Iranian Journal of Applied Animal Science

Impacts of Trace Minerals Supplementation on Reproductive Performance and Blood Parameters of Ghezel Ewes

Research Article

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Received on: 24 Dec 2024
Revised on: 9 Feb 2025
Accepted on: 21 Feb 2025
Online Published on: Mar 2025

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Online version is available on: www.ijas.ir
https://doi.org/10.71798/ijas.2025.1202957

ABSTRACT

Nutrition is critical to maximizing genetic potential. Trace minerals play an important role in animal diets and can influence productivity and reproduction. Therefore, a study was conducted to evaluate the effects of trace mineral supplementation on reproductive function, hormonal and biochemical blood parameters in Ghezel ewes. Accordingly, 30 ewes were treated with three dietary treatments for a period of five weeks: 1- Flushed with trace minerals (TM): (Fe, Cu, Mn, Co, Se, Zn, and Cr), 2- Flushed without trace minerals (NTM) 3- Control (CON): only pasture grazed only. Estrus synchronization was achieved by intravaginal fluorogestone acetate sponges for 12 days interval + 400 IU PMSG injection. The number of follicles and pregnancy were determined by ultrasound. Blood samples were also taken during the experiment. The experiment showed that the TM group had higher lambing rate, twinning, prolificacy as well as higher pregnancy rate during the first estrus than the other groups ($P<0.05$), though no effect was observed on the number of follicles groups ($P>0.05$). Total protein and triglyceride were also lower in the TM group compared to the other groups ($P<0.05$). T4 level was higher in TM group ($P<0.01$). Progesterone and glutathione peroxidase activity were also highest in the TM group ($P<0.05$). Malondialdehyde levels were highest in the NTM group ($P<0.05$). Serum estradiol and enzymes were not affected by the treatments ($P>0.05$). In general, TM supplementation improved reproductive performance and some blood biochemical parameters.

KEY WORDS ewe, reproductive performance, trace minerals.

INTRODUCTION

The maintenance of various biochemical processes in livestock requires adequate levels of trace minerals, including cobalt (Co), copper (Cu), iodine (I), iron (Fe), zinc (Zn), manganese (Mn), and selenium (Se) (Kumar *et al.* 2011). Mineral deficiencies can occur in livestock when these essential minerals are not adequately supplied (Kumar *et al.* 2011; Jin *et al.* 2023). Nutritional status is a critical factor in the reproductive success of sheep and goats due to their need for high quality and sufficient amounts of nutrients (Vázquez-Armijo *et al.* 2011). Research has shown that pastures are often deficient in essential nutrients, especially minerals, which are essential for normal growth and metabolic processes in livestock, and the nutritional quality of grass is not always guaranteed (Domínguez-Vara *et al.* 2009; Kumar *et al.* 2011; Jin *et al.* 2023; Kuru *et al.* 2024). Due to the low economic input in extensive sheep production systems, targeted mineral supplementation strategies are essential to mitigate deficiencies in a dynamic production context (Suttle, 2010; Kumar *et al.* 2011; Vázquez-Armijo *et al.* 2011).

Sheep-specific trace mineralized salt (TM salt) is essential to provide adequate levels of trace minerals while reducing the risk of toxicity (Joshi, 2022; Martínez-Morcillo *et al.* 2024). Sheep are particularly sensitive to copper and require careful supplementation to prevent toxicosis (Joshi, 2022). Strategic supplementation with TM salt can prevent deficiencies and improve animal performance, though nutrient interactions and mineral balance influence trace mineral requirements and metabolism (Suttle, 2010; Joshi, 2022; Martínez-Morcillo *et al.* 2024). Previous research suggests that organic trace minerals may enhance the absorption and processing of biological elements (Ma *et al.* 2014; Mousaie *et al.* 2014; Alimohamady *et al.* 2019; Nie *et al.* 2025). However, recent studies have reported similar results regarding the efficacy of different trace mineral sources (Abdian Samarin *et al.* 2022). Nutrient delivery to grazing animals should be timed to critical life stages, focusing on reproductive performance of dams and growth of young animals, rather than year-round supplementation. For example, pre-mating treatment of ewes with bolus supplements of vitamin E and trace minerals can improve fertility by increasing lambing percentages (Zonturlu *et al.* 2017). To maximize offspring benefits, it's often best to treat dams with Se, Co, Cu, and I, which readily cross the placenta (Grace *et al.* 2006; Gonzalez-Rivas *et al.* 2023). Flushing ewes with selenium before and after mating can increase lamb birth weight (Safdar *et al.* 2020). The benefits of trace minerals in advance of high demand periods such as pregnancy and lambing have been demonstrated in sheep (Abdollahi *et al.* 2015; Gonzalez-Rivas *et al.* 2023; Kuru *et al.* 2024). This study investigated the Ghezel sheep breed reproduction, which is affected by mineral deficiencies originated from regional environmental and nutritional conditions. The ewes exhibit suboptimal reproductive performance due to inadequate pasture minerals (Jin *et al.* 2023). This research focused on a new formulation of minerals during the flushing period (the critical window prior to mating) and emphasizes the interactions among various elements on reproduction rather than their individual effects. This innovative approach addressed the potential optimal nutritional strategies (TM supplementation during flushing period) to overcome the impact of mineral deficiencies in Azerbaijan's pasture regions on ewe reproduction. Accordingly, this study was designed to evaluate the impact of supplementing ewes with trace minerals on reproductive performance, lamb weight, lamb survival, and ewe blood parameters.

MATERIALS AND METHODS

Animals, design, synchronization, and flushing diet

All animal-related procedures were approved by the Animal Ethics Committee of the University of Tabriz. The experiment was conducted at Khalat-Pushan Agricultural Research Station, University of Tabriz, Iran (coordinates: 38° 01'54"N 46° 23'41"E) from July 2021 to April 2022. Animals (sheep, rams and ewes of Ghezel breed) were grazed on pasture and fed with flushing diet (according to treatments). During the breeding season, 30 ewes (BW=49. 04±7.5 kg) with 1 to 4 parities and a body condition score (BCS) ranging from 2 to 3. 5 were divided into three groups to ensure equal distribution of body weight and parity. The experiment lasted five weeks, including two weeks before mating and three weeks after mating. Treatments included: 1- TM group: ewes received 300 g/ewe/day flush diet containing 0.3% trace minerals (including Fe, Cu, Mn, Co, Se, Zn, and Cr in their inorganic forms as trace mineral salt powders); 2- NTM group: ewes received 300 g/ewe/day flush diet without trace mineral supplements (as positive control group); and 3- CON group: ewes grazed only on pasture (as negative control or without flushing group). The chemical composition and nutritional values of the flushing diets are shown in Table 1.

Table 1 Flushing diet components

Ingredients of flushing	Percent
Soybean meal	17. 52
Barley grain	68. 36
Wheat bran	9. 44
Salt	0. 50
Calcium carbonate	1. 19
Dicalcium phosphate	1. 19
Sodium bicarbonate	0. 50
Vitamin supplement[1]	1. 00
Mineral supplement or filler	0. 30
Chemical composition of the flushing diet	
Metabolizable energy (Mcal/kg)	2. 40
Crude protein (%)	16. 00
Ca (%)	0. 80
P (%)	0. 38

[1] Vitamin supplement composition per kg: vitamin A: 500000 IU/kg; vitamin D_3: 100000 IU/kg; vitamin E 100 mg/kg and Antioxidant: 400 mg/kg.

All ewes were synchronized with intravaginal fluorogestin acetate sponges (45 mg) sponges (Po BOX 579, UIIadulIa, NSW 2539 Australia) for 12 days + 400 IU PMSG i.m. (Gonaser, Hipra, Spain) on day 12 of the experiment (Figure 1).

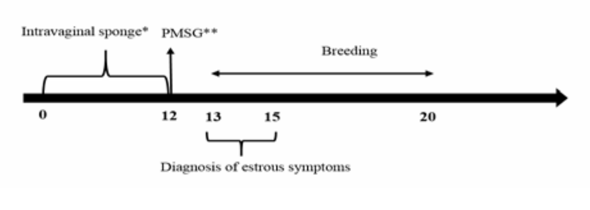

Figure 1 Estrus synchronization protocol of Ghezel ewes
* Fluorogestone acetate (45 mg)
** Equine chorionic gonadotropin (400 IU PMSG)

Rams were then introduced to the herd between days 13 to 20 of the experiment (first estrus). On day 17, the introduction of rams was repeated for the ewes that showed signs of estrus (second estrus).

Blood sampling and evaluations

Blood samples were collected from the jugular vein of each animal on days 0 (start of the flushing diet and synchronization), 11 (24 hours before removal of the intravaginal fluorogestin acetate sponges), 14 (48 hours after removal of the sponges), and 34 (at the end of the flushing diet). Blood samples were collected in non-heparinized tubes, allowed to clot for 2 h at room temperature, and centrifuged (3000×g for 20 min) to collect serum. Samples were stored at -20 °C until analysis for hormones, metabolites, enzymes, and antioxidants. In addition, on day 34 of the experiment, whole blood samples were collected by jugular venipuncture into sterile EDTA tubes and stored at -20 °C for evaluation of blood superoxide dismutase (SOD), glutathione peroxidase (GPx), and hemoglobin (Hb) concentrations.

Serum glucose, total protein, cholesterol, and triglyceride, and serum alkaline phosphatase (ALP) and cholinesterase were evaluated by enzymatic, colorimetric, and spectrophotometric methods using reagents from Sigma Diagnostics (Pars Azmon Co, Tehran, Iran). Hormone kits of estrogen (product no. 4925-300A, Monobind, USA), progesterone (product no. 4825-300A, Monobind, USA), T3 (product no. 14003, Pishtaz TEB Diagnostics, Iran), T4 (product no. 14003, Pishtaz TEB Diagnostics, Iran) were used to evaluate the relevant hormones based on the enzyme-linked immunosorbent assay (ELISA) method by ELISA reader (Hiperion Microplate Reader, Germany). The intra-assay coefficients of variation for progesterone and estrogen were 0. 42% and 0. 13%, respectively. The intra-assay coefficients of variation for serum T3 and T4 were 0. 06% and 0. 47%, respectively. Malondialdehyde (MDA) concentration was measured by thiobarbituric acid reaction (ZellBio, Germany) based on a colorimetric assay (Esterbauer and Cheeseman, 1990). Glutathione peroxidase (GPx) activity, superoxide dismutase (SOD) activity and total antioxidant capacity (TAC) were measured using a recognized commercial kit (Randox, Crumlin, UK). Absorbance was recorded using a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) based on the wavelengths suggested by the kit.

Ultrasound examination

The present study employed real-time B-mode ultrasonography (EXAGO, ECM Company, NANUK 930, French) equipped with a 7. 5 MHz linear array transducer to diagnose the number of follicles on the right ovary of ewes on day 15 of the experiment (72 hours after synchronization) (Figure 2). The linear transducer was inserted into the rectum after fecal pellets were removed. The ewes underwent ultrasound examination while restrained in a holding crate in a standing position, with the rectal probe inserted into the rectum and the ovaries viewed. Additionally, pregnancy was examined twice: the first estrus pregnancy examination was conducted via transrectal ultrasonographic method on day 40 after ram introduction; and the second estrus pregnancy examination was done on day 70 after ram introduction. It is noteworthy that only ewes that were non-pregnant in the initial examination were evaluated for pregnancy status during the subsequent ultrasonography.

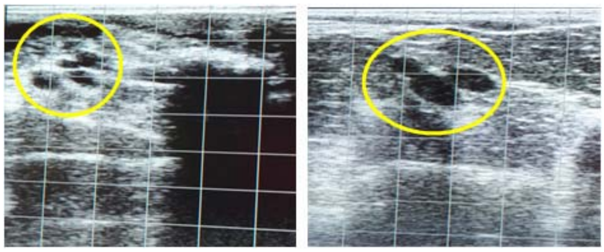

Figure 2 Ultrasonographic images of the ewes' ovaries obtained using a transrectal 7.5 MHz linear array probe displayed using a real-time B-mode ultrasonography

Calculation of reproductive parameters

Subsequent to parturition, lamb numbers and their weight were recorded. Then, all reproductive parameters were calculated based on the formula represented by Hasani *et al.* (2018).

Statistical analysis

Reproductive performance data were analyzed using LOGESTIC procedure of SAS software (SAS, 2013). Blood factors (except for estrogen and progesterone), BCS, number of follicles, and lambs' weight were analyzed by GLM procedure of SAS software and duncan's multiple range test was used for comparing treatments; in which, $P < 0.05$ was considered statistically significant. Blood estrogen and progesterone concentrations in four different times were analyzed using the MIXED procedure of SAS software (SAS, 2013).

RESULTS AND DISCUSSION

Reproductive performance

Table 2 presents the parameters of reproductive performance observed during the breeding season. The findings indicate that the treatments did not exert an influence on the number of follicles present in the right ovary ($P>0.05$). Furthermore, a non-significant pregnancy rate was observed among the treatments ($P>0.05$), though the pregnancy rate during the first estrus exhibited a numerical increase in the TM treatment. Although abortion rate was not different between treatments (P>0.05), no fetal loss or abortion was observed in the TM and NTM groups. While the treatments did not have a significant impact on the lambing rate, the TM group exhibited the highest lambing rate compared with the other groups (P>0.05). Furthermore, the TM group demonstrated the highest total twinning rate and prolificacy compared with NTM and CON groups.

The results of the present study showed that mineral supplementation of ewes did not affect the number of large follicles observed 72 hours after sponge removal in all groups. Consistent with these findings, previous studies have examined the effects of mineral supplementation on ovarian activity and follicular development in ewes (Abdollahi *et al.* 2015) and heifers (Lamb *et al.* 2008) and have shown that such supplementation does not increase the number or size of follicles. In the present study, the addition of TM before breeding increased the proportion of pregnant ewes in the first estrus (70% in TM *vs.* 50% in NTM and CON, respectively), though the difference was not statistically significant, which is similar to previous studies (Sánchez *et al.* 2008; Zonturlu *et al.* 2017; Díaz-García, 2019; Perry *et al.* 2021). However, the experiments conducted by Egan (1972) and Farrag *et al.* (2021) reported that feeding lower levels of TM for a longer period of time resulted in a significantly higher pregnancy rate compared to the control group. Trace element deficiencies have a significant impact on the reproductive health of ruminants, and adequate levels of manganese, cobalt, copper, and zinc are essential to improve fertility and reproductive success (Uslu *et al.* 2017; Joshi, 2022). Manganese deficiency can lead to reproductive problems such as irregular estrous cycles, anovulation, embryonic death, and lower pregnancy success rates (Suttle, 2010; Kumar *et al.* 2011; Joshi, 2022). These effects are likely due to manganese's role in enzymatic processes that are critical for normal ovarian function and hormone synthesis. Similarly, cobalt deficiency reduces pregnancy rates, disrupts the estrous cycle, and can negatively affect multiple lambing and lamb survival during early pregnancy (Suttle, 2010), possibly due to its role in vitamin B12 synthesis, which is essential for cellular metabolism and DNA synthesis (Hostetler *et al.* 2003). One study reported that a decrease in copper and zinc levels during mating was associated with a decrease in pregnancy rates in fat-tailed Murkaraman ewes (Uslu *et al.* 2017). Copper deficiency disrupts the hypothalamic-pituitary-gonadal axis, impairing ovarian function and reducing fertility. Copper is essential for several enzymes, including cytochrome c oxidase, superoxide dismutase, and lysyl oxidase, which are essential for energy production, antioxidant defense, and collagen formation (Suttle, 2010; Kumar *et al.* 2011; Yiqin *et al.* 2022). These functions are important for the development of reproductive tissues and embryos (Lamraoui *et al.* 2024). In addition, copper is involved in steroid hormone synthesis, particularly through the enzyme aromatase, which converts androgens to estrogens in ovarian granulosa cells (Nikhil Kumar *et al.* 2021). Zinc is essential for maintaining meiotic arrest, egg activation, and preventing polyspermy during fertilization (Garner *et al.* 2021). It also supports preimplantation embryo development, placental formation, and fetal growth (Garner *et al.* 2021). The effect of TM on embryo and fetal survival, both in terms of the number of offspring and their size at birth, is well established (Hostetler *et al.* 2003; Grace *et al.* 2006; Zimmermann, 2006; Kumar *et al.* 2011; Safdar *et al.* 2020). In the current study, though there was no significant difference in lambing rate between treatments, TM consumption two weeks before breeding and three weeks after breeding increased lambing rate numerically (130% in TM *vs.* 100% in NTM and CON), which is consistent with previous reports (Perry *et al.* 2021; Gonzalez-Rivas *et al.* 2023; Kuru *et al.* 2024). In addition, overall mating and fertility rates were improved by TM supplementation in this study. Consistent with the present results, Zonturlu *et al.* (2017) reported that administration of a vitamin E and mineral bolus to Avasi ewes 14 days prior to mating increased both proficiency and rate of multiple births (49. 9% *vs.* 20%). In another study, ewes fed a manganese-supplemented diet for five months prior to mating and during pregnancy had a lower number of services per conception than those fed a low-manganese diet (Hidiroglou *et al.* 1978). Also, Sánchez *et al.* (2008) reported higher lambing and twinning rates in ewes supplemented with 0. 5 mg/kg selenium. In the study by Abdollahi *et al.* (2015), ewes that received two mineral boluses (Ferrobloc) with 400 IU PMSG i. m. four weeks prior to CIDR showed a higher multiple lambing rate than those that received a single Ferrobloc bolus. Thus, the results of this study suggest that supplementation with TM before and after breeding may indirectly improve reproductive performance in ewes. Proposed mechanisms include hormonal regulation, maintenance of meiotic arrest, prevention of polyspermy, and promotion of embryonic growth and preimplantation development. Further research with larger sample sizes and longer duration is needed to confirm these findings.

The impact of the treatments on lambs' birth weight and ewes' body condition score of the ewes is shown in Table 3. No differences (P>0.05) in lambs' birth weight and ewes' BCS were found between treatments. According to Atashi and Izdifar (2012), the typical birth weight of Gezel lambs ranges from 4. 5 to 4.7 kg. In our experiment, the lambs born from the animals had birth weights within this normal range.

Table 2 Effect of trace minerals on reproductive parameters of Ghezel ewes

Reproductive parameters	Treatments			P-value
	TM (n=10)	NTM (n=10)	CON (n=10)	
Follicle number (mean±SE)	3. 10±0.26	2.90±0.26	3.40±0.26	0.40
Number of pregnant ewes in the first estrus (head)	7	5	5	-
Pregnancy rate in the first estrus (%)	70.00	50.00	50.00	0.58
Total number of pregnant ewes (head)	9	8	9	-
Total pregnancy rate (%)	90.00	80.00	90.00	0.76
Number of abortion (head)	0	0	1	-
Abortion rate (%)	0.00	0.00	11.11	0.90
Number of lambs in the first estrus (head)	10	7	7	-
Lambing rate in the first estrus (%)	100.00	70.00	70.00	0.59
Total number of lambs (head)	13	10	10	-
Total lambing rate (%)	130.00	100.00	100.00	0.40
Twinning rate in the first estrus (%)	60.00	28.57	57.14	-
Total twinning rate (%)	61.54	20.00	40.00	-
Prolificacy in the first estrus (%)	142.86	116.66	140.00	-
Total prolificacy (%)	144.44	111.11	125.00	-

TM: flushing with inorganic trace minerals; NTM: flushing without TM and CON: control group.

Table 3 Effect of trace minerals on lambs' birth weight and ewes' body condition score (mean±SE)

Traits	Treatments			P-value
	TM (n=10)	NTM (n=10)	CON (n=10)	
Lambs' birth weight in the first estrus (kg)	4.44±0.25	4.73±0.29	4.10±0.29	0.34
Total lamb birth weight (kg)	4.54±0.24	4.83±0.28	4.53±0.28	0.70
Ewes' body condition score	3.07±0.17	3.15±0.17	3.13±0.17	0.95

TM: flushing with inorganic trace minerals; NTM: flushing without TM and CON: control group.

However, no significant differences in birth weight were observed between the treatment groups, which is consistent with previous studies such as Sánchez *et al.* (2008), who also reported no changes in birth weight with mineral supplementation. In contrast, Kuru *et al.* (2024) found that Bakofix® supplementation increased multiple birth rates and lamb birth weights in Anatolian Merino ewes. Gonzalez-Rivas *et al.* (2023) reported improved marking rates and lamb weights following injectable trace mineral (ITM) supplementation in Australian sheep. Collectively, these studies suggest that trace mineral supplementation may positively affect reproductive outcomes, though effects may vary depending on the specific minerals, timing, and method of administration (Gonzalez-Rivas *et al.* 2023; Kuru *et al.* 2024). In the present study, BCS of ewes was not affected by TM supplementation. The lack of effect on BCS is consistent with Amanlou *et al.* (2020) and Yasui *et al.* (2014), and reinforces the idea that BCS is less responsive to short-term mineral supplementation. This suggests that longer supplementation durations or different formulations may have more pronounced effects. Future research should explore longer supplementation periods, different doses, and organic versus inorganic forms of trace minerals to better understand their role in optimizing lamb birth weight and ewe BCS, potentially leading to improved reproductive success and productivity.

Table 4 shows the results for urea, glucose, cholesterol, total protein and triglyceride concentrations. Urea, glucose, and cholesterol were not affected by the treatments ($P>0.05$). However, serum total protein and triglyceride concentrations were significantly affected by the treatments ($P<0.05$), with the lowest level observed in the TM group. In the current study, there was a decrease in triglyceride levels in the TM group. Previous research has indicated that the reduction in serum triglyceride levels following Cr supplementation is likely due to the action of insulin (Yan *et al.* 2008).

Similarly, Abdulrahman *et al.* (2017) found that increasing the dietary intake of trace element ruminal bolus in ewes and their lambs led to a reduction in plasma triglyceride levels. Furthermore, Haldar *et al.* (2009) reported that Cr supplementation reduced triglyceride concentrations in goats, reinforcing the idea that Cr improves lipid metabolism by improving insulin function. In addition, Zhou *et al.* (2013) found that dietary yeast chromium and L-carnitine supplementation had a positive effect on lipid metabolism in sheep, resulting in lower triglyceride levels. Taken together, these findings suggest that trace minerals, particularly Cr, play a pivotal role in modulating lipid profiles and improving overall metabolic health. Changes in serum biochemical markers are closely associated with changes in tissue permeability and metabolism (Zhang *et al.* 2021).

Table 4 Effect of trace minerals on blood parameters of Ghezel ewes during the flushing period (mean±SE)

Parameters	Treatments			P-value
	TM[1] (n=10)	NTM[2] (n=10)	CON[3] (n=10)	
TP[4] (g/dL)	7.07±0.16[b]	7.46±0.16[ab]	7.66±0.16[a]	0.04
Urea (mg/dL)	34.40±1.72	33.56±1.82	34.89±1.72	0.87
Glucose (mg/dL)	44.30±2.16	45.63±2.28	42.10±2.16	0.50
TG (mg/dL)	28.33±1.68[b]	35.63±1.78[a]	35.20±1.59[a]	0.01
Cholesterol (mg/dL)	55.10±4.07	56.38±4.29	61.70±4.07	0.50
T_3 (ng/dL)	1.80±0.10	1.65±0.10	1.89±0.10	0.27
T_4 (ng/dL)	5.70±0.41[a]	1.46±0.46[b]	0.73±0.43[b]	<0.01
Estradiol (ng/mL)	55.75±13.20	48.83±12.24	62.68±12.24	0.73
Progesterone (ng/mL)	3.35±0.34[a]	1.32±0.34[b]	1.60±0.35[b]	<0.01
ALP (U/L)	181.70±24.77	98.22±26.11	165.90±24.77	0.07
Cholin-esterase (U/L)	222.30±33.93	287.55±35.77	269.20±33.93	0.40
MDA (nmol/mL)	2.02±0.31[b]	3.02±0.33[a]	1.86±0.31[b]	0.03
GPx (IU/gHb)	80.54±3.07[a]	68.67±3.07[b]	66.73±3.07[b]	0.02
SOD (U /gHb)	1696.23±90.91	1520.69±90.91	1570.69±90.91	0.40
TAC (nmol/L)	0.30±0.02	0.27±0.02	0.33±0.02	0.09

TM: flushing with inorganic trace minerals; NTM: flushing without TM and CON: control group.
TP: total protein; TG: triglyceride; T3: triiodothyronine; T4: thyroxine; ALP: alkaline phosphatase; MDA: malondialdehyde; GPx: glutathione peroxidase; SOD: superoxide dismutase and TAC: total antioxidant capacity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Mineral elements are vital nutrients for ruminants, as their appropriate concentrations are essential for the optimal development of physiological, catalytic, and regulatory functions (Suttle, 2010). In the present study, treatments did not affect plasma concentrations of cholesterol and glucose. The stability of serum urea levels suggests that reproductive function remained normal and was unaffected by TM deficiency or supplementation.

This is important because urea, which is derived from protein degradation (Jin *et al.* 2023), serves as a key indicator of nitrogen metabolism. Similar studies investigating zinc sources reported no changes in serum cholesterol, urea, and glucose levels in rats (Nagalakshmi *et al.* 2017), sheep (Sethy *et al.* 2018), and Holstein dairy cows (Sobhanirad and Naserian, 2012). The results of the present study are also consistent with previous research in sheep, which found that TM supplementation did not significantly affect serum cholesterol, urea, and glucose (Zhou *et al.* 2013; Mousaie *et al.* 2014). A significant decrease in total protein levels was observed in the blood of TM-treated ewes compared to the control group. The decrease in total protein levels in the TM group may be attributed to increased fetal growth and amino acid utilization during pregnancy (Lamraoui *et al.* 2024). In agreement with the present study, Moussafir *et al.* (2023) reported that total plasma protein decreased from day 30 of pregnancy. Similarly, our results showed significantly lower levels from day 22 after the first estrus of ewes in the TM group.

The effects of TM treatment on thyroid hormonal concentrations are shown in Table 4.

Although there was no effect of TM supplementation on serum T3 concentration (P>0.05), T4 concentration was higher in the TM group (P<0.01; Table 4). In this study, T3 concentration was not significantly different between groups. However, T4 concentration was significantly higher in the TM group than in the NTM and CON groups. Zinc, iron, copper, and selenium are essential for the action of enzymes that facilitate thyroid hormone metabolism and the conversion of T4 to T3 (Ebrahimi *et al.* 2009; Yatoo *et al.* 2013; Arora *et al.* 2018). Selenium supplementation increased plasma T3 levels while decreasing T4 levels (Todini, 2007; Farrag *et al.* 2021). The lower T3 in the unsupplemented groups indicated impaired 5'-deiodinase activity due to selenium deficiency (Arthur and Beckett, 1989; Ebrahimi *et al.* 2009). Mousaie *et al.* (2014) found that lambs supplemented with Se-Met (1.5 mg/kg) and Cr-Met (0.8 mg/kg) had higher T3 levels. Similarly, Ebrahimi *et al.* (2009) reported that suckling calves receiving 0. 3 mg/kg Se from Sel-Plex had comparable results. Zimmermann (2006) also reported a decrease in hepatic deiodinase enzyme activity due to iron deficiency. Thyroid peroxidase, a heme-dependent protein essential for the initial steps of thyroid hormone synthesis, can have its activity improved by iron supplementation (Zhou *et al.* 2022). This suggests that iron plays a dual role in both hormone synthesis and activation. Zinc is essential for the activity of the enzyme 5′-deiodinase (Arora *et al.* 2018). Alimohamady *et al.* (2019) found that supplementing lambs with 30 mg Zn/kg diet (organic and/or inorganic) increased serum T3 and T4 levels.

Soil chemistry greatly influences the availability of minerals in livestock, which can lead to deficiencies or toxicity, especially for essential minerals such as iodine (Martínez-Morcillo *et al.* 2024). Imbalances can disrupt thyroid hormone production and metabolism, affecting the hypothalamic-pituitary-thyroid axis and potentially leading to hypothyroidism in ruminants (Paulíková *et al.* 2017). Increased T4 levels in the TM group suggest increased synthesis and secretion, while unchanged T3 levels may indicate a temporary adaptation or compensatory mechanism in the hypothalamic-pituitary-thyroid axis (Paulíková *et al.* 2017). While T3 levels were not significantly different, higher T4 levels indicated a possible reserve for T3 production during increased demands, such as pregnancy or lactation. Furthermore, elevated glutathione peroxidase (GPx) activity in the TM group supports the role of trace minerals in mitigating oxidative stress and maintaining thyroid function (Arthur and Beckett, 1989; Osredkar and Sustar, 2011; Zhou *et al.* 2022).

Changes in serum sex hormone concentrations in ewes are shown in Table 4. Estradiol concentration was not affected (P>0.05) by mineral supplementation. Progesterone concentration was significantly affected by treatments (P<0.05), with the highest amount of progesterone observed in TM group compared to other groups. Time had a significant effect on progesterone concentration (P<0.01; Figure 3).

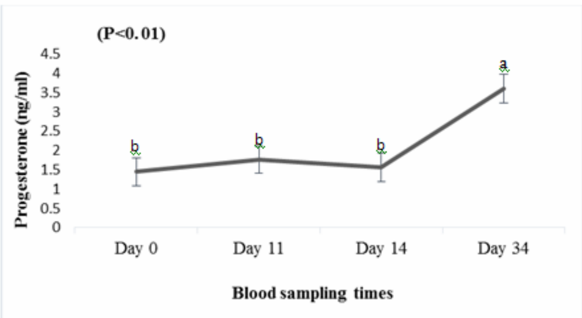

Figure 3 The effect of different blood sampling times on serum progesterone concentration in Ghezel ewes. Days 0 (Start of feeding flushing diet and synchronization), 11 (24 hours before removing the sponge), 14 (48 hours after removing the sponge), and 34 (at the end of feeding flushing diet)

[a, b, c] Means within the same row with different letter differ significantly (P<0. 05)

Also, the effect of treatment by time was significant in TM group compared with other groups (P<0.01; Figure 4). In this study, the highest non-significant level of estradiol was found in the CON group, while the concentration of progesterone was higher in the TM group. Trace minerals play an important role in proper follicular growth and pregnancy maintenance by influencing the secretion of reproductive hormones (Vázquez-Armijo *et al.* 2011).

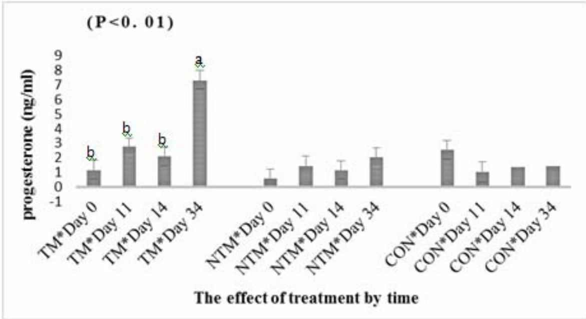

Figure 4 The effect of different blood sampling times on serum progesterone concentration in Ghezel ewes. Days 0 (Start of feeding flushing diet and synchronization), 11 (24 hours before removing the sponge), 14 (48 hours after removing the sponge), and 34 (at the end of feeding flushing diet)

TM: flushing with inorganic trace minerals; NTM: flushing without TM and CON: control group

[a, b, c] Means within the same row with different letter differ significantly (P<0.05)

Zinc is a component of a specific type of protein involved in the genetic expression of reproductive hormones. In addition, copper is essential for the synthesis and maintenance of appropriate serum follicle-stimulating hormone levels (Vázquez-Armijo *et al.* 2011). Manganese also plays a role in cholesterol synthesis, which is necessary for the synthesis of steroids such as progesterone, estrogen, and testosterone (Xie *et al.* 2014). Cu has been shown to stimulate the release of GnRH and FSH, which leads to the synthesis and release of estradiol (Roychoudhury *et al.* 2016). In addition, copper may be steroidogenic and thus promote estradiol synthesis by increasing the enzyme aromatase, which converts androgens to estradiol in ovarian granulosa cells (Nikhil Kumar *et al.* 2021). Rutigliano *et al.* (2008) found that estradiol increased with follicle diameter during Ovsynch, but selenium sources (sodium selenite and/or selenized yeast) did not affect estradiol concentration. On the other hand, dietary supplementation of trace mineral sources increased serum (Kujur *et al.* 2016; Shi *et al.* 2018; Daghigh Kia *et al.* 2019; Safdar *et al.* 2020) and follicular fluid estradiol concentrations in ruminants. Progesterone is secreted by the placenta and corpus luteum and plays an important role in establishing and maintaining pregnancy during gestation (Shi *et al.* 2018). During the formation of the corpus luteum after ovulation, the concentration of progesterone increases (Shi *et al.* 2018). Manganese plays a critical role in progesterone secretion (Hostetler *et al.* 2003). Similarly, previous studies in ewes showed that feeding selenium during the flushing period
increased progesterone concentrations three weeks after mating (Kujur *et al.* 2016; Daghigh Kia *et al.* 2019; Safdar

et al. 2020). According to the results of the current study, the highest non-significant estradiol level in the CON group, together with lower progesterone concentrations, was associated with a lower pregnancy rate in this group. On the other hand, significantly higher serum progesterone in the TM group with lower estradiol concentrations was associated with a higher pregnancy rate in this group.

The effect of the TM treatment on the concentration of ALP and cholinesterase is shown in Table 4. Serum ALP was not affected by treatments (P>0.05), though it tended to be higher in the TM group (Table 4). Research indicates that the normal activity of alkaline phosphatase (ALP) in sheep serum ranges from 70 U/L to 390 U/L (Jin *et al.* 2023). The ALP activity of all the Ghezel ewes studied was within the normal range. However, a non-significant increase in ALP levels was observed in the TM group. Alkaline phosphatases are cell membrane metalloenzymes whose activity in serum is used as a marker of bone formation to evaluate osteoblastic activity (Turan *et al.* 2011). Although ALP levels tend to decrease as pregnancy progresses in ewes (SY *et al.* 2009), the non-significant increase observed in the TM group may reflect the beneficial effects of trace minerals on bone turnover and tissue repair during this critical period. Zinc is directly involved in stabilizing the structure of ALP, while copper may influence its synthesis or activation (Liu *et al.* 2016). Similarly, in pigs, alkaline phosphatase (ALP) activity was increased in those fed micromineral-enriched diets compared to a basal diet, suggesting that ALP is responsive to zinc and copper supplementation (Liu *et al.* 2016). Furthermore, in Chinese yellow-feathered broilers, low levels of organic TM supplementation increased ALP activity, serum Fe and Cu concentrations, and improved antioxidant capacity compared to inorganic TM, likely due to improved bioavailability and absorption efficiency (Nie *et al.* 2025). Although several studies reported no significant effect of trace minerals on ALP concentration (Aksu and Ozsoy, 2010; Nagalakshmi *et al.* 2017; Yaqoob *et al.* 2020; Toghdory *et al.* 2023), other studies reported positive effects of trace minerals on serum ALP concentration in animals (Ma *et al.* 2014; Alimohamady *et al.* 2019).

Serum cholinesterase was also not affected by treatments (P>0.05; Table 4). Cholinesterase is synthesized in the liver and its low levels are caused by several diseases, especially liver disease and malnutrition (Vorhaus and Kark, 1953). This enzyme hydrolyzes acetylcholine to choline (and acetic acid), which is the main component of the cholinergic system, and this enzyme is widely distributed in the central nervous system and also in red blood cells, platelets, and lymphocytes (Çokuğraş, 2003). The results of the present study are consistent with previous studies showing that cholinesterase levels are not affected by reproductive status or selenium treatment in rats (Gokani, 2014). The stability of cholinesterase levels despite dietary interventions may be attributed to the robustness of hepatic synthesis under normal physiological conditions, where baseline liver function effectively maintains enzyme levels within a stable range (Vorhaus and Kark, 1953). In addition, El-Demerdash and Nasr (2014) observed no changes in the activity of cholinesterase in the plasma of male rats following Se-treatment. Furthermore, Antunović *et al.* (2004) indicated that blood cholinesterase was not affected by the reproductive status of ewes.

Plasma concentration of malondialdehyde, as a marker of oxidative stress, was affected by experimental treatments (P<0.05), and the amount was higher in NTM treatment compared with other groups (Table 4). The effect of treatments on the activities of GPx was significant (P<0.05), where the highest activities of GPx were observed in TM group (Table 4). The effect of treatments on the activities of SOD and the value of TAC was not significant (P>0.05), though the activity of SOD was numerically higher in TM compared with other treatments (Table 4). Oxidative stress in sheep during pregnancy and parturition adversely affects fetal development, increases neonatal mortality, and decreases lamb birth weight (Safdar *et al.* 2020; Gonzalez-Rivas *et al.* 2023). The involvement of trace minerals, including selenium, zinc, copper, and manganese in reducing oxidative stress by enhancing antioxidant defense mechanisms is well established (Arthur and Beckett, 1989; Osredkar and Sustar, 2011; Alimohamady *et al.* 2019). For example, selenium-dependent glutathione peroxidase (GPx) is essential for neutralizing hydrogen peroxide and lipid hydroperoxides, thereby protecting cells from oxidative damage (Novoselec *et al.* 2022; Qiu *et al.* 2022). Management of oxidative stress through nutritional interventions, particularly supplementation with essential trace minerals, can significantly improve animal health and productivity during critical periods such as parturition (Zimmermann, 2006; Kuru *et al.* 2024). In the present study, ewes fed NTM diets had significantly higher blood MDA levels compared with the TM and CON groups. However, the addition of trace minerals did not significantly affect lipid peroxidation or MDA levels. GPx activity was significantly increased in the TM group. Although SOD and TAC were not affected by the treatments, SOD activity showed a non-significant increase in the TM group. Essential trace minerals such as zinc, copper, and manganese are crucial for SOD, while selenium is crucial for GPx; then, sufficient amounts of these minerals enhance protection against oxidative stress (Osredkar and Sustar, 2011; Alimohamady *et al.* 2019; Yaqoob *et al.* 2020). Imbalance of elements such as Fe, Cr, Cu, and Co results in the production of reactive radicals leading to oxidative stress (Osredkar and Sustar, 2011). In rats, MDA levels were elevated under copper-deficient conditions, while increasing copper intake reduced MDA levels (Mackenzie *et al.* 2011). Similar to our findings, Seifalinasab *et al.* (2022) reported that lambs fed a diet supplemented with 3 mg Cr/kg DM for 8 weeks had higher serum GPx levels than those fed an unsupplemented diet. Higher serum GPx levels were also observed with Se supplementation in sheep (Qiu *et al.* 2022). The increased Gpx activity and higher SOD levels in the TM group observed in the present study may be related to the beneficial effect of trace minerals on antioxidant activity (Novoselec *et al.* 2022). Also, Zhang *et al.* (2021) found that organic trace minerals increased TAC and SOD activities in weaned piglets. On the other hand, feeding inorganic selenium (sodium selenite) reduced blood MDA levels in ewes and lambs (Novoselec *et al.* 2022), reinforcing the protective role of selenium against oxidative stress.

CONCLUSION

Ghezel ewes experienced an increase in reproductive performance following strategic trace mineral supplementation during the flushing period. This supplementation appears to offer potential benefits for optimizing reproductive performance and improving certain blood biochemical parameters in ewes. In conclusion, the strategic timing and formulation of trace mineral supplementation represents a promising method for improving reproductive efficiency and health in sheep production systems.

ACKNOWLEDGEMENT

The authors would like to express their profound gratitude to the University of Tabriz for providing the necessary facilities to carry out this work. They would also like to acknowledge the financial support of Sodour Ahrar Shargh company for the present study.

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