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The effect of Vitamin D Supplementation on its serum level and energy metabolic profile in Grey Shirazi ewes

Zahra Davoodabadi[a], Seyedeh Missagh Jalali[a], Meysam Makki[b], Alidad Boostani[a], Mohamad Rahim Hajikolai[a]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[b] Department of Animal Science, Fars Agricultural and Natural Resources Research and Education Center, Fars, Iran.

ABSTRACT

The present study aimed to evaluate the effects of vitamin D supplementation on serum vitamin D levels and the metabolic profile of Grey Shirazi ewes, during pregnancy. A total of sixty healthy Grey Shirazi ewes were divided into three groups: Group 1 (control); Group 2, which received a single intramuscular dose of 10,000 IU vitamin D at the time of insemination; and Group 3, which received a single dose of 10,000 IU vitamin D at mid-pregnancy. Blood samples were collected at four stages: insemination, mid-pregnancy, late pregnancy, and post-lambing. Vitamin D levels in the control group decreased significantly during pregnancy. In contrast, no such decrease occurred in the two supplement groups, so that the highest vitamin D concentrations were observed in late pregnancy and postpartum in group 3. In terms of energy balance, serum β-hydroxybutyrate (BHBA) and non-esterified fatty acid (NEFA) levels were lowest in the mid-pregnancy supplemented group. Additionally, in group 3, insulin levels increased during the final stages of pregnancy, while serum calcium and phosphorus concentrations were higher in this group compared to the other groups, reaching their maximum levels after delivery. All groups increased triglycerides and cholesterol, but Group 3 had the highest triglycerides postpartum. Additionally, serum protein and albumin levels were significantly higher in Group 3 during the postpartum period, reflecting improved nutritional status and protein synthesis. The results of this study suggest that vitamin D supplementation during mid-pregnancy has the potential to improve metabolic health in Grey Shirazi ewes, having significant implications for both maternal and neonatal health outcomes.

Keywords

Vitamin D, Energy balance, metabolic biomarkers, Pregnancy, Grey Shirazi sheep

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Abbreviations

BHBA: β-hydroxybutyrate
NEFA: non-esterified fatty acid
25(OH)D: 25-hydroxyvitamin D
1,25(OH)2D: 1,25-dihydroxyvitamin D
VDR: vitamin D receptor
NEB: negative energy balance

Corresponding author: *Seyedeh Missagh Jalali*
Email: mi.jalali@scu.ac.ir
Tel: 98(91)66134-7900

Introduction

Vitamin D is an essential nutrient for regulating calcium levels and maintaining skeletal health in ruminants [1]. However, there is increasing evidence that vitamin D plays additional roles, including effects on metabolism and overall health [2]. The body obtains the vitamin D it needs through oral intake of cholecalciferol or through its production in the skin through exposure to ultraviolet radiation. After production or consumption, vitamin D is hydroxylated in the liver to 25-hydroxycholecalciferol (calcidiol), and then converted in the kidneys to the active form 1,25-dihydroxycholecalciferol (calcitriol). The active form functions as a hormone, binding to the vitamin D receptor (VDR) to control calcium and phosphorus metabolism, which is necessary for bone formation and maintenance. The primary functions of vitamin D and its effects on calcium homeostasis are achieved by increasing calcium absorption from the intestines, regulating calcium release from bones by acting on osteoblasts and osteoclasts, and promoting calcium reabsorption from the kidneys [3].

Vitamin D deficiency has been reported in sheep. It can occur due to inadequate sunlight exposure, ineffective shearing, or insufficient vitamin intake, which can have significant implications for calcium absorption and balance, as well as overall health [4]. Published studies show that cholecalciferol supplementation in sheep can effectively increase serum vitamin D levels and is therefore effective in preventing or treating vitamin D deficiency and its subsequent complications, including calcium balance disorders, and is also associated with positive effects on the metabolic profile of these animals [5, 6]. Other evidence that can be considered for the more widespread effects of vitamin D is the presence of receptors for this vitamin in numerous tissues, such as the endocrine pancreas, liver, muscle, and adipose tissue, indicating its possible role in regulating energy metabolism [7]. Supplementation of vitamin D can result in an improvement in insulin sensitivity and glucose tolerance in ruminants, highlighting its potential role in energy metabolism [6].

The timing of vitamin D supplementation, whether it occurs during mating or throughout the gestation phase, makes a great difference in its effects [8]. Cholecalciferol supplementation should be considered during two critical stages of sheep reproduction. Administration of this vitamin at the time of mating can be effective in improving ovulation and increasing reproductive efficiency [9, 10]. In the later stages of pregnancy, due to fetal growth, multiple births, and preparation for lactation, as well as the overall increase in metabolic needs of the ewe, the use of this supplement is justified. Supplementation during this period can improve serum vitamin D concentrations, support optimal energy metabolism, reduce the likelihood of metabolic disorders in pregnant ewes, and aid fetal development [11].

Pregnancy toxemia is a common metabolic disease in ewes, which occurs especially in high-producing animals and ewes with multiple births in late pregnancy and coincides with the rapid fetal growth phase due to a mismatch between energy intake and expenditure, negative energy balance (NEB). This condition, which is associated with multiple metabolic disorders, including increased fatty acid levels and insulin resistance, can lead to significant economic losses in the sheep husbandry. Therefore, finding effective strategies that promote energy balance and metabolic health of ewes seems essential to reduce these risks [12].

Gray Shirazi breed is a native Iranian sheep breed originating from the Shiraz region of Fars Province in southern Iran. The breed is known for its high quality wool and adaptability to the arid and semi arid environments of the region, displaying strong resilience to harsh weather conditions and low quality forage. Grey Shirazi sheep are medium sized animals with a typical bluish-gray coat, hence the name. In addition to their contributions to wool production, Grey Shirazi sheep are also raised for their meat, thereby supporting the livelihoods of local farmers. Their resilient characteristics, coupled with moderate reproductive rates, position them as a vital genetic resource for promoting sustainable farming practices in the region. Even though the importance of this breed cannot be underestimated, there is a decline in their population, raising concerns over the conservation of this unique breed [13].

Regardless of known metabolic benefits, the effect of vitamin D supplementation on pregnancy-induced metabolic changes in ewes has not been adequately demonstrated. Therefore, this study was conducted to evaluate the effects of vitamin D supplementation on serum concentrations of 25-hydroxyvitamin D and energy-related metabolic biomarkers in pregnant Grey Shirazi ewes.

Abbreviations-Cont'd

FGF-23: fibroblast growth factor-23
HMGCR: hydroxymethylglutaryl-CoA reductase

Result

Vitamin D

Table 1 presents changes serum vitamin D concentrations as determined within groups across time

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Table 1.
Mean ± SE of serum Vitamin D (ng/ml) in various groups at different sampling times.

	mating	mid-pregnancy	Late pregnancy	Post-parturition
Group 1	53.16 ± 14.07	30.36 ± 13.69	39.71 ± 10.18	34.31 ± 13.12
	a	b	b	b
		A	A	A
Group 2	54.58 ± 10.27	73.24±14.52	53.25±9.31	44.09 ± 14.29
	a	b	a	a
		B	B	A
Group 3	47.13 ± 12.01	52.20 ± 13.39	67.14±13.99	50.81 ± 16.98
	a	a	b	a
		C	C	B

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

points. In Group 1 (control), a significant reduction in serum vitamin D levels was observed throughout pregnancy and postpartum compared with pre-pregnancy period (e.g., mating: 53.16 ± 14.07 ng/mL vs. mid-pregnancy: 30.36 ± 13.69 ng/mL; $p < 0.05$). In Group 2 (supplementation at insemination), a significant increase was detected at mid-pregnancy (73.24 ± 14.52 ng/mL) compared to mating (54.58 ± 10.27 ng/mL; $p < 0.05$), with levels remaining stable thereafter. In Group 3 (supplementation at mid-pregnancy), a significant rise in vitamin D was observed from mid-pregnancy (52.20 ± 13.39 ng/mL) to late pregnancy (67.14 ± 13.99 ng/mL; $p < 0.05$).

Between-group comparisons at each sampling point showed that Groups 2 and 3 had significantly higher serum vitamin D levels than Group 1 during the middle of pregnancy (Group 2: 73.24 ± 14.52 ng/mL; Group 3: 52.20 ± 13.39 ng/mL; Group 1: 30.36 ± 13.69 ng/mL; $p < 0.05$) and during late pregnancy (Group 3: 67.14 ± 13.99 ng/mL; Group 2: 53.25 ± 9.31 ng/mL; Group 1: 39.71 ± 10.18 ng/mL; $p < 0.05$). The highest postpartum level of serum vitamin D was recorded in Group 3 (50.81 ± 16.98 ng/mL), which was significantly higher than both Group 2 (44.09 ± 14.29 ng/mL; $p < 0.05$) and Group 1 (34.31 ± 13.12 ng/mL; $p < 0.05$).

BHBA and NEFA

Within-group comparisons of serum BHBA levels over time are presented in Table 2. In all three

Table 2.
Mean ± SE of serum Vitamin D (ng/ml) in various groups at different sampling times.

			mid-pregnancy	Late pregnancy	Post-parturition
BHBA (mmol/l)	Group 1	0.21 ± 0.02	0.45 ± 0.06	0.79 ± 0.29	0.44 ± 0.18
		a	b	c	b
	Group 2	0.18 ± 0.02	0.40 ± 0.03	0.91 ± 0.11	0.38 ± 0.05
		a	b	c	b
	Group 3	0.20 ± 0.02	0.38 ± 0.02	0.63 ± 0.12	0.42 ± 0.08
		a	b	b	b
NEFA (mmol/l)	Group 1	1.18 ± 0.49	0.85 ± 0.16	1.78 ± 0.09	2.22 ± 0.62
			A	AB	A
	Group 2	1.44 ± 0.17	1.61± 0.07	2.41 ± 0.30	1.73 ± 0.22
			B	A	B
	Group 3	1.31 ± 0.28	0.77 ± 0.06	1.09 ± 0.18	1.15 ± 0.29
			A	B	C

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
* Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p<0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

groups, BHBA concentrations increased significantly as pregnancy progressed. In Group 1, BHBA levels rose from 0.21 ± 0.02 mmol/L at the time of mating to 0.79 ± 0.29 mmol/L at late pregnancy ($p < 0.05$). Similarly, Group 2 exhibited a significant increase from 0.18 ± 0.02 mmol/L at the time of mating to 0.91 ± 0.11 mmol/L at late pregnancy ($p < 0.05$). In Group 3, the increase was more moderate, with levels rising from 0.20 ± 0.02 mmol/L to 0.63 ± 0.12 mmol/L by late pregnancy ($p < 0.05$).

Between-group comparisons across each time point (Table 2) revealed no significant differences in BHBA levels ($p > 0.05$).

Table 2 shows that within-group comparisons of NEFA levels did not present any significant differences ($p > 0.05$).

Between-group comparisons exhibited significant differences in NEFA levels at several time points. During mid-pregnancy, Group 2 (1.61 ± 0.07 mmol/L) had significantly higher NEFA concentrations than Groups 1 (0.85 ± 0.16 mmol/L) and 3 (0.77 ± 0.06 mmol/L; $p < 0.05$). At late pregnancy, Group 3 had significantly lower NEFA levels (1.09 ± 0.18 mmol/L) than Group 2 (2.41 ± 0.30 mmol/L; $p < 0.05$). In the postpartum period, NEFA was also lowest in Group 3 (1.15 ± 0.29 mmol/L), significantly differing from Group 1 (2.22 ± 0.62 mmol/L; $p < 0.05$). Insulin and glucose

Table 3 presents the within-group changes of serum insulin levels throughout the experiment. In the control group (Group 1), insulin levels increased significantly with the progression of pregnancy and postpartum, rising from 1.39 ± 0.14 ng/mL at the time of mating to 3.36 ± 1.33 ng/mL postpartum ($p < 0.05$). A similar increase was observed in the supplemented groups, with Group 2 from 1.44 ± 0.64 ng/mL at the time of mating to 1.99 ± 1.09 ng/mL postpartum, and Group 3 from 1.34 ± 0.16 ng/mL at the time of mating to 6.06 ± 1.06 ng/mL postpartum ($p < 0.05$ for Group 3).

Between group comparisons at each time point showed significant differences in insulin levels during late pregnancy and postpartum. At late pregnancy, insulin levels in Group 3 (4.00 ± 1.82 ng/mL) were significantly higher than in Group 1 (2.39 ± 1.44 ng/mL) and Group 2 (2.96 ± 1.42 ng/mL; $p < 0.05$). This difference became more pronounced postpartum, with Group 3 reaching the highest insulin level (6.06 ± 1.06

Table 3.
Mean ± SE of serum insulin and glucose in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-parturition
Insulin (ng/ml)	Group 1	1.39 ± 0.14	1.86 ± 0.82	2.39 ± 1.44	3.36 ± 1.33
		a	a	a	b
	Group 2	1.44 ± 0.64	1.64 ± 0.51	A	A
				2.96 ± 1.42	1.99 ± 1.09
	Group 3	1.34 ± 0.16	1.08 ± 0.79	A	A
		a	a	4.00±1.82	6.06±1.06
Glucose (mg/dl)	Group 1	111.90 ± 7.65	82.71 ± 5.42	b	b
		a	ab	B	B
	Group 2	99.14 ± 5.97	85.00 ± 7.89	71.42 ± 4.40	47.00 ± 6.96
		a	ab	b	c
	Group 3	109.00 ± 6.58	97.61 ± 5.14	75.85 ± 4.56	43.53 ± 2.95
		a	ab	b	c

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).
* Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).
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ng/mL), significantly exceeding both Group 1 (3.36 ± 1.33 ng/mL) and Group 2 (1.99 ± 1.09 ng/mL; $p < 0.05$).

Within-group comparisons of serum glucose levels over time showed a significant downward trend during pregnancy and postpartum (Table 3). In all groups, glucose concentrations decreased significantly from mating to postpartum. For example, in Group 1, glucose declined from 111.90 ± 7.65 mg/dL at mating to 47.00 ± 6.96 mg/dL postpartum ($p < 0.05$). Similar reductions were observed in Group 2 (99.14 ± 5.97 mg/dL to 43.53 ± 2.95 mg/dL) and Group 3 (109.00 ± 6.58 mg/dL to 45.71 ± 4.50 mg/dL; $p < 0.05$ in both).

Between-group comparisons did not show significant differences in glucose levels at any stage of sampling ($p > 0.05$).

Calcium and Phosphorous

Within-group comparisons over time showed stage-specific elevations in calcium levels (Table 4). In Group 2, the highest calcium concentration was recorded during mid-pregnancy showing a value of 10.41 ± 0.09 mg/dL, which was significantly higher than values at other stages of this group ($p < 0.05$). In Group 3, the maximum level of calcium was observed in the postpartum stage (10.55 ± 0.09 mg/dL), which was also significantly higher than the previous stages in this group ($p < 0.05$). In contrast, calcium levels in Group 1 showed no significant variations throughout the sampling period, and remained relatively stable ($p > 0.05$).

Between-group comparisons of serum calcium levels at each time point are presented in Table 4. At the postpartum stage, Group 3 (vitamin D supplementation at mid-pregnancy) showed a significantly higher calcium concentration (10.55 ± 0.09 mg/dL) compared to Group 1 (10.07 ± 0.12 mg/dL) and Group 2 (9.86 ± 0.10 mg/dL; $p < 0.05$). No significant intergroup differences were observed during the earlier stages of sampling (mating, mid-pregnancy, or late pregnancy; $p > 0.05$).

Table 4 presents between-group comparisons of serum phosphorus levels over time revealed a significant decline during late pregnancy and postpartum in all groups. For example, in Group 1, phosphorus decreased from 6.04 ± 0.20 mg/dL at mating to 5.03 ± 0.38 mg/dL postpartum ($p < 0.05$). Similar trends were observed in Group 2 (6.68 ± 0.28 mg/dL to 4.60 ± 0.26 mg/dL) and Group 3 (6.12 ± 0.16 mg/dL to 4.81 ± 0.33 mg/dL), with statistically significant reductions in each case ($p < 0.05$).

Between-group comparisons of phosphorus concentrations at each stage revealed no statistically significant differences among the three groups ($p > 0.05$).

Lipids and proteins

Table 5 shows the comparisons of serum triglyceride concentrations among study periods within each group. In all groups, triglyceride levels increased significantly during pregnancy. For instance, Group 1

Table 4.
Mean ± SE of serum Calcium and Phosphorous in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-parturition
Calcium (mg/dl)	Group 1	10.13 ± 0.06	10.28 ± 0.25	9.82 ± 0.19	10.07 ± 0.12
					A
	Group 2	9.90 ± 0.13	10.41 ± 0.09	10.08 ± 0.12	9.86 ± 0.10
		a	b	a	A a
	Group 3	10.11 ± 0.09	10.13 ± 0.12	10.09 ± 0.13	10.55 ± 0.09
		a	a	a	B b
Phosphorous (mg/dl)	Group 1	6.04 ± 0.20	6.48 ± 0.57	5.32 ± 0.80	5.03 ± 0.38
		a	a	b	b
	Group 2	6.68 ± 0.28	5.97 ± 0.35	5.54 ± 0.43	4.60 ± 0.26
		a	a	b	b
	Group 3	6.12 ± 0.16	6.67 ± 0.31	5.29 ± 0.26	4.81 ± 0.33
		a	a	ab	b

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

Table 5.
Mean ± SE of serum triglycerides (mg/dl) in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-parturition
Triglycerides (mg/dl)	Group 1	36.45 ± 4.00	41.85 ± 2.35	58.42 ± 7.39	46.57 ± 2.56
		a	ab	b	AB ab
	Group 2	28.21 ± 3.80	48.00 ± 2.67	61.28 ± 9.70	40.60 ± 2.58
		a	b	b	A b
	Group 3	30.75 ± 4.73	38.46 ± 2.65	55.70 ± 5.24	55.71 ± 2.62
		a	a	b	B b
Cholesterol (mg/dl)	Group 1	72.18 ± 2.76	64.00 ± 1.78	61.57 ± 4.39	64.42 ± 5.42
		a	b 70.85 ± 2.35	A b	b
	Group 2	69.14 ± 3.29	70.85 ± 2.35	76.85 ± 3.43	70.46 ± 3.20
		a	a	B b	a
	Group 3	65.62 ± 3.45	65.00 ± 2.36	74.30 ± 4.05	69.28 ± 3.23
				B	
Protein (g/dl)	Group 1	7.52 ± 0.14	6.88 ± 0.20	6.15 ± 0.42	6.98 ± 0.29
		a	ab	b	ab
	Group 2	7.22 ± 0.15	6.81 ± 0.17	6.21 ± 0.18	6.69 ± 0.12
		a	ab	b	b
	Group 3	7.55 ± 0.18	6.80 ± 0.20	6.40 ± 0.24	6.85 ± 0.22
		a	b	b	ab
Albumin (g/dl)	Group 1	3.50 ± 0.05	3.18 ± 0.06	2.71 ± 0.14	3.31 ± 0.10
		a	b	A b	a
	Group 2	3.41 ± 0.06	3.15 ± 0.07	3.04 ± 0.09	3.41 ± 0.03
		a	ab	AB ab	a
	Group 3	3.53 ± 0.07	3.30 ± 0.08	3.18 ± 0.05	3.42 ± 0.13
		a	b	B b	ab

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

increased from 36.45 ± 4.00 mg/dL at the time of mating to 58.42 ± 7.39 mg/dL at late pregnancy ($p < 0.05$), Group 2 from 28.21 ± 3.80 mg/dL to 61.28 ± 9.70 mg/dL ($p < 0.05$), and Group 3 from 30.75 ± 4.73 mg/dL to 55.70 ± 5.24 mg/dL (p < 0.05). Postpartum, triglyceride levels declined in Groups 1 and 2 but remained elevated in Group 3.

Between-group comparisons revealed a significant difference only at the postpartum stage. At this time, Group 3 exhibited a significantly higher triglyceride concentration (55.71 ± 2.62 mg/dL) compared to Group 1 (46.57 ± 2.56 mg/dL) and Group 2

(40.60 ± 2.58 mg/dL; $p < 0.05$).

Within-group comparisons over time showed different patterns in cholesterol concentrations across groups. In Group 1 (control), cholesterol levels declined significantly from mating (72.18 ± 2.76 mg/dL) to mid-pregnancy (64.00 ± 1.78 mg/dL) and remained low through late pregnancy (61.57 ± 4.39 mg/dL; $p < 0.05$). In contrast, Group 2 showed a significant increase in cholesterol concentrations from mating (69.14 ± 3.29 mg/dL) to late pregnancy (76.85 ± 3.43 mg/dL; $p < 0.05$), followed by a slight decrease postpartum.

Between-group comparisons of serum cholesterol concentrations at late pregnancy are also shown in Table 5. At this stage, both Group 2 (76.85 ± 3.43 mg/dL) and Group 3 (74.30 ± 4.05 mg/dL) had significantly higher cholesterol levels compared to the control Group 1 (61.57 ± 4.39 mg/dL; $p < 0.05$). No significant differences were observed between groups at other sampling points ($p > 0.05$).

Comparisons of serum total protein concentrations over time resulted a significant decrease in all groups during pregnancy. For example, in Group 1, protein levels decreased from 7.52 ± 0.14 g/dL at the time of mating to 6.15 ± 0.42 g/dL at late pregnancy ($p < 0.05$). Similar trends were observed in Group 2 (7.22 ± 0.15 to 6.21 ± 0.18 g/dL) and Group 3 (7.55 ± 0.18 to 6.40 ± 0.24 g/dL).

Between-group comparisons at each time point did not show significant differences in serum protein concentrations ($p > 0.05$).

Serum albumin concentrations followed a consistent within-group trend across all groups. A significant reduction in albumin was observed during pregnancy in all groups. For instance, in Group 1, albumin declined from 3.50 ± 0.05 g/dL at mating to 2.71 ± 0.14 g/dL at late pregnancy ($p < 0.05$). This pattern was also noted in Group 2 (3.41 ± 0.06 to 3.04 ± 0.09 g/dL) and Group 3 (3.53 ± 0.07 to 3.18 ± 0.05 g/dL). Postpartum, albumin levels increased in all groups, returning to near-baseline levels ($p < 0.05$).

Between-group comparisons during late pregnancy indicated significantly higher serum albumin levels in Group 3 (3.18 ± 0.05 g/dL) compared to Group 1 (2.71 ± 0.14 g/dL; $p < 0.05$).

Discussion

This study investigated the effect of vitamin D supplementation at the insemination stage compared to mid-gestation on metabolic indices of pregnant Grey Shirazi ewes. Given the extensive physiological roles of vitamin D, especially in regulating metabolism, this study investigated the effect of supplementation on serum levels of this vitamin and metabolic changes associated with pregnancy and postpartum.

According to the results, in the group that did not receive vitamin D, serum levels of this vitamin decreased significantly during pregnancy and postpartum, such that in the control group it decreased from 53.16 ± 14.07 ng/ml at the time of mating to 30.36 ± 13.69 ng/ml at mid-pregnancy and 34.31 ± 13.12 ng/ml postpartum. In contrast, in the groups receiving supplementation, vitamin D levels remained relatively stable. Ewes supplemented at insemination (Group 2) showed a peak of 73.24 ± 14.52 ng/mL at mid-pregnancy ($p < 0.05$ vs. control), while those supplemented at mid-pregnancy (Group 3) reached 67.14 ± 13.99 ng/mL at late pregnancy and maintained the highest postpartum levels (50.81 ± 16.98 ng/mL, $p < 0.05$ vs. control).

Group 3 showed higher vitamin D levels than group 2 in late pregnancy and postpartum, however the difference between the two groups was not significant ($p > 0.05$). Therefore, although supplementation in mid-pregnancy was associated with more pronounced effects, this effect should be interpreted cautiously as a clear benefit.

The decline in serum vitamin D of the control group ewes is in agreement with previous studies, demonstrating that pregnancy decreases circulating 25-hydroxyvitamin D due to increased utilization by the mother and fetus [4,14]. Comparable patterns have been reported in both humans and livestock, where maternal vitamin D status decreases as gestation progresses, particularly in the absence of supplementation [15,16].

The rise in vitamin D concentration following supplementation in mid-pregnancy may reflect increased metabolic demands of pregnancy, particularly for regulating calcium and phosphorus levels through improved intestinal absorption and renal reabsorption, which are essential for fetal skeletal formation [17]. Due to the increased maternal calcium requirement during pregnancy, upregulation of vitamin D processing occurs during this period [18]. Therefore, the possible reason why supplementation in mid-pregnancy is more effective in maintaining serum vitamin D levels is the increased physiological requirement for this nutrient during this period of fetal development.

The observed difference in response observed between the timing of supplementation (mid-pregnancy versus insemination) may also be related to the varying expression of vitamin D receptors (VDR) and the activity of enzymes responsible for vitamin D metabolism in maternal tissues and the placenta as pregnancy advances. Studies in humans and other animals have shown that placental expression of VDR and 1α-hydroxylase, in charge of converting 25-hydroxyvitamin D to its active form, calcitriol, increases during pregnancy [19]. This may help explain why vitamin D supplementation in mid-pregnancy had a more pronounced effect, as the physiological machinery needed to convert and utilize vitamin D is more active at this stage.

Comparing these results with other studies on livestock, the impact of vitamin D supplementation on serum concentrations and metabolic indices has been well-documented in various species. According to the results of a study conducted by Rodney et al. (2018), vitamin D supplementation to pregnant dairy cows increases serum levels of this vitamin and improves calcium metabolism, which in turn can prevent many important postpartum complications such as hypocalcemia [20]. It has been shown that providing of vitamin D to sheep improves the balance of calcium and phosphorus, thereby supporting better fetal bone development and maternal health [21].

Furthermore, the timing of vitamin D supplementation appears to play a significant role in maximizing its advantages. Poindexter et al. (2023) showed that administering vitamin D supplementation in late pregnancy in goats is much more effective in increasing maternal serum levels of this vitamin than when administered at earlier stages of pregnancy, which is consistent with the results of the present study [22].

In this study, the rise in serum β-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) noted in the control group ewes suggests a negative energy balance (NEB), which is frequently seen during late pregnancy due to increased energy requirements and limited nutrient consumption. During negative energy balance, due to reduced energy availability, increased fat catabolism occurs, and the rise in BHBA and NEFA is a marker of this condition [23]. Previous studies have also reported a similar trend in late pregnancy, indicating that the energy requirements for fetal growth are higher than the maternal dietary intake [24].

Vitamin D supplementation had a significant influence on these metabolic parameters, with BHBA and NEFA levels being lower in ewes that received vitamin D in comparison to the control group. Ewes supplemented with vitamin D during mid-pregnancy exhibited the lowest BHBA levels, although the differences among groups were not significant. On the contrary, changes in NEFA levels were more noticeable, with the mid-pregnancy supplementation group displaying significantly reduced NEFA levels both in late pregnancy and after delivery. These results indicate that vitamin D supplementation, especially when given during mid-pregnancy, may help alleviate NEB and decrease fat mobilization during late gestation.

The mechanisms underlying this effect may be associated with the regulatory role of vitamin D in energy metabolism. Vitamin D has been shown to enhance insulin sensitivity and glucose utilization, potentially leading to better energy partitioning and reducing reliance on fat stores during periods of high energy demand [25]. The reduction in NEFA levels in the supplemented groups is also likely due to improved insulin function and reduced NEFA release from adipose tissue. Decreases in NEFA and BHBA and improved energy balance during the transition period have been previously reported in dairy cows receiving vitamin D supplementation [25].

In the present study, the selected dose of 10,000 IU vitamin D administered at mid-pregnancy (Group 3) was sufficient to maintain stable serum 25(OH) D concentrations and showed a tendency to improve certain metabolic parameters. BHBA levels in late pregnancy were lower in group 3 (0.63 ± 0.12 mmol/L) than in group 1 (0.79 ± 0.29 mmol/L) and group 2 (0.91 ± 0.11 mmol/L), but these differences were not significant ($p > 0.05$). This suggests that the administered dose may have had a partial effect, and it is plausible that a higher dose or repeated administration could exert a stronger and more consistent influence on ketone body regulation and energy balance in late gestation.

In this study, a significant rise in serum insulin concentrations was found in ewes supplemented with vitamin D during mid-pregnancy, while supplementation at insemination did not have an effect. Previous studies have shown that during metabolic stress, such as pregnancy, vitamin D stimulates insulin secretion by improving pancreatic beta cell function [25]. Increased insulin levels, in turn, can prevent excessive fat mobilization and the development of NEB by increasing glucose uptake by maternal and developing fetal tissues. This finding reinforces the concept that vitamin D prevents NEB in pregnant ewes by a mechanism involving improvement in glucose metabolism and insulin activity.

On the other hand, serum glucose levels significantly declined as pregnancy advanced in all ewes, which aligns with the heightened use of glucose for fetal growth. Although vitamin D supplementation during mid-pregnancy was associated with an increase in serum glucose levels, this difference did not reach statistical significance. Although vitamin D supplementation is associated with increased insulin sensitivity, the lack of correspondence between changes in serum glucose and insulin suggests that it cannot significantly alter glucose regulation in the late stages of pregnancy, when significant glucose is consumed for fetal growth [6].

In total, the observed changes in insulin, glucose, and NEFA levels suggest a potential improvement in insulin sensitivity in Group 3. This group had the highest postpartum insulin concentrations (6.06 ± 1.06 ng/mL), while having relatively higher glucose levels and lower NEFA concentrations during late pregnancy and postpartum, which may indicate reduced insulin resistance. These findings align with previous studies reporting that vitamin D enhances insulin receptor expression and modulates energy metabolism in ruminants and other mammals. Although direct measures of insulin resistance were not directly measured in this study, the metabolic profile observed in Group 3 supports the hypothesis that vitamin D supplementation during mid-pregnancy may positively influence insulin action.

In this study, the highest serum calcium level, especially in postpartum sampling, were in group 3, receiving vitamin D supplementation in mid-pregnancy. Given the role of vitamin D in regulating serum calcium levels through increasing intestinal absorption and renal reabsorption and calcium transport from bone, such an effect is expected, especially during times of increased demand, including late pregnancy and lactation [17, 26].

The absence of a significant difference in serum calcium levels between the control and the group supplemented at the time of insemination may be explained by the timing of vitamin D administration. Considering the lower calcium requirements in early pregnancy compared to late pregnancy and lactation [27], vitamin D supplementation in mid-pregnancy may have been more effective in meeting the higher calcium requirements in later pregnancy and postpartum.

Conversely, the lack of significant differences between groups in serum phosphorus concentrations at any of the sampling times is likely due to the tight regulation of phosphorus levels in ruminants to support metabolic processes such as energy production and bone mineralization [28]. On the other hand, since phosphorus levels are regulated primarily by fibroblast growth factor-23 and parathyroid hormone, the concentration of this mineral is less affected by vitamin D than calcium [29]. In addition, phosphorus absorption is usually efficient in ruminants, and dietary intake may have been sufficient to maintain serum levels, regardless of vitamin D administration [28].

In this study, the administration of vitamin D to ewes was associated with a significant increase in serum cholesterol levels at the end of pregnancy and increased triglyceride levels observed after delivery. These findings propose that vitamin D may play a part in lipid metabolism, especially during periods of heightened metabolic demand,

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such as late pregnancy and lactation.

Vitamin D influences cholesterol homeostasis by controlling the expression of essential enzymes involved in cholesterol production and absorption, such as hydroxymethylglutaryl-CoA reductase (HMGCR) [30]. Cholesterol is an important part of cell membranes and is required for synthesizing steroid hormones, which are particularly important during pregnancy for supporting fetal development and preparing for lactation [31,32]. The rise in cholesterol levels observed at the conclusion of pregnancy in ewes that were given vitamin D supplements may suggest improved availability of cholesterol for these essential physiological functions.

In the same vein, the rise in serum triglyceride levels after lambing corresponds with earlier research conducted on other livestock species. Since milk is rich in fat, triglycerides serve as the main source of energy for both mother and infant during lactation [33]. The increase in triglycerides could be related to the mobilization of maternal fat stores to meet the energy needs of lactation and may also reflect increased insulin-stimulated lipogenesis. The elevation in triglyceride levels in ewes receiving vitamin D after delivery suggests that vitamin D supplementation may support energy metabolism throughout the shift from pregnancy to lactation.

In this study, total protein and serum albumin levels in all ewes decreased as pregnancy progressed. Previous studies have also shown that pregnancy can lead to changes in plasma protein concentrations due to increased fluid retention and altered protein metabolism [34]. During late pregnancy, as the demand for nutrients by the developing fetus increases, maternal protein reserves may be mobilized to support fetal growth, causing a reduction in serum protein levels [35].

Vitamin D supplementation did not result in significant alterations in total serum protein. However, it is noteworthy that the ewes receiving vitamin D injections during mid-pregnancy had higher serum albumin levels by the end of pregnancy. Albumin is a major plasma protein, which has a fundamental role in preserving oncotic pressure and facilitating the transport of different substances, such as hormones, fatty acids, and drugs [36]. The significant increase in serum albumin concentrations in the supplemented group may reflect the function of vitamin D in enhancing liver performance and protein synthesis. Vitamin D has been shown to influence the synthesis of several proteins, including albumin, and may improve the general nutritional status of the animal [37,38].

Additionally, improved serum albumin levels are often associated with better health outcomes and less incidence of metabolic disorders. This can be especially advantageous for pregnant ewes during the crucial transition phase around parturition [39].

Although vitamin D administration increased albumin levels, total protein levels did not undergo significant changes following vitamin D administration, probably due to the effect of several factors such as dietary protein intake and physiological status of the animal in ewes during late pregnancy on this analyte [40].

In conclusion, this study demonstrated that supplementing with vitamin D, especially in mid-pregnancy, considerably boosts serum vitamin D levels and favorably affects several metabolic indices in pregnant ewes of the Grey Shirazi breed. Ewes receiving vitamin D supplementation had lower serum BHBA and NEFA levels, indicating better energy balance, along with increased serum cholesterol and triglyceride levels, supporting lactation requirements. In addition, higher serum albumin levels in these ewes indicated improved protein synthesis and overall nutritional status. These findings highlight the potential of vitamin D administration to improve outcomes for both the ewe and lamb during pregnancy and lactation.

Materials and Methods

Ethical Considerations

All animal procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethics Committee of Shahid Chamran University of Ahvaz.

Animals

The research was conducted at the Fars Agricultural and Natural Resources Research Center, where 60 healthy ewes of the

Grey Shirazi breed were randomly selected. All ewes received a diet consisted of a total mixed ration containing conserved forage (corn silage and legume haylage) and commercial concentrates formulated to meet maintenance and gestational nutritional requirements. On a dry matter basis, the diet contained approximately 12.5% crude protein, 2.4 Mcal/kg metabolizable energy, 32% neutral detergent fiber (NDF), and 4.2% crude fat. Mineral and vitamin premixes were included to provide balanced micronutrient intake. Clean drinking water was available ad libitum throughout the study period.

All animal procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethics Committee of Shahid Chamran University of Ahvaz.

Estrus Synchronization

All ewes were synchronized by inserting a 40 mg progesterone-containing sponge (Esponjavet, Hipra, Spain) into the vaginal vault for 12 days. Following the removal of the sponges on the twelfth day, 400 units of PMSG (Gonaser, Hipra, Spain) were administered. The ewes were laparoscopy inseminated about 46 hours after PMSG injection to ensure control and uniformity in the reproductive process of the study.

Experimental Design

The ewes were randomly allocated into three experimental groups (n = 20 per group), ensuring balance across groups in terms of age, body weight, and parity to minimize confounding effects.

Group 1 (Control): Ewes in this group were not given any vitamin D supplement.

Group 2: Ewes in this group received a 10,000 IU vitamin D injection during the insemination period [41].

Group 3: Ewes in this group received a 10,000 IU vitamin D injection at 2.5 to 3 months of pregnancy.

The ewes which did not lamb during the trial period or suffered from any disorder or disease were excluded from the study.

Blood Sampling

Blood sampling was conducted at four stages: at the time of insemination, at 2.5 to 3 months of pregnancy, and two weeks before and after lambing. Approximately 10 ml of blood was collected through the jugular vein of each ewe at each time point using disposable syringes and subsequently transferred into plain gel tubes for analysis. Following centrifugation at 4000 rpm for 10 minutes, the serum was collected and subsequently stored at -20°C for further analysis.

Laboratory assessments

Laboratory assessments were performed to determine serum levels of various metabolites and hormones.

- Vitamin D3 and insulin concentrations were measured using ELISA kits (MonoKit, Iran).

- Nonesterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) were quantified using commercially available enzymatic colorimetric assay kits (Randox Laboratories Ltd., Ardmore, UK). For the NEFA assay, the detection limit was 0.06 mmol/L, with an intra-assay CV of <5% and an inter-assay CV of <7%. The BHBA assay had a detection limit of 0.07 mmol/L, with intra- and inter-assay CVs of <5% and <6%, respectively. All measurements were performed in duplicate following the manufacturer's instructions.

- Glucose, Triglycerides, Cholesterol, Total Protein, Albumin, Calcium, and Phosphorus were measured using colorimetric methods with commercial kits from Biorex Fars (Iran) and a biochemical autoanalyzer (Biotecnica BT-1500, Italy).

Statistical Analysis

The statistical evaluation was performed using version 22 of the SPSS software (SPSS Inc., Chicago, IL, USA). The findings were expressed as means ± standard error (SE) across various groups. Data distribution was assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. The data underwent statistical assessment utilizing Repeated Measures Analysis of Variance (ANOVA), One-way ANOVA, and Tukey's post hoc tests. The level of significance was taken at $p < 0.05$ in all analyses.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they have used AI-based writing assistance tools available in Grammarly (Grammar Check, Clarity, and Writing Enhancement features) to improve the readability, and language of the manuscript.

Authors' Contributions

S.M.J., M.M., and M.R.H. conceived and planned the experiments. A.B. and M.M. carried out the experiments. S.M.J. and Z.D. carried out the laboratory analysis. S.M.J. and M.M. contributed to the interpretation of the results. S.M.J. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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