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CYTOKINE AND OXIDATIVE STRESS PROFILES IN CERVICOVAGINAL MUCUS OF COWS AT INSEMINATION: ASSOCIATIONS WITH PREGNANCY AND CALF SEX

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ABSTRACT

This study aimed to examine the relationship between the Th1/Th2 cytokine balance and oxidative stress parameters in the cervicovaginal mucus of cows at the time of insemination. It also investigated the potential associations of these factors with conception rates and calf sex. Cervicovaginal mucus samples were collected from 90 cows at the time of insemination. Of these, 55 cows were confirmed pregnant (Group I), and 33 were non-pregnant (Group II). Ultimately, 35 female and 20 male calves were born. The mucus samples were analyzed for interleukins (IL) -2, IL -4, IL -5, IL -10, tumor necrosis factor-alpha (TNFα), interferon-gamma (IFN-γ), and oxidative stress markers including malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD). IFN-γ levels were higher in pregnant cows, while MDA levels were higher in non-pregnant cows. Among parturient cows, those that gave birth to male calves had higher levels of IL-2 and GSH compared to those with female calves. Based on these findings, cytokine and oxidative stress parameters in cervicovaginal mucus may not serve as reliable biomarkers for predicting pregnancy success or calf sex. Furthermore, no significant differences in Th1/Th2 cytokine balance were observed in relation to pregnancy status or offspring gender.

Keywords: Cow, cervicovaginal mucus, cytokine, oxidative stress

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INTRODUCTION

The reproductive performance of dairy cows is a critical determinant of productivity and profitability in the dairy industry, with fertility directly impacting both genetic progress and herd sustainability (Hanumant *et al*., 2019; Muller *et al*., 2018). Over the past four decades, advances in genetics, nutrition, and management practices have significantly increased milk yield per cow; however, these gains have often been accompanied by declines in reproductive efficiency (Bernardi *et al*., 2016; Butler, 1998; Royal *et al*., 2000; Lucy, 2001). This reduction has been linked to long-term selection for milk production (Hanuš *et al*., 2010), suboptimal herd management (Vacek *et al*., 2007), and particularly negative energy balance, which can delay postpartum estrus and impair estrus detection (Leblanc, 2010; Palmer *et al*., 2010; Řehák *et al*., 2012).Accurate estrus detection and timing of insemination are essential for maximizing conception rates. Although estrus synchronization protocols using hormonal treatments are commonly applied, their success is limited by individual variability in physiological response (Ball and Peters, 2008; Alkar *et al*., 2011; Beran *et al*., 2013). As a result, researchers have increasingly focused on cervicovaginal mucus (CVM) as a potential non-invasive biomarker for fertility assessment. CVM, produced by epithelial cells lining the cervix, undergoes both physical and biochemical changes during the estrous cycle in response to hormonal fluctuations, particularly estrogen and progesterone (Ježková *et al*., 2008; Pluta *et al*., 2011; Layek *et al*., 2013).

The physicochemical properties of CVM—such as viscosity, color, pH, crystallization (fern pattern), and spinnbarkeit—can reflect a cow's reproductive status and have been used to assess estrus and fertility (Hanumant *et al*., 2019). For instance, during ovulation, CVM becomes less viscous and more elastic, facilitating sperm transport, while during the luteal phase, it becomes denser and more hostile to spermatozoa (Hunter, 1995; Ahmadi *et al*., 2005). The presence and pattern of crystallization begin several days before estrus and peak on the day of estrus,

disappear thereafter as progesterone dominates (Rangnekar *et al*., 2002).

In addition to its physical characteristics, CVM contains numerous biochemical and immunological components, including mucins, electrolytes, antimicrobial peptides, and immune mediators such as cytokines (Rutllant *et al*., 2005; Tsiligianni *et al*., 2001; Adnane *et al*., 2018). Cytokines secreted by endometrial epithelial cells play a central role in local immune modulation. T helper (Th) cells, specifically Th1 and Th2 subsets, mediate immune balance by secreting cytokines such as IFN-γ, IL-2, and TNF-α (Th1), or IL-4, IL-5, and IL-10 (Th2) (Mosmann *et al*., 1986; Adkins *et al*., 2004). This Th1/Th2 balance is crucial in regulating inflammation, tissue remodeling, and implantation success (Noronha *et al*., 1995).

Moreover, oxidative stress has emerged as another potential factor influencing fertility. It refers to an imbalance between reactive oxygen species (ROS) and the antioxidant defense system, which can adversely affect cellular function and reproductive outcomes (Schafer and Buettner, 2001; Nazifi *et al*., 2009). Common biomarkers used to evaluate oxidative stress in reproductive contexts include superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and malondialdehyde (MDA) (Passi *et al*., 2001; Kataria and Kataria, 2005; Piccione *et al*., 2007).

Given these complex interactions, CVM provides a promising matrix for evaluating the immunological and oxidative environment of the reproductive tract. However, the relationship between CVM's cytokine profile, oxidative stress parameters, and reproductive outcomes—such as conception and calf gender—remains poorly understood. Therefore, this study aimed to investigate the Th1/Th2 cytokine balance and oxidative stress markers in the CVM of cows at the time of insemination and explore their associations with pregnancy status and offspring sex.

MATERIALS AND METHODS

The study focused on cows bred on farms in Bingöl Province, located in eastern Türkiye The study involved 90 simmental cows aged 3-4 years using their cervicovaginal mucus samples taken during insemination. Pregnancy status was confirmed through ultrasonographic checks at intervals of 17–24 days post-insemination, and then again at 1 month and 2 months later. Using the same method, embryonic mortality rates were tracked during the first month after insemination, and the absence of pregnancy was noted during the second month of the follow-up. The sex of the calves was established through ultrasonography during the second-month post-insemination check by observing the placement of the genital tract and udder teats and by monitoring the births of the pregnant cows. Due to the extremely low number of embryonic mortality cases (n=2) found in the studied cows, no statistical comparison of cytokine and oxidative stress parameters was performed.

Cervicovaginal mucus sampling: Cervicovaginal mucus samples were collected aseptically before artificial insemination using the "Pipette and Injector Method" (Reddy, 1974; Panangala *et al*., 1978). This process involved first cleaning the perineal region with antiseptic water, then advancing a sterile plastic catheter into the vagina towards the porsio vaginalis part of the cervix. A syringe, attached to the catheter, was then used to collect a 5-10 mL sample of cervicovaginal mucus. The collected samples were preserved at -20°C until they could be analyzed for cytokine content.

-Sample preparation: The cervicovaginal mucus samples had a viscous consistency. First, the samples were placed in 10 mL serum tubes and spun at 4000 rpm for 10 min for analysis. After the solid portion precipitated, 100 µl of the less dense layer on top was moved to 1.5 mL microcentrifuge tubes. We then added 400 µl of phosphate buffered saline, homogenized the samples using a vortex, and stored them at -20°C until analysis was performed (Eltohamy *et al*., 1990).

-Th1/Th2 cytokine analysis: The levels of Th1 and Th2 cytokines in cervicovaginal mucus samples were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Sahna and Risvanli, 2015).

-Determination of oxidative stress parameters: Commercial ELISA kits were used to assess the levels of oxidative stress parameters, specifically MDA, GSH, CAT (USCN Life, China), GSH-Px, and SOD (Shanghai Coon Koon Biotech Co., Ltd, China) (Li *et al*., 2013; Xu *et al*., 2019).

-Statistical analysis: Levene's test was used to evaluate variance homogeneity, and the Shapiro-Wilk test was applied to confirm the normality of the data distribution. For data that met parametric assumptions, we performed intergroup comparisons using the Student t-test. These analyses were performed using the SPSS software package (version 22.0). The data are expressed as a mean ± standard deviation, with a P value of less than 0.05 deemed significant. We utilized the G*Power software to determine the sample size, aiming for a type I error rate of 0.05, 85% statistical power, and an effect size of 0.65, which yielded a required sample size of 88.

RESULTS

Cervicovaginal mucus samples were collected from 88 cows during insemination, and the results were recorded. The cows were then divided into two groups:

those that became pregnant (pregnancy-positive; Group I, n=55) and those that did not (pregnancy-negative; Group II, n=33). Cytokine levels and oxidative stress parameters were measured for both groups and the results were compared. No significant statistical differences were observed in the levels of IL-2, IL-4, IL-5, IL-10, and TNFα when comparing the groups. The levels of interferon-gamma (IFN-γ) were substantially higher in the pregnancy-positive group (161.83±200.92 pg/mL) than in the pregnancy-negative group (60.88±41.37 pg/mL) ($p<0.01$) (Table 1, Figure 1).

Table 1. Cervicovaginal cytokine concentrations measured at insemination in Group I (pregnant) and Group II (non-pregnant) cows.

Parameter	Group I (n=55)	Group II (n=33)	P
IL-2 (pg/ml)	88.15±66.8	111.86±97.67	0.180
IL-4 (pg/ml)	966.91±230.28	969.62±226.99	0.957
IL-5 (pg/ml)	16.4±7.85	14.32±9.43	0.268
IL-10 (pg/ml)	21.69±26.53	24.62±23.78	0.603
TNFα (pg/ml)	863.25±265.38	849.22±238.09	0.804
IFN-γ (pg/ml)	161.83±200.92	60.88±41.37	0.006*

Data are presented as mean ± standard deviation.
*P < 0.05 was considered significant.

The concentrations of SOD, CAT, GSH, and GSH-Px showed no statistically significant variation between Group I and Group II. However, Group II exhibited a higher MDA level (2122.42±318.93 ng/mL) compared to Group I (1723.05± 374.01 ng/mL) ($p<0.001$) (Table 2, Figure 2)."

Table 2. Oxidative stress biomarker concentrations in cervicovaginal mucus of pregnant (Group I) and non-pregnant (Group II) cows at the time of insemination.

Parameter	Group I (n=55)	Group II (n=33)	P
SOD (U/ml)	485.41±164.2	523.44±113.46	0.244
CAT (ng/ml)	14.49±4.24	14.68±3.34	0.824
GSH (µg/ml)	71.87±26.06aaw	73.04±34.98	0.858
GSH-Px (U/ml)	469.38±243.82	391.91±265.75	0.167
MDA (ng/ml)	1723.05± 374.01	2122.42±318.93	0.001*

Data are presented as mean ± standard deviation.
*P < 0.05 was considered significant.

When comparing pregnant cows based on the gender of their calves, it was observed that cows giving birth to male calves (MALE) had significantly higher levels of IL-2 and GSH ($p<0.05$) than those giving birth to female calves (FEMALE) (Tables 3 and 4; Figures 3 and 4).

Table 3. Cervicovaginal cytokine levels by calf gender in pregnant cows (Group I).

Parameter	FEMALE (n=35)	MALE (n=20)	P
IL-2 (pg/ml)	0.11±0.04	0.15±0.11	0.039*
IL-4 (pg/ml)	3.48±0.62	3.3±0.81	0.363
IL-5 (pg/ml)	0.12±0.03	0.12±0.03	0.738
IL-10 (pg/ml)	0.18±0.1	0.22±0.19	0.224
TNFα (pg/ml)	2.47±0.76	2.73±0.68	0.214
IFN-γ (pg/ml)	0.4±0.26	0.51±0.45	0.236

Data are presented as mean ± standard deviation.
*P < 0.05 was considered significant.

Table 4. Oxidative stress parameters by calf gender in pregnant cows (Group I).

Parameter	FEMALE (n=35)	MALE (n=20)	P
SOD (U/ml)	0.32±0.2	0.27±0.12	0.329
CAT (ng/ml)	0.33±0.11	0.31±0.1	0.623
GSH (µg/ml)	0.66±0.13	0.77±0.25	0.035*
GSH-Px (U/ml)	0.68±0.36	0.54±0.32	0.151
MDA (ng/ml)	1.1±0.22	1.14±0.36	0.625

Data are presented as mean ± standard deviation.
*P < 0.05 was considered significant.

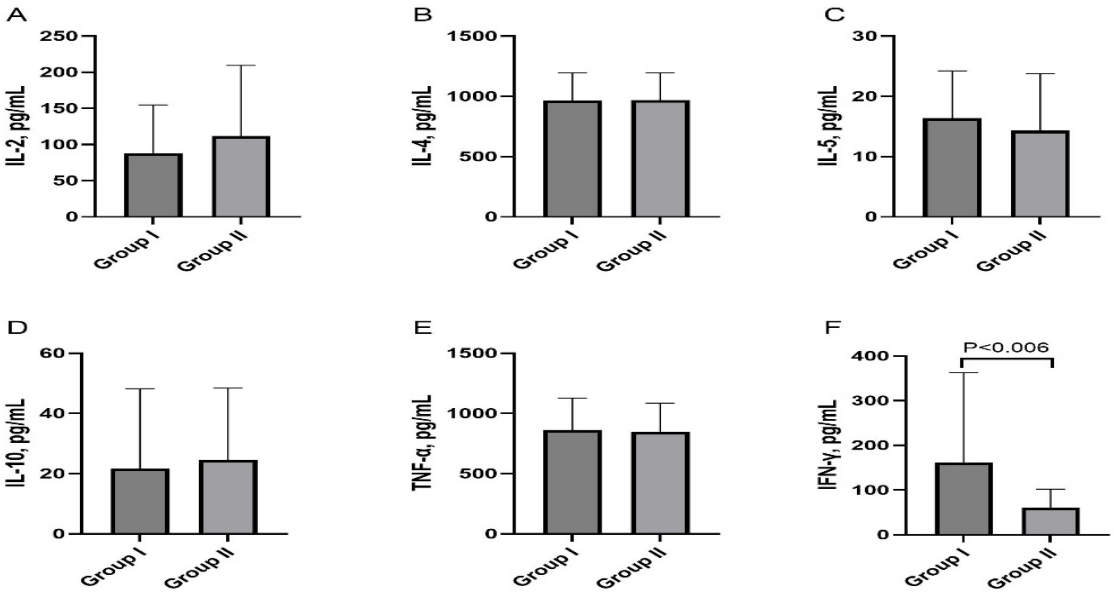

Figure 1. Cytokine concentrations in cervicovaginal mucus of cows at insemination, comparing pregnant (Group I) and non-pregnant (Group II) animals.

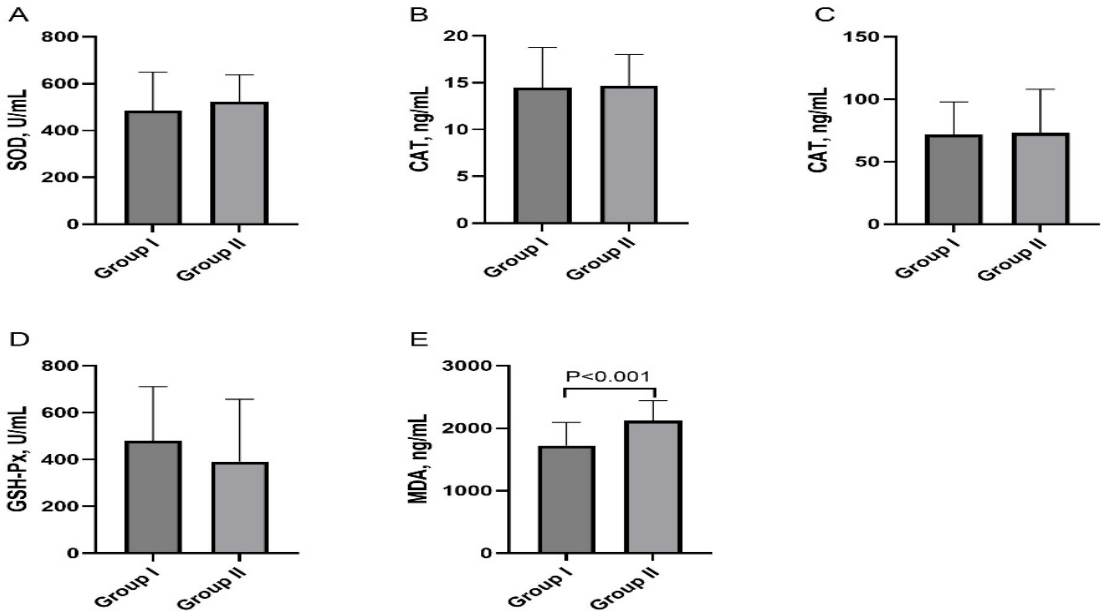

Figure 2. Oxidative stress parameters in cervicovaginal mucus of cows at insemination, comparing Group I (pregnant) and Group II (non-pregnant).

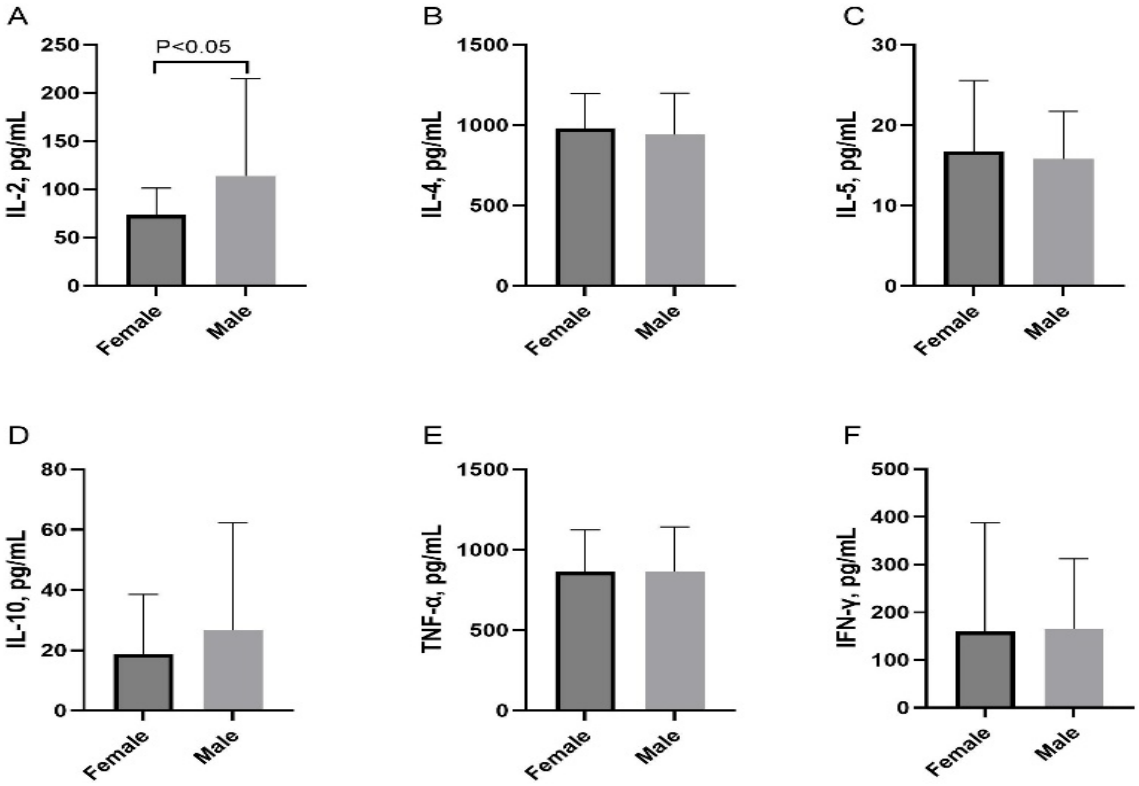

Figure 3. Comparison of cervicovaginal cytokine concentrations in cows (Group I) based on the sex of the calves born (male vs. female).

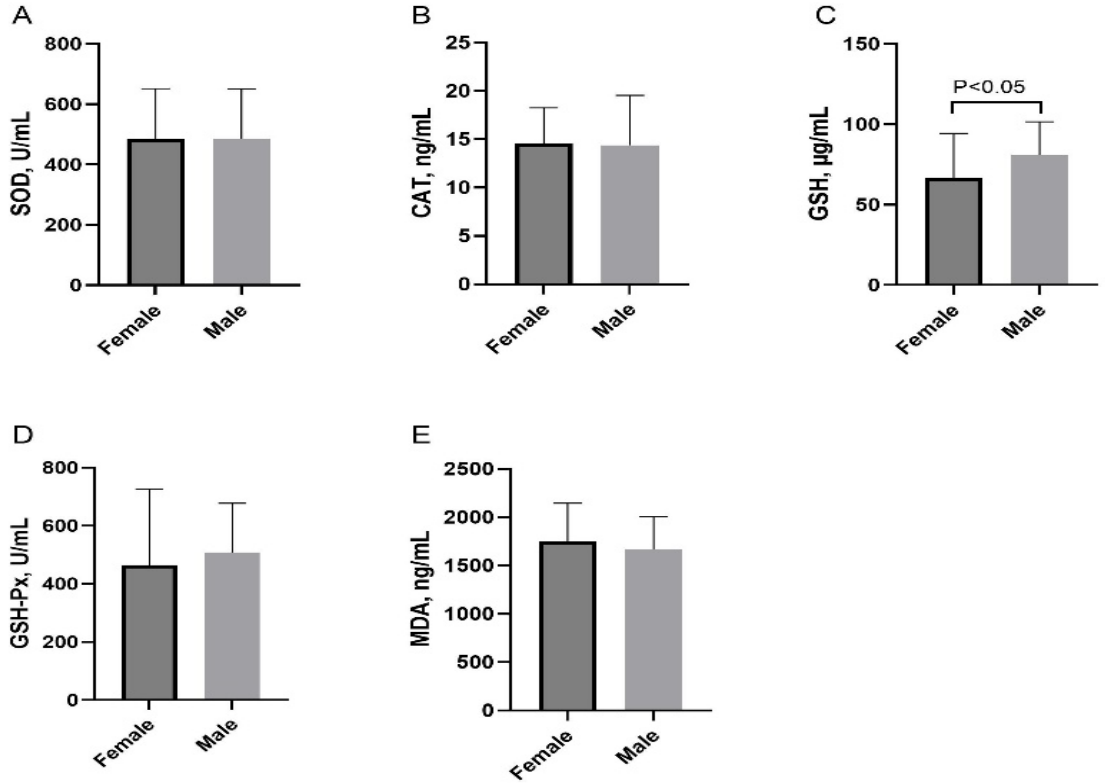

Figure 4. Comparison of oxidative stress parameters in cervicovaginal mucus (Group I cows) based on the sex of the calves born (male vs. female).

DISCUSSION

Human pregnancies are known to be sustained by Th2 cytokines, which are more predominant than Th1 cytokines (Saito, 2000). However, in cows, some pregnancies may fail due to heightened Th1 cytokine production during inflammation or immune activation, and the regulation of Th1/Th2 cytokines in ruminants remains unclear (Maeda *et al*., 2013). This study aimed to investigate the relationship between the Th1/Th2 cytokine balance and oxidative stress parameters in cervicovaginal mucus collected at the time of insemination, as well as their potential association with pregnancy outcomes in cows. A total of 88 cows were evaluated and categorized based on pregnancy status after insemination. Our findings showed no statistically significant differences in the concentrations of IL-2, IL-4, IL-5, IL-10, and TNF-α between pregnant and non-pregnant cows. This suggests that, although these cytokines are important for immune regulation and embryo tolerance, they may not play a decisive role in cervicovaginal mucus at the time of insemination in determining pregnancy success. Notably, IFN-γ levels were significantly higher in the pregnancy-positive group, suggesting a more nuanced role for Th1-associated responses in promoting successful implantation. Although IFN-γ is traditionally considered a pro-inflammatory Th1 cytokine, recent studies have highlighted its context-dependent roles in maternal immunity, including contributions to decidualization and vascular remodeling during early gestation (Murphy *et al*., 2009). In terms of oxidative stress, no significant differences were observed in antioxidant markers such as SOD, CAT, GSH, and GSH-Px between the two groups. However, malondialdehyde (MDA), a marker of lipid peroxidation and cellular oxidative damage, was significantly higher in non-pregnant cows. This finding suggests that elevated oxidative stress at the mucosal level may negatively impact fertilization or early embryonic development. These results align with previous research indicating that oxidative stress in the female reproductive tract may impair oocyte quality, sperm viability, or embryo development (Agarwal *et al*., 2005). Interestingly, a subgroup analysis of pregnant cows based on calf sex revealed that those carrying male fetuses had significantly higher IL-2 and GSH levels than those carrying female fetuses. This observation is particularly intriguing and may reflect subtle immunometabolic differences in response to fetal sex, potentially mediated by fetal-maternal signaling pathways. Although the mechanisms remain unclear, such sex-specific immune responses have been reported in studies examining maternal immune and metabolic profiles during early pregnancy (Clifton, 2010).

Conclusion: In conclusion, while most Th1/Th2 cytokines and antioxidant parameters did not significantly differ with pregnancy status, the elevated IFN-γ levels in pregnant cows and higher MDA levels in non-pregnant cows may suggest their potential roles in shaping the local reproductive environment. Furthermore, the influence of fetal sex on cytokine and antioxidant expression warrants further investigation, as it may provide new insights into maternal–fetal interactions at conception. Future studies involving larger sample sizes and longitudinal follow-up may help clarify the beneficial role of IFN-γ and the impact of oxidative stress thresholds on fertility outcomes in bovine reproduction.

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Ethical Approval: Ethical approval for this study was obtained from the Bingöl University Experimental Animals Ethics Committee (Date and Number: 11/05/2020 - 1198).

Conflict of Interest Statement: We affirm that we possess no commercial or associative interests that might constitute a conflict of interest with the submitted work.

Author's Contribution: Mehmet Akif Kılınç: Writing - review and editing; Investigation; Formal analysis; Funding acquisition; Project administration.

Ali Rişvanlı: Methodology; Writing - review & editing; Funding acquisition; Conceptualization.

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