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Effects of Calcium Supplementation on Blood Metabolites, Subclinical Mastitis and Reproductive Efficiency in Poor Performing Postpartum Dairy Cows

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ABSTRACT

Calcium supplementation is anticipated to positively impact reproductive performance, potentially enhancing fertility rates and outcomes. The current study aimed to assess the effects of varied calcium supplementation levels on milk yield, blood metabolites, udder health status, and reproductive performance. Cows (N=45) in F2 were categorized into three groups: Control (no calcium supplementation), low dietary calcium (50 g/day calcium bolus), and high dietary calcium (100 g/day calcium) over a 4-week period. Milk yield was recorded daily throughout the study. At the conclusion, blood metabolites (calcium, glucose, cholesterol, triglycerides, cortisol), somatic cell count, and reproductive performance parameters (progesterone, estrus interval, visual estrus, services per conception, and days open) were assessed. The findings indicated a linear ($P<0.01$) increase in calcium concentration in the treated groups compared to the control, demonstrating the impact of dietary calcium supplementation. Moreover, elevating the calcium dose from 50 to 100g led to a significant ($P<0.01$) increase in serum calcium concentration. Additionally, lactation performance was significantly ($P<0.01$) enhanced in both treatment groups compared to the control group, underscoring the positive influence of calcium supplementation on this aspect. Nevertheless, no statistical difference was observed between the low and high dietary calcium supplemented groups. Calcium supplementation exhibited a significant ($P<0.01$) linear decrease in somatic cell count compared to the control group. Glucose levels were significantly ($P<0.05$) higher in T1 and T2 groups than in the control; however, no significant ($P>0.05$) difference was found between the treated groups. Plasma cholesterol and triglycerides showed no significant variation over time ($P>0.05$). Similarly, there was no significant difference in cortisol levels between the treated and control groups. The data analysis revealed a significantly higher ($P<0.05$) serum progesterone concentration in the treatment groups compared to the control. Similarly, the interestrus interval (IEI) was significantly ($P<0.01$) higher in the control group compared to the treatment groups, shortening from 45 days to 24 days. However, no difference was observed between the treatment groups for the interestrus interval. The incidence of visual estrus mirrored the same pattern ($P<0.01$) as the IEI. Furthermore, the number of services required for conception significantly reduced in the treatment group. In conclusion, calcium supplementation is associated with elevated serum progesterone levels, indicative of improved fertility in highly productive postpartum dairy cows.

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Dietary calcium, Blood metabolites, Milk yield, Fertility, Somatic cells count

INTRODUCTION

The highly productive dairy cows often experience energy deficiency, leading to physiological stress due to inadequate feed intake to meet the demands for maintenance, production, and reproduction (DeFrain *et al*., 2005; Royal *et al*., 2000). Negative nutrient balance in early lactation involves substantial mobilization of body reserves for colostrum and milk production, resulting in metabolic and endocrine alterations that may lead to disturbances in postpartum dairy cows (Ceciliani *et al*., 2018; Hernández Castellano *et al*., 2017). Processes such

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as colostrum synthesis, lactogenesis, immune activation, and inflammation challenge homeostatic mechanisms responsible for maintaining blood calcium (Ca) concentration, potentially causing low blood Ca during the peripartum period (Valldecabres and Silvadel-Río, 2022). Oral Ca supplementation is based on the premise that soluble forms of Ca create a concentration gradient favoring passive absorption (Goff and Koszewski, 2018).

The synthesis of colostrum by dairy cows during the initial days of calving results in a substantial drain of serum calcium, exceeding the plasma pool. Homeostatic mechanisms must be in place to maintain blood calcium concentration during the post-calving period (Goff, 2008). Studies have shown varied associations of lower Ca concentration with health and production outcomes, with some reporting decreased milk production in cows with low Ca concentrations (Chapinal *et al*., 2011, 2012). Responses to oral Ca supplementation may depend on parity and milk production level (Martinez *et al*., 2016). The early postpartum period in dairy cows is associated with low serum calcium concentrations, categorized as clinical and subclinical hypocalcemia. Subclinical hypocalcemia is reported more frequently in multiparous compared to primiparous dairy cows, with a higher incidence in older animals (Reinhardt *et al*., 2011).

Calcium deficiency is considered a risk factor for decreased milk yield and increased incidence of reproductive and udder disorders. Subclinical hypocalcemia also affects reproductive performance and fertility, emphasizing the importance of dietary calcium supplementation to alleviate hypocalcemia (Couto Serrenho *et al*., 2021; Chapinal *et al*., 2012; Jeong *et al*., 2015). Monitoring blood metabolites in postpartum dairy cows is crucial for assessing the risk of disorders. High liver proteins have been associated with high serum calcium and cholesterol, correlating with lower disease incidence. Increased metabolites like NEFA and ketone bodies lead to changes in the GH-insulin-IGF-I-glucose signaling pathway, affecting follicular development and postpartum anestrus. Serum progesterone concentration is a key indicator of ovarian activity and is associated with improved pregnancy rates and oocyte quality (Bertoni *et al*., 2008; Lucy *et al*., 2001; Kendrick *et al*., 1999; Ferguson *et al*., 1990).

Reduced reproductive efficiency can impact the profitability of dairy production, increasing days open, calving intervals, service per conception, culling rate, replacement heifer costs, and veterinary services. Subclinical hypocalcemia has been linked to compromised leukocyte function, increasing the risk of metritis and reducing reproductive performance (Kimura *et al*., 2006; Martinez *et al*., 2012). Somatic cell count (SSC) is widely used for udder health assessment, with elevated SSC indicating mammary infection. Calcium deficiency has been associated with increased somatic cell counts in dairy cows (Halasa and Kirkbey, 2020; Rodrigues *et al*., 2020). The current study aims to investigate the effects of different levels of calcium supplementation on milk yield, blood metabolites, udder health, and reproductive performance in high-yielding F2 dairy cows.

MATERIALS AND METHODS

Experimental animals

The experiment was conducted at Al-Riza Dairy Farm, Private limited, Gujranwala, Punjab. The potential high yielding F2 dairy cows (n=45) with low calcium level that performed worsen in term of productive and reproductive efficiency and udder health were supplemented with oral calcium for a period of 4 weeks. It had been hypothesized that calcium supplementation may be the possible remedy for alleviation of poor productive, reproductive performance and udder health status in postpartum high yielding dairy cows.

Study design

High yielding F2 dairy cows (n=45) were classified into three groups (n=15; within each group): control, with no calcium supplementation; T1: low dietary calcium group, supplemented with 50 g/day/animal of calcium (Calcium carbonate) and T2: high dietary calcium group supplemented with 100 g/day/animal of calcium (Calcium carbonate) for a period of 4 weeks. The serum metabolites concentration (glucose, triglycerides, total cholesterol) and plasma progesterone and cortisol concentrations were determined among control, T1 and T2 groups along with its possible association with calcium concentration among different groups. The feed composition of all the three group is given in Table I.

Blood and milk sampling

Blood samples were collected with 20 gauge × 2.54 cm vacutainer needle from coccygeal venipuncture using a vacutainer tube without anticoagulant. Blood samples were collected from 8th week postpartum to 12th weeks postpartum on weekly intervals for a period of 4 weeks, for determination and comparison of blood metabolites (glucose, cholesterol triglycerides and serum calcium) and progesterone (P_4) to assess the presence of active corpus luteum. Serum cortisol was determined to quantify the stress level among different groups of F2 dairy cows. Similarly, milk samples were collected from 8th week postpartum to 12th weeks postpartum on weekly intervals for a period of 4 weeks, for detection of subclinical mastitis

manifested through somatic cells count. SCC exceeding 250,000 per ml of milk was considered as positive for subclinical mastitis.

Table I. Diet composition of experimental animals.

Ingredients	Proportion (%)
Total mixed ration	
Cotton seed cake	12
Sun flower cake	8
Mustard seed cake	8
Maize grain	30
Maize gluten	30
Molasses	10
Dicalcium phosphate	1
Sodium chloride	1
Green grass	
Oats	30 kg/cow/day
Chemical analyses	
Crude protein	18%
Crude fat	4.3%
Neutral detergent fiber	33%
Total calcium	0.95%

Laboratory analysis

Blood samples collected were brought to laboratory and were centrifuges at 3000 rpm for 15 min. Blood serum was harvested and was stored at -20 °C for plasma metabolites (glucose, cholesterol and triglyceride) and hormones (progesterone and cortisol) analysis. All the laboratory procedures were performed at Veterinary Research Institute, Khyber Pakhtunkhwa, Peshawar, Pakistan.

Milk yield

The dairy cows were milked thrice a day at an interval of 8h and milk production was recorded on daily basis for the period of 4 weeks.

Calcium determination

The concentration of calcium was assessed using an atomic absorption spectrophotometer. For this 1 to 2 g of samples, was added in 20-25 ml of concentrated HNO_3 and then heated for 30-45 min. After cooling, 10 ml of 70% perchloric acid was added, and the sample was heated again. Now distilled water was added to make a 100 ml volume. The determination of calcium was performed using an atomic absorption spectrophotometer, measuring absorbance at 422.7 nm with a slit width of 0.7 nm. A nitrous oxide acetylene flame was utilized. Stock standard solutions of calcium, calcium carbonate, and deionized water were prepared. After calibrating the machine, plasma calcium was analyzed by introducing the nebulizer into the samples, and the concentrations of the samples were observed on the display window.

Somatic cells count

Milk somatic cell count (SSC) was determined following the method described by Khan *et al*. (2024). An SCC exceeding 250,000 cells per ml of milk was considered positive for subclinical mastitis.

Reproductive performance

Luteal activity was assessed through the level of serum progesterone. Carpus luteum was considered functionally active when serum P_4 reached to ≥1 ng/ml.

Estrus interval was determined by counting of number of days between the two consecutive estrous cycles.

Service/conception was taken as the number of insemination required for pregnancy.

Days open were the number of days from calving to conception.

Statistical analysis

Data were analyzed using two way analysis of variance considering breed and days as main effects and their interaction with the help of statistical software (Statistix version 8.1). Statistical difference was determined using fishers LSD test. P value less than 0.05 were considered statistically significant. One way ANOVA was used for analysis of reproductive parameters.

RESULTS

Lactation performance, SCC and serum calcium concentration of the control, T1 (low dietary calcium @ 50g/day/animal) and T2 (high dietary calcium @ 100g/day/animal) groups are presented in Table II. Wherein dietary calcium supplementation showed linear (P=0.01) increase in calcium concentration in the treated groups compared to control. It was also observed that increasing the calcium dose from 50 to 100g resulted in a significant (P=0.01) increase in serum calcium concentration. Similarly, calcium supplementation significantly (P=0.01) increased lactation performance of both treatment groups compared to control group. However, there was no statistical difference between the low and high dietary calcium supplemented groups. Calcium supplementation showed a significant (P=0.01) linear decrease in somatic cells count in supplemented groups compared to control.

Table II. Effect of different levels of dietary calcium supplementation on calcium levels (mg/dl), milk yield (liters/week/animal), somatic cells count (cells/ml) plasma glucose, cholesterol, triglycerides, progesterone concentration and reproductive parameters in dairy cows.

Variables	Control	Treatment groups (Mean± SE)		P- value
		T1	T2	
Calcium (mg/dl)	4.97^{c}±0.08	7.77^{b}± 0.58	9.55^{a}±0.67	0.01
Milk Yield lit/week/animal	219.00^{b}± 1.49	226.87^{a}± 2.04	227.50^{a}± 1.75	0.01
SSC (/ml.10$^{3)}$	273.75^{a}± 1.49	137.50^{b}± 3.22	126^{c}±2.02	0.01
Glucose (mg/dl)	63.75^{b}±0.47	68.75 ^{a}± 1.75	69.25 ^{a}±1.43	0.02
Cholesterol (mg/dl)	149.50±4.33	150.25±3.44	148.50±4.26	0.99
Triglycerides (mg/dl)	21.50±0.64	22.25±0.47	21.25±0.86	0.67
Cortisol (ng/ml)	126.50±0.64	127.25±1.10	126.0.75±0.85	0.83
Progesterone (ng/ml)	1.25^{c}±0.03	2.85^{a}±0.07	2.70^{b}±0.06	0.003
Inter Estrus Interval (days)	42.57^{a}±0.55	24.33^{b}±0.42	24.74^{b}±0.35	0.001
Service/conception (n)	3.05^{a}± 1.50	1.95^{b}±0.72	1.89^{b}±0.69	0.001
Days open (days)	147.35^{a}±13.39	115.11^{b}±15.52	108.65^{b}±15.45	0.001

Control group: No supplementation with Calcium; T1: (Low dietary Calcium supplemented group), supplemented @ 50g/d/animal calcium; T2 (High dietary Calcium supplemented group: supplemented @ 100g/d/animal calcium. ^{abc}Mean values bearing different superscripts in column differ significantly (P<0.05). ^{ab}Mean values bearing different superscripts in rows differ significantly (P<0.05). IEI, Inter estrus interval; Number of days between two successive estrous cycles; Service/conception, Number of insemination(s) required for conception; Days open, Number of days from calving to conception.

Effect of dietary calcium supplementation on blood metabolites (glucose, cholesterol, triglycerides and cortisol) is shown in Table II. Results show that calcium supplementation significantly affected plasma glucose level in the current study. Glucose level was significantly (P<0.05) higher in T1 and T2 groups compared to control. However, there was no significant (P>0.05) difference between the treated groups. Plasma cholesterol, triglycerides and serum cortisol did not reveal significant (P>0.05) difference between control and the treatment groups.

Reproductive performance were recorded as luteul activity manifested through progesterone concentration, interestrus interval (days), service/conception and days open, is shown in Table II. The data analysis showed that serum progesterone concentration was significantly (P<0.05) higher in the treatment groups compared to control. Similarly, Interestrus interval was significantly (P<0.01) higher in control group compared treatment groups, where it shortened to 24 days from 42 days. However, there was no significant difference between the treatment groups for inter estrus interval. Number of services required for conception significantly reduced in the treatment group. Thus, it could be concluded that calcium supplementation is associated with elevated serum progesterone and thereby improved fertility in postpartum potential high yielding dairy cows.

DISCUSSION

The study investigated the impact of calcium supplementation on cows' blood calcium levels, finding that cows receiving 100 g/day/animal exhibited significantly higher blood calcium levels than the control group. Similar trends were observed in crossbreed cows with prophylactic intravenous calcium supplementation (Blanc *et al*., 2014). Kassio *et al*. (2019) reported fluctuations with significantly higher serum total calcium levels in treated animals compared to controls. Post-partum oral supplementation with 2 doses of 50 to 60 g of calcium was shown to increase serum mineral concentration and reduce subclinical hypocalcemia in Jersey cows (Jahani-Moghadani *et al*., 2018). Oral calcium bolus administration also increased total calcium concentrations in serum on Day 2, reducing the prevalence of severe hypocalcemia and improving first service conception rates (Jahani-Moghadani *et al*., 2018).

The swift increase in serum calcium concentration following treatment suggests that calcium chloride is a suitable alternative to intravenous calcium borogluconate, especially when the swallowing reflex is preserved (Queen *et al*., 1993). For animal welfare and convenience, oral dosing with calcium chloride appears preferable to subcutaneous administration of calcium borogluconate (Queen *et al*., 1993). Martinez *et al*. (2016) reported reduced

subclinical hypocalcemia prevalence in cows receiving an oral calcium bolus, identifying it as a significant risk factor for metritis and other diseases (Martinez *et al*., 2012). However, conflicting findings exist, as Domino *et al*. (2017) found no association between low calcium concentrations and early-lactation health status, despite increased serum calcium concentrations with oral calcium bolus administration.

In this study, cows supplemented with a calcium source exhibited significantly higher milk yield. Lactation-induced calcium transfer from blood to milk often decreases blood calcium levels, and dietary calcium may reduce calcium mobilization from bones, resulting in lower blood serum calcium (Boda and Cole, 1980). Buffaloes fed higher calcium and phosphorus levels demonstrated the highest milk yield and fat-corrected milk (FCM) production (Begam *et al*., 2010). Di-calcium phosphate supplementation also increased milk yield and fat content (Rekhis *et al*., 2001), while calcium propionate quadratically increased milk yield (Zangha *et al*., 2022). Oral calcium boluses were associated with 2.9 kg more milk in high-producing cows, emphasizing a positive response in milk yield (Oetzel and Millert, 2012).

The current study revealed increased milk yield in the treated group with continuous calcium bolus supply over a 4-week period at doses of 50 and 100 grams per day per animal. Contrary to short-term calcium supply studies, this prolonged supplementation likely contributed to the observed enhancement. Administering a bolus dose of calcium chloride at calving may not significantly boost dry matter intakes, impacting overall lactation milk yield. Previous research suggests that strongly anionic diets during the dry cow period enhance total lactation milk yields, supporting the hypothesis that sustained calcium mobilization from the skeleton has a more substantial impact on total lactation milk yield than brief post-calving calcium supplementation (Beede *et al*., 1991; Wilson and Courtney, 1997). The study highlights the importance of prolonged and strategic calcium supplementation for optimizing milk production in high-yielding cows.

In the current study, calcium supplementation led to a significant reduction in somatic cell count (SCC) in cows. Elevated SCC, indicative of mammary gland inflammation, is associated with subclinical mastitis when exceeding 200,000 cells/ml (Hillerton, 1999). Low serum calcium levels can affect muscle contraction, potentially leading to poor teat end sphincter function (Goff, 2008). Monitoring SCC aids in detecting mammary inflammation, with an increase in somatic cells being a response to pathogenic insults (Halasa and Kerkeby, 2020). The rise in SCC may serve as a protective mechanism against pathogens, and its correlation with the severity and duration of mammary inflammation has been observed (Gussmann *et al*., 2020). Amanlou *et al*. (2016) found significant effects of calcium treatment and parity on somatic cell count, indicating a more considerable challenge in overcoming intramammary infections in the control group.

Furthermore, the study revealed that blood glucose was significantly higher in calcium-supplemented cows. Postpartum elevation in blood glucose plays a crucial role in maintaining positive energy balance, initiating ovarian activity, and early postpartum estrus. Glucose, a precursor for lactose synthesis, is essential for milk production in dairy cows, and its availability is crucial for improving milk yield and immunity during the transition period (Luo *et al*., 2019). Hormonal changes around calving promote gluconeogenesis and glycogenolysis to meet the mammary gland's demands for milk production (Garverick *et al*., 2013). The findings underscore the positive impact of calcium supplementation on reducing SCC and enhancing blood glucose levels, contributing to overall mammary health and milk production in dairy cows.

In the current study, blood cholesterol, triglycerides, and cortisol did not show significant changes in calcium-supplemented cows. The observed increase in plasma total cholesterol after calving may be linked to the initiation of ovarian activity and the establishment of postpartum ovarian cyclicity. Cholesterol serves as a precursor for steroid hormones synthesis, particularly progesterone, which plays a crucial role in preparing the uterus for embryo implantation and maintaining pregnancy by nourishing the conceptus. This fluctuating pattern of plasma cholesterol during pregnancy is inversely associated with plasma progesterone levels (Abdul-Kareem *et al*., 2012).

No significant changes were observed in triglyceride levels between control and treated cows, aligning with Guedon *et al*. (1999) perspective that triglyceride levels remain relatively constant and unrelated to postpartum ovarian activity resumption. Results also correspond with Shelke *et al*. (2011), where no significant effect on plasma triglycerides was observed in buffaloes fed rumen-protected fat and protein prepartum compared to the control. However, Singh *et al*. (2012) reported significantly higher plasma triglyceride levels in buffaloes supplemented with Asparagus racemosus during prepartum, partum, and postpartum periods.

Similarly, cortisol concentrations showed no significant differences between control and calcium-supplemented cows in the current study. Negrao and Marnet (2006) found higher plasma cortisol levels in Holstein cows with higher milk yield. However, Mehdid (2009) and Diaz *et al*. (2013) suggested that severe stress increases cortisol concentration in blood, along with an increase in somatic cell count (SCC). The study

provides insights into the complex interplay of calcium supplementation and hormonal factors, highlighting the need for further exploration to fully understand their dynamics in dairy cows.

In the current study, calcium supplementation significantly increased blood progesterone concentration, estrus interval, and visual estrus in cows. This suggests potential benefits for cows susceptible to subclinical hypocalcemia, impacting fertility (Martinez *et al*., 2016). Subclinical hypocalcemia has been associated with delayed resumption of estrous cycles, reduced pregnancy rates at first artificial insemination (AI), extended days to pregnancy, and decreased pregnancies per AI up to 120 days in milk (DIM) (Ribeiro *et al*., 2013; Chapinal *et al*., 2012; Martinez *et al*., 2012, 2016).

Begam *et al*. (2010) found early commencement of estrous cycles and fewer services per conception in buffaloes supplemented with 120% calcium + phosphorus compared to lower levels. Saghar (2003) observed enhanced fertility with dietary mineral supplementation. Jahani-Moghadam *et al*. (2018) reported increased first-service conception rates with oral calcium bolus supplementation in cows with a positive dietary cation-anion difference (DCAD) before calving.

Phiri *et al*. (2007) administered calcium, phosphorus, and zinc drenches to dairy cows, resulting in significantly shorter intervals to resumption of estrus in calcium-supplemented cows. Calcium's impact on oocyte maturation and activation during fertilization suggests a carryover effect on reproductive performance (Stricker and Smythe, 2003). Inflammatory diseases' negative effects on pregnancy per artificial insemination (P/AI) and pregnancy loss can extend up to 4 months (Ribeiro *et al*., 2016). The study highlights the intricate relationship between calcium supplementation, reproductive hormones, and inflammatory factors, suggesting potential benefits for fertility in dairy cows.

In a comprehensive review, Ryan *et al*. (2019) found that cows fed non-dietary cation-anion difference (NDCA) and non-dietary (ND) diets had shorter days to first ovulation postpartum compared to those on the control diet. Caixeta *et al*. (2017) observed abnormal calcium levels within the first three days after calving were associated with delayed return to normal cyclicity.

CONCLUSIONS

The findings revealed that calcium supplementation, irrespective of the dose, led to increased milk yield, serum calcium, and glucose levels, while concurrently decreasing somatic cell count. Additionally, in cows receiving calcium supplementation, there were significant increases in blood progesterone and significant decrease in inter-estrus interval, service/conception, and days open.

DECLARATIONS

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Ethical statement

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Statement of conflict of interest

The authors have declared no conflict of interest.

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