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Original Scientific Article

BONE METABOLISM MARKERS AND BLOOD MINERALS CONCENTRATION IN DAIRY CATTLE DURING PREGNANCY AND LACTATION

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Summary: Biochemical markers of bone metabolism are substances in blood and urine that indicate how intensively bone is forming or degrading at the time of sample (blood or urine) collection. The aim of the study was to establish concentration of total calcium (Ca), inorganic phosphate (iP), magnesium (Mg), C-terminal telopeptide crosslinks of collagen I (CTx) (biochemical marker of bone resorption) and activity of total alkaline phosphatase (ALP) and bone specific alkaline phosphatase (BALP) (biochemical marker of bone formation) in blood serum of clinically healthy mature dairy cows from dry period until peak lactation. Holstein-Friesian cows (n=54) with high milk yields (average 8,463 kg/previous lactation) were enrolled in the study. Cows were divided in 5 groups according to stage of lactation: 1. approximately 1 month before calving (AP) (early dry period) (n = 10), 2. 10 to 1 day AP (close-up dry period) (n = 10), 3. 0 – 48 hours after calving (PP) (n = 10), 4. 10 – 20 days PP (n = 10) and 5. peak of lactation (35 – 55 days in milk) (n = 14). The nadir of mean Ca and iP were in the third group and significantly lower than in other groups ($P<0.05$). Mean Mg was higher in groups 3 and 5 compared to groups 2 and 4 ($P<0.05$). The lowest mean value of BALP was established in group 1. The difference was statistically significant compared to all other groups. The highest values of CTx were measured in groups 4 and 5. These values were also significantly higher compared to groups 1, 2 and 3. The lowest CTx values were measured in the 1. group. Results of the study indicate that bone tissue is the most catabolically active 10 to 20 days after calving and the most anabolically active during the dry period. Strong negative correlation ($P<0.01$) was established between BALP and minerals Ca and iP.

Key words: biochemical markers; CTx; BALP; ALP; cows; mature

Introduction

Bone metabolism in physically mature animals is a continuous dynamic process of bone resorption coupled with bone formation, so called bone remodeling. This allows adaptation of bones quality (flexibility and firmness), to physical load and during periods of minerals shortage, bone can be the main source of especially calcium (Ca) (1). In mature dairy cattle bone metabolism is especially active due to Ca requirement from parturition to peak lactation when Ca demand quickly rises to its highest. Enough Ca for maintenance and production from ration is typically very hard to obtain and they have to use also their body Ca reserve in bones to maintain normocalcemia. Ca metabolism is connected to bone metabolism and closely hormonally regulated. During bone resorption, inorganic phosphorus (iP) and magnesium (Mg) are also released, but their blood concentration is not hormonally regulated (2-4).

Biochemical markers of bone metabolism are substances in blood and / or urine that indicate the intensity of bone formation or resorption at the time of sample collection. During bone formation (anabolic activity), osteoblasts are more

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metabolically active. Byproducts of osteoblast activity are markers of bone formation, eg. bone specific enzyme bone alkaline phospahatase (BALP) in blood serum. During bone resorption (catabolic activity), osteocalsts are metabolically more active. Byproducts of osteoclast activity are markers of bone resorption, eg. C-terminal telopeptide crosslinks of collagen I (CTx) in blood serum. Markers of bone metabolism can indicate if bone metabolism is more anabolically, catabolically or in general more or less active (1, 5). In high yielding dairy cows we expect increased catabolic bone metabolism at the beginning of lactation when absorbable Ca demand suddenly increases from about 20 g to around 60 g or more per day because of colostrum and milk production concurrent with a relatively low dry matter (nutrient) intake (3, 6). Lactational osteoporosis, where around 13 % Ca can be lost from bones, can result as significant proportion of Ca is resorbed from bones at the beginning of lactation (7). This finding was confirmed also in a study conducted by *Beighle* (8) where cortical bone Ca concentration decreased as milk production increased. When milk production starts to decline body stores of Ca are replenished and bone metabolism is more anabolic (9). If these processes are not functioning properly bone disease and Ca metabolism disorders develop (hypocalcaemia and milk fever) which affect animal welfare, result in suboptimal production and other diseases in cattle (3). Very little is known about dynamics of biochemical markers of bone metabolism in dairy cattle during lactation and dry period.

The aim of this study is to investigate bone metabolism in mature dairy cows in intensive dairy production by measuring serum bone resorption marker CTx and bone formation marker BALP along with the classic mineral metabolism panel (Ca, iP, Mg and total alkaline phosphatase (ALP)) from dry period until peak lactation.

Material and methods

Herd and animals

The study was carried out in a heard of Holstein-Friesian cattle in intensive dairy production in farm setting. Clinically healthy skeletally mature cows (n=54) in appropriate body condition for their physiological period and at least in 4th lactation (range 4th to 7th lactation; mean age 7 years, median age 7 years, range 5 to 10 years) were enrolled in the study. Cows were sampled from February until mid-April when all animals were exclusively kept indoors, in tie-stall type housing system on short stalls. Average annual milk yield of investigated cows in previous lactation was 8,463 kg (rage from 7,128 kg to 10,215 kg) milk with 3.7 % milk fat and 3.17 % crude milk protein. All the cows included in the study were in dry period for 50 ± 3 days.

Cows were divided in 5 groups according to their stage of lactation:

1. 35 – 25 days before calving (AP) or at early dry period, n = 10,
2. 10 – 1 day AP (end of close-up period – mammary gland is regenerating and secreting colostrums), n = 10.
3. 0 – 48 hours after calving (PP) (the most critical time for development of milk fever (3)), n = 10,
4. 10 – 20 days PP (direct influence of calving on bone metabolism is low; milk production is steeply rising), n = 10,
5. peak lactation (35 – 55 days in milk), n = 14,

Ration

Cows were fed usual winter total mix ration (TMR), adapted to high productivity and their stage of lactation during the study, according to NRC (6) recommendations. The TMR was based on home produced forages: grass silage, maize silage, hay and straw. Anion salts were not added to feed in our study and forages were not treated in any way to reduce DCAD. DCAD of TMR was estimated to be in the range from +200 to +300 mEq/kg DM.

Composition of TMR is presented in table 1.

The ration for lactating cows was suitable for 30 kg milk yield per day. Fresh cows received about 15 kg DM of ration for peak lactation cows and hay ad libitum.

Blood sampling and analytical methods

Venous blood samples were collected in evacuated tubes (10 mL) without any additives (Venoject, plain silicone coated, Terumo Europe N.V., Belgium) from *v. caudalis mediana* according to the protocol between 9 and 11 a.m., to avoid daily fluctuations in analytes. Cows were

Table 1: Composition of TMR fed to cows during lactation and dry period

TMR ingredient	Peak lactation	Early dry period	Close-up dry period
Grass silage (33.1 % DM)	14 kg	14 kg	5 kg
Corn silage (39.2 % DM)	20 kg	7 kg	7 kg
Hay (85.3 % DM)	1 kg	/	3,5 kg
Straw	/	3 kg	/
Wheat	3 kg	/	1 kg
Commercially prepared concentrated feed (26% crude protein, 3% protected fats)	3 kg	/	1 kg
Commercially prepared energy feed (9 MJ NEL, 14% crude protein)	/	/	1kg
Rape skins	1 kg	0.5 kg	0.3 kg
Beet pulp	1 kg	/	0.3 kg
Soya bean skins	0.3 kg	/	0.1 kg
Mineral-vitamin supplement for lactating cows (Ca 18%, P 6%, Mg 4%, D_3 80.000 IE/kg)	0.1 kg	/	/
Mineral-vitamin supplement for lactating cows (Ca 19%, P 4.5%, Mg 2.5%, D_3 125.000 IE/kg)	0.1 kg	/	/
Mineral-vitamin supplement for dry cows (Ca 3%, P 8%, Mg 7%, D_3 125.000 IE/kg)	/	0.1 kg	/
Grounded calcium carbonate	0.06 kg	/	/
$NaHCO_3$	0.12 kg	/	/
Dry matter intake by animals	22 kg	11 kg	10,5 kg
Ca	165.69 g	49.99 g	58.74 g
P	86.14 g	39.54 g	33.70g
Mg	47.75g	25.53 g	43.28 g

sampled once or twice a week, during 14 sampling sessions. During this time at least 10 animals were randomly obtained for each group. Only one blood sample was taken from each animal. After blood clotting, samples were centrifuged at 3000 rpm for 10 minutes then supernatants were centrifuged again at 3000 rpm for 10 minutes at room temperature. Harvested blood serum was stored at -20° C until analyses.

Blood serum Ca, aP and Mg concentration and activity of total ALP were measured with automatized biochemical analyzer RX Daytona (Randox, Ireland) according to manufacturer's instructions. Blood serum BALP activity was measured using Alkphase-B kit (Metra Biosystems, USA) by enzyme imunoanalysis according to manufacturer's instructions on Immulite 2500 analyser (Siemens, Germany). The absorbance at the end of reaction was measured with optical reader Humareader (Human, Egypt) at 405 nm wave length. Cross reactivity of the test was validated for use in cattle (1). CTx concentration in blood serum was measured by electrochemiluminiscent imunoanalysis ECLIA. The test was conducted using Elecysis 3 – CrossLaps kit on Elecys 1010 analyzer (Roche Diagnostics, USA) according to manufacturers' instructions.

Statistical analysis

Data were statistically analyzed using SPSS verson 15.0. software (SPSS, USA). Mean and standard deviation were calculated for measured parameters. Influence of physiological period on parameters was analyzed with analysis of variance (one-way ANOVA). All obtained values were previously normalized according to Box-Cox. When statistical differences were discovered between groups, they were ascertained with Tukey's algorithm of multiple comparisons. Person's correlations were calculated for all parameters. Statistical significance was set at $P<0.05$.

Results

Descriptive statistics for Ca, iP, Mg, ALP, BALP, CTx and statistically significant differences according to stage of lactation (group) are presented in Table 2. Dynamics of investigated parameters from dry period until pick lactation are demonstrated in Graph 1 and 2.

Values of Ca and iP were statistically significantly lower in 3. group compared to other groups and under physiological range for adult cattle (reference blood serum Ca of adult dairy cattle is 2.2 – 2.5 mmol/L and iP 1.61 – 2.26 mmol/L (3)). All the animals had Mg within reference range for adult dairy cattle, > 0,82 mmol/L (3). The lowest values of BALP were measured in 1. group. The difference was statistically significant compared to all other groups. The highest values of CTx were measured in 4. group.

Statistically significant positive Pearson's correlations were found between total ALP and BALP ($r=0.585$, $P<0.01$) and also between Ca and iP ($r=0.671$, $P<0.01$) (Graph 1 and 2). Strong negative correlation was ascertained between Ca and BALP ($r=-0.372$, $P<0.01$), and between iP and ALP ($r=-0.191$, $P<0.05$) / BALP ($r=-0.352$, $P<0.01$). Negative correlation between iP and Mg was close to significance ($r=-0.162$, $P=0.08$). Ca was also positively correlated with CTx but not statistically significanty ($P>0.05$). All the other correlations were statistically insignificant.

Discussion

Sampling methodology used in this study employed original approach compared to other studies in biochemical bone markers research, which achieved minimal changes in environmental conditions despite very long production cycle in cattle (ideally of 1 year, but usually much more than a year) in farm settings. It also includes different animals, sample more general population, physically comparable though in contrast to other studies that were monitoring the same animals through the lactation. This way we wanted to test the use of biochemical markers of bone metabolism in more applied clinically relevant settings that wanted to prove their dynamics in different groups of healthy cows in appropriate body condition.

The highest mean CTx concentration was established in the 4. group in our study. This finding is in accordance with *Holtenius and Ekelund* (9) who report that CTx concentration in dairy cows was highest in the first week after calving and then decreased evenly over the next 33 weeks. *Filipović et al.* (10) also observed the highest CTx values in 21 Holstein-Friesian cows 10 days after calving compared to 14 days before calving and 30 days after calving. Similarly reported *Liesegang et al.* (11) in a study performed in 30 Brown Swiss cows when they measured bone resorption marker ICTP. The highest CTx and ICTP concentrations first week after calving are in accordance with general finding that Ca resorption from bones is the most intensive during this period (3). The highest mineral demand of cows is from calving until peak lactation, what is demonstrated in more intense bone resorption, since enough Ca can not be provided from the ration alone in this period.

Our findings suggest that bone is minimally catabolically active (very low CTx), and still has high anabolic activity (relatively high BALP) during the dry period, which indicates that there is a net gain in mineral reserves in bone tissue. We believe that reserve gained during dry period is very important for maintenance of normocalcaemia at the beginning of next lactation. It is well known that peak production is followed by maximal consumation of dry matter in dairy cow by about 2 – 3 weeks (12). During this period the deficit of nutrients (including Ca) between those required for production of milk and those available from consumed feed has to be mobilized from body reserves (3).

Liesegang et al. (11) found out that blood osteocalcine (OCN), a bone formation marker, concentration markedly decreased after calving and then started to slowly rise until the second month of lactation when it reached a plateau, then slowly decreased until calving. Similar findings about OCN were reported by *Holtenius and Ekelund* (9) and *Iwama et. al.* (13). *Filipović et al.* (10) measured the highest values of BALP 14 days before calving, but not statistically significantly higher than 10 days after calving. Our findings are not completely in agreement with findings of mentioned authors since we ascertained the highest BALP activity within 48h after calving and then in 4. and 2. group. This means that bone remodeling is very intensive around calving (both bone resorption and formation are very high). High activity of BALP PP shown in our study is negatively correlated with serum total Ca concentration

Table 2: Mean values and standard deviations of Ca, iP, Mg, ALP, BALP and CTx at each stage of lactation and statistical significance

Group	n	Ca (mmol/L)	iP (mmol/L)	Mg (mmol/L)	ALP (U/L)	BALP (U/L)	CTx (ng/L)
1	10	2.43 ± 0.12[3]	2.22 ± 0.33[3,4]	0.97 ± 0.08[2]	32.4 ± 5.9[2.3]	11.64 ± 1.64[2.3.4,5]	0.089 ± 0.044[4,5]
2	10	2.44 ± 0.09[3]	2.04 ± 0.19[3]	0.84 ± 0.05[1.3.5]	48.1 ± 7.4[1]	16.29 ± 2.1[1.3]	0.201 ± 0.131[4,5]
3	10	1.96 ± 0.27[1.2.4.5]	1.37 ± 0.30[1.2.4.5]	1.02 ± 0.13[2,4]	52.1 ± 10.7[1]	21.75 ± 4.35[1.2.4.5]	0.386 ± 0.132[4,5]
4	10	2.30 ± 0.14[3,5]	1.87 ± 0.17[1.3]	0.88 ± 0.10[5]	41.9 ± 14.7	16.91 ± 3.12[1.3]	1.174 ± 0.582[1,2,3]
5	14	2.57 ± 0.13[3.4]	1.94 ± 0.23[3]	1.05 ± 0.06[2.4]	44.1 ± 12.3	16.14 ± 4.03[1.3]	0.828 ± 0.352[1,2,3]

Legend: 1,2,3,4,5 – statistically significant differences for parameters between groups at P<0.05

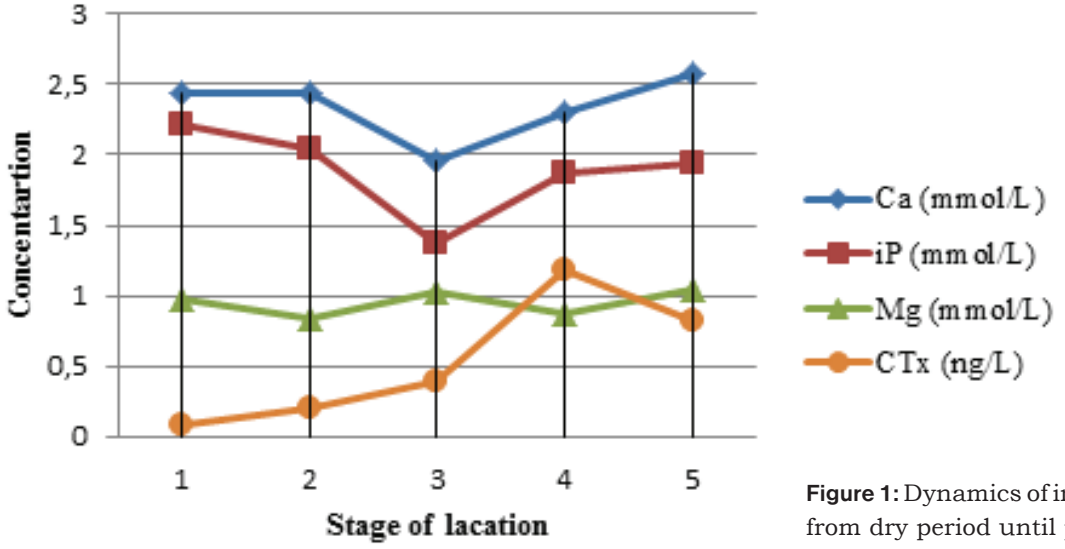

Figure 1: Dynamics of investigated parameters from dry period until pick lactation

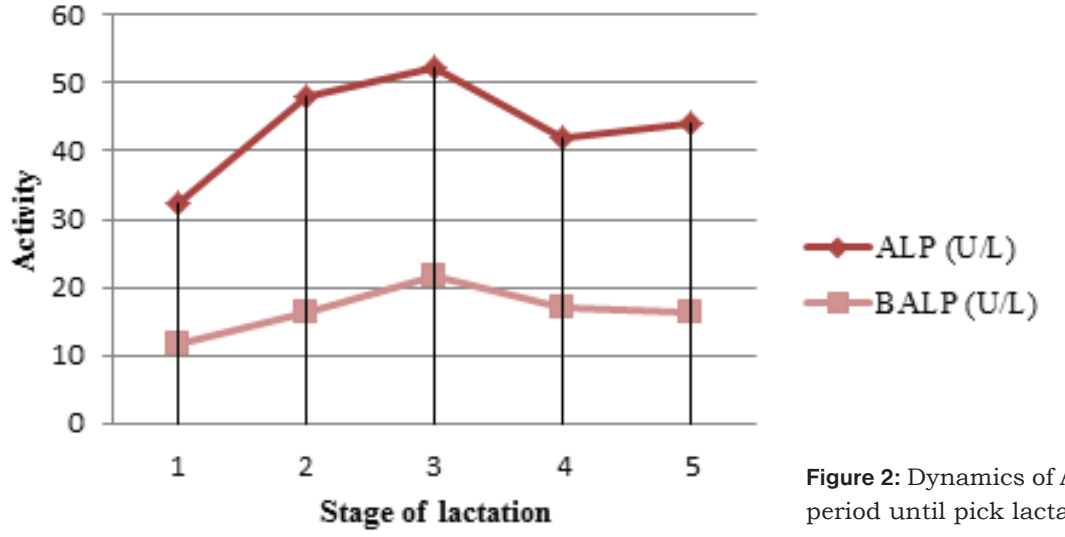

Figure 2: Dynamics of ALP and BALP from dry period until pick lactation

and indicates inappropriate response of bone metabolism to hypocalcaemia. The mechanism for maintaining normocalcaemia is likely overridden by other mechanisms, associated with calving in dairy cattle beside enormous Ca drain for milk production. Cows with milk fever had lower CTx and higher BALP than healthy cows in a study by *Starič and Zadnik* (14). Adding anionic salts to the ration would increase bone resorption (CTx) and the concentration of ionized calcium in blood (3). High BALP activity can be associated with bone metabolism that was unprepared to provide Ca for the steep increase in demand or responded inappropriately to hypocalcaemia. *Filipović et al.* (10) ascertained statistically significant ($P<0.001$) negative correlation between estradiol and CTx. Estradiol reaches the highest values before calving (15). Anabolic and osteoprotective effect of estradiol on bone tissue is well known, which can significantly contribute to the development of periparturient hypocalcaemia in dairy cows (5, 9, 10, 16). Estradiol contributed probably to the highest mean BALP activity within 48 hours after calving in this study.

Total ALP correlates with BALP and can be used in assessing bone metabolism, provided that there is no liver pathology present.

Results of Ca and iP dynamics in our study were in accordance with results of other authors (17, 18, 19). Almost all cows experience a certain level of hypocalcaemia during the first days after calving in our study and in mentioned studies. It was suggested that this is physiological in high yielding cows due to the very abrupt rise in Ca demand for milk production. After few days the homeostatic mechanisms for Ca adapt to higher Ca and iP demand and maintain blood Ca and iP concentration within the normal range until the next calving (17, 18, 19).

Mean Mg concentration was within normal range for dairy cattle in all test groups in our study which confirms appropriate Mg supply. Mg dynamics resemble those of *Bigras-Paulin and Tremblay* (17) and *Riond et al.* (20). Higher mean Mg values were also ascertained in the 3. and 5. group in our study. Higher Mg concentration around calving is consequence of hypocalcaemia and high PTH, which stimulates Mg resorption in kidney tubules. High PTH concentration lowers Mg secretion in urine and thus elevates blood Mg concentration. PTH also stimulates iP excretion via kidney and salivary glands (3), which was the reason for almost statistically significant negative correlation between Mg and iP in our study.

Further studies in bone metabolism are needed to evaluate and extend current knowledge particularly in bigger animal sample and in groups treated by different means that elevate blood Ca concentration around calving and influence bone metabolism, like anion salts.

Conclusion

Dynamics of biochemical markers of bone metabolism in groups of cows from early dry period until peak lactation were demonstrated. Results of the study indicate that bone tissue is the most catabolically active 10 to 20 days after calving and the most anabolically active during the dry period. Strong negative correlation ($P<0.01$) was established between BALP and minerals Ca and iP. Our results suggest that biochemical bone markers can be used as precalving tool for detection of cows that are at risk of having low blood Ca and iP after calving, which could be an important novelty in detection of cows at risk of milk fever. From the results of this study we can speculate that the dry period is important for filling up Ca reserves in bone tissue.

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KAZALCI METABOLIZMA KOSTI IN KONCENTRACIJA MINERALOV V KRVI PRI KRAVAH MOLZNICAH MED PRESUŠITVIJO IN LAKTACIJO

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Povzetek: Biokemijski kazalci metabolizma kosti so snovi v krvi in/ali urinu, ki kažejo kako intenzivno se kostnina izgrajuje oz. razgrajuje v času odvzema krvnih oz. urinskih vzorcev. Namen raziskave je ugotoviti gibanje celotnega kalcija (Ca), anorganskega fosfata (aP), magnezija (Mg), celotne alkalne fosfataze (ALP), kostno specifične alkalne fosfataze (BALP) kot biokemijskega kazalca izgrajevanja kostnine in C-terminalnih prečno povezanih telopeptidov kolagena I (CTx) kot biokemijskih kazalcev razgrajevanja kostnine v krvnem serumu odraslih krav molznic v času od presušitve do viška laktacije. V raziskavo je bilo vključenih 54 klinično zdravih črno-belih krav z visoko prirejo mleka (povprečno 8,463 kg v prejšnji laktaciji). Krave so bile razdeljene v 5 skupin glede na fazo laktacije: 1. približno 1 mesec do poroda (AP) oz. sredi presušitve (n = 10), 2. 10 do 1 dan AP (n = 10), 3. 0 do 48 ur po porodu (PP) (n = 10), 4. 10 do 20 dni PP (n = 10) in 5. višek laktacije (n = 14). Vrednosti Ca in aP so bile pri kravah v 3. skupini nižje kot pri tistih v ostalih skupinah ($P<0,05$). Višje vrednosti Mg smo izmerili pri kravah v 3. in 5. skupini glede na 2. in 4. ($P<0,05$). Najnižjo vrednost BALP smo izmerili v 1. skupini. Razlika je bila statistično značilna glede na ostale skupine ($P<0,05$). Najvišje vrednosti CTx so bile izmerjene v 4. in nato v 5. skupini. Statistično značilno so se razlikovale od vrednosti v 1., 2. in 3. skupini. Rezultati raziskave kažejo, da je kostno tkivo katabolično najbolj aktivno 10 - 20 dni PP, anabolično pa med presušitvijo. Ugotovljena je bila močna negativna korelacija ($P<0,01$) med BALP in rudninama Ca in aP.

Ključne besede: biokemijski kazalci; CTx; BALP; ALP; odrasle krave