

Effect of Short-term and Long-term Preservation on Motion Characteristics of Garole Ram Spermatozoa: A Prolific Microsheep Breed of India

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ABSTRACT : Garole is a prolific, rare, less known and small size Indian sheep breed found in low and humid Sunderban region of West Bengal. Although information on stored Garole ram liquid semen upto 24 h is available, but there is a need to further investigate the short-term and long-term preservability of Garole ram semen for extensive utilization of this valuable germplasm by artificial insemination. The aim of the present study was to apply computer-assisted sperm analysis technique for assessing the motion characteristics of Garole ram semen stored (i) in liquid state at refrigeration temperature for short-term preservation upto 48 h and (ii) in frozen state at -196°C for long-term preservation after packaging in mini straws. Short-term preservation had a significant effect on motility ($p < 0.01$) as the motility progressively decreased from 90.1% at 0 h to 85.5% and 73.2% after 24 and 48 h of storage, respectively. Although the decline in rapid moving sperms was also significant ($p < 0.01$) on storage but the decrease was more pronounced at 48 h as compared to 24 h of storage period. Storage of chilled semen had also a significant effect on % linearity ($p < 0.05$), % straightness ($p < 0.01$), sperm velocities ($p < 0.01$), amplitude of lateral head displacement ($p < 0.01$) and beat frequency ($p < 0.01$) of spermatozoa. The replication had a significant effect for all the variables except average path and straight line velocity. However, the interactions of short-term storage and replication were non-significant for most of the variables except % of medium moving sperms, sperm velocities and beat frequency. On long-term preservation of Garole ram spermatozoa under controlled conditions the mean post-thaw recovery of 70.4 and 71.4% motile spermatozoa was achieved having 48.8 and 48.9% of rapidly motile spermatozoa, respectively in both the replicates. The effect of replication on cryopreservation was significant ($p < 0.05$) on amplitude of lateral head displacement and beat frequency, but there was no significant effect on motility, rapidly motile spermatozoa, linearity, straightness and sperm velocities of frozen-thawed spermatozoa. It can be concluded from these results that an average 70% motility can be achieved on storage of Garole ram semen in chilled liquid state upto 48 h or in liquid nitrogen after freezing under controlled conditions in straws. However, further studies are required to evaluate the fertility of short-term and long-term preserved Garole ram semen for extensive use of this prolific sheep breed. (*Asian-Aust. J. Anim. Sci.* 2001. Vol 14, No. 11 : 1527-1533)

Key Words : Sheep, Rams, Short-Term Preservation, Long-Term Preservation, CASA Analysis

INTRODUCTION

Conservation of sheep genetic resources are primarily *in-situ* involving maintenance of breeds in their natural habitat but *ex-situ* conservation of germplasm in the form of spermatozoa, ova and embryos assume significance if a gene of major interest or effect is detected (Ponzoni, 1997). A prolific, rare, less known and small size sheep breed in India that requires logical attention for *in-situ* and *ex-situ* conservation is Garole. The natural tract of this microsheep breed is the low and humid Sunderban region of West Bengal situated within 87° to 89° E longitude and 21° to 23° N latitude spread over 4,226 sq km with an average rainfall of 1,750 mm per annum (Bose and Moitra, 1995). Garole sheep weigh 10-14 kg at maturity and are characterized by light brownish coat colour, coarse wool and well-developed udder. Apart from high prolificacy the other unique features of this breed are: ability to breed round the year, grazing habit in knee-deep water, resistance to foot rot disease and good mothering instinct (Ghalsasi

and Nimbkar, 1993; Bose and Moitra, 1995; Singh and Bohra, 1996; Sharma et al., 1999).

An average litter size of 2.27 lambs with 7.3% single births, 65.45% twins, 21.8% triplets and 5.45% quadruplets have been reported for Garole ewes (Ghalsasi and Nimbkar, 1993; Fahmy and Mason, 1996). It is speculated that prolificacy to the Booroola Merino strain evolved in Australia has been derived from this breed (Turner, 1982) due to a major gene (Piper and Bindon, 1982). The single Booroola fecundity (FecB) gene in Booroola Merino strain is known to increase ovulation rate and litter size (Montgomery et al., 1992). There is evidence that the origin of FecB gene may be traced back to early importation of sheep in the late 18th century from Bengal (Montgomery et al., 1994). Considering the importance of multiple births for augmenting the reproductive rate of non-prolific breeds, Garole sheep has been introduced in the semi-arid tropical climate for exploring the possibility of incorporating prolificacy in monotocus breeds (Sharma et al., 1999). The majority of sheep breeds in India are found in semi-arid or arid regions and are mostly monotocus. The observations recorded on agnostic, investigatory, precopulatory and ejaculatory behaviour of adult rams have shown that Garole

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rams have a potential to perform well under semi-arid environment (Maurya et al., 1999). The ejaculate volume of Garole rams is very less varying from 0.1 to 0.9 ml (Joshi et al., 1999a) averaging 0.58 ml (Joshi et al., 2000a). The computer-assisted sperm analysis (CASA) data on stored Garole ram liquid semen upto 24 h is known (Joshi et al., 1999b), but there is a need to further investigate the short-term and long-term preservability of Garole ram semen for extensive utilization of this valuable germplasm. The aim of the present study was to apply CASA technique for objective assessment of the motion characteristics of Garole ram semen preserved for (i) short-term in liquid state upto 48 h at refrigeration temperature and (ii) long-term in frozen state at -196°C after packaging in straws.

MATERIALS AND METHODS

Location

The study was conducted at the Institute's sheep farm at Avikanagar which is located at longitude of $75^{\circ}28'$ E, latitude of $26^{\circ}26'$ N and an altitude of 320 m above mean sea level in the semi-arid tropical tract of the country. The rainfall is erratic and mainly concentrates during July to August. The precipitation ranges from 400 to 700 mm per annum.

Animals

Adult Garole rams of 2-5 years of age and maintained under the semi-intensive management system were used as semen donors. The donor rams were randomly selected from the original flock procured from the Sunderban area in March 1997 or from the offsprings delivered at the institute farm. Eight rams weighing between 12.5 to 17.0 kg (14.9 ± 1.2 kg) and 10 rams weighing between 11.0 to 17.0 kg (14.6 ± 2.2) were respectively used for conducting experiments on short-term and long-term preservation of semen. The rams were allowed for 8-10 h daily grazing on natural vegetation interspersed with seasonal shrubs and forbs. In addition to grazing, they were provided a concentrate mixture of 150 g/ram/day.

Experiment 1: Short-term preservation of Garole ram semen

Semen collections were made with the aid of artificial vagina at the onset of autumn season when major breeding activities commence at the institute farm. Each ram was scheduled for single ejaculation three times at a weekly interval. After ejaculation, semen samples were immediately diluted 1:1 with egg yolk McIlvaine glucose (EYMG) diluent maintained at room temperature. Analytical grade chemicals were used for preparation of EYMG diluent. The composition of the diluent was: 100 ml McIlvaine buffer (prepared by titrating 1.78% solution of

di-sodium hydrogen orthophosphate dihydrate with 1.68% solution of citric acid monohydrate to pH 7.0); 0.8% glucose; 0.3% streptopenicillin and 20 ml egg yolk (Mathur et al., 1993). The osmolality of the diluent measured by using automatic cryoscopic osmometer (Osmomat 030, Gonotec, Berlin) was 316 mOsmol/kg. The diluted semen samples were gently mixed, transferred into round bottom screw cap glass tubes (5 ml capacity, 15×77 mm) and stored in the refrigerator ($4-7^{\circ}\text{C}$). The semen attributes of 8 samples per replicate were evaluated by CASA technique at 0, 24 and 48 h of storage at refrigeration temperature.

Experiment 2: Long-term preservation of Garole ram semen

Semen collections were made in quick succession from rams using artificial vagina during winter season when there is no breeding activity in the farm. Each ram was scheduled for two ejaculations twice within a week. The semen quality was assessed subjectively for volume, consistency and wave motion (0-5 scale). Ejaculates having ≥ 0.3 ml volume, thick consistency and rapid wave motion (≥ 4) were pooled and the rest were discarded. The pooled semen sample was assessed for sperm concentration using a previously calibrated colorimeter and diluted with Test-yolk-glycerol extender (Schmehl et al., 1986) to a final concentration of 1×10^9 /ml sperms/ml (Joshi et al., 2000b). The final composition of the diluent prepared by using analytical grade chemicals was: TES:N tris (hydroxymethyl) (methyl-2-aminoethane) sulfonic acid 4.83%; Tris (hydroxymethyl) aminoethane 1.16%; fructose 0.2%; streptopenicillin 0.3%; glycerol 6 ml; egg yolk 15 ml; triple glass distilled water added upto 100 ml. The pH and osmolality of the diluent were 7.20 and 1,553 mOsmol/kg, respectively. Diluted semen samples were aspirated into 0.25 ml French mini plastic straws and equilibrated at 5°C for 2 h. Equilibrated straws were frozen under controlled conditions in a programmable cell freezer (R-204, Planer Products Ltd., UK.) precooled to 5°C upto -125°C at the rate of $25^{\circ}\text{C}/\text{mts}$ and then plunged into liquid nitrogen for storage at -196°C until required. Twenty-five frozen straws from each replicate were thawed individually after random selection at 50°C for 10 seconds in a thermostatically controlled water bath (Joshi et al., 1998a). The semen attributes of the thawed samples were evaluated by CASA technique.

Evaluation of motion characteristics of liquid-stored and frozen-thawed Garole ram semen

The motion characteristics of liquid-stored and frozen-thawed Garole ram spermatozoa were assessed objectively by CASA technique using HTM-S version 7.2 Y motility analyzer (Hamilton Thorn, USA) by the procedure described earlier by Joshi and Mathur, 1996. Prior to analysis, the samples were diluted to approximately 25 \times

10^6 sperms/ml with normal saline solution and the semen analyzer was set-up as follows: Image type: Phase contrast; Digitization rate: 25 frames/sec; Digitization time: 0.8 sec; Minimum contrast: 8; Minimum size: 6; Low/High size gates: 0.6 to 1.8; Low/High intensity gates: 0.6 to 1.8; Magnification: 2.17. Twenty μ l of diluted sample was placed in a prewarmed Makler counting chamber (10 μ m deep, Sefi-Medical Instruments Ltd., Israel) and 3 fields per chamber were analyzed at 37°C in the analyzer (Joshi et al., 1999 b). The 11 CASA parameters recorded by the analyzer were: curvilinear velocity (CLV, μ m/sec), average path velocity (APV, μ m/sec), straight line velocity (SLV, μ m/sec), % motility, % rapid ($APV > 25 \mu$ m/sec), % medium ($10 < APV < 25 \mu$ m/sec), % slow ($0 < APV < 10 \mu$ m/sec), % linearity, % straightness, beat frequency (BF, Hz) and amplitude of lateral head displacement (ALH, μ m) of spermatozoa.

Statistical analyses

The values reported in percentages were subjected to arc sin transformation. The CASA data obtained from the experiment on short-term preservation of semen were analysed by two-way analysis of variance using mixed model least squares and maximum likelihood computer programme (Harvey, 1990). The Duncan's multiple range test was used to determine the difference between two means after analysis of variance. The CASA data from the experiment on long-term preservation of semen were analysed by student's t test of two independent means (Snedecor and Cochran, 1989). The significant differences between means were determined at $p < 0.05$ and $p < 0.01$ levels.

RESULTS

Influence of short-term preservation on spermatozoal motion

Table 1 summarizes the influence of short-term preservation on % motility, % fraction of rapid, medium and slow moving sperms, % linearity and % straightness of Garole ram semen. Prior to storage the mean motility was 91.6, 89.5 and 89.2% in first, second and third replicates and ranged from 89 to 94%, 80 to 95% and 80 to 95%, respectively. There was a significant effect on motility ($p < 0.01$) on storage as the motility progressively decreased after 24 and 48 h. Although the decline in rapid moving sperms was significant ($p < 0.01$) on storage but the decrease was more pronounced at 48 h as compared to 24 h of storage period. However, the fraction of medium and slow moving sperms increased progressively at a significant rate ($p < 0.01$) on storage. Storage had also a significant effect on % linearity ($p < 0.05$) and % straightness ($p < 0.01$) of spermatozoa. The replication had a significant effect for all

the 6 variables. However, the interactions of storage and replication were non-significant for all the variables except for percentage of medium moving sperms.

Influence of short-term preservation on velocity and track dimensions

Table 2 summarizes the effect of short-term preservation on sperm velocity and track dimensions of Garole ram semen. Velocity measurements such as CLV, APV and SLV decreased progressively after sperm were stored for 24 or 48 hours and the effect were significant ($p < 0.01$). Although, there was also a significant decrease in ALH ($p < 0.01$) but the BF was increased significantly ($p < 0.01$) on storage. The replication had significant effect on CLV ($p < 0.05$), ALH ($p < 0.01$) and BF ($p < 0.05$) but the effect on APV and SLV were non-significant. Although BF, CLV, APV and SLV accounted for significant interactions of storage and replication but it was not significant for ALH.

Influence of long-term preservation on spermatozoal motion

The mean values of spermatozoal motion of pooled semen samples obtained by CASA analysis during post-dilution in the first and second replicates were not much different and were as follows: % motility 92.0 vs 89.0; % rapid 88.0 vs 85.3; % medium 4.0 vs 3.0; % slow 0.0 vs 0.0; % linearity 58.7 vs 55.7% and straightness 73.3 vs 70.0, respectively. Similarly, the respective mean values of semen samples during post-equilibration stages in the first and second replicates were: % motility 90.0 vs 89.3; % rapid 86.7 vs 88.0; % medium 3.3 vs 0.7; % slow 0.0 vs 0.0; % linearity 53.3 vs 56.0; % and straightness 68.7 vs 71.7 (Not given in the table). The effect of long-term preservation on spermatozoal motion of frozen-thawed Garole ram spermatozoa are presented in table 3. The mean post-thaw recovery of motile spermatozoa was more than 70% in both the replicates and was not significantly different ($p > 0.05$). There was also no significant effect ($p > 0.05$) on % rapid, % medium, % slow, % linearity and % straightness of frozen-thawed spermatozoa of both the replicates. However, after post-thawing the fraction of rapid moving sperms decreased substantially in both the replicates as compared to post-dilution and post-equilibration stages.

Influence of long-term preservation on velocity and track dimensions

The mean values of velocity and track dimensions of pooled semen samples obtained by CASA analysis during post-dilution in the first and second replicates were not much different and were as follows: CLV, 153.6 vs 162.3 μ m/sec; APV, 120.6 vs 125.6 μ m/sec; SLV, 94.0 vs 93.3 μ m/sec; ALH, 8.0 vs 8.4 μ m; and BF, 8.1 vs 8.3 Hz, respectively. Similarly, the respective mean values of

Table 1. Effect of short-term preservation on spermatozoal motion of Garole ram spermatozoa

Treatment	% Motility	% Rapid	% Medium	% Slow	% Linearity	% Straightness
Storage (H)						
0	90.1 ^c	84.4 ^c	4.9 ^a	0.4 ^a	58.7 ^a	71.1 ^a
24	85.5 ^b	71.1 ^b	10.8 ^b	2.6 ^b	64.2 ^b	76.4 ^b
48	73.2 ^a	49.5 ^a	16.1 ^c	6.3 ^c	63.2 ^b	77.0 ^b
Significance	p<0.01	p<0.01	p<0.01	p<0.01	p<0.05	p<0.01
Replication (R)						
I	86.8 ^b	76.8 ^b	7.3 ^a	1.7 ^a	55.9 ^a	71.0 ^a
II	80.7 ^a	60.5 ^a	13.8 ^b	4.3 ^b	65.6 ^b	77.4 ^b
III	82.7 ^a	69.9 ^b	9.6 ^{ab}	2.1 ^a	64.5 ^b	76.1 ^b
Significance	p<0.05	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01
H × R						
H ₁ × R ₁	91.6	89.9	1.6 ^a	0.0	51.3	65.8
H ₁ × R ₂	89.5	78.6	9.7 ^{cd}	0.9	62.9	75.0
H ₁ × R ₃	89.2	83.6	4.9 ^{ad}	0.2	61.6	72.4
H ₂ × R ₁	88.7	81.1	6.2 ^d	0.9	58.1	71.5
H ₂ × R ₂	82.2	60.5	15.5 ^{bc}	4.5	69.2	80.1
H ₂ × R ₃	85.1	70.6	11.7 ^{bcd}	1.9	65.1	77.3
H ₃ × R ₁	78.6	55.0	17.1 ^b	5.4	58.3	75.5
H ₃ × R ₂	67.9	40.5	17.2 ^b	8.8	64.6	77.0
H ₃ × R ₃	72.6	53.1	14.0 ^{bc}	5.0	66.8	78.4
Significance	ns	ns	p<0.05	ns	ns	ns

Values are actual means of data; ns, non-significant. Means within columns with different superscripts are significantly different.

semen samples during post-equilibration stages in the first and second replicates were: CLV, 144.7 vs 146.3 $\mu\text{m}/\text{sec}$; APV, 114.0 vs 111.3 $\mu\text{m}/\text{sec}$; SLV, 83.3 vs 83.0 $\mu\text{m}/\text{sec}$; ALH, 8.4 vs 7.9 μm ; and BF, 9.7 vs 9.5 Hz (Not given in the table). The effect of long-term preservation on velocity and track dimensions of frozen-thawed Garole ram spermatozoa are presented in table 4. The sperm velocities (CLV, APV and SLV) of frozen-thawed Garole ram spermatozoa were not significantly different ($p>0.05$) in both the replicates but decreased considerably as compared to post-dilution and post-dilution stages. However, among the two replicates freezing and thawing had significant ($p<0.05$) effect on BF and ALH of spermatozoa.

DISCUSSION

Germplasm preservation can make a major contribution in preserving biodiversity. The importance of preserving rare breeds and establishing genetic resource banks of frozen semen, oocytes and embryos are becoming increasingly recognized, especially for their potential value in responding to environmental changes (Holt, 1997). Semen preservation enables widespread use of superior rams for artificial insemination, *ex-situ* conservation of elite or endangered breeds and international exchange of elite germplasm (Yoshida, 2000). The high incidences of multiple births in Garole sheep holds promise to increase

ewe productivity of non-prolific breeds by improving their reproductive efficiency. Although the low quantity of semen production from Garole rams makes their judicious use imperative in major breeding season but provides enormous scope to for *ex-situ* conservation of this prolific breed in the form of spermatozoa by harvesting maximum semen during non-breeding period. CASA is a rapid and precise technique for objectively monitoring sperm kinematics (Holt and Palomo, 1996) and has also been applied successfully for evaluating the motion characteristics of ram spermatozoa of different breeds stored for short-term (Briggs et al., 1996; Joshi et al., 1999b) or long-term preservation (Edward et al., 1995; Joshi et al., 1998a,b; Bag et al., 1998; 2000a,b). For obtaining the reliable CASA estimates, sperm concentration between 20 to 50 million sperms per ml and proper instrument settings are essentially required (Davis and Katz, 1992; 1993; Davis and Siemer, 1995). In this study, prior to CASA analysis, all the semen samples were diluted to approximately 25 million sperms per ml and the instrument settings were not changed for all the observations.

During liquid-storage of ram semen there is a gradual decline in motility with the passage of time (Maxwell and Salamon, 1993). A significant fall in motility of chilled ram semen extended with EYMG diluent was observed after 24 h of storage period on subjective (Srivastava et al., 1987) and objective (Joshi et al., 1999b) evaluation. The present findings were in agreement to these reports as storage and

Table 2. Effect of short-term preservation on velocity and track dimensions of Garole ram spermatozoa

Treatment	CLV ($\mu\text{m}/\text{sec}$)	APV ($\mu\text{m}/\text{sec}$)	SLV ($\mu\text{m}/\text{sec}$)	ALH (μm)	BF (Hz)
Storage (H)					
0	147.1 ^c	118.8 ^c	91.8 ^c	6.8 ^c	9.6 ^a
24	111.2 ^b	92.5 ^b	75.6 ^b	5.6 ^b	9.8 ^a
48	90.8 ^a	72.8 ^a	60.2 ^a	4.9 ^a	10.5 ^b
Significance	p<0.001	p<0.01	p<0.01	p<0.01	p<0.01
Replication (R)					
I	123.8 ^b	96.2	72.5	6.5 ^b	10.3 ^b
II	107.8 ^a	89.8	75.0	5.2 ^a	10.0 ^{ab}
III	117.4 ^b	98.0	80.0	5.6 ^a	9.6 ^a
Significance	p<0.05	ns	ns	p<0.01	p<0.05
H × R					
H ₁ × R ₁	151.0 ^b	111.9 ^b	80.6 ^{bc}	7.7	9.5 ^a
H ₁ × R ₂	137.0 ^b	115.0 ^b	93.8 ^{bd}	6.1	10.2 ^{ac}
H ₁ × R ₃	153.3 ^b	126.6 ^b	100.8 ^b	6.7	9.0 ^a
H ₂ × R ₁	133.1 ^b	107.1 ^{bc}	82.6 ^{bc}	6.6	9.6 ^a
H ₂ × R ₂	99.5 ^a	86.3 ^a	75.0 ^{acd}	5.0	9.9 ^a
H ₂ × R ₃	100.9 ^a	84.0 ^{ac}	69.1 ^{ac}	5.3	9.8 ^a
H ₃ × R ₁	87.4 ^a	66.5 ^a	54.1 ^a	5.4	11.7 ^{bc}
H ₃ × R ₂	87.0 ^a	68.4 ^a	56.4 ^a	4.5	9.8 ^a
H ₃ × R ₃	98.0 ^a	83.5 ^a	70.1 ^{ac}	4.8	10.0 ^a
Significance	p<0.05	p<0.05	p<0.05	ns	p<0.01

Values are actual means of data; ns, non-significant. Means within columns with different superscripts are significantly different.

Table 3. Effect of long-term preservation on spermatozoal motion of frozen-thawed Garole ram spermatozoa

Parameter	Replicate I (Mean±S.E.)	Replicate II (Mean±S.E.)
% Motility	70.4±2.29	71.4±2.16
% Rapid	48.9±2.60	48.8±3.15
% Medium	16.9±1.02	16.2±1.18
% Slow	4.6±0.62	6.5±0.91
% Linearity	56.9±0.67	57.8±0.76
% Straightness	74.2±0.61	74.2±0.56

Values are actual means of data derived from 25 observations per replicate.

Table 4. Effect of long-term preservation on velocity and track dimensions of frozen-thawed Garole ram spermatozoa

Parameter	Replicate I (Mean±S.E.)	Replicate II (Mean±S.E.)
CLV ($\mu\text{m}/\text{sec}$)	109.5±2.43	103.8±3.12
APV ($\mu\text{m}/\text{sec}$)	84.7±1.89	80.6±2.29
SLV ($\mu\text{m}/\text{sec}$)	68.1±1.67	65.0±1.80
ALH (μm)	6.9±0.12	6.5±0.17*
BF (Hz)	10.9±0.16	10.5±0.16*

Values are actual means of data derived from 25 observations per replicate and * Denotes significance at p<0.05 level.

replication had a significant effect on motility and rapid moving Garole ram spermatozoa preserved upto 24 h. Although the mean motility decreased from 85.5% to 73.2% on prolonging the storage period from 24 to 48 h, but the decline in the fraction of rapid moving spermatozoa was more pronounced as compared to motility and was from 71.1% to 49.5%. The buffers traditionally used in egg yolk diluents are either citrate or phosphate based (Salamon and Maxwell, 2000). The maintenance of 73.2% mean motility after 48 h of storage may be attributed to the presence of both the ions in the EYMG diluent used for short-term storage of Garole ram semen. The measurement of velocity is an indirect indicator of mitochondrial function of spermatozoon (Graham et al., 1984) and is associated with fertility (Aitken, 1990). The significant decrease in CLV, APV and SLV on storage may be possible due to impaired function of the sperm mitochondria. It has been reported that the rapidly moving spermatozoa with high values of CLV or SLV have greater ALH (Budworth et al., 1988). In the present study storage also significantly decreased ALH which may be attributed to the decrease in CLV or SLV.

Ram spermatozoa are susceptible to various stresses during freezing and thawing (Pontbriand et al., 1989; Salamon and Maxwell, 1995a) causing impaired sperm transport in the female genital tract and low fertility following cervical insemination (Salamon and Maxwell, 1995b; Watson, 2000). Under the best experimental

conditions about half of the population of motile sperms survive after the freeze-thaw process (Watson, 1995). However, the progress made in cryopreservation of ram semen has opened the possibility of conservation and utilization of frozen semen of elite rams in sheep improvement programme (Salamon and Maxwell, 2000, Naqvi et al., 2001). Cryopreservation of spermatozoa under controlled freezing conditions significantly improves sperm survival after thawing (Parkinson and Whitefield, 1987). Zwitterion buffers have been used with varying success as the basis of diluents for freezing ram semen (Salamon and Maxwell, 1995a; Joshi et al., 2000b; Salamaon and Maxwell, 2000). The good post-thaw recovery obtained following long-term preservation of Garole ram spermatozoa in this study may be attributed to the use of Zwitterion based Test-yolk-glycerol extender, the efficacy of the cryopreservation protocol, controlled rate freezing and to the criteria of processing only those ejaculates for cryopreservation which have thick consistency with rapid wave motion. The average 70% post-thaw recovery achieved also compares well with our earlier reports on freezing and thawing ram semen of exotic Awassi (Joshi et al., 1998b), crossbred Bharat Merino (Bag et al., 1998, 2000a,b; Joshi et al., 1998a) or native Malpura (Mathur and Joshi, 1996b; Bag et al., 1999, 2000a,b; Joshi et al., 2000b; Naqvi et al., 1998) sheep breeds maintained under the same semi-intensive farm management system in the semi-arid tropical climate.

It is concluded from these results that CASA information generated for Garole ram semen can be useful for (i) preservation of spermatozoa in liquid or frozen state for artificial insemination of non-prolific sheep breeds (ii) *ex-situ* conservation in the form of spermatozoa for posterity and (iii) predicting the relative fertility because spermatozoa moving rapidly after short-term or long-term preservation seems to have a high probability of crossing the cervical barrier. However, further studies are needed to evaluate the fertility of short-term and long-term stored Garole ram semen in order to extensively utilise this prolific sheep breed.

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