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Impact of equilibration on sperm quality in Malabari buck semen: A comparative study of different freezability levels#

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Abstract

This study aimed to investigate the alterations in the sperm quality parameters across high and low freezability semen samples of Malabari bucks during post-extension and pre-freeze stages of cryopreservation. A total of 32 semen ejaculates with at least 80 per cent initial progressive motility were selected, categorising four bucks as high freezability and four as low freezability based on post-thaw characteristics. Significant variation was found in progressive motility at the pre-freeze stage, with high freezability samples showing a markedly higher motility (68.25 ± 1.02 %) compared to low freezability samples (58.50 ± 1.0 %), indicating that motility is closely linked to freezability. Sperm viability was higher in the high freezability group post-extension (91.2 ± 0.56 %) and pre-freeze (79.70 ± 0.60 %) than in the low freezability group (91.00 ± 0.79 % and 78.85 ± 1.09 %, $p < 0.05$). Sperm abnormalities increased significantly from post-extension (high: 1.45 ± 0.25 %; low: 2.50 ± 0.11 %) to pre-freeze stages (high: 3.55 ± 0.34 %; low: 04.98 ± 0.36 %, $p < 0.05$). Acrosome integrity was consistently higher in the high freezability group across fresh (90.00 ± 0.49 %) and pre-freeze (82.91 ± 0.59 %) semen samples, compared to the low freezability group (87.20 ± 0.95 % and 78.58 ± 0.87 %, $p < 0.05$). Spermatozoa DNA integrity was significantly higher ($p < 0.01$) in high freezability samples (77.21 ± 0.86 %) compared to low freezability samples (67.95 ± 0.70 %) after equilibration. Pre-freeze (equilibration) phase effects on these parameters were evident, as demonstrated by the decline in motility, viability, and acrosome integrity between post-extension and pre-freeze stages. These findings suggested that selecting bucks based on freezability could improve semen quality, with tailored protocols necessary to address the unique requirements of low freezability bucks. The study also found a significant differences in the attributes of high and low freezable buck semen at the post-equilibration stage.

Keywords: *Sperm attributes, freezability, fresh, pre-freeze, equilibration*

Artificial insemination (AI) with cryopreserved semen is widely employed to enhance and preserve genetic traits across animal species. During the freezing and thawing processes, sperm cells are highly susceptible to cryo-damage, often resulting in discarding the semen samples due to reduced sperm motility and viability, both essential for successful

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fertilisation. The equilibration phase is critical, as it allows cryoprotectants like glycerol to penetrate the sperm membrane, reducing the likelihood of intracellular ice formation during freezing. Studies suggest that an optimum equilibration period of 2–4 hours balances cryoprotectant infiltration and minimises cytotoxicity, whereas prolonged equilibration can induce cryoprotectant toxicity and oxidative stress. Cryopreservation also causes irreversible membrane damage, resulting in structural alterations, protein degradation, and increased reactive oxygen species (ROS) production (Frau *et al.,* 2020). Additionally, the equilibration phase can cause sperm cells to shrink and swell, disrupting cytoskeletal structures and membranes, thus affecting head dimensions and overall sperm function (Martin *et al.,* 2023). Research has shown that the quality of fresh semen does not reliably predict cryotolerance, as animals with similar sperm parameters may exhibit substantial variability, and naturally fertile individuals can have low semen freezability (Kumar *et al.,* 2019). Significant disparities in sperm parameters between high and low freezability samples emerged during equilibration in buck semen. This phase considerably impacts low freezability samples, often reducing their viability for further processing and long-term storage, thereby lowering fertilisation potential. Therefore, evaluating sperm characteristics during equilibration is essential for predicting semen freezability. The equilibration phase significantly reduced total and progressive motility in both high and low freezability samples, influencing post-thaw outcomes (Sundararaman and Edwin, 2008). This study aimed to assess differences in sperm attributes between high and low freezable semen samples, focusing specifically on the post-extension and post-equilibration stages of cryopreservation.

Materials and methods

Semen samples were collected twice a week from eight healthy Malabari bucks maintained at the Artificial Insemination Centre of the Department of Animal Reproduction, Gynaecology, and Obstetrics, College of Veterinary and Animal Sciences, Pookode, Wayanad and Kerala Livestock Development Board, Ltd., Dhoni, Palakkad, Kerala using a Danish-type artificial vagina (12×3.8 cm, IMV technologies®, L'Aigle, France) maintained at 43ºC. The bucks were maintained under standard conditions, receiving uniform feeding, controlled housing, and consistent lighting. Records on semen quality parameters from June 2022 to October 2023 were utilised to calculate freezability. The parameter used for accepting or rejecting an ejaculate was sperm motility. Ejaculates with sperm motility <75 per cent (extended) and <35 per cent (post-thawed) were rejected. After collection, the semen samples were maintained in a water bath at 37ºC and evaluated for volume, colour, density, mass activity, and concentration (ACCUCELL (Ovine photometer), IMV Technologies®, L'Aigle, France). The semen was then extended with a Tris-based solution to achieve a concentration of 300 million motile spermatozoa per mL. The extended semen was packaged into 0.25 mL French mini straws (IMV Technologies®, L'Aigle, France) and equilibrated at 5ºC for four hours. Following equilibration, the semen was stored in liquid nitrogen vapour at $^{-}$196ºC. After 24 h of cryopreservation, the semen was thawed at 37ºC and assessed for post-thaw motility, viability, and abnormalities. Six ejaculates from each buck were test-frozen, and post-thaw spermatozoa progressive motility was evaluated. Based on the motility results, bucks were classified into two groups: Group I (High Freezability, n=4) with ≥ 35 per cent post-thaw sperm motility and Group II (Low Freezability, n=4) with < 30 per cent post-thaw sperm motility, as per Hidalgo *et al.* (2007). The post-extension (Fresh) and post-equilibration (Pre-freeze) characteristics of spermatozoa were comprehensively assessed through evaluations of progressive motility, viability and morphological integrity, acrosomal integrity and functional membrane integrity.

Statistical analyses

Statistical analysis was done using SPSS software version 24.0, applying a student's t-test to evaluate characteristics of post-extension and equilibrated semen samples.

Results and discussion

A total of thirty-two semen ejaculates, with a minimum of 80 per cent initial progressive motility, were selected for the cryopreservation studies. The present study identified four bucks as having semen with high freezability and four with poor freezability based on post-thaw semen characteristics.

No significant differences were found between high and low freezability groups in semen characteristics (volume, pH, concentration) in fresh semen samples (Table 1).

Table 1. Fresh semen characteristics

S.No	Parameters	Freezability		P – Value
		High Freezability	Low freezability	
1.	Volume	1.17 ± 0.12	0.85 ± 0.06	0.056^{ns}
2.	pH	6.66 ± 0.02	6.70 ± 0.00	0.141^{ns}
3.	Concentration	3150.75 ± 165.59	2550.70 ± 314.74	0.130^{ns}

ns- Not significant at 5% level (p>0.05)

Table 2. Sperm biophysical characteristics of fresh (extended) and pre-freeze (equilibrated) semen samples of high and low freezability groups

S. No	Parameters	Stages	High	Low	P – value
1	Progressive motility	Fresh	81.80 ± 0.49 ^{A}	82.00 ± 0.00 ^{A}	0.694
		Pre-freeze	68.25 ± 1.01^{aB}	58.50 ± 1.00 ^{bB}	<0.001**
		P-value	**<0.001****	**<0.001****	
2	Viability	Fresh	91.20 ± 0.56 ^{A}	91.00 ± 0.79 ^{A}	0.842
		Pre - freeze	79.70 ± 0.60 ^{aB}	78.85 ± 1.09 ^{aB}	0.513
		P-value	<0.001**	0.001**	
3	Abnormality	Fresh	1.45 ± 0.25^{aA}	2.50 ± 0.11^{bA}	0.005*
		Pre - freeze	3.55 ± 0.34 ^{aB}	4.98 ± 0.36 ^{bB}	0.019*
		P-value	0.009**	0.002**	
4	Acrosome integrity	Fresh	90.00 ± 0.49^{aA}	87.20 ± 0.95^{bA}	0.031*
		Pre - freeze	82.91 ± 0.59 ^{aB}	78.58 ± 0.87 ^{bB}	0.003**
		P-value	**0.001****	**0.007****	
5	Plasma membrane integrity	Fresh	86.63 ± 0.78 ^{aA}	82.93 ± 0.98^{bA}	0.015*
		Pre - freeze	73.65 ± 0.80 ^{aB}	66.35 ± 0.56 ^{bB}	<0.001**
		P-value	**0.001****	**<0.001****	
6	DNA integrity	Fresh	77.80 ± 0.36 ^{a}	71.50 ± 1.06^{bA}	<0.001**
		Pre - freeze	77.21 ± 0.86 ^{a}	67.95 ± 0.70 ^{bB}	<0.001**
		P-value	**<0.607**	**<0.019***	

Means with different lowercase superscript letters within a row (freezability groups) and uppercase letters within a column (stages) differ significantly at 5% (*) or 1% (**) level

The study observed a significant difference in the mean sperm progressive motility between high and low freezability semen groups at the pre-freeze stage (Table 2). Progressive motility was significantly higher ($p< 0.001$) in high freezability group (68.25 ± 1.02 %) compared to low freezability group (58.50 ± 1.00 %), indicating that freezability significantly impacts motility. These findings were contradictory to those by John (2016), who reported no significant differences in motility post-extension between low and high freezability of Malabari bucks. Studies in Beetal and Assam hill breeds reported comparable motility values (Goswami *et al*., 2020; Deori *et al*., 2018), whereas Black Bengal and Saanen breeds generally exhibited higher motility of 82.0 ± 3.0 and 88.5 ± 1.8 per cent, respectively in low freezability bucks (Mishra *et al*., 2010; Mojapelo *et al*., 2021; Morrell *et al*., 2022). These discrepancies underscore the influence of breed-specific factors on motility outcomes and freezability. The disparity in results may also be attributed to the cooling rate during the equilibration process, which can induce membrane fusion, degradation of ion channels, and disorganisation of lipid domains (Ahmad *et al.,* 2015). These changes can trigger cold shock, ultimately leading to decreased sperm motility, particularly in the low freezability semen samples during equilibration.

Sperm viability was significantly higher in post-extension samples (high: 91.2 ± 0.56% and low: 91.00 ± 0.79%) compared to pre-freeze semen samples (high: 79.70 ± 0.60% and low: 78.85 ± 1.09%) in both high and low freezability groups (Table 2). Notably, viability remained comparable between the two groups. However, in low freezability samples, viability decreased significantly after equilibration (78.85 ± 1.09%) compared to post-extension (91.00 ± 0.79%). These findings differ from those of John (2016), who observed no significant difference in viability during pre-freeze stage in Malabari breed, yet align with reports for Beetal and Sirohi breeds (Goswami *et al*., 2020). Species-specific and breed-specific variations in sperm membrane composition significantly influence responses to equilibration regimes, emphasizing the need for tailored evaluation protocols, especially for low freezability groups.

Sperm abnormality differed significantly ($p<0.05$) between the low and high freezability groups; however, the percentage of abnormal sperms were significantly higher ($p<0.01$) in pre-freeze stage (high: 3.55 ± 0.34%, low: 4.98 ± 0.36%) than in post extension stage (high: 1.45 ± 0.25%, low: 2.5 ± 0.11%) in both the groups (Table 2). This increase, suggesting that equilibration process affects sperm morphology beyond inherent quality variations, consistent with reports by John (2016), Pawshe (2016), and Deori *et al.* (2018). Similar findings were recorded in other studies that morphological alterations were highly variable based on cryopreservation methods (Krishnan, 2017; Wahjuningsih *et al.,* 2021).

The hypo-osmotic swelling (HOS) test showed significant differences ($p < 0.05$) in membrane integrity

between high and low freezability groups, with higher values observed in the high freezability group (post-extension: 86.63 ± 0.78%, pre-freeze: 73.65 ± 0.80%) compared to the low freezability group (post-extension: 82.93 ± 0.98%, pre-freeze: 66.35 ± 0.56%). Moreover, a highly significant difference ($p < 0.01$) was observed between pre-freeze and fresh samples. These values were consistent with findings by John (2016) and Goswami *et al*. (2020) suggesting HOS responses remain consistent across freezability groups, re-affirming its reliability as a marker for membrane integrity in buck sperm. The equilibration process resulted in a significant reduction in membrane integrity in low freezability group semen samples, which may be attributed to the presence of osmotically inactive plasma membranes in spermatozoa.

Acrosome integrity was significantly higher in the high freezability group in post-extension (90.00 ± 0.49%) and pre-freeze (82.91 ± 0.59%) semen compared to the low freezability group (87.20 ± 0.95% and 78.58 ± 0.87%, $p < 0.05$), indicating its potential as a freezability predictor (Table 2). This aligns with findings by Dorado *et al.* (2010), who emphasized acrosome integrity as crucial to cryotolerance. These results contradict those of John (2016), who reported no significant post-extension differences in acrosome integrity between low and high freezability groups of Malabari bucks, potentially due to breed-specific responses to equilibration protocols.

For DNA integrity, a highly significant difference ($p< 0.01$) was noted in post-extension as well as pre-freeze, with high freezability group displaying DNA integrity of spermatozoa (77.80 ± 0.36% and 77.21± 0.86%, respectively) compared to the low freezability group (71.50 ± 1.06% and 67.95 ±0.70 (Table 2). These results were consistent with Üstüner *et al*. (2015) who identified breed-related differences in DNA fragmentation in bull and goat spermatozoa. Additionally, studies suggested that DNA integrity is also influenced by factors such as age, season, and metabolic stress (Gonzalez-Marin *et al*., 2012; Hamilton, 2020).

The results across sperm motility, acrosome integrity, plasma membrane integrity, DNA integrity, and morphological abnormalities underscore significant differences between high and low freezability groups in the post-extension and post-equilibration. These observations correlate with studies conducted on various Malabari breed semen Bhai *et al*., 2023; John *et al*., 2022), highlighting the critical role of freezability as a factor in successful equilibration outcomes.

The findings on equilibration time (ET) further underline its influence on post-thaw survival rates. Previous research suggested that an ET of 2–4 h enhanced cryoprotectant penetration (Yi *et al*., 2002), whilst prolonged ET potentially detrimental to sperm integrity (Herold *et al*., 2006). Additionally, ET, in combination with cooling rates, has been shown to significantly impact post-thaw survival in ram and bull semen (Okano *et al*., 2004). The results of this study underscore the need for breed-specific ET protocols to improve buck semen equilibration outcomes. Cold shock induces osmotic and oxidative changes, disrupting sperm homeostasis and upregulating ROS. The severity of these effects depends on cell membrane composition, specifically PUFA ratio. This may explain the alterations in sperm attributes during equilibration, particularly in low freezability samples.

Conclusion

Equilibration process significantly altered sperm quality parameters in Malabari buck semen, irrespective of inherent freezability. High freezability bucks showed superior motility, acrosome integrity, and lower DNA damage compared to low freezability bucks. A decline in semen quality was observed between post-extension and post-equilibration stages, underscoring the adverse effects of equilibration processes. Selecting breeding bucks based on high freezability can enhance fertility outcomes in artificial insemination programs. Tailored interventions can improve cryotolerance in low freezability bucks.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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