Characteristics and prediction of subclinical hypocalcemia in dairy cows during the transition period using blood analytes*

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Summary

This study aimed to present the characteristics of and to predict subclinical hypocalcemia in dairy cows during the transition period using blood analytes. We examined fluctuations in plasma calcium (Ca), phosphorus (P), bone metabolic markers carboxy-terminal telopeptide of type I collagen (CTX), fibroblast growth factor (FGF23), 1,25(OH)₂D₃, parathyroid hormone, and other blood biochemical analytes from prepartum week 2 to postpartum day 14 in 116 multiparous high-producing Holstein cows from a free-stall barn dairy farm. With a plasma concentration of Ca < 2.0 mmol/L as a criterion for the diagnosis of subclinical hypocalcemia, 64 cows were classified as normocalcemic, and 52 cows as subclinically hypocalcemic. Among the 52 hypocalcemic cows, 50 were detected on postpartum days 1 or 3, and 2 on postpartum day. The subclinically hypocalcemic cows were in a state of low bone turnover in the prepartum period, with low plasma concentrations of Ca and CTX. The subclinically hypocalcemic cows showed signs of a P regulation disorder in the prepartum period. This was marked by high plasma concentrations of P and low concentrations of 1,25(OH)₂D₃ and FGF23, which is also considered to be the cause of the low bone turnover. The results of a multiple logistic regression model showed that prepartum plasma concentrations of FGF23, CTX, and Ca were ideal predictors of postpartum subclinical hypocalcemia in dairy cows, using the model equation $38.8 - 0.052 \cdot \text{FGF23} - 0.492 \cdot \text{CTX} - 10.645 \cdot \text{Ca}$, with a score of > 0 considered as an indication of increased risk of subclinical hypocalcemia after calving. The scoring rule had an accuracy of 79.3%, sensitivity of 76.9%, and specificity of 81.3%. The plasma concentrations of FGF23, CTX, and Ca were ideal predictors of postpartum subclinical hypocalcemia in dairy cows.

Keywords: dairy cow, subclinical hypocalcemia, blood analytes, FGF23, CTX

Hypocalcemia is a common metabolic disorder in which homeostatic mechanisms fail to maintain normal blood calcium (Ca) concentrations at the onset of lactation (1). As many as 50% of periparturient dairy cows may have subclinical hypocalcemia, which was defined in a prospective cohort study as blood Ca of 1.38-2.0 mmol/L on post-calving days 1 to 2 with no clinical symptoms (14, 20). Cows with hypocalcemia are at an increased risk of developing other periparturi-

ent problems, including displaced abomasum, ketosis (19), fatty liver, retained placenta, metritis, and mastitis (10). Furthermore, hypocalcemic cows have a reduced proportion of neutrophils with phagocytic activity (9) and an impaired mononuclear cell response to an antigen-activating stimulus (6). At the onset of lactation, the synthesis and secretion of colostrum leads to major losses of Ca, which are equivalent to 7-10 times the amount of Ca in blood (4, 10). To cope with this rapid loss of Ca, Ca resorption from bone is increased. The plasma concentration of carboxy-terminal telopeptide of type I collagen (CTX) is commonly used as a marker of bone resorption. Bone Ca mobilization is regulated by parathyroid hormone (PTH), which is activated by

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lower Ca concentrations to increase $1,25(\text{OH})_2\text{D}_3$ synthesis, improve urinary Ca reabsorption, and stimulate the intestine to efficiently absorb dietary Ca (3). In addition, fibroblast growth factor 23 (FGF23) is secreted by osteocytes in response to elevated $1,25(\text{OH})_2\text{D}_3$ and is responsible for phosphorus (P) and $1,25(\text{OH})_2\text{D}_3$ metabolism and subsequent impairment of Ca absorption (13). The aim of the present study is to present the characteristics of Ca homeostasis from late pregnancy to early lactation in dairy cows, to identify potential regulators of hypocalcemia, and to explore biomarkers predictive of subclinical hypocalcemia during the transition period.

Material and methods

Animal grouping and sampling. The study protocol was approved by the Ethics Committee for the Use and Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, China). The study cohort included 116 multiparous high-producing Holstein cows (with a mean of postpartum 3.10 ± 0.72 parities and a mean body condition score of 3.51 ± 0.06) from a free-stall barn dairy farm, which were fed a total mixed ration (TMR) (Tab. 1) during the transition period. Plasma total Ca was used as a diagnostic marker of subclinical hypocalcemia, which was defined as a postpartum serum Ca concentration of < 2.0 mmol/L

Tab. 1. Components and nutrient levels in the feeds

Item	Diet
Ingredient, g/kg	
Corn silage	380.32
Alfalfa	50.71
Oat grass	25.35
Chinese hay	22.82
Cotton seed	32.96
Soybean	21.30
Tableting corn	32.45
Concentrated feed	234.53
Water	199.34
DM (%)	58.26
Composition, % DM	
NDF	39.08
ADF	21.05
Starch	15.36
Crude protein	16.25
Crude fat	6.09
Gross energy (Mcal/kg)	2.54
Total carbon	43.45
Ca	1.06
P	0.45

Explanations: DM – dry matter; NDF – neutral detergent fiber; ADF – acid detergent fiber

(11). Blood samples were collected from the tail vein into heparinized tubes in the early morning before the morning feeding on days 7 and 14 prior to the expected calving date (1-2 weeks), on the day of calving (1 day), and on postpartum days 3, 7, and 14. The milk yield was obtained from herd DHIA records. Plasma biochemical analytes, evaluated with an automatic clinical analyzer (Synchron DXC800; Beckman Coulter, Inc., Brea, CA, USA), included total Ca, P, magnesium (Mg), potassium (K), glucose, non-esterified fatty acid (NEFA), β -hydroxybutyric acid (BHBA), aspartate aminotransferase (AST), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Plasma FGF23, $1,25(\text{OH})_2\text{D}_3$, PTH, and CTX concentrations were

measured with an enzyme-linked immunosorbent kit (Wes-tang Biotechnology Co., Ltd., Shanghai, China).

Statistical analysis. Plasma biochemical analytes are presented as mean $\pm$ SD. Differences between the subclinical hypocalcemia and normocalcemia groups were identified by repeated measures analysis of variance. $P < 0.05$ was considered statistically significant. To predict subclinical hypocalcemia, data for each plasma biochemical analyte 1 and 2 weeks prepartum were pooled. Each potential prepartum predictor was quantified using univariate logistic regression analysis with Wald's $P \leq 0.25$ entered into a backwards stepwise multivariate regression model. The selected potential predictors, that is, FGF23, CTX, Ca, P, LDL-C, NEFA, $1,25(\text{OH})_2\text{D}_3$, HDL-C, and Mg, were initially included in an overall “history model.” A reduced “history model” was consecutively extended to evaluate the added value of each parameter for prediction of subclinical hypocalcemia, with $P \leq 0.05$ considered significant. The capacity of each model to predict the occurrence of subclinical hypocalcemia was evaluated using the area under the receiver operating characteristic curve (ROC area) (5).

Results and discussion

Characteristics of blood analytes during the transition period. With plasma concentration of Ca < 2.0 mmol/L as the criterion for the diagnosis of subclinical hypocalcemia, 64 cows (with a mean of postpartum 3.06 ± 0.77 parities) were classified as normocalcemic, and 52 cows (with a mean of postpartum 3.15 ± 0.69 parities) as subclinically hypocalcemic. Among the 52 hypocalcemic cows, 50 were detected on postpartum days 1 or 3, and 2 on postpartum day. Plasma Ca concentrations were significantly lower in the subclinically hypocalcemic cows than they were in the normocalcemic cows (Tab. 2) from prepartum week 2 to postpartum day 7. Increase in bone Ca resorption and intestinal Ca absorption is a major homeostatic response to hypocalcemia (5). PTH is activated by lower Ca concentrations to increase $1,25(\text{OH})_2\text{D}_3$ synthesis and promote osteoclast formation, thereby increasing the amount of Ca mobilized from bone. There were no significant differences in the plasma concentration of PTH between the subclinical hypocalcemia and normocalcemia groups. Yamada et al. (17) report that serum biochemical markers of bone metabolism are believed to be more precise than the measurement of PTH because of interindividual variations in bone responsiveness to PTH. Intravenous treatment of subclinical hypocalcaemia has a positive effect without causing transient hypocalcemia (12). As a biochemical marker of bone resorption, plasma CTX was significantly higher in the normocalcemia group than in the subclinical hypocalcemia group. These dynamic changes might indicate a state of high bone turnover during the perinatal period among cows in the normocalcemia group.

In this study, plasma P concentrations were significantly higher in the subclinically hypocalcemic cows

Tab. 2. Characteristics of plasma biochemical analytes and milk yield in dairy cows during the transition period

Characteristic	Group	Transition period					
		prepartum week 2	prepartum week 1	postpartum day 1	postpartum day 3	postpartum day 7	postpartum day 14
Ca (mmol/L)	N	2.38 ± 0.09	2.39 ± 0.09	2.24 ± 0.18	2.20 ± 0.15	2.25 ± 0.16	2.31 ± 0.13
	SH	2.31 ± 0.08*	2.32 ± 0.08*	1.98 ± 0.21**	1.89 ± 0.17**	2.14 ± 0.15	2.21 ± 0.17
P (mmol/L)	N	2.15 ± 0.12	2.10 ± 0.17	1.55 ± 0.41	1.82 ± 0.52	1.56 ± 0.34	1.87 ± 0.32
	SH	2.26 ± 0.26	2.30 ± 0.24*	1.66 ± 0.59	1.68 ± 0.59	1.73 ± 0.29	1.81 ± 0.31
Mg (mmol/L)	N	0.87 ± 0.17	0.84 ± 0.20	0.87 ± 0.17	0.85 ± 0.19	0.78 ± 0.17	0.89 ± 0.24
	SH	0.95 ± 0.15	0.88 ± 0.13	0.96 ± 0.19	0.93 ± 0.13	0.78 ± 0.20	0.88 ± 0.19
K (mmol/L)	N	3.90 ± 0.22	3.98 ± 0.24	4.01 ± 0.37	4.06 ± 0.32	3.84 ± 0.33	3.99 ± 0.33
	SH	3.93 ± 0.19	3.93 ± 0.25	3.98 ± 0.26	3.97 ± 0.29	3.83 ± 0.38	4.05 ± 0.32
AST (U/L)	N	79 ± 22	79 ± 20	88 ± 21	107 ± 22	113 ± 33	115 ± 29
	SH	82 ± 29	82 ± 26	111 ± 49	139 ± 55*	108 ± 13	118 ± 40
GLU (mmol/L)	N	3.69 ± 0.36	3.72 ± 0.26	5.39 ± 2.55	3.23 ± 0.73	3.24 ± 1.08	3.18 ± 0.76
	SH	3.73 ± 0.30	3.60 ± 0.37	4.70 ± 2.23	3.45 ± 0.64	3.28 ± 1.17	3.14 ± 0.91
NEFA (mmol/L)	N	0.11 ± 0.09	0.19 ± 0.12	0.42 ± 0.39	0.63 ± 0.45	0.57 ± 0.40	0.55 ± 0.39
	SH	0.19 ± 0.13	0.19 ± 0.13	0.44 ± 0.31	0.38 ± 0.20	0.54 ± 0.27	0.44 ± 0.23
BHBA (mmol/L)	N	0.59 ± 0.21	0.48 ± 0.16	0.43 ± 0.15	0.83 ± 0.54	1.38 ± 1.13	1.67 ± 1.39
	SH	0.58 ± 0.20	0.57 ± 0.24	0.42 ± 0.15	0.83 ± 0.38	1.24 ± 0.90	1.15 ± 1.12
TC (mmol/L)	N	2.15 ± 0.40	1.92 ± 0.45	1.70 ± 0.42	1.79 ± 0.33	2.07 ± 0.50	2.73 ± 0.75
	SH	2.03 ± 0.34	1.98 ± 0.23	1.56 ± 0.29	1.67 ± 0.44	1.76 ± 0.56	2.41 ± 0.85
TG (mmol/L)	N	0.30 ± 0.05	0.29 ± 0.10	0.16 ± 0.05	0.15 ± 0.03	0.16 ± 0.03	0.17 ± 0.02
	SH	0.33 ± 0.08	0.28 ± 0.07	0.16 ± 0.05	0.13 ± 0.02	0.15 ± 0.02	0.18 ± 0.04
HDL-C (mmol/L)	N	1.28 ± 0.14	1.24 ± 0.19	1.17 ± 0.22	1.24 ± 0.26	1.46 ± 0.37	1.81 ± 0.33
	SH	1.23 ± 0.16	1.20 ± 0.24	1.00 ± 0.14*	1.13 ± 0.29	1.17 ± 0.32*	1.56 ± 0.48
LDL-C (mmol/L)	N	0.51 ± 0.16	0.42 ± 0.14	0.33 ± 0.13	0.34 ± 0.10	0.37 ± 0.08	0.48 ± 0.16
	SH	0.44 ± 0.10	0.40 ± 0.10	0.28 ± 0.08	0.30 ± 0.11	0.31 ± 0.13	0.39 ± 0.17
FGF23 (ng/L)	N	203 ± 26	196 ± 31	195 ± 35	183 ± 33	184 ± 33	172 ± 24
	SH	175 ± 20*	170 ± 24*	177 ± 34	179 ± 25	169 ± 19	175 ± 24
PTH (ng/L)	N	39.4 ± 3.93	40.6 ± 5.14	40.4 ± 6.11	39.3 ± 5.95	38.2 ± 6.50	38.0 ± 6.47
	SH	39.6 ± 3.55	38.0 ± 5.76	41.6 ± 7.33	40.6 ± 7.43	39.0 ± 6.06	38.8 ± 5.67
1,25(OH) ₂ D ₃ (ng/L)	N	91.6 ± 6.71	98.5 ± 14.1	95.5 ± 10.8	93.8 ± 13.8	94.5 ± 14.2	88.2 ± 10.8
	SH	89.1 ± 10.5	91.5 ± 13.5	88.2 ± 11.9	90.2 ± 13.3	89.8 ± 10.5	84.3 ± 9.2
CXT (nmol/L)	N	10.25 ± 1.77	10.8 ± 2.73	10.25 ± 2.73	11.16 ± 2.77	10.17 ± 2.77	9.93 ± 3.34
	SH	7.73 ± 1.29**	8.08 ± 2.17**	8.16 ± 2.07*	8.34 ± 2.19**	8.08 ± 2.11*	7.08 ± 1.21*
Milk yield (Kg)	N			9.13 ± 2.50	24.47 ± 4.97	32.21 ± 8.36	34.96 ± 6.59
	SH			10.04 ± 3.33	28.79 ± 4.98	36.59 ± 4.92	38.70 ± 4.52

Explanations: * significant differences between the subclinical hypocalcemia and normocalcemia groups, $P < 0.05$; ** significant differences between the subclinical hypocalcemia and normocalcemia groups, $P < 0.01$; N – normocalcemia group ($n = 64$); SH – subclinical hypocalcemia group ($n = 52$)

than they were in the normocalcemic cows 1 week prepartum. Lean et al. (8) also observe that the concentration of dietary P in dairy cows is closely related to the risk of developing milk fever. Similarly, Goff (2) suggests that a diet providing pregnant dairy cattle with more than 80 g of P per day may block the production of 1,25(OH)₂D₃. A low concentration of 1,25(OH)₂D₃ was detected from prepartum week 2 to postpartum day 7 in the subclinical hypocalcemia group, which was thought to be caused by high plasma P concentrations.

FGF23 release is markedly stimulated by 1,25(OH)₂D₃ and plays an important role in excretion of P by the kidneys (7). It seems that the increased plasma P concentration in the subclinically hypocalcemic cows was due to the lower plasma concentration of FGF23. Thus, high plasma P concentrations may be a major cause of the low bone turnover and 1,25(OH)₂D₃-mediated intestinal absorption of Ca.

A significantly increased AST concentration was detected in plasma samples from the subclini-

cally hypocalcemic dairy cows on postpartum day 3. According to Seifi et al. (15), the increased activity of AST might be attributed to a higher incidence of milk fever, and a variety of tissues have high AST activity in a state of hypocalcemia (e.g., striated muscle and the liver). However, in the subclinically hypocalcemic cows, AST activity was dramatically higher on postpartum day 1 than it was 1 week prepartum, while increased AST activity was detected on postpartum day 3. Thus, high AST activity on postpartum day 3 was suggested to attribute to hypocalcemia (15). Interestingly, plasma TC concentrations were low during the transition period, especially in the subclinically hypocalcemic dairy cows, and postpartum HDL-C in those cows was significantly lower than it was in the normocalcemic group. Yamamoto et al. (18) report that apolipoprotein C (apoC)-III concentrations were distinctly decreased in cows with milk fever. Transcription of the apoC-III gene is regulated by mitogen-activated protein kinase. Phosphorylation by this kinase of retinoid X receptor α , a member of the nuclear receptor family, results in the inhibition of $1,25(\text{OH})_2\text{D}_3$ -dependent signal transduction (16) in the regulation of Ca metabolism. Together, these findings suggest that, since apoC-III and apoC-related HDLs are involved in the development of milk fever, the monitoring of apoC-III concentrations during the peripartum period may be helpful in identifying cows susceptible to fatty liver-associated diseases, such as milk fever.

Prediction model of subclinical hypocalcemia in dairy cows. To predict subclinical hypocalcemia in the prepartum period, the data for each plasma biochemical analyte 1 and 2 weeks prepartum were pooled. As shown by the results of the univariate analysis of the pooled data presented in Table 3, plasma FGF23, CTX, P, Ca, NEFA, $1,25(\text{OH})_2\text{D}_3$, HDL-C, and Mg were significantly predictive of subclinical hypocalcemia (Plasma biochemical analytes with $P > 0.25$ are not shown). Then, multiple logistic regression analysis was performed for the subclinical hypocalcemia and normocalcemia groups. As show in Table 3, the overall history model had a ROC area of 0.91. Further exclusions and other combinations of predictors decreased the ROC area. The combination of FGF23, CXT, and Ca was selected as the final model for prediction of subclinical hypocalcemia in the prepartum period. The

Tab. 3. Independent predictors of subclinical hypocalcemia

Predictor	Univariate regression			Multivariate regression					
	Wals*	P	Odds Ratio	Model 1		Model 2		Model 3	
				Wals ^a	P	Wals ^a	P	Wals ^a	P
CXT	10.377	0.001	0.633	8.344	0.004	9.921	0.002	9.024	0.003
FGF23	10.609	0.001	0.958	4.701	0.030	8.994	0.003	8.799	0.003
Ca	4.554	0.033	0.001	5.080	0.024	5.608	0.018	–	–
P	4.890	0.027	24.147	1.647	0.199	–	–	–	–
LDL-C	3.247	0.072	0.024	0.510	0.475	–	–	–	–
NEFA	1.831	0.176	23.855	0.612	0.434	–	–	–	–
$1,25(\text{OH})_2\text{D}_3$	1.792	0.181	0.967	1.308	0.253	–	–	–	–
HDL-C	1.564	0.211	0.146	0.084	0.772	–	–	–	–
Mg	1.537	0.215	7.824	0.764	0.382	–	–	–	–
ROC area (95% CI)	–			0.91 (0.80-0.97)		0.90 (0.79-0.96)		0.86 (0.74-0.94)	

Explanations: * normocalcemia group (n = 128), subclinical hypocalcemia group (n = 104); ROC area – area under the receiver operating characteristic curve

model was then transformed to the following easily applicable scoring rule:

$38.8 - 0.052 * \text{FGF23} - 0.492 * \text{CTX} - 10.645 * \text{Ca}$, where a score of > 0 was considered as an indication of increased risk of subclinical hypocalcemia after calving. The prediction model has an accuracy of 79.3%, sensitivity of 76.9%, and specificity of 81.3%.

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