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Cryoprotective role of organic Zn and Cu supplementation in goats *(Capra hircus)* diet

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ABSTRACT

The current study focused on cryopreservation and assessment of characters of post-thaw semen of indigenous Osmanabadi bucks maintained with standard diet, supplemented with different concentrations of organic zinc (Zn), copper (Cu) or in combination, for a period of 180 days. The different doses of organic Zn and Cu were fed per kg DM basis, Zn groups (low: Zn20, medium: Zn40 and high: Zn60), Cu groups: (low: Cu12.5, medium: Cu25 and high: Cu37.5) and combination of Zn + Cu groups (low: Zn20 + Cu12.5, medium: Zn40 + Cu25 and high: Zn60 + Cu37.5) respectively. The control group bucks were maintained mainly on the basal diet without any additional mineral supplementation. Two hundred and forty (240) semen samples were collected from 40 bucks aged 11 months, through electro ejaculator method, processed and analysed for sperm quality parameters both at pre freeze and post-thaw stage. The semen samples were diluted in Tris egg yolk extender, cooled and equilibrated for 4 h at 5 °C, cryopreserved using programmable freezer (PLANER Kryo 360–1.7) and stored at −196 °C. The organic trace minerals (Zn, Cu and Zn + Cu) protected the spermatozoa against the cryoinjury and maintained higher post-thaw semen parameters except in high Zn group. Additional feeding of organic Cu and Zn to bucks had a protective role and resulted in higher sperm liveability, plasma membrane and acrosome integrities, motility and velocity and reduced oxidative stress in supplemented goats ($P < 0.05$).

1. Introduction

Organic mineral supplementations are known to improve semen production, sperm motility, male fertility through efficient utilization and absorption in the body to maintain optimum reproductive function [32,54,1]. Among the trace minerals, Copper (Cu) and Zinc (Zn) in the diets of animals have more influence in livestock production [5,29,41,55]. The requirements of Zn for testicular growth and spermatogenesis are greater than body growth and appetite [15,66]. Supplementation of Zn to animals is reported to improve the normal sperm cells percentage with more intact sperm membranes and antioxidative properties [5,27,66]. Cu deficiency is responsible for reduced libido or male infertility [21,22,42,62,67,70].

The wider application and potential use of frozen semen in goats can allow one to store gametes of superior bucks. Artificial insemination (AI) with frozen semen is known to result in low conception rate due to cryodamage to cells, reduction in morphological and physical properties of the spermatozoa due to lethal damages occurring during the process of cryopreservation [10,18,44,61,68]. Different *in vivo* approaches are being made to improve the semen quality prior to ejaculation, like supplementation of trace minerals and by enhancing the protein and energy content of the diets [28,32]. Incorporation of additives, like amino acids, seminal plasma proteins, antioxidants [6,7,19,57,71] at the time of semen dilution in fresh or frozen semen has also been tried to enhance the quality of frozen spermatozoa with more intact sperm plasma/acrosome membrane after cryopreservation. The basic reason behind these approaches is to either improve the spermatogenesis quality or to minimize the cryoinjury and or cryodamage to sperm cells [30,52,56]. The type of semen extender, cryoprotectants and their properties, duration and type of equilibration, freezing protocol and thawing are all equally important for successful cryopreservation of goat semen [31]. The goat semen contains high levels of lipases, which react with semen diluents like egg yolk and milk. Because of this unique composition, the buck semen needs more attention during freezing to obtain maximum post-thaw quality [45,50,53]. Irrespective of the method adopted it should protect the

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sperm cells against cold shock at equilibration time as well as during freezing.

Several methods are standardized to assess the semen quality both *in vitro* and *in vivo* for various species of livestock [6,8,24]. However, the routine and traditional methods have certain limitations for accurate prediction of sperm quality. Recent literature indicates that the semen quality or the fertility outcome can be predicted more accurately by using molecular biology tools like next generation sequencing of sperm DNA, seminal plasma proteins, and developmentally regulated genes and/or sperm mRNAs [7,25,26,47]. Acrosome intactness, plasma membrane integrity and sperm cell viability can directly predict the fertilizing potential, and additional feeding of trace minerals to animals have been reported to maintain the functional structure of the sperm cells [15,21]. The effect of trace minerals supplementation on sperm cells during semen equilibration, cryopreservation and post-thaw semen quality are not reported for goats and other livestock species. Therefore, in the present study we focused on the protective role of trace mineral on semen samples during cooling cum equilibration, freezing, and to assess the changes in post-thaw seminal characteristics in bucks.

2. Materials and methods

2.1. Mineral supplementation and feeding management

The institutional animal ethics committee (IAEC) of ICAR- National Institute of Animal Nutrition and Physiology, Bengaluru approved the experimental protocol. The present study was carried out to test the effect of trace minerals supplementation on semen cryopreservation.

Forty indigenous bucks (n = 40) of Osmanabadi breed, aged 11 months were used for the present experiments with four numbers of bucks randomly assigned to each of ten groups. A basal diet comprising of roughage (50%) and concentrate mixture (50%) (Table 1) was prepared and fed to the goats to meet their nutrient requirement (ICAR, 1998). Different doses (mg/kg DM) of organic Zn and Cu (Bioplex Zn proteinate and Cu proteinate, procured from ALTECH Inc., Kentucky, USA) were fed to the experimental bucks as per the individual body weight basis. Three levels of Zn: (low: Zn20, medium: Zn40 and high: Zn60), three levels of Cu: (low: Cu12.5, medium: Cu25 and high: Cu37.5) and different combinations of Zn and Cu: (low: Zn20 + Cu12.5, medium: Zn40 + Cu25 and high: Zn60 + Cu37.5), were additionally supplemented to goats from the group Zn20 to Zn60 + Cu37.5, and the control group was maintained only with basal diet without additional mineral supplementation. The concentrate mixture contained 44% maize, 17% ground nut cake (GNC), 20% wheat bran, 16% soya bean meal (SBM), 2% mineral mixture and 1% salt.

2.2. Semen collection, dilution, cooling and equilibration

After 4 months of mineral supplementation, the semen samples were collected twice in a week with three days interval period from each buck for 2–3 months. The minerals were supplemented during the semen collection period also. Two hundred and forty (240) fresh semen samples (> 60% sperm motility) were processed from a sample size of 400 for cryopreservation. The selected samples were diluted in Tris egg yolk buffer as described earlier [49] with slight modifications. The composition of the modified Tris egg yolk buffer was, Tris 0.29M, Citrate 0.1M, Fructose 0.11M, Streptomycin 100mg/100 ml, Pencillin 1 lakh IU/100 ml, Egg yolk 15% and Glycerol 6%. We used egg yolk @ 15% instead of 10 or 20% as described earlier [49]. The diluted samples were adjusted to maintain a final concentration of 100 million spermatozoa/ml of semen. The samples were then kept in semen cold handling apparatus and gradually the temperature was lowered to 5 °C (2hr) and equilibrated for 2 h at 5 °C. After completion of equilibration, semen samples were loaded into 0.25 ml French mini straws and sealed with polyvinyl alcohol powder. Finally the equilibrated semen straws were cryopreserved using controlled rate programmable freezer (PLANER Kryo 360–1.7) and stored at −196 °C till further use.

Table 1
Chemical composition of Concentrate and Ragi straw.

Component	Concentrate mixture	Ragi straw
Dry matter (g/kg)	936.1	940.1
Organic matter (g/kg)	929.2	894.6
Total ash (g/kg)	70.8	105.4
Ether extract (g/kg)	9.3	10.5
Crude protein (g/kg)	212.5	63.4
NDF (g/kg)	569.1	657.8
ADF (g/kg)	86.6	386.6
ADL (g/kg)	20.1	48.8
Zinc (mg/kg)	21.58	10.72
Copper (mg/kg)	24.93	10.21

2.3. Semen cryopreservation

With the two cooling rate protocols followed as described earlier for buck semen cryopreservation [35,59], we could not get sufficient post-thaw motility and consistency in the semen samples. Therefore, we standardized a cooling rate protocol that had been developed and used for stallion semen cryopreservation [9,14] and compared with the earlier methods [35,59]. For this standardization study, twenty four semen ejaculates (8 × 3 = 24) were collected from 8 bucks through electro ejaculator method, and were analysed for initial quality, diluted in Tris egg yolk buffer, cooled, equilibrated and cryopreserved as described in the previous paragraph. The cooling rate protocols adopted for standardization is as follows:

Protocol 1: The straws containing the diluted semen samples were cooled from 37 °C (straws were kept at 37 °C water in plastic box) to 5 °C for 2 h, and then equilibrated at 5 °C for 2 h. Then the equilibrated semen straws samples were loaded into programmable freezer and held at 5 °C for 5 min. Cooling rate adopted for the study ranged from +5 to −15 °C @ −10 °C/min, and then from −15 to −100 °C @ −19 °C/min, then the straws were held for 10 min at −100 °C and directly plunged in to liquid nitrogen (LN2) and stored at −196 °C [9,14].

Protocol 2: In this protocol the steps involved in the standardization process were similar to protocol 1 up to the equilibration step. The cooling rate adopted for the standardization of the second protocol ranged from 5 °C to −20 °C at a rate of 5 °C/min and from −20 °C to −100 °C the rate of cooling was 20 °C/min and after that the straws were plunged into liquid nitrogen (−196 °C) for storage as per Kundu et al. [35].

Protocol 3: In the third protocol, the steps followed during the standardization process were similar to both protocol 1 and protocol 2 up to the equilibration step. Thereafter, the cooling rate was increased from 5 °C to −160 °C at 25 °C/min and plunged into liquid nitrogen (−196 °C) for storage as per Sharma et al. [59]. The frozen semen samples were thawed and analysed with CASA. Among the three protocols tried for freezing, protocol 1 gave significantly ($P < 0.01$) better post-thaw semen characters in comparison to protocols 2 and protocol 3. Thus for our study, we adopted protocol 1 for cryopreservation of selected semen samples.

2.4. Thawing of semen

The frozen straw samples were removed from the LN2 container and placed in a water bath (37 °C) for 45 s and then evaluated for post-thaw semen characteristics.

2.5. Semen evaluation

The semen samples were subjected to pre freeze and post-thaw