scientific reports

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25(OH)vitamin D inflammatory and oxidative stress markers in healthy Holstein cows and cows with peri-partum diseases during the transition period

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In dairy cows, the immune system is suppressed around calving, predisposing them to infectious diseases. This study investigated vitamin D levels and various immunological and oxidative stress markers in cows suffering from peri-parturient diseases compared with healthy cows. A total of 45 cows with peri-parturient diseases (including ketosis and uterine diseases) and 23 healthy cows were selected. For statistical comparisons, diseased cows were compared with healthy cows in various ways. To perform statistical comparisons, sick cows were categorized into different stages: first, those with various peri-parturient diseases; second, those with ketosis; and third, those with uterine diseases, for comparison with the healthy groups. Blood samples were collected at three time points: 7 days pre-partum, 7 days post-partum and 21 days postpartum. 25(OH) vitamin D (25(OH) D), interleukins 4 and 10 (IL-4, IL-10), interferon-gamma (INF-γ), immunoglobulin G (IgG), and haptoglobin amounts were measured via ELISA, while total antioxidant capacity (TAC) and malondialdehyde (MDA) amounts were measured spectrophotometrically. The Friedman test revealed significant time effects on 25(OH) D levels in both the healthy and sick groups ($p \leq 0.05$). However, no significant differences were detected between healthy and sick cows at any sampling time. IL-4 levels significantly decreased in sick cows during the peri-parturient period, whereas IL-10 levels notably differed only after calving. IgG levels were significantly lower in sick cows than in healthy cows during the first week postpartum. INF-γ levels were significantly lower in sick cows, particularly one week postpartum. Haptoglobin levels were lower in cows with uterine diseases prior to calving. Although time influenced TAC and MDA levels, no significant differences were found between the groups. Overall, these findings indicate that 25(OH) D and certain immunological parameters may play a role in the health status of dairy cows around calving, warranting further investigation into their potential therapeutic effects.

Keywords 25(OH) D, Inflammatory markers, Oxidative stress, Peripartum diseases, Holstein cows

Maintaining fertility (reproductive performance) and minimizing peri-partum diseases are crucial yet complex goals in dairy herds[1,2]. Unfortunately, the transition period is disastrous for 30–50% of dairy cows, which develop at least one peri-partum disease[1,4,77,78]. This period, characterized by a significant rise in milk production, triggers a phenomenon known as homeorhesis[3], resulting in a temporary overridden homeostatic mechanisms. Concurrently, nearly all cows experience insulin resistance and decreased appetite, leading to weight loss and a negative energy balance in early lactation[5]. This period, encompassing the final weeks of gestation through the early weeks of lactation, poses a substantial risk for the development of metabolic and infectious diseases such as ketosis, metritis, and endometritis. Increased fat mobilization coupled with altered metabolic demands due to increased lactation directly affects the antimicrobial function of the immune system during early lactation[6]. Negative energy balance (NEB) reduces glucose availability, which is critical for the effective functioning of immune cells, particularly neutrophils. Additionally, the metabolites from NEB have negative effects on immune system cells, especially neutrophils[7–9]. The increase in β-hydroxybutyric acid (BHBA) observed in

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cows with ketosis disrupts neutrophil activity, increasing the risk of mastitis[10]. Consequently, leukocytes with impaired chemoattractant activity fail to eliminate cotyledons, leading to the retention of the placenta and fetal membranes. Damage to the superoxide activity of neutrophils can also contribute to conditions such as metritis and endometritis[4,5].

Vitamin D, once primarily associated with calcium and phosphorus homeostasis, is now recognized as an emerging modulator of immune function[11,12]. Vitamin D signaling suppresses certain proinflammatory factors in the adaptive immune system while enhancing some components of the innate immune system[13,14]. Intramammary injection of vitamin D improves local immune functions and reduces bacterial accumulation[15]. Furthermore, vitamin D enhances resistance to chronic and prepartum diseases in cows[16,17]. According to NRC recommendations, a daily 21,000 IU of vitamin D is necessary to maintain 25(OH) D3 levels between 20 and 50 ng/mL for optimal calcium and phosphorus balance[19]. However, the concentration of vitamin D required for overall robust immune performance remains undetermined. Recent studies have indicated that many dairy cows consume 1.5 to 2.5 times the NRC- recommended amount of vitamin D, with the serum concentration averaging between 60 and 70 ng/ml[18].

Previous studies have suggested that vitamin D plays a critical role in modulating immune responses and preventing infectious diseases. The purpose of this study was to investigate the amount of vitamin D and its relationship with several immunological indicators and oxidative stress in cows suffering from peripartum diseases compared with healthy cows. We hypothesize that there is no significant difference in vitamin D levels between healthy and affected cows (*H0*).

Materials and methods

Study design and implementation

All cows used in the study are properties of Talise Asil and Chaltasian dairy companies, Varamin, Iran, and all sampling was performed after informed consent was obtained from the owners. This study was conducted in accordance with the ethical standards for animal experimentation and was approved by the Ferdowsi University of Mashhad Ethics Committee (3/52071). Given that this study involved animals, consent to participate was not applicable. The study followed national and institutional guidelines for the care and use of animals. In addition, the authors claim that the study is reported in accordance with the ARRIVE guidelines.

This study was conducted in 2019 at two privately owned industrial dairy farms located in Varamin city, Tehran Province, Iran. The first farm housed 1,470 dairy cows with an average milk production of approximately 41.5 kg per day, whereas the second farm housed 1,540 dairy cows with an average milk production of 39.3 kg per day. All management factors and the diets of the cows were identical for the two farms. Pregnant cows were kept in open pens, and post-partum were housed in free-stall barns with sandy bedding. The cows had free access to water and were fed a total mixed ration (TMR) formulated according to NRC guidelines[19].

Feed composition

The feeding schedules for the different groups were as follows:

- Close-up cows: 6 AM and 1 PM.
- Near-calving cows: 9 AM and 2 PM.
- Fresh cows: 6 AM and 1 PM.
- Lactating cows: 6 AM, 1 PM, and 5 PM.

The chemical composition, nutritional values, and concentrate components are presented in Tables 1, 2, 3, 4.

Sample selection

A total of 330 heifers and multiparous cows were selected from the pre-calving pens 14 days before their expected calving dates for another ongoing study. All selected animals were physically healthy and had not received any medication. By the end of the study period (210 days post-partum), 162 of these cows had developed peri-parturient diseases, including ketosis (more than 1.2 mmol/L BHBA in the serum 1–21 days postpartum), mastitis (inflammatory response to infection causing visibly abnormal milk until 14 days post parturition), dystocia (assistance of two or more people or mechanical extraction or surgical procedure for calf delivery), endometritis (characterized by ultrasound examination of uterus 28 to 34 days post-partum), metritis (enlarge uterus and fetid watery red brown fluid to viscous purulent, uterine discharge within 21 days after parturition), hypocalcemia, and retained placenta (failure to expel the fetal membranes within 24 h of the end of parturition). Among these cows, 120 had only one disease, and 42 had at least two diseases. Specifically, five cows had only retained placenta, 4 had hypocalcemia, 1 had metritis, 25 had endometritis, 27 had dystocia, 2 had mastitis, and 56 had ketosis. Healthy cows did not show any clinical signs during the 210 days of study.

Comparison groups

Sixty-eight Holstein cows were selected for this study, comprising 45 cows with periparturient diseases (12 with ketosis, 5 with retained placenta, 4 with hypocalcemia, 5 with endometritis, 4 with dystocia, 1 with metritis, and 14 with multiple diseases) and 23 healthy cows. The diseased cows were further categorized on the basis of their conditions, including ketosis and uterine diseases (metritis and endometritis), ensuring that the proportion of each disease among the 45 cows matched that of all diseased cows. Additionally, the parity and body condition score (BCS) were matched between the healthy and diseased groups. The median of parity and BCS for the healthy cows were 3 and 3.5, respectively, whereas for the diseased cows, these values were 3 and 3.57, respectively. In addition, we tried to sample healthy and diseased cows from two farms equally.

Component	DM	Percentage (%)	Component	Percentage (%)
Alfalfa	1.65	16.12	CP %	11.4
Barley silo	1.48	14.46	EE %	2.8
Wheat straw	0.71	6.90	NDF %	36.1
Corn silo	2.57	25.14	ADF %	23
Concentrate	6.44	37.38	Starch Sugar	27
Forage dry matter percentage	62.62		Ash%	5.7
Concentrate dry matter percentage	37.38		ME Mj/kg	10.6
			Ca^{2+} %	1.01
			P %	0.35
			Mg^{2+} %	0.26
			Na^{+}%	0.06
			K^{+} %	1.25
			Cl^{-} %	0.74
			S %	0.29
			DCAD (mEq/kg DM)	−46

Table 1. Chemical composition and nutritional value of diets in Close-up Cows. *DM* dry matter, *DCAD* dietary cation anion difference, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber,*ME* metabolizable energy, *ADF* acid detergent fiber, *Ca* Calcium, *P* phosphorus, *Na* sodium, *K* potassium, *Mg* Magnesium, *Cl* chloride, *S* sulfur

Component	DM	Percentage (%)	Component	Percentage (%)
Alfalfa	2.3	10.67	CP %	15.7
Barley silo	1.12	5.17	EE %	4.3
Molasses	0.47	2.17	NDF %	34.4
Flaxseed	0.51	2.39	ADF %	12
Beet pulp	0.92	4.27	Starch Sugar	28.7
Alfalfa silo	5.66	26.26	Ash%	4.3
Concentrate	10.58	49.07	ME Mj/kg	12
Forage dry matter percentage	50.93		Ca2+ %	1.4
Concentrate dry matter percentage	49.07		P %	0.34
			Mg^{2+} %	0.3
			Na^{+}%	0.47
			K^{+} %	1.13
			Cl^{-} %	0.35
			S %	0.17
			DCAD (mEq/kg DM)	291

Table 2. Chemical composition, and nutritional values of diets in fresh Cows. *DM* dry matter, *DCAD* dietary cation anion difference, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber, *ME* metabolizable energy, *ADF* acid detergent fiber, *Ca* Calcium, *P* phosphorus, *Na* sodium, *K* potassium, *Mg* Magnesium, *Cl* chloride, *S* sulfur.

For statistical comparisons, diseased cows were initially grouped for analysis. Cows with ketosis and cows with uterine diseases were subsequently compared with the healthy group.

Blood sample collection and measurements

Blood samples were collected from all cows at three time points: 7 days before calving, 7 days after calving, and 21 days after calving. Sampling was performed at a fixed time interval (10.30 AM to 12 PM) relative to feeding. A total of 10 ml of blood was drawn from the coccygeal vein into serum vacutainer tubes (FL medical, Torreglia, Italy). The samples were immediately transferred to a refrigerator prior to centrifugation; blood samples in plain tubes were allowed to clot. Sera were harvested by centrifugation of blood samples (1800 g for 10 min) at room temperature, aliquoted in 1.5 mL microtubes, and stored at −80 °C in a freezer until further analysis. The total 25(OH)D concentration (25-(OH)D2 & 25-(OH)D3) in the serum samples was determined with enzyme-linked immunosorbent assay (ELIZA) kits (Monobind Inc. Lake Forest, CA92630, USA; within assay precision: 6.36%, between assay precision: 6.95%, sensitivity: 0.67 ng/ml).

Component	DM	Percentage (%)	Component	Percentage (%)
Alfalfa	0.74	2.4	CP %	16.2
Barley silo	0.76	2.48	EE %	3.6
Molasses	0.36	1.17	NDF %	31.4
Wheat straw	0.92	3	ADF %	17.3
Flaxseed	0.53	1.74	Starch Sugar	34.3
Beet pulp	1.38	4.51	Ash%	4.8
Alfalfa silo	2.23	7.29	ME Mj/kg	12.2
Corn silo	7.87	25.7	Ca^{2+} %	0.99
Concentrate	15.07	51.7	P %	0.36
Forage dry matter percentage	48.3		Mg^{2+} %	0.31
Concentrate dry matter percentage	51.7		Na^{+}%	0.46
			K^{+} %	1.14
			Cl^{-} %	0.35
			S %	0.18
			DCAD (mEq/kg DM)	

Table 3. Chemical composition, and nutritional values of diets in lactating Cows. *DM* dry matter, *DCAD* dietary cation anion difference, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber, *ME* metabolizable energy, *ADF* acid detergent fiber, *Ca* Calcium, *P* phosphorus, *Na* sodium, *K* potassium, *Mg* Magnesium, *Cl* chloride, *S* sulfur.

Concentrate Feed Components	Close-up (%)	Fresh Cows (%)	Lactating Cows (%)
Wheat Bran	2.0	–	–
Crushed Barley	36.7	21.5	29.05
Crushed Corn	20.5	20.30	24.25
Corn/Soy Mix	–	9.5	–
Soybean Meal	–	15.8	18.0
Canola Meal	18.5	5.0	9.0
High-Fat Soybean	–	16.0	9.7
Internal Fat	–	–	1
External Fat	–	1.0	1.4
Bentonite	1.0	1.4	1.5
aniofir[b]	15.0	–	–
Mineral Supplements[a]	3.0	1.67	0.8
Vitamin Supplements[a]	1.5	0.83	0.5
Magnesium Oxide	–	0.33	0.3
Bicarbonate	–	2.27	2.0
Salt	–	0.6	0.5
Calcium Carbonate	1.5	1.2	0.7
DCP	–	0.50	0.3
Propylene Glycol	–	1.95	0.7
Mycosorb	0.3	–	–
Methionine	–	0.15	–
Total percentage	100	100	100

Table 4. Concentrate feed components provided to cows during different Periods. Supplement contain/ kg: Vit A 1300,000 IU, Vit D3 360,000 IU, Vit E 12000 IU, Mn 10,000 mg, Zn 16000 mg, Cu 3000 mg, Se 80 mg, Iodine 150 mg, Fe 800 mg, Co 120 mg, antioxidant 1000 mg b Anionic salt, NFC: 30± 1, Ca: 2.7 %, p: 0.5 %, Mg: 1.1 %, Cl: 6.9%, K: 1.1 %, Na: 0.03 %, S: 1.9 %, DCAD: – 2850 meq/kg.

Measurement of immunological parameters

Immunological parameters, including interleukin 4 (IL-4), interleukin 10 (IL-10), interferon-γ (INF-γ), immunoglobulin G (IgG), and haptoglobin (hap) amounts, were measured via bovine-specific ELISA kits (Shanghai Crystal Day Biotech Co., Ltd., Shanghai, China). The assays were conducted according to the protocols provided by the manufacturer. All the measurements were performed via ELISA using an automatic washer and reader device (Bio Tek, USA, Winooski ELx-800, ELx-50) in the Laboratory of Center of Excellence in Ruminant

Abortion and Neonatal Mortality of the Ferdowsi University of Mashhad, Faculty of Veterinary Medicine. The performance characteristics of the kits are summarized in Table 5.

Assessment of oxidative stress markers

Lipid peroxidation (MDA)

Malondialdehyde (MDA) amounts were measured via the Nalondi™ Lipid Peroxidation Assay Kit (Navand Salamat Co., Urmia, Iran) with a spectrophotometer at a wavelength of 500 nm, according to the manufacturer's recommendation.

Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of the serum was assessed using the Naxifer™ Total Antioxidant Capacity (TAC) Assay Kit (Navand Salamat Co., Urmia, Iran). This assay is based on the ferric reducing ability of plasma (FRAP) and was measured at a wavelength of 593 nm via an autoanalyzer (Biotecnica, BT 1500, Rome, Italy).

Statistical analysis

The data were analyzed via SPSS for Windows (version 22; SPSS Inc.). The Kolmogorov-Smirnov test, kurtosis, and skewness indicated a non-normal distribution of the measured variables in the diseased cows. The Friedman test was used to examine the effect of time within each group, and the Mann–Whitney test was applied for comparisons between healthy and diseased cows at each sampling time. Spearman's rank correlation was used to determine correlations between variables. Statistical significance was declared at $P \leq 0.05$, and $0.05 < P < 0.10$ indicated a trend toward significance. The data are presented as medians and 25th–75th percentiles.

Results and findings

The Friedman test revealed that time had a significant effect on 25(OH) D levels in both healthy and diseased animals ($p \leq 0.05$). Pairwise comparisons at different sampling times revealed no significant differences between the healthy and diseased groups. Additionally, there were no significant differences in 25(OH) D levels between healthy cows and cows with ketosis at any sampling time. Moreover, no significant differences in 25(OH) D levels were detected between healthy cows and cows with uterine diseases.

The changes in the IL-4 levels over the study period were significant in healthy cows ($p \leq 0.05$), whereas the changes over time tended to be significant in diseased cows. IL-4 levels differed significantly between healthy and diseased cows one week before and one week after calving ($p \leq 0.05$), with lower levels in diseased cows. No significant differences in the IL-4 levels were detected between healthy and ketotic cows at any sampling time. IL-4 levels were significantly lower in cows with uterine diseases than in healthy cows one week before and one week after calving ($p \leq 0.05$).

Time had a significant effect on the IL-10 levels in healthy cows ($p \leq 0.05$) but not in diseased cows. IL-10 levels differed significantly between healthy and diseased cows one week after calving ($p \leq 0.05$) and tended to significantly differ three weeks after calving. No significant differences in IL-10 levels were found between healthy cows and cows with ketosis at any sampling time. However, significant differences in IL-10 levels were observed between healthy cows and cows with uterine diseases in the first and third weeks after calving ($p \leq 0.05$).

Time had no significant effect on the IgG levels in either group, but the IgG levels differed significantly between healthy and diseased cows one week after calving, with lower levels in diseased cows ($p \leq 0.05$). Additionally, the IgG levels tended toward significance one week before calving and three weeks after calving, with lower levels in diseased cows. IgG levels were significantly lower in cows with uterine diseases than in healthy cows at all sampling times ($p \leq 0.05$).

INF-γ levels were not affected by time in healthy cows, although time had a significant effect on diseased cows ($p \leq 0.05$). INF-γ levels differed significantly between healthy and diseased cows one week after calving, with lower levels in diseased cows ($p \leq 0.05$). Significant differences were also observed between healthy cows and cows with uterine diseases at the same time, with lower levels in cows with uterine diseases ($p \leq 0.05$). No significant differences were found in the INF-γ levels between healthy and ketotic cows.

Time had a significant effect on haptoglobin levels in diseased cows ($P < 0.05$) and tended toward significance in healthy cows. No significant differences in haptoglobin levels were found between healthy and diseased cows or between healthy cows and cows with ketosis. However, a significant tendency toward a decrease in haptoglobin levels was observed in cows with uterine diseases compared with healthy cows seven days before calving.

Time had a significant effect on TAC levels in both healthy and diseased cows ($P < 0.05$), but no significant differences were detected between the two groups at any sampling time. A tendency toward a significant decrease

Parameters	Sensitivity	Assay range	Inter-assay	Intra-assay
IgG	1.03 µg/ml	2-600 µg/ml	CV<10%	CV<8%
hap	1.36 µg/ml	3-900 µg/ml	CV<10%	CV<8%
IL-4	0.54 ng/L	1- 280 ng/L	CV<10%	CV<8%
IL-10	2.52 ng/L	5-2000 ng/L	CV<10%	CV<8%
INF-γ	2.35 pg/ml	5-2000 pg/ml	CV<10%	CV<8%

Table 5. Measurement characteristics of the kits used for assessing the immunological variables.

in TAC levels was observed in healthy cows compared with cows with uterine diseases one week after calving. No differences in TAC levels were found between healthy and ketotic cows.

Time had no effect on MDA levels in healthy cows, but a significant effect was observed in diseased cows ($p \leq 0.05$). No significant differences in MDA levels were found between healthy and diseased cows at any sampling time. A trend toward significant differences in MDA levels was observed between healthy cows and cows with uterine diseases one week after calving.

In the first week before calving, a significant inverse correlation was observed between 25(OH) D and IL-4 levels in healthy cows ($r = -0.569$, $p = 0.042$). Moreover, in diseased cows, 25(OH) D levels were negatively correlated with IL-10 ($r = -0.318$, $p = 0.031$) and INF-γ ($r = -0.336$, $p = 0.023$) levels. No correlations were detected between the 25(OH) D level and the other measured parameters in the first and third weeks after calving. All the results are presented in Tables 6, 7 and 8.

Discussion

The Friedman test revealed that time significantly affects 25(OH) D levels in both the healthy and diseased animal groups. In both healthy and diseased cows, the 25(OH) D levels were highest 7 days before calving, then significantly decreased, reaching their lowest levels on the 7th day after calving, and subsequently increased again until the 21 st day postpartum. This decrease occurs exactly when cows are most susceptible to peri-parturient diseases. Our findings are consistent with those of Holcomb et al., who reported that 25(OH) D levels during the transition period were significantly lower than those during the dry period and close-up period[11]. Another study that assessed 25(OH) D levels at different stages of lactation reported lower levels early in lactation than during mid- to late lactation[18]. Similarly, Hassanabadi et al. reported a significant reduction in 25(OH) D levels in the control group seven days after calving[20]. Another study reported a gradual increase in 25(OH) D and VDBP levels during the lactation period[21].

With the onset of lactation, the demand for calcium to produce colostrum suddenly increases. Therefore, dairy cows face a sudden drop in calcium early in lactation[22], leading to the mobilization of more body calcium reserves to maintain homeostasis. Notably, colostrum contains approximately two to three times more 25(OH) D than samples taken at the sixth milking and on the 28th day postpartum[22]. Increased parathyroid hormone secretion after calving helps regulate calcium metabolism, leading to increased $1,25(OH)_2D_3$ levels, which may be associated with decreased 25(OH) D levels in the serum. Additionally, some vitamin D is used for immune system activities. One study indicated that the decrease in 25(OH) D levels is due to renal dysfunction early in lactation, leading to the excretion of 25(OH) D and VDBP in urine[23]. However, this finding does not appear to apply to our study, as no clinical or laboratory signs of renal damage were observed in the cow population.

Dual comparisons at different sampling times revealed that, contrary to our expectations, there were no significant differences in 25(OH) D levels between the healthy and diseased groups. Our results align with those of Poindexter et al., who also reported no significant difference in the incidence of peri-parturient diseases between the treatment groups (D_2 and D_3 in the diet)[24]. Bruce et al. noted that although there is substantial evidence of the immunomodulatory properties of $1,25(OH)_2D_3$ in vitro, such evidence in vivo is limited. The authors also suggested that the role of these properties in disease processes is not yet clear[25]. Conversely,

Variable	1 Week Before Calving		1 Week After Calving		3 Weeks After Calving		*p*-value	
	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
25(OH)D (ng/ml)	52.04 (45.55-59.18)[a]	50.69 (46.53-58.60)[a]	47.27 (35.79-50.93)[a]	45.08 (39.11-50.82)[a]	53.62 (41.20-59.08)[a]	51.79 (44.48-58.54)[a]	0.013	0.000
IL-10 (ng/L)	183.22 (153.32-258.79)[a]	159.14 (122.18-232.63)[a]	194.84 (172.42-281.21)[a]	142.53 (139.31-220.17)[b]	176.58 (145.02-213.94)*	140.04 (109.73-185.71)*	0.007	0.160
IL-4 (ng/L)	30.23 (20.36-36.25)[a]	21.6 (19.27-27.87)[b]	26.74 (21.16-29.86)[a]	21.88 (18.29-25.44)[b]	23.41 (20.14-27.11)[a]	22.75 (17.71-26.67)[a]	0.012	0.065*
INF-γ (pg/ml)	205.58 (169.04-255.31)[a]	176.66 (132.51-246.94)[a]	182.24 (153.31-229.43)[a]	150.27 (121.09-201/52)[b]	171.07 (152.30-196.95)[a]	147.73 (116.02-197.97)	0.092*	0.000
Hpt (µg/ml)	83.44 (72.08-127.79)[a]	75.68 (59.60-95.91)[a]	80.67 (71.52-98.41)[a]	73.18 (52.67-93.31)[a]	84.55 (64.04-102.84)[a]	71.24 (54.06-86.07)	0.079*	0.012
IgG (µg/ml)	57.38 (44.84-61.77)*	38.94 (33.88-61.99)*	54.97 (44.38-68.88)[a]	40.15 (36.83-54.36)[b]	48.62 (34.94-56.02)[a]	40.45 (34.94-56.02)[a]	0.084*	0.589
TAC (mmol Fe^{2}-L)	0.3 3(0.30-0.34)[a]	0.33 (0.38-0.39)[a]	0.38 (0.35-0.43)[a]	0.36 (0.33-0.39)[a]	0.41 (0.36-0.44)[a]	0.39 (0.37-0.46)[a]	0.009	0.000
MDA (nmol/ml)	21.39 (19.27-26.00)[a]	22.79 (19.53-26.34)[a]	23.26 (19.02-32.56)[a]	25.85 (21.28-32.52)	27.44 (20.25-23.17)[a]	25.00 (21.93-34.85)[a]	0.146	0.004

Table 6. Median and quartile of measured variables and statistical comparisons in healthy and deceased cows. In each row, different letters indicate a significant difference ($p < 0.05$). *Indicates a tendency towards significance ($0.1 > p > 0.05$).

Variable	1 Week Before Calving		1 Week After Calving		3 Weeks After Calving		*p*-value	
	Healthy	Ketotic	Healthy	Ketotic	Healthy	Ketotic	Healthy	Ketotic
25(OH)D (ng/ml)	52.04 (45.55–59.18)[a]	51.86 (47.97–56.35)[a]	47.27 (35.79–50.93)[a]	47.45 (40.39–53.45)[a]	53.62 (41.20-59.08)[a]	51.85 (47.62–58.02)[a]	0.013	0.257
IL-10 (ng/L)	183.22 (153.32-258.79)[a]	173.25 (133.39-267.09)[a]	194.84 (172.42-281.21)[a]	159.14 (103.08-256.71)[a]	176.58 (145.02-213.94)*	125.92 (106.41-287.85)[a]	0.007	0.123
IL-4 (ng/L)	30.23 (20.36–36.25)[a]	25.87 (20.61–29.83)[a]	26.74 (21.16–29.86)[a]	21.88 (20.36–28.27)[a]	23.41 (20.14–27.11)[a]	23.48 (21.19–27.25)[a]	0.012	0.708
INF-γ (pg/ml)	205.58 (169.04-255.31)[a]	202.03 (143.93-275.61)[a]	182.24 (153.31-229.43)[a]	154.33 (125.66-259.12)[a]	171.07 (152.30-196.95)[a]	131.49 (51.43-107.83)[a]	0.092*	0.001
Hpt (μg/ml)	83.44 (72.08-127.79)[a]	71.24 (58.22-125.99)[a]	80.67 (71.52–98.41)[a]	65.42 (51.01-120.58)[a]	84.55 (64.04-102.84)[a]	67.92 (51.43-107.83)[a]	0.079*	0.030
IgG (μg/ml)	57.38 (44.84–61.77)[a]	42.27 (36.00-63.51)[a]	54.97 (44.38–68.88)[a]	42.42 (37.05–61.84)[a]	48.62 (34.94–56.02)[a]	37.89 (35.01–66.23)[a]	0.084*	0.109
TAC (mmol Fe^{2-}L)	0.33 (0.30–0.34)[a]	0.34 (0.30–0.43)[a]	0.38 (0.35–0.43)[a]	0.37 (0.33–0.43)[a]	0.41 (0.36–0.44)[a]	0.41 (0.37–0.47)[a]	0.009	0.002
MDA (nmol/ml)	21.39 (19.27-26.00)[a]	24.88 (20.81–27.79)[a]	23.26 (19.02–32.56)[a]	24.88 (18.82–26.87)[a]	27.44 (20.25–23.17)[a]	23.49 (20.25–31.39)[a]	0.146	0.931

Table 7. Median and quartile values of measured variables and statistical comparisons in healthy and ketotic cows. In each row, different letters indicate a significant difference ($p < 0.05$). * Indicates a tendency towards significance ($0.1 > p > 0.05$).

Variable	1 Week Before Calving		1 Week After Calving		3 Weeks After Calving		*p*-value	
	Healthy	Uterine Disease	Healthy	Uterine Disease	Healthy	Uterine Disease	Healthy	Uterine Disease
25(OH)D (ng/ml)	52.04 (45.55–59.18)[a]	51.53 (47.09–59.54)[a]	47.27 (35.79–50.93)[a]	43.78 (40.03–50.38)[a]	53.62 (41.20-59.08)[a]	52.17 (43.93–62.33)[a]	0.013	0.000
IL-10 (ng/L)	183.22 (153.32-258.79)[a]	153.32 (110.97–207. 30)[a]	194.84 (172.42-281.21)[a]	138.38 (110.14-220.59)[b]	176.58 (145.02-213.94)*	131.73 (109.31–174.91)[b]	0.007	0.559
IL-4 (ng/L)	30.23 (20.36–36.25)[a]	20.22 (16.62–25.87)[b]	26.74 (21.16–29.86)[a]	21.67 (18.11–24.42)[b]	23.41 (20.14–27.11)*	22.22 (16.37–26.31)*	0.012	0.054*
INF-γ (pg/ml)	205.58 (169.04-255.31)[a]	175.13 (132.00-225.88)[a]	182.24 (153.31-229.43)[a]	149.76 (117.79-192.89)[b]	171.07 (152.30-196.95)[a]	143.67 (119.32-201.52)[a]	0.092*	0.002
Hpt (μg/ml)	83.44 (72.08-127.79)[a]	75.68 (59.60-95.91)[a]	80.67 (71.52–98.41)[a]	73.18 (52.67–93.31)[a]	84.55 (64.04-102.84)[a]	71.24 (54.06–86.07)*	0.079*	0.237
IgG (μg/ml)	57.38 (44.84–61.77)*	38.04 (33.05-53.00)[b]	54.97 (44.38–68.88)[a]	39.25 (34.26–50.43)[b]	48.62 (34.94–56.02)[a]	41.06 (33.20-48.92)[a]	0.084*	0.970
TAC(mmol Fe^{2-}L)	0.33 (0.30–0.34)[a]	0.32 (0.30–0.38)[a]	0.38 (0.35–0.43)[a]	0.36 (0.32–0.38)*	0.41 (0.36–0.44)[a]	0.39 (0.36–0.47)[a]	0.009	0.000
MDA(nmol/ml)	21.39 (19.27-26.00)[a]	22.79 (19.30-25.13)[a]	23.26 (19.02–32.56)*	28.29 (22.86–35.76)*	27.44 (20.25–23.17)[a]	29.27 (22.93–36.51)[a]	0.146	0.001

Table 8. Median and quartile values of measured variables and statistical comparisons in healthy and cows with uterine diseases. In each row, different letters indicate a significant difference ($p < 0.05$). * Indicates a tendency towards significance ($0.1 > p > 0.05$).

another study reported that parathyroid hormone and 25(OH) D levels were significantly higher in cows with parturient paresis than in healthy cows[26]. Additionally, Martinez et al. demonstrated that oral cholecalciferol administration reduced disease incidence and mortality rates in cows[22]. Similarly, Lippolis et al. reported that 25(OH) D administration reduced the severity of intramammary diseases through various mechanisms. Another study revealed that 25(OH) D might be effective in modulating the innate immune system in mammary glands to improve defense against bacterial diseases[16].

Moreover, there was no significant difference in 25(OH) D levels between healthy cows and those with ketosis at any sampling time. Xu et al.'s study also supports our findings, showing no change in BHBA levels and their negative impact on cow health with vitamin D supplementation[27]. Silva et al. also reported no change in BHBA levels. This might be because the oxidative capacity of the liver in their study was not beyond the limits of the liver[28]. Typically, BHBA levels are less than 1.2 mmol. If levels are between 1.2 and 1.5, cows will show clinical and subclinical ketosis symptoms[29]. In contrast, Wisnieski et al. reported that high 25(OH)D concentrations during the dry and close-up periods were associated with increased urinary ketone body concentrations from days 2 to 10 postpartum. Wisnieski et al. suggested that high availability of 25(OH) D might increase ketone body formation[30]. Martinez et al. reported that adding cholecalciferol to the diet increased ketone bodies in the blood, but in cows receiving cholecalciferol, the risk and duration of hyperketonemia and its recurrence were similar to those in other treatment groups[22]. Another study showed that calcitriol administration mildly increased BHBA levels, leading to the conclusion that calcitriol does not immediately improve lactation and energy metabolism in the first month postpartum[31]. On the other hand, Hassanabadi et al. reported that vitamin D injection reduced BHBA and NEFA levels, which could control the negative energy balance[20]. Given the conflicting results of different studies, further research in this area is necessary.

There was no significant difference in 25(OH) D levels between healthy cows and those with uterine diseases. This result was consistent with that of Poindexter et al.'s study, which revealed no difference in peri-parturient disease incidence between treatment groups (D_2 and D_3 in the diet)[24]. However, Martinez et al. in 2018 reported that cows that consumed 3 mg of cholecalciferol daily presented significantly lower rates of retained placenta and metritis than cows that consumed the same amount of cholecalciferol daily[22]. Similarly, Wisnieski et al. reported that in early lactation, 25(OH) D levels were associated with a decrease in the incidence of uterine diseases. Calcium and vitamin D metabolism disorders can increase the risk of retained placenta and metritis, as shown by several studies[32,33]. Hypocalcemia negatively affects neutrophil function, including bacterial phagocytosis and oxidative bursts, which affect the immune system of dairy cows[32,34]. $1,25(OH)_2D_3$ regulates both the innate and acquired immune pathways[14,35]. One reason for our results might be that the antioxidant effect of vitamin D is not direct; thus, its mechanism takes time. Additionally, in other studies, dietary interventions involved adding excess vitamin D in various forms, whereas in our study, vitamin D levels were measured without any alterations, which may have influenced the discrepancy with other studies.

Multiple studies have shown that almost all cows experience some degree of inflammation after calving, with the severity and duration of inflammation directly related to pre-partum disease. In our study, the IL-10 levels significantly increased in healthy cows during the first week postpartum and decreased in the third week postpartum. The trend of IL-10 changes in our study was similar to that reported by Grawel et al.[36]. Our results were also consistent with the findings of Heiser et al., who reported increased IL-10 levels in the first week postpartum, followed by a decrease[37]. Lange et al. reported an increase in IL-10 levels postpartum across all groups and noted that IL-10 expression was lower in cows with BCS = 5 than in those with BCS = 4, suggesting that cows with BCS = 5 were less prone to systemic inflammation. Lange and colleagues reported that nutritional management significantly impacted IL-10 expression[38]. IL-10 is a cytokine that modulates the immune response of helper T (Th) cells through various anti-inflammatory mechanisms. This includes the inhibition of NF-κB; the reduction of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α; the suppression of tissue factor expression; the gene transcription of INF, IL-2, and chemokines involved in activation; T-cell expression; RANTES secretion; and the secretion of metalloproteinases in monocytes/macrophages and CD4 + T cells. IL-10 also suppresses inflammation by inducing the expression of cytokine synthesis inhibitors and COX-1, which play roles in reducing preeclampsia. Furthermore, IL-10 is involved in differentiating T cells into regulatory T cells, contributing to immune system regulation. In humans, normal pregnancy is associated with increased serum and placental IL-10 levels[39].

However, in the present study, there were no significant changes in the IL-10 levels in the diseased cows. The authors suggest that this difference between healthy and diseased cows could be due to variations in disease severity, immune status, nutrition, and herd BCS. The lack of an inappropriate IL-10 response postpartum might contribute to postpartum diseases.

In addition, no significant differences in IL-10 levels were found between healthy and ketotic cows at different sampling times. This finding contrasts with that of Karimi et al., who reported significantly higher IL-10 levels in subclinical ketosis cows than in controls[40]. Another study reported elevated IL-10 concentrations in both cows with subclinical and clinical ketosis, indicating simultaneous inflammatory and anti-inflammatory processes. Compared with those in cows with clinical ketosis, elevated IL-10 levels in cows with subclinical ketosis had a greater inhibitory effect on inflammation. The authors suggested that IL-10, produced by regulatory T lymphocytes, has a protective effect and prevents tissue damage by inhibiting immune cell activity and reducing autoimmune responses[41]. It is possible that the severity of ketosis in the current study was not high enough to induce significant IL-10 changes.

Significant differences in IL-10 levels between healthy cows and those with uterine diseases were observed in the first and third weeks postpartum, with a notable decrease in diseased cows compared with healthy cows. This finding contrasts with that of Islam et al., who reported higher IL-10 levels in cows with clinical metritis and retained placenta 15 days before calving and increased IL-10 levels on days 0 and 15 postpartum[42]. Galvao et al. reported no correlation between IL-10 gene expression and uterine diseases when *E. coli*-stimulated cells were analyzed. The gene expression of IL-10 increased at 14 and 28 days postpartum, but no relationship with uterine diseases was found. Multiple comparisons revealed significant differences in IL-10 gene expression between metritis and endometritis on days 0, 7, and 21 postpartum, with a trend toward significance on day 7. IL-10 gene expression was lowest on day 14 postpartum and highest on day 21 postpartum. There was no correlation between IL-10 gene expression and uterine diseases in cells stimulated with *E. coli*, and timing also did not significantly affect IL-10 expression[43].

Changes in IL-4 levels during the study period were significant in healthy cows, and the effect of time tended toward significance in the group of diseased cows. IL-4 is another anti-inflammatory cytokine synthesized by T-helper 2 lymphocytes and mast cells. It increases the expression of IgG and IgA and suppresses the production of IFN-γ and IL-17. IL-10 may suppress the production of cytokines such as IL-4[44]. The decrease in IL-4 production could be due to the inhibitory effect of IL-10.

IL-4 levels were significantly lower in the week before and the week after calving in the diseased cows than in the healthy cows, but no significant difference was observed on day 21 postpartum. There was also a significant difference in IL-4 levels between healthy cows and those with uterine diseases one week before and one week after calving. These results are consistent with those of Warma et al., who reported a significant reduction in IL-4 expression around calving in various groups. The increased expression of IL-4 receptors in follicular granulosa cells may mediate the ability of IL-4 activity to promote granulosa cell proliferation and steroidogenesis. The reduction in IL-4 around calving might indicate its role in cell proliferation, similar to that in lymphocytes, oocyte maturation, and ovulation. The increased IL-4 expression during oocyte maturation supports the hypothesis that IL-4 plays a crucial role in the production of superior oocytes. A decrease in IL-4 around calving may lead to limited granulosa cell proliferation and delayed oocyte maturation, resulting in prolonged periods without ovulation. Changes in IL-4 expression also lead to follicular persistence and anovulation in cows with ovarian cysts[46]. In humans, inflammation is essential for successful pregnancy, and instability of anti-inflammatory cytokines can lead to pregnancy disorders. IL-4 and IL-10 are vital for maintaining pregnancy, and evidence suggests that deficiencies in IL-4 or IL-10 can cause infertility, spontaneous abortion, preterm birth, and fetal growth disorders[45].

In the present study, no significant difference in IL-4 levels was detected between healthy and ketotic cows at the sampling times. This finding contrasts with that of Karimi et al., who reported significantly higher IL-4 levels in cows with subclinical ketosis than in control cows[40]. Since ketosis is associated with a negative energy balance and high NEFA levels, it activates the TLR-NF-κB pathway, leading to inflammation[47]. The lack of increased IL-4 in the ketotic cows in our study might be because the severity of the disease was not high enough to cause changes in the IL-4 amounts.

Time did not significantly affect the IgG levels in either group, but the IgG levels were significantly lower in the week after calving in the diseased cows than in the healthy cows. Additionally, the IgG levels tended toward significance, with lower values in the diseased cows in the week before calving and three weeks after calving. These findings are in line with those of Herr et al., who reported a persistent decrease in IgG levels from the eighth week before calving[48]. Other studies also reported a decreasing trend in IgG levels from weeks four to five before calving[49,50]. These findings suggest that a decrease in the IgG amount during the third trimester of pregnancy is a physiological phenomenon in dairy cows. The reduction in IgG may be due to the transfer of immunoglobulins to the mammary glands, with a selective influx of IgG1[51]. Studies have shown that increased expression of IgG1 receptors in the mammary glands during the dry period increases the binding of immunoglobulins to these components[52]. Herr et al. reported that the second phase of parturition (fetal expulsion) was associated with the lowest IgG concentrations, which remained low until seven days postpartum before increasing[48]. These results align with our study.

IgG levels were significantly lower at all sampling times in cows with uterine diseases than in healthy cows. Few studies have confirmed the presence of different classes of immunoglobulins in uterine secretions[54]. In addition to IgE, the presence of other major immunoglobulins in endometrial secretions reflects the inflammatory response of the endometrium following bacterial infections and clinical recovery[55]. These immunoglobulins likely act as opsonins to increase phagocytosis and stimulate the complement pathway[56,57]. The type of IgG secreted in the reproductive tract might depend on the antigenic stimulus. For example, Trichomonas fetal infection leads to a Th2 response and the production of IgG1 and IL-4[58]. Our results align with these findings, as IgG levels are lower in cows with uterine diseases, potentially because of the secretion of this immunoglobulin from the endometrium.

No significant difference in IgG levels was observed between healthy and ketotic cows. This finding contrasts with that of Karimi et al., who reported a trend toward significant differences in IgG levels between healthy and diseased groups[40]. Vasilieva et al. reported that IgG levels were lower in cows with ketonuria than in controls. Metabolic disorders in pregnant cows, associated with increased ketone bodies, negatively impact antibody production. This study revealed that ketosis had the least effect on reducing IgG levels in serum and colostrum, which is related to the half-life of different immunoglobulin classes[53]. The severity of the disease in our study may not have been sufficient to alter IgG synthesis.

IFN-γ levels were not affected by time in healthy cows, although the effect of time was significant in diseased cows. Compared with those in healthy cows, IFN-γ levels in diseased cows were significantly lower in the first week postpartum. A significant difference in the levels of IFN-γ, which decreased in the group with uterine diseases, was also detected between healthy and diseased cows. Kehrli et al. reported that pregnancy leads to the suppression of Th1 (cell-mediated immunity) and increased Th2 (humoral immunity) responses. As pregnancy progresses, the immune system's ability to produce Th1 cytokines (IFN-γ and IL-2) decreases[59]. Estrogen and progesterone play crucial roles in suppressing cellular immunity and enhancing humoral immunity in mice[60]. Corticosteroids are also known to suppress cell-mediated immune responses and increase humoral immunity by inhibiting Th1 cytokines, including IL-2 and IFN-γ[61]. Similar findings have been reported in cows[62,63]. Cytokines produced by bovine leukocytes around parturition are disrupted, and IL-2 and IFN-γ production significantly decreases, indicating Th1 response suppression[64]. These cytokine production changes might be associated with an increased incidence of peri-parturient diseases[65]. The reduction in IFN-γ levels severely impacts phagocytic cell function, which is consistent with the role of IFN-γ in restoring suppressed neutrophil function[59]. Furthermore, disruptions in B-cell capacity around calving in IgM secretion appear to result from impaired IL-2 and IFN-γ production[49]. Our results are consistent with those of Patra et al., who reported higher

IFN-γ expression in healthy cows than in diseased cows up to three weeks postpartum[66]. Immunosuppression around calving in cows leads to mastitis and postpartum metritis[67]. A study in humans also revealed fewer IFN-γ-producing Th1 cells in women with mild to severe endometritis than in healthy women[68]. Our study aligns with other researchers' findings, as a decrease in IFN-γ around calving predisposes cows to peri-parturient diseases.

The effect of time on haptoglobin levels was significant ($P<0.05$) in diseased cows and tended toward significance in healthy cows. In the group with uterine diseases, haptoglobin levels were lower seven days before calving than in healthy cows. These results contradict those of other studies. Shin et al. reported a greater occurrence of peri-parturient and postpartum diseases in cows with higher average haptoglobin levels. Pohl et al. also reported a greater incidence of postpartum diseases within five days of calving in cows with higher haptoglobin levels. Other studies confirm Shin and Pohl's findings, suggesting that high haptoglobin concentrations postpartum are associated with increased occurrence of peri-parturient and postpartum diseases[69,70]. The lack of difference between the two groups in our study might be due to the type and severity of inflammation, as haptoglobin is a major acute phase protein, and its increase correlates with the intensity and severity of inflammation.

Time had a significant effect on TAC levels ($P<0.05$) in both healthy and diseased cows, but no significant differences were detected between the groups at any sampling time. This is in contrast with another study that did not find a significant increase in TAC levels postpartum[76]. In our study, parturition in both groups likely caused oxidative stress and increased TAC levels.

Time did not affect MDA levels in healthy cows, but it had a significant effect on diseased cows. No significant difference in MDA levels was detected between healthy and diseased cows at any sampling time. A trend toward significance was observed in the one-week postpartum MDA levels in diseased cows compared with healthy cows. This finding aligns with Baithalu et al., who reported that healthy cows had higher TAC levels and lower MDA levels around calving[71]. Hanafi and Heidarpour reported that lower TAC levels are associated with endometritis in cows and buffaloes[72,73]. Cows exposed to high oxidative stress exhibit very high MDA levels and very low TAC levels around calving and suffer from acute uterine diseases ten days postpartum. Postpartum, the endometrium is exposed to multiple infectious agents and oxidative stress[74,75]. Increased oxidant production and reduced antioxidant capacity may damage uterine defense mechanisms and cause uterine disorders[71].

Conclusion

These findings indicate that cows with peri-parturient diseases exhibit altered immune responses characterized by reduced levels of key inflammatory markers, such as IL-4, IL-10, and IgG. Despite significant fluctuations in vitamin D levels over time, no notable differences were found between healthy and affected cows, suggesting that other factors may also contribute to immune dysfunction in this context.

The observed trends in oxidative stress markers further emphasize the complex interplay between nutrition, immune health, and disease susceptibility in dairy cows. Given the potential implications for herd health management, these results underscore the importance of further research into the role of vitamin D in enhancing immune function and reducing disease incidence in dairy herds. Future studies should explore targeted interventions, including vitamin D supplementation, to improve the health outcomes of cows during the vulnerable periparturient period.

Data availability

The data that support the findings of this study are not openly available and are available from the corresponding author upon reasonable request.

Received: 2 January 2025; Accepted: 2 July 2025
Published online: 06 July 2025

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Acknowledgements

The authors thank all persons that help for conducting this study in farm and laboratory.

Author contributions

Saba Ahmadi: Investigation; Data curation; Methodology; Validation Writing review & editing. Amir Afshar Bahrabad: Investigation; Data curation. Mehrdad Mohri: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing review, and editing. Nima Farzaneh: Supervision; Validation; Methodology. All authors reviewed the manuscript.

Funding

This work was supported by the Deputy of Research and Technology, Ferdowsi University of Mashhad (grant number 52071).

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The study did not include human subjects or client-owned animals. All cows used in the study are properties of Talise Asil and Chaltasian dairy companies, Varamin, Iran, and all sampling was performed after informed consent was obtained from the owners. This study was conducted in accordance with the ethical standards for animal experimentation and was approved by the Ferdowsi University of Mashhad Ethics Committee (3/52071). Given that this study involved animals, consent to participate was not applicable. The study followed national and institutional guidelines for the care and use of animals.

Additional information

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