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Asparagus racemosus improves seminal antioxidant status and sperm characteristics in buck semen at refrigeration temperature

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ABSTRACT

The present investigation was carried out to evaluate the effect of *Asparagus racemosus* (Shatavari) aqueous extract on buck semen quality during preservation. In the current study, 8 ejaculates from 8 Jakhrana bucks maintained at Jakhrana unit of ICAR-CIRG (semi-arid region) were collected (total 64 ejaculates) during the period from April to June, 2022. Good quality semen samples were pooled during each collection. Pooled semen samples were then divided into 4 equal parts, and diluted in TRIS buffer containing different concentration of Shatavari aqueous extract (Different groups, i.e. Gr1-5 mg, Gr2-2.5 mg, Gr3-1.25 mg, Gr4-0 mg of Shatavari aqueous extract/ml of semen diluent). All the diluted semen samples were kept at refrigerated temperature (5°C) for seven days, and on each day, diluted semen was evaluated for various sperm characteristics and antioxidant status. Gr3 showed significantly better results in terms of sperm viability, sperm motility, acrosomal integrity and plasma membrane integrity. Along with this, the longevity of sperm was also enhanced in Shatavari supplemented group.

Keywords: Antioxidant, Aphrodisiac, Buck semen, Fertility

Artificial insemination (AI) is most widely used technique for fast dispersal of elite animal genetics, and is more successful and economical, actually reaching to the farmers' door-step in India. Semen preservation is the indispensable step in AI, which prolongs the keeping duration and maintains sperm fertility, eases worldwide transfer of preserved semen, and enriches the reproducing potential of elite male animals (Wen *et al*. 2019). Either chilled or frozen semen, can be employed for AI however, liquid preservation leads to better pregnancy rates than that of frozen one. During semen preservation at refrigerated temperature, antioxidant in semen is exhausted and at the same time ROS is spontaneously formed by sperm aerobic metabolism (Gangwar *et al.* 2018) resulting in disparity between antioxidant and pro-oxidant activities, which eventually causes the lipid peroxidation of the polyunsaturated fatty acids of the sperm membrane. This curtails the efficient use of semen preservation technology. There is an obligatory requirement to optimize the buck semen extender to attain the better semen quality with higher fertility. The chemical antioxidants provide benefit in one way and may be harmful in another way, i.e. may stimulate inflammatory/immune reaction in females during artificial insemination (Ribeiro *et al.* 2021). However, this problem can be addressed by using the good quality antioxidants which are of natural origin and herbal antioxidants do not have such deleterious effects (Shahid *et al.* 2022).

Continuous research efforts have been directed towards investigating plants and their derivatives, particularly for their potential in treating various diseases and developing innovative therapeutic approaches (Kuo *et al.* 2018). *Asparagus racemosus* (*A. racemosus*) is one such important medicinal plant having wide range of pharmacological and therapeutic effects, and it is called as a *rasayana* (plant drug that enhances overall health by increasing cellular vitality and resistance) in the Ayurveda (Goyal *et al.* 2003). Hence, after going through the available literature on the beneficial effects and potent antioxidant property of Shatavari (Wiboonpun *et al.* 2004, Guo *et al.* 2023), the current research work was designed with the objective to study effect of Shatavari aqueous extract on buck semen preservation and seminal antioxidant potential.

MATERIALS AND METHODS

Method of aqueous extract preparation of Shatavari: A. racemosus comes under the family Asparagaceae and roots were purchased from the local market. The aqueous extract was prepared from the roots in the Veterinary Medicine Laboratory of the Institute (ICAR-CIRG, Makhdoom). Dried root powder was soaked in distilled water for 5 h at 80ºC. The sediment was separated by filtration and the filtrate was concentrated by lyophilisation. The obtained aqueous extract was processed with a small pulverizer, and

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kept at 25°C for further use.

Semen collection: The collection of semen was done as per the guidelines laid down by the Institute Animal Ethics Committee. In the present experiment, 8 Jakhrana bucks of 2 to 4.5 years of age, and weighing 35±1.8 kg were used. From each buck, 8 ejaculates were collected. Semen was collected twice at weekly intervals with the help of artificial vagina in the morning hours.

Experimental design: The semen was diluted with tris–citric acid fructose diluents having 6% (v/v) glycerol and 10% (v/v) egg yolk as cryoprotective agent (Gangwar *et al.* 2014). The methodology was developed for the addition of Shatavari aqueous extract in the above extender (Different groups, i.e. Gr1-5 mg, Gr2-2.5 mg, Gr3-1.25 mg, Gr4-0 mg of Shatavari aqueous extract per ml of semen diluent). The semen samples were diluted to maintain sperm concentration approximately 100–120 million/straw. All the diluted semen samples were kept at refrigerated temperature (5ºC) for seven days, and on each day, the semen samples were evaluated for various sperm characteristics and antioxidant status.

Semen evaluation: The semen samples were placed in water bath at 37ºC till the primary evaluation. The semen samples were evaluated for volume, *p*H, colour, consistency, concentration and mass motility just after the collection. Quantity of each ejaculate was measured with the help of the graduated collection cups. Mass motility and individual sperm motility was assessed as reported by Gangwar *et al.* (2014). The semen samples showing mass motility more than three were pooled, and diluted for further experimental study. Live sperm percentage and acrosomal integrity of buck spermatozoa was evaluated as

Table 1. Sperm characteristics (Mean±SEM) at different concentrations of Shatavari extract supplementation at different time intervals of buck semen preservation

Sperm characteristics	Day	Gr1	Gr2	Gr3	Gr4
Sperm motility (%)	Day 1	89± 1.87	89± 1.87	90± 1.58	89± 1.87
	Day 2	84± 1.01^{a}	88± 1.22^{a}	88± 1.22^{a}	79± 2.45^{b}
	Day 3	72± 2.24^{b}	76± 2.24^{ab}	78.2± 1.22^{a}	64± 3.02^{c}
	Day 4	60± 3.16^{ab}	67± 4.06^{a}	70± 3.16^{a}	52± 2.06^{b}
	Day 5	38± 3.74^{bc}	47± 3.03^{ab}	51± 3.32^{a}	33± 3.05^{c}
	Day 6	18± 2.06^{b}	28± 2.04^{a}	32± 2.07^{a}	12± 2.05^{c}
	Day 7	7± 2.06^{bc}	13± 3.10^{ab}	18± 3.74^{a}	2.5 ± 2.10^{c}
Sperm viability (%)	Day 1	92± 0.63	93.2± 0.73	91.8± 0.73	90.6± 0.81
	Day 2	87± 0.32^{b}	90.8± 0.37^{a}	92.6± 0.40^{a}	83.6± 1.12^{c}
	Day 3	76.4± 1.36^{b}	81.4± 1.29^{a}	84.6± 0.81^{a}	72± 2.50^{c}
	Day 4	64.4± 2.77^{bc}	71± 3.21^{ab}	75.8± 2.63^{a}	57± 2.05^{c}
	Day 5	42.8± 3.12^{bc}	50.8± 2.92^{ab}	56± 3.22^{a}	36.6± 3.61^{c}
	Day 6	22.6± 2.23^{b}	32.8± 1.74^{a}	37.2± 1.66^{a}	15.4± 1.66^{c}
	Day 7	11± 2.68^{bc}	16.8± 3.20^{ab}	22.2± 3.35^{a}	4.4± 2.23^{c}
Acrosomal integrity (%)	Day 1	87.4± 0.60	87.6± 1.02	88.2± 0.86	87.4± 1.08
	Day 2	84.2± 0.86^{bc}	85.8± 0.86^{ab}	86.8± 0.49^{a}	83± 0.71^{c}
	Day 3	79.2± 0.58^{c}	81.6± 0.75^{b}	84.6± 0.40^{a}	76.4± 0.50^{d}
	Day 4	72.6± 1.66^{bc}	77± 1.34^{ab}	79.8± 1.32^{a}	69± 1.58^{c}
	Day 5	65± 2.39^{bc}	71± 1.95^{ab}	75.4± 1.43^{a}	60.6± 2.38^{c}
	Day 6	56.4± 3.78^{bc}	64.4± 2.42^{ab}	69.4± 1.63^{a}	51.8± 3.26^{c}
	Day 7	49.4± 4.28^{bc}	55.4± 2.87^{ab}	61.4± 2.16^{a}	42.6± 4.83^{c}
Plasma membrane integrity (%)	Day 1	84.2± 2.08	83.8± 2.01	84.4± 1.6	83.8± 2.39
	Day 2	78.4± 1.57^{bc}	82± 1.70^{ab}	84.6± 1.6^{a}	74.6± 1.72^{c}
	Day 3	70.8± 1.36^{b}	74.4± 0.68^{a}	77.4± 0.68^{a}	65.6± 1.69^{c}
	Day 4	55.8± 2.69^{bc}	61.4± 2.94^{ab}	65.2± 2.87^{a}	48.6± 2.54^{c}
	Day 5	37.8± 3.56^{bc}	44± 2.66^{ab}	48.4± 2.09^{a}	30.8± 2.52^{c}
	Day 6	18.8± 2.73^{b}	29.2± 2.01^{a}	34± 1.38^{a}	12± 1.92^{c}
	Day 7	8.1 ± 2.70^{ab}	13.6± 3.66^{a}	17.6± 4.30^{a}	3.2± 1.96^{b}
Sperm abnormality (%)	Day 1	6.4± 0.68	6.8± 0.95	6.4± 1.03	6.6± 0.51
	Day 2	7.6± 0.75	7± 0.77	6.8± 0.8	8.2± 0.97
	Day 3	8.8± 0.8	7.2± 0.86	7.2± 0.77	9.6± 0.93
	Day 4	9.8± 0.86^{ab}	8.2± 0.84^{b}	7.6± 0.81^{b}	11± 0.95^{a}
	Day 5	10.6± 0.98^{ab}	8.8± 0.86^{b}	8.8± 0.73^{b}	12.6± 1.36^{a}
	Day 6	11.6± 0.98^{a}	9.8± 0.73^{b}	9.2± 0.77^{b}	13.6± 0.98^{a}
	Day 7	12.4± 0.75^{a}	10.2± 0.84^{b}	9.8± 0.58^{b}	14.4± 0.6^{a}

Within the same preservation time, mean values with different superscripts differ significantly ($P< 0.05$) among treatment groups.

per the method described by Gangwar *et al.* (2019). The hypo-osmotic swelling test (HOST) was done to evaluate the integrity of the sperm plasma membrane. The sperm membrane integrity was examined as per the protocol used by Jayendran *et al.* (1984). Sperm abnormalities were assessed as mentioned by Nayak *et al.* (2016).

Effect on lipid peroxidation: Lipid peroxidation in sperm plasma membrane was indirectly measured by MDA (malondialdehyde) production assay as mentioned by Arangasamy *et al.* (2018) with certain changes. MDA levels were estimated at day 1 to day 7 of preservation, respectively, and denoted as nmol/dl.

Statistical analysis: Data was given as mean±S.E., and the statistical analysis was done by SPSS-22 Software, IBM (SPSS Inc., Chicago, IL, USA). The differences in mean values were assessed using Duncan's multiple range tests and one-way analysis of variance (ANOVA). The difference between means was significant at 95% level of significance (P<0.05).

RESULTS AND DISCUSSION

After first day of preservation, all the experimental groups had similar total sperm motility, sperm viability, acrosomal integrity, sperm plasma membrane integrity, sperm abnormalities whereas on the subsequent days, total sperm motility was significantly (P<0.05) higher in Shatavari extract supplemented groups in comparison with the control group. However, significantly (P<0.05) enhanced sperm motility was observed in Gr3 containing 1.25 mg of Shatavari aqueous extract per ml of diluter. Thus, we can say the buck spermatozoa are more motile for longer duration in this group. Hence, it was concluded that Shatavari helped in maintaining the sperm motility, viability, acrosomal integrity for longer duration and it minimized the early acrosome reaction as well as protected the sperm plasma membrane for longer duration and reduced the sperm abnormality (Table 1 and Table 2).

Table 2. MDA concentration (Mean±SEM) at different concentrations of Shatavari aqueous extract at different time intervals of buck semen preservation

Day	Gr 1	Gr2	Gr3	Gr4
Day 1	2.64±0.33	2.55±0.35	2.61±0.35	2.73±0.35
Day 2	3±0.32	2.98±0.29	2.83±0.28	3.56±0.30
Day 3	3.20±0.38^{b}	3.05±0.37^{b}	3.01±0.40^{b}	4.89±0.32^{a}
Day 4	3.60±0.32^{b}	3.47±0.28^{b}	3.27±0.32^{b}	5.24±0.21^{a}
Day 5	3.92±0.30^{b}	3.74±0.32^{b}	3.67±0.36^{b}	5.47±0.33^{a}
Day 6	4.4±0.18^{b}	4.13±0.25^{b}	4.06±0.28^{b}	6.24±0.23^{a}
Day 7	4.67±0.22^{b}	4.42±0.18^{b}	4.25±0.21^{b}	6.35±0.21^{a}

Within the same incubation time, values with different superscripts differ significantly (P< 0.05) among treatment groups.

All the experimental groups showed similar MDA production for two days, whereas on the subsequent days, lipid peroxidation was significantly (P<0.05) lower in Shatavari extract supplemented groups in comparison with the control group (Table 2).

Earlier, various researchers reported that addition of antioxidants in semen diluter improved the semen quality (Gangwar *et al.* 2014, Gangwar *et al.* 2015, Gangwar *et al.* 2018). But now a days, people are trying natural antioxidants in semen extenders (Wen *et al.* 2019) and extract from *Asparagus racemosus* (Shatavari) roots have been used in many investigations as antioxidants (Goyal *et al.* 2003, Guo *et al.* 2023). However, there are no evident research findings with respect to the protective effect of Shatavari aqueous extract on buck sperm as well as inhibition of lipid peroxidation. Thus, this research work was carried out to examine the semen quality and antioxidant activity in the bucks after supplementation of Shatavari aqueous extract in buck semen diluter.

Reduced lipid peroxidation of sperm plasma membrane reflected by MDA level in Shatavari extract supplemented group was also seen. Though, this type of investigation has not been done or performed with herbal antioxidant supplementation in buck semen diluter in comparison with results of this study. Very little amount of data is present with regard to the specific use of herbs in laboratory animals, boar and human beings (Kopalli *et al.* 2015, Ansari and Khan 2017, Kumar *et al.* 2018). Thakur *et al.* (2009) reported that *Asparagus racemosus*, *Chlorophytum borivilianum*, and rhizomes of *Curculigo orchioides* improved the mating behaviour and aphrodisiac activity in male rats. In the current investigation, it was observed that Shatavari extract had negative correlation with MDA production; hence it had ability to reduce the lipid peroxidation and sperm abnormalities during semen cryopreservation and this might be due the presence of flavonoids in it (Table 3).

Similar to our findings, Wen *et al.* (2019) reported that sperm motility, acrosomal integrity, mitochondrial membrane potential, plasma membrane integrity, and total antioxidative activity in the 30 mg/L GSPE (Grape seed procyanidin extract) group was significantly enhanced, whereas MDA content was lower than the control group

Table 3. Correlation among the different sperm parameters and MDA level in different treatment groups of buck semen

	Sperm Motility	Sperm Viability	Acrosomal Integrity	HOST	Sperm Abnormality	MDA
Sperm motility	1	0.996	0.938	0.99	-0.702	-0.762
Sperm viability	0.996	1	0.945	0.992	-0.694	-0.75
Acrosomal integrity	0.938	0.945	1	0.947	-0.744	-0.745
HOST	0.99	0.992	0.947	1	-0.706	-0.778
Sperm abnormality	-0.702	-0.694	-0.744	-0.706	1	0.753
MDA	-0.762	-0.75	-0.745	-0.778	0.753	1

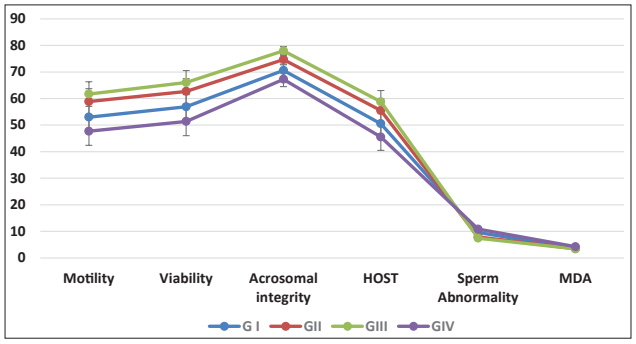

Fig. 1. Overall sperm characteristics and MDA level during the entire period of experiment in buck semen supplemented with different concentration of Shatavari extract.

(P <0.05). Similarly, Zhao *et al.* (2009) reported the significant correlation between *Rhodiola sacra* aqueous extract and concentrations of GSH and MDA in frozen-thawed boar semen. It is already known that oxidative stress is a well-documented inducer of apoptosis, and lipid peroxidation can initiate aging and a diminished longevity of the cryopreserved spermatozoa.

The results of the current study indicate that there is overall increase in sperm motility, sperm viability, acrosomal integrity, plasma membrane integrity in treatment groups as compared to the control groups whereas, there is significant increase in number of abnormal spermatozoa and MDA level in control group as compared to treatment groups which shows higher peroxidative damage in control group (Fig. 1). In a previous study, it was found that dietary azolla supplementation significantly improved the semen quality and libido in bucks (Gangwar *et al.* 2019). Earlier study showed that buck sperm motility was 68.59% after three days of storage at 5°C (Wen *et al.* 2019); it reached 78.2% (a significant increase) after three days of liquid preservation with Shatavari supplementation. Hence, Shatavari extract is capable to protect the buck sperm from various types of cryo-damages.

Malo *et al.* (2011) found that adding rosemary extract into the freezing medium enhanced post-thawed semen quality in boar. Similarly, Zhao *et al.* (2009) reported that *Rhodiola sacra* aqueous extract (RSAE) exhibited strong scavenging activity against superoxide anion radical. They observed significant improvement in progressive motility, HOST response, fertility, and safeguarding the functional integrity of sperm plasma membrane. Antecedent findings indicated that the number of hypo-osmotic swelled spermatozoa is positively correlated with the oocyte penetration rate in human oocytes.

In congruence with our results, El-Sheshtawy *et al.* (2016) demonstrated that addition of bull semen extender with 10% and 20% pomegranate Juice provides adequate chilling, and boosted frozen-thawed semen quality. Similarly, Khan *et al.* (2017) reported that addition of green tea extract in semen diluter exhibited remarkable response on the post-thawed spermatozoa motility, viability and membrane integrity of Achai bull. Vahedi *et al.* (2018) found that supplementation of *Thymus vulgaris* extract at the rate of 4-8 ml/dl of diluter improves the attributes of ram sperm after freezing and thawing. Addition of 5% *Tribulus terrestris* extract in semen diluter improved the sperm motility in Afshari rams at 5°C (Pour *et al.* 2015). Similarly, Mehdipour *et al.* (2016) reported that supplementation of *Camellia sinensis* extract at level of 10 mg/L can enhance post-thaw ram semen quality, when it is cryopreserved in a soybean lecithin extender.

In the present investigation, the enhancement of semen quality and antioxidant activity could be due to the richness of *A. racemosus* in advantageous polycarbohydrates and flavonoids (kaempferol, quercetin, and rutin).

The results of the current study showed that Shatavari aqueous extract can be used as natural antioxidant in buck semen diluter, and may be replaced with chemical antioxidants. It significantly enhanced the sperm motility, acrosomal integrity, plasma membrane integrity and increased the sperm viability for longer duration, thus improving buck semen quality during preservation at refrigerated temperature.

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