

Original Research Article

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Effect of Feeding Azolla on Sexual Behaviour, Seminal Characteristics and Freezability in Marwari Stallions

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ABSTRACT

The present investigation was carried out to evaluate the effect of feeding of Azolla on sexual behaviour, seminal characteristics and freezability in Marwari Stallions. Three adult fertile Marwari stallions were subjected to a switchover technique of feeding in which they were fed with basal feed for 45 days (T₀), then for next 45 days feeding trial 10% of total concentrate feed protein was replaced by Azolla supplementation (T₁). All the parameters were recorded during control and treatment feeding trials. Sexual behaviour and libido (erection time, ejaculation time, mounting time and number of thrust) were recorded at the time of semen collection and after exposing to the estrus mare. Six ejaculates from each stallion (18 in total) were collected during the breeding season for control and treatment stallions using AV and estrus mare as dummy. Immediately after collection, semen samples were evaluated for various quantitative and qualitative parameters. Then semen samples were diluted with primary (Citrate) extender 5% (v/v) and extended in secondary (Lactose-Glu) extender having Dimethyl formamide as cryoprotective agent and 20% (v/v) egg yolk. After equilibration, semen samples were filled in 0.5ml straws and frozen for 12 min and 4 cm above the liquid nitrogen and finally shifted into liquid nitrogen container. Post thaw seminal characteristics were studied to find out the effect of Azolla feeding on the freezability. From the above study it was observed that, there was no significant increase either in the libido or seminal characteristics of the stallions in either of the groups, but it was proved that Azolla can be used as an alternative source of protein in the diet of stallions in conserving the freezability and retaining the qualitative seminal parameters in stallions without deterioration.

Keywords

Azolla, Stallion semen, Freezability, Libido, Semen cryopreservation

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Introduction

Artificial insemination (AI) has quickened the genetic improvement due to the comfort involved in transporting the semen from the insemination center to the place where the mare is inseminated which offers a broader choice to breeders outside the country (Alamaary *et al.*, 2019). Therefore, the use of AI becomes the basic technique in the modern horse industry. Unfortunately, many stallions produce semen that is unable to provide acceptable motility after undergoing the rigors of cooling and storage, and cryopreservation further magnifies this reduction in motility (Brinsko *et al.*, 2000). High-quality semen is dependent on several major husbandry factors, including proper management, good health care, and sufficient nutrients to meet the needs of reproductive stallions (Freitas *et al.*, 2016). Supplementing the equine diet is a popular management method to improve stallion health and thereby, semen quality (Arruda *et al.*, 2010).

There are currently two strategies are being adopted for improving the semen quality and freezability across the livestock species. Supplementing the diet of livestock with energy rich (Arangasamy *et al.*, 2018a) or anti-oxidant rich (Ravi *et al.*, 2018) nutraceuticals as *in-vivo* strategy and addition of antioxidants and other cryoprotective agents during the semen dilution as *in-vitro* strategy are popular and thus being widely followed. Azolla has been fed an ideal feed substitute for cattle, buffalo, sheep, goat, pigs, poultry and fish (Becerra *et al.*, 1995; Hossiny *et al.*, 2008; Indira *et al.*, 2009; Leterme *et al.*, 2010). Supplementation of nutrient rich feed like azolla, which contains many minerals, vitamins, poly unsaturated fatty acids (PUFA) in addition to high protein content (Gupta *et al.*, 2014) may improve the reproductive performance of stallions. Azolla 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.*, 2015, Isaac *et al.*, 2017) and it has a worldwide distribution from temperate to tropical climates. Zinc (Zn) plays an important role in male reproduction like testicular steridogenesis, androgen metabolism and interaction with steroid receptors and its requirement for testicular growth is greater than body growth and appetite (Bedwal and Bahuguna, 1994). Balaji *et al.*, (2009) reported that chemical composition of sun dried and ground Azolla meal (*Azollapinnata*) contained 24.5% crude protein, 14.9% crude fibre, 3.7% ether extract, 17.0% total ash and 39.90% NFE on DM basis. Copper (Cu) deficiency is responsible for reduced libido or male infertility (Arangasamy *et al.*, 2018b). This plant multiplies rapidly and gives good dry matter yield in spite of its high water content (Singh, 1982). 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).

For stallions, so far no controlled studies on the effects of dietary supplementation of Azolla on sexual behaviour and semen quality have been conducted. Therefore the current study was performed to evaluate the effects of dietary supplementation of Azolla on quality of equine semen.

Materials and Methods

Experimental animals

Three apparently healthy, adult (5-8 years) and fertile Marwari Stallions weighing between 450-540 Kgs, and are being maintained at the ICAR- National Research Centre on Equines, Equine Production Campus, Bikaner, Rajasthan were utilized for

the current study. All the stallions were housed with uniform feeding and management schedule occurring for all animals and no special light was provided and were maintained under natural light conditions having free access to feed and water. The experiments were carried out in accordance with the guidelines and approval of Institute Animal Ethics Committee.

Azolla cultivation and feeding

Azolla had been propagated in nine ground pits of different dimensions and four cement tubs at the Azolla Production Unit established at the farm. The stallions were subjected to a switchover technique of feeding in which they were fed with basal feed for 45 days (trial I), then for next 45 days feeding trial 10% of total concentrate feed protein was replaced by Azolla supplementation (trial II). The animals were fed with concentrate, twice a day (at 9.00h and 18.00h), while dry fodder and green fodder was fed once a day at 14:00 h and 11:00 h, respectively. Water availability to the animals was throughout the day. The basal diet of a stallion was comprised of concentrate mixture (Gram-30%, Wheat bran-27%, Oats grain-40% and Salt 2% & Min. mix 1%), and *ad libitum* dry fodder (50:50 ratio of Wheat straw and Groundnut haulm). Supplemental feed comprised of *ad libitum* dry fodder (50:50 ratio of Wheat straw and Groundnut haulm) and 10% of total protein of concentrate mixture was supplemented through Azolla (fresh and green) and concentrate mixture was adjusted accordingly. The effect of Azolla supplementation on libido, semen characteristics and freezability parameters were studied.

Libido of the stallions

For evaluating the libido of the stallions various parameters like reaction time, mounting time, ejaculation and erection time

were recorded and analysed. Reaction time in the present study was recorded as the time between the stallions entered the breeding arena and the stallion mounted the dummy as described by Cavinder *et al.*, (2012). Erection time in the present study as recorded as the time between the stallions first saw the dummy mare and the stallion's penis get fully erected as described by Waheed *et al.*, (2015). Ejaculation time in the present study was recorded as the time between the intromission of the penis by stallion and the first emission of semen. Number of thrusts was noted for each of the stallion during mounting and ejaculation process.

Semen collection and processing

The semen from stallions was collected (6 collections from each stallion, a total of 18 collections each during control and treatment durations), twice per week using artificial vagina (AV) (Colorado model) in an insulated jacket protecting it from direct sun light and temperature shock. The semen samples were collected directly into a clean dry graduated plastic bottle attached at the end to the latex cone of the AV (Talluri *et al.*, 2016). Immediately after collection, the gel fraction was removed and semen was filtered through a sterile gauze. Semen collection, evaluation and processing for freezing were done as previously described (Soni *et al.*, 2012). Immediately after semen collection, seminal parameters like appearance, volume, colour, consistency and pH were recorded by visual observation. The other semen parameters that were evaluated were total and progressive sperm motility, sperm concentration, livability, Hypo-osmotic swelling test (HOST) to determine plasma membrane integrity. Gel free semen was mixed with Citrate-Glucose- EDTA primary extender in the ratio of 1:1 and centrifuged at 600g for 3 min (Talluri *et al.*, 2016). The supernatant was discarded and sperm pellet was obtained.

The sperm pellet was suspended in a clarified egg yolk mixed in Lactose EDTA secondary extender.

Semen cryopreservation

The semen was loaded into 0.5 ml polyvinyl chloride straws, sealed with an automatic filling and sealing machine, and then cooled to 4°C over 2 h as equilibration period. Freezing was performed by traditional method of freezing in liquid nitrogen vapours by spreading the straws on a straw stand at height of 4cms and then the straws were taken out after 10 min exposure and plunged into canisters of liquid nitrogen (-196°C) containers till further analysis. The straws were thawed in a water bath at 37°C for 30s immediately before semen analyses.

Statistical analysis

Statistical analysis of the results were done by paired sample t-test as per Snedecor and Cochran (1989) according to a complete randomized design using statistical software package (SPSS version 20) and correlation using standard statistical methods (Snedecor and Cochran, 1994). The mean values were compared by using Duncan's multiple range test (DMRT) described by Duncan (1955).

Results and Discussion

Azolla supplementation in the diet of stallions and its effect on libido, semen quality as well as freezability of stallion semen had not yet been investigated. Hence, in the present study, the effects of dietary supplementation of Azolla on stallion behaviour and semen quality were evaluated.

The reaction time, mounting time, no. of ejaculations/thrusts, erection and ejaculation time recorded for the stallions (control and treatment) during the semen collection

process were calculated and presented in Table. No.1. Improved Libido and steroidogenesis were reported in Azolla supplemented buck group due to the presence of high amount of Zn and Cu in Azolla (Kumar *et al.*, 2015 and Ganwar *et al.*, 2019). But in contrary, there was no significant increase was observed in the libido of the stallions fed with Azolla in the present study however, there is a non-significant decrease in the reaction time and erection time were recorded. Whereas, the mounting time and ejaculation time were found to be on higher side which were also found to be non-significant. This may be due to variation in the species and different digestibility patterns.

The seminal parameters of the stallions recorded during the control and treatment periods were summarized in Fig. 1. The overall mean value of total semen volume observed in control and treatment groups were 67.44 ± 9.36 and 75.78 ± 14.19 , respectively and they varied from 33.41 ml to 129.24ml in both the groups. The average gel volume was 23.28 ± 7.77 and 32.61 ± 10.42 ml in control and treatment group, respectively, while the mean gel free semen volume was 44.17 ± 3.73 and 43.17 ± 4.94 ml in respective groups. The results obtained in the study were in accordance with the observations of Pal *et al.*, (2009) and Soni *et al.*, (2017) reported for Marwari stallions. Great variations are observed for ejaculate volume between the ejaculates and between the stallions. The mean semen volume values were not significantly different between both groups in the present study. Similarly, Kumar *et al.*, (2015) and Gangwar *et al.*, (2019) did not found any significant change in ejaculate volume between control and treatment groups fed with Azolla in Barbari bucks. The overall mean spermatozoa concentration in control and treatment group was 253.05 ± 31.36 and $373.52 \pm 52.85 \times 10^6$ /ml per ejaculate, respectively. In present study, spermatozoa

concentration was not significantly different between both the groups. Tallur *et al.*, (2017) observed mean value of spermatozoa concentration 253.33 ± 8.52 and $262.33 \pm$

$15.89 \times 10^6/ml$ in Marwari stallion and Poitujacks, respectively. The results of present study show no statistically significant differences between both the groups (Fig. 2).

Table.1 Mean \pm S.E. values of different libido examination parameters in control and treatment groups (n=18)

Parameters	Control group	Treatment group	Significance
Reaction Time (sec)	64.17 \pm 3.02	66.31 \pm 5.17	NS
Erection time (sec)	138.10 \pm 3.67	132.91 \pm 5.12	NS
Ejaculation time (sec)	30.43 \pm 0.33	31.54 \pm 0.39	NS
Mounting time (sec)	28.93 \pm 0.39	29.80 \pm 0.44	NS
Number of thrust	8.78 \pm 0.36	9.89 \pm 0.34	NS

Fig.1 Fresh Seminal characteristics of stallions of control and treatment groups

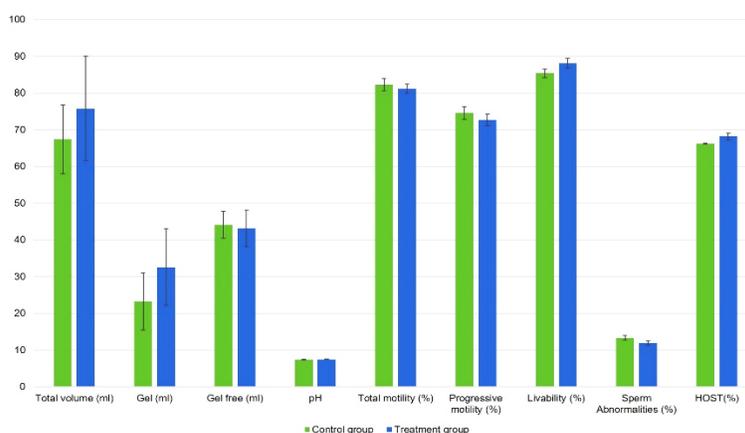
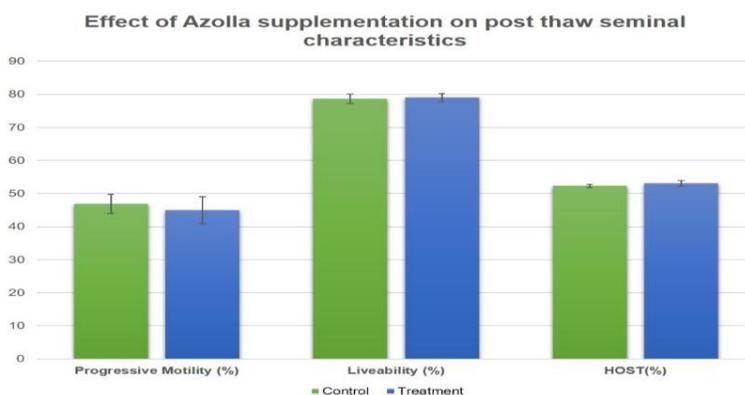


Fig.2 Pre-freeze and post-thaw seminal characteristics of stallions of control and treatment groups



Mean total motility of spermatozoa observed in control and treatment group was 82.23 ± 1.71 and 81.21 ± 1.25 , respectively. The difference was statistically non-significant between control and treatment group. Pal *et al.*, (2009) observed mean total motility 82.60 and 79.76% in Marwari stallions and French donkey stallions, respectively. The overall mean progressive motility of horse spermatozoa observed in control and treatment group was 74.54 ± 1.74 and $72.69 \pm 1.48\%$, respectively. The results revealed that there was no significantly difference between both the groups. Ravi *et al.*, (2013) in Kathiawari stallions and Soni *et al.*, (2017) in Manipuri stallions observed mean progressive motility 77.0 ± 1.51 and $75.00 \pm 2.84\%$, respectively. Pal *et al.*, (2009) observed mean progressive sperm motility $73.33 \pm 0.94\%$ and $77.80 \pm 1.0\%$ in Marwari stallions and French donkey stallions, respectively. Talluri *et al.*, (2017) observed the mean value 77.00 ± 2.22 and $81.46 \pm 0.87\%$ in Marwari stallion and Poitu jacks, respectively.

The overall mean livability of spermatozoa (%) in fresh semen sample was 85.36 ± 1.22 and 88.11 ± 1.36 in control and treatment group, respectively. The results revealed that the overall mean livability of spermatozoa does not differ significantly between both the groups. The similar findings were reported by Kumar *et al.*, (2015) in Barbari bucks who also did not find any significant change in live and dead ratio after Azolla feeding. The HOST positive spermatozoa in control and treatment groups were recorded as 66.18 ± 0.17 and 68.19 ± 0.94 respectively and there is non-significant increase in the plasma membrane integrity of the spermatozoa. In a recent study carried on Bucks supplemented with Azolla diet reported a positive and significant improvement in the progressive sperm motility and HOST positive sperms (Ganwar *et al.*, 2019) which is not the case with the stallions.

The overall mean post thaw motility observed in control and treatment groups were 46.89 ± 2.93 and $45.03 \pm 4.02\%$, respectively. The difference was statistically non-significant between control and treatment groups. The overall mean post thaw livability (%) observed in control and treatment group was 78.65 ± 1.40 and $79.04 \pm 1.25\%$, respectively. The mean HOST positive spermatozoa for control and treatment groups were found to be 52.29 ± 0.57 and 53.09 ± 0.88 . The results revealed that there was no significant difference between both the groups. Betterment in some semen quality parameters like post thaw motility, livability, HOST% may be due to protective role against reactive oxygen species (ROS), and membrane stabilizing action of Copper & zinc, other trace minerals and thereby influencing the fluidity of lipids, which in turn protects the sperm membrane during cryopreservation process.

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. The effect of Azolla supplementation on sexual behaviour, libido, semen quality as well as freezability of stallion semen had not yet been established. Present experiment indicated the beneficial effect of Azolla supplementation in the stallions on semen preand post thaw quality and the same results were also observed in bucks' fed with supplemented Azolla feeding (Kumar *et al.*, 2015 and Gangwar *et al.*, 2019). Positive effect on libido is a good indication for improve reproductive performance in breeding stallions. The improvements in semen quality might be due to the different minerals, vitamins, poly unsaturated fatty acids and amino acids

present in the Azolla responsible for spermatogenesis. Mandal *et al.*, (2012) reported that Azolla contains all important amino acids, β -carotene, minerals which are required in spermatogenesis and steroidogenesis, and these may be helpful in improving semen quality.

It can be concluded that Azolla can be used as an alternative for protein supplementation in the diet of stallions as no negative effect was found on parameters taken for study.

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