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Assessing post-thaw sperm quality in Malabari bucks: Impact of freezing resilience on cryo-survival

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Abstract
Cryopreservation of Malabari buck semen provides valuable genetic preservation, but its effectiveness is hindered by cryodamage, with low-freezability (LF) bucks being particularly susceptible. This study evaluated fresh and post-thaw sperm quality in high-freezability (HF) and LF bucks. HF bucks demonstrated significantly higher post-thaw motility (47.50±1.53% vs. 15.75±1.16%), viability (78.45±0.41% vs. 73.35±0.97%), acrosomal integrity (62.65±0.91% vs. 44.60±1.67%), and hypo-osmotic swelling (HOS) response (62.65±0.92% vs. 38.65±1.91%) compared to LF bucks ($p < 0.05$). DNA integrity (57.10±1.08% vs. 35.60±1.82%) was also superior in HF bucks. Malondialdehyde (MDA) levels, indicative of oxidative stress, were lower in HF bucks (7.88±0.33% vs. 11.48±0.29%). These findings highlight the resilience of HF bucks and the importance of selective breeding and optimising cryopreservation protocols to enhance artificial insemination outcomes in Malabari bucks.

Keywords: Malabari, cryopreservation, freezability, cryodamage, sperm.

Introduction
The application of Frozen Thawed (FT) semen in Malabari bucks offers genetic advantages for breeding programs but has not gained extensive commercial use in AI due to the high success rate and low cost associated with fresh or chilled semen. The freezing-thawing process causes irreversible damage to sperm membranes, altering structure, degrading proteins and generating reactive oxygen species (Frau *et al.*, 2020) [5]. Evaluating semen quality both pre-freeze and post-thaw is essential for understanding factors that affect sperm viability during cryopreservation. Key quality parameters, including motility, plasma membrane integrity (PMI), and acrosomal integrity, can indicate semen resilience to cryopreservation. Bucks with low semen freezability tend to experience significant declines in these parameters following thawing, reducing fertilisation potential (Kumar *et al.*, 2019) [12]. In contrast, bucks with high semen freezability maintain better motility and viability, suggesting an inherent biological resilience to cryodamage (Leboeuf *et al.*, 2000) [13].
Cryopreservation places considerable stress on sperm cells, primarily due to osmotic changes, ice crystal formation, and oxidative stress. These effects are most pronounced in semen with low freezability, where the sperm membrane experiences heightened structural and functional damage. Fertility studies by John *et al.* (2022) [9] showed that animals with lower semen freezability had decreased fertility rates. The aim of the study was to evaluate and compare the fresh and post-thaw sperm quality of Malabari bucks with varying levels of cryosurvivability.

Materials and Methods
Bucks maintained at Department of Animal Reproduction, Gynecology and Obstetrics, College of Veterinary and Animal Sciences, Pookode, Wayanad, and Kerala Livestock development Board Ltd., Dhoni, Palakkad, Kerala, India were used for the present study. Parameters used for accepting or rejecting an ejaculate were sperm motility. Ejaculates with sperm motility less than 75 percent (extended) and less than 40 percent (post-thaw) were discarded.

Records on semen quality from the date of June 2022 to October 2023 were utilized to calculate freezability. Semen samples were collected twice a week from eight healthy Malabari bucks using a Danish-type artificial vagina at 43ºC (Urmila *et al.*, 2023) [18]. After collection, the semen was maintained at 37 ºC and the semen was then extended with a Tris-based solution to achieve a concentration of 300 million motile sperm per ml. Extended semen was packaged into 0.25 ml French mini straws and equilibrated at 5ºC for 4 hours. Following equilibration, the semen was frozen in liquid nitrogen vapour and stored in liquid nitrogen. After at least 24 hours, the semen was thawed at 37ºC and bucks were classified based on post-thaw motility: those with more than 40 percent motility were considered high freezability, while those with less than 40 percent were deemed poor freezability (Hidalgo *et al*, 2007) [7]. Sperm viability and morphological abnormalities were evaluated using eosin-nigrosine staining, while acrosomal integrity was determined through Giemsa staining (John *et al*., 2022) [9]. Functional membrane integrity was assessed using the hypo-osmotic swelling test with a 100 mOsm/L hypoosmotic solution. Lipid peroxidation status was measured by quantifying malondialdehyde concentrations (µmol/L) through the thiobarbituric acid assay, following the method described by Lone *et al*. (2017) [14].

Results and Discussion

This study found that initial progressive motility in fresh semen was comparable between high (81.80±0.49%) and low (82.00±0.00%) semen freezability bucks, aligning with earlier findings in Black Bengal (Mohanty *et al.*, 2008) [15] and Malabari bucks (Behera, 2012) [1]. However, post-thaw motility showed significant differences (Table 1 and Fig 1), with high freezability bucks (47.50±1.53%) outperforming low freezability bucks (15.75±1.16%) ($p<0.05$), consistent with disparities reported in Black Bengal (Mohanty *et al.*, 2008) [15] and Beetal bucks (Goswami *et al.*, 2020) [6]. These findings underscore the susceptibility of low freezability bucks to cryodamage and highlight the critical role of sperm membrane composition in preserving motility during cryopreservation (Krishnan, 2017; Pini *et al.*, 2018; Bhai *et al.*, 2023) [11, 16, 2].

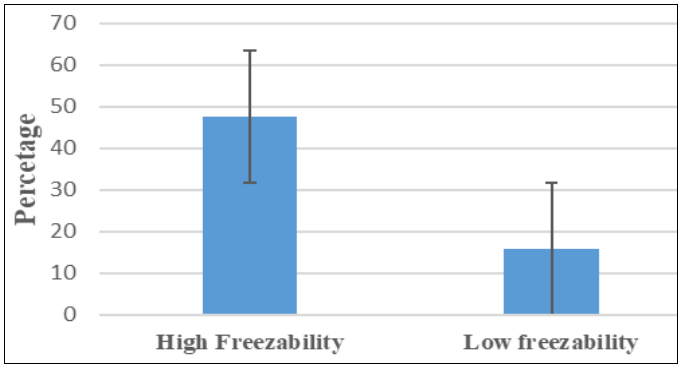

Fig 1: Post-thaw progressive motility of Malabari Buck semen of high and low freezability

Table 1: Post-thaw characteristics of spermatozoa of high and low freezability semen

S. No	Parameters	Stages	High	Low	P-Value
1.	Progressive motility	Fresh	81.80±0.49 ^{A}	82.00±0.00 ^{A}	0.694
		Post-thaw	47.50±1.530^{aB}	15.75±1.16^{bB}	<0.001
		p-value	< 0.001	< 0.001	
2.	Viability	Fresh	91.20±0.56 ^{A}	91.00±0.79 ^{A}	0.842
		Post-thaw	78.45±0.413 ^{aB}	73.35±0.97^{aB}	<0.001
		p-value	< 0.001	< 0.001	
3.	Abnormality	Fresh	1.45±0.25^{aA}	2.50±0.11^{bA}	0.005
		Post-thaw	04.90±0.36 ^{aB}	07.15±0.47^{bB}	0.005
		p-value	0.003	< 0.001	
4.	Acrosome integrity	Fresh	90.00±0.49^{aA}	87.20±0.95^{bA}	0.031
		Post-thaw	62.65±0.91 ^{aB}	44.60±1.67^{bB}	<0.001
		p-value	< 0.001	< 0.001	
5.	Plasma membrane integrity	Fresh	86.63±.78 ^{A}	82.93±.98^{bA}	0.015
		Post-thaw	62.65±0.92^{aB}	38.65±1.91^{bB}	<0.001
		P-value	< 0.001	< 0.001	
6.	DNA integrity	Fresh	77.80±0.36 ^{aA}	71.50±1.06^{bA}	<0.001
		Post-thaw	57.10±1.09 ^{aB}	35.60±1.83^{bB}	<0.001
		p-value	< 0.001	< 0.001	
7.	MDA	Post-thaw	07.88±0.34^{a}	11.48±0.29^{b}	<0.001

Means with different superscript letters within rows and columns indicate significant differences ($p<0.05$). Lowercase: freezability group differences. Uppercase: fresh vs pre-freeze stage differences

A significant difference in spermatozoa viability was observed between high and low semen freezability bucks after extension (Table 1). This contrasts with John *et al.* (2022) [9], who reported no significant difference. The sperm viability in this study aligns with Bhai (2012) [3], who recorded viability percentages of 90.14±0.53% and 90.83±0.95%, respectively. Post-thaw viability was significantly higher in high freezability bucks (78.45±0.41%) compared to low freezability bucks (73.35±0.97%) ($p<0.05$, Figure 2). This difference aligns with Shiny (2011) [17] and Bhai (2012) [3]. High freezability bucks may retain greater membrane stability due to higher cholesterol content, reducing cryodamage and supporting better viability (Pini *et al*., 2018; Bhai *et al*., 2023) [16, 2].

Bucks with low semen freezability had a significantly higher percentage of sperm abnormalities compared to those with high semen freezability. This observation aligns with John (2016) [10], who reported abnormal spermatozoa percentages of 1.70±0.13% and 1.9±0.12% in high and low freezability bucks, respectively. The percentage of abnormal sperm observed in this study is comparable to values reported by Shiny (2011) [17] (2.50±0.56%) and Behera (2012) [1] (1.41±0.23%). At the post-thaw stage, high semen freezability bucks exhibited significantly lower spermatozoa abnormalities (4.90±0.35%) compared to low semen freezability bucks (7.15±0.46%) (Table 1 and Fig 3).

Comparable trends were reported by Shiny (2011) [17] (11.17±0.60%). Breed-specific variations, cryopreservation protocols, and differences in semen quality may contribute to these discrepancies (Ismail *et al*., 2020 and Bhai *et al*., 2023) [8, 2].

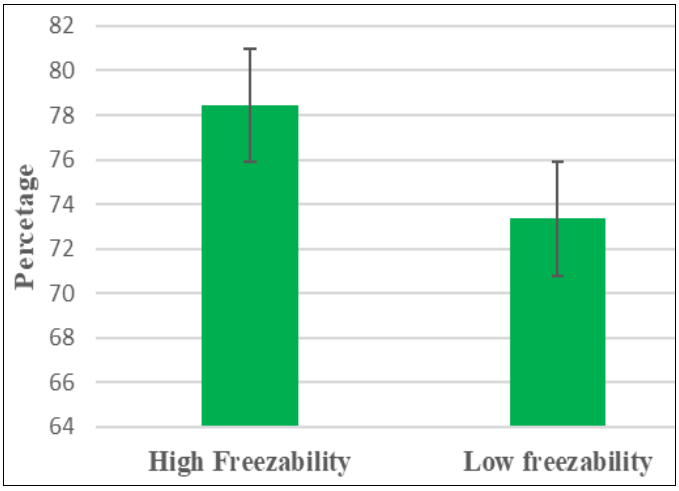

Fig 2: Post-thaw Viability of Malabari Buck semen of high and low freezability

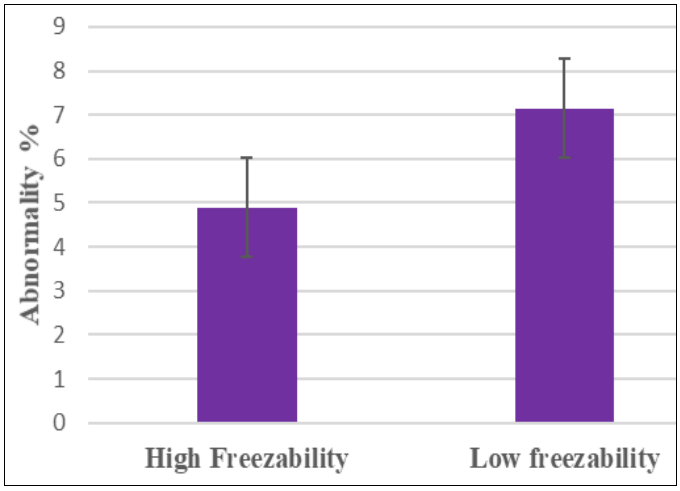

Fig 3: Post-thaw Abnormality of Malabari Buck semen of high and low freezability

The current study found a significant difference in acrosome integrity of fresh semen between bucks with high and low semen freezability. This result is consistent with John (2016) [10], who reported mean intact acrosome percentages of 91.13±0.36 and 91.04±0.54 in high and low freezability bucks, respectively. Similar levels of acrosome integrity have been reported in previous studies, including Behera (2012) [1] (91.58±0.54%), Bhai (2012) [3] (94.67±0.78%), and Shiny (2011) [17] (89.67±0.95%). Notably, post-thaw acrosomal integrity was significantly higher in high freezability bucks (62.65±0.91%) than in low freezability bucks (44.60±1.67%) (p<0.05), consistent with Bhai *et al*. (2023) [2]. Cryopreservation can cause acrosomal damage, affecting fertilizing capability (John, 2022) [9]. The results suggest that high freezability bucks may benefit from a greater abundance of proteins involved in acrosome stability, providing resilience during freezing and thawing (Table 1 and Fig 4).

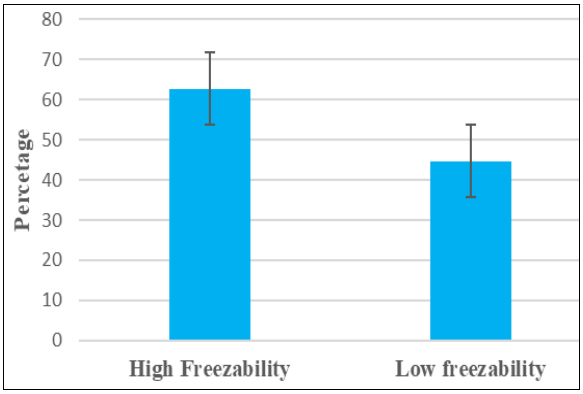

Fig 4: Post-thaw acrosome integrity % of Malabari Buck semen of high and low freezability

The mean hypo-osmotic swelling (HOS) test response after semen extension did not differ significantly between bucks with high and low semen freezability, consistent with John (2016) [10]. However, other studies reported lower HOS responses, such as Behera (2012) [1] (54.53±1.99%), and Bhai (2012) [3] (60.73±2.30%). The relatively higher HOS responses observed in this study indicate improved membrane integrity and functionality. Post-thaw, high freezability bucks had a significantly higher mean HOS response (62.65±0.92%) than low freezability bucks (38.65±1.91%) (p < 0.05), indicating better membrane functionality (Table 1 and Fig 5). This trend is supported by Krishnan (2017) [11], who noted improved HOS response in bucks with higher freezability, suggesting that membrane integrity and osmotic resilience contribute to improved post-thaw semen quality (Goswami *et al*., 2020) [6].

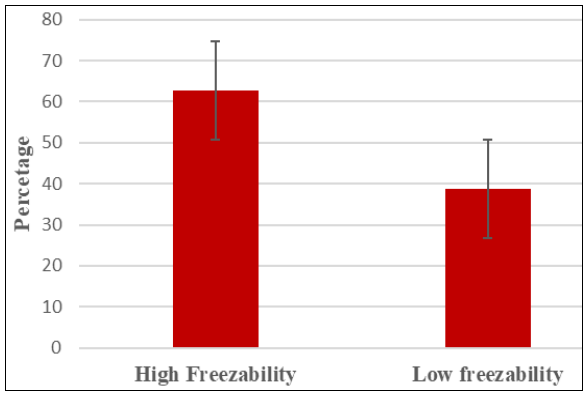

Fig 5: Post-thaw functional membrane integrity% of Malabari buck semen of high and low freezability

The analysis of DNA integrity in fresh semen revealed a significant difference between bucks with high and low semen freezability. High freezability bucks exhibited higher DNA integrity, indicating DNA integrity as a crucial factor in semen freezability. Factors like season, age, and metabolic activity influence DNA fragmentation (Bogdaniuk *et al*., 2022) [4]. High freezability bucks maintained superior DNA integrity (57.10±1.08%) compared to low freezability bucks (35.60±1.82%) at the post-thaw stage (p<0.05), consistent with Bhai *et al*. (2023) [2] (Table 1 and Figure 6). The maintenance of DNA integrity in high freezability bucks is essential for successful fertilization and embryo development, suggesting an inherent resilience to cryodamage (Krishnan, 2017) [11].

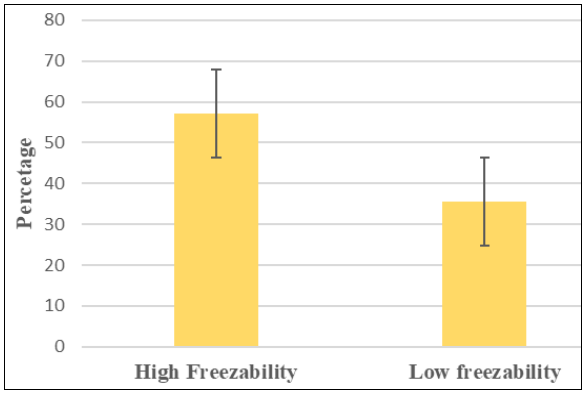

Fig 6: Post-thaw DNA integrity% of Malabari Buck semen of high and low freezability

Finally, high freezability bucks demonstrated significantly lower MDA levels (7.88±0.33%) compared to low freezability bucks (11.48±0.29%) post-thaw ($p< 0.05$) (Table 1 and Fig 7). Elevated MDA levels are indicative of lipid peroxidation and oxidative stress, which are known to compromise semen quality. This observation reinforces that low freezability bucks may lack sufficient antioxidant defenses to mitigate cryodamage, leading to higher levels of oxidative stress and subsequent impairment of sperm function (Kumar *et al.*, 2019) [12].

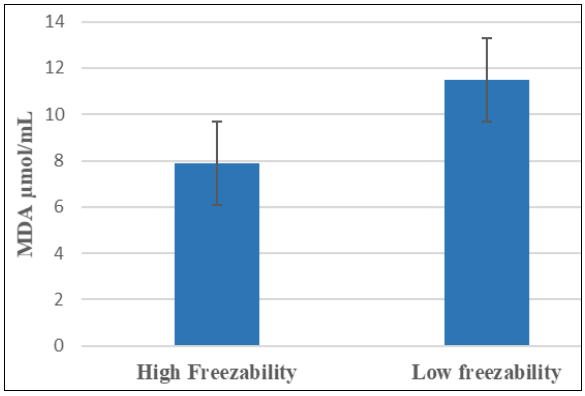

Fig 7: Post-thaw Malondialdehyde concentration of Malabari Buck semen of high and low freezability

Conclusion

Cryopreservation of Malabari buck semen revealed significant variations in post-thaw quality between high and low freezability samples. High freezability bucks exhibited enhanced sperm motility, viability, and acrosome integrity, along with reduced DNA damage and malondialdehyde level. Moreover, a substantial decline in sperm quality was observed between fresh and post-thaw samples, highlighting the adverse impact of cryopreservation on sperm integrity during the freeze-thaw process.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Not available

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