \text{ mmol/L}$) for dairy cows in this physiological period (Goff, 2008). Mildly subclinically hypocalcemic were also most of the healthy cows, but the mean serum Ca concentration in this group was statistically significantly higher than in cows with MF. Similar findings were observed by many authors (Lapeteläinen *et al.*, 1993; Liesegang *et al.*, 1998; Bigras-Poulin and Tremblay, 1998; Beighle, 1999). Our results are also in agreement with the findings of Larsen *et al.* (2001) where typical clinical signs of MF (muscle weakness, depression of the cardiovascular system, hypothermia, recumbancy, loss of consciousness) begin to show when Ca concentration in the blood decreases below 1.60 mmol/L .

Marked hypophosphatemia in MF cows is associated with PTH secretion during hypocalcemia. Even though PTH also stimulates via calcitriol the absorption of phosphates from the intestines and resorption from bones, it looks like the increase in renal and salivary secretion of phosphates is overwhelming. PTH primary function is in maintaining normocalcemia. This is often a reason why hypocalcemic cows tend to be also hypophosphatemic (Goff, 2000). Connection of Ca and iP metabolism our study is also evident as both analytes correlate significantly (Table 2). Mild hypophosphatemia was noted also in healthy cows, but significantly less than in MF cows ($p = 0.03$). Jazbec *et al.* (1970), Lapeteläinen *et al.* (1993), Horst *et al.* (1994), Liesegang *et al.*, (1998), Bigras-Poulin and Tremblay (1998) obtained similar results.

Serum Mg concentration was not significantly different in both groups ($p = 0.849$). In our study higher values in most of the investigated animals were obtained than is the normal reference range for Mg in adult cattle, $\text{Mg} = 0.75\text{-}$

1,00 mmol/L (Goff, 2004). Jazbec *et al.* (1970), Riond *et al.* (1995) and Bigras-Paulin and Tremblay (1998) also reported higher serum values of Mg on the first and second day after calving than later in lactation. Higher Mg values after calving are associated by hypocalcemia and release of PTH, which stimulates tubular resorption of Mg in kidneys (Oetzel, 1988; Fontenot *et al.*, 1989) and possibly regulates ionic equilibrium. Liesegang, *et al.* (1998) did not establish statistically significantly higher values of Mg on the first day after calving compared to the next 2 weeks of lactation in cows with and without MF.

Total ALP activity was higher in MF cows than in healthy cows in our study, but not significantly ($p = 0.120$). One of the reasons could be the recumbence of MF cows that could have a negative effect on hepatic tissue and consequently resulted in a rise of ALP activity originating from liver. Our opinion is that the rise in ALP is not associated with more intensive osteoblast activity, since there was no correlation between ALP and bALP ($r = 0.260$, $p = 0.269$). Lappeteläinen *et al.* (1993) on the contrary measured lower values of ALP in cows with MF than in healthy ones and concluded that cows with MF had impaired osteoblast and osteoclast function.

Mean bALP activity was slightly higher in cows with MF, but not significantly ($p = 0.502$). It looks like bone anabolic activity was at the same rate in both groups of cows. Interestingly, we measured much higher values of bALP in our cows than Filipović *et al.* (2008) 14 days before calving and 10 days after calving. Our opinion is that the anabolic effect in our study is due to very high estrogen levels in cows just before calving and estrogen is known to be a osteoprotective anabolic hormone (Riggs *et al.*, 2002).

CTx concentration was much higher in healthy cows than in MF cows in our study. The difference was almost statistically significant ($p = 0.057$). A statistically significant positive correlation was established between CTx and Ca ($r = 0.497$, $p = 0.026$) and also iP ($r = 0.549$, $p = 0.012$). It looks like adequate bone resorption after calving is essential for maintaining sufficient blood Ca and iP and prevention of MF. Holetenius and Ekelund (2005) obtained highest CTx values at the beginning of lactation in a study conducted on 11 dairy cows, which supports our opinion. What triggers adequate bone resorption in healthy cows after calving is not known. The reason could be by optimal response of osteoblasts to PTH and activation of osteoclasts. This does not happen in MF cows. Estrogen could be another reason. In the study conducted by Lappeteläinen *et al.* (1993) mean estrogen value was higher in cows with MF than in healthy cows during the same physiological period. Estrogen is known to decrease osteoclast formation and activity (Riggs *et al.*, 2002). Sechen *et al.* (1988) also suggested that high estradiol concentration in late pregnancy inhibits bone resorption and predisposes cows to MF.

CONCLUSION

It looks like response of bone metabolism in cows with MF was inappropriate. Instead of a more intensive resorption of bone tissue in cows with MF, there was less intensive resorption compared to healthy cows and slightly

more intensive bone formation. Reasons for such bone metabolism response still have to be elucidated. We suggest that hormonal status of cows at calving is one of the suspects for this situation. For example the sex hormone estrogen, which has osteoprotective and anabolic effects on bone metabolism, reaches highest blood values at the time of calving.

We also propose that there is a potential in measuring biochemical markers of bone metabolism before calving at close up dry period to predict if a cow is at risk of developing MF after calving. Further studies in the field of biochemical markers of bone metabolism in periparturient cows are needed.

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Address for correspondence:

Starič J
Clinic for ruminants
Veterinary faculty
University of Ljubljana
Gerbičeva 60
1115 Ljubljana, Slovenia
E-mail: joze.staric@vt.uni-lj.si

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BIOHEMIJSKI MARKERI METABOLIZMA U KOSTIMA KRAVA OBOLELIH OD PUERPERALNE PAREZE

STARIĆ J i ZADNIK T

SADRŽAJ

U ovom radu su izneti rezultati ispitivanja metabolizma u kostima 8 zdravih krava i 12 krava obolelih od puerperalne pareze. Uzorci krvi su prikupljeni od zdravih krava, 48 sati posle teljenja, a od bolesnih, neposredno pre terapije.

Metabolizam u kostima i metabolizam minerala procenjavani su određivanjem koncentracije biomarkera resorpcije kostiju (C-terminalni telopeptid kolagena tipa I - CTx) i biomarkera formiranja kostiju (alkalna fosfataza specifična za kosti - bALP). Osim toga, u serumu je određivana i koncentracija kalcijuma, neorganskih fosfata, magnezijuma i aktivnost alkalne fosfataze (ALP).

Prosečna koncentracija kalcijuma u krvi krava obolelih od puerperalne pareze je iznosila $1,01 \pm 0,29$ mmol/l, dok je kod zdravih jedinki ova vrednost bila značajno veća ($p < 0,05$) i imala je vrednost od $1,94 \pm 0,04$ mmol/l. Sve krave su imale malu koncentraciju neorganskih fosfata i ona je bila značajno manja kod obolelih jedinki ($p < 0,05$). Pojedine plotkinje sa puerperalnom parezom su imale i sniženu koncentraciju magnezijuma ali razlike između grupa nisu bile statistički značajne. Srednje vrednosti za ukupnu aktivnost ALP i bALP su bile veće kod obolelih krava ($65 \pm 17,7$ U/l i $21,3 \pm 8,5$ U/l) nego kod zdravih ($55,9 \pm 7,0$ U/l i $20,5 \pm 8,9$ U/l). Koncentracija CTx u serumu obolelih krava ($0,212 \pm 0,091$ ng/l) je bila manja nego kod zdravih jedinki ($0,417 \pm 0,252$ ng/l) ali ove razlike, kao ni za bALP, nisu bile statistički značajne.