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RELATIONSHIPS AMONG MACRO-MINERALS, OTHER SELECTED SERUM MARKERS OF BONE PROFILE AND MILK COMPONENTS OF DAIRY COWS DURING LATE LACTATION*

Ryszard Mordak[1♦], Zbigniew Dobrzański[2], Robert Kupczyński[2]

[1]Department of Internal Medicine, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Pl. Grunwaldzki 47, 50-366 Wrocław, Poland
[2]Department of Environmental Hygiene and Animal Welfare, Faculty of Biology and Animal Science, Wrocław University of Environmental and Life Sciences, Chełmońskiego 38c, 51-630 Wroclaw, Poland
♦Corresponding author: ryszard.mordak@upwr.edu.pl

Abstract

Testing blood and milk parameters as well as analysing the relationships among these markers is very useful for monitoring the internal homeostasis and health in high-yielding dairy cows during various production periods. The aim of the study was to assess the correlations (relationships) among macro-minerals, such as calcium (Ca), inorganic phosphorus (P), magnesium (Mg), other selected bone profile markers, such as total protein (TP), albumin, activity of alkaline phosphatase (ALP) measured in serum and selected milk components such as number of somatic cells (SCC), colony-forming units (CFU), milk fat (MF), milk protein (MP), milk lactose (ML), solids, solids non-fat (SNF) and milk production in late-lactation cows. Both blood and milk samples were collected from 11 clinically healthy milking cows during the late-lactation period. The cows were examined once a day for 3 consecutive days resulting in 33 sets of blood and milk samples for laboratory and statistical analysis. Significant correlations were observed between: Mg and MP, Mg and SNF, ALP and SCC, TP and SCC, TP and MP, TP and SNF, albumin and MP, albumin and SNF, P and Mg, Mg and albumin, and between TP and albumin. When monitoring macro-mineral homeostasis and mammary gland health, especially in intensively fed high-yielding dairy cows correlations between these markers should be considered. The revealed correlations can allow for deeper comparative laboratory diagnostics of homeostasis and can be especially useful for laboratory monitoring of the potential risk of subclinical macro-mineral deficiency in high-yielding dairy cows.

Key words: cows, serum macro-minerals, serum proteins, serum ALP, milk components, correlations

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Background

The evaluation of haematochemical parameters is one of the most common investigations performed in clinical practice. The comparison to an established reference interval is necessary for meaningful interpretations of haematological, biochemical and hormonal variables as well as milk components. However, during particular life phases such as peripartum, and particularly lactation, female mammals have to adapt to physiological demands and adaptation, thus the comparison with established reference intervals becomes complicated (Arfuso et al., 2016; Fiore et al. 2018). Relationships between contents of biochemical metabolites in blood and milk in highly productive dairy cows are important not only during transition or early lactation period but also during other stages of milk such as mid-lactation and late lactation when the daily values of milk are also high (Nozad et al., 2012; Djokovic et al., 2019; Mordak et al., 2020).

Calcium, phosphorus and magnesium are very important macro-minerals in the body that maintain health and milk yield in cows, and should be carefully monitored (Whitaker et al., 2005; Cook et al., 2006; Kendall and Bone, 2006; Mordak and Nicpoń, 2006; Filipejová and Kováčik, 2009; LeBlanc, 2011; Kupczyński et al., 2011; Jóźwik et al., 2012; Mordak et al., 2017; Kołacz et al., 2017; Djokovic et al., 2019). Appropriate macro-mineral homeostasis ensures proper biochemical processes and normal functions of various body systems, including bone structure mobilising which accounts for about 98% calcium, 85% phosphorus, 55% magnesium of the body, as well as extracellular space and blood serum that bind about 1% of these body macro-minerals (Downey and Siegel, 2006; Datta et al., 2008). These macro-minerals are necessary for the normal functioning of nerves, muscles, heart and other organs, and are important in laboratory diagnostics especially during increased metabolism in lactating cows (Oezel, 2004; Whitaker et al., 2005; Djokovic et al., 2014).

Bone is composed of 20 to 40% organic matrix – osteoid (produced by osteoblast cells and containing organic compounds, mainly proteins called osseomucoid and osteoalbuminoid), 50 to 70% minerals, 5 to 10% water, and around 3% lipids (Clarke, 2008). In bone structure, the main minerals such as calcium, phosphorus and their inorganic compounds or ions are represented by calcium-phosphate-hydroxide also known as the hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]; however, significant amounts of magnesium and many other minerals are also present (Florencio-Silva et al., 2015). In addition, bone is a dynamic tissue that is constantly remodelled through the coordinated action of osteoblasts, osteoclasts and bone lining cells, where osteoblasts cause matrix production and mineralisation, and osteoclasts degrade this tissue (Robling et al., 2006).

Osteogenesis is controlled by local factors such as: growth factors, cytokines and prostaglandins produced by bone cells as well as by systemic factors important for maintaining bone homeostasis such as parathyroid hormone (PTH), calcitonin, 1,25-dihydroxyvitamin D_3, glucocorticoids, androgens and estrogens (Manolagas, 2000; Phan et al., 2004). However, PTH stimulators are also macro-minerals: reduced serum Ca and Mg or increased serum P, whereas PTH inhibitors may be the elevated serum calcium levels, or significantly reduced serum magnesium concen-

trations (Heron, 2018). In addition, osteoblasts synthesize collagenous proteins, non-collagenous proteins and glycosylated proteins, including alkaline phosphatase (ALP), and secrete them into the extracellular matrix (Clarke, 2008). According to the quoted author, total serum ALP consists of approximately 50–60% from bone, 30% from intestinal and 10–20% from the liver fraction, although this enzyme can also be produced in other tissues, including the mammary gland.

An imbalance in macro-minerals in dairy cows can cause specific health problems such as hypocalcaemia, hypophosphataemia, hypomagnesaemia, and also osteopathy, such as osteoporosis, osteomalacia, and epiphysiolysis, or be the cause of many disorders resulting in impaired welfare, health, reproduction and milk production, which usually create financial losses in the farm (Mee, 2004; Kendall and Bone, 2006; Mulligan et al., 2008; Bodarski et al., 2013).

The macro-minerals present in the blood have a significant influence on biochemical processes, and the performance of individual organs and tissues including the functioning of the immune system, which is essential for health and productivity, especially in high-yielding dairy cows (Goff, 2003, 2006; Kimura et al., 2006; Gray et al., 2007; Spears and Weiss, 2008). Important for human laboratory diagnostics is the knowledge that about 48% of serum calcium is ionised, 46% is bound to proteins such as albumin and immunoglobulin, and 7% is formed as complexes with anions (phosphate, citrate, sulphate, bicarbonate or other). Moreover, in the case of serum magnesium, 55% remains unbound, 30% is bound with proteins such as albumin, 15% is bound to anions such as phosphate and citrate, while in the case of serum phosphate, approximately 55% is free, 35% is bound with small cations, and 10% is protein bound (Risteli et al., 2012; Heron, 2018).

In veterinary diagnostics, the examination of serum minerals, proteins and other parameters is also important, especially in high-yielding dairy cows, to monitor their internal blood homeostasis and the relation to milk production and milk composition during the long lactation period (Kida, 2003; Mordak and Nicpoń, 2006; Nozad et al., 2011; Djokovic et al., 2014; Prado et al., 2019). It is also important to know the relationship between blood and milk parameters in dairy cows, to better assess their metabolism, and to aid in early diagnoses of subclinical metabolic health problems, that may occur at various stages of the lactation period (Patil et al., 2015; Djokovic et al., 2019).

Milk yield and composition, especially in terms of protein, lactose, fat, number of somatic cells and many other components depends on many factors such as the type of feed dose, the season, and the cow's health, including the udder, which can significantly affect the state of homeostasis of individual organs and tissues including blood (Cozzi et al., 2011; Bobbo et al., 2017; Mordak et al., 2020). Numerous blood parameters are commonly used in diagnostics, but blood proteins, including albumin, can be very important in relation to milk composition in both physiological and pathological conditions (Bondan et al., 2018). Especially albumin, which is produced in the liver and present in significant amounts in both blood serum and milk. Albumin plays an important role as a molecular transporter and carrier for low water content molecules, including fat-soluble hormones, bile salts, unconjugated bilirubin, free fatty acids, cholesterol, but also electrolytes and minerals (Prado et al., 2019). In addition, there are many nutritional (López-Alonso et al., 2016; Khachlouf

et al., 2019) and environmental factors such as heat stress (Hall et al., 2018), which affect the composition of milk in cows.

There is not much detailed literature data on the simultaneous monitoring of blood and milk markers during later lactation in dairy cows, and there are no comprehensive analyses of the correlations between these markers forming different diagnostic profiles. We hypothesize that milk yield and milk composition as well as the levels of serum macro-minerals and other selected blood markers used in the bone profile during the late lactation period in cows are associated with significant correlations, which can serve as important indicators of health and aid in the laboratory diagnostics in high-producing dairy cows.

The aim of the study was to assess the correlations (relationships) among macro-minerals, such as calcium, phosphorus, magnesium, other selected bone profile markers, such as total protein, albumin, ALP tested in serum, and selected milk components in late-lactation cows.

Material and methods

Animals and study design

The study protocol was approved by the 2nd Local Bioethics Committee in Wrocław (decision no. 24/2007). Randomly selected 11 Black-and-White Holstein Friesian cows aged 4–5 years, with a similar body condition scoring (BCS 3.5–4.0 in a five-point scale system) were included in the study. The cows were monitored in a similar later stage of lactation (mean 218 days after calving) on the same dairy farm in the autumn season. The herd of dairy cows was free of common infectious diseases and reached the average milk yield about 8500 kg per lactation. Cows were clinically healthy, but they were in a herd with typical risk for metabolic diseases and mastitis, usually present in high-yielding animals. Each of the 11 selected cows was clinically examined as well as milk and blood samples were collected once a day for three consecutive days. Thus, from 11 cows (within three days), 33 sets of blood and milk samples were obtained for analyses. All milk samples were collected during morning milking using special devices – milk meters, while blood samples were collected immediately after the morning milking at around 5:00. Blood and milk samples were delivered to two appropriate laboratories within 1 hour.

The cows were kept in the hall for 90 cows with free access to the lairs. These animals had high-quality environmental conditions (mattresses, automatic ventilation system) and nutrition (table feed – with total mixed ration and stations with electronic system for dry ration). The cows were fed using total mixed ration (TMR), supplemented with a commercial concentrate mixture for lactating cows based on daily milk yield. The cows had free access to fresh water. Fresh TMR was offered twice daily in the morning and afternoon. TMRs were formulated according to INRA system using INRAtion 4.0 software, based on chemical analyses of ingredients (Table 1). TMR samples were analyzed according to AOAC methods (AOAC, 2005). The Delaval Alpro milking system (parallel 2×4) was used for cows. They were milked twice a day at 5:00 and 15:00, and milk yield was automatically recorded.

Table 1. Detailed ingredients and nutrient composition of TMR

Item, unit	Cows in lactation
Ingredients (% of DM)	
Corn silage	34.5
Alfalfa-grass silage	24.5
High moisture corn	15.6
Sugar beet pulp	7.1
Rapeseed meal	1.9
Soybean meal	1.5
Alfalfa/grass hay	3.9
Barley straw	1.5
Complete mixture	5.20
Fodder chalk	0.15
Calcium bicarbonate	0.2
Vitamins and minerals (mixture)*	0.3
Chemical composition, unit	
NEL (MJ/kg)	6.12
Crude protein (%)	16.8
Crude fat (%)	3.80
NDF (%)	36.2
ADF (%)	22.4
calcium (%)	0.78
phosphorus (%)	0.36

*Contained: 11% Ca; 15% P; 12.5% Mg; 2% S; 2% K; 1,200 mg/kg of Fe; 100 mg/kg of I; 1,500 mg/kg of Cu; 3,300 mg/kg of Mn; 5,000 mg/kg of Zn; 36 mg/kg of Co; 22 mg/kg of Se; 550,000 IU/kg of vitamin A; 220,000 IU/kg of vitamin D; and 3,100 IU/kg of vitamin E.

Blood analysis

In cows, 10-ml polypropylene tubes (Sarstedt, Germany) were used to collect blood from the external jugular vein. Blood tests were performed in a specialized laboratory of the University of Environmental and Life Sciences in Wrocław. Concentrations of macro-minerals in the serum such as calcium (Ca), magnesium (Mg) and inorganic phosphorus (P) were measured using the Microlab 300 analyzer (ELITech Group France). Serum concentrations of total protein (TP) and albumin, as well as alkaline phosphatase (ALP) activity were measured using the Pointe 180 analyzer (Pointe Scientific, USA).

Milk analysis

Sterile 50-ml polypropylene containers (Prolab, Poland) were used to collect representative milk samples. Milk samples were taken automatically from all phases of milking. These milk samples were collected in special tanks analogous to those used in the trial milking. The collected milk samples were analysed including ba-

sic morphological and biochemical parameters of milk such as number of somatic cells (SCC), colony-forming units (CFU), milk fat (MF), milk protein (MP), milk lactose (ML), solids and solids non-fat (SNF). Biochemical analyses of milk were performed in a specialized laboratory for milk of the University of Environmental and Life Sciences in Wrocław. The SCC assay was performed on a Somacount-150 analyzer (Bentley, USA) using the fluoro-opto-electronic method. CFU determination was performed by flow cytometry on a Bactocount-70 analyzer (Bentley, USA). Determinations of the milk fat, protein, lactose, solids, and SNF fractions were done by a Milko-Scan 133 B analyzer (Foss Electric, Denmark). Milk yields were measured during the morning milkings, when blood and milk samples were also taken (at 5:00 a.m.). Milk yield as a production parameter was recorded by the electronic Delaval Alpro system. For statistical analyses, the yield value of milk was measured only during morning milking.

Statistical analysis

Statistical analysis was performed using the Statistica 10 StatSoft Inc. program. The samples were tested for normal distribution using the Shapiro-Wilk test. Linear regression was used to determine the level of linear relationships between changes in the values of the individual and Pearson's correlation coefficient (r) was calculated. Each determined coefficient was also tested for statistical significance with the Student's t-test for n-2 degrees of freedom. The number of samples was 33 and the value of the correlation coefficient defining the threshold for a statistical significance level of 5% ($P<0.05$) was $r = 0.344$. All the values of Pearson's correlation coefficients between the variables, including those indicating their statistical significance, are presented in the appropriate sets of results (1–2).

Results

The results of the linear relationships between macro-minerals, other selected markers of bone profile in blood serum and parameters of milk in tested dairy cows during late lactation period are shown in the results sets 1–2. The values of Pearson's correlation coefficients between variables exceeding the limit of significant statistical correlations are specially marked. For each assessed parameter, the normality of distribution was checked by the Shapiro-Wilk test. The average values and standard deviations for blood and milk parameters tested are presented in Table 2.

Table 2. The mean values and standard deviations of the tested samples of blood serum and milk parameters in cows

Parameter of blood or milk	Mean $\bar{x}$	SD ±	Unit	Reference values (Winnicka, 2011)
1	2	3	4	5
Serum calcium	2.23	0.25	mmol/L	2.4–3.0
Serum magnesium	1.12	0.16	mmol/L	0.78–1.23

Table 2 – contd.

1	2	3	4	5
Serum phosphorus	1.59	0.25	mmol/L	1.81–2.1
Serum alkaline phosphatase	53.54	17.85	U/L	41–116
Serum total protein	66.75	4.71	g/L	51–71
Serum albumin	29.78	2.36	g/L	32–49
Milk production (1 milking)	16.53	2.63	liters	
Milk CFU*	82.77	52. 12	× 1000/ml	
Milk SCC*	114.36	63.74	× 1000/ml	
Milk fat	4.30	1.81	%	
Milk protein	3.57	0.42	%	
Milk lactose	4.64	0.22	%	
Milk solids	13.14	1.56	%	
Milk SNF*	8.82	0.48	%	

*CFU – colony-forming units, SCC – somatic cell count, SNF – solids non-fat in milk.

Result set 1. Results of the linear relationships between serum macro-minerals and other selected serum markers of bone profile in dairy cows during late-lactation period

	Ca	P	Mg	ALP	TP	Albumin
Ca						
P	–0.203					
Mg	0.148	**0.379***				
ALP	0.020	–0.098	–0.134			
TP	0.123	–0.257	0.148	0.150		
Albumin	–0.144	–0.029	**0.530***	–0.213	**0.418***	

*significant statistical correlation at r = 0.344 (P<0.05).

Result set 2. Results of the linear relationships between macro-minerals, other selected markers of bone profile in serum and parameters of milk in dairy cows during late-lactation period

	Ca	P	Mg	ALP	TP	Albumin
Production	–0.010	–0.208	–0.320	–0.117	0.003	–0.185
CFU	–0.082	0.209	0.217	0.092	–0.323	–0.056
SCC	0.078	–0.084	–0.062	**0.421***	**0.395***	–0.033
MF	–0.116	0.258	–0.175	0.066	–0.282	–0.165
MP	–0.131	0.084	**0.487***	0.238	**0.452***	**0.449***
ML	0.066	–0.235	0.095	–0.192	–0.048	0.042
Solids	–0.165	0.286	–0.061	0.123	–0.127	–0.062
SNF	– 0.085	–0.037	**0.474***	0.150	**0.374***	**0.413***

*significant statistical correlation at r = 0.344 (P<0.05).

Calcium showed no significant correlation with any of the studied other mineral and bone markers. Significant positive correlations were observed between: P and

Mg (r=0.379), Mg and albumin (r=0.530), TP and albumin (r=0.418). There were no significant negative correlations between the tested markers.

Calcium and phosphorus showed no significant correlation with any of the studied milk parameters. The significant and positive correlations were observed between: Mg and MP (r=0.487), Mg and SNF (r=0.474), ALP and SCC (r=0.421), TP and SCC (r= 0.395), TP and MP (r=0.452), TP and SNF (r=0.374), albumin and MP (r=0.449), albumin and SNF (r=0.413). Significant negative correlations were not observed among tested parameters. The relationships and correlations between only milk parameters were published in a separate article indicated later in the text.

Discussion

In our studies, serum calcium did not show a significant correlation with any of the other macro-mineral and bone markers tested in the blood. In addition, serum calcium did not show a significant correlation with any of the tested milk components. Similarly, other authors also did not find a significant correlation between serum calcium and other milk components, such as fat or protein in cows tested in different periods of lactation (Djokovic et al., 2019). In addition, according to the cited authors, they found no correlation between serum calcium and calcium tested in milk. Similarly, as in the case of calcium, the cited authors did not find analogous correlations for magnesium and inorganic phosphorus. Moreover, other authors, who tested cows during the first week after calving (not during late-lactation) observed that most phenotypic correlations among Ca, P, Mg concentrations were positive and low (r=0.09 – r=0.16), whereas the correlation between P and Mg was negative and low at the level r=−0.16 (Tsiamadis et al., 2016). However, the positive correlations between phosphorus and magnesium in blood serum observed in our study in cows during late lactation do not correspond with the cited results obtained in cows during different lactation periods.

The significant positive correlations observed between Mg and albumin in the blood serum of our examined cows may be due to the fact that approximately 20% of the serum magnesium binds to plasma proteins, especially to albumin and globulin, but total serum magnesium is dependent on albumin content (Kaneko et al., 1997; Evans and Parsons, 1988). This may also be confirmed by a significant positive correlation between total protein and serum albumin, but at a slightly lower level than the relationship between magnesium and serum albumin content. These relationships may also apply to milk, where our results showed significant positive correlations between serum Mg and MP, and, as a consequence, significant positive correlations between serum Mg and SNF. Interestingly, in other studies performed during different lactation periods in cows, other authors recorded a significant positive correlation between blood magnesium and blood protein at r=0.36 (P<0.01), but did not record a significant correlation between serum magnesium and protein in milk (Nozad et al., 2012). However, in other studies of cows, also during various lactation periods, there were no significant correlations between magnesium and serum protein or between

magnesium and serum albumin, and also none between serum magnesium and protein in milk (Djokovic et al., 2019).

The significant and positive correlation (r=0.418) between total serum protein and albumin recorded in the results seems to be expected, especially in healthy cows, because albumin is made by the liver and has a significant contribution to total protein value – up to about 60% according to normal, reference limits for cattle (Winnicka, 2011). The significant and positive correlations between serum TP and MP (r=0.452), as well as between serum albumin and MP (r=0.449) obtained in our studies show a clear metabolic and structural relationship between blood and milk proteins. The significant and positive correlation between total serum protein and milk protein was also noted by other authors on the level r=0.23 (P<0.01), but they did not compare albumin specifically (Nozad et al., 2012). Other authors reported that they compared serum albumin with milk protein with insignificant result (r=0.107), but they tested cows in different lactation periods with different statuses (Djokovic et al., 2019). Significant positive correlations between total serum protein and milk protein as well as between serum albumin and milk protein obtained in our study resulted in positive significant correlations between TP and SNF, as well as between albumin and SNF, because solids non-fat in milk can also contain different types of these proteins.

Significant and positive correlations were observed between TP and SCC (r= 0.395). This relationship can be caused by the natural stimulation of microorganisms in the mammary gland which increases the number of leukocytes and epithelial cells in milk, and also increases the level of globulins causing a higher concentration of all proteins in the serum. Other authors also confirmed that the number of somatic cells in milk was significantly associated with the higher levels of total protein and globulin in the blood (Bobbo et al., 2017). In addition, according to the cited authors, serum proteins are significant indicators of animal health, including the risk of mastitis in cows.

The significant and positive correlation observed between serum ALP and milk SCC (r=0.421) shows a measure of the relationship between the stimulation of leukocyte proliferation, their influx into the mammary gland, as well as the level of degeneration of mammary secretory epithelial cells and enzyme activity in clinically healthy dairy cows during later lactation. Other scientific studies evaluated the concentration of milk somatic cells (SCC) and ALP activity in the milk of buffaloes (Patil et al., 2015). According to the cited authors, a higher concentration of milk SCC was accompanied by higher activity of alkaline phosphatase in milk as a consequence of cell structural damage, and therefore, ALP activity can be used along with SCC for early diagnosis of subclinical mastitis. Other authors found no significant correlation between ALP activity in blood and milk serum, which suggests that the usefulness of this enzyme in milk as a marker of early diagnosis of subclinical metabolic diseases in dairy cows, is low (Djokovic et al., 2019).

Levels of linear interrelationships between selected parameters of milk such as: CFU, SCC, MF, MP, ML, solids, SNF and milk production were also analysed in tested cows. Results of these analyses were also presented and discussed in the previous research (Mordak et al., 2020).

Conclusions

The presented diagnostic model for both blood and milk turned out to be a useful tool for detailed assessment of the internal body homeostasis in cows. Deeper diagnostics is better than a single analytical model, because it not only allows assessing the current state of health, but also its trends, the direction of potential changes, as well as the correlations of blood and milk parameters tested in a highly homogeneous group of dairy cows.

Significant positive correlations were observed between: Mg and MP, Mg and SNF, ALP and SCC, TP and SCC, TP and MP, TP and SNF, albumin and MP, albumin and SNF, P and Mg, Mg and albumin, and between TP and albumin. Significant correlations between the studied markers should be considered in practice in the context of monitoring macro-mineral homeostasis and mammary gland health in dairy cows.

These results may be particularly useful for practical diagnosing of subclinical health problems regarding an imbalance of macro-minerals having their aetiology on the axis of bone tissue, blood and mammary gland in cows.

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