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Short Communication
Genetics

Assessment of systemic inflammation following oral calcium supplementation in healthy postpartum multiparous dairy cows – a randomized controlled trial

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Abstract: Around parturition, dairy cows inevitably and perhaps necessarily experience some degree of systemic inflammation, but when excessive or dysregulated, it may contribute to health disorders. As immune activation decreases blood Ca, greater extracellular Ca availability may potentiate or sustain inflammation. We hypothesized that in clinically healthy multiparous cows, postpartum administration of supplemental Ca would increase serum concentrations of inflammatory markers. The objective of this randomized controlled trial was to investigate a possible effect of supplementing calcium (Ca) on postpartum systemic inflammation in dairy cows. Healthy cows (n = 101) from 2 commercial dairy farms in Ontario calving into parity 2, 3, or 4 were enrolled. Cows were blocked by parity and randomly assigned to receive an oral bolus of Ca (42 g of Ca) within 12 h after calving and a second bolus 12 h later (TRT; n = 51), or no Ca supplementation (CON; n = 49). Concentrations in serum of total Ca (tCa), haptoglobin (Hp), and albumin (ALB) were assessed at d 0 (within 12h postpartum), 0.5 (12 h later), 1, 2, 4, 6, and 8 postpartum; ionized calcium (iCa) was assessed at d 0, 0.5, 2, and 4 and lipopolysaccharide binding protein (LBP) and serum amyloid A (SAA) were assessed at d 0, 2, and 4. Multivariable linear regression models of each outcome accounting for repeated measures included treatment, parity (2 vs. 3 or 4), farm, sampling day, baseline concentration (d 0), and interactions of treatment with farm, parity, and day. Results are presented as least squares means and 95% confidence intervals. Concentration of tCa tended to be greater at d 0.5 (TRT 2.07 mmol/L [2.03–2.12]; CON 2.01[1.96–2.06]) but was lesser at d 2 (TRT 2.18 [2.13–2.23]; CON 2.27 [2.23–2.32]) in TRT than CON cows. Concentrations of LBP were greater in TRT (2.28 ng/mL [2.06–2.50]) than CON (1.99 [1.77–2.21)] in parity 2, but not different in older cows (TRT 2.28 ng/mL [2.06–2.50]; CON 1.99 [1.77–2.21). Concentrations of SAA were greater in TRT than CON cows at d 2 (TRT 135 ug/mL [124 −146]; CON 114 [75 −106]). Treatment had no effect on ALB or Hp. In clinically healthy cows, oral Ca supplementation had a small transient effect on blood tCa and little indication of increasing inflammation based on the analytes evaluated.

Subclinical hypocalcemia [SCH; serum total calcium (tCa) < 2.15 mmol/L] can occur in the days after calving in 55 to 85% of multiparous dairy cows and is associated with impaired innate immune function and increased risk of metritis and other clinical diseases (Rodríguez et al., 2017; Martinez et al., 2018). Therefore, it is commonly assumed that greater tCa concentration in early postpartum would be associated with improved health outcomes. Accordingly, approximately 70% of dairy farms in the USA use Ca supplementation in postpartum cows (USDA, 2016). The results of controlled trials of Ca supplementation to reduce the risk of disease or improve production or reproduction are equivocal (Miltenburg et al., 2016; Valldecabres et al., 2023). Several studies point to selective supplementation of multiparous cows based on previous milk yield or other risk factors, such as lameness or BCS (Martinez et al., 2016; Leno et al., 2018). Moreover, (Martinez et al., 2016) conducted a study with 450 cows in the United States and demonstrated unexplained detrimental effects on uterine health and reproductive performance of primiparous cows supplemented with oral Ca postpartum. Transient hypocalcemia (≤1.77 mmol/L at 1 DIM but ≥2.20 mmol/L at 4 DIM) was associated with greater milk yield, while delayed or persistent hypocalcemia (≤2.20 mmol/L at 4 DIM) was associated with lesser milk yield and worse reproductive performance (Seely and McArt, 2023). Therefore, Ca supplementation strategies should at least be selective and perhaps fundamentally rethought.

Recent data have underlined a link between inflammation and Ca metabolism (Al-Qaisi et al., 2020; Horst et al., 2020). Around parturition, dairy cows inevitably and perhaps necessarily experience some degree of systemic inflammation (Bradford et al., 2015), but excessive or dysregulated inflammation likely contributes to inflammatory disease such as metritis and endometritis (Pascottini and LeBlanc, 2020). The degree and duration of both postpartum inflammation and hypocalcemia are associated with health, production, and reproductive performance (Bertoni et al., 2008; Neves et al., 2018; Venjakob et al., 2018), although the relationship between postpartum Ca metabolism and inflammation is not fully understood (Al-Qaisi et al., 2020; Horst et al., 2020). Immune activation decreases blood Ca (Al-Qaisi et al., 2020), so greater extracellular Ca availability might potentiate or sustain inflammation (Hendy and Canaff, 2016; Klein, 2018). Horst et al. (2020) infused Ca intravenously to maintain blood Ca following a lipopolysaccharide (LPS) challenge in 12 non-pregnant lactating

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cows. Unexpectedly, maintaining eucalcemia intensified inflammation and tended to worsen milk production. This supports the notion that decreased Ca availability during an inflammatory response could be a protective mechanism (Eckel and Ametaj, 2016). Further investigation is needed on whether early postpartum Ca supplementation, especially when administered to all cows, can heighten systemic inflammation. The objective of this randomized controlled trial was to investigate the role of supplemental Ca in postpartum systemic inflammation (SI) in healthy multiparous dairy cows. We hypothesized that in clinically healthy cows, administration of supplemental Ca would increase serum concentrations of markers of inflammation.

The experimental protocol was reviewed and approved by the University of Guelph Animal Care Committee (AUP 4633). We conducted a randomized clinical trial of oral postpartum Ca supplementation on 2 commercial dairy farms with 475 (Farm A) and 460 (Farm B) lactating cows in Ontario, Canada, between September and December 2021. Within each farm, treatments were balanced for cows entering parity 2 vs. 3 or 4, and in sequential blocks of 4. To avoid confounders of the inflammatory response, cows with twins or dystocia, or presenting signs of clinical disease (e.g., milk fever, retained placenta (RP), displaced abomasum (DA), metritis, or clinical mastitis (CM), as described by Kelton et al., 1998) were not included or excluded if diagnosed after enrollment. Cows that received Ca supplementation outside the experimental protocol or anti-inflammatory medication due to farm management decisions were excluded. At calving, eligible cows were randomly assigned to one of 2 treatment groups: one oral Ca supplementation bolus (42 g of Ca, Bovikalc, Boehringer Ingelheim Animal Health) within 12 h after calving and a second Ca bolus approximately 12 h later (TRT group), or no Ca supplementation (CON, control group). Treatment assignments were generated with a randomization tool (Sealed Envelope, London, UK). Older cows (starting ≥ fifth lactation) were purposely not included because of their greater risk of milk fever (MF) (Degaris and Lean, 2009).

Farm staff was blinded to treatment allocation; blinding of the research team members was not possible, but the outcome measures did not depend on subjective evaluation (objective laboratory measurements). The research team visited each farm twice daily (Farm A: 0600 and 1600; Farm B: 0700 and 1700) and were responsible for the treatment allocation, oral Ca bolus administration, body condition score (BCS) assessment at enrollment, and blood sampling.

We measured postpartum serum concentrations of total Ca (tCa) and ionized Ca (iCa) and 4 acute phase proteins (APP) as markers of inflammation: haptoglobin (Hp; positive APP), serum amyloid A (SAA; positive APP), LPS binding protein (LBP, positive APP) and albumin (Alb; negative APP). Our primary outcome was serum Hp concentration because it is the most studied APP in dairy cows in the early postpartum period. Based on Dubuc et al. (2010), a difference of 0.3 g/L was expected between cows that do or do not develop reproductive tract inflammatory disease. To detect a difference in mean ± SD blood Hp of 0.3 ± 0.5 g/L with 95% confidence and 80% power, 44 cows per group were required. We aimed to enroll at least 46 cows per group to cover losses to follow-up, for a total of 92 multiparous cows.

Body condition score (BCS; 1-to-5 scale, measured in 0.25-point increments; (Ferguson et al., 1994) was recorded at enrollment (within 12h after calving; d 0). Blood samples for ionized calcium (iCa) were collected at d 0 (before Ca bolus administration), and 12 h (d 0.5), 48 h (d 2), and 96 h (d 4) after enrollment. Blood samples to measure tCa, BHB, albumin (ALB), and haptoglobin (Hp) were collected at d 0, 0.5, 1, 2, 4, 6, and 8. Serum amyloid A and LBP were assessed at d 0, 2, and 4. Blood for tCa, ALB, Hp, and BHB was collected into a 10-mL evacuated tube without anticoagulant (Vacutainer Precision Glide, Becton Dickinson); an additional 10-mL tube without anticoagulant was collected at d 0, 2, and 4 for SAA and LBP concentration assessment. A 3-mL tube with lithium heparin (Vacutainer, Becton Dickinson) was used to collect whole blood for iCa assessment. Analysis of iCa and BHB was done on-farm immediately after blood sampling with a validated portable clinical analyzer (i-Stat System, Abbott Laboratories, Abbott Park, IL) and hand-held meter (Precision Xtra Meter and β-ketone test strips, Abbott), respectively. Blood samples collected into tubes without anticoagulant were placed on ice and, within 2 h of collection, centrifuged (1500 × g for 15 min) at the University of Guelph. Aliquots of serum were stored at −20°C (for tCa, ALB, Hp) or −80°C (for SAA, LBP) until analysis. Serum tCa, Hp, and ALB were measured at the Animal Health Laboratory (University of Guelph). Concentrations of SAA and LBP were measured in serum samples, thawed at room temperature (~22°C), using commercial ELISA kits (Multispecies SAA, Tridelta Development Ltd.; LBP various species, Hycult Biotech Inc., Wayne, USA) as per manufacturers' instructions. The assay lower limit of quantification was 9.40 µg/mL for SAA and 0.39 µg/mL for LBP, and the inter- and intra-assay coefficients of variation were 15 and 22% for SAA and 7.2 and 7.8% for LBP, respectively. Differences at d 0 (baseline data) for BCS and the outcome variable were assessed with t-tests. For each outcome, a multivariable linear regression model (PROC MIXED, SAS, version 9.4) accounting for repeated measures was built. Treatment, parity, farm, sampling day, baseline value, and the 2-way interactions of treatment × farm, treatment × parity, and treatment × sampling day were included as covariates. The distributions of the model residuals were assessed by graphic and analytic (Shapiro-Wilk test) methods. The residuals of BHB, iCa, and Hp were not normally distributed, so these variables were transformed to the natural log scale. For each model, the covariance structure for repeated measures was selected based on the lowest Akaike Information Criterion (AIC; tCa, iCa, SAA, LBP: unstructured; BHB and Hp Toeplitz; ALB: heterogeneous Toeplitz). Models were built using a backward elimination approach, with variables removed when $P > 0.1$. In the presence of an interaction ($P \leq 0.1$), differences between treatments by sampling day or within parity group were assessed with multiple comparisons. Results are presented as LSM and 95% CI; LSM and 95% CI were back-transformed when the transformation was applied to the dependent variable. Graphical representation of treatment × sampling day interactions includes baseline data (d0; raw means and 95% CI).

Of 115 cows enrolled, 14 were excluded: 1 with MF, 7 with RP, 1 with metritis, 2 with CM, and 3 with twins. The analyses included 101 healthy multiparous Holstein cows, 31 from Farm A (16 2nd parity and 15 3rd or 4th parity) and 70 from Farm B (27 2nd parity and 43 3rd or 4th parity). At calving, BCS was not different between treatment groups (BCS ≥3.25: CON 21/50; TRT 19/51, Chi-Square $P = 0.6$). Data from all included cows were used in the analyses of tCa, BHB, ALB, HP, SAA, and LBP but only 79 cows were used for the iCa analysis as the remaining cows had at least one iCa value missing because of problems with supply of

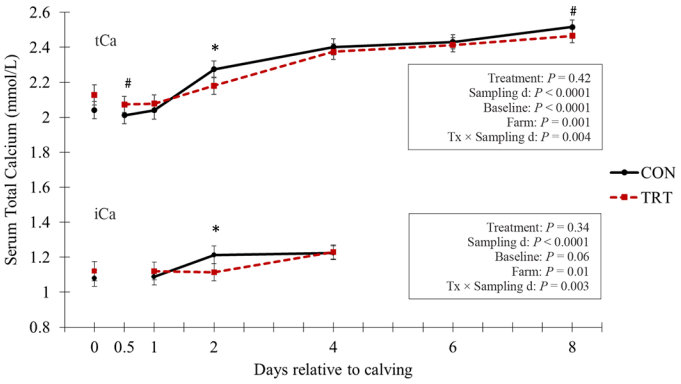

Figure 1. Least squares means (and 95% confidence intervals) of serum total Ca (tCa; TRT n = 51; CON n = 50) and ionized Ca (iCa; TRT n = 39; CON n = 40) concentrations in healthy postpartum multiparous cows in a randomized controlled trial of oral Ca bolus supplementation administered at calving (d 0) and 12 h (d 0.5) later (TRT) or control (CON; no Ca supplementation). Day 0 represents the baseline means. Within a sampling day, * denotes a difference between treatment groups ($P < 0.05$), and # denotes a tendency ($P < 0.1$).

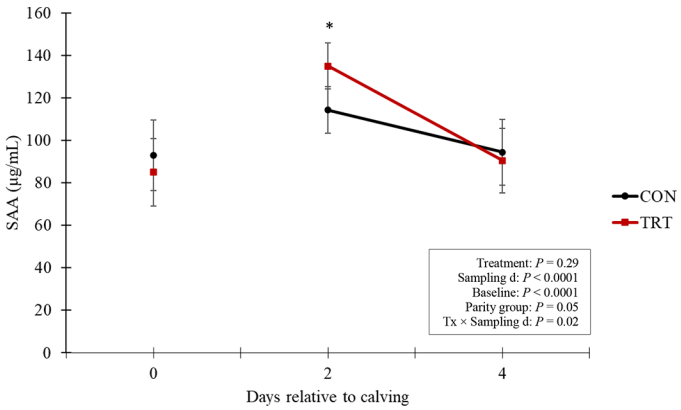

Figure 2. Least squares means (and 95% confidence intervals) of Serum Amyloid A (SAA) in healthy postpartum multiparous cows in a randomized controlled trial of oral Ca bolus supplementation administered at calving (d 0) and 12 h (d 0.5) later (TRT; n = 51) or control (CON; no calcium supplementation; n = 50). Day 0 represents the baseline means. Within a sampling day, * denotes difference between treatment groups ($P = 0.009$).

the test cartridges. The nadir of tCa was observed at 0.5 d after enrollment (mean ± SD, 2.05 mmol/L ± 0.21). Ionized Ca was not collected at this time point; the iCa nadir was observed at d 0 (1.10 mmol/L ± 0.15).

At enrollment, tCa and iCa were not different between treatments ($P > 0.26$). We did not detect an effect of treatment on BHB (CON: 0.6 [0.51–0.60]; TRT 0.5 [0.50–0.59] mmol/L; $P = 0.75$), ALB (CON 35.6 [35.17 - 35.99]; TRT 35.6 [35.15 - 35.96] g/L; $P = 0.93$), or HP (CON 0.335 g/L [0.304 - 0.368]; TRT 0.334 g/L [0.303 - 0.367]; $P = 0.96$) concentrations. A treatment by time interaction was detected for tCa ($P = 0.004$) and iCa ($P = 0.003$; Figure 1). At 0.5 d, TRT tended to have greater tCa than CON (2.07 [2.03- 2.12] and 2.01 [1.96–2.06] mmol/L, respectively; $P = 0.06$). At 1 d there were no differences between treatments, and 2 d, TRT had lower tCa than CON (2.18 [2.13–2.23] vs. 2.27 [2.23–2.32] mmol/L, respectively; $P = 0.007$). There were no differences in tCa detected at 4 and 6 d ($P > 0.38$). At 8 d, we observed a tendency for TRT to have lower tCa than CON (2.47 [2.43–2.51] vs. 2.52 [2.48–2.56] mmol/L, respectively; $P = 0.08$; Figure 1a). The iCa followed the same pattern as tCa, with a difference between treatments detected at 2 d (TRT 1.11 [1.07–1.16]; CON 1.21 [1.16–1.26] mmol/L, $P = 0.005$; Figure 1).

Of the assessed inflammatory markers, we detected a treatment × sampling day ($P = 0.02$) and treatment × parity interaction ($P = 0.09$) for SAA and LBP, respectively. At 2 d, TRT had greater SAA concentrations than CON (135.0 [124.1–145.8] vs. 114.4 [103.5-125.3] µg/mL, respectively, $P = 0.009$); no differences were detected at 4 d (Figure 2). In 2nd parity cows, TRT tended to have greater LBP concentrations (2.28 [2.06–2.50] vs. 2.11 [1.92–2.30] µg/mL, respectively; $P = 0.07$); differences were not detected in older cows (Figure 3).

By supplementing oral Ca postpartum to healthy multiparous cows, we observed small transient effects on serum tCa and some indications of modest increases in blood markers of inflammation. We hypothesized that providing supplemental Ca to transition cows without apparent transition challenges would increase peripartum inflammation. This hypothesis was based on previous findings that maintaining Ca levels during an inflammatory challenge was associated with greater inflammation and reduced milk yield (Horst et al., 2020), and greater risk of uterine disorders with oral Ca administration in primiparous cows (Martinez et al., 2016). Here, treatment had small effects on blood Ca and correspondingly small or no effects on markers of inflammation, so we found little evidence under the conditions of this trial that oral Ca supplementation exacerbates systemic inflammation.

Serum tCa concentrations within 1 d of parturition tended to be greater (+0.06 mmol/L) in TRT cows but we did not detect differences at 1 d. Conversely, at d 2, tCa concentrations were significantly, albeit modestly, lower in TRT than in CON (−0.09 mmol/L). Domino et al. (2017), assessed the same Ca bolus administration protocol (n = 10 cows; parity 2 to 5) vs. a negative control

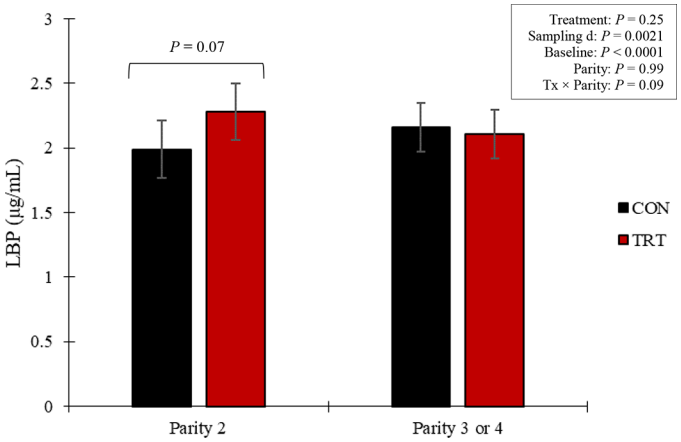

Figure 3. Least squares means (and 95% confidence intervals) of serum lipopolysaccharide binding protein (LBP) in healthy postpartum multiparous cows in a randomized controlled trial of oral calcium bolus supplementation administered at calving (0d) and 12 h (0.5d) later (TRT; n = 51) or control (CON; no calcium supplementation; n = 50).

(n = 10 cows; parity 2 to 4) and observed a greater tCa difference at 12 h (approximately 0.2 vs. 0.06 mmol/L in our study). However, these differences were not statistically significant in either study. At 1 d, Domino et al. (2017) observed a statistically significant tCa increase of approximately 0.3 mmol/L in treated cows. The fact that we included only healthy and ≤4 lactation multiparous cows may explain the differences. However, both studies align in showing modest transient effects of oral Ca supplementation as used on circulating Ca concentrations. In postpartum multiparous cows, a decrease in tCa the day after calving, followed by an increase by 4 DIM may be part of, and perhaps necessary for successful metabolic adaptation to lactation (Neves & McArt, 2020). We speculate that Ca supplementation may have disrupted Ca homeostatic mechanisms. Further studies are needed to confirm this hypothesis.

Measuring APP, nonspecific markers of inflammation, provides partial characterization of SI (Cray et al., 2009). In dairy cows, some degree of inflammation, characterized by changes in APP (Bertoni et al., 2008) and cytokine concentrations, is expected around parturition (Bradford et al., 2015; Pascottini and LeBlanc, 2020). The studied APP (ALB, Hp, SAA, and LBP) are among the better-described SI markers in dairy cows (LeBlanc, 2023). We observed a treatment effect on SAA and LBP but did not detect an effect of treatment on ALB and Hp. Haptoglobin had moderate to high specificity (~80%) and low sensitivity (~40 to 50%) when measured in the first week postpartum to predict metritis or purulent vaginal discharge (Dubuc et al., 2010). Others have evaluated associations of blood Hp in the early postpartum period with uterine disease (Huzzey et al., 2009; Schneider et al., 2013; Bogado Pascottini and LeBlanc, 2020), calving-related disorders (e.g., need for assisted calving or retained placenta; Pohl et al., 2015), or responses to feed restriction (Pascottini et al., 2019) and systemic LPS challenges (Chandler et al., 2022). We used APP to evaluate systemic inflammation related to a management practice in healthy cows. The Hp concentrations observed here were similar to previous reports in healthy cows. For example, Huzzey et al. (2009) reported Hp concentrations in clinically healthy cows of 0.58 ± 0.12 and 0.31 ± 0.08 g/L on d 3 and d 6 postpartum, respectively.

Although SAA and HP increase proportionally to the severity of the challenge (Jacobsen et al., 2004), previous work has demonstrated that SAA is a more sensitive marker than Hp in an acute inflammatory episode (Heegaard et al., 2000; Chandler et al., 2022). For example, in a LPS challenge (n = 14 cows), SAA concentration peaked about 24 h post challenge (4-fold increase) and was above baseline concentration for 3 d, while Hp peaked later, at 48 h with a smaller magnitude of difference (2-fold increase; Chandler et al., 2022). This could explain why we observed an effect of Ca supplementation on SAA but not on Hp. We observed a greater SAA concentration in supplemented than CON cows at d 2, which correspond to 48 and 36 h after the first and second bolus administrations, respectively. At d 4, there were no differences between treatments in SAA.

An interaction of treatment with parity was detected in the LBP model. Younger multiparous cows receiving Ca boluses tended to have greater tCa concentration than controls. The magnitude of the tCa decline postpartum is smaller in younger cows (Szenci et al., 1994; Venjakob et al., 2017). Therefore, cows in parity 2 would have less potential need for Ca supplementation than 3rd or 4th lactation cows. We speculate that in cows resilient to the natural postpartum drop in Ca, the potential contribution to inflammation by oral Ca bolus administration may be relevant. The physiological mechanisms that explain both the potential benefits and detrimental effects in different cow populations following Ca bolus supplementation are not completely understood. Previous work suggested that postpartum oral Ca supplementation should be targeted e.g., to cows entering parity ≥3, over-conditioned cows, and lame cows (Leno et al., 2018). A recent meta-analysis of 8 studies of postpartum oral Ca bolus administration concluded that in the entire population, milk production, and reproductive performance did not improve following supplementation (Valldecabres et al., 2023). There were too few studies to investigate effects of treatment within subgroups of cows.

A third treatment group with sham bolus administration but without supplementation of Ca would have allowed us to rule out any potential inflammatory response caused by handling. However, our goal was to assess possible unintended effects of the practice as employed in the field. We are not aware of published data evaluating the effects of handling alone on inflammatory markers.

To avoid confounding, we only included cows that did not experience health disorders during the study period. We speculate that if the study included healthy and unhealthy cows an interaction of treatment and health status may be present. Because we included only Holstein cows, the results cannot necessarily be generalized to other dairy breeds.

In this randomized controlled trial, oral Ca supplementation had a small transient effect on blood Ca and indications of modest increases in some markers of inflammation. Under the conditions of this trial, we did not detect evidence of meaningful exacerbation of SI by administration of oral Ca supplementation to clinically healthy multiparous cows. We encourage selective, evidence-based assessment of the use of Ca supplements.

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NOTES

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