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SYNERGISTIC EFFECTS OF INTENSIVE EARLY MILKING AND POST-TAI MELOXICAM ADMINISTRATION ON DAIRY COW PRODUCTION AND PREGNANCY RATES

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ABSTRACT

Optimizing early-lactation milk yields while maintaining high reproductive efficiency remains a major management challenge in high-producing dairy herds. This study evaluated the effects of an intensive milking regimen (6x vs. 3x daily) during the first 21 days postpartum, combined with anti-inflammatory administration after timed artificial insemination (TAI), on milk production and reproductive efficiency in Holstein cows. Forty Holstein cows were assigned to either 6x or 3x milking for 21 days before reverting to a standard 3x schedule. All cows followed a Double Ovsynch (DO) protocol for TAI with sex-sorted semen. Cows in the milking group were further subdivided into two groups receiving an anti-inflammatory injection on day 16 post-TAI. Data collected included daily milk yield (90 days), 305-day total yield, body condition scores (BCS), milk composition, progesterone (P4) and prolactin (PRL) concentrations, and pregnancy. BCS did not differ significantly between groups. Daily milk yields were comparable at 33.75 ± 0.8 kg for the 6x group and 34.27 ± 0.8 kg for the 3x group during the first 21 days postpartum. However, the 6x group had a significantly higher total milk yield over 305 days (11,555 kg vs. 11,008 kg, P = 0.04) and Lower total solids content (12.13 ± 0.12 vs. 12.49 ± 0.12, P<0.05). The administration of anti-inflammatory drugs failed to enhance pregnancy outcomes and was associated with a numerical decline in pregnancy rates within the 6x milking group. Second-parity cows showed higher conception rates, particularly in the 3x group. Increasing milking frequency to 6x during early lactation enhances long-term milk production without compromising cow welfare or milk composition. Post-TAI anti-inflammatory treatment does not appear to provide a reproductive advantage.

Keywords: Dairy cows, Milk frequency, Anti-inflammatory, Milk yield, TAI.

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1. INTRODUCTION

The dairy cattle industry is a cornerstone of food security and sustainable agriculture in Jordan, comprising approximately 556 farms and nearly 89,000 head of cattle and producing around 440,000 tons of milk annually (Alekish & Ismail, 2022). However, Jordanian dairy enterprises face persistent pressure to maximize technical and biological efficiency on-farm (Al-Sharafat, 2013). To optimize performance, many high-producing herds have transitioned from traditional twice-daily (2x) milking to more frequent intervals (Hovinen & Pyörälä, 2011; Murphy et al., 2023). In particular, doubling milking frequency to 6x daily during early lactation followed by a return to standard schedules has emerged as a powerful management tool to stimulate mammary gland remodeling and enhance long-term milk yields throughout the entire lactation curve (Shoshani et al., 2017; Hanling et al., 2023).

Despite these lactational benefits, intensive early-lactation milking regimens heavily partition energy toward milk synthesis, which can exacerbate negative energy balance and severely compromise subsequent reproductive efficiency and fertility (Amma et al., 2024). More importantly, another study notes an alarming trend: repeat breeder syndrome, in which cows fail to conceive even after more than three inseminations (Jeong & Kim, 2022; Pérez-Marín & Quintela, 2023; Villar et al., 2025). To overcome these reproductive bottlenecks, advanced synchronization protocols such as Double Ovsynch (DO) are widely utilized in primiparous and multiparous cows to eliminate the need for estrus detection and facilitate timed TAI (El-Zarkouny et al., 2004; Astiz & Fargas, 2013; Li et al., 2024). For a successful pregnancy after fertilization, adequate progesterone (P4) secretion from the corpus luteum (CL) is crucial for uterine preparation and for inhibiting prostaglandin F2α ($PGF_{2\alpha}$) (Thatcher et al., 2001; Choi et al., 2023). The embryo must secrete interferon-tau between days 14 and 16 after fertilization for maternal recognition of pregnancy (MRP) (Spencer & Bazer, 2004; Talukder et al., 2023). Insufficient P4, slowed embryonic growth, and/or delayed MRP signaling can lead to early $PGF_{2\alpha}$ secretion and embryo loss (Thatcher et al., 2001). Ongoing research

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is exploring $PGF_{2\alpha}$ inhibitors and anti-inflammatory drugs, such as Meloxicam, to mitigate early embryonic loss (Spencer et al., 2020).

Crucially, how an intensive early postpartum milking regimen (6x) interacts with highly controlled synchronization strategies (Double Ovsynch + sex-sorted semen) and post-AI anti-inflammatory therapy to influence maternal endocrine profiles (progesterone and prolactin) and pregnancy outcomes remains largely unexplored. Therefore, this study evaluated the effects of an intensive 6x milking regimen during the first 21 days postpartum, combined with post-TAI anti-inflammatory administration, on long-term milk yields, milk composition, endocrine profiles, and reproductive efficiency in Holstein cows under Jordanian management conditions.

2. MATERIALS AND METHODS

2.1. Study Location

This study was conducted from 20 November 2023 to April 20, 2024, at a private commercial dairy farm (Adel Haroun & Partners Farm) housing approximately 600 Holstein Friesian lactating dairy cows located in Zarqa-Al-Dulayl, Jordan.

2.2. Animals and Experimental Design

Forty Holstein cows (24 primiparous or 16 second-lactation) were randomly assigned to two main groups: an intensive regimen milked six times daily (6x; n = 20) for the first 21 days postpartum, and a control regimen milked three times daily (3x; n = 20). Following the 21-day treatment, all cows reverted to the 3x schedule. To evaluate the interaction with anti-inflammatory treatment, each main group was further divided into two sub-treatments (n = 10/group) after the DO protocol:

T1: 6x milking + anti-inflammatory treatment with METACAM® (Boehringer Ingelheim, Germany). This medication contains meloxicam at a concentration of 20 mg/mL and was given once on day 16 after TAI, at a dose of 15 mL per cow (2.5 mL of Meloxicam /100 kg of body weight).

T2: 6x milking + No Meloxicam.

T3: 3x milking + anti-inflammatory treatment (same as treatment 1).

T4: 3x milking + No Meloxicam (Control).

Meloxicam was given on Day 16 after TAI to align with maternal recognition of pregnancy in cattle. Around Days 15–17 post-fertilization, the conceptus releases interferon tau (IFN-τ), which inhibits endometrial oxytocin receptor expression and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) release, thus sustaining corpus luteum function (Erdem & Guzeloglu, 2010).

Holstein–Friesian lactating dairy cows were housed in naturally ventilated free-stall barns equipped with shade and a source of water *ad libitum*. All cows received the same basal diet during the 21-day period, formulated to meet National Research Council (2001) requirements and containing 18 % crude protein (CP) and an estimated net energy for lactation of 1.40 Mcal/kg of dry matter (the reported DMI was 22.41 kg/day per cow) as shown in Table 1. The roughage component consisted of equal parts dry matter from thermally dried alfalfa and corn silage (5.60 kg DM/head each). After this initial 21-day period, both groups were returned to the standard three-times-daily milking schedule for the remainder of the three-month study period. Every cow underwent a DO treatment starting at 42 ± 3 days postpartum (Fig. 1). The protocol utilized 500 µg of PGF2α (Cloprostenol; Estrumate, Intervet) and 10 µg of a GnRH agonist (Buserelin; Receptal®, Hoechst Roussel Vet GmbH). All cows were inseminated via TAI by a single experienced technician using sex-sorted semen (ABS Global, Inc.). Pregnancy status was confirmed by rectal palpation days 45 and 50 after insemination as described by Romano et al. (2007).

Table 1: Ingredients and Nutrient Composition of the Basal Diet (Percentage of Diet on dry matter 90%)

Ingredients	Percentage
Barley	32.42
Corn	16.22
Soybean Meal	16.22
Wheat Bran	16.22
Limestone	0.89
Salt	0.81
Methionine (Mepron®)	0.081
Protected Glycerin	2.25
Potassium Carbonate	0.18
Sodium bicarbonate	1.46
Magnesium oxide (Phix-Up)	0.225
Protected Choline	0.54
Avila4 (organic zinc, manganese, copper, and cobalt)	0.081
Avila organic chromium	0.045
Premix (protected Vitamin A, D, E, and minerals' sulphate copper, iron, and minerals' oxide zinc, manganese)	0.36
Yeast Culture (Diamond V XPC)	0.234
Calcium soaps	1.764

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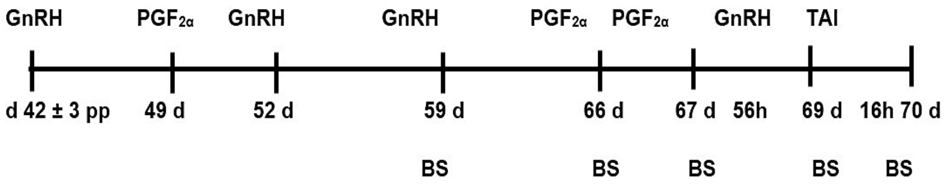

Fig. 1: Schematic diagram of the Double Ovsynch (DO) protocol and blood samples (BS) initiated on d 42 ± 3 postpartum (PP) and concluding on d 70 ± 3 PP in treatment (6x and 3x).

2.3. Blood Sample Collection and Analysis

Blood samples were collected from all cows via coccygeal venipuncture into heparinized tubes for subsequent measurement of Prolactin (PRL) and Progesterone (P4). For PRL, samples were collected on days 0, 7, 14, 21, 28, and 35 after calving. To monitor the effectiveness of the DO protocol, samples were collected on days 59, 66, 67, 69, and 70 postpartum, corresponding with the date of injection during the second ovsynch or second arm of the DO protocol. Samples were centrifuged at 3000 rpm for 15 minutes, and plasma was collected and stored at −20°C until analysis of P4 and PRL levels. Plasma PRL concentrations were determined using a bovine quantitative ELISA kit (ELK Biotechnology Company, Wuhan, China). The plasma standards included 200, 100, 50, 25, 12.5, 6.25, 3.13, and 0 ng/mL. The inter- and intra-assay coefficients of variation were 6.27 and 3.97%, respectively. Plasma P4 concentrations were determined using a quantitative ELISA system from DiaMetra (DiaMetra S.r.I., Milan, Italy) utilizing highly specific polyclonal antibodies. The plasma standards included 0, 0.2, 1, 1.5, 15, and 40 ng/mL. The inter- and intra-assay coefficients of variation were 7.67 and 4.79%, respectively.

2.4. Milk Samples

Representative milk samples were collected automatically during the morning milking on days 14, 21, and 35 postpartum using integrated proportional in-line samplers (3 mL per 300 mL of milk flow from the udder) to obtain a composite sample of the entire milking. These samples were immediately analyzed with the MILKANA analyzer to determine their fat, total solids, and protein contents (Mayasan Biotech Milk Analyzers, Milkana Istanbul, Turkey) (Alan et al., 2023).

2.5. Body Condition Score (BCS)

The body condition score (on a 5-point scale) for all cows was measured at 0, 21, and 49 days post-calving to monitor metabolic recovery and negative energy balance during the critical transition period as described previously (1 = 1-1.5 Emaciated, 2 = 1.6-2.5 Thin, 3 = 2.6-3.5 Good, 4 = 3.6-4.5 fat, and 5 = ≥ 4.6 Obese) by Roche et al. (2009).

2.6. Milk Yield

Milk yield data were recorded daily for all cows using the specialized dairy management software, ALPRO™. Every cow received a transponder from Delaval International Inc. (Tumba, Sweden) that sent milk yield data to the computer system every fifteen minutes. This configuration made it possible to track daily milk output precisely and promptly. The ALPRO™ software was used to collect the data after the measurements were gathered between Day 3 and Day 90 post-calving.

2.7. Statistical Analysis

The experiment was a completely randomized design (CRD) arranged as a 2 × 2 factorial. The cow was considered an experimental unit. Statistical analysis was performed using SAS (version 9.4, SAS Institute, USA). Analysis of variance (ANOVA) was conducted with PROC GLM, and PROC FREQ in SAS was used to evaluate overall differences between groups for milk frequency (6 times and 3 times), milk composition (fat, protein, and total solids), BCS, PRL, and P4 levels. A t-test was employed to determine significant differences between means as a repeated measurement. The PROC MIXED procedure in SAS was used to analyze the data. In the model statement, milking frequency was the main plot effect, and anti-inflammation injection was the subplot effect nested within milking time. We included interaction terms between milking time and injection to assess whether their effects on the outcome variable depend on each other. We used PROC FREQ to compare pregnancy per artificial insemination (P/AI) rates between days 45 and 50 after insemination for first pregnancies. We also evaluated the correlation between PRL concentration, anti-inflammatory drug injection, and average milk yield. All statistical tests were considered significant at $P<0.05$ and as a tendency at $P<0.01$ using the t-test.

3. RESULTS

3.1. Effect of Milking Frequency 6x or 3x on Body Condition Score (BCS)

Milking frequency (6x vs. 3x) did not significantly affect BCS at any time point ($P>0.05$), as shown in Table 2.

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Table 2: Body condition score (BCS) of cows on days 0, 21, and 49 after calving by treatment (6x and 3x)

	BCS[1]		
Treatment[2]	d0	d21	d49
6x	3.06 ± 0.02	2.95 ± 0.03	2.86 ± 0.02
3x	3.07 ± 0.02	2.89 ± 0.03	2.88 ± 0.02
P-value*	0.67	0.18	0.48

[1]BCS (mean ± SEM) on days 0, 21, and 49 after calving. [2]treatment: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving. *P-value: Not significant at P>0.05.

3.2. Effect of Milking Frequency 6x or 3x on Milk Composition

Least square means for milk fat, protein, and total solids percentages of cows on days 14, 21, 35, and the overall average after calving for treatments 6x and 3x are shown in Table 3. Across all milk fat measurements, the two treatment groups showed no significant effects (P>0.05) on milk fat percentage during early lactation. Furthermore, the treatment groups also did not affect the average milk fat percentage over the evaluation period (P=0.81). These findings clearly show that the treatment had no effect on milk fat percentage in early lactation, with both groups maintaining similar compositional quality throughout the measurement period. For all milk protein measurements, the expected pattern of protein reduction in the first few weeks after calving was found. Additionally, treatment groups did not influence average milk protein percentage (P>0.05) on days 14, 21, or 35 after calving. These findings show that the treatment had no effect on milk protein percentage throughout early lactation. In addition, least square means (± SEM) for the percentage of total solids of cows on days 14, 21, 35, and the overall average after calving for treatment 6x and 3x are shown in Table 3. On day 14, the 6x treatment group had a significantly lower total solids percentage (12.19 ± 0.16) than the 3x group (12.71 ± 0.16) (P = 0.02). This indicates an early impact of the 6x treatment in lactation. However, no significant differences were observed on days 21 and 35 after calving. When averaging total solids from d14 to d35, the 6x group (12.14 ± 0.12). The 6x group also showed a significantly lower percentage (P = 0.03) than the 3x group (12.52 ± 0.12). These results indicate that, although treatment did not consistently affect total solids at every time point, the 6x treatment showed a significant early (d14) and overall decrease in total solids % during the first 35 days after calving.

Table 3: Least squares mean ± SEM for the milk fat, protein, and total solids percentages of cows on days 14, 21, 35, and the average after calving by treatment (6x and 3x)

		Treatments[1]		
Item[2]	Days	6x	3x	P-value
Fat (%)				
	14	4.20 ± 0.15	4.04 ± 0.15	0.41
	21	3.78 ± 0.22	4.10 ± 0.22	0.38
	35	4.23 ± 0.26	4.03 ± 0.26	0.77
	Average[3]	4.04 ± 0.02	4.02 ± 0.02	0.99
Protein (%)				
	14	3.10 ± 0.03	3.13 ± 0.03	0.54
	21	3.03 ± 0.02	2.99 ± 0.02	0.32
	35	2.97 ± 0.03	2.92 ± 0.03	0.27
	Average	3.03 ± 0.02	3.01 ± 0.02	0.44
Total Solid (%)				
.	14	12.19 ± 0.16[b]	12.73 ± 0.16[a]	0.02
	21	12.11 ± 0.17	12.38 ± 0.17	0.30
	35	12.10 ± 0.23	12.43 ± 0.23	0.25
	Average	12.13 ± 0.12[b]	12.49 ± 0.12[a]	0.04

[1]Treatments: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving. [2]Fat, Protein, and Total solids percentages: on days 14, 21, and 35, and the averages from day 14-35 after calving measurements (± SEM). A value that summarizes days 14, 21, and 35. [3]Average: *P- value: significant level at P<0.05; Mean ± SEM within a row with different superscripts differ (P<0.05).

3.3. Effect of Milking Frequency 6x or 3x on Milk Production

Least-squares means (± LSM) for average milk yield in cows during the experimental period for the 6x and 3x treatment groups and for first and second lactations are shown in Fig. 2 & 3, respectively. From Day 3 to Day 21 after calving, the average daily milk yield was similar between the 6x and 3x groups (33.75 ± 0.8 kg and 34.27 ± 0.8 kg, respectively; P=0.67; Table 4). Milk yields from Day 3 to Day 21 after calving during the second lactation were significantly higher than those during the first lactation (37.88 ± 0.9 kg vs. 30.13 ± 0.8 kg; P=0.001), indicating that early lactation production was mainly influenced by parity rather than the treatment. However, a significant interaction (P=0.04) was observed between the treatment and parity; no figures for the interaction were provided. Since there was an interaction, the effect of the treatment was different between parities. It may be that 2nd parity has tolerated more milking (6x) than 1st parity. From Week 4 to Week 9, a significant effect of treatment was observed, with milk yield in the 6x group lower (P<0.05) than in the 3x group (Fig. 2). From Week 10 to Week 12, both treatment groups showed similar milk yields. However, in Week 13, significance reappeared, with milk production lower (P<0.05) in cows in the 6x treatment group than in the 3x group (P=0.03) (Fig. 2).

Despite a non-significant effect on average daily yield in the first three weeks and a significant reduction over the next weeks (4-9), there was a significant difference in the adjusted/expected Total Milk Yield over 305 days (MY305d) (P = 0.04). The treatment group had significantly greater MY305d (6x: 11555 kg vs 3x: 11008 kg; Table 4). These findings imply that, while the treatments had no effect on short-term average daily yield, the 6x treatment resulted in significantly increased long-term milk production. The primary benefit of 6x milking appears to manifest in the lactation 'tail' rather than the peak. This suggests that early-lactation frequency induced a regenerative effect or mammary remodeling that enhanced udder persistency. Notably, these cows remain in the herd, and this functional recovery has proven to be a permanent physiological shift, with increased productivity persisting into subsequent lactations. Least squares mean (± SEM) of milk yield across the 13-week trial period, stratified by parity (lactation number): Parity 1 (First Lactation) and Parity 2 (Second Lactation) (Fig. 3). From week one to week thirteen, the second lactation yield was considerably higher (P<0.05) than the first (Fig. 3).

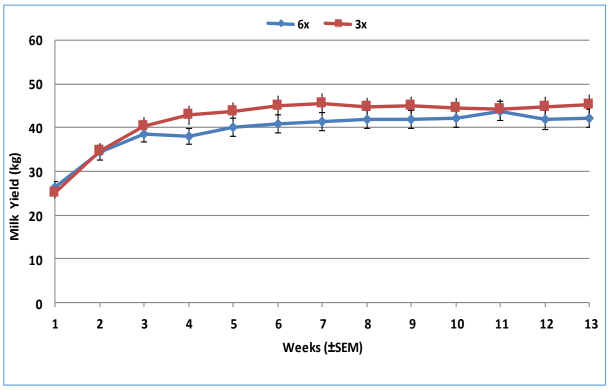

Fig. 2: Milk yield (Liters: mean ± SEM) for 6x and 3x treatments during the experimental period.

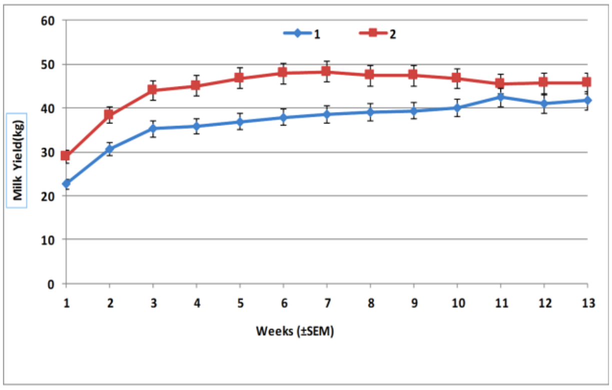

Fig. 3: Milk yield (Liters: means ± SEM) for first and second lactations during the experimental period.

Table 4: The milk yield (kg) of cows during weeks 1, 2, 3, the average of the first three weeks after calving, and the total milk yield adjusted to 305 days by treatment (6x and 3x)

	Milk yield (kg)				
Treatments[1]	wk1	wk2	wk3	Average[2]	Total MY305d[3]
6x	26.4 ± 0.97	34.3 ± 0.96	38.6 ± 0.93	33.75 ± 0.8	11555 ± 182[a]
3x	25.1 ± 0.97	34.7 ± 0.96	40.4 ± 0.93	34.27 ± 0.8	11008 ± 182[b]
P- value*	0.33	0.72	0.19	0.67	0.04

[1]Treatments: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving (mean ± SEM). [2]Average: a value that summarizes (weeks 1, 2 and 3). [3]Total MY305d: The standard criterion for measuring milk production in dairy cows, as it begins on the first day after calving and continues for 305 days. *P- value: significant level at P<0.05. Values within a column with different superscripts differ (P<0.05).

3.4. Effect of Milking Frequency 6x or 3x on Prolactin Levels

Least squares means (± SEM) for prolactin concentrations (ng/mL) on days 0, 7, 14, 21, 28, and 35 following calving for the 6x and 3x treatment groups are shown in Table 5. The analysis indicates no significant difference (P>0.05) in plasma prolactin levels between the two treatment groups (6x and 3x). These findings indicate that the treatment (6x vs. 3x) did not affect circulating prolactin concentrations throughout the early post-calving period.

Table 5: The PRL concentrations (mean ± SEM) on days 0, 7, 14, 21, 28, and 35 and the average after calving by treatment (6x and 3x)

	PRL (ng/mL)[1]						
Treatment[2]	D0	D7	D14	D21	D28	D35	Average PRL[3]
6x	31.2 ± 3.2	28.4 ± 2.4	32.4 ± 3.6	29.3 ± 3.6	30.4 ± 2.6	28.7 ± 2.3	30.2 ± 1.3
3x	30.3 ± 3.2	27.5 ± 2.4	27.1 ± 3.7	28.5 ± 3.6	25.7 ± 2.6	26.5 ± 2.4	27.5 ± 1.3
P- value*	0.84	0.79	0.30	0.87	0.21	0.51	0.14

[1]Prolactin concentrations on days 0, 7, 14, 21, 28, and 35 after calving (± SEM). [2]treatment: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving. [3]Average PRL: a value that summarizes on (days 0, 7, 14, 21, 28, and 35). *P- value: Not significant at P>0.05.

3.5. Reproductive Performance

The effects of treatment, pregnancy status, and parity on plasma P4 concentration (ng/mL) are shown in Table 6. Measurements were taken at key time points during the second Ovsynch of the DO protocol: the first GnRH, first PGF2α, second PGF2α, second GnRH, and TAI. The proportions of cows with functional CL (plasma P4 [≥1 ng/mL]) at the first GnRH and first PGF2α were 82.5 and 80.0%, respectively. Plasma P4 concentrations did not differ significantly (P>0.05) between the 6x and 3x treatment groups at any measured time point during the DO protocol.

Pregnant cows had significantly (P<0.05) higher P4 concentrations than non-pregnant cows at the first GnRH and first $PGF_{2\alpha}$, while lower at the second GnRH and TAI. No difference was observed at the second PGF2α. Regarding parity, second-lactation cows showed significantly (P<0.05) higher P4 concentrations only at the first $PGF_{2\alpha}$ compared to first-lactation cows.

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Table 6: P4 concentrations across different treatments (6X and 3X), Pregnancy status, and Parity during the second Ovsynch of a double Ovsynch protocol

Variable/Treatment[2]	P4 concentration ng/mL (mean ± SEM) at different time[1]				
	GnRH (1)	$PGF_{2\alpha}$ (1)	$PGF_{2\alpha}$ (2)	GnRH (2)	TAI
6x	2.72 ± 0.33	2.33 ± 0.30	0.473 ± 0.10	0.737 ± 0.14	0.340 ± 0.09
3x	2.33 ± 0.34	1.66 ± 0.30	0.322 ± 0.10	0.621 ± 0.14	0.316 ± 0.09
P- value	0.40	0.12	0.30	0.58	0.54
Pregnancy[3]					
+	3.70 ± 0.27	2.52 ± 0.33	0.379 ± 0.12	0.214 ± 0.13	0.160 ± 0.10
-	1.72 ± 0.23	1.647 ± 0.27	0.410 ± 0.09	0.989 ± 0.11	0.488 ± 0.08
P- value	0.001	0.05	0.84	0.001	0.01
Parity[4]					
1	2.37 ± 0.30	1.66 ± 0.27	0.33 ± 0.10	0.54 ± 0.13	0.31 ± 0.08
2	2.77 ± 0.37	2.59 ± 0.32	0.50 ± 0.11	0.88 ± 0.16	0.37 ± 0.10
P- value*	0.40	0.03	0.26	0.12	0.64

[1]GnRH (1) = Day 59 GnRH injection; $PGF_{2\alpha}$ (1) = Day 66 $PGF_{2\alpha}$ injection; $PGF_{2\alpha}$ (2) = Day 67 $PGF_{2\alpha}$ injection; GnRH (2) = Day 69 afternoon (pm) GnRH injection; TAI = 16 hours after GnRH (2) injection. [2]Treatment: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving. [3]Pregnant: + / - = Pregnant or not pregnant, respectively. [4]Parity: 1 = first lactation, 2 = second lactation; *P- value: significant level at $P<0.05$.

Table 7 shows the pregnancy rate at 48 ± 3 days after TAI across different treatments, anti-inflammatory groups, and parities. The main treatment groups (6x vs. 3x) showed no statistically significant association with pregnancy at the first TAI (P=0.53), with both groups achieving a 40 % success rate (8/20) that aligns with farm standards. However, outcomes varied when accounting for anti-inflammatory administration. In the 6× treatment, cows that received the anti-inflammatory treatment tended to have a lower pregnancy rate (20 %; 2/10) compared to those that did not receive anti-inflammatory treatment (60 %; 6/10). In contrast, anti-inflammatory administration had no effect in the 3x group, where both subgroups maintained a 40 % pregnancy rate. Regarding parity, second-lactation cows (Parity 2) demonstrated a numerically higher overall pregnancy rate compared to first-lactation cows (Parity 1) when treatments were combined. Although the overall difference between the two parities was minimal, the 3x treatment appeared to be more beneficial for second-lactation cows than the 6x treatment.

Table 7: Pregnancy rates (%) at day 48 ± 3 after Timed Artificial Insemination across different treatments, anti-inflammatory, and parity groups

Variable/	PR % (n/n)[1]					
Treatment[2]	Anti[3]	Non-Anti	*P*-value	Parity[3] 1	Parity[4] 2	P-value
6x	20 (2/10)	60 (6/10)	0.068	41.7 (5/12)	37.5 (3/8)	0.85
3x	40 (4/10)	40 (4/10)	1.000	33.3 (4/12)	50 (4/8)	0.46
All	30 (6/20)	50 (10/20)	0.197	37.5 (9/24)	43.8 (7/16)	0.69

[1]PR: Pregnancy Rate, n = number of pregnant cows/number of inseminated cows. [2]treatment: 6x = milking 6 times daily for the first 21 days after calving; 3x = milking 3 times daily for the first 21 days after calving. [3]Anti: Non-steroidal anti-inflammatory drug (Meloxicam) at a concentration of 20 mg/mL, given once on day 16 after insemination; Non-Anti: without receiving any anti-inflammatory treatment. [4]Parity: 1 = first lactation, 2 = second lactation.

4. DISCUSSION

This study examined the effects of using the anti-inflammatory medication meloxicam after insemination and high-frequency milking (six times a day, or 6x) throughout the first 21 days of lactation. It evaluated dairy cow productivity and reproductive performance using a DO protocol and sexed semen artificial insemination (AI). The main goals were to assess the efficacy of post-synchronization meloxicam therapy in increasing pregnancy rates and to determine whether increasing milking frequency from three times daily (3x) to six times daily would improve milk output. The results showed no significant difference in BCS between cows milked six times a day and those milked three times a day during the early stages of lactation. This result is consistent with Shoshani et al. (2017), who reported no negative impacts on energy balance or general health, but it differs from Eslamizad et al. (2010), who noted a modest weight loss in high-frequency milked Holstein cows over a 42-day period. An adequate diet, good management practices, and the relatively short (21-day) high-frequency milking period likely explain the stable BCS in the current study. Overall, the data indicate that high-frequency milking during early lactation can increase milk production without adversely affecting cow welfare or future lactation performance with proper diet and management (Shoshani et al., 2017; Khan et al., 2026).

During the first 21 days, milking cows six times a day had no apparent effect on milk fat or protein contents as compared to three times a day. On the other hand, by day 14 postpartum, total solids had dramatically decreased and continued to do so for 35 days. These findings are similar to what was previously reported by Eslamizad et al. (2010).

During the first 21 days of lactation, cows milked six times as much as those milked three times did not significantly enhance their daily milk production, according to this study. This runs counter to previous research by Dahl et al. (2004) and Shoshani et al. (2017), who found notable increases in milk yield. Compared with previous

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studies, the current study's sample size of 40 cows may make it harder to detect small changes in daily productivity. Contrary to the short-term results, long-term data supported Dahl et al. (2004) and Shoshani et al. (2017) by demonstrating a significant positive influence on 305-day milk production for cows milked six times daily. This implies that early lactation milking frequency has a positive long-term effect on overall lactation performance. Prolactin, which is necessary for mammary cell growth, may be responsible for this effect. This could lead to more differentiated cells and a greater total milk production (Akers et al., 1981; Capuco et al., 2001; Lacasse, 2025).

The current study supports Eslamizad et al. (2010) by finding no difference between 6x and 3x milking and no significant change in plasma PRL concentrations at any sample time point. Around parturition, plasma PRL levels usually peak before progressively declining. This is consistent with research by Tong et al. (2018) and Lanctôt et al. (2025). Blood samples may not have been taken at the optimal time to capture the brief peak prolactin release that takes place during and soon after milking, which could explain the lack of change in PRL levels despite more frequent milking (Akers, 2016). Additionally, environmental conditions, such as photoperiod, can affect PRL secretion, making assessments more difficult (Lacasse et al., 2014; Flis et al., 2026).

Throughout the second half of the DO program, plasma progesterone concentrations did not differ significantly between the 6x and 3x treatment groups at any time point. This supports the findings of Carruthers & Hafs (1980) and Amos et al. (1985) that increased milking frequencies have little effect on circulating reproductive hormones by indicating that the intense milking frequency during the first three weeks after calving has no detrimental effect on the protocol's primary hormonal regulatory systems. The DO regimen appears to be efficient in normalizing hormonal profiles and reducing small metabolic or endocrine differences caused by increased milking frequency (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013). The only variation was seen at the first $PGF_{2\alpha}$ injection, where parity influenced P4 levels. Cows in their second lactation showed a stronger luteal response early in synchronization, which could be attributed to a better established hypothalamic-pituitary-ovarian axis. However, the protocol's constant hormonal control in subsequent stages minimized the differences between parities (Souza et al., 2008; Herlihy et al., 2012).

Regardless of treatment, higher concentrations of P4 were observed at the first GnRH and first $PGF_{2\alpha}$ injections, while lower levels appeared at the second GnRH in pregnant cows compared to non-pregnant cows. According to previous studies, pregnancy rates in dairy cows are often improved by having higher P4 levels at the first GnRH injection and lower P4 levels at the second GnRH injection under a DO protocol (Abay et al., 2026). This strategy creates a more favorable hormonal environment for early pregnancy and helps ensure ovulation of a more developed follicle (Souza et al., 2008; Giordano et al., 2012; Herlihy et al., 2012; Kohsari et al., 2024; Li et al., 2024). Recent protocol adjustments that increase P4 at the first PGF2α have been linked to improved pregnancy rates, notably in primiparous cows (Kohsari et al., 2024).

The DO technique was used to assess PR, followed by TAI using sex-sorted semen and meloxicam administration to half of the cows on day 16 post-AI. Overall, the PR was 30% (6/20) in the meloxicam group and 50% (10/20) in the non-meloxicam group, with no significant difference. This suggests that meloxicam did not improve overall fertility, consistent with Spencer et al. (2020), who found insufficient evidence of meloxicam's beneficial effects on conception. Further studies are needed to show if earlier administration of meloxicam, on day 13-14 post AI, would have a different effect, as it might precede the expected $PGF_{2\alpha}$ peak in non-pregnant cows. Meloxicam treatment tended to reduce PR in the 6x group (20% vs. 60%) but had no effect in the 3x group (both at 40%). Intensive milking elevates metabolic stress (Mwaanga & Janowski, 2000). The lower PR in the high-frequency 6x group implies that, in cows already under metabolic stress, meloxicam suppression of prostaglandin pathways (Agrawal & Gupta, 2010; de la Puente et al., 2024) may negatively affect reproduction. Other studies have shown lower PR after meloxicam use (Erdem & Guzeloglu, 2010). However, Özyurtlu et al. (2016) found good effects in stable postpartum conditions.

The evaluation of pregnancy rates based on milking frequency (6× vs. 3×) and parity (1 vs. 2) showed meaningful differences, though not statistically significant. Overall, second-lactation cows had a higher pregnancy rate (43.75%) compared to first-lactation cows (37.5 %). In the 3× treatment, parity-2 cows had a pregnancy rate of 50% versus 33.3% for parity-1. In contrast, first-lactation cows showed a slightly higher rate (41.7%) than parity-2 animals (37.5%) under the 6× treatment. These findings suggest that milking frequency affects metabolic load and parity-related physiological responses (Cattaneo et al., 2023). According to several studies (Souza et al., 2008; Herlihy et al., 2012; Astiz & Fargas, 2013; Dirandeh et al., 2015; Priyanto et al., 2026), primiparous cows often exhibit more consistent ovulatory and luteal responses, improving reproductive outcomes under increased metabolic demands. The 6× group shows this pattern, with first-lactation cows having a statistically greater conception rate. High milking frequency might raise energy requirements and physiological stress, which may reduce older cows' ability to reproduce (Mwaanga & Janowski, 2000; Kusaka, 2026). Supporting the hypothesis that multiparous cows are susceptible to high metabolic pressure, parity-2 cows under 6× have a reduced pregnancy rate. This pattern highlights the need to consider parity and metabolic load when creating management and fertility plans.

5. CONCLUSION

During the first 21 days after giving birth, increasing milking frequency from three to six times per day had no effect on cow welfare or metabolic stability; protein, milk fat, and BCS percentages remained constant. The 6x regimen raised 305-day milk yield (MY305d), indicating long-term productivity gains, even though short-term milk yields were comparable. Additionally, the Double Ovsynch protocol effectively synchronized ovulation between groups. Overall pregnancy rates were not statistically changed by post-TAI meloxicam treatment on day 16. Although there is no obvious reproductive benefit to meloxicam therapy in this study, more extensive research is required to find out whether high-frequency milking stress and early pregnancy actually interact negatively.

Declarations

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Data Availability: All relevant data are included within the manuscript. The raw data will be available from the corresponding author upon reasonable request.

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