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CORRELATION BETWEEN HYPO-OSMOTIC SWELLING TEST (HOST) AND OTHER SEMINAL CHARACTERISTICS OF DECCANI RAM SEMEN

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Inter-relationship

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ABSTRACT

Integrity of sperm plasmalemma plays a pivotal role in the process of fertilization. The present study was undertaken to investigate the plasma membrane integrity by Hypo-osmotic swelling test (HOST) of Deccani ram sperm during liquid storage of semen (5°C) and its inter-relation with other seminal characteristics (Acrosomal integrity, livability, motility, sperm abnormalities and methyl blue reduction test (MBRT)). Semen was collected from 8 Deccani rams (18 ejaculates/ram) and diluted (1:4; 200millions/ml) with egg yolk citrate extender (EYC) after assessment of semen volume (ml), concentration (millions/ml), colour, consistency and mass activity (0-5 scale). Seminal characteristics were evaluated immediately after dilution, after 24 and 48 h of storage at 5°C. The Pearson's (r) correlation test was used to calculate correlations between Hos-test and other sperm parameters (Motility, Livability, Acrosome integrity, abnormalities and MBRT). The findings of the present investigation showed highly significant positive correlation between HOS coiling and motility ($P<0.01$), sperm livability ($P<0.01$) and acrosome integrity ($P<0.01$) which indicate its efficiency as feasible screening method for evaluation of male fertility prior to Artificial insemination.

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1 Introduction

The Plasma membrane integrity (PM) is essential for survival of sperm in the female reproductive tract and to maintain the fertilizing ability by acting as a selective barrier between components of the intracellular and extracellular environment (Gwathmey et al., 2006). Hypo-osmotic swelling test (HOST) is one of the simplest methods to evaluate the sperm cells plasmalemma stability (Padrik et al., 2012). This method complements with the routine sperm analysis (Nie & Wenzel, 2001). A positive correlation was reported between the results of the HOST and the non-return rate of female animals (Correa et al., 1997), which potentially makes it one of the most appropriate and simple methods for semen quality evaluation (Padrik et al., 2012). Reaction of spermatozoa against the HOS solution varies from animals to animals and it depending on animal species, composition, osmolality and time of incubation (Amorim et al., 2009).

Till now, the variation of plasma membrane integrity within Deccani breed when semen was cooled to 5°C was not investigated. The present investigation was aimed at assessment of the correlation between Hypo-osmotic swelling test (HOST) and other sperm quality parameters of Deccani ram semen.

2 Materials and methods

The present investigation was conducted after getting the approval of Institutional Animal Ethics Committee.

2.1 Semen collection

A total of 144 ejaculates (18 ejaculates/ ram) was collected from January to May with the intensity of twice a week from eight Deccani rams (2-4 years age) selected from the sheep herd maintained at ILFC (Instructional Livestock Farm Complex), Rajendranagar (longitude: 78.4018° E, latitude: 17.3203° N) trained to serve an Artificial vagina (AV) at a temperature of 45°C. Each ram fed on green fodders along with concentrate feed (300 gm/day) throughout the experimental period

2.2 Semen extension and Evaluation

Immediately after semen collection, macroscopic parameters like semen volume, colour, consistency and mass activity (0-5 score) were recorded. Semen was diluted with sodium citrate extender [2.9% aqueous solution of Sodium citrate dihydrate (Fisher scientific - 6132-04-3; 294.1g/mol); 20% (v/v) of egg yolk, streptomycin (1000 µg/ml) and pencillin (1000 IU/ml); pH - 6.8] (dilution rate- 1:4; final concentration- 200 million sperms/ml) and preserved at 5°C. Semen analysis was done immediately after dilution and after cooling (5°C) at 24 and 48 h of storage.

2.2.1 Sperm concentration

Sperm concentration in semen was determined by using improved Neubauer counting chamber after a dilution of semen with 1:200 with a diluting fluid [2% eosin y-solution dissolved in 1%(v/v) formalin, Saturated NaCl solution (1%w/v), Distilled water] and expressed in millions/ml.

2.2.2 Individual Motility

10µl semen was mixed with 200µl normal saline on a clean glass slide and motility was observed under high power at 400X magnification and expressed in terms of percentage of progressively (0-100) motile sperms.

2.2.3 Supravital staining

Semen smears were made by mixing it with Eosin-nigrosin stain (Eosin -1.67 gm, Nigrosin -10 gm, Distilled water -100 ml) and examined under high power (1000X) objective. Minimum of 200 spermatozoa were counted for determining the livability of the spermatozoa.

2.2.4 Acrosomal Integrity

Acrosome integrity was assessed using Giemsa staining procedure (Watson, 1975) with slight modifications. Semen smeared glass slides were put into 5% formaldehyde solution for fixing at 37°C for 30 min. After washing and air drying, smeared slides of spermatozoa were put into the working solution (Giemsa's stock-3 ml, Sorenson's phosphate buffer-2 ml and dist. water- 45 ml) and kept at 37°C for 3 hrs. Sperm with intact, partially damaged and fully damaged acrosome were determined at 1000X magnification.

2.2.5 Sperm abnormalities assessment by Rose bengal staining

One drop of diluted semen was placed on glass slide and air dried, this was followed by the staining with 3% rosebengal stain (3 gm - Rose Bengal powder, 1ml - Formalin added to 100 ml Distilled water) for 15-20 minutes. Then, observed under light microscopy at 1000X magnification. Atleast 100 sperms were counted.

2.2.6 Hypo-osmotic Swelling Test (HOST)

1 ml of pre-incubated (at 37°C) hypo-osmotic solution (7.35 g sodium citrate trihydrate, 13.51 g fructose in 1,000 ml of distilled water; 150 mOsm/L) was mixed with extended semen (0.1 ml) in a test tube. Simultaneously, control solution (1ml; 300 mOsm/L) was prepared and added with 0.1 ml of same semen in another test tube. The test tubes were incubated for 30 minutes (37°C). 2 drops of 2% eosin Y solution was added to the test tubes for better visualisation of spermatozoa. A drop of incubated semen was examined at 1000X magnification and observed for curling pattern of sperm tails.

The proportion of swollen spermatozoa was determined by method given by Sharma et al. (2012)

Host reactive spermatozoa (%) = Proportion of swollen spermatozoa in hypo - osmotic solution (150 mOsm/L) - Proportion of swollen spermatozoa in control sample (300 mOsm/L)

2.2.7 Methylene Blue Reduction Test (MBRT)

To 1ml of diluted semen, 100 μ l of 1 % methylene blue solution (50mg of methylene blue dissolved in 100ml of 2.9% sodium citrate dihydrate solution) was added and then mixed gently. Mineral oil (1cm thick) was layered above the mixture to ensure anaerobic condition and then incubated in a water bath at 47°C. The time required for the change of semen colour was recorded.

2.3 Statistical analysis

The data obtained from conventional parameters were analysed by ANOVA (Analysis of Variance) for diluted (0 h) and chilled semen (24 and 48 h). Data was statistically analyzed by using Statistical Package for Social Science (SPSS, version 16). Duncans test was used to compare the means at a probability level of 5%. The Pearson's (r) correlation test was used to calculate correlations between HOST and other sperm parameters (Motility, Livability, Acrosome integrity, abnormalities and MBRT).

3 Results

The Deccani ram semen characteristics like colour, volume, consistency, mass activity, concentration which were analysed immediately after collection was represented in Table 1.

Table 1 Seminal traits of Deccani Rams

Seminal traits	Mean $\pm$ S.E (n=144)
Volume	0.63 $\pm$ 0.27
concentration	10825.00 $\pm$ 537
Mass activity	3.64 $\pm$ 0.58
Colour	Creamy white
Consistency	Thick

[Values in parenthesis are the no. of ejaculates collected from the eight rams]

Figure 1 HOS test – (→) indicating the tail curling pattern and (↓) showing HOS test non reactive spermatozoa (1000X).

Figure 2 Spermatozoa stained with Rose Bengal stain (1000X)

Figure 3 Eosin Nigrosin staining - (→) indicating partially stained (DEAD) sperm (1000X)

Motility livability, abnormalities, acrosome integrity and HOS-test, MBRT were (Figure. 1, 2 and 3) analysed immediately after dilution (0 h) and during storage period at 24 and 48 h (5°C) were given in the Table 2. The sperm motility, HOST and acrosome integrity decreased significantly ($P \leq 0.05$) while mean MBRT time increased significantly ($P \leq 0.05$) as the period of storage increased from time of dilution to 48 h of storage at 5°C (Table 2). Correlation between Hos-test and other seminal attributes of Deccani ram semen diluted in Egg yolk citrate extender was represented in the Table 3. HOST showed significant positive correlation ($P < 0.01$) with livability ($r = 0.878$) and motility ($r = 0.513$) and acrosome integrity ($r = 0.613$). Significant negative correlation was found between HOST and MBRT ($r = -0.738$, $P < 0.01$).

4 Discussions

The individual motility percentage of native rams semen of Bangladesh (75.3%) (Pervage et al., 2009) was higher compared to the present study. The period of semen collection and ejaculation frequency may cause some variation in sperm motility (Yotov et al., 2011). The overall mean live spermatozoa percentage of Deccani ram semen was lower compared to the Churra ram semen of Spain diluted with Tris based extender (75%) (Mata-Campuzano et al., 2014). The variation may be due to variation in extender used, breed, storage x extender interaction (Soltanpour et al., 2014) and semen collection method (Matthews et al., 2003). The sperm abnormalities percentage obtained in the present study was comparable to that of Bangladesh ram semen (6.63%) (Pervage et al., 2009) and Awassi ram semen of Iraq (Azawi & Ismael, 2012). While Akkaraman ram semen of Turkey obtained higher percentage of sperm abnormalities (Kulaksiz et al., 2012). The variation may be due to breed (Gil et al., 2003) and age of the rams.

The mean percentage of acrosomal integrity obtained in the present study was in accordance with that of Pathanwadi ram semen of India (Kakadiya et al., 1995) and was lower compared to that of Churra ram semen of Spain (Mata-Campuzano et al., 2014). The variation in acrosome integrity might be due to difference in the age of the rams, season, breed and extender (Paulenz et al., 2002).

Table 2 Different microscopic examination parameters and HOS test characteristics of diluted semen (0 h) of Deccani Rams

Parameters	Duration of storage			Overall mean
	Fresh semen diluted	24 hr	48 hr	
Individual Motility (%)	70.93±1.86 ^a	58.43±1.69 ^b	47.70±1.27 ^c	58.85±1.23
Live spermatozoa (%)	77.00±1.34 ^a	66.66±1.26 ^b	47.18±1.19 ^c	63.61±1.26
Acrosome Integrity (%)	93.58±0.58 ^a	88.64±0.74 ^b	82.50±0.79 ^c	88.24±0.55
Sperm Abnormalities (%)	4.72±0.23 ^a	4.95±0.19 ^a	5.41±0.21 ^a	5.03±0.12
HOS test Reactive sperm (%)	69.22±1.88 ^a	60.20±1.18 ^b	44.64±0.92 ^c	58.02±1.16
MBRT (min)	1.57±0.06 ^c	3.59±0.07 ^b	6.17±0.08 ^a	3.78±0.16

Given values in table are average of eighteen replicates; Mean $\pm$ S.E value followed by different letter in same horizontal rows are significantly different according to Duncan's multiple range test ($p < 0.05$)

Table 3 Correlation matrix showing coefficients of correlation among seminal attributes of Deccani ram semen diluted in Egg yolk citrate (EYC) extender

	Motility	Livability	Acrosome integrity	Host	Sperm abnormalities	MBRT
Motility						
Livability	0.581**					
Acrosome integrity	0.381**	0.676**				
Host	0.513**	0.878**	0.613**			
Sperm abnormalities	-0.132	-0.183*	-0.119	-0.167*		
MBRT	-0.665**	-0.832**	-0.708**	-0.738**	0.207*	

Significant at ** $p < 0.01$, * $p < 0.05$ level

Figure 4 Graph representing the significant positive correlation between HOS coiling % age and motility % age of spermatozoa of Deccani ram species

Figure 5 Graph representing the significant positive correlation between HOS coiling % and livability % of spermatozoa of Deccani ram species

Figure 6 Graph representing the significant positive correlation between HOS coiling % and acrosome integrity % of spermatozoa of Deccani ram species

Figure 7 Graph representing the negative correlation between HOS coiling % and abnormalities % of spermatozoa of Deccani ram species

Figure 8 Graph representing the significant negative correlation between HOS coiling % and MBRT (min) of spermatozoa of Deccani ram species

HOST is used to characterize the plasma membrane integrity of ram semen by means of “curling” pattern of sperm tail, which occurs due to influx of water when maintained under hypotonic environment. Nalley & Arifiantini (2013) evaluated plasma membrane integrity of Indonesian Garut ram semen (fresh) and reported 69.47% of swollen sperm when subjected to HOST. In the present study, the mean percentage of the sperm positive to HOST was lower compared to that of Indigenous ram semen of Bangladesh (69%) diluted with Tris based extender (Azizunnesa et al., 2014). The variation might be due to difference in the seminal plasma biochemical constituents (Barrios et al., 2000). Higher values of mean MBRT time (min) was obtained in ArkharMerino x Moghani and Baluchi x Moghani ram semen (Moghaddam et al., 2012) while compared to that of the present study. The difference was attributed to the variation in motility, livability and metabolic activity of spermatozoa.

The findings of the present investigation showed highly significant correlation between HOS coiling and motility ($P < 0.01$) (Figure 4), sperm livability ($P < 0.01$) (Figure 5) and acrosome integrity ($P < 0.01$) (Figure 6) in deccani rams which was in accordance with that of the goat spermatozoa (Fonseca et al., 2005) and Nili-ravi Buffalo and sahiwal cow bull (Lodhi et al., 2008) semen samples, Jersey x local hill cattle crossbred Indian bull semen (Sharma et al., 2012) and ram (Bohlooli et al., 2012) semen samples. However the parameters HOST and Sperm livability correlated well compared to HOST and motility which was of similar trend to that of crossbred cattle bull semen. In contrast, no correlation was found between sperm reactive to the hypoosmotic test and the other semen characteristics of goats (Oliveira et al., 2013) which might be due to species variation and difference in the sperm plasma membrane composition leading to the reduced response of the sperm to Hypo-osmotic solution. Highly significant negative correlation was found between Hos test and MBRT ($P < 0.01$) (Figure. 8) indicating the reduced membrane integrity of sperm plasma membrane leading to loss of metabolic enzymes in the spermatozoa as the duration of storage increased. Low significant ($P < 0.05$) negative correlation was observed between Hos-test and

sperm abnormalities (0.167^*) (Figure. 7) indicating the impaired plasma membrane activity in the defective or abnormal spermatozoa.

Thus, the correlation of HOST with other seminal parameters indicates its efficacy as screening test for routine semen evaluation which is easy and inexpensive.

Conclusion

The present investigation demonstrates that the Deccani ram semen could maintain motility, viability and functional integrity of spermatozoa upto 48 h of storage when diluted with extender EYC and chilled at 5°C and indicates the importance of the HOST in assessing the functionality of Deccani ram spermatozoa which can be used for screening of semen samples prior to artificial insemination.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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