Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows

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ABSTRACT

The objectives were to study the effects of induced subclinical hypocalcemia [SCH, blood ionized Ca (iCa^{2+}) <1.0 mM, without recumbency] on physiological responses and function of immune cells in dairy cows. Ten nonpregnant, nonlactating Holstein cows were blocked by lactation and assigned randomly to a normocalcemic (NC; intravenous infusion of 0.9% NaCl i.v. plus 43 g of oral Ca, as Ca sulfate and Ca chloride, at -1 and 11 h) or an induced SCH [SCH, 5% ethylene glycol tetraacetic acid (EGTA), a selective iCa^{2+} chelator, intravenous infusion] treatment for 24 h, using a crossover design. The sequence of treatments was either NC–SCH or SCH–NC, with a 6-d washout period. Ionized Ca was evaluated before, hourly during the infusion period, and at 48 and 72 h, to monitor concentrations and adjust the rate of infusion, maintaining blood $iCa^{2+} <1.0$ mM in SCH throughout the 24-h infusion period. Additional measurements included heart and respiratory rates, rectal temperature, dry matter intake, rumen contractions, whole-blood pH, concentrations of glucose and K in whole blood, concentrations of total Ca, Mg, nonesterified fatty acids, β -hydroxybutyrate, and insulin in plasma, and urinary excretion of Ca. Total and differential leukocyte count in blood was also performed. The concentration of cytosolic iCa^{2+} in neutrophils and lymphocytes was quantified and neutrophil function was assayed in vitro. Infusion of a 5% EGTA solution successfully induced SCH in all SCH cows, resulting in decreased blood iCa^{2+} concentrations throughout the 24-h treatment period (0.77 ± 0.01 vs. 1.26 ± 0.01 mM iCa^{2+}). Induction of SCH reduced dry matter intake on the day of infusion (5.3 ± 0.8 vs. 9.1 ± 0.8 kg/d) and rumen contractions (1.9 ± 0.2 vs. 2.7 ± 0.2 contractions/2 min) for the last 12 h of infusion. Cows in SCH had decreased plasma insulin concentration (1.44 ± 0.23 vs. 2.32 ± 0.23 ng/mL) evident

between 6 and 18 h after the beginning of the infusion, accompanied by increased concentrations of glucose (4.40 ± 0.04 vs. 4.17 ± 0.04 mM). Plasma nonesterified fatty acids concentration was greater for SCH than NC cows (0.110 ± 0.019 vs. 0.061 ± 0.014 mM). Neutrophils of cows in SCH had a faster decrease in cytosolic iCa^{2+} after stimulation with ionomycin (9.9 ± 1.0 vs. 13.6 ± 1.4 Fluo-4:Fura Red post-end ratio) in vitro. Furthermore, induction of SCH reduced the percentage of neutrophils undergoing phagocytosis (22.1 ± 2.1 vs. $29.3 \pm 2.1\%$) and reduced the oxidative burst response after incubation of pathogenic bacteria (16.1 ± 1.7 vs. $24.2 \pm 1.7\%$). Subclinical hypocalcemia compromised appetite, altered metabolism, and impaired function of immune cells in dairy cows.

Key words: dairy cow, immune function, metabolism, subclinical hypocalcemia

INTRODUCTION

Dairy cows have between 2 and 4 g of Ca in blood, half of which is in the ionized form (iCa^{2+}). On the first day of lactation, synthesis and secretion of colostrum impose major losses of Ca equivalent to 7 to 10 times the amount of Ca present in blood (Horst et al., 2005). To cope with this rapid Ca loss and maintain normocalcemia, homeostatic mechanisms have to be in place to increase the influx of Ca into the blood (Goff, 2008). However, the inability of the cow to fully reestablish concentrations of Ca in blood, through intestinal absorption or bone resorption, likely explain the high prevalence of subclinical hypocalcemia (SCH) in primiparous (25%) and multiparous (47%) cows (Reinhardt et al., 2011). Change in cytosolic iCa^{2+} is a component of important cellular messenger systems, and increased cytosolic iCa^{2+} concentration is required for regulation of important processes such as neurotransmission, muscle contraction, cell metabolism, cell growth, cell proliferation, and activation of immune cells, among others (Saris and Carafoli, 2005; Parekh, 2006; Vig and Kinet, 2009). Cows with milk fever are at increased risk of developing other periparturient

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problems, including dystocia and ketosis (Curtis et al., 1983), displaced abomasum (Massey et al., 1993), uterine prolapse (Risco et al., 1984), and retained placenta (Melendez et al., 2004). The increased risk of developing these conditions is likely mediated by several factors, including the effects of low iCa^{2+} suppressing smooth muscle contractions (Hansen et al., 2003). Furthermore, hypocalcemic cows have increased plasma concentrations of cortisol (Horst and Jorgensen, 1982), reduced proportion of neutrophils with phagocytic activity (Ducousin et al., 2003; Martinez et al., 2012), and impaired mononuclear cell response to an antigen-activating stimulus (Kimura et al., 2006). This reduction of immune response has linked hypocalcemia to infectious diseases of bacterial origin such as metritis (Martinez et al., 2012) and mastitis (Curtis et al., 1983).

Although the consequences of SCH have not been fully established, affected cows during the first 3 DIM had marked increases in lipid mobilization and a decreased percentage of neutrophils with phagocytic and killing activities, and were at much greater risk of developing metritis and puerperal metritis compared with normocalcemic cows (Martinez et al., 2012). In corroboration with these findings, lactating grazing cows with SCH in the first 7 DIM had increased incidence of metritis and endometritis, along with higher concentrations of NEFA and BHBA in plasma (Ribeiro et al., 2013). Descriptive data from cows induced to have SCH indicated depressed feed intake and rumination (Hansen et al., 2003). Collectively, these studies suggest that SCH is associated with immune dysfunction, exacerbated negative nutrient balance, and greater incidence of uterine diseases in early postpartum dairy cows. Despite the strong link between SCH and peripartum diseases, most studies have been observational in nature and performed during the early postpartum period, when it is difficult to isolate the effect of inadequate concentrations of Ca and iCa^{2+} in blood from the combined metabolic and endocrine disturbances that might influence immune function.

The hypotheses of the current study were that SCHI, defined as blood iCa^{2+} concentration <1.0 mM without recumbency, would reduce DMI and compromise measures of energy metabolism, and reduce cytosolic concentrations of iCa^{2+} in leukocytes, thereby depressing measures of white blood cell function in dairy cows. Therefore, the objectives were to study the effects of SCHI on physiological responses and function of immune cells in dairy cows.

MATERIALS AND METHODS

All procedures involving cows in the study were approved by the University of Florida Institutional Ani-

mal Care and Use Committee and by the University of Florida Institute of Food and Agricultural Sciences Animal Research Committee.

Cows and Housing

Ten nonpregnant, nonlactating Holstein cows from the University of Florida Dairy Unit (Gainesville) were enrolled in the study. The mean ($\pm$ SD) age, lactation number, and BW at enrollment were, respectively, 4.4 ± 0.4 yr, 2.4 ± 0.3 lactations, and 696.8 ± 29.2 kg. Selection criteria involved being healthy to a physical exam at the time of enrollment and no history of disease within the last 30 d. Cows were dried off at least 1 wk before the beginning of the study. Cows were moved to individual stalls in the Large Animal Clinic of the Veterinary Teaching Hospital at the University of Florida (Gainesville) at least 4 d before initiating the study to acclimate to the experimental facilities.

Intravenous Solutions

A sterile commercial solution containing 0.9% NaCl (Hospira Inc., Lake Forest, IL) was used. Ethylene glycol-bis (2-aminoethylether)- N,N,N',N' -tetraacetic acid (**EGTA**, cat. no. E3889, Sigma Aldrich, St. Louis, MO) 5% solution was prepared aseptically under a laminar flow hood using 900 mL of sterile water (Baxter Healthcare Corp., Deerfield, IL), 50 g of EGTA, and 50 mL of 5 M NaOH. The solution was continuously mixed using a vortex until salts were fully dissolved and pH was measured. Additional 5 M NaOH solution was added until the pH was adjusted to 7.4. The fully dissolved solution was then filtered using a 0.45- μ m bacteriological filter. The solution was then labeled and stored at 4°C until use within 10 d.

Experimental Design and Treatments

The study followed a crossover design in which the 2 treatments were applied to each cow in 2 different periods. Cohorts of cows were blocked based on lactation number and, within each block, cows were assigned randomly to 1 of 2 treatment sequences. The treatments were (control) normocalcemia (**NC**) or induced subclinical hypocalcemia (**SCHI**). Therefore, the sequence of treatments for a given cow was either NC-SCHI or SCHI-NC. Treatments were administered in sequence with a 6-d washout period between treatment administrations to minimize carryover effects.

The day of intravenous infusion was considered study d 0. On study d -1 , all cows had the jugular groove area shaved and surgically disinfected, and an intravenous indwelling catheter (14-gauge, 140 mm, Abbocath-T,

Hospira) was inserted in each jugular vein. One catheter was used for continuous infusion and the other for blood sampling. Catheters were continuously flushed with heparinized solution (30 USP units of heparin sodium/mL; Sigma Aldrich) to maintain patency and they were removed on d 3.

Intravenous infusion was performed for 24 h on study d 0, starting at 0900 h and finishing the next morning at 0900 h. Normocalcemic cows received a continuous infusion of a solution containing 0.9% NaCl, whereas SCHI cows received a continuous infusion of a solution containing 5% EGTA with an adjusted pH of 7.4. The infusion was performed using a controlled infusion pump (Heska Vet IV, Loveland, CO) and rate of infusion was maintained equally between NC and SCHI cows and determined based on blood $i\text{Ca}^{2+}$ concentration of SCHI cows. Cows in SCHI received an initial infusion rate of 500 mL/h until blood $i\text{Ca}^{2+}$ concentrations fell below 1.0 mM, after which the infusion rate was modified hourly according to $i\text{Ca}^{2+}$ concentrations. The goal was to maintain an $i\text{Ca}^{2+}$ concentration between 0.7 and 0.9 mM throughout the 24-h infusion period. Concentrations of blood $i\text{Ca}^{2+}$ were monitored hourly throughout the infusion period using CG8+ cartridges read by a cow-side handheld biochemical analyzer (i-Stat System, Abbott Laboratories, Abbott Park, IL).

Cows in the NC treatment were supplemented with one bolus containing 43 g of Ca as sulfate and chloride salts (Bovikalc, Boehringer Ingelheim Vetmedica, St. Joseph, MO) at -1 and 11 h relative to the beginning of the infusion period. Based on a pilot study, we observed that infusion of 5% EGTA decreased blood pH of cows from 7.44 to approximately 7.39 (data not shown). Therefore, the rationale for supplementing cows with a bolus containing Ca chloride and sulfate was to ensure normocalcemia and to equalize the blood pH to those receiving the 5% EGTA infusion, because of the strong anions in the salts.

Urinary Excretion of Ca and Creatinine

Urine was sampled by massaging the perineal area and waiting for a continuous stream of urine. Samples were taken immediately before the beginning of infusion and 12, 24, 48, and 72 h later. The pH was immediately measured using a portable pH meter and samples were frozen at -20°C for later analyses of concentrations of creatinine and total Ca.

Urine samples were analyzed individually for creatinine using a commercial colorimetric method (Creatinine Urinary Detection Kit, Arbor Assays, Ann Arbor, MI). Urinary Ca was measured using an atomic absorption spectrophotometer after 1:400 dilution with 0.5% lanthanum chloride. Each assay was performed as a

single run, and the intraassay coefficients of variation (CV) for creatinine and urinary Ca were 8.3 and 3.4%, respectively. Urinary creatinine has been validated as a marker to estimate daily urinary volume, assuming urinary creatinine excretion of 29 mg/kg of BW/d (Valadares et al., 1999). Total excretion of urinary Ca was calculated based on the urinary volume per day multiplied by the concentration of urinary Ca.

Feeding and Measurements of DMI

The diet offered to cows was mixed and fed every 12 h as a TMR and formulated to meet or exceed the MP and ME requirements of a 650-kg nonpregnant, nonlactating Holstein cow consuming 10 kg of DM/d (Table 1). Diets were offered at 0900 and 2100 h, and orts were measured immediately before each feeding. The DMI was calculated by adding the amounts consumed in two 12-h periods multiplied by the DM content of the diet after sequential drying of samples at 55°C and then at 105°C.

Feed was sampled daily and dried in an air circulating oven at 55°C for 48 h and moisture loss was recorded. Dried samples were ground to pass a 1-mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ). Ground samples were composited for each period and then analyzed for DM (105°C for 12 h), OM (512°C for 8 h), sequential analysis of NDF using a heat-stable α -amylase and ADF (Van Soest et al., 1991) with the Ankom Fiber Analyzer system (Ankom Technology, Macedon, NY), and N using an automated quantitative

Table 1. Ingredient composition (% of DM) and nutrient profile of ration offered to cows

Item	Value
Ingredient	
Corn silage	73
Soybean hulls	18
Soybean meal, 44% CP	9
DM, % ($\pm$ SD)	53.9 $\pm$ 0.5
Nutrient profile, DM basis ($\pm$ SD)	
NE _L , ¹ Mcal/kg	1.68 $\pm$ 0.02
OM, %	96.0 $\pm$ 0.2
Ash, %	4.0 $\pm$ 0.2
CP, %	10.6 $\pm$ 0.2
NDF, %	38.8 $\pm$ 0.6
ADF, %	24.5 $\pm$ 0.3
Ether extract, %	2.9 $\pm$ 0.1
Ca, %	0.24 $\pm$ 0.02
P, %	0.24 $\pm$ 0.01
Mg, %	0.17 $\pm$ 0.01
K, %	1.04 $\pm$ 0.02
Na, %	0.02 $\pm$ 0.01
Cl, %	0.16 $\pm$ 0.01
S, %	0.11 $\pm$ 0.01
DCAD, mEq/kg	160.4 $\pm$ 4.6

¹Calculated at 1.8 $\times$ maintenance (12 kg/d) and based on the chemical analysis of dietary ingredients (NRC, 2001).

combustion digestion method (Leco 628, Leco Corp., St. Joseph, MO). The energy density of the diet was estimated using analyzed feed values and calculated at $1.8 \times$ maintenance intake (Table 1; NRC, 2001). Minerals were analyzed by inductively coupled plasma mass spectrometry in a commercial laboratory (Dairyland Laboratory, Arcadia, WI).

Physiological Responses

Heart and respiration rates and rumen contractions were evaluated by auscultation using a stethoscope (Littmann Classic II, 3M, St. Paul, MN) at -48 , -24 , 0 , 6 , 12 , 18 , 24 , 30 , 36 , 48 , 60 , and 72 h relative to the beginning of infusion. Vaginal temperature was measured every 15 min using a data logger (Temperature Logger iButton, Maxim Integrated, San Jose, CA) attached to a blank intravaginal insert. Data on vaginal temperature were then averaged hourly from -48 to 72 h relative to the beginning of infusion. The average vaginal temperature between -48 and 0 h was considered the baseline for each cow.

Concentrations of Minerals, Metabolites, and Hormones, and Blood pH

Blood was sampled in a 2-mL syringe rinsed with 1,000 USP units/mL of sodium heparin at -24 and -0.5 h, and then at hourly intervals from 1 to 24 h relative to infusion, and then at 26, 30, 36, 48, and 72 h, for measurements of iCa^{2+} , K, glucose, and pH using an i-Stat analyzer (Abbott Laboratories). At each sampling point, an additional blood sample was collected in heparinized evacuated tubes (Vacutainer system, Becton Dickinson Co., Franklin Lakes, NJ), placed in ice, and then centrifuged within 2 h at $2,000 \times g$ for 15 min for plasma separation. Plasma was frozen at $-20^{\circ}C$ until analyses, and plasma samples were analyzed for concentrations of total Ca, Mg, BHBA, NEFA, and insulin. Total Ca and Mg were analyzed using an atomic absorption spectrophotometer (AAAnalyst 200, Perkin-Elmer Inc., Waltham, MA) with the flame technique and acetylene gas as described previously (Martinez et al., 2012). Intra- and interassay CV were, respectively, 1.9 and 5.1% for Ca, and 0.9 and 2.6% for Mg. Commercial kits were used for analyses of plasma concentrations of NEFA (NEFA-C kit; Wako Diagnostics Inc., Richmond, VA; as modified by Johnson and Peters, 1993) and BHBA (Wako Autokit 3-HB; Wako Diagnostics Inc.). Intra- and interassay CV were, respectively, 4.7 and 10.6% for NEFA, and 2.6 and 7.0% for BHBA. Insulin was analyzed using a commercial ELISA kit for bovine insulin (Mercodia Bovine Insulin

ELISA, Mercodia Inc., Uppsala, Sweden). Intra- and interassay CV were 3.3 and 8.7%.

Measurements of Cytosolic iCa^{2+} in Neutrophils and Lymphocytes

Cytosolic concentrations of iCa^{2+} of isolated leukocytes were measured in vitro before and after stimulation with ionomycin at 0, 24, 48, and 72 h after the beginning of infusion with a high-sensitivity, low-noise assay. Changes in iCa^{2+} concentration were recorded using a combined cellular loading with 2 nonratiometric Ca probes: Fura Red (Fura Red, AM, cell permeant, Life Technologies, Grand Island, NY) and Fluo-4 (Fluo-4, AM, cell permeant, Life Technologies), according to a protocol adapted from June et al. (2001). The ratio between Fluo-4 and Fura Red measures cytosolic iCa^{2+} and is more accurate than either probe by itself, because it controls for differences in cell size and cell loading (June et al., 2001).

For each sample, 2 extracellular media containing Hanks' balanced salt solution (HBSS) were used, one with iCa^{2+} concentration equivalent to that of normal cows (1.26 mM iCa^{2+}) and one equivalent to cows with SCH (0.80 mM iCa^{2+}), to evaluate differences according to expected extracellular iCa^{2+} concentrations in NC and SCH cows. Briefly, peripheral blood mononuclear cells (PBMC) and neutrophils were isolated by gradient density centrifugation using 6 mL of blood carefully overlaying 8 mL of Ficoll-Paque Premium 1077 (GE Healthcare, St. Giles, UK) and 8 mL of Histopaque 1119 (Sigma-Aldrich Co., St. Louis, MO), with the highest gradient at the bottom of the 50-mL conical tubes. After 30 min of centrifugation at $2,000 \times g$ and $21^{\circ}C$, the entire granulocyte layer and 0.3 mL of the PBMC layer were separately transferred into 2 empty 50-mL vials, and each tube containing both cell types was then assigned to an HBSS medium containing 0.80 or 1.26 mM iCa^{2+} for the remainder of the assay. The tubes were washed with the respective medium and centrifuged at $1,400 \times g$ for 10 min at $21^{\circ}C$. Supernatant was discarded and erythrocytes in the pellet were lysed using 20 mL of sterile distilled water, mixing gently for 20 s, after which 5 mL of $5 \times$ PBS was added to restore isotonicity. Samples were centrifuged at $400 \times g$ for 5 min, the supernatant discarded, the lysis and centrifugation process repeated, and the pellet resuspended in 1 mL of the respective designated HBSS medium. The concentration of leukocytes was determined using a Coulter counter (Z Series Coulter counter, Beckman Coulter Inc., Brea, CA), samples were diluted to a final concentration of 4×10^6 cells/mL, and 1 mL of each sample was transferred into 5-mL polystyrene tubes.

Five microliters of Fluo-4 and 12.5 μL of Fura Red were added to 4×10^6 cells, samples were protected from light, and incubated at 37°C for 30 min. Samples were then centrifuged at $180 \times g$ for 6 min, and resuspended in their respective HBSS iCa^{2+} medium, incubated in the dark at room temperature for 15 min, and then incubated at 37°C for 12 min before flow cytometric analysis. An Accuri C6 digital analyzer flow cytometer (Accuri C6, Becton Dickinson Immunocytometry Systems, San Jose, CA) was used for data acquisition. Fluo-4 and Fura Red emission were both excited using a 488-nm blue laser. Neutrophils and lymphocytes were then identified using forward and side scatter. The ratio of Fluo-4 to Fura Red in both cell populations was recorded for 1 min to obtain the prestimulation cytosolic iCa^{2+} ratio, and then for 10 min following the addition of the ionophore ionomycin at 1 mM concentration (Calbiochem, San Diego, CA) in the HBSS medium. The ionophore activates Ca channels in the endoplasmic reticulum, increasing cytosolic iCa^{2+} concentrations, and inducing a secondary sustained store operated iCa^{2+} entry. Kinetic data from acquisition of a least 300,000 cells detected in 11 min per sample were processed using FacsKin software (version 0.6.5, FacsKin, Budapest, Hungary; Kaposi et al., 2012). The prestimulation, post-maximum, and post-end ratios, as well as the area under the curve, were recorded for neutrophils and lymphocytes, using the same extracellular iCa^{2+} concentration that the cow had at the moment the sample was taken.

Neutrophil Phagocytosis and Oxidative Burst

The percentage of neutrophils exhibiting phagocytosis and oxidative burst activities after *Escherichia coli* challenge was measured in vitro at 0, 24, 48, and 72 h after the beginning of infusion, according to procedures described in detail by Martinez et al. (2012). Briefly, whole blood was analyzed for total and differential cell counts using an automated hematology analyzer (ProCyte Dx Hematology Analyzer, Idexx Laboratories, Westbrook, ME). Neutrophils were loaded with dihydrorhodamine 123 (Sigma-Aldrich Co.) and then cocultured with propidium iodide-labeled *E. coli* isolated from milk from a cow with mastitis at a bacteria:neutrophil ratio of 40:1. The samples were run within 2 h of preparation using an Accuri C6 digital analyzer flow cytometer. Neutrophils were identified according to their size and granularity using forward and side scatter detectors, and data from acquisition of a least 10,000 cells per sample were analyzed using Flowjo software (version 7.6.5., Tree Star Inc., Ashland, OR). The parameters quantified were the percentage of neutrophils containing phagocytized propidium iodide-

labeled *E. coli*, the percentage of neutrophils with oxidative burst activity, the mean fluorescence intensity (MFI) for phagocytosis as a proxy for the number of bacteria phagocytized per neutrophil, and the MFI for oxidative burst, as an indicator for the amount of oxygen reactive species generated per neutrophil.

Statistical Analysis

Data were analyzed using the GLIMMIX procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) for a crossover design. Distributions of the residuals of continuous data were evaluated for normality using graphical diagnostics, and data transformation was performed when appropriate. In the latter case, least squares means were back transformed and the standard errors of the means calculated. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the *F*-tests in the mixed models. Baseline measurements were calculated using the average of 1 or 2 d preceding the initiation of treatments, depending upon the response, and baseline measurements were included as covariate for the respective analysis.

For responses that required hourly measurements during the infusion period, 2 different analyses were performed to utilize the proper covariance structure according to sampling spacing, one during the 24-h infusion period, including hourly measurements, and another including measurements taken immediately after the infusion period and thereafter until 72 h. The covariance structure that results in the lowest Akaike's information criterion was selected for each model.

Responses with repeated measures within the same cow for a given period were analyzed with models that included the covariate value, the fixed effect of treatment (NC, SCHI), block (1 to 5), period (1 and 2) nested within block, hour of measurement, and the interaction between treatment and hour. Random effects included cow nested within block and cow nested within treatment to account for the repeated measurements. Model fitting was evaluated using the fit statistics. When the *F*-test for an interaction was significant, means were then partitioned using the SLICE command in SAS. Treatment differences with $P \leq 0.05$ were considered significant, whereas tendencies for differences were reported if $0.05 < P \leq 0.10$.

RESULTS

Induction of Subclinical Hypocalcemia

The rate of solution infused, adjusted hourly according to blood iCa^{2+} concentrations in SCHI cows and matched for the NC cow in the same block, averaged

8.57 ± 0.41 for the 24-h infusion period, and ranged from 150 to 600 mL/h for individual cows (357 ± 7.2 mL/h). Infusion of EGTA or saline solutions never stopped during the 24-h treatment period. As anticipated, the rate of infusion was exactly the same for both treatments. The amounts of solution infused resulted on an average of 1.13 mol of EGTA administered to SCHI cows (range = 0.74 to 1.39 mol/cow).

All cows had blood $i\text{Ca}^{2+} > 1.10$ mM before treatments were applied. As anticipated, infusion of 5% EGTA solution successfully induced SCH in SCHI

cows (Figure 1a). All SCHI cows had blood $i\text{Ca}^{2+} < 1$ mM within 2 h of infusion, and it remained < 1 mM in all cows for the next 24 h as all cows had blood $i\text{Ca}^{2+} < 1$ mM in the 2 h after cessation of infusion. The concentration of $i\text{Ca}^{2+}$ in blood remained lower ($P < 0.001$) for SCHI than NC cows during the infusion period (0.77 ± 0.01 vs. 1.26 ± 0.01 mM $i\text{Ca}^{2+}$) and only increased above 1.10 mM 6 h after the end of infusion, at h 30 in the study. Similarly, total Ca concentration in plasma decreased in SCHI cows (1.74 ± 0.03 vs. 2.07 ± 0.03 mM Ca) during the infusion period (Figure 1b),

Figure 1. Calcium concentrations of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of 2 measures taken in the 24 h preceding infusion. (A) Concentration of ionized Ca ($i\text{Ca}^{2+}$) in whole blood. The mean concentrations of $i\text{Ca}^{2+}$ in whole blood for the 72-h experimental period were 1.26 ± 0.01 and 0.77 ± 0.01 mM for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P < 0.001$), hour ($P < 0.001$), and interaction between treatment and hour ($P < 0.001$). (B) Concentration of total Ca in plasma. The mean concentrations of total Ca in plasma for the 72-h experimental period were 2.05 ± 0.03 and 1.94 ± 0.03 mM for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P < 0.001$), hour ($P < 0.001$), and interaction between treatment and hour ($P < 0.001$).

Figure 2. Urinary Ca excretion of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of d -2 and -1 relative to infusion. The mean daily urinary Ca excretions between d 0 and 2 were 3.9 ± 0.9 and 10.8 ± 0.8 g/d for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P < 0.001$), day ($P < 0.001$), and interaction between treatment and day ($P < 0.001$). *Within a day, treatments differ ($P < 0.001$).

Figure 3. Dry matter intake of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of d -2 and -1 relative to infusion. The mean DMI between d 0 and 2 were 10.3 ± 0.6 and 9.0 ± 0.6 kg/d for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P = 0.15$), day ($P < 0.001$), and interaction between treatment and day ($P = 0.02$). *Within a day, treatments differ ($P < 0.01$).

but at a slower rate compared with blood $i\text{Ca}^{2+}$. In addition, the percentage of $i\text{Ca}^{2+}$ from total Ca decreased ($P < 0.001$) in SCHI compared with NC cows during the 24-h infusion period (44.2 ± 0.7 vs. $61.0 \pm 0.7\%$ of total Ca).

Urinary Excretion of Ca

Daily urinary volume was calculated based on the constant creatinine excretion of 29 mg/kg of BW (Valadares et al., 1999). We observed no difference ($P = 0.33$) between SCHI and NC cows for the amount of urine produced (14.6 ± 1.4 vs. 12.6 ± 1.3 L/d) during the first 72 h of the study, and both groups had increased ($P < 0.001$) urinary output on the day of infusion (18.2 ± 1.8 L/d) compared with the day before infusion (10.1 ± 1.5 L/d). Urinary excretion of Ca increased in both treatments on the day of infusion and was greater ($P < 0.001$) for SCHI compared with NC cows (30.2 ± 1.3 vs. 9.4 ± 1.3 g/d) on that day (Figure 2). Therefore, induction of SCH with 5% EGTA chelated approximately 30 g of Ca on the day of the infusion, based on the difference between urinary excretion of Ca on the day of infusion compared with that observed on the day preceding infusion, which averaged 0.21 ± 0.04 g/d. This amount of Ca chelated and excreted is similar to that secreted in colostrum when cows initiate lactation (Horst et al., 2005).

DMI and Physiological Responses

The DMI of cows decreased ($P < 0.001$) on the day of infusion and was lower ($P < 0.01$) for SCHI compared with NC cows (5.3 ± 0.8 vs. 9.1 ± 0.8 kg/d; Figure 3). Cows in both treatments recovered their DMI on the day following treatment to an amount comparable

to the pretreatment intakes. Induction of subclinical hypocalcemia reduced ($P = 0.01$) the number of rumen contractions (1.9 ± 0.2 vs. 2.7 ± 0.2 contractions/2 min) during the second half of the infusion period.

Respiration and heart rates and vaginal temperature were not affected by treatment (Table 2). Cows in both treatments had a decrease in urine pH, with a nadir at 24 h after beginning of infusion. However, urine pH was less ($P = 0.001$) for cows with SCHI at 12 h of the infusion period ($\text{pH } 6.2 \pm 0.3$ vs. $\text{pH } 7.6 \pm 0.3$) compared with NC cows, after which no differences were detected.

Blood pH, Minerals, Metabolites, and Hormones

Blood pH declined ($P < 0.01$) in both treatments from an average of 7.428 ± 0.008 at 0 h to 7.391 ± 0.007 within 1 h of infusion. For the first 13 h of infusion, blood pH was not different between treatments and averaged 7.387 ± 0.007 . However, cows with NC had a further decrease ($P = 0.04$) in pH in the last 11 h of infusion and remained lower until 72 h in the study, averaging 7.377 ± 0.004 and 7.392 ± 0.004 for NC and SCHI, respectively. The further decline in pH for NC coincided with the administration of the second bolus of Ca at h 11. Blood pH returned to baseline values in both treatments within 48 h after the end of the infusions. Similar to blood pH, blood HCO_3^- did not differ between NC and SCHI before treatments (27.2 ± 0.6 mM), but decreased ($P < 0.01$) within 1 h of infusion in both treatments. During the 24-h infusion, blood HCO_3^- averaged 22.0 ± 0.7 and 22.2 ± 0.7 mM for NC and SCHI, respectively. Bicarbonate concentrations quickly returned to baseline within the first 24 h after the end of the infusion.

Table 2. Effect of inducing subclinical hypocalcemia on physiological measures of dairy cows in the first 72 h after treatment

Item	Treatment ¹		P-value ²		
	NC	SCHI	Trt	Hour	Trt × Hour
Respiration rate, no./min	43.7 ± 1.1	42.5 ± 1.1	0.38	0.20	0.96
Heart rate, no./min	68.7 ± 1.1	68.8 ± 1.1	0.98	0.02	0.87
Vaginal temperature, °C	38.9 ± 0.1	38.8 ± 0.1	0.70	<0.01	0.74

¹NC = normocalcemia (i.v. infusion of 0.9% NaCl 24 h plus 43 g of oral Ca, as Ca sulfate and Ca chloride, at -1 and 11 h); SCHI = induced subclinical hypocalcemia (i.v. infusion of 5% ethylene glycol tetraacetic acid solution for 24 h to maintain blood iCa^{2+} <1.0 mM, without recumbency).

²Trt = effect of treatment; Trt × hour = interaction between Trt and hour after the beginning of infusion.

Concentrations of K in blood were less ($P < 0.001$) for SCHI compared with NC cows during the 24-h infusion period (2.91 ± 0.04 vs. 3.48 ± 0.04 mM) but returned to baseline within the first 24 h after the end of the infusion. Concentrations of Mg in plasma increased ($P < 0.001$) during infusion in SCHI cows compared with NC cows and averaged 0.93 ± 0.01 and 0.67 ± 0.01 mM, respectively.

Interestingly, cows with SCHI had greater ($P < 0.01$) blood glucose concentrations than NC cows throughout the infusion period (4.40 ± 0.04 vs. 4.17 ± 0.04 mM; Figure 4). In addition, cows subjected to SCHI had reduced ($P < 0.01$) concentrations of insulin in plasma between 6 and 18 h after the beginning of the infusion (1.44 ± 0.23 vs. 2.32 ± 0.23 ng/mL; Figure 5). Concurrently, plasma concentrations of NEFA increased ($P < 0.05$) compared with NC cows (0.110 ± 0.02 vs. 0.061 ± 0.01 mM; Figure 6). Finally, concentrations of BHBA in plasma did not differ ($P = 0.56$) between SCHI and NC cows and averaged 0.374 ± 0.13 and 0.357 ± 0.13 mM, respectively.

Complete Blood Cell Count and Neutrophil Function

The number of erythrocytes, platelets, and total leukocytes per milliliter of blood did not differ between SCHI and NC cows. Similarly, the differential leukocyte count was not affected by treatment, and SCHI and NC had similar numbers of lymphocytes and neutrophils. However, SCHI cows had a tendency for increased number of monocytes per milliliter of blood (Table 3).

Neutrophil function was suppressed by SCHI. Cows induced to develop SCHI had a reduced ($P = 0.01$) percentage of neutrophils with phagocytic activity (22.1 ± 2.1 vs. $29.3 \pm 2.1\%$; Figure 7a), with similar ($P = 0.46$) numbers of bacteria phagocytized per neutrophil between SCHI and NC cows ($78,842 \pm 1,927$ vs. $80,975 \pm 1,979$ MFI, respectively). Furthermore, SCHI cows had a reduction ($P < 0.01$) in the percentage of neutrophils with oxidative burst activity (16.1 ± 1.7 vs. $24.2 \pm 1.7\%$; Figure 7b), with lower ($P < 0.01$) production of reactive oxygen species per neutrophil ($54,778 \pm 2,403$ vs. $65,552 \pm 2,237$ MFI) compared with NC

Figure 4. Blood glucose concentrations of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of 2 measurements taken in the 24 h preceding infusion. The mean concentrations of whole-blood glucose during the 24-h infusion were 4.17 ± 0.04 and 4.40 ± 0.04 mM for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P = 0.01$), hour ($P = 0.11$), and interaction between treatment and hour ($P = 0.25$).

Figure 5. Plasma insulin concentrations of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of 2 measurements taken in the 24 h preceding infusion. The mean values for plasma insulin for the 72-h experimental period were 2.18 ± 0.17 and 1.66 ± 0.17 ng/mL for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P = 0.06$), hour ($P = 0.05$), and interaction between treatment and hour ($P < 0.05$). *Within an hour, treatments differ ($P = 0.02$).

cows. In fact, relative to the baseline values at h 0, both phagocytosis and oxidative burst declined ($P < 0.05$) in SCHI ($73.7 \pm 18.2\%$ and $69.4 \pm 20.4\%$ for phagocytosis and oxidative burst), whereas both increased in NC cows (127.8 ± 18.2 and $160.6 \pm 20.4\%$ for phagocytosis and oxidative burst) at 72 h after the beginning of the infusion.

Cytosolic Measurements of iCa^{2+} in Neutrophils and Lymphocytes

For neutrophils, SCHI and NC cows had similar ($P > 0.30$) amounts of cytosolic iCa^{2+} before stimulation (2.0 ± 0.2 vs. 2.2 ± 0.2 Fluo-4:Fura Red prestimulation ratio) and at the peak (16.6 ± 1.4 vs. 18.8 ± 1.4 Fluo-

Figure 6. Plasma NEFA concentrations of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Base = mean of 2 measurements taken in the 24 h preceding infusion. The mean concentrations of plasma NEFA during the 24-h infusion were 0.061 ± 0.01 and 0.110 ± 0.02 mM for NC and SCHI, respectively. Statistical analysis revealed effects of treatment ($P = 0.02$), hour ($P < 0.74$), and interaction between treatment and hour ($P = 0.11$).

Table 3. Effect of inducing subclinical hypocalcemia on counts of erythrocytes, leukocytes, and platelets in blood of dairy cows in the first 72 h after treatment

Item	Treatment ¹		P-value ²		
	NC	SCHI	Trt	Hour	Trt × Hour
Erythrocytes, 10 ⁶ /μL	6.09 ± 0.23	6.04 ± 0.23	0.61	0.61	0.53
Platelets, 10 ³ /μL	286.1 ± 20.4	284.0 ± 20.4	0.95	0.03	0.97
Leukocytes, 10 ³ /μL	15.34 ± 0.68	14.85 ± 0.68	0.58	0.02	0.80
Lymphocytes, 10 ³ /μL	10.48 ± 0.40	10.06 ± 0.40	0.31	0.09	0.46
Neutrophils, 10 ³ /μL	3.16 ± 0.34	2.52 ± 0.34	0.20	0.08	0.92
Monocytes, 10 ³ /μL	1.21 ± 0.17	1.73 ± 0.17	0.08	0.89	0.33

¹NC = normocalcemia (i.v. infusion of 0.9% NaCl 24 h plus 43 g of oral Ca, as Ca sulfate and Ca chloride, at -1 and 11 h); SCHI = induced subclinical hypocalcemia (i.v. infusion of 5% ethylene glycol tetracetic acid solution for 24 h to maintain blood iCa²⁺ <1.0 mM, without recumbency).

²Trt = effect of treatment; Trt × hour = interaction between Trt and hour after the beginning of infusion.

4:Fura Red post-maximum ratio) after stimulation with ionomycin (Figure 8). However, cows with SCHI had less ($P = 0.05$) cytosolic iCa²⁺ during the post-end phase of the assay (9.9 ± 1.0 vs. 13.6 ± 1.4 Fluo-4:Fura Red post-end ratio), which typically characterizes entry of iCa²⁺ from the extracellular space. Indeed, the SCHI cows tended ($P = 0.07$) to have a faster decline in cytosolic concentrations of iCa²⁺ after the post-maximum ratio based on the difference between measurements taken at the post-maximum and post-end phases of the assay compared with NC cows (-4.7 ± 0.5 vs. -3.3 ± 0.5 Fluo-4:Fura Red ratio decline). As a result of less cytosolic iCa²⁺, the area under the curve for all phases of the assay tended ($P = 0.08$) to be smaller in SCHI compared with NC cows ($5,382 \pm 910$ vs. $7,013 \pm 702$ relative intensity units/s).

The amount of cytosolic iCa²⁺ in lymphocytes did not differ ($P > 0.26$) between SCHI and NC cows before (0.23 ± 0.1 vs. 0.39 ± 0.1 Fluo-4:Fura Red pre-stimulation ratio), at the peak concentration (25.3 ± 1.6 vs. 27.4 ± 1.7 Fluo-4:Fura Red post-maximum ratio), and at the end of the measurement period (20.8 ± 1.7 vs. 22.8 ± 1.7 Fluo-4:Fura Red post-end ratio). Similarly, no differences were found in the area under the curve ($11,166 \pm 734$ vs. $12,520 \pm 855$ relative intensity units/s) between SCHI and NC cows.

DISCUSSION

Continuous infusion of a solution containing 5% EGTA successfully induced SCH in all SCHI cows within 2 h, which compromised DMI and rumen contractions, altered insulin concentrations, increased lipolysis, and decreased cellular replenishment of cytosolic iCa²⁺ in neutrophils, thereby suppressing phagocytosis and bacterial killing.

The onset of lactation is accompanied by a state of negative nutrient balance and immune dysfunction, and the severity and duration of these conditions render

cows susceptible to infectious diseases and metabolic disorders (Sordillo and Raphael, 2013). Subclinical hypocalcemia has been associated with disturbances in measures of energy metabolism and reduced immune function (Larsen et al., 2001; Martinez et al., 2012), which has been linked to increased risk of peripartum

Figure 7. Neutrophil function of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). Baseline refers to phagocytosis at hour -0.5, before treatments were applied. (A) Phagocytosis of *Escherichia coli*; mean phagocytosis in the 72 h following treatments were 29.3 ± 2.1 and $22.1 \pm 2.1\%$ for NC and SCHI cows, respectively. Statistical analysis revealed effects of treatment ($P = 0.01$), hour ($P = 0.17$), and interaction between treatment and hour ($P = 0.58$). (B) Oxidative burst; mean oxidative burst in the 72 h following treatments were 24.2 ± 1.7 and $16.1 \pm 1.7\%$ for NC and SCHI cows, respectively. Statistical analysis revealed effects of treatment ($P < 0.01$), hour ($P = 0.23$), and interaction between treatment and hour ($P = 0.25$).

Figure 8. Neutrophil cytosolic ionized Ca (iCa^{2+}) of cows subjected to normocalcemia (NC) or induced subclinical hypocalcemia (SCHI). For the prestimulation stage, effects of treatment ($P = 0.54$), hour ($P = 0.07$), and interaction between treatment and hour ($P = 0.84$). For the post-maximum ratio after ionophore stimulation, statistical analysis revealed effects of treatment ($P = 0.30$), hour ($P = 0.07$), and interaction between treatment and hour ($P = 0.92$). For the post-end ratio, statistical analysis revealed effects of treatment ($P = 0.05$), hour ($P = 0.06$), and interaction between treatment and hour ($P = 0.71$). *Treatments differ ($P < 0.05$).

diseases (Martinez et al., 2012). Because of the high prevalence of SCH in the first week of lactation (Reinhardt et al., 2011) and the high incidence of peripartum problems in dairy cows, it is important to document and understand the effects of subnormal concentrations of iCa^{2+} on energy metabolism and immune function. These findings support some of the results obtained in a previous epidemiological study in early postpartum dairy cows (Martinez et al., 2012) and corroborate the idea of a direct mechanism by which SCH further depresses energy balance and innate immune function in early postpartum dairy cows.

The rationale for using nonpregnant, nonlactating dairy cows as a model to induce SCH was to isolate effects inherently related to SCHI, therefore avoiding the typical hormonal and metabolic adaptations that occur with parturition and the onset of lactation in dairy cows. Nevertheless, the amount of iCa^{2+} chelated by EGTA, based on increased urinary excretion during the day of infusion, was equivalent to the amount of Ca secreted in colostrum on the day of calving in dairy cows (Horst et al., 2005). This indicates that the model utilized in SCHI cows resulted in an average daily loss of iCa^{2+} similar to that of early postpartum dairy cows after the first milking postpartum. The amount of EGTA infused per cows averaged 1.13 mol, which is sufficient to chelate approximately 45 g of iCa^{2+} , based on the chelating ability of a 1:1 molar ratio of EGTA and iCa^{2+} . Therefore, the average amount of Ca excreted in the urine of SCHI cows on the day of infusion, although high (30.2 g), represented only

67.1% of the potential chelating ability of the EGTA administered.

The first hypothesis of the study was that cows with SCHI would have decreased DMI compared with NC cows. Infusion of either saline or EGTA solutions reduced DMI, but the magnitude of decline doubled in SCHI compared with controls. Induction of SCH suppressed DMI when blood iCa^{2+} was low, but appetite quickly recovered as concentrations of iCa^{2+} were reestablished in the subsequent days. Subclinical hypocalcemia reduces rumen contractions, thereby affecting rumination and particulate matter passage rate, which might influence rumen fill. In lactating and dry cows, diets with excessive content of NDF are known to suppress appetite by increasing rumen fill because of a reduced rate of digestion (Allen, 2000). However, given the relatively low dietary NDF of the diet fed to dry cows, it is unlikely that rumen fill was a major factor for the reduced intake. Others have observed a reduction in chewing activity and smooth muscle contractility of rumen and abomasum in cows with induced subclinical or clinical hypocalcemia (Daniel, 1983; Hansen et al., 2003). Perhaps an overall reduction in digestive tract motility, which likely affected the forestomachs but also the abomasum and intestine, reduced appetite independently of pressure sensors typically activated during distension of the gut (Allen, 2000).

The second hypothesis of the study was that cows with SCHI would have increased NEFA and BHBA compared with NC cows. The animals used in the present study were nonpregnant, nonlactating dairy cows and, even though these cows were not under negative caloric balance at the time of SCH induction, SCHI cows exhibited an increased concentration of NEFA in plasma compared with NC cows. This finding agrees with results of previous studies in early lactating dairy cows in which the typical elevation in plasma concentrations of NEFA and BHBA in blood were exacerbated in cows diagnosed with SCH (Martinez et al., 2012; Ribeiro et al., 2013). However, the results of the present study with dry cows did not indicate an effect of SCHI on concentrations of BHBA in plasma. The latter is likely related to the relatively low baseline (0.100 ± 0.02 mM) NEFA concentrations in SCHI cows, and because BHBA typically increases when hepatic uptake of NEFA exceeds the energy requirements of the hepatocytes (Fukao et al., 2004). It is possible that even after the concentrations of NEFA in plasma of SCHI increased by 50% at the end of the EGTA infusion period (0.148 ± 0.03 mM), the amounts of NEFA taken up by the liver and oxidized were still below the concentrations necessary to overwhelm hepatocytes and stimulate an increased production of ketone bodies

by diverting fatty acid carbon from complete oxidation to ketogenesis.

Interestingly, cows with SCHI had an immediate and constant elevation in whole-blood glucose concentrations compared with NC throughout the infusion day, concurrently with a decreased insulin concentrations in plasma. An increase in blood glucose can be attributed to a decrease in insulin production with a corresponding decrease in circulating insulin (Hayirli, 2006). Spontaneous hypocalcemia is known to impair insulin release in dairy cows with milk fever (Littledike et al., 1968). In addition, experiments with different animal species and in vitro with human β cells demonstrated that the pancreatic cells require iCa^{2+} influx into the cytosol to release insulin granules and to respond to insulin after glucose tolerance tests (Witzel and Littledike, 1973; Rorsman et al., 2012). This could explain the lower concentrations of insulin in plasma observed in SCHI compared with NC cows. Collectively, these results suggest that SCHI impaired pancreatic insulin secretion, which in turn increased glucose concentrations in blood by reducing peripheral tissue glucose uptake. The increase in glucose concentration observed in SCHI cows is consistent with a study that evaluated chemical profiles in cows within 12 h of calving, finding a negative correlation between blood glucose and total blood Ca (Larsen et al., 2001). Another consequence of decreased insulin secretion is the removal of the inhibition of hormone sensitive lipase, which promotes lipolysis, thus increasing lipid mobilization and, ultimately, NEFA concentrations in blood (Fukao et al., 2004). Such changes in plasma insulin likely explain the elevation in plasma NEFA observed in the present study. These results have important implications for early lactation cows that typically develop insulin resistance (Hayirli, 2006). Further impairment in insulin and glucose metabolism might increase lipolytic signals and promote additional tissue catabolism, thus predisposing cows to lipid-related disorders. Indeed, hypocalcemia has been linked to increased risk of ketosis in dairy cows (Curtis et al., 1983).

The main hypothesis of the study was that immune cells of SCHI cows would have less cytosolic iCa^{2+} available for activation and, as a consequence, function would be affected. The association between decreased neutrophil function and SCH (Martinez et al., 2012) or clinical hypocalcemia (Ducusin et al., 2003) has been reported before. Cows with milk fever have been shown to have reduced cytosolic concentrations of iCa^{2+} in PBMC, which has been suggested to impair immune function (Kimura et al., 2006). The theory underlying the hypothesis is that upon engagement of immune cell antigen receptor with an antigen, a signaling cascade is activated, resulting in an increase in cytosolic iCa^{2+} and

depletion of iCa^{2+} stores from the endoplasmic reticulum (Brécard and Tschirhart, 2008). The depletion of endoplasmic reticulum iCa^{2+} stores is soon followed by activation of store-operated iCa^{2+} entry, which induces an additional influx of iCa^{2+} from the extracellular space, generating a transient peak (Brécard and Tschirhart, 2008). The final replenishment of iCa^{2+} is regulated by activation of the sarcoplasmic and endoplasmic reticulum iCa^{2+} ATPase pumps, which allows for iCa^{2+} entry from the cytosol into the endoplasmic reticulum (Burgos et al., 2011). Lack of adequate concentrations of extracellular iCa^{2+} are expected to decrease the entry of iCa^{2+} through store-operated iCa^{2+} channels, which would compromise cell organelle replenishment of Ca concentrations. Additionally, not only is the iCa^{2+} increase required for the initiation of multiple immune cell functions, but also the dynamics of magnitude, amplitude, and duration of changes in cytosolic iCa^{2+} control the destiny of the immune cells (Quah and Parish, 2012).

In this study, after inducing activation of neutrophils, the levels of cytosolic iCa^{2+} declined faster in SCHI cows than in NC cows. Therefore, the results indicate that the influx of extracellular iCa^{2+} was unable to sustain the use of cytosolic iCa^{2+} , or to replenish the endoplasmic reticulum stores, or both. An increase in cytosolic iCa^{2+} is required to trigger adhesion to endothelial cells, migration, phagocytosis, and killing in neutrophils (Cohen, 1994). Therefore, it is plausible that the observed reduction in cytosolic iCa^{2+} replenishment explains the reduced phagocytic and killing activities of neutrophil in SCHI cows. The negative effects on neutrophil function persisted for 72 h after the end of infusion with EGTA, even though the extracellular iCa^{2+} concentrations returned to baseline at 30 h in the study. It is known that the mature bovine neutrophil is stored in the bone marrow until released into the circulation, where it remains an average of 9 h before mobilizing into organs to undergo apoptosis (Paape et al., 2003). Hence, these results suggest that SCHI cows had not only mature circulating neutrophils affected, but also those stored in the bone marrow or undergoing final maturation.

In contrast to the findings in neutrophils, no statistical differences were detected for the concentrations of cytosolic iCa^{2+} in lymphocytes between SCHI and NC cows at different stages of induced activation, although they were numerically less for SCHI. These results disagree from those of others (Kimura et al., 2006), who observed a decline in PBMC cytosolic iCa^{2+} in cows with milk fever. Recently calved cows with milk fever are expected to have much less circulating iCa^{2+} than dry cows with SCHI, which likely explains the differences between the findings of the current study

and those of Kimura et al. (2006). In addition, it is possible that adaptations during onset of lactation in dairy cows that are not present in dry cows further suppress cytosolic stores of iCa^{2+} or the ability of leukocytes to uptake extracellular iCa^{2+} . Also, the maximum ratio of cytosolic iCa^{2+} in lymphocytes was achieved at 470 ± 34 s, relatively near the end of the 660-s measurement interval in the current study, in contrast with the time observed for neutrophils (191 ± 11 s). Therefore, the possibility exists that the 660-s measurement interval might not have been sufficient to evaluate differences in the dynamics of cytosolic iCa^{2+} concentrations in lymphocytes.

The present study demonstrated the direct link between SCH and DMI, glucose metabolism, lipid mobilization, and innate immune function in dairy cows. These findings corroborate epidemiologic studies that connect early lactation Ca metabolism and risk of peripartum diseases, particularly those that affect the reproductive tract (Martinez et al., 2012). The ramification of these results includes the need to maintain proper blood Ca concentrations to minimize excessive tissue catabolism in early lactation.

The immediate increases in concentrations of plasma NEFA and glucose concurrent with decreased insulin indicate that insulin release by the pancreas was compromised in SCH cows. Therefore, it is plausible that early lactation cows that undergo SCH face additional challenges to balance glucose metabolism and tissue catabolism. When extrapolated to early lactation cows, which typically have decreased DMI, negative energy balance, and immune dysfunction, development of SCH would further contribute to the severity of these conditions. The application of the current model to recently calved cows warrants further investigation to determine the role of SCH on metabolism and immune function of postpartum cows when undergoing adaptation to lactation.

CONCLUSIONS

Infusion of a solution of 5% EGTA successfully induced SCH in dairy cows by chelating iCa^{2+} in blood and increasing the urinary excretion of Ca. Cows subjected to SCH excreted an amount of Ca in urine similar to that observed in colostrum during the first day postpartum. Cows with SCH had reduced DMI and rumen contractions and decreased concentrations of insulin in plasma, likely by compromised pancreatic release of the hormone, which explains the increased concentrations of blood glucose and lipid mobilization based on elevation in plasma NEFA concentrations. Cows induced to develop SCH had a faster decline of cytosolic iCa^{2+} in neutrophils after ionophore stimulation,

which likely explains the compromised phagocytic and oxidative burst activities of these cells against pathogenic bacteria. Collectively, our results document the negative effects of SCH on DMI, energy metabolism, and function of innate immune cells in nonpregnant, nonlactating dairy cows and serve as a model to study the role of Ca metabolism on increased susceptibility to peripartum diseases. Increased risk of diseases observed in cows that develop SCH in early lactation might, in part, be caused by suppressed function of immune cells mediated by reduced cytosolic iCa^{2+} concentrations.

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