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Post-thaw quality of Malabari buck semen with different freezing resilience#

K. Lekshmi Bhai[1], B. Bibin Becha[2*], C. Jayakumar[3], Hiron, M. Harshan[2], M. Shynu[4] and R. Thirupathy Venkatachalapathy[5]
Department of Animal Reproduction, Gynaecology and Obstetrics
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University
Kerala, India

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Abstract

The study was conducted to assess the difference in post-thaw quality parameters of Malabari buck spermatozoa of good and poor semen freezability. The ejaculates collected from Malabari bucks of good (n=3) and poor (n=3) semen freezability were pooled separately as group A and group B, respectively after the initial evaluation. The extended semen in each group was processed and cryopreserved by the conventional method of static vapour freezing. After thawing, the quality of semen in both groups was assessed in terms of progressive motility, viability, morphological abnormalities, acrosome integrity, functional membrane integrity and lipid peroxidation status (MDA) of spermatozoa. Results showed that there was no significant difference between the fresh semen characteristics of group A and B bucks except for concentration, progressive motility and functional membrane integrity of spermatozoa. But, post-thaw quality of groups A and B differed significantly in terms of progressive motility (48.89 ± 0.64 % vs 18.06 ± 1.08 %), viability (55.17 ± 0.57 % vs 26.33 ± 0.95 %), acrosome integrity (62.44 ± 0.62 % vs 33.42 ± 0.34 %), functional membrane integrity (47.00 ± 0.36 % vs 23.36 ± 0.63 %) and MDA concentration (7.83 ± 0.29 nmol/mL vs 13.95 ± 0.08 nmol/mL) whereas morphological abnormalities (2.11 ± 0.08 % vs 2.39 ± 0.09 %) did not differ significantly. The findings of the present study showed that even with similar fresh semen characteristics, spermatozoa from different Malabari bucks have varying degrees of cryosurvivability.

Keywords: *Malabari buck spermatozoa, good semen freezability, poor semen freezability, freezing resilience, cryopreservation*

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1. *PhD scholar*
2. *Associate professor*
3. *Associate professor and head*
5. *Professor, Department of Veterinary Biochemistry*
6. *Professor, Department of Animal Genetics and Breeding*

**Corresponding author: bibinbecha@gmail.com, Ph. 9447971872*

Cryopreservation of spermatozoa has been proven as the most essential tool for the long-term preservation of germplasm of genetically superior males even after their lifetime, relevant transgenic lines and endangered species. It also makes it possible to use the preserved semen over a distance. However, the process of freezing and thawing inflicts irreversible damage to the spermatozoan membrane through alteration of membrane structure, loss of proteins and production of reactive oxygen (Frau *et al*., 2020). These factors negatively affect the post-thaw fertilisation potential.

There exists inter-male variability in the response to cryopreservation and the semen of certain males consistently freezes with less cryodamage compared to others irrespective of the freezing protocol adopted, so they can be considered as 'good freezers' (Leboeuf *et al*., 2000). Studies revealed that the animals with identical sperm quality parameters in the neat semen had varying cryotolerance of spermatozoa and also some of the naturally fertile animals were found to be of poor semen freezability (Kumar *et al*., 2019).

Thus, the present study was carried out to evaluate and compare the post-thaw quality parameters of the spermatozoa from Malabari bucks of different semen cryosurvivability.

Materials and methods

The study was conducted at the Artificial Insemination Centre, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy using 108 semen ejaculates collected from Malabari bucks of good and poor semen freezability (three each) aged two to three years and weighed 42-46 kg, maintained under uniform feeding, housing and other managemental conditions. The animals with previous records of semen freezability were selected for the study following a minimum of six confirmatory cryopreservations and post-thaw evaluation for each buck. Thus, three animals with more than 35 per cent post-thaw progressively motile spermatozoa were included in the group of good semen freezability (Group A) and those with less than 30 per cent post-thaw progressively motile spermatozoa were considered as of poor semen freezability (Group B).

Semen ejaculates were collected from the selected bucks using Danish-type artificial vagina (12×3.8 cm) twice weekly at an interval of two to three days (Urmila *et al*., 2023). After the initial evaluation (Behera *et al*., 2015a), those ejaculates with more than 80 per cent progressively motile spermatozoa of group A were pooled and the same procedure was adopted for group B. The pooled semen was extended to 400 million progressively motile spermatozoa (Bhai and Joseph, 2015) in the conventional tris egg yolk-based extender, packed in French medium straws (IMV, France), sealed with polyvinyl alcohol powder (IMV, France) and equilibrated for 4h at 5°C in the cold handling unit. The conventional method of freezing was carried out using static vapour freezing in a styrofoam box for 10 min and the straws were plunged in liquid nitrogen and stored in a cryogenic liquid nitrogen container (BA 7).

Post-thaw quality was evaluated in terms of progressive motility, viability, morphology, acrosome integrity, functional membrane integrity and lipid peroxidation status of spermatozoa in each group. Per cent of viability and morphological abnormalities of spermatozoa were assessed with eosin-nigrosine staining and acrosome integrity was assessed with Giemsa staining. The functional membrane integrity of spermatozoa was assessed with hypo-osmotic sperm swelling test using a hypoosmotic solution of 100 mOsm/L. The lipid peroxidation status of spermatozoa was assessed in terms of malondialdehyde concentration (nmol/L) using thiobarbituric acid assay method (Lone *et al*., 2017).

The data was statistically analysed using SPSS (Statistical Package for Social Studies) software version 24.0 in which the fresh and post-thaw semen characteristics were analysed with a student t-test.

Results and discussion

After the initial evaluation of the ejaculates, it was observed that there was

no significant difference in the semen characteristics of group A and B bucks except for concentration, progressive motility and functional membrane integrity of spermatozoa (Table 1). However, no significant difference was observed in the fresh semen characteristics of Malabari bucks with different semen freezability by John (2016) and Krishnan (2017). Regarding the values of fresh semen characteristics of Malabari buck semen, similar and lower results were previously reported (Behera *et al*., 2015b; Bhai *et al*., 2015). The difference in observations within the breed itself can be substantiated according to Hidalgo *et al*· (2006) who reported that animal-to-animal variation could happen in the semen characteristics of bucks of a particular breed·

After freezing and thawing, progressive motility (48·89 ± 0·64 % *vs* 18·06±1·08 %) differed significantly (p<0.01) in groups A and B (Fig 1). In accordance with the present observations, John (2016) and Krishnan (2017) reported a significant difference in the per cent of post-thaw progressive motility (40·63 ± 1·98 % *vs* 17·17 ± 1·00 %; 51·67 ± 1·67 % *vs* 19·17 ± 0·83 %). Lower cholesterol content in the sperm membrane of bucks with poor semen freezability (Krishnan, 2017) might have made those spermatozoa more susceptible to cryodamage and in turn loss of motility. The difference in the semen proteome of good and poor semen freezability group of bucks also might have contributed to this. During the process of cryopreservation, proteins associated with motility like Actin β are lost or decreased in abundance (Pini *et al*·, 2018). The degree of cryoelution of such proteins might have been higher in the poor semen freezability group. Considering the per cent of post-thaw progressive motility, the previously recorded values in Malabari buck semen cryopreserved with tris egg yolk-based extender such as 40·50 ± 1·65 per cent (Behera *et al*., 2015c) and 46·50 ± 0·93 per cent (Bhai *et al*., 2015) are similar to the present result of good semen freezability group· But, higher values were reported in other breeds as 72·77 ± 0·65 per cent in Jamunapari (Reza *et al*·, 2021).

Post-thaw viability (Fig 2) was also significantly higher (55·17 ± 0·57 %) in group A (p<0.01) compared to group B (26·33 ± 0·95 %). The same trend (46·11 ± 1·02 % *vs* 21·36 ± 1·04 %; 57·00 ± 1·13 % *vs* 22·67 ± 0·80 %) was noticed by John (2016) and Krishnan (2017). Comparatively higher cholesterol content in the sperm membrane of good semen freezability bucks might have stabilized the sperm membrane and maintained better viability after cryopreservation. Following cryopreservation, the proteins associated with membrane integrity were found to be retained more in spermatozoa of good freezability. The proteins related to ROS protection like superoxide dismutase decrease in abundance with freezing (Pini *et al.,* 2018) which affects the integrity of the sperm membrane. The degree of such protein loss might have been lesser in the good semen freezability group. A similar percentage of viable spermatozoa could be obtained as 58·77 ± 1·89 per cent in Malabari bucks by Behera *et*

Table 1· Characteristics of fresh semen ejaculates from Malabari bucks of good and poor semen freezability

Seminal parameters	N	Type of semen freezability	
		Good	Poor
Volume (mL)	54	1·00 ± 0·02	0·96 ± 0·02
Progressive motility (%)	54	85·56 ± 0·43[a]	82·31 ± 0·37[b]
Concentration (millions/mL)	54	3955·19 ± 38·97[a]	3776·48 ± 38·32[b]
Viability (%)	8	90·88 ± 0·36	89·81 ± 0·46
Morphological abnormalities (%)	8	0·75 ± 0·09	0·81 ± 0·13
Functional membrane integrity (%)	8	82·44 ± 0·24[a]	80·38 ± 0·16[b]
Acrosome integrity %)	8	92·00 ± 0·33	91·81 ± 0·16

Values having different superscripts in a row differ significantly

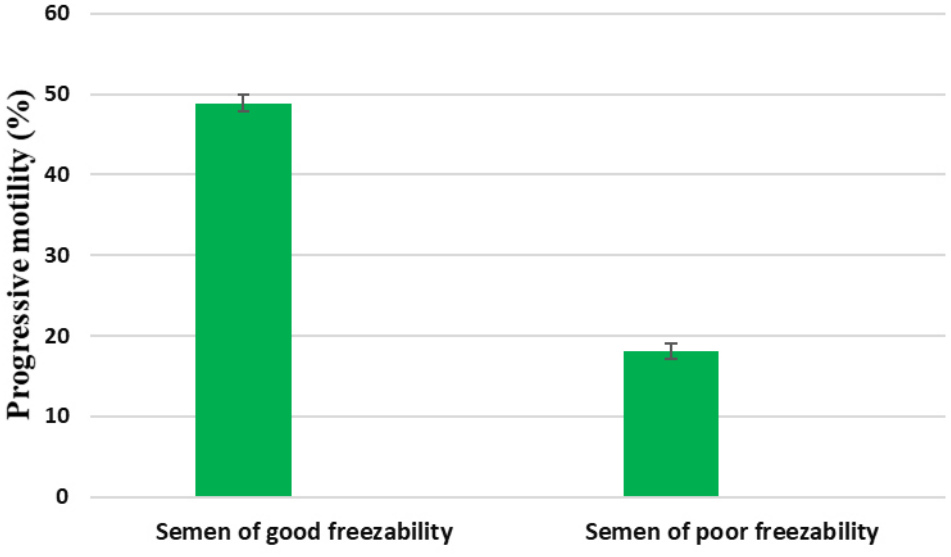

Fig.1. Post-thaw progressive motility of Malabari buck spermatozoa of good and poor freezability

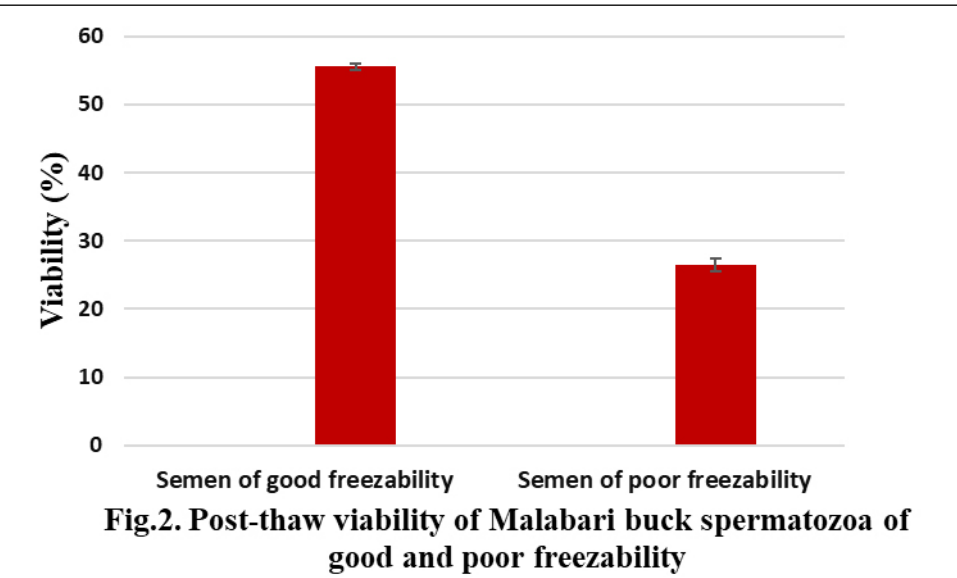

Fig.2. Post-thaw viability of Malabari buck spermatozoa of good and poor freezability

al. (2015d) whereas Bhai and Joseph (2015) reported higher values (68·28 ± 1·25 %)· Lower per cent of viable spermatozoa like that of the poor semen freezability group in the present study was also reported previously as 29·7 ± 1·43 per cent in Baladi bucks (Ismail *et al*·, 2020). The difference in values can be justified by the animal-to-animal variation and difference in processing before cryopreservation which may affect the post-thaw viability of spermatozoa (Memon *et al*·, 2011)·

Cryopreserved semen of groups A and B had 62·44 ± 0·62 per cent and 33·42 ± 0·34 per cent spermatozoa with intact acrosome (Fig 3), respectively where the values differed significantly ($p < 0·01$)· Similarly, John (2016) and Krishnan (2017) reported significant

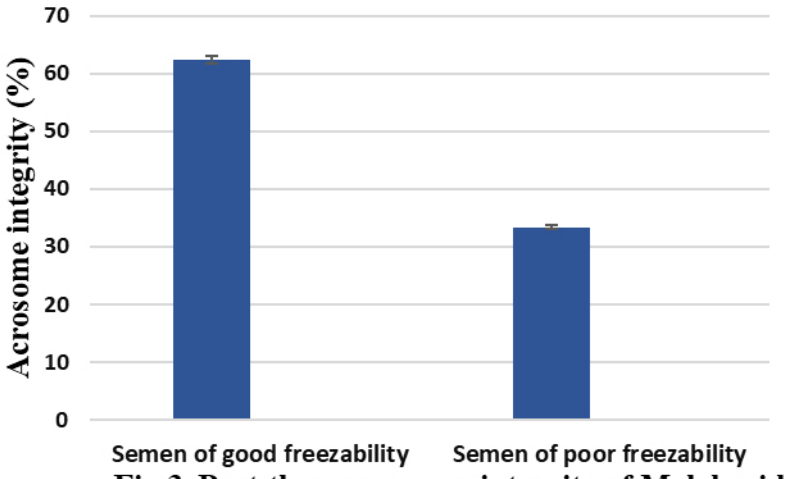

Fig.3. Post-thaw acrosome integrity of Malabari buck spermatozoa of good and poor freezability

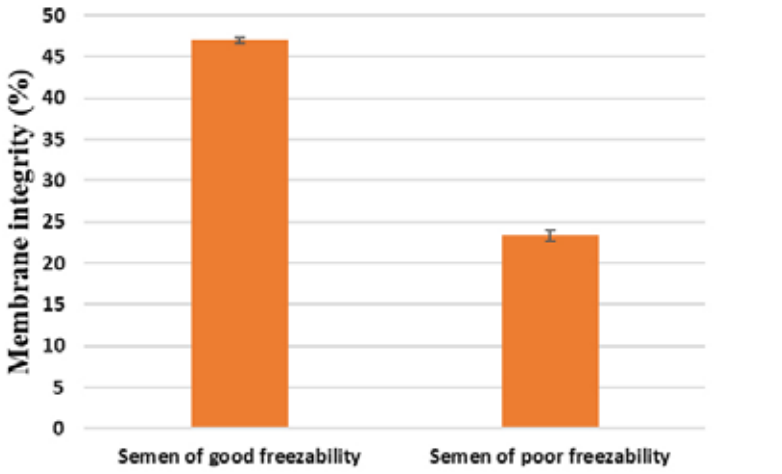

Fig.4. Post-thaw functional membrane integrity of Malabari buck spermatozoa of good and poor freezability

differences in the percentage of acrosome integrity (56·99 ± 0·82 % *vs* 31·71 ± 1·22 %; 56·33 ± 1·12 % *vs* 29·50 ± 1·23 %) of Malabari buck spermatozoa of good and poor freezability. In the bucks of poor semen freezability, the sperm membrane cholesterol level might be inadequate to maintain the membrane integrity which might have enhanced its fusion with outer acrosomal membrane and thereby acrosome reaction. With the same extender, the per cent of post-thaw acrosome integrity of spermatozoa was 63·33 ± 1·05 per cent in the crossbred Malabari bucks (Shiny, 2011) which is similar to the present observation in the good semen freezability group whereas higher values such as 75.84 ± 1.25 per cent (Behera *et al*., 2015d) were obtained in Malabari bucks. However, post-thaw acrosome integrity was found to be lower as 20.30 ± 1.05 per cent in the Beetal breed (Iqbal *et al*., 2015).

There was a significant difference ($p<0·01$) in the per cent of spermatozoa with functionally intact membrane (Fig.4) in the cryopreserved semen of Malabari bucks with good and poor semen freezability (47·00 ±

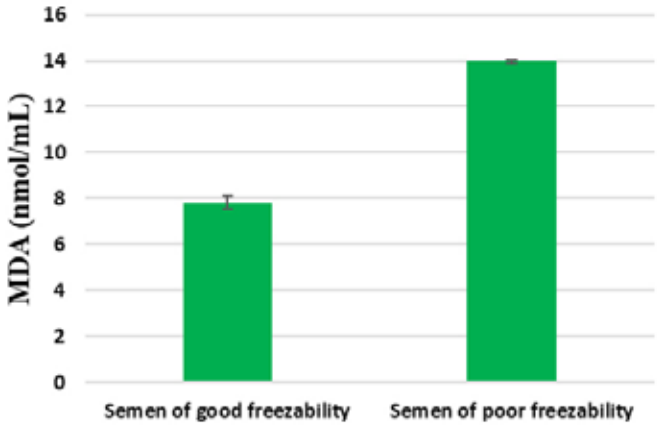

Fig.5. Post-thaw malondialdehyde concentration of Malabari buck spermatozoa of good and poor freezability

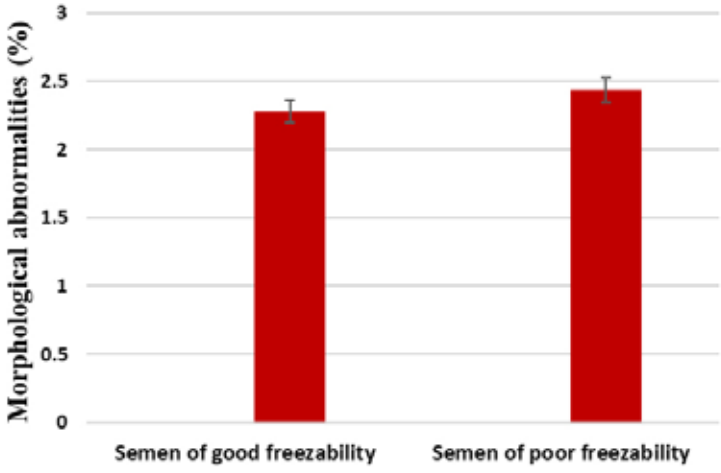

Fig.6. Post-thaw morphological abnormalities of Malabari buck spermatozoa of good and poor freezability

0·36 % *vs* 23·36 ± 0·63 %). During freezing, redistribution of membrane phospholipids and lipid phase separation occurs which disturbs the lipid-protein interaction required for intact membrane. This results in the loss of membrane intactness of spermatozoa. Additionally, the redistribution of antioxidant enzymes in the sperm membrane and the reduction in their activity predispose the membrane to oxidative stress (Frau *et al*., 2020). All these together contribute to the loss of the semipermeable nature of the sperm membrane. In the spermatozoa of the poor freezability group, such changes might have been intense and hence, the loss of functional membrane integrity was more.

Malondialdehyde concentrations of cryopreserved Malabari buck semen of good and poor semen freezability were significantly different ($p<0·01$) (7·83 ± 0·29 nmol/mL *vs* 13·95 ± 0·08 nmol/mL). Malondialdehyde is an indicator of oxidative stress. Due to the excess production of ROS during cryopreservation of spermatozoa, lipid peroxidation of the polyunsaturated fatty acids in the sperm

membrane (Kumar *et al*., 2019) occurs resulting in the formation of malondialdehyde. Loss of antioxidant system might be severe in the poor semen freezability group which might have resulted in more ROS production and thereby high concentration of malondialdehyde.

In the present study, the morphological abnormalities of spermatozoa (2.11 ± 0.08 % *vs* 2.39 ± 0.09 %) had no significant variation in the two groups of semen freezability (Fig 6). These results are in accordance with the previous reports of John (2016) and Krishnan (2017) that the percentage of morphological abnormalities of spermatozoa of good and poor semen freezability were not significantly different (4.96 ± 0.33 % *vs* 5.70 ± 0.25 %; 6.67 ± 0.61 % *vs* 7.67 ± 0.42). Regarding the values, Ismail *et al.* (2020) reported higher values (11.2 ± 0.49 %).

The variation observed in the post-thaw quality parameters might be due to the pronounced difference existing among individuals in resistance of spermatozoa to the process of cryopreservation. Spermatozoa present in the ejaculates of good and poor semen freezability group of bucks might be differed in membrane fluidity (John, 2016) and the protein composition of spermatozoan membrane which are defined during spermatogenesis, epididymal maturation and post-ejaculation events (Pini *et al.,* 2018). Such integral variations might have contributed to the difference in the freezing induced reduction of antioxidant mechanism and loss of proteins and thereby freezing resilience of spermatozoa, which was reflected in the post-thaw seminal parameters.

Conclusion

Fresh semen characteristics were similar in Malabari bucks of good and poor semen freezability except concentration, progressive motility and functional membrane integrity. But, post-thaw quality of cryopreserved Malabari buck semen of good and poor semen freezability differed in terms of progressive motility, viability, acrosome integrity, functional membrane integrity and lipid peroxidation status of spermatozoa.

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Conflict of interest

The authors declare that they have no conflict of interest.

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