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

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Abstract

This 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. 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 significantly decreased in the control group during pregnancy, while Groups 2 and 3 maintained stable levels, with Group 3 exhibited the highest levels by late pregnancy and postpartum. In terms of energy balance, serum β-hydroxybutyrate (BHBA) and non-esterified fatty acid (NEFA) levels were lowest in the mid-pregnancy supplemented group. Insulin concentrations also rose in Group 3 in the later stages, indicating better glucose metabolism. Calcium and phosphorus levels were most favorable in Group 3, peaking postpartum. All groups experienced 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 enhance metabolic health in Grey Shirazi ewes, with significant implications for both maternal and neonatal health outcomes. Further research is necessary to identify optimal dosing and timing for broader application in practice.

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

FGF-23: fibroblast growth factor-23

HMGCR: hydroxymethylglutaryl-CoA reductase

Introduction

Vitamin D is a vital nutrient for calcium homeostasis and skeletal health in mammals, including sheep [1]. However, its roles extend beyond calcium regulation, influencing metabolic pathways and overall health [2]. Vitamin D is obtained through dietary intake or produced in the skin upon exposure to UV rays. Once produced or ingested, vitamin D undergoes two critical hydroxylation steps. The initial step takes place in the liver, resulting in the formation of 25-hydroxyvitamin D (25(OH)D). Subsequently, in the kidneys, the biologically active form, 1,25-dihydroxyvitamin D (1,25(OH)2D), is produced. 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. It affects multiple organs, such as bones, intestines, kidneys, and parathyroid glands, to regulate calcium balance. For example, vitamin D improves calcium absorption in the intestines, manages calcium concentrations in bones by acting on osteoblasts and osteoclasts, and affects calcium reabsorption in the kidneys [3].

Sheep may be at risk for vitamin D deficiency related to insufficient sunlight exposure or inadequate dietary intake [4]. However, the potential benefits of supplementation are significant. It can cause a significant rise in serum 25(OH)D levels, thereby enhancing optimal calcium absorption and promoting skeletal health [5]. Furthermore, vitamin D supplementation may not only exert a positive influence on bone health but also affect the metabolic profiles of ruminants [6]. VDRs are found in multiple tissues—such as pancreatic islets, liver, muscle, and adipose tissue—indicating their involvement in glucose and energy metabolism [7]. The administration of vitamin D can result in an enhancement in insulin sensitivity and glucose tolerance in ruminants, highlighting its potential role in energy metabolism [6].

The timing of vitamin D intake—whether it occurs during mating or throughout the gestation phase—substantially affects its impacts [8]. Providing vitamin D during mating can enhance reproductive outcomes and metabolic conditions, creating a solid groundwork for successful pregnancy [9,10]. In late pregnancy, the demands on the ewe's body increase significantly due to fetal growth and preparation for lactation. 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, or ketosis, is a common metabolic disease in ewes during late pregnancy, characterized by elevated levels of free fatty acids and insulin resistance. This condition often results from a mismatch between energy intake and expenditure, resulting in negative energy balance (NEB). In the final months of pregnancy, when about 70% of fetal growth takes place, ewes are especially susceptible to NEB, which can lead to pregnancy toxemia and significant economic losses [12]. Implementing effective vitamin D supplementation strategies may help alleviate these risks by promoting better energy balance and enhancing metabolic health.

The Grey Shirazi breed, a traditional variety of sheep indigenous to Iran, is known for its exceptional wool quality and adaptability to regional climates. Originating from the Shiraz region in southern Iran, this breed is primarily recognized for its semi-fine wool, which holds significant value in the textile industry. Grey Shirazi sheep are medium-sized animals characterized by their unique bluish-gray coat, which is the source of their name. They are well-suited to the arid and semi-arid environments of the region, displaying strong resilience to harsh weather conditions and low-quality forage. 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. Despite their significance, the Grey Shirazi sheep population is declining, raising concerns over the conservation of this unique breed [13].

Despite known metabolic benefits, the effect of timed vitamin D supplementation on pregnancy-specific metabolic changes in indigenous sheep breeds remains underexplored. 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. The metabolic profile assessed included glucose, insulin, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), calcium, phosphorus, triglycerides, cholesterol, total protein, and albumin. It was hypothesized that vitamin D supplementation, particularly when administered at physiologically critical stages such as mating and late pregnancy, would result in elevated serum 25(OH)D levels, improved glucose-insulin dynamics, reduced lipolysis (as reflected by lower NEFA and BHBA levels), and more stable calcium and phosphorus homeostasis. These changes

were anticipated to reflect enhanced energy balance and metabolic adaptation during pregnancy, potentially contributing to improved maternal physiological status in the peripartum period.

Results

Vitamin D

Within-group comparisons across time points revealed significant changes in serum vitamin D concentrations, as shown in Table 1. In Group 1 (control), a significant reduction in serum vitamin D levels was observed during all stages of pregnancy and postpartum compared to pre-pregnancy (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 demonstrated that Groups 2 and 3 had significantly higher serum vitamin D levels than Group 1 at mid-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 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; $p < 0.05$). Postpartum, the highest serum vitamin D level 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 groups, BHBA concentrations increased significantly as pregnancy progressed. In Group 1, BHBA levels rose from 0.21 ± 0.02 mmol/L at 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 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 at each time point (Table 2) revealed no statistically significant differences in BHBA levels ($p > 0.05$).

Within-group comparisons of NEFA levels (Table 2) did not show any statistically 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

Within-group comparisons of serum insulin concentrations over time are shown in Table 3. 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 mating to 3.36 ± 1.33 ng/mL postpartum ($p < 0.05$). A similar pattern was observed in the supplemented groups, with Group 2 increasing from 1.44 ± 0.64 ng/mL at mating to 1.99 ± 1.09 ng/mL postpartum, and Group 3 rising from 1.34 ± 0.16 ng/mL at mating to 6.06 ± 1.06 ng/mL postpartum ($p < 0.05$ for Group 3).

Between-group comparisons at each time point revealed statistically significant differences in insulin concentrations 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 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 statistically 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 (10.41 ± 0.09 mg/dL), which was significantly greater than values at other time points in the same group ($p < 0.05$). In Group 3, the peak calcium level was observed postpartum (10.55 ± 0.09 mg/dL), also representing a significant increase compared to earlier stages ($p < 0.05$). In contrast, calcium levels in Group 1 remained relatively stable throughout the sampling period, with no significant within-group differences ($p > 0.05$).

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; $p > 0.05$).

Within-group comparisons of serum phosphorus levels over time revealed a significant decline during late pregnancy and postpartum in all groups, as shown in Table 4. 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

Within-group comparisons of serum triglyceride concentrations over time are shown in Table 5. In all groups, triglyceride levels increased significantly during pregnancy. For instance, Group 1 increased from 36.45 ± 4.00 mg/dL at 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 ($p > 0.05$).

Within-group comparisons of serum total protein concentrations over time demonstrated a significant decline in all groups during pregnancy. For example, in Group 1, protein levels

decreased from 7.52 ± 0.14 g/dL at 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 showed no statistically 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 impacts of vitamin D supplement administration during two critical reproductive stages—insemination and mid-pregnancy—on the metabolic profiles of pregnant Grey Shirazi ewes. Given the extensive physiological roles of vitamin D, particularly in regulating metabolism, this study aimed to assess how supplementation affects serum vitamin D levels and related metabolic factors during pregnancy and postpartum.

The results showed that, in the absence of supplementation, serum vitamin D concentrations declined progressively during pregnancy and postpartum, with the control group exhibiting a

drop from 53.16 ± 14.07 ng/mL at mating to 30.36 ± 13.69 ng/mL at mid-pregnancy and 34.31 ± 13.12 ng/mL postpartum. In contrast, vitamin D supplementation significantly mitigated this decline. 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).

Although Group 3 consistently showed higher 25(OH)D levels than Group 2 at late pregnancy and postpartum, the differences between the two supplemented groups were not statistically significant ($p > 0.05$). Therefore, while a trend favoring mid-gestation supplementation was observed, caution is warranted in interpreting this as a definitive advantage. Further studies with larger sample sizes are needed to confirm whether mid-pregnancy offers superior timing for sustained vitamin D status.

The decline in serum vitamin D levels observed in the control group ewes is consistent with previous studies, which have shown that pregnancy can lead to a reduction in circulating 25-hydroxyvitamin D due to increased utilization by the mother and fetus [4,14]. Comparable patterns have been observed in both humans and livestock, where maternal vitamin D levels decline as gestation progresses, particularly in the lack of supplementation [15,16].

The observed elevation in serum vitamin D levels following supplementation during mid-pregnancy could be attributed to the enhanced metabolic demands of pregnancy, particularly in supporting calcium and phosphorus homeostasis, which are critical for fetal skeletal formation. Vitamin D is essential for regulating these minerals by improving intestinal absorption and renal reabsorption [17]. During pregnancy, the maternal demand for calcium increases, resulting in the

upregulation of vitamin D processing [18]. The higher efficacy of supplementation during mid-pregnancy in maintaining serum vitamin D levels may reflect the heightened physiological requirement for this nutrient during this critical period of fetal growth.

The discrepancy in response observed between the timing of supplementation (mid-pregnancy versus insemination) may also be attributed 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 clarify 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. A study by Rodney et al. (2018) demonstrated that administering vitamin D supplements to pregnant dairy cows resulted in increased serum vitamin D levels and improved calcium metabolism, which is vital for preventing postpartum complications like hypocalcemia [20]. Research has shown that in sheep, the provision of vitamin D can enhance 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 be crucial in maximizing its advantages. A study by Poindexter et al. (2023) conducted on goats revealed that giving supplements during late gestation markedly enhanced maternal vitamin D levels compared to earlier supplementation, aligning with the findings of our 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. Elevated BHBA and NEFA are well-known markers of NEB, reflecting increased fat mobilization as energy stores are depleted [23]. This pattern is similar to former studies that have demonstrated a rise in these indicators during late gestation, as the energy requirements of fetal growth exceed maternal dietary intake [24].

Vitamin D supplementation significantly influenced these metabolic indicators, as levels of BHBA and NEFA were 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 statistically significant. Conversely, the variations 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 imply that vitamin D supplementation, especially when given during mid-pregnancy, may help alleviate NEB and decrease fat mobilization in 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 reduced reliance on fat stores during periods of high energy demand [25]. Improved insulin action may also suppress the release of NEFA from adipose tissue, which could explain the lower NEFA levels in the supplemented groups. In dairy cows, similar effects have been observed, where vitamin D supplementation reduced NEFA and BHBA concentrations and improved overall energy balance during the periparturient period [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. However, while Group 3 exhibited numerically lower BHBA concentrations during late pregnancy (0.63 ± 0.12 mmol/L) compared to Group 1 (0.79 ± 0.29 mmol/L) and Group 2 (0.91 ± 0.11 mmol/L), the differences were not statistically 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. Future studies should evaluate dose-response relationships to determine the optimal vitamin D level required to suppress lipolysis and BHBA production more effectively.

In this study, a significant rise in serum insulin concentrations was found in ewes receiving vitamin D supplementation during mid-pregnancy, while supplementation at insemination had no effect. This result aligns with previous studies indicating that vitamin D stimulates insulin secretion and improves pancreatic β-cell function, particularly during times of metabolic stress such as pregnancy [25]. Elevated insulin levels may help promote glucose uptake by maternal tissues and the growing fetus, thereby preventing excessive fat mobilization and reducing the risk of NEB. This finding reinforces the concept that vitamin D contributes to preventing NEB in pregnant ewes by enhancing 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 accompanying an increase in serum glucose levels, this difference did not reach statistical significance. The absence of a significant change in glucose levels may indicate that while vitamin D enhances insulin sensitivity, it does not drastically alter

glucose homeostasis in late pregnancy, where the majority of glucose is likely directed toward 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), along with 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 included 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, a significant increase in serum calcium levels was recorded in the group receiving vitamin D supplementation during pregnancy, particularly after delivery. This finding aligns with the critical role of vitamin D in maintaining calcium balance. Vitamin D improves intestinal calcium absorption and promotes the transfer of calcium from bone to sustain adequate serum calcium concentrations, particularly throughout times of increased demand, including late pregnancy and lactation [17]. The increased serum calcium concentrations in the supplemented group could be due to improved calcium absorption from the diet, facilitated by elevated serum levels of active vitamin D (calcitriol). Additionally, vitamin D may have supported calcium reabsorption in the kidneys, preventing excessive loss through urine, which is critical during the postpartum period when calcium demands are high for lactation [26].

The absence of a significant difference in serum calcium levels between the control group and the group supplemented at the time of insemination could be described by the timing of vitamin D administration. In the early stages of pregnancy, the demand for calcium is relatively lower compared to late pregnancy and lactation, when fetal skeletal development and milk production require substantial calcium reserves [27]. Therefore, vitamin D supplementation during mid-pregnancy might have been more effective in meeting the higher calcium demands during the later stages of pregnancy and postpartum.

In contrast, no significant differences were noted in serum phosphorus concentrations across the groups at any stage of the investigation. This result may reflect the tight regulation of phosphorus levels in ruminants, which are maintained within a narrow limit to support metabolic processes such as energy production and bone mineralization [28]. Phosphorus levels are less directly influenced by vitamin D compared to calcium, as phosphorus homeostasis is primarily regulated by fibroblast growth factor-23 (FGF-23) and parathyroid hormone (PTH), with vitamin D playing a more secondary role [29]. In addition, phosphorus absorption is generally 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 accompanied by a significant rise in serum cholesterol levels at the end of pregnancy and elevated 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, 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. Triglycerides serve as a major energy source for both the dam and the neonate during lactation, as milk is rich in fat content [33]. The rise in triglycerides can be related to the mobilization of fat stores in the dam to meet the energetic demands of lactation and may also reflect enhanced lipogenesis stimulated by insulin. 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. This decline in protein levels is consistent with previous outcomes, which suggest that the physiological demands of pregnancy can lead to changes in plasma protein concentrations due to increased dilutional effects from 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 administration did not result in significant alterations in total serum protein levels. However, it is noteworthy that the ewes receiving vitamin D injections during mid-pregnancy exhibited elevated serum albumin levels by the end of pregnancy. Albumin, a major plasma protein, plays a fundamental role in preserving oncotic pressure and facilitating the transport of

different substances, such as hormones, fatty acids, and drugs [36]. The significant rise 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 demonstrated 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 reduced incidence of metabolic disorders. This can be especially advantageous for pregnant ewes during the crucial transition phase around parturition.

The absence of notable alterations in total protein levels with vitamin D administration may suggest that while vitamin D administration can enhance albumin levels, the overall protein status in ewes during late pregnancy is influenced by multiple factors, such as dietary protein intake and the animal's physiological condition. Further investigation is required to clarify the specific mechanisms through which vitamin D affects serum albumin levels and to explore the impact of dietary protein on the overall protein status in pregnant ewes.

This research 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. Supplemented ewes exhibited reduced serum β-hydroxybutyrate (BHBA) and non-esterified fatty acid (NEFA) levels, indicating a lower negative energy balance, alongside increased serum cholesterol and triglyceride levels, which support lactation energy demands. Additionally, higher serum albumin levels in these ewes suggest 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, warranting further research to explore optimal dosing strategies and underlying mechanisms.

Materials and Methods

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. The ewes' diet consisted of a total mixed ration composed of conserved forage—including 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 underwent synchronization, a process that involved 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. Approximately 46 hours after the PMSG injection, the ewes were inseminated via laparoscopy, ensuring a controlled and uniform reproductive process for the study.

Experimental Design

The ewes were randomly allocated to 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 [39].

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

Ewes that 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. A significance threshold of $p < 0.05$ was considered statistically significant.

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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 a A	30.36 ± 13.69 b A	39.71±10.18 b A	34.31±13.12 b A
Group 2	54.58 ± 10.27 a A	73.24±14.52 b B	53.25±9.31 a B	44.09 ± 14.29 a A
Group 3	47.13 ± 12.01 a A	52.20 ± 13.39 a C	67.14±13.99 b C	50.81 ± 16.98 a 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 2. Mean ± SE of serum BHBA and NEFA in various groups at different sampling times.

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

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 a	1.86±0.82 a	2.39±1.44 a A	3.36±1.33 b A
	Group 2	1.44±0.64	1.64±0.51	2.96±1.42 A	1.99±1.09 A
	Group 3	1.34±0.16 a	1.08±0.79 a	4.00±1.82 b B	6.06±1.06 b B
Glucose (mg/dl)	Group 1	111.90±7.65 a	82.71±5.42 ab	71.42±4.40 b	47.00±6.96 c
	Group 2	99.14±5.97 a	85.00±7.89 ab	75.85±4.56 b	43.53±2.95 c
	Group 3	109.00±6.58 a	97.61±5.14 ab	84.20±4.88 b	45.71±4.50 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 ($p<0.05$).

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 a	10.41±0.09 b	10.08±0.12 a	9.86±0.10 A a
	Group 3	10.11±0.09 a	10.13±0.12 a	10.09±0.13 a	10.55±0.09 B b
Phosphorous (mg/dl)	Group 1	6.04±0.20 a	6.48±0.57 a	5.32±0.80 b	5.03±0.38 b
	Group 2	6.68±0.28 a	5.97±0.35 a	5.54±0.43 b	4.60±0.26 b
	Group 3	6.12±0.16 a	6.67±0.31 a	5.29±0.26 ab	4.81±0.33 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 a	41.85±2.35 ab	58.42±7.39 b	46.57±2.56 AB ab
	Group 2	28.21±3.80 a	48.00±2.67 b	61.28±9.70 b	40.60±2.58 A b
	Group 3	30.75±4.73 a	38.46±2.65 a	55.70±5.24 b	55.71±2.62 B b
Cholesterol (mg/dl)	Group 1	72.18±2.76 a	64.00±1.78 b	61.57±4.39 A b	64.42±5.42 b
	Group 2	69.14±3.29 a	70.85±2.35 a	76.85±3.43 B b	70.46±3.20 a
	Group 3	65.62±3.45	65.00±2.36	74.30±4.05 B	69.28±3.23
Protein (g/dl)	Group 1	7.52±0.14 a	6.88±0.20 ab	6.15±0.42 b	6.98±0.29 ab
	Group 2	7.22±0.15 a	6.81±0.17 ab	6.21±0.18 b	6.69±0.12 b
	Group 3	7.55±0.18 a	6.80±0.20 b	6.40±0.24 b	6.85±0.22 ab
Albumin (g/dl)	Group 1	3.50±0.05 a	3.18±0.06 b	2.71±0.14 A b	3.31±0.10 a
	Group 2	3.41±0.06 a	3.15±0.07 ab	3.04±0.09 AB b	3.41±0.03 a
	Group 3	3.53±0.07 a	3.30±0.08 b	3.18±0.05 B b	3.42±0.13 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 ($p<0.05$).

عنوان مقاله: اثر مکمل ویتامین D بر سطح سرمی آن و پروفایل متابولیسم انرژی در میش‌های کبوده شیراز

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خلاصه فارسی:

این مطالعه با هدف بررسی اثرات مکمل ویتامین D بر سطح سرمی این ویتامین و پروفایل متابولیک میش‌های نژاد کبوده شیراز در دوره آبستنی انجام شد. 60 میش سالم به‌طور تصادفی به سه گروه تقسیم شدند: گروه 1 (کنترل)، گروه 2 که در زمان تلقیح IU 10,000 ویتامین D دریافت کردند، و گروه 3 که در میانه دوره آبستنی مکمل ویتامین D دریافت کردند. سطح ویتامین D در گروه کنترل (گروه 1) طی آبستنی به‌طور قابل‌توجهی کاهش یافت، در حالی که گروه‌های 2 و 3 سطح پایدار ویتامین D را حفظ کردند و گروه 3 در اواخر آبستنی و پس از زایمان بالاترین سطح را داشت. از نظر تعادل انرژی، غلظت-β هیدروکسی‌بوتیرات (BHBA)و اسید چرب غیر استریفیه (NEFA) در گروه دریافت کننده مکمل در میانه آبستنی کمترین میزان را نشان داد. غلظت انسولین نیز در پایان آبستنی در گروه 3 افزایش یافت. سطوح کلسیم و فسفر در گروه 3 پس از زایمان به اوج خود رسید. هر سه گروه افزایش تری‌گلیسرید و کلسترول را تجربه کردند، اما گروه 3 پس از زایمان بالاترین سطح تری‌گلیسرید را داشت. همچنین، سطح پروتئین سرم و آلبومین در گروه 3 پس از زایمان به‌طور قابل‌توجهی بالاتر بود که نشان‌دهنده وضعیت تغذیه‌ای بهتر و سنتز پروتئین بود. یافته‌های این مطالعه نشان می‌دهد که تجویز ویتامین D در میانه آبستنی می‌تواند سلامت متابولیک و تعادل انرژی میش‌های کبوده شیراز را بهبود بخشد و پیامدهای مثبتی برای سلامت میش و بره داشته باشد. تحقیقات بیشتر برای تعیین دوز و زمان‌بندی بهینه جهت کاربرد گسترده ضروری است.

واژگان کلیدی: ویتامین D، سلامت نشخوارکننده، توازن انرژی، متابولیسم آبستنی