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Effects of maintaining eucalcemia following immunoactivation in lactating Holstein dairy cows

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ABSTRACT

Periparturient hypocalcemia is a common metabolic disorder and it is ostensibly associated with negative health and production outcomes. Acute infection also markedly decreases circulating Ca, but the reasons for and consequences of it on physiological and immunological parameters are unknown. Objectives were to evaluate the effects of maintaining eucalcemia on production, metabolic, and immune variables following an intravenous lipopolysaccharide (LPS) challenge. Twelve multiparous lactating Holstein cows (717 ± 20 kg of body weight; 176 ± 34 d in milk; parity 3 ± 0.2) were enrolled in a study containing 2 experimental periods (P); during P1 (3 d), cows consumed feed ad libitum and baseline values were obtained. At the initiation of P2 (4 d), cows were randomly assigned to 1 of 2 treatments: (1) LPS administered (LPS-Con; 0.5 μg/kg of body weight LPS; n = 6) or (2) LPS administered + eucalcemic clamp (LPS-Ca; 0.5 μg/kg of body weight LPS; Ca infusion; n = 6). Cows were fasted for the first 12 h during P2. After LPS administration, ionized Ca was determined every 15 min for 6 h and every 30 min for an additional 6 h and intravenous Ca infusion was adjusted in LPS-Ca cows to maintain eucalcemia. Blood ionized Ca was decreased 23% for the first 12 h postbolus in LPS-Con cows, and by design, Ca infusion prevented hypocalcemia. To maintain eucalcemia for the 12 h, 13.7 g of Ca was infused. The total Ca deficit (including Ca not secreted into milk) accumulated over the 12 h was 10.4 and 20.2 g for the LPS-Con and LPS-Ca treatments, respectively. Mild hyperthermia (0.8°C) occurred for ~6 h post-LPS administration relative to P1. From 6 to 7 h postbolus rectal temperature from LPS-Ca cows was increased (0.6°C) relative to LPS-Con cows. On d 1 of P2, milk yield decreased (61%) in both treatments relative to P1. Relative to LPS-Con cows, milk yield decreased (15%) in LPS-Ca cows during P2. Overall, circulating LPS-binding protein continuously increased postbolus, and at 24 h LPS-binding protein levels in LPS-Ca cows were increased (80%) relative to LPS-Con cows. During P2, serum amyloid A increased (4-fold) in both treatments relative to P1. Administering LPS initially decreased circulating neutrophils, then cell counts progressively increased with time. Calcium infusion decreased neutrophil counts (40%) from 9 to 12 h postbolus relative to LPS-Con cows. Neutrophil function, as assessed by oxidative burst and myeloperoxidase production, did not differ due to treatment. In summary, maintaining eucalcemia (via intravenous Ca infusion) during an immune challenge appeared to intensify inflammation and adversely affect lactation performance.

Key words: calcium, endotoxin, inflammation, infection

INTRODUCTION

At the onset of lactation, the mammary gland's use of plasma Ca is so extensive that it often exceeds the capacity of homeostatic mechanisms (i.e., parathyroid hormone and vitamin D) to replenish it and, consequently, cows develop clinical or subclinical hypocalcemia (**SCH**; Horst et al., 2005; Goff, 2008). Hypocalcemia has been loosely associated with reduced milk production and increased disease incidence (Chapinal et al., 2011; Martinez et al., 2012, 2014; Venjakob et al., 2018) and is believed to be a causative factor in periparturient immunosuppression (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2012, 2014). Despite the purported immune "dysfunction," nearly all transition cows (even seemingly healthy ones) experience some degree of inflammation postpartum (Humblet et al., 2006). The magnitude and persistency of the inflammatory state appears dependent on the frequency and type of immune insults (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018) and is predictive of transition cow performance (Ohtsuka et al., 2001; Ametaj et al., 2005, 2010; Abuajamieh et al., 2016).

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Interestingly, independent of the transition period, immune activation decreases circulating Ca and the response is conserved across species including humans (Cardenas-Rivero et al., 1989; Dias et al., 2013), calves (Tennant et al., 1973; Elsasser et al., 1996), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2005), pigs (Carlstedt et al., 2000), and sheep (Naylor and Kronfeld, 1986). We and others have repeatedly demonstrated LPS-induced hypocalcemia in lactating cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018). Thus, because most periparturient cows are inflamed, immunoactivation may be contributing (at least to some extent) to hypocalcemia during the transition period, a theory originally proposed by early investigators (Thomas, 1889; Hibbs, 1950). Inflammation's effect on Ca homeostasis may explain why SCH remains common, despite successfully implementing pre-partum prophylactic and therapeutic strategies (i.e., acidifying rations, low Ca diets, Ca chelators, and so on) aimed at preventing milk fever (Reinhardt et al., 2011).

Postcalving oral Ca supplementation is an additional approach to mitigate SCH (Oetzel and Miller, 2012; Oetzel, 2013). Aside from improving plasma Ca concentrations and despite some inconsistencies (Domino et al., 2017), oral Ca boluses have been shown to improve milk production and some health metrics (Oetzel, 1996; Oetzel and Miller, 2012). We recently demonstrated that oral Ca (in combination with live yeast) before and following LPS administration ameliorates hypocalcemia (although it did not prevent it) and improves production (feed intake and milk yield) performance (Al-Qaisi et al., 2020). Unfortunately, we were unable to distinguish the effects of Ca and live yeast; however, considering the presumed role of Ca in immune function and transition cow performance, we conjectured the beneficial effects of the oral bolus were primarily due to alleviating hypocalcemia. Therefore, we hypothesized that maintaining eucalcemia during immunoactivation in lactating dairy cows would improve production and inflammatory outcomes. Study objectives were to evaluate the effects of preventing hypocalcemia on production parameters, inflammatory biomarkers, and neutrophil function in intensely immune-activated lactating dairy cows.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twelve nonpregnant lactating Holstein cows (717 ± 20 kg of BW; 176 ± 34 DIM; parity 3 ± 0.2; SE) were housed in individual box-stalls (4.57 × 4.57 m) at the Iowa State University Dairy Farm (Ames). Cows were allowed 3 d to acclimate during which they were implanted with bilateral jugular catheters. Cows were fed ad libitum once daily (0600 h) with a diet formulated to meet or exceed the predicted requirements (NRC, 2001) of energy, protein, minerals, and vitamins (Table 1). Cows were milked twice daily (0600 and 1800 h) throughout the experiment and yield was recorded. A sample for composition analysis (milk fat, protein, lactose, MUN, and SCC) was obtained at each milking and stored at 4°C with a preservative (bronopol tablet, D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC-approved infrared analysis equipment and procedures (AOAC International, 1995). Additional milk samples were collected and stored at −20°C until analysis by the Iowa State University Veterinary Diagnostic Lab (Ames) for total Ca concentrations using inductively coupled plasma MS (Analytic Jena Inc., Woburn, MA). Rectal temperature was measured using a digital thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA) and recorded after each milking.

The trial consisted of 2 experimental periods; **P1** lasted 3 d and served as the baseline, which yielded data for covariate analysis. During **P2**, which lasted 4 d, animals were randomly assigned to 1 of 2 intravenous bolus treatments: (1) LPS administered in which hypocalcemia was allowed to develop (**LPS-Con**; 0.5 μg/

Table 1. Ingredients and composition of diet[1]

Item[2]	Value
Ingredient (% of DM)	
Corn silage	21.4
Alfalfa hay	4.1
Baleage	16.4
Ground corn	29.7
Corn gluten feed	9.4
SoyPlus[3]	2.6
Soybean meal	5.9
Whole cottonseed	3.6
Molasses	1.8
CP mix	5.1
Chemical analysis (% of DM)	
Starch	27.9
CP	17.2
NDF	29.2
ADF	18.4
NE_L (Mcal/kg of DM)	1.64

[1]Values represent an average of ration nutrient summary reports collected throughout the trial. Diet moisture averaged 51.8%.

[2]Average nutrient levels: 4.86% fat, 0.95% Ca, 0.43% P, 0.36% Mg, 0.23% S, 1.38% K, 0.49% Na, 0.65% Cl, 88.06 mg/kg Zn, 51.31 mg/kg Mn, 4.06 mg/kg Fe, 15.64 mg/kg Cu, 0.87 mg/kg Co, 0.19 mg/kg Se, 0.87 mg/kg I, 6,810.1 IU/kg vitamin A, 643.6 IU/kg vitamin D, and 18.9 IU/kg vitamin E.

[3]Mechanically processed soybean meal, Dairy Nutrition Plus, Ralston, IA.

kg of BW of LPS; n = 6) and (2) LPS administered in which eucalcemia was maintained (**LPS-Ca**; 0.5 μg/kg of BW of LPS; Ca infusion; n = 6). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 89.6 μg/mL and passed through a 0.2-μm sterile syringe filter (Thermo Scientific, Waltham, MA). The total volume of LPS solution administered i.v. was approximately 4 mL. Each respective treatment bolus was administered immediately following the AM milking and 0 min blood sample collection. In the LPS-Ca treatment, we performed a eucalcemic clamp, where a 23% Ca gluconate solution (Agri Laboratories Ltd., St. Joseph, MO) was i.v. infused at a known and adjustable rate utilizing a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) to maintain the pre-LPS administration blood iCa concentrations (±10% of baseline). To ensure infusion rate accuracy, 250 mL of the Ca gluconate solution was further diluted in 750 mL of sterile saline. Cows were tethered during the 12-h challenge period (but allowed to stand up and lay down) to allow for frequent sampling. Water was provided ad libitum and feed was removed from all cows ~0.5 h before treatment administration. Animals remained fasted during the 12-h data collection period to eliminate the confounding effect of dissimilar nutrient intake.

To establish baseline iCa levels, blood samples were obtained at −30 and 0 min relative to LPS administration and immediately analyzed using an iSTAT handheld machine and cartridge (CG8+, Abbott Point of Care, Princeton, NJ). Blood iCa was measured hourly for LPS-Con cows. For cows in the LPS-Ca treatment, blood samples were obtained every 15 min for the first 6 h and every 30 min for the next 6 h thereafter. Ionized Ca infusion began when blood iCa content declined below baseline concentrations. The rate of Ca infusion (**ROCI**) was transformed from mL/h to g/h. The total Ca infused for each cow was calculated using the ROCI for each interval (36 intervals in total) according to the following equation, where i is an index of time:

$$\sum_{i=0}^{24}\mathrm{ROCI}\left(\mathrm{g/h}\right)_i \times \frac{1\ \mathrm{h}}{60\ \mathrm{min}} \times 15\ \mathrm{min} + \sum_{i=0}^{12}\mathrm{ROCI}\left(\mathrm{g/h}\right)_i \times \frac{1\ \mathrm{h}}{60\ \mathrm{min}} \times 30\ \mathrm{min}.$$

Milk total Ca content was assessed at the AM and PM milkings on d 1 of P2 and analyzed for total Ca concentrations. Additional plasma samples were collected from all treatments daily at 0600 h during P1 and at −0.5, 0, 6, 12, 24, 48, and 96 h relative to bolus administration during P2. Plasma was harvested following centrifugation at 1,500 × *g* for 15 min at 4°C and was subsequently frozen at −20°C until analysis.

Plasma insulin, glucagon, nonesterified fatty acids (**NEFA**), BHB, BUN, LPS-binding protein (**LBP**), and serum amyloid A (**SAA**) concentrations were determined using commercially available kits according to the manufacturers' instructions (insulin, Mercodia AB, Uppsala, Sweden; Glucagon, RD Systems Inc., Minneapolis, MN; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc., Canton, MI; BUN Teco Diagnostics, Anaheim, CA; LBP, Hycult Biotech, Uden, the Netherlands; SAA, Tridelta Development Ltd., Kildare, Ireland). The inter- and intraassay coefficients of variation for insulin, glucagon, NEFA, BHB, BUN, LBP, and SAA were 4.3 and 6.0%, 10.5 and 14.1%, 3.0 and 3.6%, 4.2 and 7.1%, 3.4 and 3.6%, 11.3 and 8.6%, and 22.3 and 5.3%, respectively. For complete blood count analysis, a 3-mL blood sample was collected at 0, 1, 2, 3, 4, 5, 6, 9, 12, 24, 36, 48, 72, and 96 h relative to bolus administration (K2EDTA, BD Franklin Lakes, NJ) and stored at 4°C for ~12 h before submission to the Iowa State University's Department of Veterinary Pathology.

PMN Isolation and Function

Blood samples (32 mL) for neutrophil isolation were collected on d 4 of P1, and at 6, 12, 24, and 48 h post-LPS administration into 50-mL conical tubes containing acid citrate dextrose (8 mL). Samples were immediately transported to the laboratory for neutrophil isolation as previously described (Kimura et al., 2014). In brief, samples were centrifuged at 1,000 × *g* for 20 min at room temperature and the plasma, buffy coat, and upper portion of the packed red blood cell pellet were removed. The remaining red blood cells were lysed twice using hypotonic phosphate-buffered deionized water, and neutrophils were pelleted following centrifugation at 300 × *g* for 5 min at 23°C. Neutrophils were resuspended in PBS and standardized to 5.0×10^7 cells/mL. Neutrophil function was assessed by extracellular release of myeloperoxidase (**MPO**) and oxidative burst (cytochrome C reduction) using methods previously described by Kimura et al. (2014).

To assess oxidative burst, 2.5×10^6 neutrophils were treated with either Hanks' balanced salt solution (**HBSS**) or HBSS plus phorbol myristate acetate followed by treatment with cytochrome C. Cells were incubated at 39°C for 15 min and immediately read at 2 wavelengths (550 and 650 nm) using a spectrophotometer.

Three cell preparations were used to assess MPO activity: (1) cells were lysed by treatment with cetyltrimethylammonium bromide solution as a measure of

total MPO, (2) PMN were stimulated with equal parts Ca ionophore A23187 and cytochalasin B in HBSS to assess release of MPO with stimulation, and (3) PMN treated with HBSS alone as a measure of unstimulated MPO release. Each preparation was loaded with 1.25 $\times$ 10^6 cells in a microtiter plate and incubated at 39°C for 20 min. Following incubation, 50 μL/well of 3,3′, 5,5′-tetramethylbenzidine dihydrochloride was added (3.25 m*M*) followed promptly with 50 μL/well of H_20_2. The reaction was stopped after a 2-min incubation with the addition of 50 μL/well 2 *N* H_2SO_4. Plates were centrifuged for 1 min at 600 $\times$ *g* at 23°C, and the supernatant was transferred to a second plate. Optical density (**OD**) was determined at 405 nm using a spectrophotometer. Percentage of MPO released from PMN was determined using the following equation:

$$\text{exocytosis (\%)} = [(\text{OD of stimulated PMN})/(\text{OD of lysed PMN})] \times 100.$$

Calculations and Statistical Analysis

Administering LPS decreases milk yield and therefore decreases Ca uptake by the mammary gland for milk synthesis. The decrease in milk yield permitted us to estimate the amount of Ca conserved (milk Ca deficit) in cows administered LPS and allowed to develop hypocalcemia and those maintained at eucalcemia. Total Ca output in milk before the challenge was calculated to establish a baseline. Milk Ca content at 12 h was subtracted from the baseline to calculate the milk Ca deficit. For LPS-Con cows, milk Ca deficit was used solely to calculate the total Ca deficit. For LPS-Ca cows, milk Ca deficit plus the amount of Ca infused to maintain eucalcemia were combined to obtain the total Ca deficit, and this approach is similar to how we calculated the glucose deficit in our previous LPS-euglycemic clamp papers (Kvidera et al., 2017; Horst et al., 2018, 2019).

Sample size determination (6 animals/treatment) was based on previous reports and logistical constraints (Horst et al., 2019; Al-Qaisi et al., 2020). Post-hoc power analysis (PROC POWER, SAS Inst. Inc., Cary, NC) based on the results of our primary objective (ionized Ca) indicated a statistical power of >90% ($\alpha = 0.05$). Each animal's respective parameter was analyzed using a repeated measures ANOVA with an autoregressive covariance structure for milk yield, DMI, and milk composition and a spatial power law covariance structure for blood metabolites, inflammatory biomarkers, neutrophil function assays, complete blood count, iSTAT, and rectal temperature data. The repeated effect was time relative to LPS administration. Each specific variable's pre-bolus values (i.e., P1 average or 0 h when available) served as a covariate for analysis of P2. Effects of treatment, time (h or d relative to bolus administration), and treatment × time interactions were assessed as a completely randomized design using PROC MIXED (SAS Inst. Inc.). To evaluate effects of LPS administration, a separate analysis (using the same model) was used to make statistical comparisons with baseline (pre- vs. post-LPS) in which the average P1 value (DMI, milk yield, and neutrophil function) or 0 h (rectal temperature and blood variables) for each parameter was included as an additional time point. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

RESULTS

Calcium and Blood Gas Analysis

Administering LPS decreased blood iCa (23%) for the first 12 h postbolus in LPS-Con cows and, by design, Ca infusion prevented hypocalcemia in LPS-Ca cows ($P < 0.01$; Figure 1). Collectively, 13.7 g of Ca was infused to maintain eucalcemia during the 12-h clamp. Milk Ca concentrations averaged 1.24 ± 0.04 and 0.94 ± 0.04 at the AM milking and 1.55 ± 0.13 and 1.71 ± 0.09 at the PM milking for LPS-Con and LPS-Ca cows, respectively. Milk yield averaged 18.1 and 20.2 kg at the AM milking and 7.3 and 7.9 kg at the PM milking for LPS-Con and LPS-Ca cows, respectively. The total Ca deficit accumulated over the 12 h was 10.4 and 20.2 g for the LPS-Con and LPS-Ca treatments, respectively. During P2, Ca infusion increased HCO_3, base excess, and pH relative to LPS-Con cows ($P \leq 0.03$; Supplemental Table S1; https://doi.org/10.3168/jds.2020-18268). Mild hyperthermia (0.8°C) was observed for ~5.5 h post-LPS administration relative to P1 ($P \leq 0.05$). From 6 to 7 h postbolus, rectal temperature in LPS-Ca cows increased (0.6°C) relative to LPS-Con cows ($P < 0.01$; Figure 2A).

Production Metrics

Overall, DMI did not differ between treatments ($P > 0.25$); however, hypophagia was observed for 2 d postbolus relative to baseline (34 and 45% for LPS-Con and LPS-Ca, respectively; $P < 0.01$; Figure 2B). On d 3 postbolus, DMI in LPS-Ca cows tended to remain below baseline (15%; $P = 0.07$), whereas it returned to pre-LPS infusion levels in LPS-Con cows (Figure 2B). Relative to baseline, milk yield decreased (45%) in both treatments for the first 2 d postbolus (61% on d 1 and 29% on d 2; $P < 0.01$; Figure 2C). Overall during P2,

milk yield from LPS-Ca cows tended to be decreased (15%) relative to LPS-Con cows ($P = 0.07$; Figure 2C) and was decreased on d 1 of P2 (37%; $P = 0.04$; Figure 2C). Milk lactose content decreased in both treatments postbolus, but the magnitude of decrease was greater in LPS-Ca cows relative to LPS-Con ($P < 0.01$; Table 2). Milk lactose gradually returned to baseline in both treatments with time ($P < 0.01$). Milk fat, protein, and MUN content increased in both treatments at 24 h following LPS administration ($P < 0.01$). From 12 to 24 h milk fat, protein, and MUN content were increased in LPS-Ca compared with LPS-Con cows (26, 11, and 17%, respectively; $P \leq 0.05$; Table 2). Milk SCC did not differ due to treatment ($P > 0.24$; Table 2).

Metabolic Variables

Regardless of treatment, administering LPS increased circulating glucose for 2 h postbolus relative to P1 (28%; $P < 0.05$), after which hypoglycemia developed (Figure 3A). Glucose concentrations returned to baseline at 24 h postbolus. Relative to P1, insulin concentrations increased post-LPS (60%) similarly among treatments ($P \leq 0.04$; Figure 3B). Overall, circulating glucagon increased for 12 h postbolus, then gradually decreased below baseline with time ($P = 0.04$; Figure 3C) and was unaffected by Ca infusion. Regardless of infusing Ca, NEFA concentrations increased (3-fold, relative to P1; $P < 0.01$) at 12 h postbolus, then progressively decreased with time ($P < 0.01$; Figure 4A). Administering LPS decreased circulating BHB (33%, relative to P1) for 12 h postbolus, after which levels returned to baseline ($P \leq 0.05$). At 48 h postbolus, BHB concentrations from LPS-Ca cows tended to be decreased (20%) relative to controls ($P = 0.07$; Figure 4B). Blood urea nitrogen increased (41% for first 24 h; $P \leq 0.01$) similarly in both treatments postbolus relative to P1. Throughout P2, BUN concentrations from LPS-Ca cows increased (8%) relative to LPS-Con cows ($P = 0.05$; Figure 4C).

Inflammatory Biomarkers and Immune Metrics

Overall during P2, circulating LBP and SAA increased post-LPS administration (3- and 4-fold, respectively; $P < 0.01$; Figure 5A and 5B) relative to P1. At 24 h postbolus, circulating LBP from LPS-Ca cows increased (80%) relative to controls ($P = 0.02$; Figure 5A). Lipopolysaccharide administration initially decreased (66% for 12 h) circulating WBC then they progressively increased with time (33% from 24 to 48 h relative to baseline; $P < 0.01$). Circulating monocytes and lymphocytes initially decreased following LPS administration and gradually increased with time ($P < 0.01$). Maintaining eucalcemia had no effect on circulating WBC, monocytes, or lymphocytes (Table 3). The pattern of circulating neutrophils reflected the WBC, however, from 9 to 12 h postbolus neutrophils from LPS-Ca cows were decreased (40%) relative to LPS-Con ($P \leq 0.05$; Figure 5C). During P2, there were no overall treatment effects on neutrophil oxidative burst. However, when compared with P1, oxidative burst from

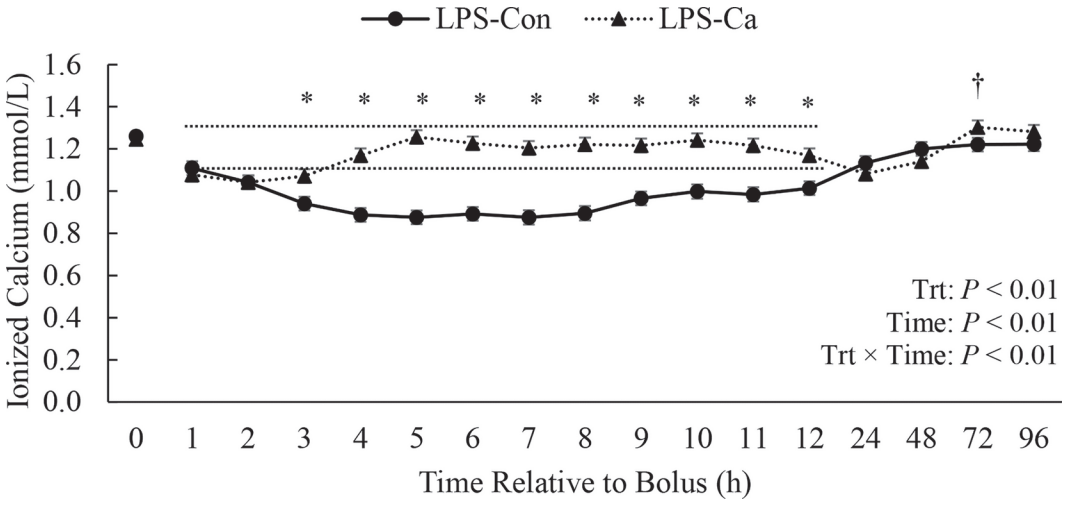

Figure 1. Ionized Ca concentrations from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Horizontal dashed lines represent ±10% of baseline. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained before LPS administration (−0.5 and 0 h) and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. † represents a tendency for a difference between treatments. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

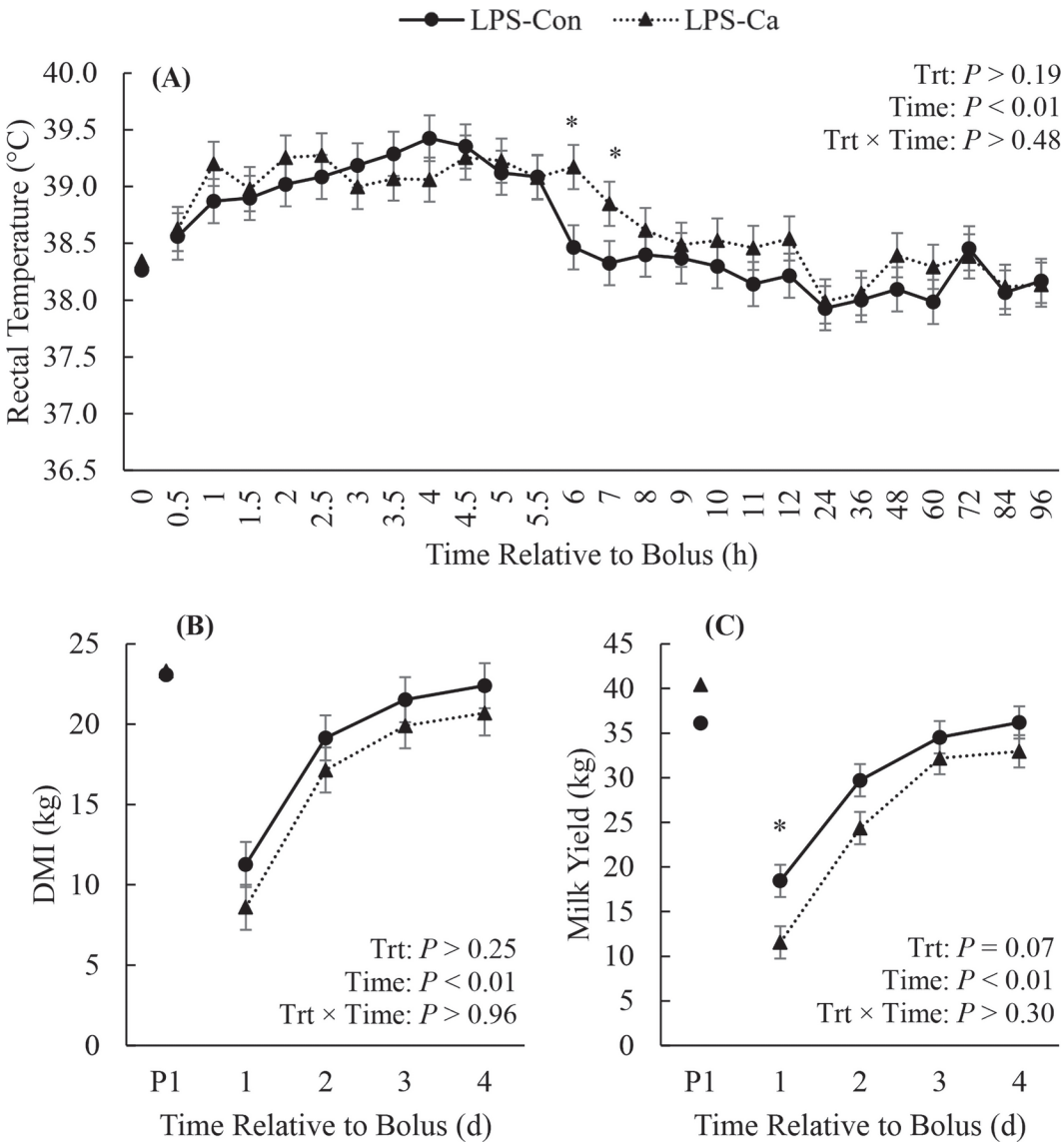

Figure 2. (A) Rectal temperature, (B) DMI, and (C) milk yield from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, time (h or d), and their interaction. Hour 0 represents an average of measurements obtained before LPS administration (−0.5 and 0 h) and was used as a covariate for rectal temperature. P1 represents an average of measurements obtained during the 3 d of period 1 and was used as a covariate for DMI and milk yield. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

LPS-Ca cows tended to increase at 6 h postbolus (31%; $P = 0.07$), whereas in LPS-Con cows it remained similar to baseline (Figure 6A). Similarly, MPO exocytosis and total MPO were not influenced by Ca infusion during P2. Neutrophil MPO exocytosis increased (76%) in both treatments for 12 h postbolus relative to P1 ($P \leq 0.10$; Figure 6B). Despite increased exocytosis, total MPO did not differ from P1 in LPS-Con cows; however, it was increased (33%) 6 h postbolus in LPS-Ca cows ($P = 0.03$; Figure 6C). In agreement with the other neutrophil function metrics, stimulated MPO release did not differ between treatments during P2. However, relative to P1, MPO release increased in both treatments, although the increase was prolonged in LPS-Con cows (66% over the 48 h; $P \leq 0.05$; Figure 6D).

DISCUSSION

At lactation onset, dairy cows experience a marked increase in Ca requirements (>65%; DeGaris and Lean, 2008) to support colostrum and milk synthesis (Horst et al., 2005). Mammary Ca uptake is so acute and ex-

tensive it often outweighs the homeostatic mechanisms employed to replenish it and consequently clinical hypocalcemia or SCH occurs (Horst et al., 2005; Goff, 2008). Implementing therapeutic and prophylactic strategies has markedly reduced the incidence of clinical hypocalcemia (Charbonneau et al., 2006), but SCH continues to afflict ~25% of primiparous and ~50% of multiparous cows in the United States (Reinhardt et al., 2011). Although asymptomatic, SCH has been loosely associated with reduced production performance (i.e., milk yield and feed intake), increased risk of displaced abomasum, immunosuppression (and consequently an increased susceptibility to infectious disease), impaired reproduction, and an overall higher culling risk (Seifi et al., 2011; Oetzel and Miller, 2012; Martinez et al., 2014). Interestingly, the severity of the maladies associated with hypocalcemia appear to be dependent on the magnitude, persistency, and timing of SCH (Caixeta et al., 2017; McArt and Neves, 2020), such that transient hypocalcemia was unrelated to health metrics, whereas persistent and delayed hypocalcemia were associated with detrimental outcomes. These different hypocalcemia "types" may explain why inconsistencies exist regarding the association between hypocalcemia and transition cow health and performance (Martinez et al., 2012; Jawor et al., 2012; Gidd et al., 2015; Venjakob et al., 2018). Although it is becoming evident that not all cases of SCH are equivalent, it remains unclear what underlying factors explain the different hypocalcemia classifications.

Aside from the transition period, we and others have repeatedly demonstrated that experimental immune activation (via LPS infusion) markedly reduces circulating Ca in lactating cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018, 2019; Al-Qaisi et al., 2020) and the response is conserved across species (Tennant et al., 1973; Cardenas-Rivero et al., 1989; Elsasser et al., 1996; Carlstedt et al., 2000; Dias et al., 2013). Immune insults are prevalent in the transition period and it is likely that nearly all cows experience repeated challenges leading up to parturition (Trevisi and Minuti, 2018) and the frequency of these challenges likely relates to the magnitude of inflammation (Trevisi and Minuti, 2018). Interestingly, immune activation was originally hypothesized by early investigators to be involved with milk fever (Thomas, 1889; Hibbs, 1950), but only a few studies in recent literature have considered it to be a contributing factor (Aiumlamai et al., 1992; Eckel and Ametaj, 2016). It is of interest to fully understand the relationship between immune activation and Ca homeostasis and how providing intravenous Ca may alter animal health and productivity as this likely has practical implications to the transition period and farm profitability.

Recently, we demonstrated that oral Ca (in combination with live yeast) before and following LPS administration ameliorates hypocalcemia and improves production performance in lactating cows (Al-Qaisi et al., 2020). However, because the oral bolus contained both Ca and live yeast we were unable to isolate how each component influenced the response. Yeast has previously been shown to be immunomodulatory (Yuan et al., 2015) and improve production performance (Desnoyers et al., 2009; Zaworski et al., 2014; Broadway et al., 2015); however, some inconsistencies exist (Swartz et al., 1994; Robinson, 1997; Yuan et al., 2015). Given the marked changes in Ca homeostasis that occur following LPS administration and the role of Ca in regulating processes such as gastrointestinal motility (Daniel, 1983), metabolism (Martinez et al., 2012, 2014), and immune function (Lewis, 2001; Kimura et al., 2006), we hypothesized that Ca was the primary mediator of the beneficial effects observed by Al-Qaisi et al. (2020). Thus, study objectives were to evaluate the effects of maintaining eucalcemia following LPS administration on production parameters, inflammatory biomarkers, and PMN function in lactating cows.

In agreement with others (Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018; Al-Qaisi et al., 2020), we observed marked and sustained hypocalcemia

Table 2. Milk composition from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion during period 2 (P2)[1]

	Treatment[2]			*P*-value		
Item	LPS-Con	LPS-Ca	SEM	Treatment	Time	Treatment × time
Fat (%)	4.38	4.80	0.20	0.18	<0.01	0.12
Lactose (%)	4.64	4.67	0.06	0.73	<0.01	<0.01
Protein (%)	3.39	3.45	0.09	0.67	<0.01	0.06
MUN (mg/dL)	10.87	12.02	0.52	0.14	<0.01	0.45
SCC ($\times 10^3$ cells/mL)	401	244	91	0.24	0.53	0.58

[1]Data presented as an average value from samples collected during the 4 d of P2.

[2]LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

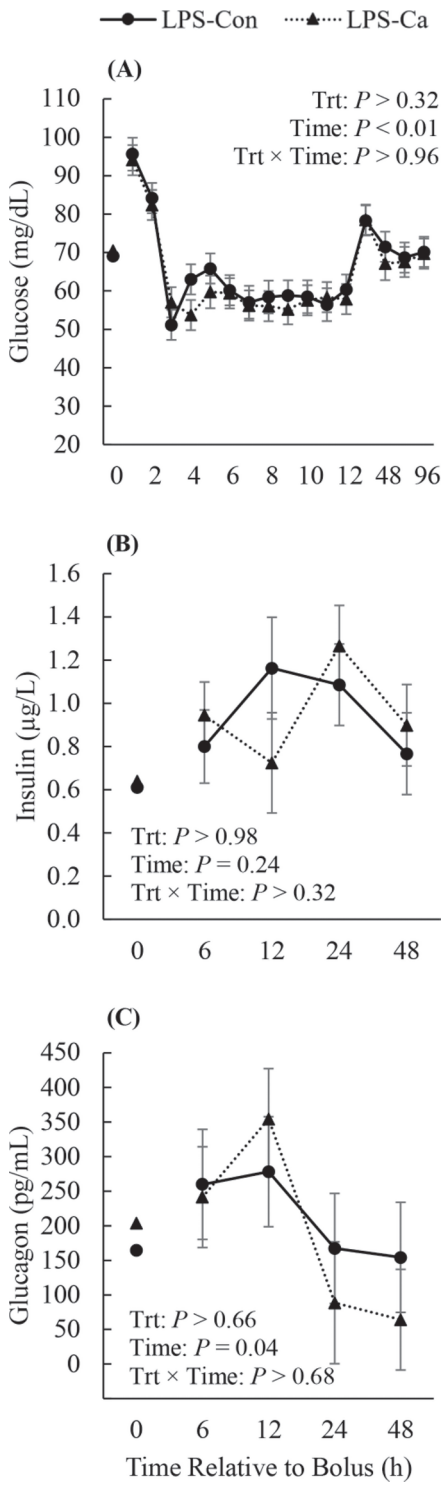

Figure 3. Circulating (A) glucose, (B) insulin, and (C) glucagon from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained before LPS administration (−0.5 and 0 h) and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

(in the LPS-Con cows) in response to LPS administration. Furthermore, we successfully maintained eucalcemia for 12 h post-LPS administration via continuous intravenous Ca infusion in the LPS-Ca treatment. The decrease in milk yield following LPS infusion allowed us to calculate the amount of Ca not going toward milk synthesis (milk Ca deficit). The milk Ca deficit was 10.4 and 6.5 g for LPS-Con and LPS-Ca cows, respectively. In LPS-Con cows the milk Ca deficit makes up the entirety of the total Ca deficit calculation. In LPS-Ca cows the amount of Ca infused during the 12-h eucalcemic-clamp was 13.7 g, which provides a total Ca deficit of 20.2 g in LPS-Ca cows. Even though we and others have repeatedly demonstrated LPS-induced hypocalcemia, little is known regarding where the 20.2 g of Ca is going and what mechanisms are regulating the disappearance.

Circulating Ca is normally under tight homeostatic control; however, during inflammation key Ca homeostatic regulators are markedly altered to favor hypocalcemia. For example, although not always consistent (Toribio et al., 2005; Merriman et al., 2018), hypoparathyroidism (Nielsen et al., 1997; Holowaychuk et al., 2012), increased circulating calcitonin and procalcitonin (Müller et al., 2000; Bonelli et al., 2018), and decreased 1,25-$(OH)_2$D concentrations (Waldron et al., 2003; Holowaychuk et al., 2012) are observed in immunoactivated states. Cytokines released during infection upregulate the parathyroid Ca sensing receptor, reducing the threshold necessary for suppression of parathyroid hormone secretion (Hendy and Canaff, 2016; Klein, 2018), resulting in hypocalcemia concurrently with hypoparathyroidism. Even if hormonal regulation favors hypocalcemia during immune activation, the acute decrease in Ca is likely too rapid to be explained by these mechanisms (Waldron et al., 2003). Rather it is probably mediated by changes in cellular/tissue uptake, systemic clearance, or both.

Extracellular Ca uptake by leukocytes is a key initial feature of activation and is necessary for cell proliferation and function (Lewis, 2001; Bréchard and Tschirhart, 2008). Leukocyte stimulation (i.e., interaction with antigen) triggers a signaling cascade resulting in Ca influx from the endoplasmic reticulum to the cytosol. This increase in cytosolic Ca signals influx from the extracellular space via Ca^{2+} release-activated Ca^{2+} channels (Lewis, 2001). Given the large gradient between extra- and intracellular Ca stores (extracellular concentrations 800-fold greater than intracellular; Goff, 1999) and the relatively small number of leukocytes in circulation (Roland et al., 2014) it is unlikely that increased cellular Ca uptake contributes to decreased circulating concentrations (Waldron et al., 2003). Urinary Ca clearance is another probable source of Ca loss;

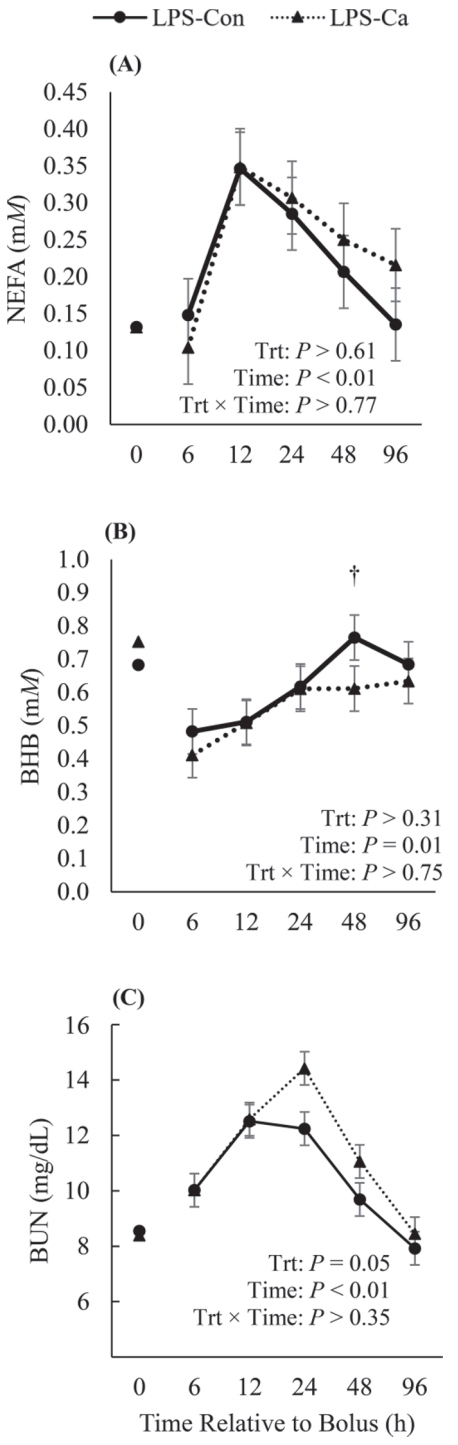

Figure 4. Circulating (A) nonesterified fatty acids (NEFA), (B) BHB, and (C) BUN from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained before LPS administration (−0.5 and 0 h) and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. † represents a tendency for a difference between treatments. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

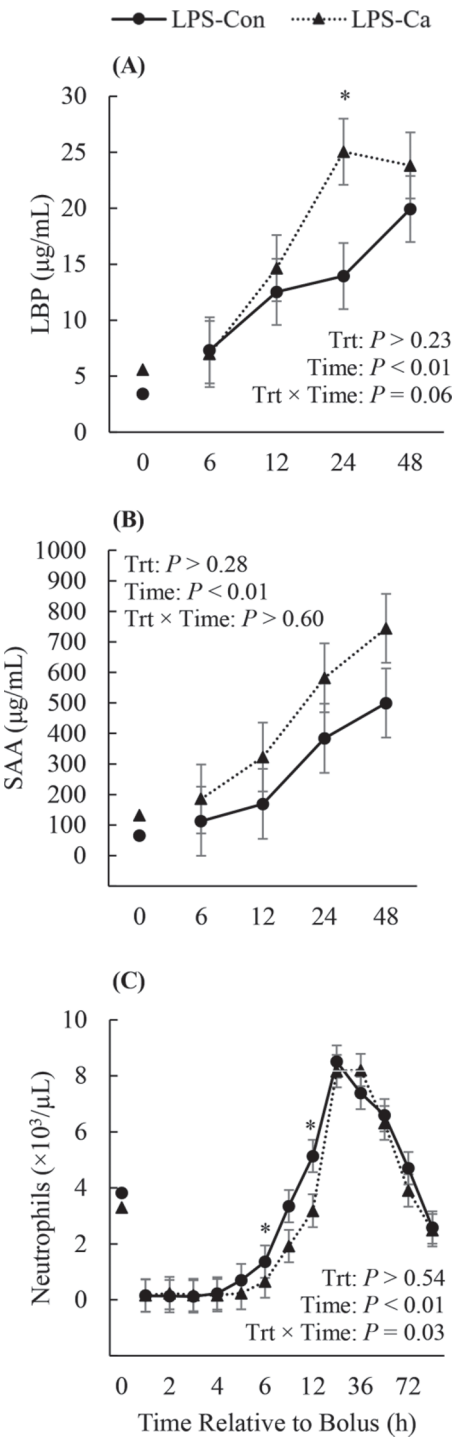

Figure 5. Circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), and (C) neutrophil counts from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained before LPS administration (−0.5 and 0 h) and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

however, reports in rodents demonstrated enhanced renal Ca conservation in response to LPS administration (Proksch et al., 1996; Ikeda et al., 2014; Meurer and Höcherl, 2019), and whether the same is true in ruminants remains to be fully elucidated. Interestingly, milk became more Ca concentrated (50%) following LPS administration and this effect was most pronounced in the LPS-Ca cows; however, the marked milk yield reduction eliminated the mammary gland as a meaningful Ca sink. Interestingly, increased Ca accumulation in ascites and liver has been observed following endotoxin administration in pigs (Carlstedt et al., 2000). Whether Ca sequestration at these sites explains the magnitude of plasma Ca reduction observed and whether it occurs similarly in ruminants is not fully understood and warrants further investigation, especially considering its practical importance to transition cows.

Decreased production performance (i.e., milk yield and DMI) is a typical response during infection and the magnitude of milk yield and DMI reduction observed herein agrees with previous LPS bolus studies (Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018). Interestingly, and in contrast to expectations, we observed an exacerbated decrease in milk synthesis and a delayed return to euphagia when eucalcemia was maintained. Pernicious phenotypic responses were surprising considering improved productivity occurred when oral Ca was provided before and following LPS administration (Al-Qaisi et al., 2020). Reasons for the aforementioned discrepancies are unclear, but may be explained by administration route, effects of secondary signals, or the confounding effects of yeast. Intravenous Ca is apparently detrimental to hormonal regulation of Ca when compared with oral delivery, and studies suggest it should not be used to treat SCH (Wilms et al., 2019). However, because Ca was purposely infused to avoid hypercalcemia, it is unlikely that signaling pathways controlling Ca status were meaningfully altered herein. The release of secondary secretagogues in response to oral Ca is an intriguing explanation for the improved performance observed by Al-Qaisi et al. (2020). For example, Ca-sensing receptors initiate a plethora of signaling pathways regulating appetite, gut motility, metabolism, immune function, and inflammation (Brennan et al., 2014; Rehfeld, 2017; Liu et al., 2018). It remains unclear whether the absence of these secondary mediators with intravenous Ca can explain the marked differences between our former (Al-Qaisi et al., 2020) and current experiment. Future research investigating the mechanisms of oral versus intravenous Ca administration following immunoactivation is warranted.

Successful immune activation was induced herein as indicated by changes in leukocyte dynamics and function, mild hyperthermia, and a marked increase in acute phase proteins (**APP**). In response to LPS-administration we observed a biphasic response in circulating neutrophils with initial neutropenia followed by neutrophilia, which agrees with past reports (Bannerman et al., 2008; Horst et al., 2018). Reduced circulating neutrophil counts likely represent leukocyte endothelial adherence, tissue migration, or increased appearance in the marginated pool (Hoedemaker et al., 1992; Lang et al., 1992; Walther et al., 2000). Interestingly, neutropenia was more pronounced in Ca-infused cows from 9 to 12 h postbolus compared with LPS-Con cows. Although not entirely clear, this may indicate increased neutrophil extravasation or reduced production from bone marrow. Calcium is well known for its roles in regulating leukocyte function (i.e., migration, endothelial adherence, phagocytosis, and so on; Cohen, 1994; Immler et al., 2018). Although not always consistent (Miltenburg et al., 2018), decreased circulating Ca concentrations have been associated with reduced leukocyte activity in cows (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2012; Zhang et al., 2019) and increasing Ca concentrations improved leukocyte activity (Vieira-Neto et al., 2017). Surprisingly, despite severe hypocalcemia in response to LPS we observed either no change or actually improved neutrophil function (i.e., MPO activity and oxidative burst). Additionally, Ca infusion had no effect on neutrophil function, which agrees with others (Kehrli and Goff, 1989; Miltenburg et al., 2018). Although our model is acute and is not

Table 3. Circulating leukocytes from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion during period 2 (P2)[1]

	Treatment[2]			*P*-value		
Item	LPS-Con	LPS-Ca	SEM	Treatment	Time	Treatment × time
White blood cells ($\times 10^3/\mu L$)	6.71	6.49	0.58	0.80	<0.01	0.93
Monocytes ($\times 10^3/\mu L$)	0.21	0.17	0.05	0.57	<0.01	0.73
Lymphocytes ($\times 10^3/\mu L$)	3.11	2.94	0.20	0.55	<0.01	0.57

[1]Data presented as an average value from samples collected during the 4 d of P2.
[2]LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused.

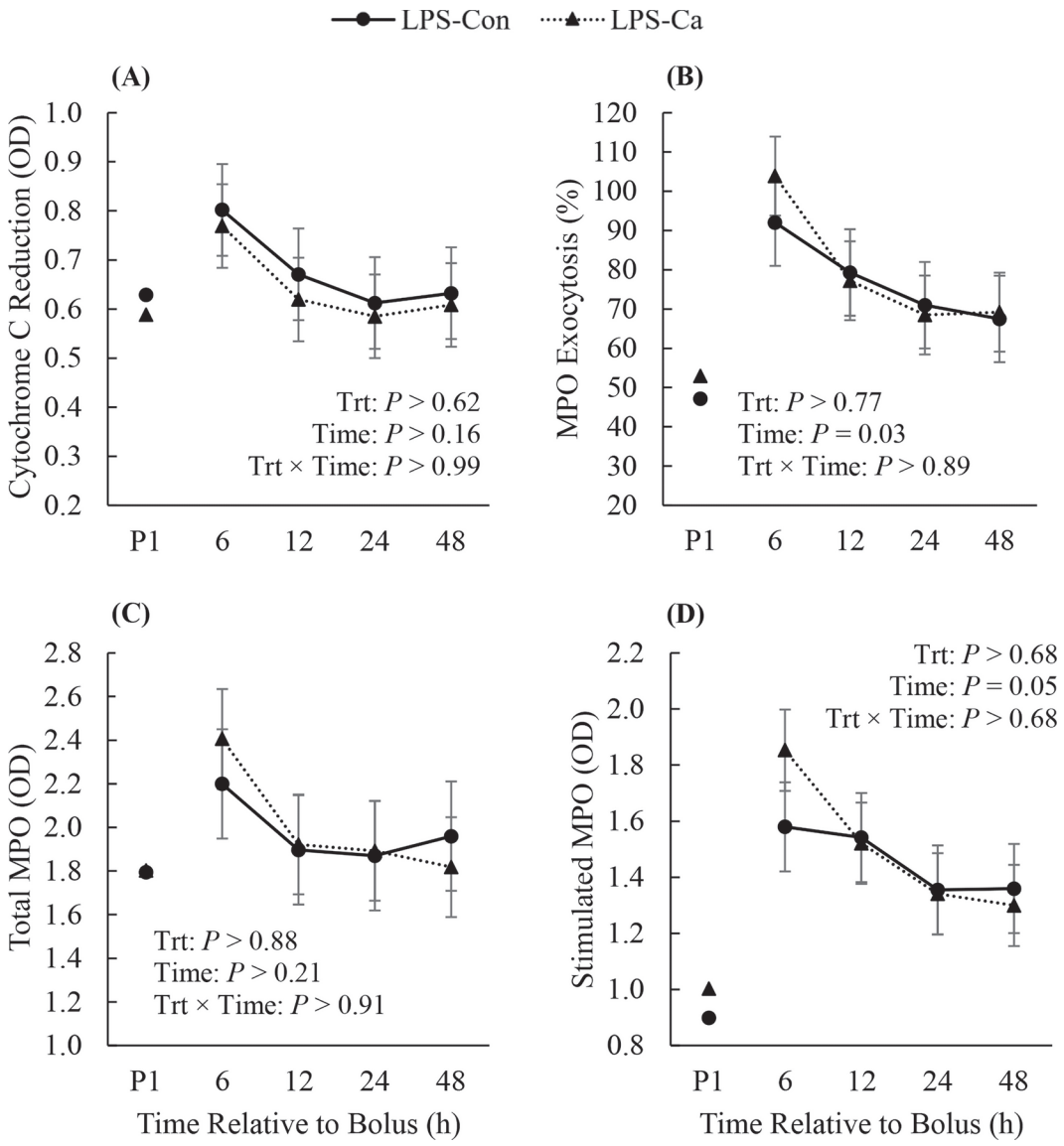

Figure 6. Neutrophil (A) cytochrome C reduction, (B) myeloperoxidase (MPO) exocytosis, (C) total MPO, and (D) stimulated MPO release from cows allowed to develop hypocalcemia versus cows maintained at eucalcemia following LPS infusion. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and included fixed effects of treatment, hour, and their interaction. P1 represents an average of measurements obtained during the 3 d of P1 and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. LPS-Con = LPS bolus; LPS-Ca = LPS bolus, Ca infused. OD = optical density.

accompanied with periparturient physiological changes, our results challenge the tenet that hypocalcemia is the primary cause of postcalving immunosuppression (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2014). It is likely that the proposed transition cow immunosuppression is multifactorial and cannot be explained by a single factor.

Interestingly, cows maintained at eucalcemia post-LPS administration had significantly increased LBP and numerically increased SAA when compared with cows allowed to develop hypocalcemia. This contradicts our previous report in LPS-infused cows (Al-Qaisi et al., 2020) as well as reports in inflamed transition cows (Benzaquen et al., 2015) provided oral Ca. Reasons for observing differences in APP are not entirely clear, but may be explained by the proposed role of Ca in LPS detoxification and its downstream effects on the inflammatory response (Skarnes and Chedid, 1964). During hypocalcemia, LPS aggregation is inhibited, allowing LBP to transfer LPS monomers to cluster of differentiation 14 and eventually to acute-phase high-density lipoproteins (**ap-HDL**) and apparently excreted by

the liver. Formation of ap-HDL is mediated by SAA displacement of apolipoprotein from normal HDL (as reviewed by Eckel and Ametaj, 2016). When eucalcemia is maintained, LPS monomers remain aggregated and this prevents its transfer to lipoproteins (Skarnes and Chedid, 1964). Maintaining extracellular Ca levels increases chemokine and cytokine release by circulating leukocytes, thereby intensifying the inflammatory response (Rossol et al., 2012; Klein et al., 2016). For these reasons, LPS-induced hypocalcemia was opined to serve as a protective strategy during immune activation (Malcolm et al., 1989; Collage et al., 2013; Klein, 2018). This tenet is supported by the fact that Ca administration in septic states increases rates of organ failure and mortality (Malcolm et al., 1989; Hastbacka and Pettila, 2003; Dias et al., 2013). Thus, increased LBP observed herein may represent impaired lipoprotein-mediated LPS detoxification. While Ca infusion may have prevented LPS disaggregation, the numerical increase in SAA concentrations from LPS-Ca cows may suggest formation of the ap-HDL molecule was not disrupted. Whether the changes in APP we observed with Ca infusion reflect an improved capacity to clear infection via pro-inflammatory mechanisms or an overly exaggerated immune response remain unclear, but the production responses (i.e., decreased milk yield and delayed return to baseline DMI) and heightened febrile response suggest the latter. The reasons why oral Ca administration in LPS-administered cows did not alter APP production are unclear, but may be explained by the quantity of Ca appearing in circulation and the rate at which it entered. Further investigation into the role of Ca in inflammation and how ameliorating hypocalcemia may influence animal health and wellbeing is of obvious interest.

The energetic requirements of immunoactivation are substantial and supporting the response requires marked alterations in nutrient partitioning (Johnson, 2012; Kvidera et al., 2017; Horst et al., 2018). The metabolic and hormonal changes implemented in response to LPS have been detailed in many of our recent publications (Kvidera et al., 2017; Horst et al., 2018, 2019). In agreement with our previous work, LPS administration increased circulating insulin, glucagon, and NEFA, but the patterns did not differ when eucalcemia was maintained. Not observing differences in circulating insulin was surprising considering the role of Ca in insulin secretion from pancreatic β cells (Rorsman and Trube, 1986). Additionally, others have previously demonstrated hypoinsulinemia when administrating the Ca chelator ethylene glycol tetraacetic acid in cows (Martinez et al., 2014). Interestingly, we observed increased circulating BUN in LPS-Ca cows, which agrees with changes in MUN, and as aforementioned may be connected to the changes observed in circulating APP as skeletal muscle proteolysis is an important source of AA needed for APP synthesis (Johnson, 2012). Circulating BHB was decreased with Ca administration; however, the biological significance of this decrease is unclear. The mechanisms driving decreased BHB concentrations during infection remain largely unexplained; however, increased peripheral utilization (Zarrin et al., 2014; E. A. Horst and L. H. Baumgard, unpublished data) and reduced production from the rumen epithelium (Pennington, 1952) due to decreased substrate are possible contributors. Interestingly, the return to baseline BHB concentrations occurred before the return of euphagia, which suggests reduced rumen epithelial production is not the primary reason for decreased circulating ketones.

CONCLUSIONS

Our results validate LPS administration as an effective model for inducing hypocalcemia. In contrast to oral Ca supplementation, we have demonstrated potential negative consequences (i.e., decreased milk yield and slower return to baseline DMI) of intravenous Ca infusion during immunoactivation and demonstrated little to no benefit of maintaining eucalcemia on leukocyte function. Additionally, Ca infusion increased circulating LBP concentrations, which may suggest an impaired capacity to detoxify LPS via noninflammatory routes. Future work should focus on the direct effect of Ca on lipoprotein-mediated LPS detoxification and how it may affect an inflammatory response.

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