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The effect of prepartum negative dietary cation-anion difference and serum calcium concentration on blood neutrophil function in the transition period of healthy dairy cows

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ABSTRACT

Our objectives were to assess the effects of a diet with a negative dietary cation-anion difference (DCAD) before calving on phagocytosis (Pc) and oxidative burst (OB) function of circulating neutrophils, and to determine the associations of serum ionized (iCa) and total calcium (tCa) concentrations with Pc and OB in transition dairy cows. We hypothesized that multiparous cows fed a negative DCAD diet prepartum would have greater iCa and tCa, and thus improved Pc and OB. From 3 wk before expected parturition until calving, 38 healthy multiparous cows from 3 farms were assigned to negative DCAD treatment (TRT; −100 mEq/kg of diet dry matter; n = 21) or a control (CON; 95 mEq/kg of dry matter; n = 17) diet. Each farm was on one treatment or the other at a time, but all farms contributed cows to both groups. Urine pH was measured weekly and in TRT was 6.1 ± 0.8 with 80% of 50 samples <7 and 74% ≤ 6.5. Phagocytosis, OB, iCa, and tCa were measured at d −7, 1, and 4 relative to calving. Median fluorescence intensity for Pc (MFIP) and OB (MFIOB), and the shift of percentage of cells active for Pc (PPc) and OB (POB) were measured in isolated, stimulated neutrophils via flow cytometry. Outcomes were assessed with mixed linear regression models accounting for repeated measures. There were no differences between treatments in the 4 neutrophil function outcomes. Although MFIOB varied over time, there were no interactions of treatment with time for any outcome. Serum ionized and tCa did not differ between TRT and CON. The least squares means ± standard deviation for iCa were: d −7, 1.23 ± 0.12 vs. 1.21 ± 0.12; d 1, 1.07 ± 0.12 vs. 1.02 ± 0.12; d 4, 1.16 ± 0.12 vs. 1.17 ± 0.12 mmol/L for TRT and CON, respectively; and for tCa: d −7 2.39 ± 0.25 vs 2.44 ± 0.31; d 1, 2.01 ± 0.25 vs 1.97 ± 0.31; d 4, 2.33 ± 0.25 vs 2.32 ± 0.31 mmol/L, respectively. The proportion of blood samples with tCa <2.15mmol/L at d −7, 1 and 4 was 5, 76, and 13%, respectively, with no differences between TRT and CON. Correlations of iCa or tCa with each of the 4 polymorphonuclear leukocyte (PMN) function outcomes were weak (r < |0.3|). We did not observe the hypothesized differences in aspects of innate immunity in clinically healthy multiparous cows fed a negative DCAD. We underline that cows that experienced clinical disease were excluded from this study, which is important for interpretation of the results.

Key words: hypocalcemia, immune function, transition cow, nutrition

INTRODUCTION

At the onset of lactation, calcium homeostasis is disrupted by a massive redirection of calcium for colostrum production (Goff et al., 2000), which triggers a marked decrease in the blood calcium concentration (Goff et al., 2002; Megahed et al., 2018) that can take up to 4 d to recover (Megahed et al., 2018). Subclinical hypocalcemia (**SCH**) is defined as a blood calcium concentration below a threshold associated with increased risk of an undesirable outcome, but without visible signs. It affects around 25% of primiparous and more than 50% of multiparous cows (Reinhardt et al., 2011; Caixeta et al., 2015; Venjakob et al., 2017).

In animals, calcium exists as ionized calcium (**iCa**) or bound to proteins or anions (Oetzel, 1988). Ionized calcium represents 50 to 60% of the total blood calcium (**tCa**) and is frequently referred to as the biologically active form because it is the fraction capable of intracellular access. Calcium status is assessed through the measurement of iCa or tCa (the sum of its 3 forms). Because measuring iCa is costly and requires extra care in handling due to sensitivity to pH (Baird, 2011;

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McArt and Neves, 2017), tCa is more commonly assessed (McArt and Neves, 2017). However, the distribution of iCa and tCa varies around parturition, and iCa is the more reliable measure of Ca concentration and its relationship with neutrophil function (Leno et al., 2017a; Overton et al., 2017).

Calcium is crucial for neutrophil function in inflammation and response to infectious disease (Lundqvist-Gustafsson et al., 2000; Martinez et al., 2012). Activated neutrophils can eliminate bacteria through killing mechanisms including phagocytosis (**Pc**) and intracellular degradation, or extracellular degranulation and traps. During Pc, pathogens are encapsulated and internalized in a phagosome, leading to neutralization and digestion (Kolaczkowska and Kubes, 2013). The phagocytic process itself does not depend on calcium (Lundqvist-Gustafsson et al., 2000), but neutrophil activation depends on the influx of calcium from the extracellular space, and only the ionized portion of serum calcium is capable of crossing Ca^{2+} channels present in the plasma membrane (Lundqvist-Gustafsson et al., 2000; Clemens and Lowell, 2015). Intracellular calcium concentration depends mainly on the increased flux of iCa from the extracellular space (Mailhot et al., 2000), but iCa is also obtained from intracellular stores (Lundqvist-Gustafsson et al., 2000; Ayub and Hallett, 2004). Reduced intracellular calcium stores lead to impaired mononuclear cell function, contributing to peripartum immunosuppression (Kimura et al., 2006). Following Pc, phagosomes may be disposed of by reactive oxygen molecules (e.g., superoxide, hydrogen peroxide, and hypochlorite) generated in the cytosol as part of the neutrophils' bactericidal activity. Oxidative burst (**OB**) testing is an indicator of oxygen-dependent killing capacity, which is impaired after calving (Kehrli et al., 1989). Reduced OB has been associated with uterine disease (Hammon et al., 1996; Martinez et al., 2012). Martinez et al. (2014) concluded that neutrophils from cows with induced SCH (iCa <1.0 mmol/L, without recumbency) had lower cytosolic iCa concentrations, reduced Pc, and impaired OB response. However, Miltenburg et al. (2018a) found no improvement in PMN, OB, or Pc function with administration of parenteral calcium after calving.

Negative DCAD diets fed during the prepartum period reduce the risk of clinical hypocalcemia (milk fever) and SCH (Charbonneau et al., 2006). Although the physiology behind this preventive strategy is not completely understood, the induced, compensated, mild metabolic acidosis increased postpartum blood calcium concentrations (Leno et al., 2017b; Rodney et al., 2018), reduced risk of disease, and improved lactation performance in multiparous cows (Lean et al., 2019; Santos et al., 2019). Multiparous cows fed acidogenic diets prepartum (DCAD of −130 mEq/kg of DM) tended to have improved Pc and OB (Martinez et al., 2018).

Thus, our objectives were to assess the effects of a negative DCAD on Pc and OB function of circulating neutrophils and to assess the associations of iCa and tCa with Pc and OB. We hypothesized that multiparous cows fed a negative DCAD prepartum would have greater iCa and tCa, and thus improved Pc and OB.

MATERIALS AND METHODS

This work is reported using the REFLECT Guideline (Sargeant et al., 2010). The experimental protocol was reviewed and approved by the University of Guelph Animal Care Committee.

Experimental Design and Treatment Groups

This experiment used a subset of healthy animals from an ongoing pen-level randomized controlled trial conducted on 4 commercial freestall dairy farms in Ontario, Canada. The present data were collected between July and October 2018. A convenience herd selection was made based upon geographic location, herd size, and willingness to comply with the experimental protocol. Data from each farm's computerized records (Dairy Comp 305; Valley Ag Software, Tulare, CA) were collected weekly.

Cows were enrolled 3 wk before the expected calving date and started on the assigned treatment. Expected calving date was defined 280 d after the last breeding date. From 3 wk before calving to the day of calving, a feed supplement (anionic supplement or placebo) was included in the TMR of the close-up pen group. The treatments consisted of a diet formulated with a negative DCAD (**TRT**; Soychlor, Landus Cooperative, Ames, IA; −100 mEq/kg of DM, weighted average of the 3 farms; Table 1) or a control (**CON**) diet using a placebo supplement nutritionally similar to TRT but with a positive DCAD (+95 mEq/kg DM, weighted average). The DCAD level of the CON group was set similar to the DCAD in the herd's diet before the study. The supplement was delivered in moisture-barrier bags labeled A or B (the only noticeable package difference) to ensure farm staff were blind to the treatment. The sample size calculation was based on detecting the effect of the DCAD using 80% power and 95% confidence. The primary outcome of neutrophil OB function required at least 13 cows per group based on detection of 10% points (SD = 9) difference between treatments for the proportion of cells responding to stimulation with phorbol myristate acetate (**PMA**). Cows were excluded if they received treatment with any calcium

Table 1. Ingredient composition and nutrient profile of prepartum diets for each farm, control (CON) and treatment (TRT) groups

	Farm A		Farm B		Farm C	
Item	CON	TRT	CON	TRT	CON	TRT
Ingredients (% of DM)						
Corn silage	22.3	23.2	39.5	44.0	43.5	46.4
Straw	50.2	48.8	27.9	31.9	25.2	23.0
Soybean meal, 48%	—	—	10.6	13.2	9.4	9.5
Protein blend[1]	19.4	19.4	—	—		
Corn grain	—	—	—	—	3.0	2.9
Canola meal, solvent extracted	—	—	—	—	9.4	9.5
Haylage	—	—	8.5	—		
Prepartum mineral[2]	2.8	—	4.2	3.5	2.5	2.6
Dry cow study supplement[3]	5.4	—	9.4	—	7.0	—
Acidogenic supplement[4]	—	8.6	—	7.5	—	6.1
Nutrients, DM basis						
DM (%)	56.9	54.2	43.7	45.1	54.9	56.9
CP (%)	14.8	14.8	14.1	14.0	15.0	15.0
ADF (%)	33.8	33.4	29.7	28.0	27.1	24.5
NDF (%)	48.1	46.5	41.7	44.3	43.6	41.8
NFC (%)	18.5	19.2	31.3	30.2	31.7	32.7
Ca (%)	1.1	1.1	1.7	1.6	0.7	1.3
P (%)	0.3	0.3	0.2	0.3	0.4	0.4
K (%)	1.0	1.0	1.3	1.3	1.0	1.0
Mg (%)	0.5	0.5	0.5	0.4	0.5	0.5
Na (%)	0.1	0.1	0.1	0.1	0.1	0.1
Cl (%)	0.3	0.9	0.3	1.1	0.3	0.8
S (%)	0.2	0.3	0.3	0.3	0.3	0.3
NE_L (Mcal/kg)	1.4	1.4	1.3	1.1	1.5	1.5
DCAD[5] (mEq/kg)	102	−100	135	−103	48	−112

[1]Contained corn dry distillers (430 kg/t), Hi-Pro soybean meal (370 kg/t; Trouw Nutrition Canada, St. Marys, ON, Canada), SoyPlus (200 kg/t; Dairy Nutrition Plus, Landus Cooperative, Ames, IA).

[2]Farm A, Propulsion Dry Care 1612 Premix medicated with Rumensin (Trouw Nutrition Canada); Farm B, Eco Lac Plus Premix Monensin (Grand Valley Fortifiers, Cambridge, ON, Canada); Farm C, contained calcium carbonate, calcium iodate, cobalt sulfate, copper sulfate, ruminant protein supplement, magnesium oxide, manganous oxide, mineral oil, monocalcium phosphate, ferrous sulfate, salt, sodium bicarbonate, sodium selenite, vitamin A, vitamin D, vitamin E, wheat middlings, zinc oxide, monensin sodium.

[3]Contained corn distillers grain (300 kg/t), soyhulls (330 kg/t), wheat shorts (120 kg/t), canola meal (110 kg/t), calcium carbonate (100 kg/t), magnesium oxide (40 kg/t).

[4]SoyChlor (Dairy Nutrition Plus, Landus Cooperative, Ames, IA).

[5]DCAD calculated as mEq of $[(Na^+ + K^+) - (Cl^- + S^{-2})]$/kg of DM.

supplement, showed signs of clinical disease during the study period, or did not receive the treatment for ≥2 weeks before calving.

Cows, Housing, Management

A convenience sub-sample of 44 dry Holstein dairy cows in late gestation (in second lactation or greater) from 3 farms (A, B, and C) were selected in 2 periods of approximately 3 wk each (1 in July and 1 in October 2018) from cows enrolled on a randomized controlled trial on the effect of a negative DCAD on clinical health disorders, with treatments administered at the pen level with replication within each farm. Between study periods, farms switched between TRT and CON. Within the 2 periods of the present study, all available healthy multiparous cows from each farm were included. Dry cows were housed in a straw pack with a feed rail and moved after calving (farm A) or were housed in a sand-bedded freestall pen and moved to a straw pack pen for calving (farms B and C). After calving, cows were moved into a freestall milking pen and milked twice daily in a herringbone parlor (farm A) or in automatic milking systems (farms B and C).

The close-up group diet was delivered daily (between 0800 and 0900 h). One TMR sample was collected from each farm for each TRT and CON diet. The nutrient content of the close-up TMR were analyzed by a commercial laboratory (Agri-Food Laboratories, Guelph, ON, Canada). Apart from the close-up DCAD level between treatments, the pre and postpartum TMR were similar across all farms. Descriptions of the pre and postpartum diets, ingredients, and chemical analyses are presented in Tables 1 and 2, respectively. The target feed intake for close-up cows was 14, 13, and 13 kg/cow per day for farm A, B, and C, respectively.

Prepartum BCS and Urine pH

At enrollment, BCS was assessed on a 1 to 5 scale, measured in 0.25-point increments (Ferguson et al., 1994). Urine pH was monitored weekly from enrollment to calving. Perineal stimulation was performed and mid-stream urine samples were collected into a 20-mL plastic collection vial to measure pH with a portable meter (Laquatwin, Horiba, Piscataway, NJ). The target range for TRT was pH 6.0 to 6.5. If the average pH was out of the range, adjustments of the amount of the supplement included in the TMR were made, but that did not occur during collection of these data.

Urine pH assessment and BCS were performed by the same research assistant, who was not blind to the treatment given the results of monitoring urine pH, but did not participate in measuring neutrophil function.

Table 2. Ingredient composition and nutrient profile of postpartum diets for each farm

Item	Farm A	Farm B	Farm C
Ingredients (% of DM)			
Corn silage	44.2	38.1	33.5
Haylage	18.5	36.9	25.0
High-moisture ear corn, 70%	—	6.3	—
Canola meal, solvent extracted	—	—	4.5
Soybean meal, solvent extracted	—	4.3	4.5
Protein blend[1]	16.2	—	—
Corn grain	7.1	—	5.9
Straw	—	2.2	1.4
Whole cottonseed	6.4	—	3.7
Dry hay	4.2	—	—
Brewers grains, wet	—	—	5.5
By-pass fat[2]	0.9	1.0	—
Dairy supplement[3] (Robot)	—	8.5	14.5
Dairy premix[4]	2.6	2.8	2.4
Nutrients, DM basis			
DM (%)	45.8	49.8	51.6
CP (%)	17.3	16.1	17.4
ADF (%)	18.2	22.4	21.0
NDF (%)	24.4	30.9	34.4
NDF from forage (%)	21.0	27.7	25.7
NFC (%)	33.0	39.6	36.9
Ca (%)	0.7	1.0	0.8
P (%)	0.4	0.3	0.4
K (%)	1.3	1.7	1.4
Mg (%)	0.3	0.4	0.3
Na (%)	0.4	0.6	0.4
Cl (%)	0.4	0.6	0.5
S (%)	0.2	0.2	0.3
NE_L (Mcal/kg of DM)	1.7	1.7	1.7
DCAD[5] (mEq/kg)	258	355	232

[1]Contained corn dry distillers (430 kg/t), Hi-Pro soybean meal (370 kg/t; Trouw Nutrition Canada, St. Marys, ON, Canada), SoyPlus (200 kg/t; Dairy Nutrition Plus, Landus Cooperative, Ames, IA).

[2]Farm A, Energizer RP10 (Scothorn Nutrition, Hortonville, NS, Canada); Farm B, APF Plus, Palm dairy fat (Trouw Nutrition Canada).

[3]Farm B, Robolac (Grand Valley Fortifiers, Cambridge, ON, Canada); Farm C, contained bakery waste, calcium carbonate, canola meal, corn grain, dairy trace vitamin/mineral pak, dried molasses, flavor, pellet binder, talc, salt, sodium bicarbonate, soybean hulls, soybean meal, wheat, wheat middlings, wheat shorts.

[4]Farm A, contained calcium carbonate (limestone) (239.88 kg/t), sodium sesquicarbonate (S-Carb SQ-810, 226.11 kg/t; Arm and Hammer, Princeton, NJ), salt (204.50 kg/t), urea (153.38 kg/t), magnesium oxide (98.31 kg/t), RM 104 (31.46 kg/t), selenium FSP 500 (20.42 kg/t), SG Dairy Ultra 1.0 NS 2018 (20.04 kg/t), Rumensin 200 (3.58 kg/t; Elanco Canada, Guelph, ON, Canada), Vitamin E FSP 50000 (2.32 kg/t); Farm B, Ecolac (Grand Valley Fortifiers); Farm C, contained calcium carbonate, calcium iodate, cobalt sulfate, copper sulfate, ruminant protein supplement, magnesium oxide, manganous oxide, mineral oil, monocalcium phosphate, ferrous sulfate, salt, sodium bicarbonate, sodium selenite, vitamin A, vitamin D, vitamin E, wheat middlings, zinc oxide, monensin sodium.

[5]DCAD calculated as mEq of $[(Na^+ + K^+) - (Cl^- + S^{-2})]$/kg of DM.

Blood Sampling

Whole blood was collected from the coccygeal vessels 7 ± 3 d (mean ± SD) before calving (d −7), within 24 h after calving (d 1), and 72 h later (d 4). Blood was collected into a 10-mL tube without anticoagulant (BD Vacutainer Precision Glide; Becton Dickinson, Franklin Lakes, NJ), 2 tubes (8.5 mL) containing acid citrate dextrose (**ACD**; Vacutainer, Becton Dickinson), and a 4-mL sodium heparin tube (Vacutainer, Becton Dickinson). Blood collected into sodium heparin tubes was used to measure iCa. Immediately after sampling, a point-of-care handheld biochemical analyzer with CHEM8+ cartridges (i-Stat System, Abbott Laboratories, Abbott Park, IL) was used to measure iCa values. Within 3 h of sampling, tubes without anticoagulant were centrifuged at 1,500 × *g* for 15 min and serum was harvested and stored (−20°C) in 2 aliquots until analysis (tCa); blood collected into ACD tubes was used to isolate PMN and subsequently measure PMN function (Pc and OB). Samples were not collected, and cows were excluded from the study, if clinical disease occurred during the sample collection period.

Serum Calcium Concentration

Total calcium measurement was done at the Animal Health Laboratory, University of Guelph, using the Cobas Calcium Gen 2 kit (Roche Diagnostics, Indianapolis, IN) with a lower limit of quantification of 0.2 mmol/L, and an intra-assay coefficient of variation of 1.9%.

Neutrophil Assays: Isolation, Phagocytosis, and Oxidative Burst

Neutrophil isolation and Pc and OB assays were performed according to the procedures described in detail by Miltenburg et al. (2018b). All samples were processed and run by the same operator who was blinded to the treatment group. Blood samples (ACD-blood) were first diluted with 1 × PBS (20 mL) at room

temperature. After layering diluted ACD-blood over a density gradient medium (Ficoll-Paque PLUS, density 1.077 g/mL; General Electric Healthcare Bio-Sciences AB, Uppsala, Sweden) and subsequent centrifugation, plasma, monocytes, and the density gradient medium were removed. Water was used for erythrolysis, and this step was repeated until we achieved a neutrophil layer without visible hemoglobin. Isolation efficiency assessment was performed via trypan blue staining and only samples with >90% neutrophils were included. After isolation, neutrophils were diluted in PBS (1×10^6 cells in 200 μL) and allocated to 4 flow cytometer tubes.

Phagocytosis was assessed by adding 50 μL of thawed bovine serum with Zymosan A (previously prepared from *Saccharomyces cerevisiae*; Sigma-Aldrich, St. Louis, MO) as described by Miltenburg et al. (2018b) to neutrophils (1×10^6 cells diluted in 200 μL of PBS) and then incubated with fluorescently labeled beads [TransFluoSpheres Carboxylate-Modified Microspheres, Thermo Fisher Scientific, Mississauga, ON, Canada; 1.0 μm (488/560 nm, excitation/emission), 2% solids] in the dark for 30 min at 37°C. An aliquot free of beads (control) was simultaneously analyzed for each sample. After 30 min, 200 μL of cold 1× PBS was added to each tube and placed on ice until flow cytometry analysis. For the OB assay, 2 flow cytometry tubes, each containing 200 μL of neutrophils (1×10^6 cells) diluted in PBS containing 10% filtered fetal bovine serum (Invitrogen, Burlington, ON, Canada), were incubated at 37°C with 2 μL of 1 m*M* 2′,7′-dihydro-dichlorofluroscein-diacetate (H_2DCFDA; Molecular Probes, Eugene, OR). After 15 min, OB stimulation was performed by adding 200 μL of 25 ng/mL PMA (Sigma-Aldrich) to 1 tube. Simultaneously, 200 μL of 1× PBS with fetal bovine serum were added to the control tube. Both samples were further incubated for 15 min and then placed on ice until flow cytometry analysis.

Flow Cytometry

Each sample was run on a flow cytometer (BD FACSCanto cell analyzer; BD Biosciences, San Jose, CA). Physical properties of 10,000 cells per sample were characterized using concurrent forward and side scatter detectors. Data were analyzed using FlowJo software (version 10.5.3, Tree Start Inc., Ashland, OR). Four function outcomes were analyzed: median fluorescence intensity for Pc (**MFIP**) and OB (**MFIOB**), and the shift of percentage of cells active (stimulated minus unstimulated) for Pc (**PPc**) and OB (**POB**). The MFIP and MFIOB represent the number of beads phagocytized and the amount of oxygen reactive species produced by neutrophils, respectively. The PPc and POB represent the percentage of neutrophils that engulfed at least one bead, and shift of activated PMN after PMA stimulation for OB activity, respectively.

Statistical Analysis

The outcomes were the 4 measures of PMN function (MIFP, MIFOB, POB, and PPc), with treatment as the main effect. Parity (categorical variable: 2nd vs 3rd or greater), BCS at enrollment (continuous), and prepartum urine pH were only used to describe baseline data of the treatment and control groups. For the TRT group, urine pH was categorized at thresholds of 7 and 6.5. Acid-base status changes calcium metabolism when urine pH ≤7 (Charbonneau et al., 2006), and a pH of ≤6.5 is recommended for negative DCAD diets to account for individual variation and to better prevent SCH (Goff, 2008). Our target urine pH was 6.0 to 6.5.

In the overlying clinical trial, the single close-up pen on each farm was randomly assigned to a starting treatment, which was alternated approximately every 3 mo for a total of 4 periods (2 TRT and 2 CON). Statistical analyses were performed using SAS, version 9.4 (SAS Institute, Cary, NC), with cow as the unit of observation, but with pen as the experimental unit. Continuous outcomes were ln or $\log_{10}$ transformed after the distribution analysis with Shapiro-Wilk's test determined that they were not normally distributed. A natural log-transformation was used to normalize POB, MIPOB, and PPc. The MIXED model function of SAS was used to fit mixed linear regression models. Repeated measures were accounted for with an autoregressive covariance structure, selected based on the lowest Akaike information criterion, although the measurements were not equally spaced in time. In the present analysis, there were 2 experimental periods (summer and fall). Pen-level randomization was accounted for with a random effect (farm × treatment × period) to correctly specify the experimental unit as pen (farm) (Bello et al., 2016; Bello and Renter, 2018) with 4 degrees of freedom for treatment in the final models. To account for clustering of animals with farm, a separate random effect of farm was also included in all models. The effect of TRT, the time relative to calving (d −7, 1, and 4), and their interactions were tested for each outcome.

In separate models, serum calcium concentration (iCa and tCa) were treated either as the dependent (DCAD effect on calcium concentration models) or independent (calcium concentration effect on neutrophil function models) continuous variable, depending on the linear model assessed, with sample day (−7, 1, and 4) and their interaction as covariates. Total Ca and iCa were also categorized as > or ≤ 2.15 mmol/L (Martinez et al., 2012) and > or ≤ 1.0 mmol/L (Martinez et al.,

2018), respectively, and these categorical variables were also tested for associations with neutrophil function responses. A random effect of farm was included in all models.

In all models, the interaction term was removed when $P > 0.1$. A Tukey test was used to adjust for multiple comparisons. Results are expressed as least squares means with their standard errors (on the logarithmic scale). Pearson correlation coefficients between iCa and tCa and each neutrophil function outcome were calculated using the CORR procedure in SAS.

RESULTS

Six cows were excluded, 2 because they were exposed to both feed supplements within 2 wk of calving, and 4 (all from CON) because they received calcium supplementation (n = 1), retained placenta (n = 1), had metritis (n = 1), or had milk fever (n = 1). The final data set consisted of 38 multiparous cows (TRT, n = 21; CON, n = 17) across the 3 herds (A = 10, B = 17, and C = 11). Farms A, B, and C provided 2, 13, and 6 cows in TRT and 8, 4, and 5 in CON, respectively. The BCS at enrollment was 3.4 ± 0.1 and 3.4 ± 0.2 for TRT and CON, respectively. Three cows had BCS of 2.75 or 3, 30 cows had BCS of 3.25 or 3.5, and 5 cows (all from farm A) had BCS of 3.75. Parity (3.3 ± 1.8) was not different between treatment groups ($P = 0.4$), and was not different among the farms (65 to 70% of cows in parity ≥3). The difference in DCAD between TRT and CON varied somewhat among farms (A: 208 mEq/kg; B: 238 mEq/kg; C: 160 mEq/kg; Table 1). The precalving urine pH in CON was 8.2 ± 0.2 and in TRT was 6.1 ± 0.8 with 80% of 50 samples <7 and 74% of samples ≤ 6.5. Among urine samples from cows in TRT, 5 of 11 on farm A, 11 of 14 on farm B, and 21 of 25 on farm C had pH ≤6.5.

Effect of Treatment on Neutrophil Function

There were no differences ($P > 0.36$) between treatment groups in the 4 PMN function outcomes. The MFIOB varied ($P < 0.01$) over time, and MFIP tended ($P = 0.07$) to vary over time, independently of treatment, and thus the sampling day covariate was retained in these models. There were no treatment by time interactions for any outcome ($P \geq 0.15$). The PMN function results are illustrated in Figure 1.

Effect of Treatment on Serum Calcium Concentrations

The mean concentrations of iCa and tCa (Figure 2) were not different between treatments ($P > 0.5$). The proportions of blood samples with tCa <2.15 mmol/L at d −7, 1, and 4 were 5% (2/38), 76% (29/38), and 13% (5/38), respectively, with no differences between TRT and CON ($P = 0.74$). The proportions of blood samples with iCa ≤1.0 mmol/L at d −7, 1, and 4 were 0%, 32% (12/38) and 3% (1/38), with no differences between TRT and CON ($P = 0.27$).

Effect of Serum Calcium Concentrations on Neutrophil Function

No associations were found between iCa ($P > 0.17$) or tCa ($P > 0.18$) with Pc or OB, although there tended to be an interaction of iCa with time for MFIOB ($P = 0.07$). When calcium was used as a categorical variable, there was no association of iCa ≤1.0 mmol/L ($P > 0.3$) or tCa ≤2.15 mmol/L ($P > 0.1$) with any of the neutrophil function outcomes assessed. Correlation coefficients between iCa or tCa and the 4 neutrophil function outcomes were weak ($r < |0.3|$) and varied in direction across time.

No adverse events were experienced throughout the trial.

DISCUSSION

To our knowledge, this is the first study assessing the effect of negative DCAD diets on neutrophil function on commercial dairy farms. Contrary to our hypothesis, feeding a negative DCAD diet for 3 wk before expected calving did not improve Pc or OB activity in healthy cows. Calcium plays an important role in neutrophil function (Martinez et al., 2014), and negative DCAD diets have been shown to increase peripartum blood calcium concentration and reduce the risk of SCH (Leno et al., 2017b; Lopera et al., 2018). Therefore, the premise was that a negative DCAD diet would improve PMN, Pc, and OB around parturition through increased serum calcium concentrations. However, unexpectedly, neither iCa nor tCa was different between treatment groups. As discussed below, the fact that we studied only clinically healthy cows should inform interpretation of these results.

Martinez et al. (2018) reported moderately increased Pc and OB intensity in multiparous cows fed negative DCAD diets (−130 mEq/kg) in comparison to cows fed a positive DCAD (+130 mEq/kg). The authors attributed this to higher iCa concentrations obtained at calving and d 1 postpartum in the negative DCAD group (iCa: positive DCAD = 0.97 vs. negative DCAD = 1.11 ± 0.008 mmol/L). Conversely, in the current study, no difference in neutrophil function was found when comparing negative (−100 mEq/kg of DM) and positive (approximately +95 mEq/kg of DM) DCAD groups or

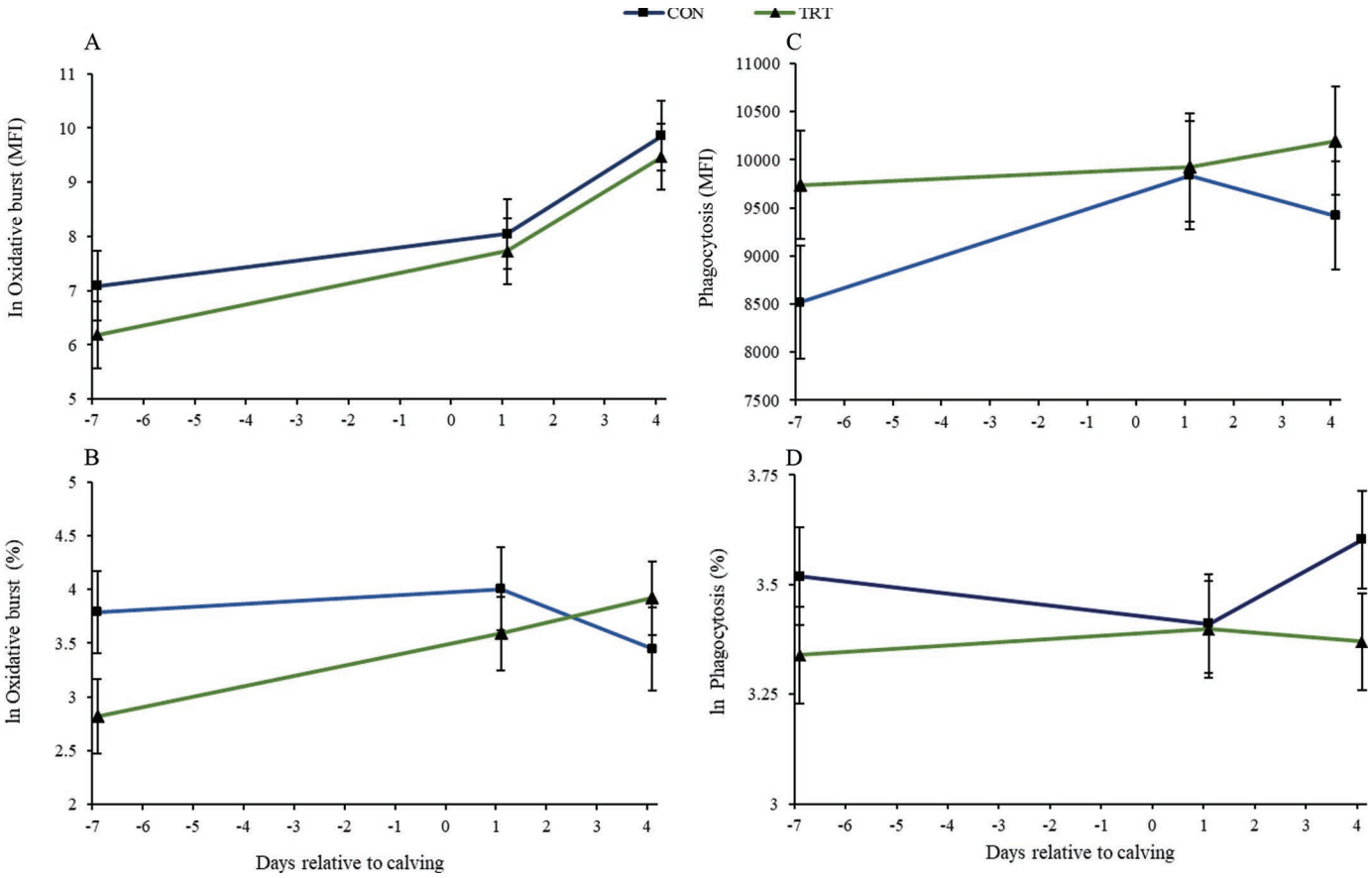

Figure 1. Median fluorescence intensity (MFI) and percentage of neutrophils positive for oxidative burst (A and B, respectively) and MFI and percentage of neutrophils positive for phagocytic activity (C and D, respectively) in cows fed negative DCAD (TRT; −100 mEq/kg of DM; n = 21) or placebo (CON; +95 mEq/kg of DM; n = 17). Error bars represent the SE. TRT = treatment, CON = control.

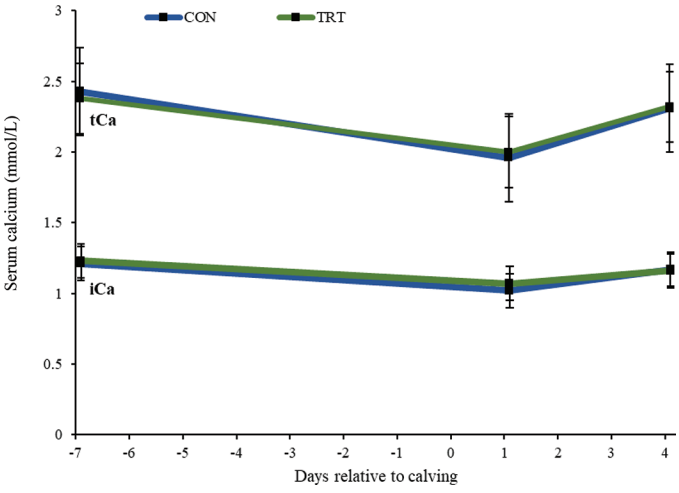

Figure 2. Mean ± SD serum calcium concentrations for ionized (iCa) and total calcium (tCa) in multiparous cows fed negative DCAD (TRT; −100 mEq/kg of DM; n = 21) or placebo (CON; n = 17; +95 mEq/kg of DM; n = 17). TRT = treatment, CON = control.

peripartum iCa and tCa concentrations. There was a smaller difference in DCAD between treatment groups in the present study (~200 mEq/kg of DM) when compared with treatment groups from Martinez et al. (2018) (260 mEq/kg of DM). In a meta-analysis of the effect of reduced (but not necessarily negative) DCAD, the mean difference in DCAD was around 230 mEq/kg of DM, which increased milk yield in multiparous cows, and decreased the odds of clinical disease (Santos et al., 2019). Therefore, it is possible that a greater absolute difference in DCAD between treatment and control (rather than only a sufficiently negative DCAD) is necessary to observe the hypothesized results. However, the lack of difference in blood Ca concentrations between TRT and CON groups, despite apparently sufficient acidification based on urine pH, was unexpected. In a meta-analysis of DCAD experiments, the mean effect was to increase blood Ca concentration on the day of calving, but there was substantial heterogeneity in this response; in 27 of 41 comparisons of blood Ca

concentration in multiparous cows, the 95% confidence interval included zero (no difference; Lean et al., 2019).

When assessing neutrophil function, Martinez et al. (2018) included both healthy and cows with clinical diseases in the analyses. In the present study, 4 cows with signs of disease, all from CON group, were excluded. Although necessary to avoid confounding the effect of treatment, considering the association between postpartum hypocalcemia and disease (Chapinal et al., 2011; Martinez et al., 2018), the exclusion of sick animals could bias our results (both neutrophil function and calcium concentrations) toward the null. We decided before the start of this experiment not to include diseased cows, and we did not collect samples once a cow was identified as diseased. Neutrophils in the blood stream become activated in response to inflammatory stimuli associated with disease conditions such as metritis and mastitis. Both the Pc and OB assays rely on measurement of responses to in vitro stimulation of neutrophils. In vivo activated neutrophils from sick cows may aggregate ("clump") in vitro, making isolation impossible, or may fail to respond to further in vitro stimulation, producing a false-negative result. Furthermore, in the present study all 4 cows with disease were from the CON group, so even if their data were available, the effects of treatment could not be disentangled from the effects of disease.

Dairy cows with induced SCH (iCa: 0.77 ± 0.01 mmol/L; tCa: 1.94 ± 0.03 mmol/L) had neutrophils with a faster decrease in intracellular iCa, and reduced Pc and OB activity, compared with normocalcemic cows (iCa: 1.26 ± 0.01 mmol/L; tCa: 2.05 ± 0.03 mmol/L; Martinez et al., 2014). Conversely, in the present study, iCa and tCa were greater than in induced SCH in Martinez et al. (2014) or in the positive DCAD group in Martinez et al. (2018). Perhaps consequently, the correlations between serum calcium concentration and neutrophil function were weak.

Due to logistical constraints of time and availability of the flow cytometer when we could carry out the neutrophil function assays, convenience sampling was used to select multiparous cows for the present subset study. Although BCS and parity were not different between groups, limitations of this study are that, based on calving patterns, different numbers of cows per treatment group were available from each farm, and the DCAD of the CON group diets varied somewhat among the farms. We did not hypothesize a treatment by farm interaction and this study did not have the statistical power to address that question. Future studies should be sized to explore possible differences in response to negative DCAD diets among farms. Conversely, a strength of this study is that it was conducted on several farms under commercial conditions. This introduces potential confounding variables, but also reflects the variation of conditions inherent to feeding a negative DCAD diet in the field.

In conclusion, we did not observe the hypothesized differences in aspects of immune function in healthy multiparous cows fed a negative DCAD, likely because the treatment did not improve blood calcium concentrations after calving. Associations of iCa and tCa with neutrophil function were weak. We underline the possibility that inclusion of cows with clinical disease would potentially have altered our results.

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