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RESEARCH ARTICLE

Production Performance, Metabolic Profile and Calcium-Regulating Hormones of Transition Dairy Cows with Different Blood Calcium Status after Parturition

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ABSTRACT

The transition period, from 21d before to 21d after calving, is viewed as a critical stage in reproductive cycle of dairy cows and influences their subsequent production performance, fertility and health. To test the hypothesis that subclinical hypocalcemia (SCH) may impair the metabolic adaptation to this period and relate to Ca homeostatic dysfunction, production performance, serum metabolic profile and calcium-regulating hormones of transition cows with different post-parturient blood Ca status were monitored from July to November 2018. Of 30 observed Holstein dairy cows, 8 which had serum Ca concentration ranging between 1.38 and 2.0 mmol/L at 24h after parturition were classified into the subclinical hypocalcemic group (LC). Normocalcemic cows were pair matched with LC ones on the basis of their initial BW and BCS (NC, 2.0<Ca<2.5 mmol/L, n=8). Body weight, BCS, milk yield, colostral and milk composition, Ca and immunoglobulin G during transition period were recorded. Blood samples were collected on -7, 1, 7, 14 and 21d relative to calving for determination of serum metabolites and calcium-regulating hormones. LC cows showed significantly lower milk production (P<0.05) during transition stage with no difference in colostral and milk composition. Cows in LC group also had significantly lower postpartum serum Ca, total protein, globulin and cholesterol concentrations than NC cows (P<0.05), while reverse was true for NEFA and BHBA. Moreover, perinatal serum Ca concentration revealed significantly negative correlations with NEFA and BHBA, and positive correlation with cholesterol and triglycerides (P<0.05). Despite no difference in blood PTH and calcitonin at 24h postpartum, significantly lower $1.25(OH)_2D$ was recorded in LC group (P<0.05). Our results suggest that SCH has a detrimental impact on lactation performance and post-parturient low blood Ca status is associated with energy metabolism of transition dairy cows, resulting in aggravation of lipid mobilization. These also provide evidence that suppressed $1,25(OH)_2D$ biosynthesis, instead of PTH, is involved in the endocrine pathogenesis of hypocalcemia.

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INTRODUCTION

Transition period, from 21d before to 21d after parturition, is a high-risk stage for dairy cows that undergo stress due to rapid fetal development, mammary gland remodelling, high nutritional requirement, as well as suppressed feed intake (Esposito *et al.*, 2014). A transition cow is characterized by dramatic changes in endocrine, metabolic and immune status. A poor metabolic adaptation to the periparturient period causes remarkable economic losses to dairy industry owing to increasing susceptibility to health problems, and reduction of subsequent productive and reproductive performance of dairy cows (Wankhade *et al.*, 2017).

Particularly, the substantial demand of calcium for initiation of lactation can result in dramatic blood Ca loss around parturition. Blood Ca level of a normocalcemic dairy cow in the transition period commonly ranges from 2.0 to 2.5 mmol/L (Larsen *et al.*, 2001; Blanc *et al.*, 2014). However, failure to cope with the challenge may bring about hypocalcemia, for instance, milk fever that has acute symptoms and is life-threatening to dairy cows. The incidence of subclinical hypocalcemia (SCH) with serum Ca between 1.38 and 2.0 mmol/L is about 50% in multiparous dairy herds (Reinhardt *et al.*, 2011), receiving less concern due to absence of overt clinical signs. Subclinical hypocalcaemia has also been reported to be linked with impaired function of immune cells (Martinez *et al.*, 2014) and other post-parturient diseases such as ketosis, displaced abomasum and retained placenta (Rodríguez *et al.*, 2017).

Blood Ca homeostasis in dairy cows is regulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] and Calcitonin (CT). When blood Ca drops, Ca homeostasis mechanism responds efficiently with the hormonal secretions acting on kidneys, bones and gastrointestinal tract (Oehlschlaeger *et al.*, 2014). Nevertheless, blood Ca concentration in some transition cows cannot accomplish to reach the normal range after parturition since the Ca homeostatic mechanism fails or is not initiated timely. Very few researchers have tried to compare these calcium-regulating hormones among transition cows with different blood Ca status.

Owing to negative energy balance (NEB) during the transition period, dairy cows need to mobilize body reserves in order to fulfill energy deficit. However, a large amount of lipid mobilization for energy supply, accompanying by elevated circulating non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHBA), can lead to hepatic damage, clinical diseases (e.g. ketosis, displaced abomasum and metritis) and lower production performance of dairy cows. Although hypocalcemic cows were reported to be more susceptible to lipid-related disorder ketosis (Rodríguez *et al.*, 2017), the association of SCH with postpartum metabolic profile, particularly energy metabolism is still not established. According to Jawor *et al.* (2012), feeding, drinking visits and standing time of transition cows were adversely affected by hypocalcemia. Moreover, SCH cows lost more body weight during first the 30 d postpartum, indicating a potential correlation between blood Ca and body tissue mobilization (Caixeta *et al.*, 2015). Understanding the relationship of SCH with perinatal energy metabolism may help to improve metabolic adaptability to transition period and ensure subsequent production performance. Hence, the objectives of present study were to investigate the effects of different blood Ca status following parturition on milk production, serum metabolic profile and calcium-regulating hormones of transition dairy cows.

MATERIALS AND METHODS

Animal and experimental design: This study was conducted from July to November 2018 on a dairy farm located at Chengdu city, Sichuan Province, China. All experimental procedures were approved by Institutional Animal Care and Use Committee of Sichuan Agricultural University. A total of 30 clinically healthy, pregnant Holstein dairy cows (Parity=3, BW=811.7±72.1 kg) were dried off at 50d prior to their expected parturition and observed from 28d before to 28d after calving the experimental dry cows and post-parturient ones were free-stall housed in pre- and postpartum pens, respectively. All cows had free access to water, the same pre- and post-partum diets (Table 1) offered to cows twice daily as TMR.

Blood sample from each cow was collected at 24h following parturition for serum Ca measurement via commercial kit (Jiancheng Bioengineering Institute, China), and SCH was defined on the basis of serum Ca threshold (1.38<Ca<2.0 mmol/L) (Goff, 2008). Of the 30 cows, 8 had serum Ca in SCH range, with no clinical sign of milk fever and were then assigned to low Ca group (LC). These 8 LC cows were pair matched with other 8 normocalcemic cows (2.0<Ca<2.5 mmol/L) as normal Ca group (NC) on the basis of initial BW and body condition score (BCS).

Table 1: Ingredients and chemical composition of diets during dry and fresh period (DM basis)

	Prepartum diet	Postpartum diet
Ingredients (% DM)		
Alfalfa hay	-	24.20
Beet pulp	5.91	1.17
Cottonseed	2.28	6.02
Oat grass	30.99	9.82
Corn Silage	14.77	10.24
Apple pomace	2.24	-
Sodium bicarbonate	-	0.33
Molasses	-	1.50
Fatty acid calcium	-	1.01
Commercial close-up concentrate	43.81	-
Commercial lactation concentrate	-	45.71
Nutrient level		
DM (%)	58.78	58.68
CP (%)	13.10	15.15
Ether extract (%)	2.56	3.08
NDF (%)	37.04	26.06
ADF (%)	18.93	14.14
Ash (%)	7.13	8.22
Ca (%)	0.69	1.08
P (%)	0.41	0.46
Na (%)	0.20	0.55
K (%)	1.30	1.48
Cl (%)	0.47	0.43
S (%)	0.31	0.33
DCAD (mEq/100g DM)	9.41	29.12

Calculated based upon DCAD=[(%Na/0.023+%K/0.039)-(%Cl/0.0355+%S/0.016)].

Sampling and measurements

Body weight, BCS and calving data: Body weight of each cow was measured on 28d before calving, immediately after calving and 28d after calving. At the same time, BCS was scored by 2 researchers independently and then averaged (Edmonson *et al.*, 1989). Calving difficulty scored 1 to 3 (1, no assistance needed; 2, calving needed minimum assistance; 3, complicated calving with invasive assistance). Calves were also weighed after birth and then moved to calf house.

Colostrum and milk samples: Colostrum samples were taken from experimental cows and analyzed for immunoglobulin G (IgG) using ELISA kit (R&D system) and Ca content via atomic absorption spectrophotometer (Hitachi ZA3000, Japan). Milking was conducted thrice

daily. Milk production was recorded at each milking up to 28 days in milk (DIM) and summarized by weeks. Milk samples collected from 3 consecutive milking on 28 DIM were composited in proportion to milk yield of each milking. Compositions of colostrum and milk were determined by Combi Foss FT$^+$ instrument (Foss, Denmark).

Serum analysis: Blood samples were collected from experimental cows by caudal vein puncture before morning feeding on -7d, 24h, 7, 14 and 21d relative to calving. Whole blood after clotting was centrifuged at 2000×g for 15 min (4°C), serum was separated, stored at -80°C in aliquots and then analyzed for energy metabolites [glucose, cholesterol, triglycerides and NEFA], protein metabolites [total protein, albumin, urea], using Hitachi Automatic Analyzer 3100 (Hitachi, Tokyo, Japan). Globulin was computed as total proteins-albumin. Postcalving serum BHBA was determined with commercial kit (Lengton Bioscience Co., LTD, Shanghai, China) via microplate spectrophotometer (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

For calcium-regulating hormones at 24h postpartum, ELISA kits supplied by Meimian Biotechnology Co. Ltd (China) were used for PTH, CT, 1,25(OH)$_2$D and Osteocalcin (OC) measurements with the same instrument described above. The inter-assay and intra-assay CV of these serum hormones assays were below 15% and the lowest detection limit was 10 pg/mL (for PTH), 1.0 pg/mL (for CT), 0.1 ng/mL [for 1,25(OH)$_2$D)] and 0.1 ng/mL (for OC).

Statistical analysis: PROC MIXED procedure (SAS ver. 9.4, SAS Institute Inc., Cary, NC) was performed for the data analysis. Pre- and postpartum data were analyzed separately. Data were transformed to achieve normal distribution for statistical analysis, if necessary, and results were reported as least square means. The nonparametric analysis of Kruskal-Wallis was applied to calving difficulty data. Correlations between serum Ca concentration and energy metabolites were analyzed using PROC CORR.

RESULTS

BW, BCS, calving data and milk production: Body weight and BCS at 28d before calving, after parturition and 28d after calving did not differ significantly between NC and LC groups (Table 2). LC showed a negative effect on milk yield during 28 DIM compared with NC (P=0.02, Fig. 1). Whereas, the milk fat, protein, lactose percentages, SCC, MUN, Ca, IgG contents in colostrum and milk, and calving data differed non-significantly between NC and LC groups (Table 3).

Serum Ca concentration: Serum Ca concentration decreased and reached a nadir at the onset of lactation in both groups (Fig. 2). Despite non-significant difference prepartum, LC cows showed higher reduction in serum Ca at 24h (1.64 vs. 2.11 mmol/L, P<0.01). Meanwhile, the LC group showed lower Ca throughout the fresh period compared to NC group (P<0.01).

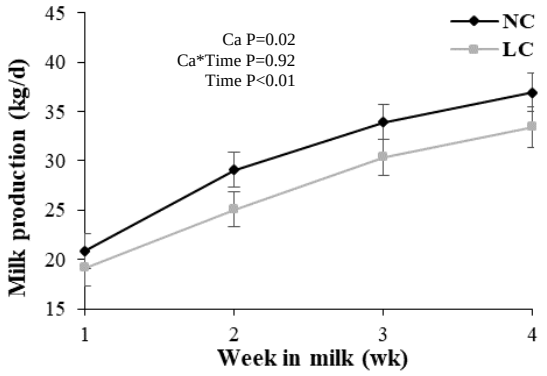


Fig. 1: Least squares means (±SE) of milk production during fresh period (1-28d) of cows with different serum Ca status at 24h after calving.

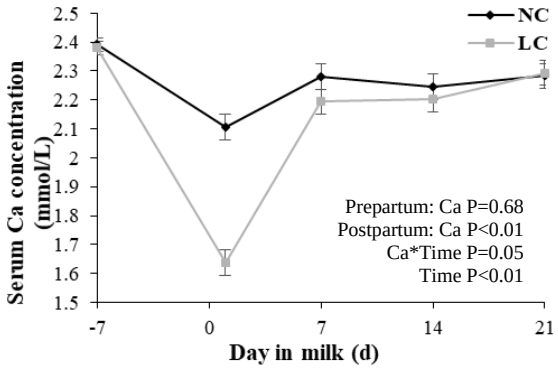


Fig. 2: Least squares means (±SE) of serum Ca concentration during transition period of cows with different serum Ca status at 24h after calving.

Table 2: BW and BCS during transition period of cows with different serum Ca concentration at 24h after calving

Items	Group		SEM	P-value
	NC	LC		
BW (kg)				
28d before calving	809.35	813.98	26.37	0.90
24h after calving	753.54	754.54	28.68	0.98
28d after calving	707.54	682.53	28.99	0.55
BW change (1-28d)	-46.00	-72.01	9.75	0.08
BCS				
28d before calving	3.88	3.94	0.22	0.85
24h after calving	3.66	3.72	0.25	0.86
28d after calving	3.25	3.13	0.19	0.64
BCS change (1-28d)	-0.41	-0.59	0.10	0.20

Serum metabolic profile and correlation analysis: Being similar before calving, serum TP and globulin were significantly higher in NC than in LC group during 21 DIM (P<0.01, P=0.01, Table 4). The low Ca status did not affect serum glucose level (Fig. 3A) but decreased post-calving cholesterol when compared with the NC group (P=0.03). Although blood triglyceride levels fluctuated during postpartum period (P<0.01), blood Ca concentration had no effect on triglycerides. On the other hand, NEFA and BHBA showed similar chronological trend (Fig. 3D & E). Significant increase in NEFA level was found in LC (P<0.01) which also had an increased BHBA in serum matched with NC (P<0.05). As can be seen from Table 5, blood Ca concentration after parturition tended to have a positive correlation with glucose (r=0.22, P=0.06), and showed significant positive correlations with triglycerides and cholesterol (r=0.30, P<0.01; r=0.57, P<0.01). Increase in NEFA and BHBA were negatively correlated with Ca levels (r=-0.49, P<0.01; r=0.26, P=0.04).

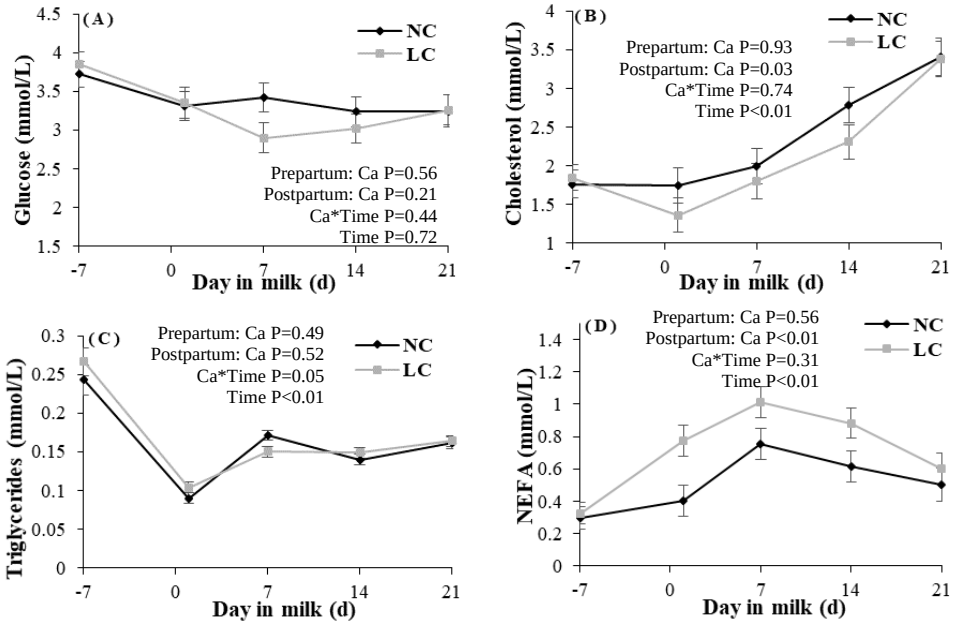
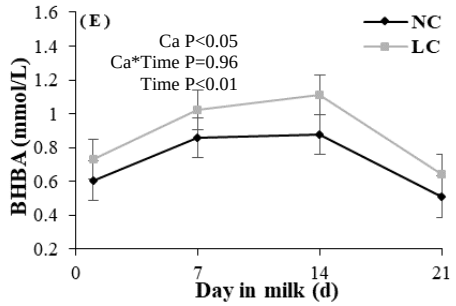


Fig. 3: Least squares means (±SE) of serum energy metabolites Glucose (A), Cholesterol (B), Triglycerides (C), NEFA (D), BHBA (E) during transition period of cows with different serum Ca status at 24h after calving. NEFA=non-esterified fatty acid, BHBA=β-hydroxybutyrate.



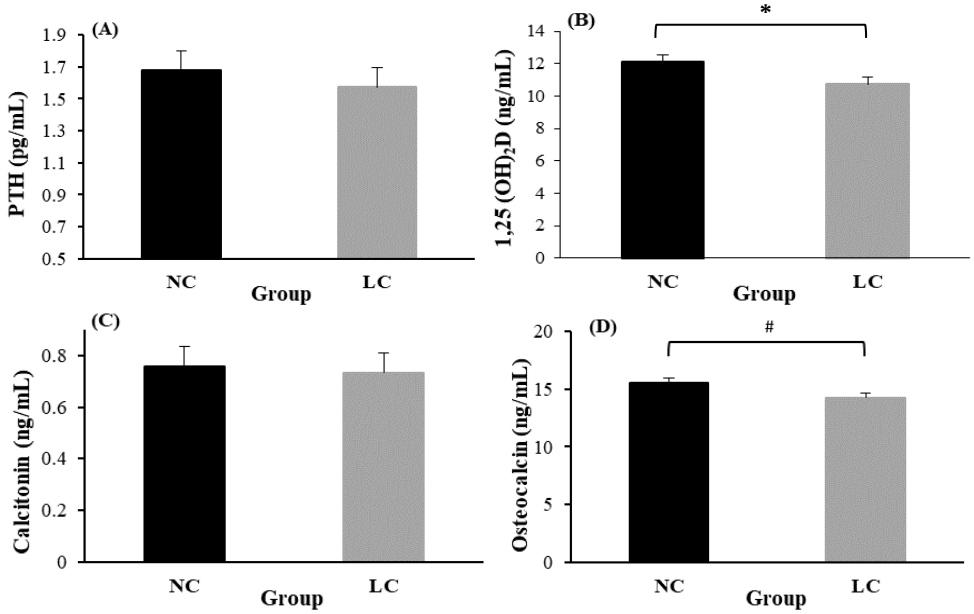


Fig. 4: Least squares means (±SE) of PTH (A), 1,25(OH)$_2$D (B), Calcitonin (C) and Osteocalcin (D) of cows with different serum Ca status at 24h after calving. * P<0.05, and # 0.05≤P<0.10.

Table 3: Milk composition and calving data of transition cows with different serum Ca status at 24h after calving

Items	Colostrum				Milk (28 DIM)			
	NC	LC	SEM	*P*-value	NC	LC	SEM	P-value
Fat (%)	6.51	6.40	0.74	0.92	3.86	3.37	0.19	0.09
Protein (%)	18.24	18.19	0.93	0.97	3.15	3.07	0.11	0.60
Lactose (%)	2.23	2.20	0.18	0.91	3.89	3.80	0.07	0.34
SCC (×1000/mL)	563.29	1953.5	673.71	0.50	405.13	476.50	189.41	0.95
MUN (mg/dL)	47.90	43.53	3.41	0.38	12.01	11.85	0.71	0.88
Ca (mg/mL)	1.92	1.87	0.03	0.25	1.15	1.15	0.014	0.79
IgG (g/L)	49.44	48.76	2.74	0.86	-	-	-	-
Calf BW (kg)	39.71	39.38	1.66	0.89	-	-	-	-
Calving difficulty	1.25	1.63	0.22	0.30	-	-	-	-

MUN=milk urea nitrogen.

Table 4: Least squares mean for serum protein metabolites during transition period of cows with different serum Ca status at 24h after calving

Items	Group		SEM	P-value		
	NC	LC		Ca	Ca* time	Time
Prepartum						
TP (g/L)	76.79	72.90	2.30	0.25	-	-
Albumin (g/L)	26.86	28.41	0.77	0.18	-	-
Globulin (g/L)	49.93	44.49	2.71	0.18	-	-
Urea (mmol/L)	4.04	4.27	0.22	0.54	-	-
Postpartum						
TP (g/L)	79.01	73.93	1.12	<0.01	0.86	<0.01
Albumin (g/L)	26.86	26.05	0.41	0.17	0.17	0.20
Globulin (g/L)	52.17	47.88	1.18	0.01	0.49	<0.01
Urea (mmol/L)	4.24	4.25	0.15	0.97	0.03	0.05

Table 5: Pearson correlation coefficients between serum Ca and glucose, triglycerides, cholesterol, NEFA and BHBA of transition dairy cows

Items	Serum Ca	
	r	P-value
Glucose	0.22	0.06
Triglycerides	0.57	<0.01
Cholesterol	0.30	<0.01
NEFA	-0.49	<0.01
BHBA	-0.26	0.04

r=correlation coefficient; NEFA=non-esterified fatty acid; BHBA=β-hydroxybutyrate.

Calcium-regulating hormones and Osteocalcin: Among the calcium-regulating hormones, PTH and Calcitonin in transition dairy cows with different blood Ca status showed non-significant difference (Fig. 4). Reduced $1,25(OH)_2D$ concentration was observed for the LC group (P=0.04), which also showed a tendency towards lower serum osteocalcin than those with normal Ca status (P=0.08).

DISCUSSION

Cows in the transition stage are prone to NEB due to lack of synchronization between milk secretion and appetite recovery. Milk production, which is closely related to energy balance, differed between cows with different Ca status in the current study. SCH cows did not produce as much milk as normocalcemic ones. It has been reported that feed intake, chewing time and rumination could be depressed by induced SCH (Hansen *et al.*, 2003), leading to less energy intake, which may explain the lower milk yield. The low Ca status at 24h after calving appeared to be a risk factor for milk yield reduction of transition dairy cows.

In the current study, colostral Ca was 1.65 times higher than that in early lactation milk. Although substantial circulating Ca mobilization into colostrum turned out to be blood Ca deficiency after delivery, normocalcemic cows were capable to cope with the Ca demand having normal blood Ca (2.11 mmol/L). SCH ones, by contrast, experienced a mean serum Ca level of 1.64 mmol/L at 24h post-calving. There is now a general consensus that 2.0 mmol/L can be the threshold of hypocalcemia (Reinhardt *et al.*, 2011). To distinguish sub- and clinical hypocalcemia, a lower bound (1.38 mmol/L) was used to differentiate SCH (Horst *et al.*, 2003), as cows with blood Ca <1.38 mmol/L frequently have severe clinical signs (e.g. postpartum paralysis). Therefore, the cut-points ranging from 1.38 to 2.0 mmol/L was chosen for identification of SCH. In addition, we observed an upswing of serum Ca in both groups at 7 DIM and Martinez *et al.* (2012) also elucidated a similar gradual rise during 7 days postpartum. This implies that the first 7 DIM is the critical period for monitoring and prevention of hypocalcemia in order to preserve production and health in the ensuing lactation.

Our results showed a decrease in serum TP and globulin of SCH dairy cows, which is supported by the previous study of Basbugan *et al.* (2015). Following blood protein mobilization for milk protein synthesis, this relationship may be explained by suppressed protein intake and digestion owing to the critical role of Ca in gastrointestinal tract. Accordingly, worse metabolic adaptation to transition period could result from the weakened blood protein turnover. The reduced blood globulin is also an indication of immunoglobulins involvement in immune response (Bionaz *et al.*, 2007).

Serum cholesterol during transition stage is associated with the occurrence of severe metritis or other diseases like retained placenta, hypocalcemia, mastitis (Sepulveda-Varas *et al.*, 2015), and its concentration reduces around parturition (Kessler *et al.*, 2014). This coincides with the time profile observed in both SCH and NC groups. Our work thereby confirmed that SCH cows had lower serum cholesterol, the latter had a positive correlation with post-parturient Ca level. Decrease in cholesterol, which was suggested to be linked with body condition loss of transition dairy cows (Kim and Suh, 2003), can be a predictor for worse NEB (Sepulveda-Varas *et al.*, 2015). Most transition cows exhibit NEB due to transition challenges that stimulate lipolysis, producing NEFA and BHBA. Increased NEFA and BHBA also occurred with SCH postpartum in the present work. Martinez *et al.* (2014) observed that cows with induced SCH exhibited higher blood NEFA level. It is noteworthy that serum Ca concentration was negatively corelated with NEFA and BHBA, suggesting a potential relationship between postcalving blood Ca status and NEB. One possible rationalization for this is limited energy intake as discussed above.

Dramatical reduction of cytosolic ionized Ca (iCa) derived from hypocalcemia has adverse effect on smooth muscle contraction of gastrointestinal tract (Goff, 2008), and poor rumen and abomasal motility ensues leading to compromised rumination, digesta passage rate and appetite (Hansen *et al.*, 2003). Another reason summarized by Chamberlin *et al.* (2013) is inadequate intracellular Ca that can inhibit mitochondrial oxidation in hepatic energy metabolism and consequently deteriorates NEB. Following that, greater lipid mobilization with heightened NEFA and BHBA production responded to the state of NEB. Besides the detrimental effects of NEFA and BHBA on immune defence shown in previous studies (Ingvartsen and Moyes, 2013), incidence of ketosis can also be increased. Further, reduction in feed intake, milk yield and greater BW loss ensue with hyperketonemia (Duffield, 2000). The associations of post-parturient Ca status with increased NEFA and BHBA lead us to understand that SCH is not limited to Ca metabolism. Rather, it has a subsequent detrimental effect on cow's productive performance, energy metabolism and health.

In the presence of calcium-sensing receptors, PTH secretion by parathyroid glands can be stimulated by fall in blood Ca. However, we did not find any difference of PTH in SCH and NC cows to explain the etiological agent, contrary to a recent report where PTH differed between low and high SCH cows (Rodríguez *et al.*, 2016).

One possible explanation for our results is that the noticeable blood Ca drop around parturition in both groups boosted PTH release in a similar manner. As reported by Goff (2004), even a slight decline in blood Ca can stimulate PTH secretion. Furthermore, one important endocrine function of PTH in Ca homeostasis is triggering 1-α-hydroxylase activity in renal proximal tubules and thus increasing $1,25(OH)_2D$ biosynthesis, which can facilitate the intestinal Ca transport and bone resorption positively (Bass and Chan, 2006). In the current work, calcium-deficient cows appeared to have lower $1,25(OH)_2D$ concentration at 24h postpartum. Similarly, Wilkens *et al.* (2012) demonstrated blood Ca rise in response to increased $1,25(OH)_2D$ concentration of transition dairy cows. Rodríguez *et al.* (2016) detected lower blood $1,25(OH)_2D$ in cows with higher degree of SCH. Our data suggest that, after parturition, cows with SCH somehow showed suppressed renal $1,25(OH)_2D$ biosynthesis which depends on PTH's activation. Goff (2008) proposed that metabolic alkalosis in transition period could alter the conformation of PTH receptors, leading to lower tissue sensitivity to PTH and failure of 1,25(OH)D hydroxylation in kidneys. The lower 1,25 $(OH)_2D$ results in impaired function of Ca replenishment from intestine and bone mobilization, which may be the cause of SCH. Additionally, SCH cows tended to have a lower osteocalcin concentration in this study, which is in agreement with Larsen *et al.* (2001), where osteocalcin was significantly correlated with blood Ca. As a biochemical marker of bone formation, osteocalcin was previously shown to decline in periparturient cows during first 5 DIM indicating arrested osteoblast activity. On the basis of our results, the inferior Ca status of SCH cows appears to exert a feedback on osteocalcin secretion from osteogenesis, and less circulating Ca will be embedded in bone accordingly.

Conclusions: Dairy cows with low blood Ca status (1.38<Ca<2.0 mmol/L) postpartum had lower milk production during first 28 DIM. Higher serum NEFA and BHBA concentrations, and their significantly negative correlations with blood Ca, suggest that SCH aggravates lipid mobilization and exerts negative influence on energy balance of transition cows. Among Ca-regulating hormones, impaired $1,25(OH)_2D$ biosynthesis around parturition is associated with failure of Ca homeostasis which explains the pathogenesis of SCH. A better understanding of how SCH and Ca homeostasis mechanism interact to impact production performance, energy metabolism and health of transition cows will facilitate the development of nutritional strategy to improve their metabolic adaptation to transition period.

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Authors contribution: XZ and ZW conceived and designed this study. XZ drafted this manuscript. MFH, AMS and QP gave critical review/revision and language proofread. XZ, XD, CT, RH and CW executed the experiment and analyzed the samples. XZ and HZ analyzed the data. BX, LW, and YJ gave suggestions on the study and manuscript.

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