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Centrifugation: A step towards the improving Murrah semen quality parameters

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Abstract
For buffalo bulls, the ejaculate having a sperm motility of 70% is only processed for sperm cryopreservation. But we can utilize the ejaculates which have lower motility by centrifugation. Therefore, in our study, we assessed the effect of single-layer centrifugation (SLC) and double-layer centrifugation (DLC) on before-freezing motility and quality of frozen semen. We selected 7 ejaculates of low sperm motility and divided them into three categories Cat-I without centrifugation (WC), Cat-II SLC, and Cat-III DLC. After that, we checked before freezing motility and cryopreserved them. After 24 hours of cryopreservation analyzed the post-thaw motility, sperm morphology, sperm plasma membrane integrity, and sperm acrosome integrity. On the basis of the results of these parameters DLC and SLC was better in semen quality than without centrifugation of lower motility ejaculates.

Keywords: Buffalo, ejaculate, sperm, acrosome integrity semen

1. Introduction
The maximum buffalo population of the world is found in India, 56.5% (109.9 million. India is the world's largest milk producer, accounting for approximately 221.06 MT (22% of global production), (DAHD, and annual report 2021-22). To increase milk production of the buffalo, there is a continuous breeding program ongoing through artificial insemination using cryopreserved semen of elite buffalo bulls. But there is only 2093 breeding buffalo bulls in our country, NDDB 2020-21 Report. Artificial Insemination (AI) is the most affordable and convenient biotechnological tool for the dissemination of improved genetics at farmers' doorstep (Singh *et al*., 2013) [16]. However, AI coverage of bovines in the country is about 30 percent ranging from 71 percent to even less than 1 percent for different states. That means 65 percent of animals are still bred through natural services either because the services are not available at farmers' doorstep or they are not convinced with the efficacy of the existing services. About 80 million inseminations are carried out in the country annually with the estimated overall conception rate of 35 percent as against 60 percent plus AI success rate in dairy-developed countries (NDDB, 2022) [11]. Benefits of AI can be doubled by improving the quality of AI services. So, due to less number of breeding bulls, we need to develop some techniques or strategies to increase semen production. Therefore, the study has been designed to evaluate fresh ejaculates motility and those ejaculates were below the prescribed guideline (below 70% motile) were divided into three categories CAT- I (WC) without centrifuged, CAT-II (SLC) single-layer centrifuged at 1500 rpm for 5 minutes and CAT-III (DLC) Double layer centrifugation and analyzed again total motility and impact of SLC and DLC on frozen semen quality.

2. Materials and Methods
2.1 Ethics statement
This article does not contain any studies with human or animal subjects.

2.2 Experimental Location and Design.
The proposed work was carried out in the Semen Freezing Laboratory of the Accuvance Dairy Genetics and Research Centre, Patauda Jhajjar Haryana.

2.3 Semen collection
The semen was collected by artificial vagina in the early hours of the morning before feeding, and from each buffalo bull, two ejaculates were taken on the day of collection, and the interval between two collections was 30 minutes.

2.4 Estimation of sperm concentration
The sperm concentration in the freshly collected semen was determined by using an Accu Cell bovine photometer (IMV, LAigla, and France) at 530 nm wavelength Semen dilution and individual sperm motility**.** Initial semen dilution was performed with an equal quantity of diluent and kept in the same water bath at 32 °C (Kumar *et al*., 2013) [9]. After the estimation of sperm concentration, the dilutor was added to pre-diluted semen, so that the final concentration became 80 million sperm/mL. After final dilution, sperm motility was assessed by placing a small drop of diluted on a clean, grease-free warm slide (37 °C) with a cover slip over the drop and examining it under 200x magnification by a phase contrast microscope (Yadav *et al*., 2023) [20]. The ejaculates had a minimum of 70% sperm motility when considered for cryopreservation.

2.5 Centrifugation
Those fresh ejaculates having sperm motility below the 70% were considered for single-layer and double-layer centrifugation at 1500 rpm for 5 minutes and seminal plasma was removed gently and then diluted with Tris-Egg Yolk Citrate (TECY) dilutor. After dilution so that the final concentration became 80 million sperm/mL (Yadav *et al*., 2023) [20].

2.6 Post-thaw sperm motility
After 24 h of cryopreservation, the semen straws were thawed at 37 °C for 30 s, and sperm motility was estimated immediately. The ejaculates that had a minimum of 50% sperm motility were selected for an artificial insemination program.

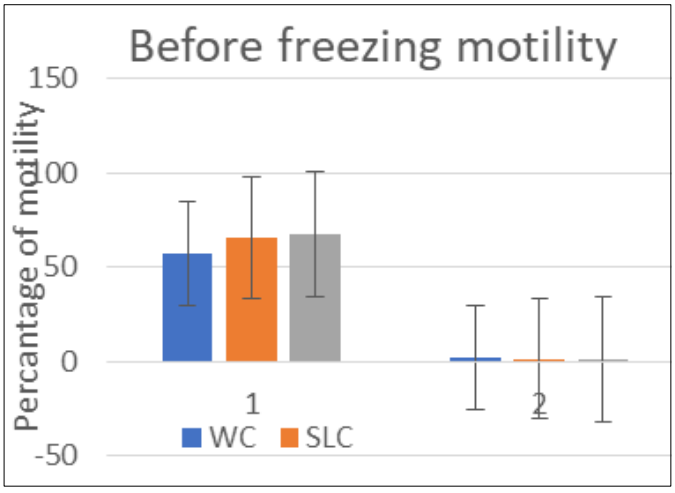
2.7 Sperm morphology
To evaluate the sperm morphology, eosin-nigrosin staining was performed in cryopreserved semen samples (Kumar *et al*., 2016) [8]. A semen straw was thawed at 37 °C, and one small drop of thawed semen was placed on prewarmed slide. After that, one small drop of each eosin and nigrosin stain was placed over the semen drop. A thin smear was prepared by spreader slide at a 30-degree angle to disperse the semen suspension over the slide length and fixed with air drying. A total of 200 spermatozoa were evaluated for each sample in different fields at 100X objective under a phase-contrast microscope and percentage of abnormal spermatozoa (bent tail, coiling of tail over mid piece, proximal protoplasmic effect, and eccentric thickening of acrosome) was determined.

2.9 Sperm plasma membrane integrity
To assess the functional integrity of the sperm plasma membrane, a hypo-osmotic swelling test was performed (Jeyendran *et al*., 1984; Dalal *et al*., 2020) [7, 4]. For the test, 500 µL hypo-osmotic solution (0.735g sodium citrate dihydrate and 1.351g fructose in 100 mL of double distilled water, 100mOsm/L) was mixed with 50µL of frozen-thawed semen, and for the control group, 500µL PBS mixed with 50µL frozen-thawed semen and incubated for 60 min at 37 ºC. After incubation, 40µL of 2% eosin solution was added to increase visibility. Semen drop was added on slide and covered with a coverslip and 200 spermatozoa were counted further coiled tails (HOST positive) were identified. A drop of diluted semen was placed on a clean sterilized dry glass slide and covered with a coverslip. A total of 200 spermatozoa were counted in different fields at 100x objective under a phase-contrast microscope and the percentage of spermatozoa positive to HOST (having coiled tails) was determined. The percentage of coiled-tail spermatozoa in PBS were subtracted from that of the HOST to get the true HOST-reactive sperm.

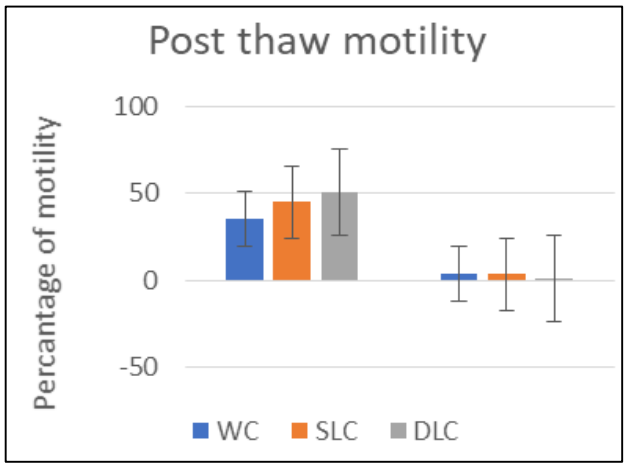
2.10 Acrosome integrity
Intactness of the acrosome of spermatozoa was assessed by using Giemsa staining method (Almadaly *et al*., 2014) [1]. A rewarmed slide was used and a drop of semen was added to prepare a smear. Fixation was done by using methanol for about 10 minutes further slide was rinsed with the help of running distilled water. Geimsa solution was prepared (3 mL Giemsa, 2 mL PBS and 35 mL distilled water) used for staining followed by rinsing and drying. At 1000x magnification about two hundred cells were assessed, spermatozoa having intact acrosome had purple heads whereas spermatozoa with damaged acrosome have pale lavender heads. Acrosome integrity was determined by a total number of spermatozoa with intact acrosomes out of the total number of spermatozoa multiplied by 100.

3. Results
3.1 Effect of SLC and DLC before freezing motility
As per Minimum standard protocol for Bovine frozen semen the fresh ejaculate must have 70% motility for further processing (MSP, 2022 GOI). Our target was to find out the effect of centrifugation on lower motility ejaculates (number of samples in each category 7), and we found the total motility (Mean±S.E) in fresh ejaculates of SLC and DLC categories were significantly higher (p<0.05) than WC (Figure 1A).

3.2 Effect of SLC and DLC Post thaw motility
After 24 hours of cryopreservation, we checked the post-thaw motility (Figure 3) in different categories of ejaculates (number of samples in each category – 7) and found the post-thaw motility (Mean±S.E) in frozen ejaculates of SLC and SLC categories were significantly higher (p<0.05) than WC (Figure 1 B).



(A)



(B)

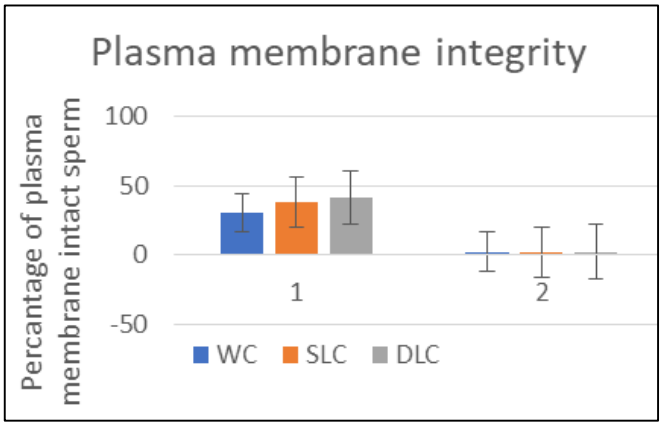
Fig 1 A & B: Showing graphical results of before-freezing and post-thaw motility

3.3 Sperm plasma membrane integrity

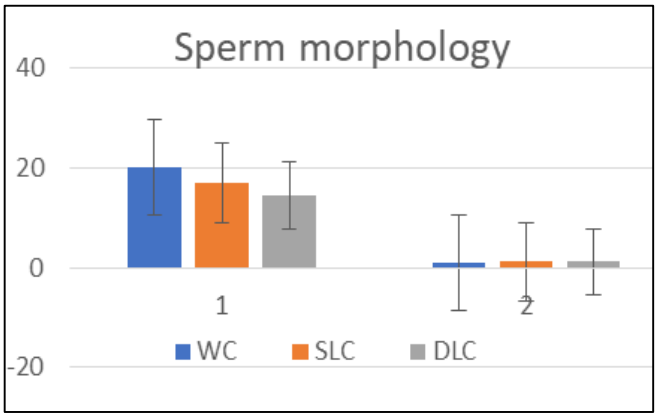
We assessed the plasma membrane integrity in all three categories (Figure 2A) and found maximum (p<0.05) plasma membrane intactness was in category DLC than WC and SLC was between WC & DLC.

3.4 Effect of SLC and DLC on sperm morphology

The sperm morphology in cryopreserved samples of all three categories (7 samples in each category) were evaluated by eosin nigrosin staining. The sperm abnormalities of category WC were higher (p<0.05) than the DLC and IN SLC category it was between DLC & WC. (Figure 2B).



(A)



(B)

Fig 2 A & B: Showing graphical results of sperm plasma membrane integrity and percentage of abnormal sperm

3.5 Effect of SLC and DLC on acrosome intactness

The live intactness of the acrosome is essential step for realising of acrosomal enzyme like acrosin, and hyaluronidase for binding ZP3 receptors of zona pellucida for successful fertilization (Sun *et al.*, 2021) [17]. The sperm acrosome intactness in cryopreserved samples of all three categories (7 samples in each category) were evaluated by Giema staining. The sperm acrosome intactness of category DLC were higher (p<0.05) than the WC while in the SLC category, it was between DLC & WC (Figure 3).

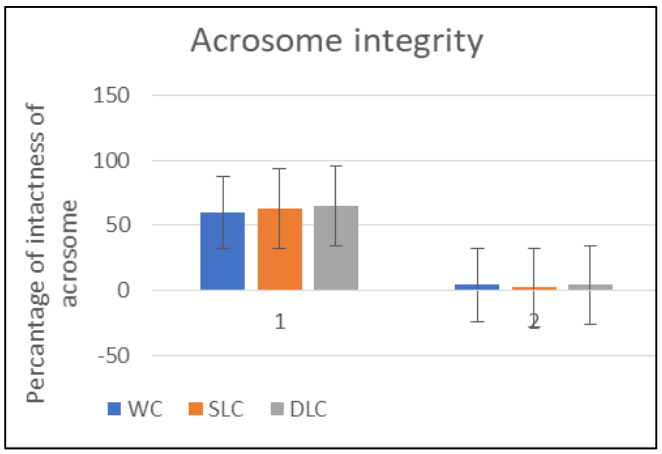


Fig 3: Showing the graphical result of acrosome integrity

4. Discussion

Perm motility is an essential parameter for achieving successful conception because, sperm motility is only way to reach ejaculated spermatozoa up to the female reproductive tract and at the site of fertilization (Vijayaraghavan *et al.*, 2003) [19]. We found the SLC and DLC ejaculates were have high motility than without centrifugation and same result found by Ortiz *et al.*, 2014 in donkeys. The buffalo sperm is more vulnerable for cryopreservation due to high amount of polyunsaturated fatty acid (Mittal *et al.*, 2019) [10]. After cryopreservation we found that post-thaw motility were significantly higher in DLC and SLC ejaculates than WC ejaculates and it was agreed by Gloria *et al.*, 2016[6]. The functional integrity of the plasma membrane is of primary importance for the fertilizing abilities of a spermatozoon (Dalal *et al.*, 2020) [4]. The plasma membrane intactness of sperm of all three categories were evaluated by HOST because many times sperm those have weak plasma membrane but under normal physiological condition show normal sperm motility but hypo-osmotic condition make them vulnerable (Correa and Zavos, 1994; Rasul *et al.*, 2001) [2, 14]. Comparatively, higher number of sperm with intact plasma membranes was in categories DLC than in the category WC and SLC. The reported percentage of buffalo frozen-thawed spermatozoa with intact sperm membranes is 32.63% (Rasul *et al.*, 2001) [14]. The recommended minimum number of frozen-thawed sperm with intact sperm membranes is 40% (MSP, 2022). Considering the recommendation of a minimum of 40%, the ejaculates of categories WC are not suitable for the use of AI. The present study also indicates that spermatozoa of category WC possess a weaker plasma membrane morphologically abnormal sperm can reduce rates of fertilization and embryonic development (Thundathil *et al.*, 2001) [18]. In our finding the maximum abnormal sperm were in categories of WC and SLC than DLC and same result were found BY Gloria *et al.*, 2016 [6] in their experiment. Acrosome

integrity is essential for penetration of zona pellucid of ovum. we revealed first time that DLC and SLC ejaculates is having more acrosome integrity than WC ejaculates.

5. Conclusion
We indicated single layer centrifugation and DLC can be useful techniques to enhance the semen quality of bulls. These techniques can be further used in field practice to get good semen quality.

6. Acknowledgments
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7. Conflict of interest
No conflict of interest.

8. References
1. Almadaly E, Foad F, Mostafa S, and Tetsuma M. Plasma membrane integrity and morphology of frozen-thawed bull spermatozoa supplemented with desalted and lyophilized seminal plasma Global Veterinaria. 2014;13(5):753-766.
2. Correa JR, Zavos PM. The hypo-osmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology. 1994;42(2):351-36
3. DAHD Annual Report. Department of animal husbandry and Dairying (DAHD), Ministry of Fisheries, animal husbandries and Dairying, Government of India; c2020-21. https://dahd.nic.in/annual-reports/dahd-annual-report-2020-21
4. Dalal J, Chandolia RK, Pawaria S, Kumar A, Kumar D, Selokar NL, *et al.* Low-density lipoproteins protect sperm during cryopreservation in buffalo: Unraveling mechanism of action. Mol. Reprod. Dev. 2020;87(12):1231-1244.
5. Gloria A, Carluccio A, Wegher L, Robbe D, Befacchia G, Contri A. Single, and double layer centrifugation improve the quality of cryopreserved bovine sperm from poor quality ejaculates. Journal of animal science and Biotechnology. 2016;7(1):1-9.
6. Gloria A, Carluccio A, Wegher L, Robbe D, Befacchia G, Contri A. Single, and double layer centrifugation improve the quality of cryopreserved bovine sperm from poor quality ejaculates. Journal of Animal Science and Biotechnology. 2016;7(1):1-9.
7. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Reproduction. 1984;70(1):219-228.
8. Kumar P, Saini M, Kumar D, Jan MH, Swami DS, Sharma RK. Quantification of leptin in seminal plasma of buffalo bulls and its correlation with antioxidant status, conventional and computer-assisted sperm analysis (CASA) semen variables. Anim. Reprod. Sci. 2016;166:122-127.
9. Kumar P, Yadav PS, Sethi RK. Applied Veterinary Andrology and Artificial insemination. Kalyani publisher, India. Mammalian Reproduction. Springer, Boston, MA; c2013. p. 79-91.
10. Mittal PK, Madan AK, Sharma V, Gottam GS, Gupta B. Cryopreservation of buffalo bull semen-restriction and expectation: A review. International Journal of Current Microbiology and Applied Sciences. 2019;8(1):1351-1368.
11. NDDB annual report on 2021-22. Ministry of Fisheries, Animal Husbandry, and Dairying. www.nddb.cop.
12. Ortiz I, Dorado J, Ramírez L, Morrell JM, Acha D, Urbano M, *et al.* Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses. Animal. 2014;8(2):308-315.
13. Ramu S, Jeyendran RS. The hypo-osmotic swelling test for the evaluation of sperm membrane integrity. Spermatogenesis: Methods and protocols; c2013. p. 21-25.
14. Rasul Z, Ahmad N, Anzar M. Changes in motion characteristics plasma membrane integrity and acrosome morphology during cryopreservation of buffalo spermatozoa. Journal of Andrology. 2001;22(2):278-283.
15. Singh I, Balhara AK. New approaches in buffalo artificial insemination programs with special reference to India. Theriogenology. 2016;86(1):194-199.
16. Singh P, Kumar D, Kumar P, Singh I, Yadav PS. Cryopreservation and quality assessment of buffalo bull semen collected from farmer's doorstep. Agricultural Research. 2013;2(2):148-152.
17. Sun W, Jiang S, Su J, Zhang J, Bao X, Ding R, Li X. The effects of cryopreservation on the acrosome structure, enzyme activity, motility, and fertility of bovine, ovine, and goat sperm. Animal Reproduction; c2021. p. 17.
18. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. The use of *in vitro* fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. Animal Reproduction Science. 2001;(65):181-192.
19. Vijayaraghavan S. Sperm motility: patterns and regulation. In Introduction to Mammalian Reproduction. Springer, Boston, MA; c2003. p. 79-91.
20. Yadav U, Dutt R, Bansal K, Gupta A, Bala R, Bhardwaj S, Kumar P. Epsilon poly-lysine in buffalo semen extender: A step towards reducing the development of antibiotic resistance. Reproduction in Domestic Animals; c2023.