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Does co-supplementation of zinc, manganese, and copper affect plasma testosterone, sperm quality, and anti-oxidant enzyme activities of ram?

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Abstract

Adding trace minerals to animal diets has a positive impact on reproductive performance. This study aimed to investigate whether supplementing zinc, manganese, and copper affects the testosterone level, sperm quality, and anti-oxidant enzyme activities in rams. Ten mature Afshari rams (2.50 years old; weighing 100 ± 3.29 kg) were fed a nutritionally adequate diet for 11 weeks, half of which receiving a sulfated zinc, manganese, and copper in their daily concentrates (supplemented group), while the other half served as a control group receiving no mineral in the concentrates. Ejaculate volume, as well as sperm concentration, motility, viability, morphology, and membrane integrity were assessed. From week five onwards, all parameters were significantly higher in the supplemented group. Additionally, the percentage of abnormal sperm was lower, anti-oxidant enzymes activities in the seminal plasma were improved, and plasma testosterone concentration was higher in the supplemented group compared to the control group. However, alkaline phosphatase activity was not significantly different. Furthermore, seminal plasma concentrations of copper, zinc, and manganese at the end of the study were higher in the supplemented group compared to pre-treatment levels, while these parameters were decreased in the control group. Overall, co-supplementing copper, zinc, and manganese in rams maintained the sperm quantity and quality, as well as seminal plasma anti-oxidant capacity, emphasizing the importance of studied elements in ram reproduction.

Introduction

Micro-nutrients are crucial for reproductive success in domestic animals. Minerals supplementation helps in maintaining more percentage of sperm membranes integrity and enhancing anti-oxidant activity in sperm cells.[1] Zinc, for instance, plays a vital role in sperm function and quality, and spermatogenesis.[2] Additionally, zinc is a component of the enzymes involved in arachidonic acid metabolism, affecting testosterone production.[3] Manganese has been shown to have anti-oxidant and protective effects against lipid peroxidation in various biological systems,[4] and its deficiency can inhibit enzymes required for sperm motility.[5] Moreover, copper deficiency can adversely affect sperm production and male infertility,[6] and it is also an integral part of the cytochrome oxidase enzyme being actively involved in the maintenance of spermatozoa motility.[3] In addition to their individual effects, zinc, manganese, and copper are all essential components of superoxide dismutase (SOD), an enzyme with potent anti-oxidant effects on sperm function.[7]

Trace mineral feeding has been shown to influence the functional structure of the sperm cells, semen quality, and fertility.[8] Sulfated forms of minerals are commonly used as supplements due to their high solubility and relatively low cost. They have an unstable molecular bond attached to the metal ion, conferring high solubility in aquatic and acidic solutions.[9] Diets may not contain sufficient amounts of specific minerals to meet the animal's requirements, minerals may not be in a biologically available form, and anti-nutritional factors may reduce the proportion of nutrients available in the diet.[10] As minerals are involved in structural, physiological, catalytic, reproductive, and enzymatic functions in animals, their inclusion in the animal diets as supplements is necessary for various reasons.[11]

The reports pertaining to the co-supplementation of zinc, copper, and manganese, and the effects on ram fertility are limited. Therefore, the aim of this study was to

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investigate whether co-supplementation of these three minerals affects sperm parameters and anti-oxidant enzyme activities in ram semen.

Materials and Methods

Location and rams. This study was carried out from September to November 2021 at the Animal Husbandry Unit of the Ferdows Agro-industrial Complex in Zanjan, Iran. The location had a latitude of 48°31'21"N, a longitude of 36°40'13"E, and an altitude of 1,663 meters. Animal welfare was given top priority throughout the experiment (Animal Care and Use Committee of the University of Tehran, Animal care and welfare were prioritized and ARRIVE guidelines were followed). Ten healthy, mature (2.5years old), and fertile Iranian Afshari breed rams, weighing 100 ± 3.29 kg, were randomly selected from the breeding flock and divided into two groups (n = 5). All rams underwent a standard breeding soundness examination[12] to ensure they were suitable for breeding. Both groups received a basal diet met their nutritional requirements according to the National Research Council [13] along with supplement daily for 11 weeks (Table 1). The rams were kept individually in pens and had free access to water. Before treating the rams, the first semen and blood samples were collected from all rams to assess testosterone concentration, and sperm and semen characteristics. After the initial sampling, half of the rams were randomly assigned into the supplemented group, received 49.00 g of a vitamin/mineral supplement in their concentrate feed daily, expected to fully meet the mineral and vitamin requirements according to the National Research Council.[13] The dietary supplement for the supplemented group contained zinc, copper, and manganese sulfate. The other half received the same supplement without the aforementioned elements (Table 1). The supplement was sprinkled on the basal diet.

Semen collection and evaluation. Before sampling, the rams were habituated for semen collection using an artificial vagina (IMV Technology, L'Aigle, France) for 2 weeks. Semen samples were collected individually with 2 weeks interval period from each ram for 6 weeks (total of six ejaculates *per* ram). The volume of each ejaculate was measured using a calibrated semen collection tube, and sperm concentration was determined using a Neubauer hemocytometer (HBG Company, Giessen, Germany). The hemocytometer is a device for counting cells and its surface has a grid etched into it (25 large squares in which each large square is divided into 16 smaller squares). For counting sperm cells, 25.00 µL semen was diluted with 5.00 mL water to kill sperms and then, sperm cells were counted in the five large squares. The motility characteristics of the sperms, including percentages of total and progressive motilities, velocity of curved line (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH), were analyzed using a computer-assisted semen analysis system named Sperm Class Analyzer (version 5.1; Microptic, Barcelona, Spain). The percentages of viable and abnormal sperms (primary sperm abnormalities) were evaluated using Eosin-Nigrosin staining and phase-contrast microscopy (Opika, Milan, Italy) at 400× magnification, with 200 sperms evaluated in each sample. Sperms without Eosin staining of the sperm head were deemed viable, whereas those with any Eosin staining were deemed dead.[14] Sperm membrane integrity was measured using a hypo-osmotic swelling test. For this, 500 µL of a 100mOsm hypo-osmotic solution was mixed with 50.00 µL of semen and incubated in an incubator at 37.00 ˚C for 30 min. One droplet of the incubated sample was placed on a pre-warmed slide, covered with a cover slip, and evaluated using phase-contrast microscopy at 400× magnification. Five microscopic fields (total of 200 sperms) were assessed to determine the percentage of intact sperms.[15]

Table 1. Ingredients and nutrient composition of the basal diet fed to rams.

Ingredients	Dry matter (%)
Alfalfa hay	47.60
Wheat straw	18.40
Barley grain (finely ground)	10.70
Corn (finely ground)	10.50
Soybean meal	3.00
Wheat bran	8.00
Supplement*	1.795
Nutrient composition	
Crude protein (%)	12.00
Metabolizable energy (Mcal kg^{-1} DM)	2.97
Neutral detergent fiber (%)	49.50
Calcium (%)	0.65
Phosphorus (%)	0.30
Zinc (ppm)	19.50
Manganese (ppm)	48.90
Copper (ppm)	5.00

*Content per kg: Vitamin A: 110,000 IU; Vitamin D3: 11,000 IU; Vitamin E: 750 mg; Copper: 500 mg; Zinc: 1,650 mg; Manganese: 1,000 mg; Iron: 2,000 mg; Selenium: 15.00 mg; Cobalt: 5.00 mg; Iodine: 25.00 mg.

Each ram received 49 g of supplement daily, expected to fully meet the mineral and vitamin requirements according to the National Research Council.[13] The dietary supplement for the supplemented group contained Zn, Cu, and Mn sulfate, whereas the dietary supplement for the control group did not contain Zn, Cu, and Mn.

Biochemical analyses. Biochemical analyses were performed on seminal plasma of each ram, being separated and stored at – 20.00 ˚C after centrifuging an aliquot of semen (0.50 mL) at 8,000 *g* for 30 min. Commercial kit (Navand Salamat, Urmia, Iran) was used to evaluate the SOD. Superoxide anions were determined by a chromogen solution, and the results were expressed as U mL^{-1} for 10^9 spermatozoa per mL. Catalase (CAT) activity was also

determined using commercial kit (Navand Salamat). The CAT activity is directly proportional to the conversion of H_2O_2 into the molecular oxygen and water. Briefly, the samples were incubated with a known concentration of H_2O_2, and the reaction was stopped by adding sodium azide after 1 min. Remaining H_2O_2 was determined by its reaction, and the results were expressed as nmol mL^{-1} for 10^9 spermatozoa per mL. Glutathione reductase (GR) activity was also assessed using a commercial kit (Navand Salamat). The activity of GR was determined by monitoring the reduction in absorbance at 340 nm, resulting from the oxidation of NADPH during the reduction of oxidized glutathione (Navand Salamat).[16] Seminal plasma concentrations of zinc, manganese, and copper were determined by inductively coupled plasma atomic emission spectroscopy (Varian 240, Agilent Technologies, Melbourne, Australia), after deproteinating 1.00 mL of seminal plasma by mixing with 9.50 mL of 1.00 N HNO_3.[17,18] Alkaline phosphatase activity was also evaluated using a commercial kit (Pars Azmoon, Karaj, Iran).[16]

Plasma testosterone concentrations. At the beginning and end of the trial, venous blood samples were collected *via* jugular venipuncture into heparinized vacuum tubes. The blood samples were centrifuged at 1,000 *g* for 20 min, and plasma was separated and stored at – 20.00 ˚C. Plasma testosterone concentration was determined using a commercial enzyme-linked immunosorbent assay kit (Pars Peyvand, Tehran, Iran) with an intra-assay coefficient of variation (CV) of 2.90 %, inter-assay coefficient of variation of 4.50%, and sensitivity of 0.06 ng mL^{-1}.[19]

Statistical analyses. The Proc Mixed was used to analyze the repeated measures data.[20] Least-squares means were calculated and tested for differences using the Tukey's test (version 9.1, SAS Institute, Cary, USA). Results were considered significant at $p < 0.05$ and presented as means ± standard error of the mean.

Results

Semen. In the control group, the volume of ejaculate began to decrease after week five and reached its lowest point at the end of the study. In contrast, the supplemented group had a relatively stable ejaculate volume being significantly higher than the control group from weeks seven to 11 (Fig. 1). Sperm concentration and total sperm *per* ejaculate in the control group were significantly lower than the supplemented group from week five onwards, while sperm concentration in the supplemented group remained relatively stable (Fig. 1). Both total and progressive sperm motilities started to decrease after week five in the control group, but remained relatively stable in the supplemented group, with a significant difference between two groups from week five onwards (Fig. 2).

Other sperm motility characteristics, such as VCL (µm per sec), VSL (µm per sec), VAP (µm per sec), and ALH (µm), began to decrease from week five in the control group and were lower ($p < 0.05$) than the supplemented group from week seven onwards (Fig. 3).

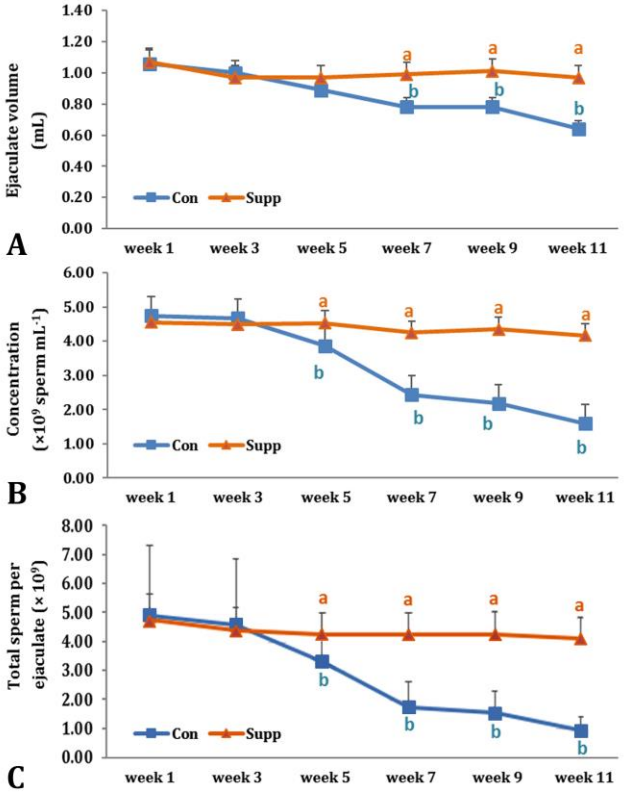

Fig. 1. Ejaculate and sperm parameters in the control (Con) and supplemented (Supp) rams.
[ab] For each characteristic and week, means without a common superscript differ significantly ($p < 0.05$).

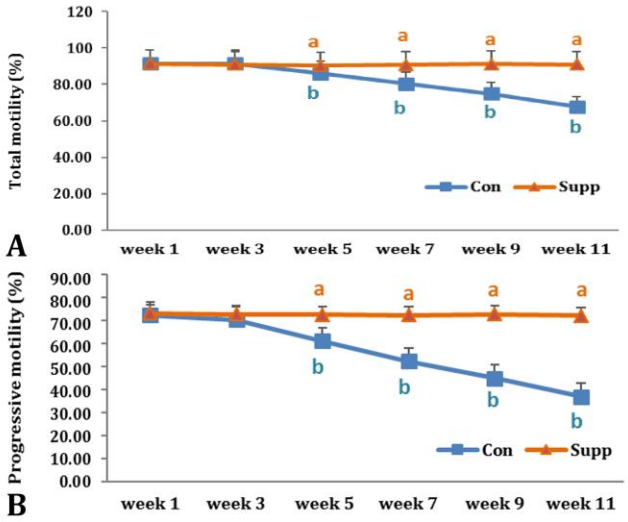

Fig. 2. A) Total motility and **B)** progressive motility in the control (Con) and supplemented (Supp) rams.
[ab] For each characteristic and week, means without a common superscript differ significantly ($p < 0.05$).

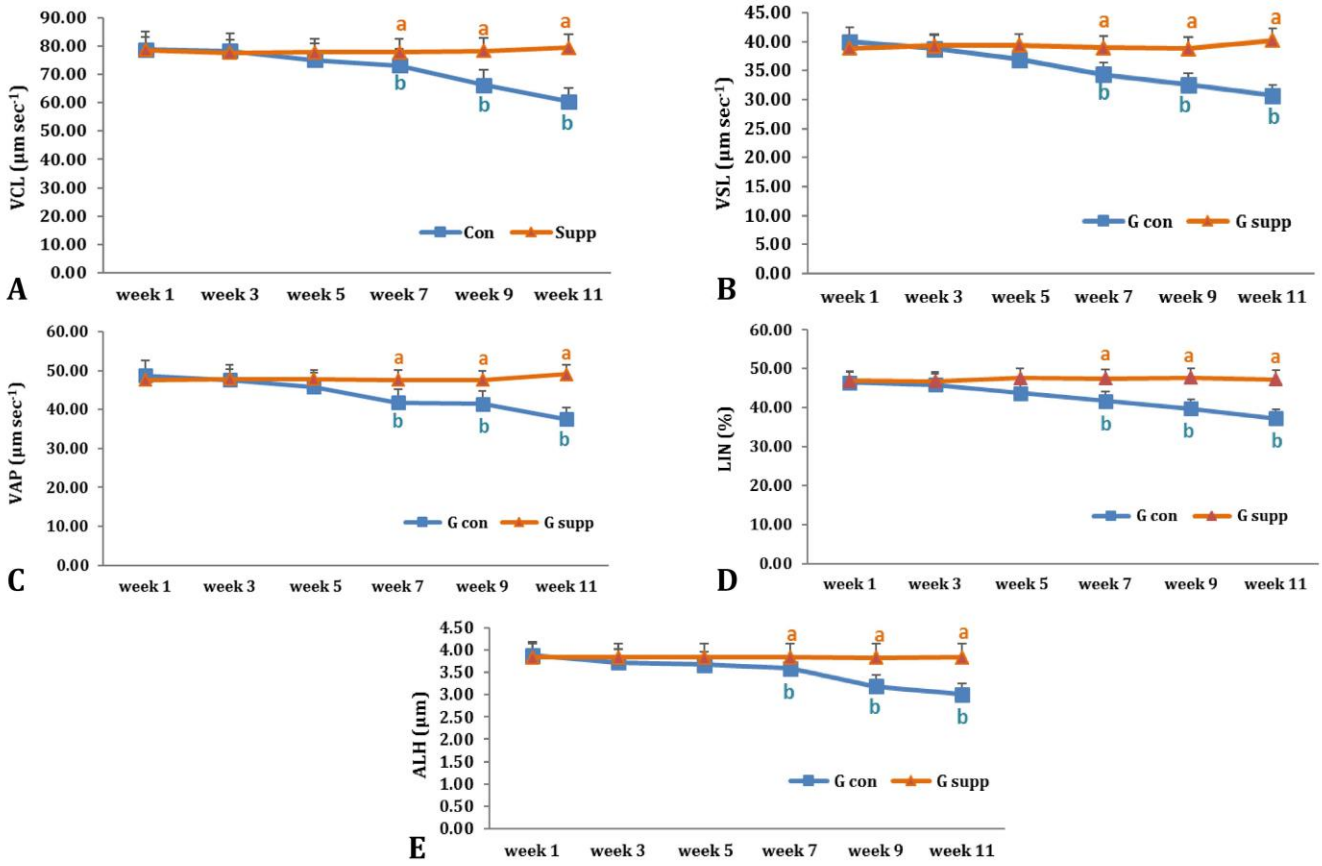

Fig. 3. A) Sperm track velocity (VCL), **B)** progressive velocity (VSL), **C)** path velocity (VAP), **D)** linearity (LIN), and **E)** lateral amplitude (ALH) in the control (Con) and supplemented (Supp) rams.
[ab] For each characteristic and week, means without a common superscript differ significantly ($p < 0.05$).

Sperm viability and membrane integrity in the control group began to decrease from week five and reached their lowest level at the end of the trial ($p < 0.05$; Fig. 4). Additionally, percentages of viable sperm and sperm membrane integrity were significantly higher in the supplemented group from week 5 to 11 and remained constant ($p < 0.05$). Morphologically abnormal sperms in the control group began to increase from week 5 and were higher ($p < 0.05$) than the supplemented group until week 11 (Fig. 4). However, in the supplemented group, morphologically abnormal sperms increased only slightly from week five to the end of the experiment.

Biochemical properties of seminal plasma. The activities of CAT, GR, and SOD were found to be lower ($p < 0.05$) in control group compared to the supplemented group of rams (Table 2). Alkaline phosphatase activity was not significantly different ($p > 0.05$). However, the anti-oxidant capacity in the seminal plasma of the supplemented group remained sustained until the end of the experiment. Also, in week 11, the concentrations of copper, zinc, and manganese in the seminal plasma had significantly increased ($p < 0.05$) compared to the pre-treatment levels; however, these concentrations had significantly decreased in control group ($p < 0.05$; Table 2).

Table 2. Enzyme activities and mineral concentrations in seminal plasma, and plasma testosterone concentration in the control and supplemented rams.

Parameters	Week 1		Week 11		*p*-value		
	Control	Supplemented	Control	Supplemented	Treatment	Time	Treatment × time
Alkaline phosphatase (IU L^{-1})	289.08 ± 10.57	291.49 ± 11.14	287.43 ± 7.55	294.84 ± 11.04	0.249	0.320	0.208
Catalase (nmol mL^{-1})	28.65 ± 0.10^{a}	28.59 ± 0.31^{a}	26.33 ± 0.09^{b}	28.50 ± 0.30^{a}	0.004	0.001	0.001
Glutathione reductase (U mL^{-1})	1.28 ± 0.00^{a}	1.28 ± 0.02^{a}	1.20 ± 0.00^{b}	1.27 ± 0.02^{a}	0.015	0.002	0.005
Superoxide dismutase (U mL^{-1})	255.14 ± 0.45^{a}	255.16 ± 0.51^{a}	241.52 ± 0.52^{b}	255.41 ± 0.57^{a}	0.024	0.420	0.021
Copper (mg L^{-1})	1.23 ± 0.01^{b}	1.25 ± 0.03^{b}	1.12 ± 0.01^{c}	1.42 ± 0.02^{a}	0.040	0.009	0.008
Zinc (mg L^{-1})	1.20 ± 0.03^{b}	1.20 ± 0.02^{b}	1.02 ± 0.02^{c}	1.40 ± 0.02^{a}	0.006	0.002	0.003
Manganese (mg L^{-1})	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.02 ± 0.01^{c}	0.08 ± 0.00^{a}	0.079	0.040	0.040
Plasma testosterone (ng mL^{-1})	15.71 ± 1.05^{a}	14.71 ± 0.49^{a}	11.92 ± 0.17^{b}	14.69 ± 0.53^{a}	0.094	0.032	0.033

[ab] Within rows, mean values with common letter are not significantly different ($p < 0.05$).

Plasma testosterone concentrations. In the control group, plasma testosterone concentrations were found to be lower ($p < 0.05$) in week 11 compared to week one. Conversely, there was no significant change in the supplemented group (Table 2).

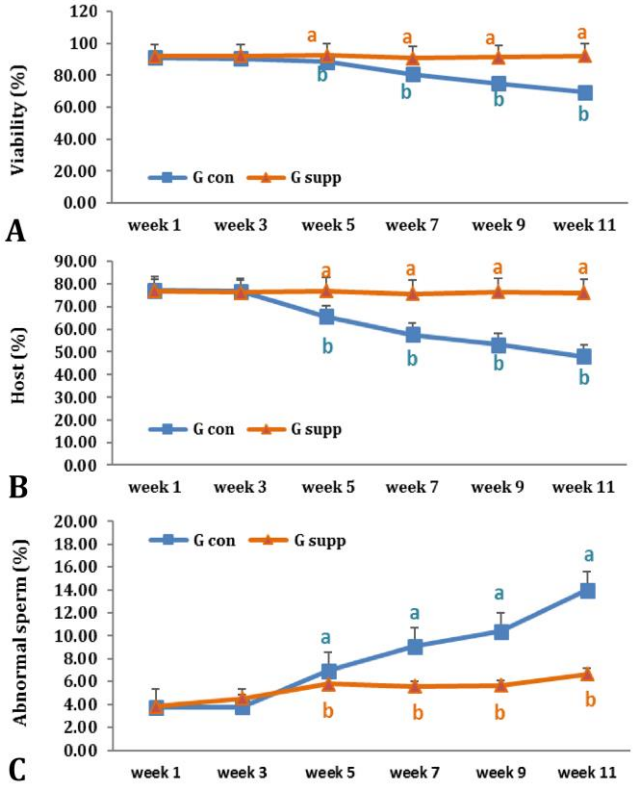

Fig. 4. Sperm viability, hypo-osmotic swelling test (HOST), and abnormality in the control (Con) and supplemented (Supp) rams. [ab] For each characteristic and week, means without a common superscript differ significantly ($p < 0.05$).

Discussion

In this study, ejaculate volume, as well as sperm concentration, motility, morphology, viability, and membrane integrity in rams being fed diets with or without copper, manganese, and zinc were evaluated. The supplemented group maintained consistent semen and sperm characteristics throughout the study, while the control group experienced a decline from week 5 onwards. The supplemented group had significantly higher concentrations of zinc, manganese, and copper in the seminal plasma at the end of the experiment, and mineral supplementation helped to maintain anti-oxidant capacity in the seminal plasma, and blood testosterone concentrations.

The control group's basal feed ingredients only provided 19.50, 5.00, and 48.90 ppm of the zinc, copper, and manganese requirements, respectively. As a result, the animals in this group were more susceptible to deficiencies caused by interactions between these elements. Additionally, basal diets may not contain sufficient amounts of trace minerals to meet an animal's needs, and anti-nutritional factors can reduce the availability of dietary nutrients. The competing interests of elements for binding sites on enzymes, such as zinc and copper, can also compromise their availability for the animal. Therefore, it is important to exercise caution when supplementing excess zinc without copper to avoid compromising an animal's copper status.[21]

Interactions between molybdenum and sulfur can affect the availability of copper for absorption, as they can form rumen thiomolybdates making copper un-available.[11,22] This suggests that the basal diet of the control group may not have provided sufficient copper, and that co-supplementation of zinc, copper, and manganese in the supplemented group may have prevented antagonistic effects of molybdenum, zinc, and sulfur on copper. This may explain why trace elements, like copper, zinc, and manganese are often added at levels above requirements in ruminants.[23] As copper and zinc are crucial for livestock reproduction,[24] the decrease in sperm characteristics observed in the control group may be related to the removal of these elements. Sub-optimal concentrations of trace minerals can affect sperm production, maturation, and fertilizing capacity.[25]

From the 5th week onwards, the control group had significantly lower ejaculate volume, sperm concentration, and total sperm *per* ejaculate compared to the supplemented group. In contrast, these endpoints remained relatively stable in the supplemented group. This study, supplemented manganese, zinc, and copper simultaneously, and did not investigate the effects of individual minerals. However, other studies have reported positive effects on sperm characteristics in farm animals following supplementation with one or more of these minerals.[26,27]

The supplemented group maintained significantly higher total and progressive sperm motilities, sperm viability, and sperm membrane integrity percentage compared to the control group. This may be due to the protective role of zinc, copper, and manganese against oxidative damage.[28,29] Zinc also plays a role in stabilizing ribosomes, lysosomes, DNA, and RNA, contributing to the sperm survival and normal function.[30] Zinc deficiency can reduce reproductive performance,[31] being consistent with the results observed in the control group at the present study. Copper has a positive correlation with ejaculate volume and sperm motility.[28,32] Therefore, positive effects of mineral co-supplementation on characteristics of ram semen in our study are consistent with previous studies.

In animals, multiple factors contribute to reproductive function, such as genetics, environment, and nutrition.[33] The important factor affecting reproductive success is trace mineral nutrition, because small fluctuations in the

concentration of trace minerals can have a significant impact on reproduction.[34] Manganese, zinc, and copper have considerable effects on reproduction.[33] According to these reasons, the parameters reduction in the control group can be explained.

In rams received mineral supplementation, there was a slight increase in morphologically abnormal sperms from week five until the end of the experiment, but this change was not significant. This finding is consistent with other studies that have linked higher concentrations of zinc, copper, and manganese in seminal plasma with normal sperm morphology. [35-37] The increases in manganese, zinc, and copper concentrations in seminal plasma observed in the supplemented group, along with stabilized sperm concentration, total motility, morphological abnormalities, and viability, are in agreement with previous studies conducted on goats and rams.[8,38] These results suggest that adding trace elements, such as zinc, copper, and manganese to the diet may prevent deficiencies in rams.[23]

Since zinc, copper, and manganese are structural components of SOD,[39,40] it is possible that higher availability of these elements may prevent the decrease in SOD activity.[41] Additionally, manganese is essential for the activity of CAT.[18] Therefore, the reduction in anti-oxidant capacity observed in the control group may be related to the absence of these elements.

The results of this study indicated that co-supplementation of zinc, copper, and manganese prevented the decrease in plasma testosterone concentration. Deficiencies in zinc, copper, and manganese may disrupt steroid synthesis pathways,[31] affect smooth endoplasmic reticulum in Leydig cells (the site of testosterone synthesis),[28] or cause a malfunction of the luteinizing hormone receptor control mechanism, and storage and release of testosterone.[42] Thus, an existing association between deficiencies in the above elements and lower plasma concentrations of testosterone in control rams is plausible.

Several studies have reported positive effects of mineral supplementation on the reproductive performance,[18,43] while a few reports have shown either no effect or an undesirable effect.[44] The results of this study reinforced the positive effects of dietary co-supplementation of zinc, manganese, and copper on ram semen characteristics.

To sum up, providing a combination of zinc, manganese, and copper supplements resulted in a notable increase in the levels of these minerals in the seminal plasma of rams. Furthermore, it led to enhancements in the sperm quality and quantity, as well as improvements in the biochemical properties of the semen. Future research could explore the role of this supplementation in sperm cryopreservation or other reproductive traits of rams.

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Conflict of interest

There is no conflict of interest.

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