Original Article
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Ultrasonographic ovarian dynamic, plasma progesterone, and non-esterified fatty acids in lame postpartum dairy cows

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The objective of this study was to compare ovulation rate, number of large ovarian follicles, and concentrations of plasma progesterone (P4) and non-esterified fatty acids (NEFA) between lame (n = 10) and non-lame (n = 10) lactating Holstein cows. The study was conducted in an organic dairy farm, and cows were evaluated by undertaking ultrasonography and blood sampling every 3 days from 30 days postpartum for a period of 34 days. Cows which became lame during the first 30 days postpartum experienced a lower ovulation rate determined by the presence of a corpus luteum (50% presence for lame cows and 100% for non-lame cows, $p \leq 0.05$). The number of large ovarian follicles in the ovaries was 5 for lame cows and 7 for non-lame cows ($p = 0.09$). Compared to non-lame cows, lame cows had significantly lower ($p \leq 0.05$) concentrations of plasma P4. Furthermore, NEFA concentrations were lower ($p \leq 0.05$) in lame cows than in non-lame cows. It is concluded that lameness in postpartum dairy cows is associated with ovulation failure and lower concentrations of P4 and NEFA.

Keywords: lameness, non-esterified fatty acids, ovulation, postpartum dairy cows, progesterone

Introduction

Lameness is a serious health condition affecting dairy cattle and has a reported incidence ranging between 2% and 50% [5]. This disorder is more likely to occur during the first third of lactation [35], affecting profitability of dairy operations due to losses in milk production, mastitis, impaired fertility, increased culling rate, and higher labor and treatment costs [1,12,33,34]. Impaired fertility has also been reported in lame cows under grazing conditions [37].

The time of resumption of postpartum ovarian activity is one of the major factors related to subsequent fertility in dairy cattle. If the resumption of ovarian activity is delayed, subsequent fertility is impaired. Several factors, such as nutrition, season, management, and diseases, to name a few, have been related with postpartum ovarian cyclicity in dairy cows [23]. Because hoof problems are acute and painful conditions that generally occur during the postpartum period, they are considered one of the major factors impairing fertility in dairy cattle. In a study conducted to examine the relationship between lameness and delayed ovarian cyclicity during the first 60 days postpartum in Holstein cattle, lame cows were 3.5 times more likely than cows classified as non-lame to have delayed ovarian cyclicity [14]. In an earlier study [25], lame cows had a lower conception rate at first service (l7.5% *vs*. 42.6%) and a higher incidence of ovarian cysts (25.0% *vs*. 11.1%) than those in non-lame cows.

Elucidation of mechanisms affecting fertility would help to understand and manage impaired fertility in lame cattle. Awareness of the potential effects of lameness during the early postpartum period on failure of ovulation and energy balance in lactating dairy cows could be useful to clinicians considering prevention and treatment strategies. The hypothesis of this study was that postpartum lame cows that were not under the influence of exogenous hormonal protocols would show higher

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incidence of failure of ovulation, lower progesterone levels, and lower non-esterified fatty acids (NEFA) concentrations than those in non-lame cows when followed for 34 days postpartum. A novel aspect of this study was the assessment of the pure effect of lameness on ovarian activity (*i.e.*, no exogenous hormonal treatments or other pharmacological products were used) for a period longer than the normal estrous cycle in cattle. Therefore, the objective of this study was to compare ovulation rates assessed by ultrasonography and plasma progesterone (P4) concentrations, as well as energy status as determined by NEFA concentrations, between lame and non-lame postpartum Holstein cows managed under organic conditions and evaluated over a period of 34 days.

Materials and Methods

Farm and animals

The study was conducted in a large organic commercial dairy farm (4,000 milking cows) in Plattville, CO, USA. This farm was selected because of its avoidance of the use of hormones and antibiotics. Additionally, postpartum management of cows at this farm did not involve the use of synchronization of estrus protocols or other hormonal treatments that may act as confounders. The study was approved by the Institutional Animal Care and Use Committee of the University of Florida (protocol No. 200509897).

Cows were milked twice a day and were housed in a dry-lot/grazing combined system. The herd had a rolling herd average milk production of 8,500 kg/cow/year. Diets were formulated to meet or exceed the requirements of the US National Research Council [29] at each stage of the milk production cycle. Dry cows were moved and housed in a dry-lot for 21 days before expected parturition.

Within a week prior to expected parturition, cows were moved to a maternity barn. At calving, the calf was immediately separated from the dam. Cows were evaluated within 12 h after parturition to determine body condition score (BCS), udder score, reproductive tract status, and the presence of retained fetal membranes. Postpartum examination was conducted in every cow between 25 and 35 days after calving. Conditions such as retained fetal membranes, uterine infections, mastitis, lameness, and pyometra were diagnosed and recorded consistently by farm veterinarians. Reproductive management consisted of a voluntary waiting period of 60 days and use of natural service with a bull to cow ratio of 1:35.

Study design

In this study, both lame and non-lame cows were compared prospectively for different outcome variables. Because the concentration of P4 during the postpartum period is a good indicator of resumption of ovarian activity [4,23], this hormone was used as a major outcome variable for sample size determination; consequently, in order to find a difference of 1.5 ng/mL (SD = 1.0 ng/mL) in plasma P4 between lame and normal cows, with 95% confidence and 80% power, a sample size of 8 cows per group was calculated [36]. The lame study group consisted of 10 randomly selected mature cows with an abnormal and painful gait attributable to claw origin between 20 and 30 days postpartum and without other health disorders (Group 1, n = 10). Matched by parity and number of days in milk, 10 healthy non-lame cows were selected at random to form the control group (Group 2, n = 10). Inclusion criteria for lameness were cases of laminitis-related disorders such as white line disease, sole ulcer, and sole hemorrhage [7,22]. Lame cows were animals with a clinical lameness score $\geq$ 3.0 (scale 1−6) [14]. The lame cows were put in a chute and the affected limb was exhaustively evaluated. If the lesion was a laminitis-type disorder the cow was assigned to the lame study group. In addition, the lame cows were hoof-trimmed and maintained in a copper sulfate foot bath for 30 min every other day. Hoof trimming, foot disease diagnosis, and treatments were carried out by a farm veterinarian. Exclusion criteria were interdigital dermatitis, digital dermatitis, or trauma.

Ultrasonography, blood sampling, and laboratory analyses

At assignment and every 3 days for 34 days, lame (n = 10) and non-lame (n = 10) cows were subjected to ultrasonography (Real McCoy; EI Medical Imaging, USA) of their ovaries. Because of animal welfare issues, the Institutional Animal Care and Use Committee of the University of Florida did not consent to evaluation of ovaries more frequently or for a longer period.

In addition, every 3 days, a blood sample was obtained from tail blood vessels by using a vacutainer system for plasma collection. Blood samples were centrifuged at 4,000 × g for 10 min, after which plasma was separated and stored in plastic tubes and frozen at −20°C until analysis was performed. During analysis, plasma P4 (ng/mL) and NEFA (μEq/L) concentrations were determined. Concentration of P4 was determined by using a radioimmunoassay as previously described [20]. The P4 concentration intra- and inter-assay coefficients of variation were 9.4% and 5.7%, respectively. Plasma NEFA concentration was determined by applying an enzymatic-colorimetric method [18] within a commercially available kit (NEFA-C; Wako Diagnostics, Japan).

Statistical analysis

Comparisons were conducted between lame and non-lame cow groups. The outcome variables were proportions of cows that ovulated as evidenced by the presence of a corpus luteum (CL) and number of large (> 17 mm) follicles. The criteria used to determine the presence of a CL included the following definition: distinctly echogenic areas within the ovarian stroma that may also contain fluid-filled cavities [19]. Other outcome variables were the concentrations, over time, of plasma P4

(ng/mL) and NEFA (μEq/L).

The proportion of cows that developed a CL was analyzed by using the Chi-squared test. The number of large (> 17 mm) follicles was analyzed by assessing the Poisson distribution. Blood metabolites were analyzed by applying ANOVA with repeated measures and constructing a mixed model considering the cow nested within group (lame/non-lame) as a random effect.

The mixed model ANOVA for repeated measures was defined as:

$$Y_{ijklm} = \mu + G_i + \text{time}_j + \text{Cow}_k(G) + P_l + \text{BCS}_m + (G \times \text{time})_{ij} + e_{ijklm}$$

where Y_{ijklm} is the dependent variable (P4 or NEFA), μ is the overall mean, G_i is the fixed effect of group (lame, non-lame), time_j is the fixed effect of time (day), $\text{Cow}_k(G)$ is the random effect of cow nested within group, P_l is the fixed effect of parity number (2, ≥ 3), BCS_m is the random effect of BCS at assignment (in a 1/4 point scale), $(G \times \text{time})_{ij}$ is the fixed effect of the interaction of group by time, which is the most important effect of the model because it compares the parallelism of the curves between groups over time, and e_{ijklm} is the experimental error term.

Table 1. Summary of ultrasonographic observations for lame and non-lame cows

Cow No.	Treatment	Largest follicle diameter (mm)	Corpus luteum	Ovulation
1	C	26	Yes	Yes
2	C	20	Yes	Yes
3	L	12	No	No
4	L	12	Yes	Yes
5	C	19	Yes	Yes
6	L	17	Yes	Yes
7	L	15	No	No
8	L	19	No	No
9	L	25	No	No
10	C	20	Yes	Yes
11	C	22	Yes	Yes
12	L	28	Yes	Yes
13	L	16	Yes	Yes
14	C	15	Yes	Yes
15	C	17	Yes	Yes
16	C	15	Yes	Yes
17	C	22	Yes	Yes
18	L	15	Yes	Yes
19	L	18	No	No
20	C	15	Yes	Yes

C, control (non-lame); L, lame.

For all models, the best goodness of fit was specified according to the best covariance structure, based on applying Schwarz's Bayesian Criterion [21]. Least squares mean ± SEM values are presented. Effects were considered significant when p value was ≤ 0.05. A tendency was considered present with a p value between 0.05 and 0.1. Statistical analysis was conducted using SAS 9.1 [36].

Results

Cows that became lame during the first 30 days postpartum had a lower ovulation rate, as determined by the proportion of cows with a CL, than that in non-lame cows (50% for lame cows and 100% for non-lame cows; $p \leq 0.05$) (Table 1). The number of large ovarian follicles in the ovaries was similar in the two groups (5 for lame cows and 7 for non-lame cows; $p = 0.09$). In addition, lame cows had a lower ($p \leq 0.05$) concentration of plasma P4 than that in non-lame cows (Fig. 1). Furthermore, NEFA concentrations were lower ($p \leq 0.05$) in lame cows than in non-lame cows (Fig. 2).

Discussion

This study was conducted in an organic dairy farm, which allowed the study to avoid some potential confounders since the enrolled cows did not receive any hormonal treatment at parturition or during the early postpartum period that could affect resumption of cyclicity. The objective was to monitor ovarian structure and P4 concentration in order to determine the rate of failure of ovulation. In addition, NEFA concentrations were monitored to provide information on energy status, which is an important factor affecting ovulation in high-production dairy cows. The results demonstrated that cows with lameness during the first 30 days of lactation had a higher rate of failure

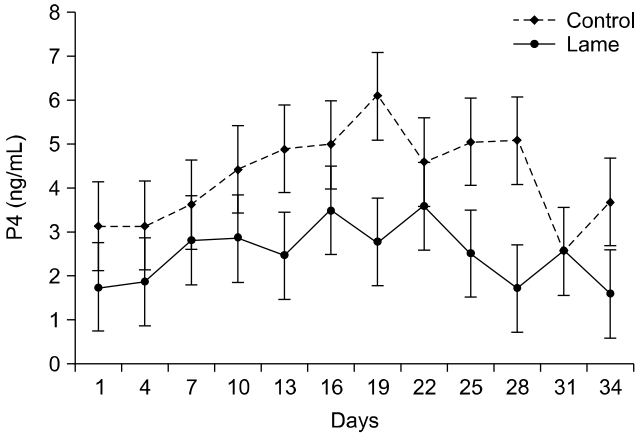

Fig. 1. Progesterone (P4) concentration in lame (n = 10) and non-lame (control; n = 10) postpartum dairy cows. Interaction days by group was significant ($p \leq 0.05$).

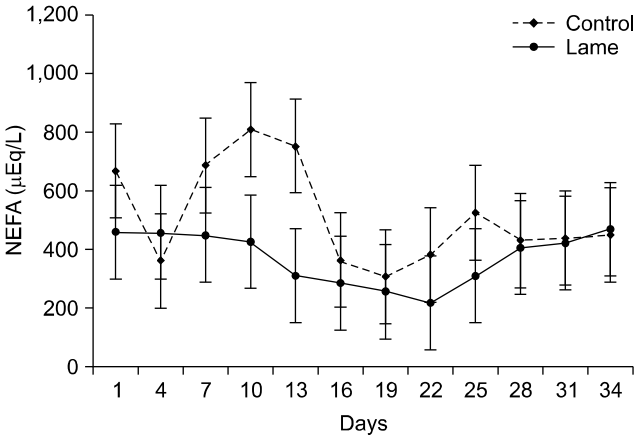

Fig. 2. Non-esterified fatty acids (NEFA) concentration in lame (n = 10) and non-lame (control; n = 10) postpartum dairy cows. Interaction days by group was significant ($p \leq 0.05$).

of ovulation and, consequently, a lower P4 concentration during the 34 day study period.

In a previous study [25], an association between lameness and ovarian activity was reported. That study was an epidemiological retrospective study in which lame cows, compared to control normal cows, were more likely to develop ovarian cysts and infertility. In order to investigate this association further, the present study was designed. Although the study's low sample size was a concern, as it is related to a potential lack of power of the statistical tests used, the number of animals per group was calculated based on a continuous variable (*i.e.*, P4 concentrations), and 10 cows per group was determined to be sufficient to have confidence in the significance of differences in the ovulation rate, plasma P4 concentration, and NEFA levels between lame and non-lame cows. This controversial topic was rigorously deliberated by the Institutional Animal Care and Use Committee of the University of Florida, due to its concerns about animal welfare issues related to lame cows. As a result, approval of this study proposal was conditional on using the smallest possible sample size to test the established hypothesis.

Ultrasonographic ovarian assessment is a powerful technology that can be used to evaluate ovarian activity in dairy cattle [13]. In this study, only half of the lame cows were observed ultrasonographically to have undergone ovulation and development of a CL. In addition, compared to non-lame cows, the lame cows tended to have smaller follicles. These results are similar to those in other studies [26,27]; however, those previous reports were confounded by the presence of other diseases (mastitis) and, most importantly, by exogenous hormonal treatments that might alter ovarian responses to the pure effect of lameness. In addition, in the aforementioned studies, lame cows were assigned only by locomotion score and not by clinical assessment of the claw. In the present study, only laminitis-type lesions were considered for inclusion. The effect of other claw conditions, not related to endotoxin release, such as digital or interdigital dermatitis, might influence the ovarian responses differently. The smaller follicular diameter in lame cows may be attributed to a deficiency of luteinizing hormone (LH) pulses caused by a more pronounced negative energy balance [4]. In addition, claw lesions related to laminitis may be associated with subacute rumen acidosis, in which endotoxins released by lysis of Gram-negative bacteria in rumen could inhibit reproductive cyclicity. Indeed, bacterial endotoxins have prevented the LH surge and affected ovarian follicular waves in dairy cows [15,32], and ewes [2,3,6]. Another mechanism associated with the observed altered ovarian function may be related to pain and stress [42]. This type of effect may result in high plasma levels of catecholamines and cortisol [28,41]. Cortisol is released as a function of adrenocorticotropic hormone (ACTH). Cortisol and/or ACTH have been related to a suppression of gonadotropin-releasing hormone and/or LH surge, which can inhibit normal follicular and ovarian activity [9-11,16,28,30].

Regarding P4, the results in the present study are consistent with those in other studies in which concentrations of P4 were lower in lame cows than in normal cows [14,40]. In addition, lame cows experienced a more marked decrease in BCS [39], spent less time eating [17], and were less likely to be cyclic than non-lame cows [14,25]. In the present study, P4 levels were used to quantify luteal function, and lame cows consistently maintained low levels of P4. Because lame cows exhibit a more pronounced loss of body condition and, therefore, a prolonged state of negative energy balance, they are at higher risk of delayed ovarian activity than that in non-lame cows [14]. Energy balance status greatly affects follicular waves, and the lower levels of NEFA observed in lame cows than in non-lame cows might be associated with the abnormal patterns of cyclicity observed in the present study. These results are similar to those in a study assessing several blood metabolites, including minerals, in lame cows [38]. That study also observed a lower concentration of NEFA, as well as the presence of ketone bodies. Moreover, in that study, NEFA level had a significant correlation with the degree of lameness experienced by the cows. Lame cows also had lower levels of minerals (Ca, Cu, I, Se, and Fe) and increased concentrations of glucose and blood urea nitrogen along with haptoglobin, histamine, and IgG than the levels in normal control cows. It has been shown that negative energy balance and weight loss have an inhibitory effect on ovarian follicular waves [8,24]. Pulses of LH and plasma concentrations of insulin-like growth factor 1 (IGF-1) are decreased in cows losing weight [23]. In addition, during periods of negative energy balance, dominant follicles spent more time and grew larger to reach a concentration of estradiol sufficient to trigger the ovulation process [4]. If lame cows lose more weight and experience a more marked negative energy balance it is reasonable to suggest that the inhibitory effect on their ovarian follicular waves is more pronounced than that in

healthy cows. The observation that lame cows in the present study had lower NEFA than that in non-lame cows may be explained by the lack of sufficient mobilizable adipose reserves due to the extent of weight loss and poor BCS in lame cows. In addition, the statistical model for NEFA concentrations covaried with BCS; as a result, the model tested a purer effect of lameness on NEFA levels. This could indicate a chronic catabolic state in which a lack of energy impedes reproductive function and results in acyclic ovaries.

In a study similar to the present one but conducted in India [31], it was observed that lame cows have a lower ovulation rate, but the P4 and NEFA concentrations were similar in the lame and non-lame cows. One of the major differences between the two studies was that in the Indian study both lame and non-lame cows were in anestrus; consequently, it is reasonable to suggest that P4 should be similar between their two groups. Furthermore, the breed evaluated by both studies was different (crossbred *vs*. Holstein), which might explain the large differences in the diameters of the largest follicles. In the Indian study, the largest follicle diameters were around 10 mm, whereas, in our study, the largest follicle diameters were around 18 mm. Finally, in our study, it was not practical to evaluate milk yield, and certainly, assessment of milk production could have been an important explanatory variable for the differences in NEFA concentration between groups. In the Indian study, unexpectedly, the milk yields from the lame and non-lame cows were not significantly different.

In conclusion, lameness in postpartum dairy cows was associated with a negative effect on follicular growth and with significant reductions of plasma P4 and NEFA concentrations. Because of these significant negative effects on reproductive physiology and, most importantly, animal welfare, lameness in cattle must be considered a priority in preventive health programs.

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Conflict of Interest

The authors declare no conflicts of interest.

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