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Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows

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ABSTRACT

The objective of this study was to determine the effects of subclinical hypocalcemia on reproductive performance in dairy cows. In a prospective cohort study, 97 cows on 2 dairy farms with automatic milking systems were monitored for subclinical hypocalcemia. Animals were enrolled 7 ± 3 days prior to estimated calving date and three parity groups were defined based on the lactation that the animals were going to start: lactation = 1, lactation = 2, and lactation ≥3. Serum calcium concentration (**Ca**) was measured in all animals in the first 3 DIM and subclinical hypocalcemia (**SCH**) was defined as Ca ≤ 8.6 mg/dL; animals that presented a low Ca level during all 3 days were classified as chronic SCH (**cSCH**). Return to cyclicity during the voluntary waiting period was analyzed based on weekly progesterone concentrations measured in serum. Information on reproductive outcomes (i.e., number of breedings, pregnancy status, days open, etc.), were collected from on-farm software after all study cows had completed their study period. Chronic SCH was present in all parity groups with higher incidence in multiparous animals (20% of parity = 1, 32% of parity = 2; and 46% of parity ≥ 3 animals). The cSCH animals took longer to show active ovaries when compared to eucalcemic and SCH animals. In a multivariable Cox's Proportional Hazard model animals with normal Ca were 1.8 times more likely to return to cyclicity by the end of the voluntary waiting period when compared to cSCH animals. Animals with cSCH also had 0.27 odds of being pregnant at first service compared to eucalcemic cows when analyzed by multivariable logistic regression. Subclinical hypocalcemia had a negative effect on return of ovarian function during the voluntary waiting period and decreased the odds of pregnancy at first service. Those cows with cSCH had an even more pronounced impaired reproductive function than those with one subclinical measurement.

1. Introduction

High producing dairy cows face a challenging period when transitioning from late pregnancy to early lactation. Energy demand increases by 2.5-fold [1,2] and mineral requirements, especially calcium, are increased by over 65% to support lactogenesis in early lactation [3]. As a result, homeorhetic adaptations take place to adjust for such increased demands [4]. Unsuccessful adaptation to transition period challenges has been associated with increased occurrence of diseases [5–7], decreased milk production [6,8] and impaired reproductive performance [6,7].

Hypocalcemia has been reported as a problem in the dairy industry for over two centuries, especially clinical cases also known as milk fever [9]. Nutritional management of cations and anions during dry period and early lactation, along with an increased understanding of transition period physiology have been the key to decreasing the incidence of milk fever to rates as low as 1% [10,11]. However, despite the low incidence of clinical cases in modern dairy cattle, reports have shown that prevalence of subclinical hypocalcemia is high in the US [10] with as many as 73% of animals of parity ≥ 3 experiencing subclinical hypocalcemia during the first 3 DIM [12]. Subclinical hypocalcemia is defined as a low calcium concentration without the development of clinical signs (e.g. recumbency, lethargy, hypothermia, and rumen atony). Several

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thresholds have been used to define subclinical hypocalcemia and they range from 8.0 mg/dL to 8.8 mg/dL [3,6,10,13,14].

Even though, hypocalcemic animals may not develop clinical signs, further metabolic and health consequences have been associated with the occurrence of this mineral imbalance during early lactation and it has a great economic impact in modern dairy enterprises [11]. Traditionally, hypocalcemia has been associated with occurrence of dystocia, uterine prolapse, retained placenta, mastitis and decreased rumen and abomasum motility [13,15,16], as well as impaired immune cell functions [17]. More recently, research has shown that subclinical hypocalcemia is associated with an increased risk of metritis [14] and displaced abomasum [6] as well as an increase in culling rates [18,19] in dairy cows. Additionally, it has been reported that grazing animals that have low calcium concentrations within the first week post-partum have increased chances of developing multiple clinical disorders during lactation [20]. Although hypocalcemia has been associated with impaired reproductive performance by delaying resumption of ovarian cyclicity [21] and impaired response to estrus synchronization protocols [22], the effect of prolonged low blood calcium concentration in early lactation on reproductive performance have not been described. The objective of the present study is to evaluate the association between subclinical hypocalcemia in the first 3 days of lactation with reproductive performance during the first 120 DIM.

2. Materials and methods

2.1. Study population, study design, and sample size calculation

A prospective cohort study was conducted from a convenience sample of 2 commercial farms in Central New York. The 2 herds were selected as part of another study [12] because of the use of automatic milking systems (**AMS** - Astronaut A3 and A4, Lely Industries N.V, Rotterdam, The Netherlands). Herd A milked over 700 cows using 14 AMS while herd B used 7 AMS to milk 400 cows. Within herd, all cows and heifers calving between June 11th of 2012 and August 8th of 2012 were enrolled in the study. These animals were followed forward for the first 120 days of lactation.

In both farms cows were housed in free-stall barns; concrete stalls were covered with mattresses. Herd A bedded with waste paper-pulp while herd B used sand bedding. In both farms, animals from different parity groups were co-mingled in the fresh cow pen where only one milking unit was available; after leaving the fresh cow pen cows were separated according to their parity group into pens with at least 2 milking units. A ratio of 60 animals/AMS was observed regardless of the lactation period of the cows. Animals were fed partial mixed ration (**PMR**) consisted of 80% forage and 20% concentrate during the dry period, and 55% forage and 45% concentrate for the lactation groups. The diet was formulated to meet or exceed the NRC nutrient requirements for lactating Holsteins according to farm conditions. A part of the total diet was offered to the animals as a grain mixture in the form of pellets in the AMS, the amount of pellets fed depended on both DIM and milk being produced at the cow level. In the North Eastern United States, dairy herds, like the study herds, routinely manage the potassium content of dry cow diets through manipulation of manure application fields and forage choice, e.g. limiting alfalfa feeding to dry cows.

The sample size was calculated in order to detect a minimum of 1.3 kg difference in milk production between eucalcemic and hypocalcemic animals with a standard deviation of 3.5 kg, power of 80% and a 95% confidence interval [12]. A post-hoc evaluation determined that the 97 animals enrolled in this study would achieve 80% power and 95% confidence interval, given the 15% difference between the proportions of animals returning to cyclicity at the end of the voluntary waiting period (**VWP**) between the two calcium status groups.

2.2. Data collection and case definition

Animals were enrolled 7 ± 3 days prior to expected calving date and body condition score (**BCS**) was determined using a 5-point scale [23]. A blood sample was collected from coccygeal vessels using a Vacutainer tube without anticoagulant and a 20-gauge x 2.54 cm Vacutainer needle (Becton, Dickson and Company, Franklin Lakes, NJ) at the following time points: pre-partum, at 1, 2, 3, 5, and 7 days in milk (**DIM**); and weekly thereafter during the VWP (between second (14 ± 3 DIM) and seventh week (49 ± 3 DIM) post-partum).

Total calcium concentration was determined pre-partum and at d 1, 2, and 3 after calving to assess calcium status around parturition. Negative energy balance was determined by the concentrations of serum non-esterified fatty-acid (**NEFA**) concentration measured pre-partum and 5 days postpartum; and serum β-hydroxybutyrate (**BHB**) concentration measured on d 3 and 5 post-partum. Finally, serum progesterone (**P4**) concentration was used to determine the presence of active corpus luteum, and consequently return to cyclicity, at d 7 and weekly starting at the second week of lactation (14 ± 3 DIM) until the seventh week post-partum (49 ± 7 DIM).

All blood samples were spun for 15 min at 2,000×*g* at the farm in a portable centrifuge within 30 min of collection and immediately placed in ice to be transported back to the laboratory where samples were kept at −20 °C until analysis were performed according to recommendations [24]. Serum samples were sent to Cornell University Animal Health Diagnostic Center (Ithaca, NY) for determination of calcium and NEFA concentration. Total calcium concentration was determined using chemical reactions (Roche Diagnostic reagents) and NEFA was determined using a commercial kit (NEFA-C, Wako Chemicals USA Inc., Richmond, VA). Serum β-hydroxybutyrate (**BHB**) concentration was evaluated cow side using the Precision Xtra meter (Abbott Laboratories, Abbott Park, IL) [25]. And progesterone concentrations were determined using radioimmunoassay as previous described by Beam and Butler [26].

Negative energy balance (**NEB**) was defined using pre- and post-partum measurements of NEFA and BHB as described by previous reports [27,28]. Briefly, metabolites concentrations were dichotomized as follows: pre-partum NEFA ≥0.3 mEq/L, post-partum NEFA ≥0.7 mEq/L, and post-partum BHB ≥1.2 mmol/L. Return to cyclicity during the VWP was determined by measurement of P4 during the VWP. Cows having P4 concentration ≥1 ng/mL were considered to have a functional corpus luteum, thus cyclic [29]. Subclinical hypocalcemia was defined based on serum total calcium concentration (**Ca**). A cut-off point of Ca ≤ 8.6 mg/dL was used to dichotomize each individual sample into hypocalcemic or eucalcemic [14].

Occurrence of any diseases (e.g., displaced abomasum, clinical ketosis, metritis, and retained placenta) during the first 10 DIM and reproductive performance data was recorded in Dairy Comp 305 (Valley Ag. Software, 2009) and retrieved at the end of the study period. Standard disease definitions as described elsewhere [12] were discussed with the farms at the start of the study; however, each farm implemented their own standardized care.

2.3. Statistical analysis

The sequential Ca concentrations were used to divide the animals into three groups based on total serum calcium concentration: eucalcemia, subclinical hypocalcemia (**SCH**), and chronic subclinical hypocalcemia (**cSCH**). Eucalcemic animals did not have an

abnormal Ca; SCH animals had at least one low Ca measurement during the first 3 DIM; while cSCH animals had low Ca concentration for all three days measured.

Descriptive statistics were generated using FREQ and MEANS procedures of SAS version 9.3 (SAS Institute Inc., Cary, NC). Comparison between the proportions of SCH animals among parity groups and within DIM, as well as cSCH between parity groups, were obtained with procedure GENMOD of SAS.

Dynamics of serum calcium concentration during the first 3 DIM for the different groups, according to calcium status, was analyzed by MIXED procedure in SAS, with repeated statement and Bonferroni adjustment for multiple comparisons. A Kenward-Roger degrees of freedom approximation was used to calculate the denominator degrees of freedom.

A general linear mixed model was carried out using the GLIMMIX procedure of SAS was used to determine the association between early lactation calcium status and the reproductive variable: days open during the first 120 DIM. The *t*-test statement was used to evaluate the mean difference, between groups with different calcium status. Statistical analysis for pregnancy at first service was carried out using multivariable logistic regression with the LOGISTIC procedure of SAS.

The effect of subclinical hypocalcemia on return to cyclicity during the VWP and time to pregnancy before 120 DIM were determined by multivariable Cox's Proportional Hazard models of MedCalc version 14.12.0 for Windows (MedCalc Software, Mariakerke, Belgium). Cows that were not pregnant and were sold or died before 120 DIM were right censored.

Throughout statistical analysis herd was included in the models as random effect while other potential explanatory variables (i.e. calcium status group, parity, disease during the first 10 DIM, NEB, and total milk production during the first 60 DIM) were included as fixed effects. All interactions between calcium status group and the other explanatory variables were analyzed. Calcium status was forced into all models and other explanatory variables and interactions were removed in a manual backwards stepwise fashion if $P > 0.10$.

3. Results

3.1. Descriptive statistics

In total, 101 animals were enrolled during the study period, but only 97 were used for statistical analysis. Four animals were excluded from the study for the following reasons: one animal died, one was culled by farm personnel during the first week of lactation, and two were excluded because of incomplete data collection due to software problems between AMS system and farm management software.

Nineteen animals (20%) did not have low Ca concentration during the first 3 DIM, while 78 were hypocalcemic; of those 45 were classified as SCH (46%) and 33 as cSCH (34%). The percentage of animals presenting abnormal Ca decreased with time, independent of parity group. Despite the elevated number of animals presenting low calcium concentrations in early lactation, no clinical hypocalcemia, i.e. milk fever, cases were diagnosed during the study period. Chronic SCH increased with parity; parity ≥3 (46%) when compared to parity = 1 (20%) ($P = 0.03$; Table 1). Serum calcium concentrations decreased to levels below cut-off values for both SCH groups at 1 DIM while eucalcemic animals continued to have normal levels, despite reaching Ca nadir (8.8 ± 0.21 mg/dL) at 1 DIM. Calcium levels had an upwards trend during the following days and after 3 DIM SCH animals had normal Ca concentrations (9.0 ± 0.14 mg/dL) while cSCH (7.9 ± 0.16 mg/dL) still presented low calcium concentrations (Fig. 1).

Table 1
Proportion of animals with low blood calcium concentration (Ca[1]) during the first 3 DIM[2] by parity. Data: number of animals with low blood calcium concentration divided by the total number of animals in a given parity (percent).

	Parity = 1	Parity = 2	Parity ≥3	*P*-value
DIM[2]				
1	11/30 (37)[a]	21/28 (75)[b]	38/39 (97)[c]	<0.001
2	12/30 (40)[a]	17/28 (61)[a]	32/39 (82)[b]	0.001
3	10/30 (33)[a]	12/28 (43)[a]	20/39 (51)[a]	0.3
cSCH[3]	6/30 (20)[a]	9/28 (32)[a,b]	18/39 (46)[b]	0.07

[1] Ca = low blood calcium concentration was defined as Ca ≤ 8.6 mg/dL.
[2] DIM = days in milk.
[3] cSCH = Chronic subclinical hypocalcemia was defined as total blood calcium concentration ≤8.6 mg/dL for all the first 3DIM.
[a,b,c] Different letters in the same row indicate P < 0.05 between percentage of animals presenting low blood calcium concentration for the different parity groups.

When controlling for parity group and occurrence of disease in the first 10 DIM, no statistical differences in the total milk production during the first 60 DIM were observed when comparing the groups with different calcium status (2006.7 ± 101.7 kg vs. 2169.0 ± 68.3 kg vs. 2259.9 ± 80.7 kg for eucalcemia, SCH, and cSCH respectively; $P = 0.18$). No statistical differences between the different calcium status groups were observed when comparing the concentration of circulating NEFA pre-partum ($P = 0.77$) and at 5 DIM ($P = 0.14$); as well as BHB at 3 DIM ($P = 0.61$) and 5 DIM ($P = 0.60$). Nonetheless, when these metabolites were analyzed together, i.e. NEB variable, the proportion of animals considered to be in NEB was elevated in both hypocalcemic groups when compared to eucalcemia animals (eucalcemia = 21% vs. SCH = 76% vs. cSCH 85%; $P = 0.007$).

The frequency of disease occurrence during the first 10 DIM was different when comparing the eucalcemic animals to both hypocalcemic groups ($P = 0.006$). When comparing the different calcium status groups separately, it was observed that fewer eucalcemic animals developed any disease when compared to cSCH animals (16% vs. 57%; $P = 0.009$) but not when compared to SCH (16% vs. 42%; $P = 0.14$). Additionally the proportion of animals developing any disease in early post-partum was not different when comparing

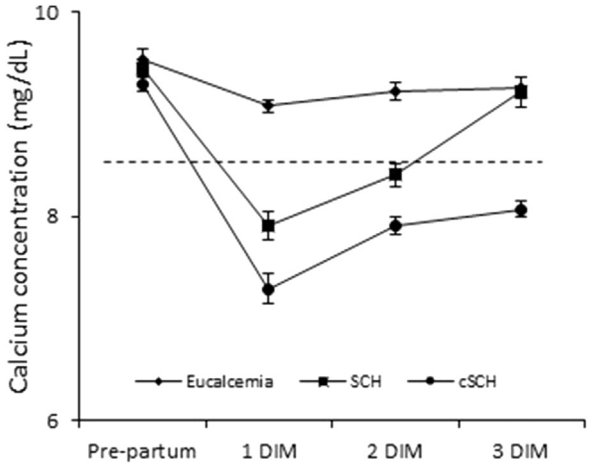

Fig. 1. Dynamics of serum calcium concentration (mg/dL) in the first 3 DIM according to calcium status group. Cows were classified as having eucalcemia (Ca > 8.6 mg/dL) or subclinical hypocalcemia (Ca ≤ 8.6 mg/dL). Subclinical hypocalcemia was categorized according to persistence of low serum calcium concentration: subclinical hypocalcemia (SCH) animals had 1 or 2 low measurements while chronic subclinical hypocalcemia (cSCH) animals presented low calcium concentration during all first 3 DIM. Horizontal dashed line represents serum calcium concentration cut-off point (8.6 mg/dL). Data are presented in LSM ± SEM and effects of the interaction between calcium status and DIM was analyzed ($P < 0.01$).

both hypocalcemia groups to each other (42% vs. 57% for SCH and cSCH respectively; $P = 0.49$).

3.2. Reproductive performance

Out of the 97 animals enrolled in the study, nine (9.3%) were right censored being culled not pregnant before reaching 120 DIM; three animals died in the first 30 days of the trial (1 presumptive listeriosis case, 1 back injury and 1 non-defined), two animals were culled due to mastitis, two animals were culled due to lameness, and two animals were culled due to low milk production possible as a consequence of clinical ketosis during early lactation. From the rest of the study population 29 animals (~30%) did not get pregnant until after 120 DIM, therefore they were considered open for the purpose of this study.

Days open during the first 120 of lactation was similar between the three groups, however days to return to cyclicity was longer for cSCH. Only a numerical difference on days open was observed when comparing the three calcium groups during the first 120 days of lactation: 85 ± 9 days for eucalcemia; 87 ± 8 days for SCH; and 89 ± 8 days for cSCH animals ($P = 0.89$). Days to return to cyclicity during the VWP was similar when comparing eucalcemia (28 ± 3 DIM) and SCH (29 ± 2 DIM), but cSCH (36 ± 2 DIM) tended to take longer to return to cyclicity ($P = 0.07$). No interaction between calcium status and other explanatory variables were observed for these reproductive outcomes ($P > 0.20$ and $P > 0.30$ for days open during the first 120 DIM and days to return to cyclicity, respectively).

The odds of pregnancy at first service for the different calcium status groups was determined by multivariable logistic regression and including parity, NEB, milk production during the VWP, and development of any disease during the first 10 DIM as covariates; however, none of these variables nor the interaction terms between these variables and calcium status were significantly associated with the outcome (P > 0.16), and were removed. Chronic SCH animals had lower odds of pregnancy at first service (OR = 0.27; 95% CI = 0.082–0.936; $P = 0.04$) when compared to eucalcemic animals (Table 2).

Cox Proportional-Hazard models were calculated to show the risk of return to ovarian function during the VWP and time to pregnancy based on calcium groups (Figs. 2 and 3). During the voluntary waiting period SCH animals had similar hazard to return to cyclicity as eucalcemic animals (HR = 0.86; 95% CI = 0.48–1.52; $P = 0.6$), while the cSCH group tended to have lower hazard for return to cyclicity (HR = 0.55; 95% CI = 0.30–1.02; $P = 0.06$). There was no difference between the eucalcemic and subclinical hypocalcemia animals ($P = 0.2$) and no difference between the

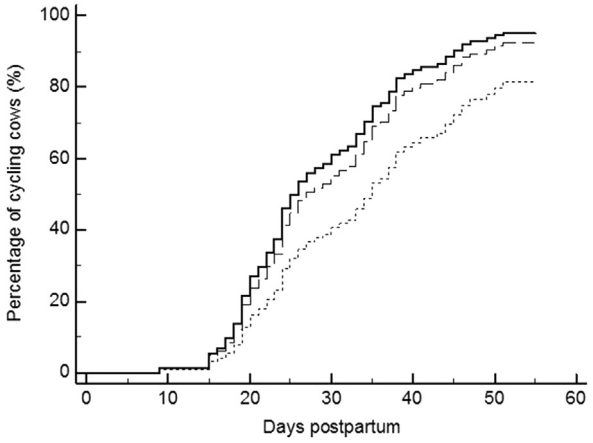

Fig. 2. Cox Proportional-Hazard curves for time to detection of active corpus luteum (progesterone > 1.0 ng/mL) for eucalcemic animals (eucalcemia; n = 19; solid line), subclinical hypocalcemia (SCH; n = 45; dashed line) or chronic subclinical hypocalcemia (cSCH; n = 33; dotted line). Subclinical hypocalcemia animals had similar hazard to return to cyclicity when compared to eucalcemic animals (HR = 0.86; 95% CI = 0.48–1.52; $P = 0.6$). Chronic SCH animals tended to take longer to return to cyclicity when compared to eucalcemia and SCH animals (HR = 0.55; 95% CI = 0.30–1.02; $P = 0.06$). The median days to active corpus luteum were, respectively, 25, 27, and 35 for eucalcemic, SCH, and cSCH animals.

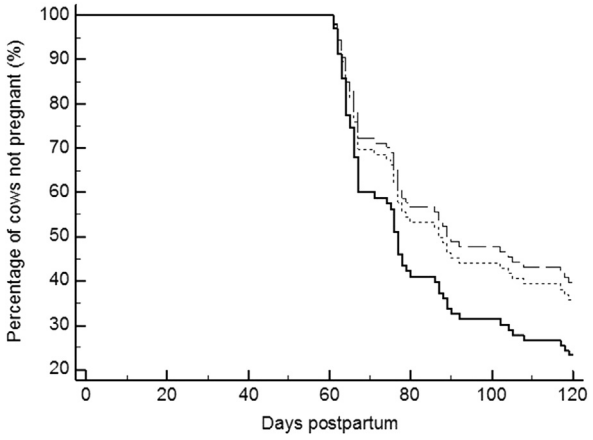

Fig. 3. Cox Proportional-Hazard curves for time to pregnancy in the first 120 DIM for eucalcemic animals (eucalcemia; n = 19; solid line), subclinical hypocalcemia (SCH; n = 45; dashed line) or chronic subclinical hypocalcemia (cSCH; n = 33; dotted line). Compared to the eucalcemic group the hazard ratio for subclinical hypocalcemic cows was 0.63 (95% CI = 0.33–1.23; $P = 0.2$) and 0.71 for chronic SCH (95% CI = 0.36–1.39; $P = 0.3$).

Table 2
Logistic regression of the association of subclinical hypocalcemia, measured within 3 DIM, with pregnancy at first service.

Variable	n[1]	Pregnant at 1st Service (%)	Odds ratio	95% CI	*P*-value
Pregnant at first service					
Calcium Status[2]					
Eucalcemia[3]	19	63[a]	*Ref.*	–	–
SCH[4]	45	44[a,b]	0.46	0.152–1.401	0.2
cSCH[5]	33	31[b]	0.27	0.080–0.876	0.03

[1] Total number of animals in each group according to total blood calcium concentration.
[2] Calcium status was defined according to the number of days the animals were classified as subclinical hypocalcemic.
[3] Eucalcemia = eucalcemic animals had all measurement of total blood calcium concentrations >8.6 mg/dL.
[4] SCH = Subclinical hypocalcemia. Subclinical hypocalcemia was defined as at least one measurement of total blood calcium concentration <8.6 mg/dL.
[5] cSCH = Chronic subclinical hypocalcemia. Chronic subclinical hypocalcemia was defined as total blood calcium concentration <8.6 mg/dL for all the first 3DIM.
[a,b,c] Different letters in the same column indicate P < 0.05.

eucalcemia and cSCH ($P = 0.3$) when evaluating pregnancy by 120 DIM as the outcome.

4. Discussion

The objective of this study was to evaluate the effects of subclinical hypocalcemia during the first 3 DIM on the reproductive performance of dairy cows. It is important to note that although there are several factors that may be associated with observed calcium concentration of a cow in the current lactation (e.g., previous lactation milk production, dry cow diet formulation, breed, and the use of anionic salts [30–32]) the objective was to evaluate the effect of subclinical hypocalcemia, irrespective of the cause. Several cut-points ranging from 8.0 to 8.8 mg/dL have been reported [6,13] for defining subclinical hypocalcemia; the cut-off used in this study was within the mentioned range, 8.6 mg/dL. Martinez et al. [14] identified 8.59 mg/dL as the value with the highest sensitivity (88.5%) and specificity (55.2%) to predict cows that would develop metritis post-partum; therefore this cut-off was chosen to dichotomize blood calcium concentration and analyze reproductive performance of dairy cows in this study.

During this study only total calcium was measured. It is important to highlight that only part of the blood Ca pool is free and readily available for biological activities, also referred to as ionized calcium (**iCa**), the rest is transported in blood bonded to albumin [33]. Additionally, increased blood pH can influence iCa concentration in blood, since alkaline environment determine a stronger binding of this ions to albumin [34]. Therefore the determination of the iCa:Ca ratio has been studied under different conditions. In humans, iCa has been shown to correspond to half of Ca circulating calcium under normal conditions [35,36], but during periods of abnormal calcium states such association is not maintained and measurement of iCa is necessary to improve calcium status diagnostics accuracy [37]. Similarly, a slight change in the iCa-Ca ratio, high iCaA:Ca due to increased percentage of Ca being ionized, was observed on dairy cows immediately after parturition depending on calcium status [38]. Despite this discrepancy, measurements of Ca were considered adequate when predicting neutrophil function, and therefore acceptable as am index of calcium status in periparturient dairy cow [38]. Ionized Ca represents the bioactive calcium in blood, but its determination is complicated and costly. Moreover iCa does not predict functional outcomes significantly better than Ca. Accordingly, total calcium is commonly measured in dairy cattle research.

The sample size was estimated to compare two groups: hypocalcemic animals, defined as low calcium concentration within the first 3 DIM, and a eucalcemic group, defined as no low calcium concentrations within the first 3 DIM. However, the incidence of cSCH animals was alarming and this led to an additional level of analysis. The consequence being that the inclusion of the third group in the analysis, decreased the statistical power. It was not an objective of this study to detect differences in the reproductive performance of the difference hypocalcemia groups, but the prevalence of cSCH led to the division into two hypocalcemic groups. A 45% power was determined by post-hoc power analysis when accounting for three calcium status groups instead of two groups (hypocalcemic vs. eucalcemic). The reduced number of individuals per group and lower power of the statistical model may explain the higher p-values. There is a chance that type II errors, i.e. indicating no difference when there truly is one, could be interpreted from some of the results presented in this study where the numerical differences where intriguing and important. If the prevalence of cSCH was known to be as elevated as encountered, more animals would have been enrolled decreasing the chances of a type II error.

In the current study, the 80% prevalence of subclinical hypocalcemia during the first 3 DIM was higher than results obtained in a survey of dairy farms in the United States [10]. However, the cut-off point to determine subclinical hypocalcemia in the survey and previous studies were lower than the cut-off used in the current study (8.0 mg/dL vs 8.6 mg/dL). Differently from previous reports [6,10,14,39], the current study also defined subclinical hypocalcemia based on the duration of low calcium concentration during the first 3 DIM and not only based on a single low blood calcium concentration. Nonetheless, when SCH was defined as low calcium concentrations throughout the first 3 DIM the numbers of affected animals were surprisingly high indicating that calcium homeostatic mechanisms were not sufficient to overcome the challenge imposed by the increased milk production for more than 30% of animals in this study [40,41].

Some degree of hypocalcemia is expected in early lactation with a nadir between 12 and 24 h after calving [40]. Nonetheless, several metabolic adaptations are triggered to overcome this challenge including enhanced absorption of dietary calcium, increased mobilization of calcium from bones, and enhanced renal re-absorption of calcium [3,13,42], as a consequence serum calcium concentration should rise to normal values within 2–3 DIM [14,43–45]. However, when these mechanisms fail and cSCH is the result we have an indication of a decreased capacity to adapt to the new physiological state in early lactation. The persistence of abnormal metabolite levels may substantiate the exacerbated negative outcomes in cSCH animals. The increased prevalence of any disease during the first 10 DIM in the cSCH animals observed in this study agrees with results from previous reports [17,46] that described an association between hypocalcemia and immune suppression due to impaired neutrophil function in periparturient animals.

The dynamics of total serum calcium concentration presented in this study are in agreement with previous reports in which Ca nadir is reached in the first day of lactation and normal values are regained by 3 DIM in the subclinical hypocalcemia groups [14,43,44]. The analysis of cSCH is a novel concept used by our group showing that some of animals do not adapt as expected to the mineral imbalances caused by the beginning of lactation. The length of time that dairy cows remain in a SCH state in early lactation might be more detrimental to health, milk production, and reproductive performance than the actual calcium concentration nadir in circulation.

Various factors throughout lactation have been associated with impaired reproductive performance in dairy cows. Among those factors, subclinical hypocalcemia happening during the very early stages of lactation have been associated with poor reproduction. In an attempt to minimize the effect of other confounding factors and isolate the association between subclinical hypocalcemia and the reproductive performance of dairy cows the analysis of the reproductive performance during the current study was restricted to the first 120 DIM.

The association between occurrence of subclinical hypocalcemia in early lactation and days open have been previously reported with inconsistent results. Martinez et al. [14] reported that hypocalcemic animals (both SCH and cSCH groups) tended to stay open 15 days longer than eucalcemic animals. On the other hand, Chamberlin et al. [44] reported no difference in the mean days open when comparing eucalcemic and subclinical hypocalcemic animals. Even though the results of the current study are in agreement with the latter, a distinction between these results must be made because time to pregnancy was only analyzed up to 120 DIM and not for the whole lactation period. It is possible that other confounders, related or not to calcium metabolism, play a role in reproductive performance later than 120 of lactation leading to the variable results reported to date.

In the present study return to cyclicity tended to be different ($P = 0.07$) when comparing the calcium status groups; with animals having abnormal Ca levels for longer being negatively affected. Similar results were reported in hypocalcemic animals [47] while no difference has been reported in hypocalcemic cows with high risk of developing uterine disease [14]. Abnormal blood calcium concentrations during the peri-parturient period has also been associated to reproductive impairment decreasing response to synchronization protocols [22] and decreased odds of pregnancy for animals [48]. Similar results were observed in this study, with animals presenting abnormal concentration of Ca during the first 3 DIM; especially cSCH animals which were less likely to have active ovaries by the time ovulation synchronization protocols were started influencing the efficiency of protocols and contributing to the lower odds of pregnancy at first service [49,50].

Impaired reproductive performance in hypocalcemic animals is, in part, explained by the association between mineral and metabolic adaptations during the transition period. Hypocalcemia can be detrimental to reproductive performance through two different interconnected pathways. First, subclinical hypocalcemia reduces calcium availability to immune cells [17] impairing neutrophil function [14] leading to an increased risk of infectious uterine diseases; aggravated by the incapacity of the uterus to expel uterine content due to suppressed smooth muscle contraction caused by the calcium deficiency [51]. Secondly, subclinical hypocalcemia exacerbates negative energy balance [10,47] and impairs lipid metabolism [44]. The decreased energy availability further intensifies immune cell dysfunction and increased occurrence of uterine diseases [52]. Clinical and subclinical diseases are associated with reduced pregnancy per artificial insemination rates and delayed return to cyclicity [47]. Even though, the mechanisms by which calcium delays ovarian activity has not been described, the negative impacts of the early lactation metabolic challenges in high producing dairy cows can have effects that are carried over on fertility months later [53].

The successful transition by eucalcemic cows is confirmed by improved early lactation reproductive performance, increased odds of getting pregnant at first service and increased hazard of getting pregnant before 120 DIM of these animals when compared to their subclinically hypocalcemic counterparts. These results are confirmed by previous reports showing that increased odds of pregnancy [14,48] and decreased reproductive disorders [14,47] are observed in animals that maintain normal serum blood concentration during the periparturient period.

The incidence and persistency of SCH during the first week of lactation is associated with impaired reproductive performance. Identification and appropriate management of these animals is important to overcome the metabolic challenge the animal is facing during early lactation.

4.1. Conclusions

Approximately 1/3 of animals of all parities experienced low Ca concentration during the first 3 DIM; with incidence increasing directly associated with parity. Subclinical hypocalcemia had a negative effect on return of ovarian function during the voluntary waiting period and decreased the odds of pregnancy at first service. Those cows with chronic subclinical hypocalcemia had an even more pronounced impaired reproductive function than those with one subclinical measurement.

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