



## Effect of azolla supplementation in feed on semen freezability in bucks

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### ABSTRACT

The present study was conducted to evaluate the effect of azolla supplementation on semen freezability in Barbari bucks. Ten adult Barbari bucks (2–4 years old) were selected, and divided into control and treatment group (5 bucks in each group) as per completely randomized design. Bucks of control group were fed with 400 g concentrate pellet/day along with 6–7 h grazing while bucks of treatment group were supplemented with 100 g fresh azolla along with 400 g concentrate pellet and 6–7 h grazing. Semen ejaculates (8) were collected from each buck of control and treatment groups after 60 days of azolla supplementation. Immediately after collection, semen samples were evaluated for colour, volume, density, mass motility, progressive motility, live sperm count, acrosomal integrity and hypo-osmotic swelling positive spermatozoa. Then semen samples were diluted with tris–citric acid fructose diluents having 6% (v/v) glycerol as cryoprotective agent and 10% (v/v) egg yolk. After equilibration, semen samples were filled in straws and vapour frozen for 10 min 4 cm above the liquid nitrogen and finally stored into liquid nitrogen container. Post thaw motility, live sperm count, abnormalities, acrosomal integrity and hypo-osmotic swelling test were conducted to check the effect of azolla feeding on the freezability. Post thaw motility, live sperm count, acrosomal integrity and hypo-osmotic swelling positive spermatozoa were significantly higher in treatment group. It could be concluded that dietary azolla supplementation to breeding bucks improved the quality and freezability of semen favouring the use in artificial insemination program.

**Key words:** Azolla, Buck semen, Freezability, Libido, Semen cryopreservation

In India, goats are mainly reared on grazing and all the required nutrients are met by grazing alone. Supplementation of nutrients is rare because of cost involved as the goats are reared by people mainly from marginal and poor economic status. Due to urbanization, increase of human population and decrease of grazing land, goat keepers are trying to get low cost nutrient supplements. Moreover, breeding bucks require more attention in feeding of balanced nutrients to maintain semen quality for better fertility. Breeding bucks are used for natural mating and their semen samples are also preserved for artificial insemination. During cryopreservation process, sperm undergo various stresses leading to cryoinjuries and reduction in their fertilization ability. Incorporation of additives at the time of semen dilution has also been tried to enhance the quality of frozen spermatozoa with more intact sperm plasma/acrosome membrane after cryopreservation (Isaac *et al.* 2017). The basic reason behind

these approaches is to either improve the spermatogenesis quality or to minimize the cryodamage to sperm cells (Manohar *et al.* 2018). Different *in vivo* approaches are also 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 (Arangasamy *et al.* 2018a). Azolla is a small fern that can easily be grown with low cost of investment in stagnant water of river, canal and artificial pond or any water bodies. It has been reported to be good source of micro minerals required for reproduction like zinc and copper along with other beneficial nutrients (Kumar *et al.* 2015a). Zinc (Zn) plays a pivotal role in male reproduction like testicular steroidogenesis, androgen metabolism and interaction with steroid receptors and its requirement for testicular growth is greater than body growth and appetite (Bedwal and Bahuguna 1994). Copper (Cu) deficiency is responsible for reduced libido or male infertility (Arangasamy *et al.* 2018b). Moreover, Azolla is an accumulator of heavy metals, vitamin A, beta-carotene and encompasses all macro and micro elements accountable for animal growth, production and are required in spermatogenesis (Srinivas *et al.* 2012). Therefore, the present study was conducted to investigate the effect of azolla supplementation on semen freezability in Barbari bucks.

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## MATERIALS AND METHODS

*Location of study:* The present study was conducted on 10 Barbari bucks aged between 2 and 4 years, weighing 25–35 kg, reared at experimental shed of Animal Nutrition and Product Technology Division of the institute.

*Management of animals:* The experimental animals were raised under semi intensive system of management. Bucks were fed with 400 g concentrate pellet (having 17.5% CP) and 6–7 h grazing on anjana (*Cenchrus ciliaris*) grass pasture to meet their nutrient requirements. The animals were dewormed regularly for internal parasites. There was free access of clean drinking water to animals.

*Experimental design:* The bucks were divided into two groups with 5 animals each. Bucks of control group were fed with 400 g concentrate pellet/day along with 6–7 h grazing while bucks of treatment group were supplemented with 100 g fresh azolla along with 400 g concentrate pellet and 6–7 h grazing. After 60 days of azolla supplementation, animals were subjected to semen collection. From each buck of both the groups, eight ejaculates were collected for study. Semen samples were evaluated at fresh and post thaw stage, simultaneously semen quality was also assessed at 0, 15 and 30 min post thaw incubation time.

*Semen collection and evaluation:* Semen ejaculates from each buck were collected twice at weekly intervals with the help of artificial vagina in the morning hours. Semen samples were evaluated immediately after collection for colour, consistency, mass motility, pH and concentration. Immediately after collection, semen was maintained in hot water bath at 37°C and subjected to evaluation. Volume of each ejaculate was recorded with the graduated collection cup. Mass motility was estimated at low power magnification (10×) using a compound microscope with neat semen on thermo stage maintained at 37°C. Semen samples having mass motility more than 3 were diluted and after dilution evaluated for progressive motility, live/dead count, abnormalities, acrosomal integrity and hypo-osmotic swelling test.

*Semen diluter and dilution:* The semen samples were diluted with tris–citric acid fructose diluents (tris, 3.604 g; citric acid, 1.702 g; fructose, 1 g; streptomycin, 100 mg; penicillin, 100,000 IU; triple distilled water, 100 ml; pH, 6.8–6.9) having 6% (v/v) glycerol as cryoprotective agent and 10% (v/v) egg yolk (Gangwar *et al.* 2015). The semen samples were extended to maintain sperm concentration approximately 100–120 million/straw.

*Semen cryopreservation:* Semen samples were equilibrated for 4 h at 5°C in cold handling cabinet. Filling of straws was done at 5°C in cold handling cabinet and sealing of straws was done with polyvinyl alcohol powder. After filling and sealing, straws were vapour frozen for 10 min above 4 cm of liquid nitrogen and finally stored into liquid nitrogen container. Post-thaw motility, live sperm count, abnormalities, acrosomal integrity and hypo-osmotic swelling test were conducted to check the effect of azolla feeding on the freezability.

*Evaluation of progressive motility:* Diluted semen (10 µl) was placed on a clean grease free pre warmed slide (37°C) with cover slip and observed under 40× of phase contrast microscope for assessing the progressive motility. The average values of two experts were considered for calculating the progressive motility as described by Gangwar *et al.* (2014).

*Live and dead sperm count:* This was estimated as per standard staining procedure described by Hancock (1951).

*Acrosomal integrity:* Giemsa stain was used to assess the acrosomal integrity of frozen thawed buck spermatozoa as per Watson (1975).

*Plasma membrane integrity:* Hypo-osmotic swelling test was carried out as described by Jayendran *et al.* (1984).

*Statistical analysis:* The data collected during study were analyzed by independent sample t-test as per Snedecor and Cochran (1989) according to a complete randomized design using statistical software package (SPSS version 20). Individual animals were considered as experimental units. The difference between means was significant at 95% level of significance ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

In the present study, the effects of dietary supplementation of azolla on buck semen was evaluated in terms of fresh and post-thaw semen quality. Findings of the experiment indicate (Table 1) that progressive motility and percentage of hypo-osmotic swelling positive spermatozoa increased significantly ( $P < 0.05$ ) after dietary supplementation of azolla in breeding bucks. Also the libido increased as compared to control group. Libido was measured in terms of reaction time and reaction time was reduced significantly after azolla supplementation (Fig. 1). Semen volume/ejaculate also increased significantly in treatment group as compared to control group. The percentage of motile spermatozoa, live and dead spermatozoa, hypo-osmotic swelled spermatozoa and acrosomal integrity for frozen semen of both the groups are summarized in Table 1. After azolla supplementation, semen freezability and post thaw semen quality were also improved. Higher percentage of progressively motile spermatozoa, live spermatozoa, and spermatozoa with intact plasma membrane after post thaw incubation for longer duration were observed after azolla supplementation in treatment group of goats.

The effect of azolla supplementation on semen quality as well as freezability of buck semen had not yet been established. Present experiment indicated the beneficial effect of azolla supplementation in the bucks on semen pre- and post thaw quality. The improvements in semen quality might be due to the different macro and micro minerals, vitamins and amino acids present in the azolla responsible for spermatogenesis. Mandal *et al.* (2012) pointed out that azolla contains all important amino acids, carotene, macro and micro minerals which are required in spermatogenesis and steroidogenesis, and these may be helpful in improving semen quality. Kumar *et al.* (2015a) reported that azolla

Table 1. Effect of dietary azolla supplementation on freezability of Barbari buck semen at post thaw stage

Parameter	Control group	Treatment group
Progressive motility (%)	37.78±0.90 <sup>b</sup>	51.25±0.78 <sup>a</sup>
Live (%)	70.52±1.03 <sup>b</sup>	77.39±0.76 <sup>a</sup>
Dead (%)	30.03±0.99 <sup>a</sup>	22.61±0.76 <sup>b</sup>
Acrosomal integrity (%)	79.81±0.69	81.08±0.64
HOS test (%)	43.50±1.09 <sup>b</sup>	52.17±0.98 <sup>a</sup>

Data are presented as the mean±SEM (n=40). <sup>a,b</sup>Different superscripts indicate significant differences between groups for each parameter at 5% level (P<0.05).

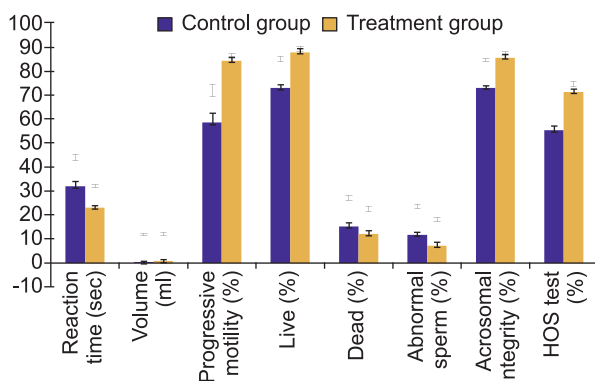


Fig 1. Effect of dietary azolla supplementation on fresh semen in Barbari bucks.

contained 0.60% sodium, 0.73% potassium, 0.11% calcium, 16.12 ppm copper and 71.47 ppm zinc which indicate azolla a good source of macro as well as micro minerals. It is scientifically established that zinc is essential in normal growth, development and reproduction. It has diverse function in male reproductive system such as testicular development and steroidogenesis (Issac *et al.* 2017). In agreement with these findings, Arangasamy *et al.* (2018a) recommended that Zn supplementation either in the inorganic or organic form in the feed of bucks improved the seminal attributes qualitatively and quantitatively. They also reported that goat semen samples supplemented with organic Zn and Cu maintained significantly higher live spermatozoa percentage (P<0.01) as compared to control group. Findings of the present study indicated that there is significant increase in the progressive motility, sperm

viability and plasma membrane integrity at 30 min post thaw incubation time in azolla supplemented group (Table 2). The enhancement in liveability percentage and plasma membrane integrity may be due to protective role against oxidative damages, and membrane stabilizing action of zinc and thereby influences the fluidity of lipids, which in turn protects the sperm membrane during cooling and freezing. This ultimately translates into better post thaw semen parameters after dietary azolla supplementation.

Libido was improved in azolla supplemented group due to the presence of high amount of Zn and Cu in azolla which is responsible for improved steroidogenesis. Low Cu levels in the diets are responsible for reduction in reproductive efficiency; these may be due to modification of enzyme systems affected by Cu deficiency and may impede libido, male fertility and even lead to infertility. Kumar *et al.* (2006) and Arangasamy *et al.* (2018b) also reported that testosterone concentration in blood serum was significantly higher in animals of Zn and Cu treated groups as compared to control group. The present results indicate that there is significant reduction in sperm abnormalities in treatment group as compared to control group. The reason behind this reduction may be the improvement at spermatogenesis level or various nutrient present in azolla provide healthy environment at germinal epithelial level. Recently, Manohar *et al.* (2018) reported that organic zinc and copper supplementation have antioxidant protective effect and it enhances sperm functional characteristics in goats. There was a pronounced influence of trace minerals on the cleavage rate, sperm-zonapellucida binding ability and the fertility related properties in goats (Hemalatha *et al.* 2018). An augmentation in the serum Cu and Zn levels can be achieved by their dietary supplementation which ultimately translates into improvement in overall hormone production, semen quality at different stages such as fresh semen, equilibrated and post thawed semen (Arangasamy *et al.* 2018a, b). The vitality of Zn and Cu has been reported for antioxidant enzymes activities such as SOD, CAT, etc. which in turn prevent the oxidative cellular damage to the sperms and thereby improves the fertility (Manohar *et al.* 2018). These findings further potentiate the present results that azolla supplementation improves the semen freezability and post thaw survivability of sperms for longer duration. This improvement may be due the reduction in cold shock

Table 2. Effect of dietary azolla supplementation on semen quality of Barbari bucks at various post thaw incubation time.

Parameter	Control group			Treatment group		
	0 min	15 min	30 min	0 min	15 min	30 min
Progressive motility (%)	37.78±0.90 <sup>b</sup>	29.37±1.13 <sup>b</sup>	13.75±1.25 <sup>b</sup>	51.25±0.78 <sup>a</sup>	41.12±1.23 <sup>a</sup>	19.45±0.41 <sup>a</sup>
Live (%)	70.52±1.03 <sup>b</sup>	52.50±1.46 <sup>b</sup>	32.87±1.80 <sup>b</sup>	77.39±0.76 <sup>a</sup>	61.35±1.71 <sup>a</sup>	38.08±1.34 <sup>a</sup>
Dead (%)	30.03±0.99 <sup>a</sup>	48.50±1.06 <sup>a</sup>	67.12±1.73 <sup>a</sup>	22.61±0.76 <sup>b</sup>	38.86±0.84 <sup>b</sup>	61.67±1.73 <sup>b</sup>
Acrosomal integrity (%)	79.81±0.69	79.25±0.67	74.75±0.45	81.08±0.64	79.88±0.67	76.88±0.79
HOS test (%)	43.50±1.09 <sup>b</sup>	37.87±1.48 <sup>b</sup>	19.75±1.33 <sup>b</sup>	52.17±0.98 <sup>a</sup>	43.51±1.31 <sup>a</sup>	26.67±0.97 <sup>a</sup>

Data is presented as the mean±SEM (n=40). <sup>a,b</sup>Different superscripts indicate significant difference between groups at same post thaw incubation time for each parameter at 5% level (P<0.05).

induced cryoinjuries and/or cryo damages to sperm cells. Hemalatha *et al.* (2018) reported enhancement in *in vitro* fertilizing capacity of frozen semen after dietary supplementation of organic zinc and copper to the male goats. A significantly higher zona pellucida binding capacity of sperm and cleavage rate percent were observed in mineral treated groups as compared to control group. No traceable literature is available regarding the dietary azolla supplementation and its effect on semen quality in bucks. However, it was observed and hypothesized that, minerals, vitamins and protein present in azolla play a critical role in spermatogenesis followed by proper maintenance during cryopreservation and post thaw process.

It could be concluded that dietary azolla supplementation in breeding bucks improved the quality and freezability of semen favouring the use in artificial insemination program.

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