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# Quality Assurance of Cryopreserved Buck Semen by Assessing Structural and Functional Integrity of Spermatozoa

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**ABSTRACT**

A total of 81 ejaculates collected from 4 Tellicherry and 2 Boer bucks were utilized to evaluate the structural and functional integrity of spermatozoa as a measure for quality assurance of cryopreserved buck semen. The semen samples were diluted with tris-egg yolk-glycerol based extender and frozen in straws. Only samples having 40% or more of post thaw motility (PTM) were regarded as "acceptable" samples for artificial insemination. The "acceptable" samples were further evaluated by hypo osmotic swelling test (HOST), sperm morphology and acrosome integrity assessment. Individual genotypes have shown significant variations ($P < 0.01$) for PTM. Significant variations ($P < 0.01$) were seen between bucks and between ejaculations of two Tellicherry bucks for hypo osmotic reacted spermatozoa. The differences in mean values for hypo osmotic reacted spermatozoa between I and II ejaculations of Tellicherry bucks were significant ($P < 0.01$). Significant variations ($P < 0.05$) were also observed for hypo osmotic reacted spermatozoa between I ejaculations of Tellicherry and Boer bucks. The variations in means of intact acrosome percent between I and II ejaculations of Tellicherry bucks was significant ($P < 0.05$). Besides post thaw motility, incorporation of structural and functional integrity tests like HOST and acrosome integrity in semen evaluation protocol add value to quality assurance of frozen buck semen.

**Keywords:** Buck semen, cryopreservation, quality assurance

Goat husbandry is one of the important livelihood options for small and marginal farmers of the developing counties especially of the Indian subcontinent. However, in comparison with bovines, not much genetic improvement has been achieved in goats. Artificial insemination (AI) with frozen semen can play a vital role in not only to progress towards the goal of augmenting genetic potential of goats but also to facilitate extension of reproductive potential of superior males beyond its life time and to conserve the endangered breeds by cryoconservation of germ-plasm in haploid form. In general, the fertility of frozen semen is lower than the fresh semen (Lemma, 2011; Apu *et al.*, 2012) because the irreversible changes in the sperm structure result in impairment of motility, membrane integrity and fertilizing ability of spermatozoa (Talaei *et al.*, 2010; Forero-Gonzalez *et al.*, 2012) . Therefore adoption of a suitable protocol for cryopreservation and assuring the quality of cryopreserved semen are the prerequisites for successful implementation of AI in goat breeding.

Under practical conditions there is a bias towards sperm motility estimation for assessing the quality of frozen semen. The subjectivity and the high variations existing in sperm motility evaluation among observers limit the accurate assessment of fertility of frozen semen. One of the structures of the spermatozoon which plays a key role in maintaining its viability, thereby its function namely fertilizing ability is the plasma membrane (Jayendran *et al.*, 1984; Ansari *et al.*, 2010). And therefore the test of plasma membrane integrity could be a measure of fertility of the spermatozoa (Jayendran *et al.*, 1984; Padrik *et al.*, 2012). Further, reduction in fertility may also be due to decrease in morphologically normal sperm in semen. The acrosome of spermatozoa has a significant role during fertilization by helping the penetration of zona pellucida of the oocyte. Therefore, acrosome integrity is essentially related to fertility (Brick *et al.*, 2010). There is no evidence to suggest a single *in-vitro* test that accurately predicts the

fertility of a semen sample. Therefore, a combination of tests is required at least to reasonably assure the quality of frozen semen. With this background, this study has been undertaken to assess the value of certain structural and functional integrity tests in addition to routine sperm motility evaluation to assure the quality of cryopreserved buck semen.

## MATERIALS AND METHODS

### Animal population and management

A total of six adult bucks, four Tellicherry and two Boer bucks maintained at Frozen Semen Bank of Madras Veterinary College, Chennai, India under standardized management conditions were involved in the study. The bucks were provided with *ad lib* green fodder and water. In addition, 0.5 kg of concentrate mixture/day/animal was given. The experiment was undertaken during June-July 2014.

### Semen collection, evaluation and cryopreservation

Semen samples were collected twice a week using artificial vagina. Each collection schedule consisted of two ejaculations per buck. A total of 81 ejaculations were collected from six bucks. The ejaculates were evaluated for volume, colour, consistency and presence of any foreign bodies and mass activity by conventional methods. Equal volume of tris based extender containing 20% of egg yolk and 7% glycerol were added to the ejaculates for evaluation of progressive sperm motility using phase contrast microscope.

Samples having 70% or more of sperm motility were further processed for freezing. The extended semen was equilibrated at 5ºC for 3 hrs, filled in French straws (0.25 ml) and frozen by exposing the semen filled straws horizontally to liquid nitrogen vapour, at 5 cm above the liquid nitrogen level for 20 minutes. The frozen straws were immersed in liquid nitrogen for final freezing and storage. After 24 hrs of storage, the samples were thawed in a water bath at 37ºC for 30 seconds and the freezability was assessed by estimation of post thaw motility (PTM) using phase contrast microscope. Samples having 40% PTM were graded as “acceptable” for AI use.

### Hypo osmotic swelling test

The structural integrity of plasma membrane was tested by hypo osmotic swelling test (HOST). Hypo osmotic medium of 100 mOsm was prepared by mixing 0.450 g of fructose and 0.245 g of sodium citrate in 50 ml of distilled water (Khalili *et al.*, 2009) and 200 µl of hypo osmotic medium was mixed with 20 µl of semen, incubated at 37°C for 30 minutes. After incubation 100 µl of the mixture was spread on a glass slide, dried in air and stained with 5% Rose Bengal stain for 10 minutes. After washing and drying, a total of 200 spermatozoa were counted using phase contrast microscope. Spermatozoa showing varying degrees of tail curling were considered as hypo osmotic reacted spermatozoa.

### Sperm morphology estimation

Thin smears of frozen thawed semen were prepared, air dried and stained with 5% Rose Bengal stain for 10 minutes. After washing and air drying, a total of 200 spermatozoa were counted for sperm morphology assessment using phase contrast microscope. Sperm with normal shape and size of head, mid piece and tail were considered as morphologically normal.

### Acrosome integrity evaluation

Semen smears were air dried and fixed in 5% formaldehyde for 30 minutes. After fixing, the slides were washed, air dried and stained with 3% Giemsa stain at 37°C for 48 hrs. The stained slides were washed, dried and 200 spermatozoa were counted under phase contrast microscope. Spermatozoa with tightly adhered intact acrosome with a smooth surface and periphery and a distinct uniformly shaped apical ridge were classified as sperm with intact acrosome (Blom, 1992).

### Statistical analysis

The experiment was based on completely randomized design and the statistical analysis of the data was done using the software package SPSS v20. The results, presented as mean ± SE, were analysed for significance ($P < 0.05$) by independent sample t-test and one way analysis of variance. The means within groups were also tested for significance (Duncan, 1995).

## RESULTS AND DISCUSSION

We found that the overall PTM was 47.53% and it varied from 45.09% in Tellicherry bucks to 51.67% in Boer bucks. Individual genotypes have shown significant variations ($P < 0.01$) for PTM (Table 1). Significant ($P < 0.05$) differences were seen between ejaculations of most of the Tellicherry and Boer bucks for PTM (Table 2).

The mean value for hypo osmotic reacted spermatozoa was 43.03% and significant variations ($P < 0.01$) were seen between bucks (Table 1) and between ejaculations of two Tellicherry bucks for hypo osmotic reacted spermatozoa (Table 2). Further, the variations observed in the means for hypo osmotic reacted spermatozoa between I and II ejaculations of all the bucks were significant ($P < 0.01$) (Table 3).

The differences in mean values for hypo osmotic reacted spermatozoa between I and II ejaculations of Tellicherry bucks were significant ($P < 0.01$) (Table 4). Significant variations ($P < 0.05$) were also observable for hypo osmotic reacted spermatozoa between I ejaculations of Tellicherry and Boer bucks (Table 5).

**Table 1: Mean (±SE) values for semen characteristics of cryopreserved buck semen**

| Breed | SireID | Ejaculation | n | Postthaw motility (%) | Hypoosmotic reacted spermatozoa (%) | Morphologically normal spermatozoa (%) | Intact acrosome (%) |
|---|---|---|---|---|---|---|---|
| Tellicherry | 39 | I | 5 | 40.00 ± 0.00 | 42.2 ± 0.86 | 85.24 ± 1.17 | 88.30 ± 2.48 |
| | | II | 4 | 42.50 ± 2.50 | 30.38 ± 3.39 | 87.05 ± 0.96 | 83.38 ± 1.84 |
| | | I & II | 9 | 41.11 ± 1.11 | 36.94 ± 2.54 | 86.04 ± 0.79 | 86.11 ± 1.74 |
| | 40 | I | 7 | 41.43 ± 1.43 | 47.21 ± 2.01 | 86.37 ± 1.62 | 85.64 ± 1.73 |
| | | II | 7 | 44.29 ± 2.02 | 46.14 ± 3.04 | 89.39 ± 1.39 | 85.36 ± 0.74 |
| | | I & II | 14 | 42.86 ± 1.25 | 46.68 ± 1.75 | 87.88 ± 1.11 | 85.50 ± 0.91 |
| | 41 | I | 7 | 42.86 ± 1.84 | 44.93 ± 1.58 | 89.36 ± 1.79 | 84.86 ± 1.09 |
| | | II | 7 | 50.00 ± 3.78 | 39.64 ± 4.59 | 87.35 ± 1.59 | 86.21 ± 1.45 |
| | | I & II | 14 | 46.43 ± 2.25 | 42.29 ± 2.45 | 88.35 ± 1.18 | 85.54 ± 0.89 |
| | 44 | I | 7 | 51.43 ± 3.40 | 44.29 ± 3.81 | 87.67 ± 1.75 | 86.57 ± 2.87 |
| | | II | 7 | 45.71 ± 2.97 | 45.00 ± 6.39 | 88.45 ± 1.59 | 84.79 ± 0.92 |
| | | I & II | 14 | 48.57 ± 2.31 | 44.64 ± 3.58 | 88.06 ± 1.14 | 85.68 ± 1.47 |
| | All sires | I | 26 | 44.23 ± 1.38 | 44.85 ± 1.22 | 87.31 ± 0.84 | 86.19 ± 1.03 |
| | | II | 25 | 46.00 ± 1.53 | 41.48 ± 2.54 | 88.18 ± 0.74 | 85.12 ± 0.59 |
| | | I & II | 51 | 45.09 ± 1.02 | 43.19 ± 1.40 | 87.74 ± 0.56 | 85.67 ± 0.59 |
| Boer | 2121 | I | 7 | 54.29 ± 2.97 | 41.64 ± 3.78 | 86.36 ± 1.37 | 84.93 ± 1.28 |
| | | II | 8 | 51.25 ± 4.41 | 46.81 ± 3.34 | 86.04 ± 1.21 | 88.06 ± 1.55 |
| | | I & II | 15 | 52.67 ± 2.67 | 44.40 ± 2.51 | 86.19 ± 0.88 | 86.60 ± 1.07 |
| | 2157 | I | 8 | 51.25 ± 3.50 | 41.94 ± 3.92 | 87.02 ± 1.25 | 86.50 ± 1.45 |
| | | II | 7 | 50.00 ± 3.09 | 40.14 ± 4.36 | 87.13 ± 1.76 | 83.71 ± 0.82 |
| | | I & II | 15 | 50.67 ± 2.28 | 41.10 ± 2.82 | 87.08 ± 1.02 | 85.20 ± 0.91 |
| | All sires | I | 15 | 52.67 ± 2.28 | 41.80 ± 2.64 | 86.71 ± 0.89 | 85.77 ± 0.97 |
| | | II | 15 | 50.67 ± 2.67 | 43.70 ± 2.75 | 86.55 ± 1.02 | 86.03 ± 1.05 |
| | | I & II | 30 | 51.67 ± 1.73 | 42.75 ± 1.88 | 86.63 ± 0.67 | 85.90 ± 0.70 |
| Overall (Both Breeds) | All sires | I | 41 | 47.32 ± 1.35 | 43.73 ± 1.24 | 87.09 ± 0.62 | 86.04 ± 0.73 |
| | | II | 40 | 47.75 ± 1.41 | 42.31 ± 1.88 | 87.57 ± 0.60 | 85.46 ± 0.54 |
| | | I & II | 81 | 47.53 ± 0.97 | 43.03 ± 1.12 | 87.33 ± 0.43 | 85.75 ± 0.45 |

**Table 2:** Mean (± SE) values for sperm characteristics of cryopreserved goat spermatozoa between I and II ejaculation within bucks

| Breed | Buck | I Ejaculation | | | | II Ejaculation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PTM | HOST | Morphology | Acrosome | PTM | HOST | Morphology | Acrosome |
| Tellicherry | 39 (5) | 40.00 ± 0.00$^{a}_{*}$ | 42.2 ± 0.86$^{b}_{**}$ | 85.24 ± 1.17 | 88.30 ± 2.48 | 42.50 ± 2.50$^{b}_{*}$ | 30.38 ± 3.39$^{a}_{**}$ | 87.05 ± 0.96 | 83.38 ± 1.84 |
| | 40 (7) | 41.43 ± 1.43$^{a}_{*}$ | 47.21 ± 2.01 | 86.37 ± 1.62 | 85.64 ± 1.73 | 44.29 ± 2.02$^{b}_{*}$ | 46.14 ± 3.04 | 89.39 ± 1.39 | 85.36 ± 0.74 |
| | 41 (7) | 42.86 ± 1.84$^{a}_{*}$ | 44.93 ± 1.58$^{b}_{**}$ | 89.36 ± 1.79 | 84.86 ± 1.09 | 50.00 ± 3.78$^{b}_{*}$ | 39.64 ± 4.59$^{a}_{**}$ | 87.35 ± 1.59 | 86.21 ± 1.45 |
| | 44 (7) | 51.43 ± 3.40 | 44.29 ± 3.81 | 87.67 ± 1.75 | 86.57 ± 2.87 | 45.71 ± 2.97 | 45.00 ± 6.39 | 88.45 ± 1.59 | 84.79 ± 0.92 |
| Boer | 2121 (7) | 54.29 ± 2.97$^{b}_{*}$ | 41.64 ± 3.78 | 86.36 ± 1.37 | 84.93 ± 1.28 | 51.25 ± 4.41$^{a}_{*}$ | 46.81 ± 3.34 | 86.04 ± 1.21 | 88.06 ± 1.55 |
| | 2157 (8) | 51.25 ± 3.50 | 41.94 ± 3.92 | 87.02 ± 1.25 | 86.50 ± 1.45 | 50.00 ± 3.09 | 40.14 ± 4.36 | 87.13 ± 1.76 | 83.71 ± 0.82 |

Figures in parenthesis indicate number of observations

PTM: Post thaw sperm motility percent, HOST: Hypo osmotic reacted sperm percent, Morphology: Morphologically normal sperm percent, Acrosome: Intact acrosome percent

Figures with different alphabets as superscripts within a row for each characteristic between I and II ejaculation differ significantly

*= P ≤ 0.01

**= P ≤ 0.05

Morphologically per cent normal sperm was observed to be 87.33 and the difference between bucks/ejaculations was non-significant (P > 0.05). The per cent intact acrosome was 85.75 (Table 1) and variations in means of per cent intact acrosome between I and II ejaculations of Tellicherry bucks were significant (P < 0.05) (Table 4).

Most of the previous results on PTM of buck semen ranged from 31.67 to 38.80% (Dorado *et al.*, 2007; Sundararaman and Edwin, 2008; Khalili *et al.*, 2009; Dorado *et al.*, 2010; Mohammad *et al.*, 2012). Ahmed *et al.* (2014) and Ustuner *et al.* (2015) have given higher values for PTM. Very low PTM (13.2%) for caprine semen was also reported for which the authors (Ramukhithi *et al.*, 2011) attribute the acidic pH of semen. The reason for consistently higher PTM values in the present study was that only samples with “acceptable” quality (40% or more for PTM) alone were considered for the analysis and the present results simulate the findings for “good” samples in an earlier experiment conducted in our laboratory (Sundararaman and Edwin, 2008).

Significant variation seen between individual genotypes for PTM in this study might be due to the fact that the

**Table 3: Mean (±SE) values for semen characteristics of cryopreserved goat semen – between ejaculations**

| Ejaculation | n | PTM (%) | Hypoosmotic reacted spermatozoa (%) | Morphologically normal spermatozoa (%) | Intact acrosome (%) |
|---|---|---|---|---|---|
| I | 41 | 47.32 ± 1.35 | 43.73 ± 1.24 | 87.09 ± 0.62 | 86.04 ± 0.73 |
| II | 40 | 47.75 ± 1.41 | 42.31 ± 1.88 | 87.57 ± 0.60 | 85.46 ± 0.54 |
| Significance of difference between means | | Non-significant | Significant (P < 0.01) | Non-significant | Non-significant |

**Table 4: Mean (±SE) values for semen characteristics of cryopreserved buck semen – between overall I and II ejaculation within breeds**

| Breed | Ejaculation | N | PTM (%) | Hypoosmotic reacted spermatozoa (%) | Morphologically normal spermatozoa (%) | Intact acrosome (%) |
|---|---|---|---|---|---|---|
| Tellicherry | I | 26 | 44.23 ± 1.38 | 44.85 ± 1.22 | 87.31 ± 0.84 | 86.19 ± 1.03 |
| | II | 25 | 46.00 ± 1.53 | 41.48 ± 2.54 | 88.18 ± 0.74 | 85.12 ± 0.59 |
| Significance of difference | | | NS | Significant (P≤0.01) | NS | Significant (P≤0.05) |
| Boer | I | 15 | 52.67 ± 2.28 | 41.80 ± 2.64 | 86.71 ± 0.89 | 85.77 ± 0.97 |
| | II | 15 | 50.67 ± 2.67 | 43.70 ± 2.75 | 86.55 ± 1.02 | 86.03 ± 1.05 |
| Significance of difference | | | NS | NS | NS | NS |

N= No of Observations

NS = Non-significant

ability of spermatozoa to withstand the effects of freezing and thawing varies with individual bucks (Watson, 1995; Dorado, *et al.,* 2010; Baren *et al.,* 2012). And genetic bases also exist for variations in post thaw semen quality between individuals (Thurston *et al.,* 2002).

### Hypo osmotic reacted spermatozoa

Plasma membrane plays an important role in maintaining the structural and biochemical function integrity of spermatozoa within the limits of osmotic stress. When exposed to hypo osmotic conditions, the biochemically active sperm cell membrane absorbs fluid from the extracellular medium to maintain equilibrium between fluid compartments, intracellular and extracellular. Absorption of fluid results in swelling of the cell due to expansion of the cell membrane which in turn culminates in curling of the tail (Jayendran *et al.,* 1984). Therefore, the proportion of hypo osmotic reacted spermatozoa in semen indicates the presence of spermatozoa with normal structural and functional integrity. In our study, the mean value for hypo osmotic reacted spermatozoa in "acceptable" samples was higher (Khalili *et al.,* 2009) but lower (Ahmed *et al.,* 2014) than the previous reports. Significant variations observed between individual bucks and between ejaculations for hypo osmotic reacted spermatozoa per cent in the present experiment were also similar to the findings of Fonseca *et al.* (2005). Further the significant difference between first ejaculation of Tellicherry and Boer bucks validate the existence of breed difference.

### Sperm morphology

Association of increased morphological abnormalities of sperm with reduced reproductive efficiency has been documented in bucks (Skalet *et al.,* 1988). The size of the sperm heads also vary greatly among bucks (Gravance *et al.,* 1995). The mean percentage of morphologically normal spermatozoa recorded in this study was higher and lower than the reports of Ustuner *et al.* (2009) and Dorado *et al.* (2010) respectively but similar to the observation of Ahmed *et al.* (2014). Although buck effect has significant

**Table 5: Mean (±SE) values for semen characteristics of cryopreserved buck semen – between ejaculation and breeds**

| Ejaculation | Breed | N | PTM (%) | Hypoosmotic reacted spermatozoa (%) | Morphologically normal spermatozoa (%) | Intact acrosome (%) |
|---|---|---|---|---|---|---|
| I | Tellicherry | 26 | 44.23 ± 1.38 | 44.85 ± 1.22 | 87.31 ± 0.84 | 86.19 ± 1.03 |
| | Boer | 15 | 52.67 ± 2.28 | 41.80 ± 2.64 | 86.71 ± 0.89 | 85.77 ± 0.97 |
| Significance of difference | | | NS | Significant (P≤0.05) | NS | NS |
| II | Tellicherry | 25 | 46.00 ± 1.53 | 41.48 ± 2.54 | 88.18 ± 0.74 | 85.12 ± 0.59 |
| | Boer | 15 | 50.67 ± 2.67 | 43.70 ± 2.75 | 86.55 ± 1.02 | 86.03 ± 1.05 |
| Significance of difference | | | NS | NS | NS | NS |

N= No of Observations

NS= Non-significant

influence on morphological characteristics (Dorado *et al.*, 2010) we could not find any significant variation between bucks or ejaculations for sperm morphology.

### Acrosome integrity

Results obtained in this experiment for acrosome intact spermatozoa percent corresponds well with the report of (Khalili *et al.*, 2009). However, some of the authors (Ahmed *et al.*, 2014; Ustuner *et al.*, 2009; Dorado *et al.*, 2009; Ustuner *et al.*, 2015) have recorded the intact acrosome in the range of 45.20 to 60.8%. The significant variation between ejaculations in Tellicherry bucks observed in our experiment was similar to the finding of Dorado *et al.* (2010).

From this study, we found that despite non-existence of any significant difference for PTM between ejaculations, the hypo osmotic reacted spermatozoa percent showed significant difference. Furthermore, significant differences were seen for per cent hypo osmotic reacted spermatozoa and per cent intact acrosome between I and II ejaculations of Tellicherry bucks without exhibiting any significant difference between PTM. These observations in samples considered as "acceptable" quality for AI, emphasize the fact that post thaw motility alone cannot considered as a good indicator of frozen semen quality and further suggest that HOST and acrosome integrity estimation may add value to the quality assessment of frozen semen of bucks. Therefore, for quality assurance of cryopreserved buck semen for AI, as in the case of bovines, a combination of tests has to be evolved essentially incorporating structural and functional integrity evaluations like HOST and acrosome integrity besides post thaw motility and other tests, perhaps to achieve better fertility on AI. Nevertheless, further research on *in-vivo* fertility to find the difference if any in fertility on AI with "acceptable" quality frozen semen but differing in hypo osmotic reacted spermatozoa and intact acrosome per cent, is required to validate the findings of the present study.

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