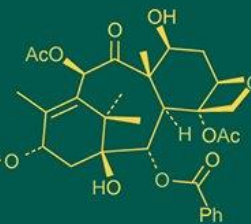
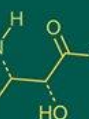
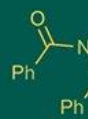


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Impact of different sperm concentration per dose on morphological attributes of cryopreserved Black Bengal buck semen

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Abstract

The present study was conducted to evaluate the effect of different sperm concentrations on sperm morphology and abnormalities during cryopreservation of Black Bengal buck semen. Semen from four fertile bucks was diluted in Tris-fructose-citrate egg yolk extender to achieve sperm concentrations of 25, 50, 75, and 100 × 10⁶ spermatozoa per dose. Sperm abnormalities like head, middle piece and tail were assessed after dilution, equilibration, and post-thaw stages using Rose Bengal staining. Sperm abnormalities increased significantly with each stage of cryopreservation across all concentration groups. The lowest abnormality percentages were recorded at 25 × 10⁶ sperm/dose (5.10±0.26, 6.80±0.25, and 10.35±0.27% after dilution, equilibration, and post-thaw, respectively), whereas the highest values were observed at 100 × 10⁶ sperm/dose (8.80±0.37, 10.70±0.37, and 14.90±0.34%, respectively). Tail defects constituted the major proportion of abnormalities. Significantly higher abnormal sperm counts were observed in the 75 and 100 × 10⁶ sperm/dose groups compared with the 25 and 50 × 10⁶ sperm/dose groups. The study concluded that semen diluted to 25 × 10⁶ spermatozoa per dose better preserved sperm morphology and minimized cryo-induced abnormalities, making it a suitable concentration for cryopreservation of Black Bengal buck semen.

Keywords: Buck, semen, cryopreservation, dilution, abnormality

Introduction

The trend of decreasing sperm concentration in semen cryopreservation is steadily increasing. One mechanism to achieve such low dose semen is by increasing the dilution of semen. However, higher dilution reduces seminal plasma components, ultimately leading to premature capacitation and reduced post-thaw viability (Maxwell and Johnson, 1997; Garner *et al.*, 2001) [29, 17]. Moreover, excessive dilution causes leakage of intracellular enzymes such as glutamic-oxaloacetic transaminase from spermatozoa (Pace and Graham, 1970) [31], leading to the so-called dilution effect (Ashworth *et al.*, 1994; Centurion *et al.*, 2003; Garner *et al.*, 2001) [5, 10, 17]. High dilution rates have been reported to decrease sperm quality across species including rabbit (Harrison *et al.*, 1982) [20], ram (Ashworth *et al.*, 1994) [5], bull (Garner *et al.*, 1997, 2001) [17, 16], boar (Centurion *et al.*, 2003) [10], dog (Peña and Linde-Forsberg, 2000; England, 1999; Wales and White, 1963) [32, 14, 42] and stallion (Jasko *et al.*, 1992) [23]. While sperm concentration is a key determinant of cryosurvival, semen quality and fertility largely depend on sperm morphology, particularly the proportion of morphologically normal spermatozoa in the ejaculate. Even at an optimum sperm concentration, the presence of dead, immature, and abnormal spermatozoa adversely affects semen quality and fertility by contributing to the accumulation of debris and toxic substances such as ROS (Shannon and Curson, 1972) [36], including hydrogen peroxide released from dead sperm, which has a lethal effect on sperm cells (Alvarez *et al.*, 2012) [3]; these factors induce apoptotic changes in viable spermatozoa, impair fertilizing ability (Wang *et al.*, 2003) [43], negatively affect fertility (Heuer and Tahir, 1982) [21], and further compromise both fresh and cryosurviving sperm through enhanced intracellular ROS generation. Thus, removal of dead and abnormal spermatozoa prior to cryopreservation can improve semen quality and enhance cryosurvival. (Graham and Graham, 1990; Cisale *et al.*, 2001, Alvarez *et al.*, 1993; Bhakat *et al.*, 2014)

[18, 12, 2, 8]. Any deviation from the normal structure of spermatozoa is considered abnormal (Sullivan, 1978) [38], and such morphological abnormalities are broadly classified as primary (originating in the testis during spermatogenesis) and secondary (arising during epididymal transit or semen handling). semen freezing increased the rate of sperm morphological abnormalities. (Kulaksiz *et al* 2012) [25]. Abnormality is one of the indicators in determining the quality of spermatozoa because abnormal cell structures can cause disturbances and obstacles during fertilization, further causing low rates of implantation and pregnancy (Wajdi, 2021) [41]. Although ejaculated semen normally contains some abnormal spermatozoa (Faulkner and Pineda, 1980) [33], fertility is generally unaffected unless abnormalities exceed 20%; however, higher prevalence is associated with reduced fertility (Lagerlof, 1934; Sullivan, 1978) [27, 38]. These abnormalities may be hereditary or acquired due to environmental or pathological factors, making sperm morphology evaluation an essential component of breeding soundness examination (Turner *et al.*, 2005) [39]. Despite extensive research on semen dilution and low sperm dose cryopreservation, most studies have primarily focused on sperm viability (Garner *et al.*, 2001; Maxwell and Johnson, 1997) [17, 29], motility (Ballester *et al.*, 2007) [6], acrosomal integrity, and hypo-osmotic swelling response (Karan, 2018) [24] with fewer studies focusing specifically on sperm abnormalities and factors affecting it in relation to low-dose semen cryopreservation are scarce in goat farming (Fernandez-Santos *et al.*, 2006) [15]. Therefore, the present study was designed to evaluate the effect of dilution and sperm concentration on post-thaw semen quality with special emphasis on sperm morphology and abnormalities in Black Bengal buck semen.

Materials and Methods

The study was conducted at ICAR-National Dairy Research Institute (Eastern Regional Station) in Kalyani, located on the eastern bank of the Hooghly River in the lower Gangetic basin. The region has a hot and humid climate (12-39 °C; 58-91% RH; ~1250 mm rainfall). The study period extended from December 2024 to July 2025. Four bucks were randomly selected from a group of 13 fertile males, aged between 2 to 4 years and weighing 20-30 kg, reared at the experimental Goat Unit. Semen was collected twice weekly using an artificial vagina with the aid of a doe (mount method). A total of 144 ejaculates (4 bucks × 36 replicates) were obtained during the study. The collected semen from four bucks was immediately placed in insulated thermo-flask maintained at 37 °C and taken to semen analysis laboratory. The samples with >3 mass motility, > 80% live sperm, >70% progressive motile sperms were selected. Only ejaculates meeting the following eligibility criteria were selected for cryopreservation: mass activity score of at least + 3, sperm concentration ≥ 2, 500 million/mL, ejaculate volume ≥ 0.5 mL, and individual motility ≥ 70%. Four such qualifying ejaculates were randomly allocated into four groups and diluted with Tris fructose-citrate egg yolk extender (Fig.1). The volume of extender was calculated specifically for each group to achieve the target sperm concentrations of 25, 50, 75, and 100 × 10⁶ sperm/dose, respectively. Sperm morphological characteristics were assessed across all sperm concentration groups at three stages: immediately after dilution, after 3 hours of equilibration, and after 48 hours of cryopreservation (post-thaw).

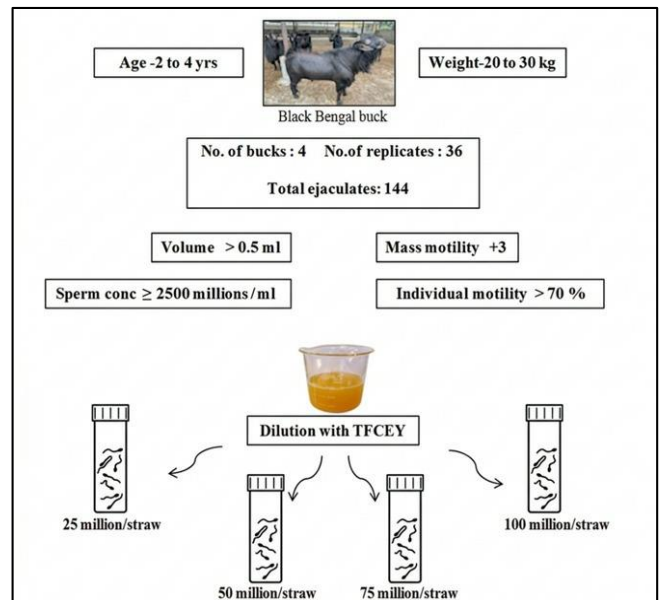


Fig 1: Schematic representation of initial quality assessment and dilution to different sperm concentration per dose of Black Bengal buck semen

Abnormal sperm count was determined by fixing a 10 µL of semen sample in Rose Bengal stain describes by Silva *et al.* (2009) [37] with slight modifications and sperm abnormalities such as head, mid piece and tail were studied (Fig .2). A minimum of 200 sperm from each slide was observed for morphological abnormalities and classified accordingly. (Figure 2.)

$$\text{Abnormal sperm\%} = \frac{\text{Sperm counted with abnormalities}}{\text{Total sperm counted}} \times 100$$

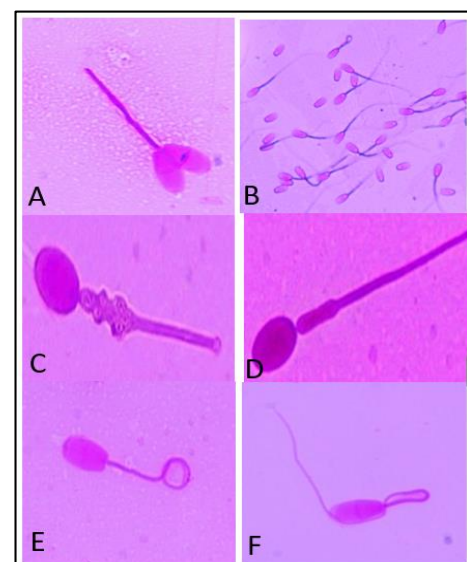


Fig 2: Rose Bengal staining A-Double head , B-Detached head, C-Irregular mid piece, D-enlarged mid piece, E-Coiled tail, F-Dag defect

Statistical analysis

The data were analyzed by One-way ANOVA using SPSS v.23 software, and means having significant differences were subjected to post-hoc comparisons (Tukey's multiple range test) and considered as significantly different when $p < 0.01$.

Results and Discussion

During the experiment, semen was diluted to variable sperm concentration for evaluating the dilution factor with least interactive losses for better post thaw semen quality particularly morphology. The mean percent value of fresh seminal attributes viz. volume (ml), concentration (million/ml), mass motility (0 to 5 grade), and progressive motility of Black Bengal buck semen was found to be 0.69 ± 0.03 , 3553.74 ± 77.89 , 4.45 ± 0.05 and 86.42 ± 0.65 respectively. The percentage of sperm abnormality in Black Bengal buck semen significantly ($p < 0.01$) increased with every stage of cryopreservation in all the four sperm concentrations per dose of semen. (Table. 1, 2, 3; Fig.3, 4, 5). At the lowest concentration of 25×10^6 sperm/straw, sperm abnormality (%) was minimal, with values of 5.10 ± 0.26 after dilution, 6.80 ± 0.25 post-equilibration, and 10.35 ± 0.27 post-thaw. Slightly higher abnormality levels were observed at 50×10^6 sperm/straw, reaching 5.95 ± 0.34 , $7.65 \pm 0.25\%$, and $10.95 \pm 0.33\%$, respectively. The lower values observed for morphological attributes in 25 million/dose may be the result of a better counter balance between the lethal reactive losses, protective seminal proteins, extender constituents and egg yolk during the process. A notable increase was found at 75×10^6 sperm/straw, where values rose to 6.75 ± 0.27 after dilution, 8.60 ± 0.25 after equilibration, and 12.75 ± 0.31 after freezing. The highest concentration group (100×10^6 sperm/straw) showed the greatest degree of abnormality at all stages $8.80 \pm 0.37\%$, $10.70 \pm 0.37\%$, and $14.90 \pm 0.34\%$, respectively. Significantly ($p < 0.01$) higher proportion of abnormal sperm population were observed in 75 and 100×10^6 sperm/straw when compared to 25 and 50×10^6 sperm/straw at all the three stages of cryopreservation while no significant difference in the abnormal sperm count was found between the 25 and 50 million sperm/straw concentrations. The probable reason may be attributed to higher concentration of reactive seminal proteins at lower dilution that interacted with egg yolk in extender to form more lethal compound affecting sperm (Brinsko *et al.*, 2000; Miro *et al.*, 2009) [9, 30]. Current findings are in agreement with the observations of Rijsselaere *et al.* (2005) [34], who reported that higher sperm concentrations were associated with a greater proportion of morphological defects. Ahmad *et al.* (2015) [1], reported that at sperm concentration of 100 million/ml, morphologically normal sperm declined significantly from $96.8 \pm 0.36\%$ in fresh semen to $96.4 \pm 5.2\%$ after dilution, $87.6 \pm 7.5\%$ after equilibration, and further to $81.0 \pm 5.7\%$ post-thaw. Deori *et al.* (2018) [13] and Asaduzzaman *et al.* (2021) [4] also reported that sperm abnormalities increased during cryopreservation due to structural damage and osmotic stress. Similarly, Vigneshwaran *et al.* (2024) [40] observed significantly higher sperm abnormalities at the pre-freeze stage with 75×10^6 /dose sperm concentration, highlighting that the equilibration process itself may induce morphological defects. These findings suggest that elevated sperm concentrations are associated with a higher incidence of abnormal spermatozoa, likely due to greater metabolic waste accumulation, oxidative stress, and inadequate cryoprotectant distribution. Such stressors can impair structural integrity and promote morphological defects during the cryopreservation process. Defective sperm morphology has been associated with compromised fertilization potential and embryo quality (Chenoweth, 2009; Lewis-Jones *et al.*, 2003) [11, 28], emphasizing the relevance of minimizing abnormalities through optimized cryopreservation protocols.

Table 1: Effect of various sperm concentration per dose on different sperm abnormality after dilution of Black Bengal bucks' semen

Sperm abnormalities	Sperm concentration per dose			
	25	50	75	100
Head	1.53 ± 0.08	1.79 ± 0.10	2.03 ± 0.08	2.64 ± 0.11
Mid piece	1.02 ± 0.05	1.19 ± 0.07	1.35 ± 0.05	1.76 ± 0.07
Tail	2.55 ± 0.13	2.98 ± 0.17	3.38 ± 0.14	4.40 ± 0.19
Total	5.10 ± 0.26^a	5.95 ± 0.34^a	6.75 ± 0.27^b	8.80 ± 0.37^c

Means with dissimilar superscripts in a column (a, b, c) differ significantly ($p < 0.01$)

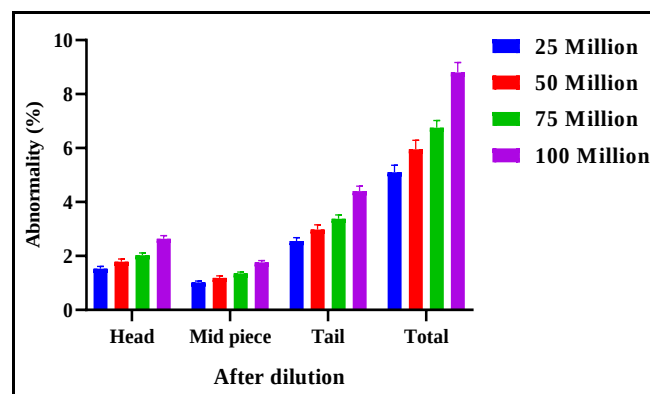


Fig 3: Effect of various sperm concentration per dose on different sperm abnormality after equilibration of Black Bengal bucks semen

Table 2: Effect of various sperm concentration per dose on different sperm abnormality after equilibration of Black Bengal bucks semen

Sperm abnormalities	Sperm concentration per dose			
	25	50	75	100
Head	2.04 ± 0.08	2.30 ± 0.08	2.58 ± 0.08	3.21 ± 0.11
Mid piece	1.36 ± 0.05	1.53 ± 0.05	1.72 ± 0.05	2.14 ± 0.07
Tail	3.40 ± 0.13	3.83 ± 0.13	4.30 ± 0.13	5.35 ± 0.19
Total	6.80 ± 0.25^a	7.65 ± 0.25^a	8.60 ± 0.25^b	10.70 ± 0.37^c

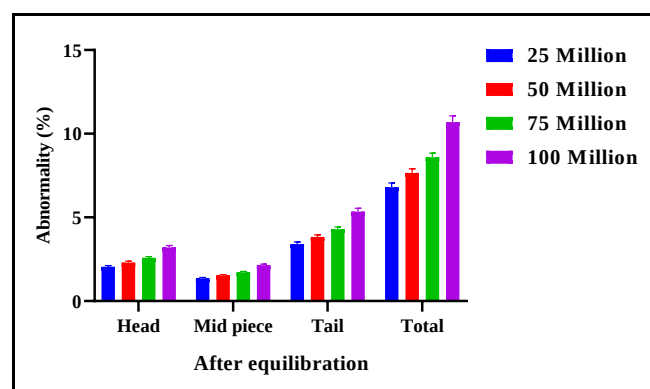


Fig 4: Effect of various sperm concentration per dose on different sperm abnormality after equilibration of Black Bengal bucks semen

Table 3: Effect of various sperm concentration per dose on different sperm abnormality after Post freeze-thaw of Black Bengal bucks semen

Sperm abnormalities	Sperm concentration per dose			
	25	50	75	100
Head	3.11 ± 0.08	3.29 ± 0.10	3.83 ± 0.09	4.47 ± 0.10
Mid piece	2.07 ± 0.05	2.19 ± 0.07	2.55 ± 0.06	2.98 ± 0.07
Tail	5.18 ± 0.14	5.48 ± 0.17	6.38 ± 0.16	7.45 ± 0.17
Total	10.35 ± 0.27^a	10.95 ± 0.33^a	12.75 ± 0.31^b	14.90 ± 0.34^c

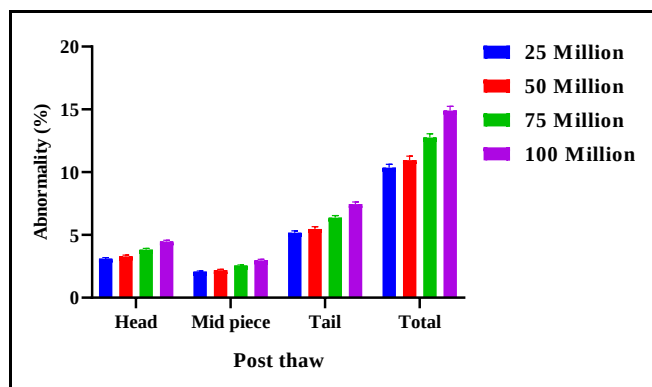


Fig 5: Effect of various sperm concentration per dose on different sperm abnormality after Post freeze-thaw of Black Bengal bucks semen

According to Rengan *et al.* (2012) ^[25], spermatozoa abnormalities that exceed 14% indicate the presence of symptoms of infertility or infertility in a male, whereas according to Kumar and Singh (2015), abnormal sperm counts in semen up to 20% will not cause a decrease in fertility rates. Spermatozoa head abnormalities are caused by the presence of free radicals that enter at the time of ejaculation (Benko *et al.*, 2022) ^[7]. Saake *et al.* (1998) ^[35] reported that abnormal head morphology of spermatozoa could have both compensable and an uncompensable component. And concluded that morphological abnormal spermatozoa are excluded from the accessory sperm population based upon severity of head shape distortion. Reduced sperm motility and membrane integrity were associated with increased DNA damage and abnormal sperms (Gundogan *et al.*, 2010) ^[19]. Januskauskas *et al.* (2001) ^[22] reported that the occurrences of abnormal spermatozoa are positively correlated with DNA damage. A high percentage of abnormal and dead spermatozoa (Shannon and Curson, 1972, Heuer and Tahir, 1982) ^[36, 21] exert toxic and lytic effect on motile sperm cells in the ejaculate, which leads to lower fertility.

Conclusion

Semen dilution to 25 million spermatozoa/dose establish a better counterbalance between different constituents influencing the morphological sperm characters and can be efficiently used in cryopreservation. Whereas quality semen also, possesses large numbers of abnormal and dead spermatozoa which exert a toxic and lytic effect on motile live sperm cells in the ejaculate, which leads to lower fertility which can be managed by the filtration technique to remove dead and abnormal spermatozoa to improve the cryosurvival of spermatozoa even at higher dilutions.

References

- Ahmad M, Ahmad N, Riaz A, Anzar M. Sperm survival kinetics in different types of bull semen: progressive motility, plasma membrane integrity, acrosomal status and reactive oxygen species generation. *Reprod Fertil Dev.* 2015;27(5):784-793.
- Alvarez JG, Lasso JL, Blasco L, Nuñez RC, Heyner S, Caballero PP, *et al.* Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Hum Reprod.* 1993;8(7):1087-1092.
- Alvarez M, Tamayo-Canul J, Anel E, Boixo JC, Mata-Campuzano M, Martinez-Pastor F, *et al.* Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. *Theriogenology.* 2012;77(6):1111-1118.
- Asaduzzaman M, Jha PK, Saha A, Akter S, Alam M, Bari F. Assessment of semen quality of two ram breeds at pre-freeze stage of cryopreservation. *Int J Livest Res.* 2021;11(2):37-44.
- Ashworth PJ, Harrison RA, Miller NG, Plummer JM, Watson PF. Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. *Reprod Fertil Dev.* 1994;6(2):173-180.
- Ballester J, Johannisson A, Saravia F, Håård M, Gustafsson H, Bajramovic D, *et al.* Post-thaw viability of bull AI-doses with low-sperm numbers. *Theriogenology.* 2007;68(6):934-943.
- Benko F, Mohammadi-Sangcheshmeh A, Ďuračka M, Lukáč N, Tvrdá E. *in vitro* versus cryo-induced capacitation of bovine spermatozoa, part 1: Structural, functional, and oxidative similarities and differences. *PLoS One.* 2022;17(10):e0276683.
- Bhakat M, Mohanty TK, Gupta AK, Mohanty AK, Abdullah M. Effect of filtration of low grade ejaculates on semen quality parameters at refrigerated temperature (4-7 °C). *Adv Anim Vet Sci.* 2014;2(11):625-631.
- Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenology.* 2000;54(1):129-136.
- Centurion F, Vazquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, *et al.* Influence of porcine spermathecae on the susceptibility of boar spermatozoa to high dilution. *Biol Reprod.* 2003;69(2):640-646.
- Chenoweth P. The semen examination. In: Australian Cattle Veterinarians (ACV), Australian Sheep Veterinarians (ASV) and Australian Reproduction Veterinarians (ARV) Conference; 2009. p. 179-187.
- Cisale HO, Fischman ML, Blasi CD, Fernandez HA, Gledhill BL. Enrichment of high-quality spermatozoa in bovine semen: relative effectiveness of three filtration matrixes. *Andrologia.* 2001;33(3):143-150.
- Deori S, Deka BC, Biswas RK, Nahardeka N, Arangasamy A, Bhuyan D, *et al.* Characteristics and freezability of Assam Hill goat semen. *Indian J Anim Res.* 2018;52(1):25-28.
- England GCW. Semen quality in dogs and the influence of a short-interval second ejaculation. *Theriogenology.* 1999;52(6):981-986.
- Fernández-Santos MR, Estes MC, Montoro V, Soler AJ, Garde JJ. Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa: effects of egg yolk, glycerol and cooling rate. *Theriogenology.* 2006;66(8):1931-1942.
- Garner DL, Thomas CA, Allen CH. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. *J Androl.* 1997;18(3):324-331.
- Garner DL, Thomas CA, Gravance CG, Marshall CE, DeJarnette JM, Allen CH. Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology.* 2001;56(1):31-40.

18. Graham EF, Graham JK. The effect of whole ejaculate filtration on the morphology and the fertility of bovine semen. *J Dairy Sci.* 1990;73(1):91-97.
19. Gundogan M, Yeni D, Avdatek F, Fidan AF. Influence of sperm concentration on the motility, morphology, membrane and DNA integrity along with oxidative stress parameters of ram sperm during liquid storage. *Anim Reprod Sci.* 2010;122(3-4):200-207.
20. Harrison RAP, Dott HM, Foster GC. Bovine serum albumin, sperm motility, and the "dilution effect". *J Exp Zool.* 1982;222(1):81-88.
21. Heuer C, Tahir MN. Experiment on deep freeze preservation of water buffalo semen using sephadex filtration as semen assay. In: *Proc Buffalo Seminar on Reproduction and Meat Production*, Tanuku; 1982. p. 55-63.
22. Januskauskas A, Johannisson A, Rodriguez-Martinez H. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology.* 2001;55(4):947-961.
23. Jasko DJ, Hathaway JA, Schaltenbrand VL, Simper WD, Squires EL. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. *Theriogenology.* 1992;37(6):1241-1252.
24. Karan P, Mohanty TK, Kumaresan A, Bhakat M, Baithalu RK, Verma K, *et al.* Improvement in sperm functional competence through modified low-dose packaging in French mini straws of bull semen. *Andrologia.* 2018;50(5):e13003.
25. Kulaksiz R, Çebi Ç, Akçay E. The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4 C. *Turk J Vet Anim Sci.* 2012;36(2):177-182.
26. Kumar P, Kumar D, Sikka P, Singh P. Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. *Anim Reprod Sci.* 2015;152:26-31.
27. Lagerlöf N. Morphologische Untersuchungen über Veränderungen im Spermabild und in den Hoden bei Bullen mit verminderter oder aufgehobener Fertilität. Vol. 19. *Levin & Munksgaard*; 1934.
28. Lewis-Jones I, Aziz N, Seshadri S, Douglas A, Howard P. Sperm chromosomal abnormalities are linked to sperm morphologic deformities. *Fertil Steril.* 2003;79(1):212-215.
29. Maxwell WMC, Johnson LA. Membrane status of boar spermatozoa after cooling or cryopreservation. *Theriogenology.* 1997;48(2):209-219.
30. Miró J, Taberner E, Rivera M, Peña A, Medrano A, Rigau T, *et al.* Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalanian donkey semen. *Theriogenology.* 2009;72(8):1017-1022.
31. Pace MM, Graham EF. The release of glutamic oxaloacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. *Biol Reprod.* 1970;3(2):140-146.
32. Peña A, Linde-Forsberg C. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology.* 2000;54(5):703-718.
33. Pineda MH, Faulkner LC. Biology of sex. In: *Veterinary endocrinology and reproduction*. Philadelphia: Lea & Febiger; 1980. p. 208-234.
34. Rijsselaere T, Van Soom A, Tanghe S, Coryn M, Maes D, de Kruif A. New techniques for the assessment of canine semen quality: a review. *Theriogenology.* 2005;64(3):706-719.
35. Saacke RG, DeJarnette JM, Bame JH, Karabinus DS, Whitman SS. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super-and single-ovulating cattle? *Theriogenology.* 1998;50(1):117-128.
36. Shannon P, Curson B. Toxic effect and action of dead sperm on diluted bovine semen. *J Dairy Sci.* 1972;55(5):614-620.
37. Silva AR, Fontenele-Neto JD, Cardoso RDC, Silva LD, Chiniréa VH, Lopes MD. Description of ultrastructural damages in frozen-thawed canine spermatozoa. *Ciênc Anim Bras.* 2009;10(2):595-601.
38. Sullivan JJ. Characteristics and cryopreservation of stallion spermatozoa. *Cryobiology.* 1978;15(3):355-357.
39. Turner RM. Moving to the beat: a review of mammalian sperm motility regulation. *Reprod Fertil Dev.* 2005;18(2):25-38.
40. Vigneshwaran B, Chacko L, Promod K, Sebastain R, Magnus Paul K, Aslam M, *et al.* Impact of equilibration on sperm quality in Malabari buck semen: a comparative study of different freezability. *J Vet Anim Sci.* 2024;56(2):280-284.
41. Wajdi SA, Utomo B, Rimayanti R, Safitri E, Suprayogi TW, Wurlina W. Supplementation of Kelor leaf (*Moringa oleifera*) aqueous extract increase on post-thawed Limousin bull sperm quality. *J Medik Vet.* 2021;4(2):249-255.
42. Wales RG, White IG. Viability of diluted dog spermatozoa *in vitro*. *Reproduction.* 1963;5(1):67-76.
43. Wang D, King SM, Quill TA, Doolittle LK, Garbers DL. A new sperm-specific Na⁺ /H⁺ exchanger required for sperm motility and fertility. *Nat Cell Biol.* 2003;5(12):1117-1122.