Asian-Aust. J. Anim. Sci.
Vol. 19, No. 12 : 1716 - 1721
December 2006

www.ajas.info

# Influence of Osmolality of Complete Semen Extender on Motion Characteristics of Frozen-thawed Ram Spermatozoa

**Anil Joshi*, A. K. Mathur[1], S. M. K. Naqvi and J. P. Mittal**

Semenology laboratory, Division of Physiology and Biochemistry, Central Sheep & Wool Research Institute
Avikanagar, Via-Jaipur, Rajasthan-304501, India

**ABSTRACT :** The present study was conducted to observe the effect of osmolality of glycerolated TEST-yolk glycerol extenders on post-thawing sperm kinematics of ram spermatozoa of the native Malpura breed maintained in a semi-arid tropical environment. Good quality semen obtained from adult rams was pooled, split and diluted to 1,000 million spermatozoa per ml in complete TEST-yolk-glycerol extenders of 900, 1,200, 1,500 and 1,800 mOsm/kg osmolality. Diluted semen samples were loaded in 0.25 ml straws and cooled down to -125°C freezing temperature at the rate of -25°C per minute under controlled conditions before plunging into liquid nitrogen for storage. The thawing of straws was performed at 50°C in a water bath for 10 seconds and sperm kinematics of the frozen-thawed spermatozoa were assessed by a computer-assisted sperm analysis technique. Osmolality of diluent had no significant effect on post-thawing % motility, % rapid, % medium and % slow moving frozen-thawed spermatozoa but significantly ($p< 0.05$) affected the % linearity and % straightness.The post-thawing % motility and % rapid motile spermatozoa were highest in samples extended in diluent of 1,500 mOsm/kg osmolality and lowest in 900 mOsm/kg. The curvilinear velocity of spermatozoa was significantly ($p<0.05$) higher for samples extended in 1,800 mOsm/kg, compared to those in 900 and 1,200 mOsm/kg, but the effect was not significantly different to those extended in diluent of 1,500 mOsm/kg osmolality. The study indicated that ram spermatozoa could tolerate a wide osmolality range for dilution in the complete TEST-yolk-glycerol extender for their cryosurvival. The highest recovery of motile spermatozoa following thawing was achieved in samples extended in the TEST-yolk-glycerol diluent of 1,500 mOsm/kg osmolality. (**Key Words :** Sheep, Extender, Osmolality, Spermatozoa, Cryopreservation, Computer-aided Semen Analysis)

## INTRODUCTION

Cryopreservation of mammalian spermatozoa has specific advantages for germplasm selection, evaluation, artificial insemination (AI) and *ex situ* conservation of livestock genetic resources (Yoshida, 2000). Extensive research has been conducted in the last few decades on ram semen diluents, semen processing, freezing and thawing methods for improving the post-thaw viability and membrane integrity of motile sperm cells (Salamon and Maxwell, 1995a, 1995b, 2000; Gillan et al., 2004). The biggest obstacle in the exploitation of frozen ram semen is that freezing and thawing reduces motility and membrane integrity, which leads to poor fertility following cervical AI (Salamon and Maxwell, 2000). For achieving an acceptable lambing rate following AI with frozen ram semen, it is desirable to achieve good post-thaw survival of spermatozoa. The combined effect of various factors (Fiser and Fairfull, 1984, 1986; Pontbriand et al., 1989) has an adverse effect on motility (Edward et al., 1995; Joshi et al., 2001; Bag et al., 2002a, b) plasma membrane (Holt et al., 1992; Holt and North, 1994) and acrosomal integrity (Aisen et al., 2000; Bag et al., 2004; Joshi et al., 2005) after freezing and thawing of ram spermatozoa. The degree of damage depends also on the composition of the semen diluent and the nature of the cryoprotectant (Salamon and Maxwell, 1995a; Maxwell and Watson, 1996; Sanchez-Partida et al., 1998; Salamon and Maxwell, 2000; Zhou et al., 2004). Previous studies have shown that freezing of ram spermatozoa in hypertonic diluents improved the cryosurvival of spermatozoa wherein the osmolality of the diluents was determined before the addition of the required volume of glycerol (Fiser et al., 1981, 1982; Fiser and Fairfull, 1986, 1989). However, there is no information on the effect of osmolality of complete glycerolated diluents on

* Corresponding Author: Anil Joshi. Tel: +91-01437-220165, Fax: +91-01437-220163, E-mail: ajoshi2k@yahoo.com

[1] Project Directorate on Cattle, Meerut, Uttar Pradesh-250 110, India.

Received July 21, 2005; Accepted October 19, 2005

post-thaw survival of ram spermatozoa. The aim of the present study was therefore, to compare the cryosurvival of ram spermatozoa frozen under controlled conditions in the glycerolated TEST-yolk-glycerol diluents of varying osmolality by computer-assisted sperm analysis (CASA) technique.

## MATERIALS AND METHODS

### Location

The study was conducted at the Central Sheep and Wool Research Institute, Avikanagar which is located at longitude 75°-28°E, latitude 26°-26°N and at an altitude of 320 m above sea level in the semi-arid zone of subtropical India. The rainfall is erratic and mainly concentrates during July to August. The precipitation ranges from 400 to 700 mm per annum. The minimum and maximum ambient temperatures range from 7 to 37°C and from 25 to 46°C, respectively while the mean relative humidity varies between 15 and 90%.

### Experimental animals and their management

Ten adult rams of the Malpura breed were used for the experiment before the onset of the autumn, when major breeding activities commence at the farm. Malpura is a hardy native sheep of the semi-arid tropical India and reared for wool and mutton production. The body weight of semen donor rams ranged between 42 and 54 kg averaging 49.4± 4.12 kg aged between 3 to 5 years. The experimental rams were maintained in the semi-intensive system under preventive and clinical veterinary care. They were grazed for 8-10 h daily on natural vegetation interspersed with seasonal shrubs and forbs. In addition to grazing, the rams were provided with 200 g concentrate (barely 65%, groundnut cake 32%, mineral mixture 2% and common salt 1%) per head, daily.

### Preparation of TEST-yolk-glycerol extenders

Two stock TEST-yolk-glycerol extenders with 0 or 10% glycerol were prepared by using analytical grade chemicals. The composition of the stock diluents, except glycerol, was as described by Schmehl et al., 1986 having TES:N tris (hydroxymethyl)(methyl-2-aminoethane) sulfonic 4.83%; Tris (hydroxymethyl) aminoethane 1.16%; fructose 0.2%; streptopenicillin 0.3%; egg yolk 15 ml; glycerol 0 (A) or 10 ml (B) and triple glass distilled water added up to 100 ml. The osmolality of the diluents A and B, measured by automatic cryoscopic osmometer (Osmomat 030, Gonotec, Berlin), was 370 and 2,096 mOs/kg, respectively. Twenty-five ml each of TEST-yolk-glycerol extenders of 900, 1,200, 1,500 and 1,800 mOs/kg osmolality were prepared by adding the stock extenders A and B in the following manner: (i) Diluent of 900 mOs/kg (17.32 ml of A+7.68 ml of B); (ii) Diluent of 1,200 mOs/kg (12.02 ml of A+12.98 ml of B); (iii) Diluent of 1,500 mOs/kg (8.63 ml of A+16.37 ml of B) and (iv) Diluent of 1,800 mOs/kg (4.29 ml of A+ 20.71 ml of B). The pH and final glycerol concentration of the diluents of 900, 1,200, 1,500 and 1,800 mOs/kg osmolality were 7.23, 7.21, 7.22, 7.22 and 3.1, 4.8, 6.5 and 8.3%, respectively.

### Semen collection, evaluation and freezing

On the day of freezing, semen samples were collected from rams in quick succession by artificial vagina and were evaluated for volume, consistency, wave motion (0-5 scale), concentration (photometrically) and % motile spermatozoa (0-100%) (Evans and Maxwell, 1987). Ejaculates having thick consistency, rapid wave motion (4 or 5 scale), 90% initial motility and ≥3,000 million spermatozoa per ml were pooled for experimentation and the rest were discarded. Sperm concentration of pooled sample was assessed using a colorimeter, previously calibrated by hemocytometry. Immediately after evaluating the sperm concentration, the pooled sample was divided into 4 equal portions and diluted further using 4 TEST-yolk-glycerol extenders of 900, 1,200, 1,500 and 1,800 mOs/kg to a final concentration of 1,000 million sperm per ml. Diluted samples were aspirated into 0.25 ml straws (IMV, France), sealed with polyvinyl alcohol, progressively cooled to 5°C in a cold handling cabinet and equilibrated at this temperature for 2 h. After equilibration, straws precooled to 5°C, were processed for freezing under controlled conditions using a programmable cell freezer (Planer Biomed R-204, UK) upto -125°C temperature at the rate of 25°C/min and then plunged into liquid nitrogen for storage, until required.

### Assessment of post-thawing semen characteristics

The study was carried out in a 4×2 factorial design with 4 TEST-yolk-glycerol extenders (900, 1,200, 1,500, 1,800 mOs/kg) and 2 freezings (I, II replicates). Frozen straws of each treatment group were randomly selected and thawed individually at 50°C for 10 seconds in a water bath. Prior to semen analysis, each thawed sample was diluted to approximately $25\times10^6$ spermatozoa/ml in a normal saline solution (Joshi et al., 2001). The analyzer (Hamilton-Thorne Sperm Motility Analyzer HTM-S version 7.2 Y, USA) was briefly 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. Ten µl aliquots of diluted semen were placed in a prewarmed 10 µm Makler counting chamber (Sefi Medical Instrument, Haifa, Israel), and loaded into the analyzer at 37°C. At least 3 fields were counted for each sample. The sperm motility characteristics included in the

**Table 1.** Effect of osmolality of diluent on sperm motility and motion characteristics of frozen-thawed ram spermatozoa

| Effect | Motility (%) | Rapid (%) | Medium (%) | Slow (%) | Linearity (%) | Straightness (%) |
|---|---|---|---|---|---|---|
| Osmotic pressure (OP, mOs/kg) | | | | | | |
| 900 | 64.4 | 36.9 | 12.7 | 8.0 | 57.3[a] | 74.9[a] |
| 1,200 | 68.7 | 39.1 | 17.8 | 7.7 | 57.2[a] | 74.4[a] |
| 1,500 | 71.2 | 41.7 | 18.9 | 7.7 | 55.2[a] | 72.6[b] |
| 1,800 | 69.2 | 42.0 | 16.6 | 7.5 | 60.2[b] | 75.8[a] |
| Significance | ns | ns | ns | ns | ($p<0.05$) | ($p<0.05$) |
| Replication (R) | | | | | | |
| I | 69.8 | 40.8 | 17.7 | 7.9 | 60.5[a] | 77.2[a] |
| II | 66.8 | 38.9 | 16.9 | 7.6 | 58.5[b] | 75.8[b] |
| Significance | ns | ns | ns | ns | ($p<0.05$) | ($p<0.05$) |
| OP×R | | | | | | |
| 900×I | 67.8 | 40.4 | 18.9 | 9.8 | 62.7[a] | 78.5[a] |
| 900×II | 65.0 | 37.3 | 18.1 | 10.1 | 56.1[b] | 75.3[b] |
| 1,200×I | 72.6 | 43.2 | 19.1 | 10.4 | 60.9[a] | 77.8[a] |
| 1,200×II | 68.6 | 38.9 | 20.6 | 9.2 | 57.4[b] | 75.1[b] |
| 1,500×I | 74.3 | 43.5 | 22.2 | 9.3 | 57.3[b] | 74.9[b] |
| 1,500×II | 72.1 | 43.9 | 19.0 | 10.2 | 57.3[b] | 74.5[b] |
| 1,800×I | 72.1 | 44.6 | 18.9 | 10.1 | 61.1[a] | 77.4[a] |
| 1,800×II | 70.1 | 43.3 | 18.3 | 9.1 | 63.4[a] | 78.3[a] |
| Significance | ns | ns | ns | ns | ($p<0.05$) | ($p<0.05$) |

analysis included the % motility, % rapid motile spermatozoa (fraction of all cells moving with average path velocity >25 μm/sec), % medium motile spermatozoa (fraction of all cells moving with 10<APV<25 μm/sec), % slow motile spermatozoa (fraction of all cells moving with 0<APV<10 μm/sec), % linearity (LIN, ratio of VSL/VCL), % straightness (STR, ratio of SLV/APV), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), straight line velocity (VSL, μm/sec), beat cell frequency (BCF, Hz) and lateral head displacement (ALH, μm) of spermatozoa (Joshi and Mathur, 1996) for 15 observations per osmolality per replicate.

**Statistical analysis**

The CASA values recorded in percentages were subjected to arc sin transformation. The data was analysed by two-way analysis of variance for osmolality of diluent, replication and their interactions using Harvey's mixed model least squares and maximum likelihood computer programme (Harvey, 1990). The Duncan's multiple range test, as modified by Kramer (1956) was used to determine the difference between two means after analysis of variance. The level of significance differences between means was set at $p<0.05$.

## RESULTS

In Table 1, the effect of osmolality of TEST-yolk-glycerol diluent on sperm motility and motion characteristics of frozen-thawed spermatozoa of Malpura rams is depicted. After thawing the % motility % rapid motile sperms were not significantly different when freezing was performed after dilution in TEST-yolk-glycerol extenders of 900, 1,200, 1,500 or 1,800 mOs/kg osmolality but were highest in 1,500 mOs/kg extender. Similarly, the % medium and slow motile sperms did not differ significantly after extension in either of the diluent. The % LIN of spermatozoa was significantly ($p<0.05$) higher at 1,800 mOs/kg diluent but the differences between 900, 1,200 and 1,500 mOs/kg osmolality diluents were not significant. However, the % STR was significantly ($p<0.05$) higher in diluents of 900, 1,200 and 1,800 mOs/kg, compared to diluent of 1,500 mOs/kg. The effect of replication was significant ($p<0.05$) on % LIN and % STR but was not significant on sperm motility attributes. There was no significant interaction between osmolality and replication in all the sperm motility traits except for % LIN and % STR.

Table 2 summarizes the effect of osmolality of TEST-yolk-glycerol diluent on the velocity and track dimensions of frozen-thawed ram spermatozoa. The osmolality of diluent had significant ($p<0.05$) effect on sperm velocities, ALH and BCF of frozen-thawed ram spermatozoa. The VCL of frozen-thawed spermatozoa extended in diluents of 1,500 and 1,800 mOs/kg osmolality were significantly ($p<0.05$) higher compared to diluents of 900 and 1,200 mOs/kg. However, the VAP and VSL of 1,800 mOs/kg osmolality were significantly ($p<0.05$) higher than the other diluents. The ALH and BCF were also significantly ($p<0.05$) in diluents of 1,500 and 1,800 mOs/kg osmolality, compared to diluents of 900 and 1,200 mOs/kg. The effect of replication was significantly ($p<0.05$) different in all the traits except BCF. The interaction between osmolality and replication were significant ($p<0.05$) for VAP, VSL and

**Table 2.** Effect of osmolality of diluent on the velocity and track dimensions of frozen-thawed Malpura ram semen

| Effect | VCL (μm/s) | VAP (μm/s) | VSL (μm/s) | ALH (μm) | BCF (Hz) |
|---|---|---|---|---|---|
| Osmotic pressure (OP, mOs/kg) | | | | | |
| 900 | 81.7[a] | 62.9[a] | 51.2[a] | 5.4[a] | 11.5[a] |
| 1,200 | 83.9[ad] | 63.7[a] | 52.6[a] | 5.4[a] | 11.3[b] |
| 1,500 | 87.0[bde] | 66.2[a] | 52.8[a] | 5.8[b] | 11.1[b] |
| 1,800 | 91.2[ce] | 72.3[b] | 60.0[b] | 5.8[b] | 10.5[b] |
| Significance | (p<0.05) | (p<0.05) | (p<0.05) | (p<0.05) | (p<0.05) |
| Replication (R) | | | | | |
| I | 88.0[a] | 68.8[a] | 56.6[a] | 5.8[a] | 11.2 |
| II | 84.0[b] | 63.8[b] | 51.7[b] | 5.5[b] | 11.0 |
| Significance | (p<0.05) | (p<0.05) | (p<0.05) | (p<0.05) | ns |
| OP×R | | | | | |
| 900×I | 84.9 | 68.3[a] | 57.0[a] | 5.2[a] | 11.3 |
| 900×II | 78.8 | 57.5[b] | 45.5[b] | 5.5[a] | 11.8 |
| 1,200×I | 86.9 | 67.7[a] | 56.0[a] | 5.5[a] | 11.5 |
| 1,200×II | 81.0 | 59.7[bc] | 49.3[bc] | 5.2[a] | 11.1 |
| 1,500×I | 85.5 | 65.0[ac] | 52.1[ac] | 6.0[bc] | 11.0 |
| 1,500×II | 88.6 | 67.3[a] | 53.5[ac] | 5.6[ac] | 11.2 |
| 1,800×I | 94.9 | 74.0[a] | 61.3[a] | 6.3[b] | 10.8 |
| 1,800×II | 87.5 | 70.6[a] | 58.7[a] | 5.4[a] | 10.2 |
| Significance | ns | (p<0.05) | (p<0.05) | (p<0.05) | ns |

ALH but were not significant for VCL and BCF.

## DISCUSSION

Objective analysis of frozen-thawed ram spermatozoa provides precise information of the sperm motion (Joshi et al., 2001; Bag et al., 2002a, b; 2004; Joshi et al., 2005) provided that care is taken in preparing the sample (Davis and Katz, 1992; 1993) and settings of the instrument (Davis and Siemers, 1995). In this study, prior to analysis all the samples were diluted to approximately $25\times10^6$ sperms/ml and settings for the instrument were kept constant for all the observations so as to ensure reliability of the results. This ensured that the time lapse between semen dilution and CASA analysis was brief thereby enabling the spermatozoa to survive until completion of analysis and the spermatozoa concentration was within the recommended range for getting an accurate CASA measurement (Bag et al., 2002b).

Cryopreservation adversely affects the cryosurvival of ram spermatozoa (Salamon and Maxwell, 2000) and under the best experimental conditions about half of the population of motile sperms survive the freeze-thaw process (Watson, 1995; 2000). The organic amine TES being Zwitterion has a good buffering capacity and has been used as a main component in TEST-yolk-glycerol diluents with varying success for freezing ram semen (Schmehl et al., 1986; Salamon and Maxwell, 1995a). The good post-thaw recovery obtained following long-term preservation of ram spermatozoa in this study may be attributed to the selection of high-quality ejaculates for cryopreservation with high initial motility, choice of the range of osmolality of TEST-yolk-glycerol diluent and the efficacy of the controlled-rate freezing protocol. The post thaw recovery of motile spermatozoa was in agreement to our earlier findings obtained after freezing controlled conditions by the same protocol in mini-size (Joshi et al., 2001; Bag et al., 2002a; Joshi et al., 2005) and medium-size French plastic straws (Bag et al., 1998, 1999; Joshi et al., 2000; Bag et al., 2004).

Freezing of ram spermatozoa in hypertonic diluents has a beneficial effect on the post-thaw survival of ram spermatozoa (Fiser et al., 1981, 1982; Fiser and Fairfull, 1986, 1989). In this study for the final osmolality of the 4 TEST-yolk-glycerol extenders, the solutes and egg yolk components were constant while the difference in the osmolality was due to the contribution of permeating glycerol used as a cryoprotectant. The results of this study showed that the osmolality of hypertonic TEST-yolk-glycerol diluent had no significant effect on the post-thawing % motility and % rapidly motile ram spermatozoa indicating that glycerol associated changes in the osmolality of the diluents had no influence on motion characteristics of frozen-thawed ram spermatozoa. The highest mean post-thaw motility of spermatozoa was obtained in TEST-yolk-glycerol extender of 1,500 mOs/kg osmolality. The post-thaw motility was also comparable with the results obtained in our earlier study, where the osmolality of the TEST-yolk-glycerol extender used was 1,553 mOs/kg (Joshi et al., 2001, 2005). For ram semen freezing the optimal glycerol concentration reported by most investigators is the range of 6-8% (Salamon and Maxwell, 1995a). In this study the glycerol concentration of TEST-yolk-glycerol extender of 1,500 mOs/kg osmolality was 6.5% and was within this

range.

Sperm velocities are also important parameters because they are correlated with fertility (Budworth et al., 1988; Aitkin, 1990). In the present study the VCL was highest in TEST-yolk-glycerol extender of 1,800 mOs/kg but it was not significantly different, compared to the TEST-yolk-glycerol extender of 1,500 mOs/kg. It has been reported that rapidly moving spermatozoa with higher values of VCL have greater values of ALH (Budworth et al., 1988). In the present study ALH was significantly higher in TEST-yolk-glycerol extender of 1,500 and 1,800 mOs/kg, compared to diluents of lower osmolality.

The study indicated that ram spermatozoa could tolerate a wide osmolality range for dilution in the complete TEST-yolk-glycerol extender for their cryosurvival. The highest recovery of motile spermatozoa following thawing was achieved in samples extended in the TEST-yolk-glycerol diluent of 1,500 mOsm/kg osmolality.

## ACKNOWLEDGEMENTS

The authors are thankful to the Director of the Institute for providing all the research facilities and Shri Munir Ahmed for rendering technical assistance during this study.

## REFERENCES

Aitken, R. J. 1990. Motility parameters and fertility (Ed. C. Gagnon). Controls of sperm motility: Biological and Clinical Aspects, CRC Press, Boca Raton, pp. 285-302.

Bag, S., A. Joshi, A. K. Mathur, P. S. Rawat and J. P. Mittal. 1998. Effect of thawing temperature on motion characteristics of frozen-thawed ram spermatozoa. Indian J. Anim. Sci. 69:16-18.

Bag, S., A. Joshi, A. K. Mathur, P. S. Rawat and J. P. Mittal. 1999. Effect of thawing temperature on motion characteristics of frozen-thawed ram spermatozoa. Indian J. Anim. Sci. 69:16-18.

Bag, S., A. Joshi, P. S. Rawat and J. P. Mittal. 2002a. Effect of initial freezing temperature on the semen characteristics of frozen-thawed ram spermatozoa in a semi-arid tropical environment. Small Rumin. Res. 43:23-29.

Bag, S., A. Joshi, S. M. K. Naqvi, P. S. Rawat and J. P. Mittal. 2002b. Effect of freezing temperature, at which straws were plunged into liquid nitrogen, on the post-thaw motility and acrosomal status of ram spermatozoa. Anim. Reprod. Sci. 72:175-183.

Bag, S., A. Joshi, S. M. K. Naqvi and J. P. Mittal. 2004. Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenol. 62:415-424.

Budworth, P. R., R. P. Amann and P. I. Chapman. 1988. Relationship between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J. Androl. 9:41-54.

Davis, R. O. and D. F. Katz. 1992. Standardization and comparability of CASA instruments. J. Androl. 13:81-85.

Davis, R. O. and D. F. Katz. 1993. Operational standards for CASA instruments. J. Androl. 14:385-394.

Davis, R. O. and R. J.Siemers. 1995. Derivation and reliability of kinematic measures of sperm motion. Reprod. Fertil. Dev. 7:857-869.

Edward, A. Y., D. P. Windsor, I. W. Purvis, L. G. Sanchez-Partida and W. M. C. Maxwell. 1995. Distribution of variance associated with measurement of post-thaw function in ram sperm. Reprod. Fertil. Dev. 7:129-134.

Evans, G. and W. M. C. Maxwell. 1987. Handling and examination of semen. In: Salamon's artificial insemination of sheep and goats. Butterworths Pty Ltd., Australia, pp. 93-104.

Fiser, P. S., L. Ainsworth and G. A. Langford. 1981. Effect of osmolality of skim-milk diluents and thawing rate on cryosurvival of ram spermatozoa. Cryobiol. 18:399-403.

Fiser, P. S., L. Ainsworth and R. W. Fairfull. 1982. Cryosurvival of ram spermatozoa in hypertonic and isotonic diluents. Can. J. Anim. Sci. 62:425-428.

Fiser, P. S. and R. W. Fairfull. 1984. The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws. Cryobiol. 21:542-551.

Fiser, P. S. and R. W. Fairfull. 1986. Combined effects of glycerol concentration, cooling velocity and osmolality of skim milk diluents on cryopreservation of ram spermatozoa. Theriogenol. 25:473-484.

Fiser, P. S. and R. W. Fairfull. 1989. The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. Cryobiol. 26:64-69.

Gillan,, L., W. M. C. Maxwell and G. Evans. 2004. Preservation and evaluation of semen for artificial insemination. Reprod. Fertil. Dev. 16:447-454.

Harvey, W. A. 1990. Mixed mode least squares and maximum likelihood computer programme. In: User's Guide for LSMLMW, Ohio State University, Columbus, USA.

Holt, W. V., M. F. Head and R. D. North. 1992. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: Observation with experimental cryomicroscopy. Biol. Reprod. 46:1086-1094.

Holt, W. V. and R. D. North. 1994. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol. Reprod. 51:414-424.

Joshi, A., S. Bag and J. P. Mittal. 2000. Freezability of ram spermatozoa packaged in mini and medium size straws. Indian J. Anim. Prod. 32:25-26.

Joshi, A. and A. K. Mathur. 1996. Assessment of sperm kinematics of frozen-thawed ram spermatozoa. Indian J. Anim. Reprod. 17:42-44.

Joshi, A., S. Bag, S. M. K. Naqvi, R. C. Sharma, P. S. Rawat and J. P. Mittal. 2001. Effect of short-term and long-term preservation on motion characteristics of Garole ram spermatozoa: A prolific microsheep breed of India. Asian-Aust. J. Anim. Sci. 14:1527-1533.

Joshi, A., S. Bag, S. M. K. Naqvi, R. C. Sharma and J. P. Mittal. 2005. Effect of post-thawing incubation on sperm motility and acrosomal integrity of cryopreserved Garole ram semen. Small Rumin. Res. 56:231-238.

Kramer, C. Y. 1956. Extension of multiple range test to group

means with unequal numbers of replicates. Biomet. 12:307-310.

Maxwell, W. M. C. and P. F. Watson. 1996. Recent progress in the preservation of ram semen. Anim. Reprod. Sci. 42:55-65.

Pontbriand, D., J. G. Howard, M. C. Schiewe, L. D. Stuart and D. E. Wildt. 1989. Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa. Cryobiol. 26:341-354.

Salamon, S. and W. M. C. Maxwell. 1995a. Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. 37:189-249.

Salamon, S. and W. M. C. Maxwell. 1995b. Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. Anim. Reprod. Sci. 38:1-36.

Salamon, S. and W. M. C. Maxwell. 2000. Storage of ram semen. Anim. Reprod. Sci. 62:77-111.

Sanchez-Partida, L. G., B. P. Setchell and W. M. C. Maxwell. 1998. Effect of compatible solutes and diluent composition on the post-thaw motility of ram sperm. Reprod. Fertil. Dev. 10:347-357.

Schmehl, M. K., S. P. Anderson, I. A. Vazques and E. F. Graham. 1986. The effect of dialysis of extended ram semen prior to freezing on post-thaw survival and fertility. Cryobiol. 23:406-414.

Watson, P. F. 1995. Recent developments and concepts in the conservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7:871-891.

Watson, P. F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61:481-492.

Yoshida, M. 2000. Conservation of sperms: current status and new trends. Anim. Reprod. Sci. 60-61:349-355.

Zhou, J. B., K. Z. Yue, M. J. Lou, Z. L. Chang, H. Liang, Z. Y. Wang and J. H. Tan. 2004. Effect of extenders and temperatures on sperm viability and fertilizing capacity of Harbin White boar semen during long-term liquid storage. Asian-Aust. J. Anim. Sci. 17:1501-1508.