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ORIGINAL ARTICLE

Effects of Vitamin D3 Supplementation during Mating or Gestation on Antioxidant Status and Reproductive Parameters in Gray Shirazi Ewes

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Vitamin D3 Effects on Antioxidants and Reproduction in Ewes

Abstract

Recent studies suggest that vitamin D3 may have preventive and therapeutic effects on autoimmune disease, cancer, and type 1 and 2 diabetes beyond the skeletal condition and calcium metabolism. To demonstrate the effects of a single 10,000 IU/kg vitamin D3 IM injection during mating and pregnancy on reproductive and antioxidant factors, 21 Gray Shirazi ewes with a parity of 1 to 3, which were kept under industrial conditions, were used for this research. All ewes underwent estrus synchronization by placing a sponge containing Flurogestone acetate for 13 days, followed by an injection of 400 units of Pregnant Mare Serum Gonadotropin hormone on the 13th day. Then, ewes were divided into three groups: the first group served as control (without any supplementation), the second group, called pre-pregnancy, received vitamin D3 10 days before laparoscopic insemination, and the third group, which received the supplement in mid-pregnancy (at the 15th week of pregnancy). Blood samples were collected from the ewes at four different stages: the first stage was 10 days after vitamin D3 injection (at the time of insemination), the second stage occurred about 15 weeks after insemination (about 10 days after vitamin D3 injection), the third stage was 2 weeks before lambing, and the fourth stage took place 2 weeks after lambing. The study's findings indicated that vitamin D3 supplementation has no significant effect on reproductive factors in Gray Shirazi ewes ($p \geq 0.05$). However, a significant decrease in blood cortisol levels was observed during both pre-pregnancy and mid-pregnancy. Additionally, there was a significant increase in antioxidant factors, such as superoxide dismutase and glutathione peroxidase, during mid-pregnancy. This conclusion can be related to the racial differences in sheep, the supplement's dose, and the timing and frequency of injections.

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Introduction

Vitamin D3, as a micronutrient, is essential for maintaining skeletal and bone health by regulating the absorption of calcium and phosphorus, promoting bone formation, and facilitating mineral circulation (1). Recent research has revealed that a deficiency in this vitamin is associated with various medical conditions such as autoimmune diseases, hypertension, and cancer (2, 3).

Vitamin D3 plays a significant role in reproductive function, and insufficient levels during pregnancy have been associated with lower reproductive success and a greater risk of fetal abnormalities, such as inadequate weight gain, low birth weight, and reduced head circumference (4, 5, 6, 7). Vitamin D3 status can also impact redox balance and subclinical pregnancy toxemia in sheep (8).

Research indicates that administering vitamin D3 to ewes during late pregnancy improves their reproductive performance and increases lamb survival rates. Additionally, vitamin D supplementation contributed to the increase in the yield of offspring obtained from the synchronization protocol in Romanov sheep in the breeding season (9).

Vitamin D supplementation in ewes, both pregnant and non-pregnant, generally has a wide safety window, but excessive doses can lead to toxicity, particularly hypercalcemia. The safety of vitamin D during pregnancy in ewes is an area of ongoing research, but some studies suggest that doses up to 4000 IU daily are safe and effective. Toxicity is more likely with prolonged exposure to very high doses (e.g., 50,000 IU/day or higher) (10).

The fetus relies entirely on the mother's vitamin D. 25-hydroxyvitamin D passes through the placenta and is converted into 1, 25-dihydroxyvitamin D (1, 25(OH) $_2$D) by the fetal kidneys. Additionally, the placenta produces 1, 25(OH) $_2$D to help regulate its metabolism. Vitamin D plays a crucial role in pregnancy by maintaining calcium balance in the mother's body and supporting the development of the fetal bones. However, there is ongoing discussion about how a mother's vitamin D levels affect pregnancy outcomes, fetal development, and the long-term health of newborns (11). Recently, a positive correlation was observed between the serum concentrations of 25(OH) D in ewes during mating and the pregnancy rate (12). Furthermore, a recent study on Scottish Blackface sheep indicated that vitamin D levels before mating positively correlated with lamb weight in both single and twin pregnancies (13).

Antioxidants may offer therapeutic promise for reproductive issues (14). Reactive oxygen species (ROS) significantly affect various reproductive processes, including fertilization, embryonic development, and fertility (15). Reduced levels of vitamin D can impair mitochondrial function and increase oxidative stress, which can lead to metabolic disorders and negatively affect fertility (16).

In a study by Lacetera et al. (2001), it was found that in ewes with subclinical ketosis, the concentration of immunoglobulin G in blood and colostrum was reduced by half and one-fifth, respectively, compared with ewes from the same flocks with normal conditions (17). Therefore, subclinical ketosis, with its significant effects on the immune system, increases the susceptibility of ewes and lambs to infectious diseases, which is also a result of the increased effect of ROS. It has been reported that administering a mixture of AD3E to pregnant ewes improved the antioxidant status in leukocytes and reduced negative energy balance during the transition period (18).

To effectively assess oxidative stress, it's essential to choose the right biomarkers. Key enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) serve as critical indicators (19). Lipids are particularly vulnerable to oxidation due to their structure, making lipid peroxidation a central aspect of oxidative stress. Notable markers for this process include isoprostanes (IsoPs) and malondialdehyde (MDA) (20). On the other hand, cortisol can increase the production of ROS and lipid peroxidation, while also depleting antioxidant defenses like glutathione (GSH). This can lead to cellular damage and contribute to various health issues (21). Moreover, total antioxidant capacity (TAC) provides valuable insight, reflecting the overall influence of antioxidants in the bloodstream and offering a comprehensive measure of the body's oxidative status (22). Collectively, the assessment of these biomarkers after vitamin D3 administration provides a thorough insight into the presence of ROS and the efficacy of the antioxidant system. Therefore, this study aimed to investigate the effects of vitamin D3 supplementation during mating and pregnancy on reproductive and antioxidant factors in Gray Shirazi ewes.

Materials and Methods

Study Population

The study was performed from September 2022 to March 2023. The animals were raised at the research station of Aliabad Kamin, affiliated with Fars Agricultural and Natural Resources Research and Training Center, in the north of Fars province. To demonstrate the effects of a single 10,000 IU/kg vitamin D3 IM injection (23) during mating or pregnancy on reproductive and antioxidant factors, 21 non-pregnant, fertile, healthy Gray Shirazi ewes with a parity of 1 to 3, which were kept under industrial conditions, were used for this research. The average weight of the ewes was about 50 to 55 kg, and their average age was about 2 years. They were fed manually, which included barley, straw, and alfalfa. In addition, lick bricks and bicarbonate were freely provided to these sheep (to prevent possible acidosis and mineral deficiencies). All ewes underwent estrus synchronization by placing a sponge containing Flurogestone Acetate for 13 days, followed by an injection of 400 units of Pregnant Mare Serum Gonadotropin (PMSG) hormone on the 13th day. Then, ewes were divided into three groups: the first group served as the control (without any supplementation), the second group was called pre-pregnancy and received vitamin D 10 days before laparoscopic insemination, and the third group received the supplement in mid-pregnancy (at the 15th week of pregnancy) (Figure 1). Pregnancy was diagnosed by ultrasonography approximately 5 weeks after insemination.

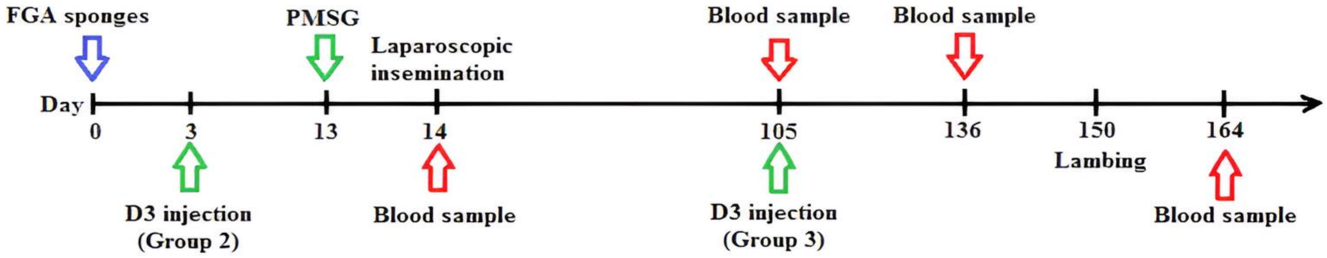

Figure 1. Mode of action and treatment plan.

Sample Collection

Blood samples were collected from the ewes at four different stages: the first stage was 10 days after vitamin D injection (at the time of insemination), the second stage occurred about 15 weeks after insemination (about 10 days after vitamin D3 injection), the third stage was 2 weeks before lambing, and the fourth stage took place 2 weeks after lambing (Figure 1).

Evaluation of Antioxidant Activity and Reproductive Parameters

To separate the blood serum, the samples were centrifuged for 10 minutes at 3000 rpm. The serum samples were subsequently kept at -20°C until laboratory assessments. The activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) was determined using spectrophotometric assay kits from Randox Laboratories Ltd. (Crumlin, UK), following the manufacturer's instructions. Total antioxidant capacity (TAC) and malondialdehyde (MDA) levels were assessed by spectrophotometric methods using kits from Zellbio GmbH (Ulm, Germany). Serum cortisol and progesterone concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using kits from Monobind Inc (Lake Forest, CA, USA). During parturition, ewes were observed for the frequency of multiple births, in addition to gender and lamb weight, and the data were recorded.

Statistical Analysis

Statistical analysis was conducted using SPSS 26 software. All data were presented as mean ± standard deviation (SD). All parameters were statistically analyzed by two-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests. A statistically significant difference between different experimental groups was represented as $p<0.05$.

Results

Serum Progesterone

The comparison results of serum progesterone levels are shown in Table 1. These results indicate no significant differences between the treatment groups ($p=0.31$). However, we found significant differences in each group between sampling stages, with the highest progesterone levels observed 15 weeks after insemination ($p<0.05$) (Figure 2).

Cortisol Levels

We observed significant differences in cortisol levels between the treatment groups ($p=0.034$) (Table 2). In both the pre-pregnancy and mid-pregnancy groups, cortisol levels were significantly lower compared to the control group during the third and fourth sampling stages ($p<0.05$). The highest cortisol level was recorded in the control group at 2 weeks after lambing ($p<0.05$) (Figure 2).

Antioxidant Activity

MDA Levels

The evaluation of MDA levels revealed no significant differences between the treatment groups ($p=0.304$). However, there were significant differences in each group between sampling stages, and levels were lower at 10 days after injection than at other stages ($p<0.05$) (Table 3). As shown in Figure 2, all groups exhibited an increase in MDA levels, followed by a decrease at 2 weeks after lambing.

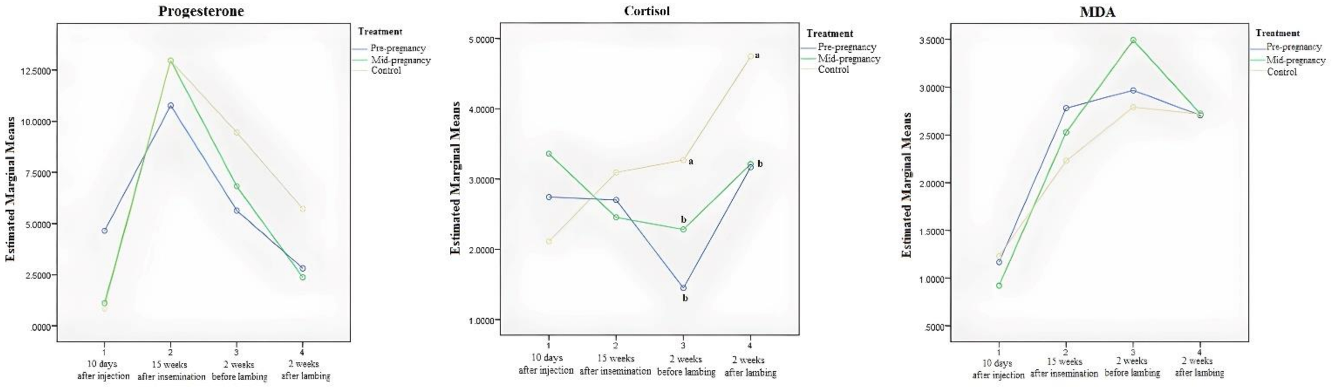

Figure 2. Time-related changes (LSM ± SE) and pairwise comparisons for the amounts of progesterone, cortisol, and MDA in trial groups.

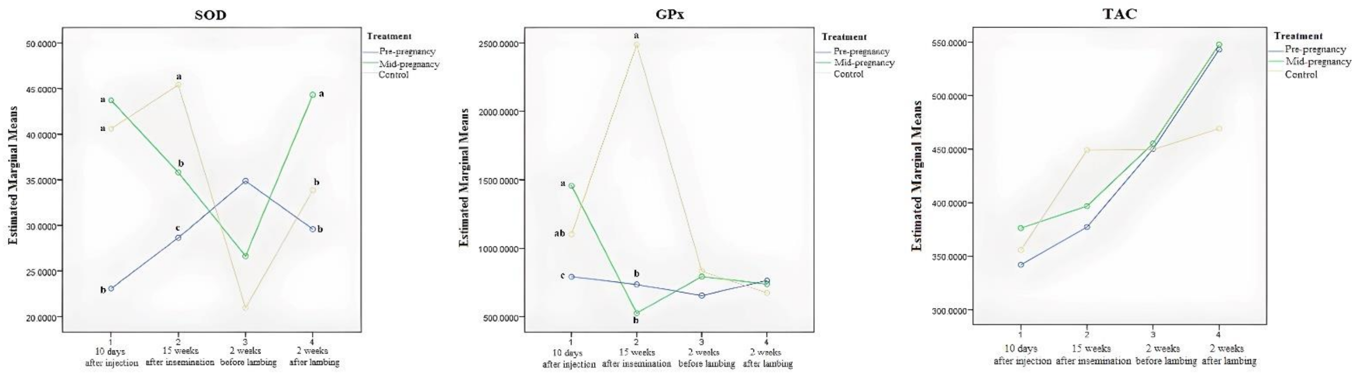

Figure 3. Time-related changes (LSM ± SE) and pairwise comparisons for the amounts of Superoxide dismutase (SOD), glutathione peroxidase (GPx), and TAC in trial groups.

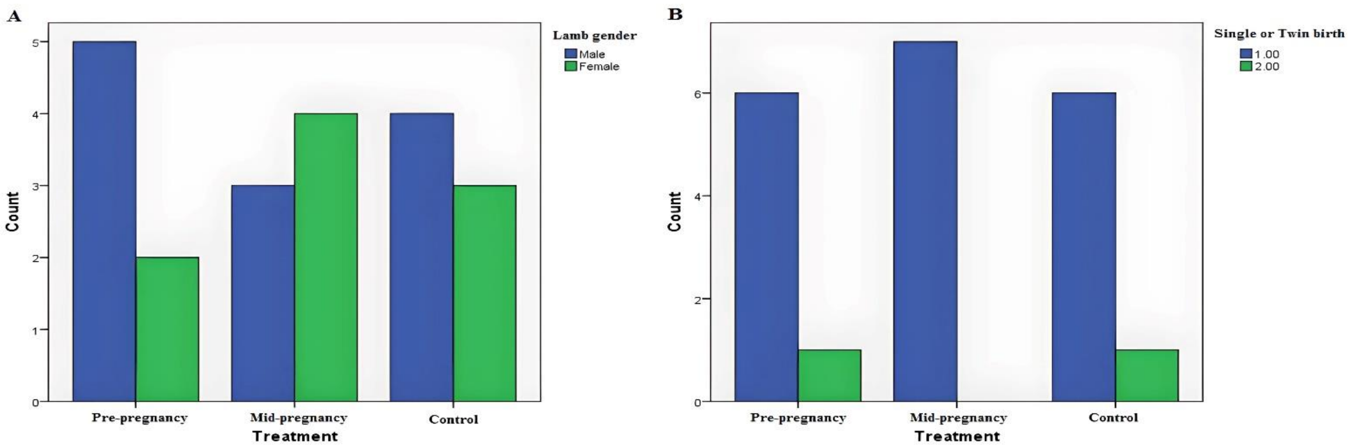

Figure 4. Lamb gender (A) and pregnancy status (B) in trial groups. Vitamin D supplementation has no significant effect on reproductive factors (p≥0.05).

Superoxide Dismutase (SOD)

A significant difference in superoxide dismutase (SOD) levels between the treatment groups was observed (p=0.001) (Table 4). The highest level was in the mid-pregnancy group at 2 weeks after lambing (p<0.05), as shown in Figure 3.

Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) assessment indicated a significant difference between the treatment groups (p<0.05) (Table 5). In the control group at 15 weeks after insemination, and the mid-pregnancy group at 10 days after injection, we found a significant difference in GPx level (p<0.05) (Figure 3).

TAC

Unlike superoxide dismutase (SOD) and glutathione peroxidase (GPx), we did not find any significant difference in TAC levels between the treatment groups (p=0.528) (Table 6). Still, we observed significant differences in treatment groups between sampling stages, and TAC levels at 2 weeks after lambing were higher than other stages (p<0.05). As shown in Figure 3, all groups exhibited increased TAC levels.

Reproductive Factors

Lamb Gender

The chi-square test was used to compare the gender of lambs in different treatment groups, with no significant difference observed (p=0.558) (Table 7), as shown in Figure 4A.

Pregnancy Status

According to Table 8, there wasn't a significant difference in the distribution of ewes with single or twin births between the study groups based on the chi-square test (p=0.575), as seen in Figure 4B.

Weight and Number of Lambs

Table 9 indicates no significant difference between the treatment groups regarding weight (p=0.938) and the number of lambs (p=0.615).

Table 1. The comparison of serum progesterone levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Pre-pregnancy	7	4.646717± 5.4940241	10.768281 ±9.4113806	5.637973 ±6.6153993	2.813791 ±4.2312897
Mid-pregnancy	7	1.115960 ±.9360247	12.964903 ±8.6016916	6.823104 ±7.3162261	2.369708 ±4.7214029
Control	7	8.43841 ±.5372090	12.964698 ±12.5730734	9.458044 ±7.2899115	5.723122 ±9.2266764

Table 2. The comparison of cortisol levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Pre-pregnancy	7	2.744290 ± 1.7457968	2.702663 ± 1.5496733	1.448891 ±1.0609481	3.169880 ±1.6804286
Mid-pregnancy	7	3.360813 ±.7288826	2.455083 ±1.8791798	2.283005 ±.4761575	3.212292 ±1.4129265
Control	7	2.112129 ±1.0782240	3.094098 ±1.9377158	3.269545 ±1.9707677	4.744064 ±3.7249079

Table 3. The comparison of MDA levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Pre-pregnancy	7	1.166667± .5365376	2.781136± .2052760	2.967033±.3345521	2.705128 ±.1356291
Mid-pregnancy	7	.922161± .4107140	2.527473±.1626199	3.492674±1.5812923	2.722527 ±.3466030
Control	7	1.228938±.6177363	2.228938±.6177363	2.791209 ±.5945187	2.715201 ±.1121860

Table 4. The comparison of SOD levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Prepregnancy	7	23.082023± 2.5960938	28.653452±5.5077546	34.869301±16.5265313	29.593593±5.1813514
Midpregnancy	7	43.713339± 10.1240171	35.809152±2.9478654	26.640248±5.4194598	44.318896±23.5744734
Control	7	40.603895±13.4030082	45.407284±9.0941420	20.964006±7.2651745	33.868792±9.4959884
Total	21	35.799752±13.1631554	36.623296±9.2729445	27.491185±11.8659819	35.927094±15.5564477

Table 5. The comparison of GPx levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Prepregnancy	7	791.669557± 295.967582	735.098129±540.4392181	654.495664±293.897464	764.034687±814.8414
Midpregnancy	7	1456.138749± 1153.6478	525.787312±225.0817591	792.788681±203.224595	738.019169±402.3656
Control	7	1102.236422±354.20058	2487.950708±793.0907925	832.496577±340.286124	673.664993±204.5762
Total	21	1116.681576±735.22982	1249.612049±1050.8397190	759.926974±281.341217	725.239617±511.6930

Table 6. The comparison of TAC levels between the treatment groups.

Treatment	Number	10 days after injection	15 weeks after insemination	2 weeks before lambing	2 weeks after lambing
Prepregnancy	7	342.000000± 83.6660027	377.238095± .2052760	450.095238±42.3764934	542.952381±94.47151
Midpregnancy	7	376.285714± 70.8601723	396.761905±.1626199	455.333333±113.316992	547.714286±125.8558
Control	7	355.809524±69.5868381	449.142857±52.9300227	449.619048 ±111.947644	469.142857±117.5116
Total	21	358.031746±72.5765423	407.714286±73.8176647	451.682540±90.3198315	519.936508±113.7124

Table 7. The comparison of lamb gender between the treatment groups.

			Lamb Gender		Total
			Male	Female	
Treatment	Pre-pregnancy	Count	5	2	7
		% within Treatment	71.4%	28.6%	100.0%
	Mid-pregnancy	Count	3	4	7
		% within Treatment	42.9%	57.1%	100.0%
	Control	Count	4	3	7
		% within Treatment	57.1%	42.9%	100.0%
Total		Count	12	9	21
		% within Treatment	57.1%	42.9%	100.0%

Table 8. The comparison of single or twin birth between the treatment groups.

			Single or Twin birth		Total
			1.00	2.00	
Treatment	Pre-pregnancy	Count	6	1	7
		% within Treatment	85.7%	14.3%	100.0%
	Mid-pregnancy	Count	7	0	7
		% within Treatment	100.0%	0.0%	100.0%
	Control	Count	6	1	7
		% within Treatment	85.7%	14.3%	100.0%
Total		Count	19	2	21
		% within Treatment	90.5%	9.5%	100%

Table 9. The comparison of weight and number of lambs between the treatment groups.

		Number	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Lamb Weight	Pre-pregnancy	7	4.8000	.87178	.32950	3.9937
	Mid-pregnancy	7	4.9143	.25448	.09619	4.6789
	Control	7	4.8286	.57071	.21571	4.3007
	Total	21	4.8476	.58959	.12866	4.5792
Number of lamb	Pre-pregnancy	7	1.1429	.37796	.14286	0.7933
	Mid-pregnancy	7	1.0000	.00000	.00000	1.0000
	Control	7	1.1429	.37796	.14286	0.7933
	Total	21	1.0952	.30079	.06564	0.9583

Discussion

Nutritional and environmental conditions are crucial during different stages of sheep reproduction. However, there is limited research on micronutrients such as vitamin D3 and its impact on fetal development and pregnancy, particularly in native Iranian breeds such as the Gray Shirazi (24). This study aimed to investigate the effect of vitamin D during mating or pregnancy on reproductive and antioxidant factors in Gray Shirazi ewes.

Using vitamin D metabolites before delivery can be a scientifically supported approach to managing post-partum hypocalcemia (25). Vitamin D undergoes a two-step metabolic process involving the liver and kidneys to become its biologically active form, calcitriol. Initially, vitamin D, whether obtained from the diet or synthesized in the skin, is converted to 25-hydroxyvitamin D (25OHD) in the liver. This 25OHD then circulates in the blood bound to vitamin D binding protein. In the kidneys, 25OHD is further converted to 1, 25-dihydroxyvitamin D, also known as calcitriol, the active form of vitamin D (26).

Wilkens demonstrated that administering 3 mg of 25-hydroxyvitamin D at the end of the dry period, along with an acidogenic diet, effectively reduced subclinical hypocalcemia in dairy cows. This study suggests a direct relationship between the dose of vitamin D and certain blood factors during pregnancy (27). Consequently, the supplement's dose is a significant factor influencing the interaction between these factors and vitamins. In the current study, there was no significant difference in blood progesterone levels between the treatment groups, which may be partly attributed to the vitamin D3 dose administered. Additionally, racial differences could also contribute to observed effects.

Cortisol serves as a reliable physiological indicator of stress in sheep. Conditions associated with pregnancy and the post-partum period, the impact of having a single or twin birth, and simultaneous milking may represent significant sources of stress for both the mother and her lambs. A recent study examined the adrenal response in ewes during late pregnancy and post-partum, taking into account the potential effects of single or twin births on fluctuations in circulating cortisol levels. The cortisol levels measured were consistent with the normal physiological ranges reported for sheep and aligned with previous data obtained from lactating ewes. Significantly higher cortisol levels were observed during the post-partum period compared to pregnancy, with similar trends noted in both Comisana and Pinzirita crossbreds. Additionally, post-partum cortisol levels were elevated in ewes regardless of whether they gave birth to a single lamb or twins. Notably, ewes that gave birth to male lambs or both female and male lambs also exhibited increased post-partum cortisol levels (28).

In our study, we observed a significant decrease in blood cortisol levels during both pre-pregnancy and mid-pregnancy. However, given the factors of single or twin births and the specific breed studied, this research cannot definitively conclude that vitamin D3 injections led to lower cortisol levels. To better understand the complex relationship between vitamin D3 and cortisol, it would be beneficial to investigate the effects of this vitamin on cortisol levels across different breeds and in ewes with both single and twin births.

Recent research indicates that vitamin D may extend its beneficial effects beyond bone health and calcium metabolism, potentially playing a role in preventing and treating autoimmune diseases, cancer, and both type 1 and type 2 diabetes. These effects may stem from vitamin D's ability to modulate the immune system, influence cell growth and differentiation, and impact metabolic processes (29). Recent research investigated the effects of an over-supplemented single injection of 8 million IU of vitamin D3 administered intramuscularly on the modulation of immune responses and oxidative/antioxidative variables in transition dairy cows. The results indicated that the vitamin D3 injection increased levels of IL-6 and enhanced hemolysate GPx activity. It also showed a trend towards affecting serum GPx activity (30). In another study, Merino ewes were supplemented with cholecalciferol, which resulted in elevated plasma concentrations of 25(OH) D in both the ewes and their lambs at birth compared to the control group. However, this supplementation did not have a significant impact on the phagocytic capacity of monocytes or polymorphonuclear leukocytes, nor did it affect the concentration of IgG in the colostrum or plasma of lambs or the vaccine-specific antibody response against tetanus toxoid (31). In this study, there was a significant increase in antioxidant factors, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), during mid-pregnancy. However, we found no significant difference in TAC levels between the treatment groups (p=0.528).

A study was conducted to examine the effects of administering vitamin D3 before mating on the reproductive performance of Rahmani ewes, focusing on its impact on biomarkers associated with uterine receptivity during the implantation period. Administering a single dose of vitamin D3 (300,000 IU per ewe) led to a less significant increment in serum pro-inflammatory cytokines (TNF-α and IL-6) and a higher significant elevation in serum level of anti-inflammatory cytokines (IL-4 and IL-10), insulin-like growth factor-1 (IGF-1), steroidogenic regulatory protein (StAR), macrophage inflammatory protein-1β (MIP-1β) and progesterone as compared to the ewes who did not receive vitamin D on the 20th day after injection. The treated group also showed a higher number of pregnancies on the 40th-day post-mating, along with an increased incidence of twin fetuses at delivery. In conclusion, administering vitamin D3 to ewes before mating significantly enhances their reproductive performance by inducing biochemical changes that improve fertility and uterine embryo receptivity (32). However, our study indicated that vitamin D3 supplementation has no significant effect on reproductive factors in Gray Shirazi ewes ($p \geq 0.05$), including lamb gender, pregnancy status, weight, and number of lambs. This conclusion can be related to the racial differences in sheep, the supplement's dose, and the timing and frequency of injections.

Conclusion

In conclusion, although reproductive outcomes remained unaffected, vitamin D3 supplementation significantly improved the oxidative profile during pregnancy, suggesting its potential role in metabolic modulation in breed-specific contexts.

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Not applicable.

Author contributions

Seyed Ehsan Sadat Mandani: Investigation, methodology, writing the original draft. **Meysam Makki:** Conceptualization, supervision, visualization, review & editing. **Seyedeh Misagh Jalili:** Data analysis, investigation, methodology. **Alidad Boostani,** Methodology, validation, visualization. **Saad Goorani Nejad** Conceptualization, validation, visualization.

Data availability

All data analyzed during this study are included in this published article.

Ethical Approval

The study protocol, incorporating sample collection and analyses, complied with ARRIVE guidelines, and all the procedures were strictly followed under the Animal Scientific Procedures Act (1986).

Conflict of Interest

The authors declare that there is no potential conflict of interest.

Consent for Publication

Not applicable.

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