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Comparative evaluation of quality parameters in cryopreserved conventional and sex-sorted semen of Sahiwal

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Abstract

This study evaluated the potential effect of sex-sorting procedure on post thaw structural and functional attributes of Sahiwal bull spermatozoa. Cryopreserved semen samples were examined after thawing for quality parameters like viability, motility, acrosomal integrity, and combined morphological abnormalities. Hierarchical clustering distinguished two groups: structural parameters (acrosomal integrity and abnormalities) and functional parameters (motility and live sperm count). Spearman correlation analysis revealed a moderate positive correlation between motility and viability, while both exhibited negative associations with acrosomal integrity and abnormalities. Non-parametric analysis showed no significant differences ($p \geq 0.05$) with small effect sizes for all the studied parameters. These findings indicate that sex-sorting does not significantly compromise the basic post-thawed sperm quality, supporting its application in artificial insemination programs.

Keywords: Sahiwal bull, cryopreservation, sex-sorting, motility, acrosomal integrity, live sperm

1. Introduction

The livestock sector is essential to global agriculture, considerably enhancing food security, rural livelihoods, and economic development. In India, the industry provides for about two-thirds of rural households and 8.8% of the country's total population. In terms of the economy, it makes up 30.87% of the GVA from agriculture and related sectors and provides 6.17% to the national Gross Value Added (GVA). India presently produces the most milk worldwide, accounting for about 24% of the total. India has the greatest cattle population in the world (192.49 million), accounting for 16% of the total (20th Livestock Census-2019)^[1]. Of these, 26.18% are crossbred and 73.82% are native or non-descript breeds.

Cattle are fundamental to Indian farming system and serves as an asset for marginal farmers. Sahiwal (*Bos indicus*) is one of the most prolific and genetically valuable native dairy breeds. It is well known for its exceptional milk quality and quantity, low maintenance needs, disease resistance, and heat tolerance. Because of these characteristics, the Sahiwal breed is useful in crossbreeding initiatives and very appropriate for low-input dairy systems. However, there remains a dearth of comprehensive data on Sahiwal bulls, particularly regarding semen quality parameters. There is a growing importance of reproductive techniques like artificial insemination and semen sexing, to effectively increase breeding efficiency.

Cryopreserved semen has enhanced the cattle breeding by enabling long-term storage of viable sperm at -196°C , while preserving their functional integrity. Sex-sorting procedures has further advanced this field, allowing selective use of X- bearing sperm cells and thereby increasing the chances of female offspring to about 90%, compared to that of 50% in conventional semen (Cerchiaro *et al.*, 2007)^[4]. Use of sorted semen ultimately enhances herd productivity, minimizes the production of unwanted males, promotes faster genetic improvement, and increases economic gains while promoting better animal welfare (Holden

and Butler, 2018)^[9].

Nonetheless, despite these advancements, both cryopreservation and semen sorting impose potential physiological and structural pressure on sperm cells. As spermatozoa has low cytoplasmic volume and membrane composition, they are more resilient to osmotic variations (Agca *et al.*, 2002)^[2]. Cryopreservation adversely affects the sperm structure and function, majorly due to thermal stress, freezing and thawing, osmotic stress from high cryoprotectants and crystals of ice formation (Chantler *et al.*, 2004)^[5]. Although straws are globally standardized for semen storage, successful cryopreservation relies on preserving sperm motility, viability and fertilizing capacity. Studies highlighted high sensitivity of plasma and acrosomal membranes of sperm to cold shock, resulting in ultrastructural damage and impaired function (Khalil *et al.*, 2018; Ozimic *et al.*, 2023)^{[10] [15]}. In sex-sorting too, factors like mechanical shear stress, fluorescent DNA labelling, laser excitation in flow cytometry, ultimately reduces sperm quality and yield per AI dose, leading to lower conception rates compared to conventional semen (Seidel *et al.*, 1999)^[18]. Therefore, the synergistic effects of both cryopreservation and sorting significantly alters sperm normal behavior.

Given these factors, this study aims to comprehensively evaluate the post-thaw semen quality of cryopreserved unsorted and cryopreserved sex-sorted Sahiwal bull semen in order to help standardize evaluation protocols for semen quality assessment in this economically and genetically significant indigenous dairy breed and to clarify the morphological and qualitative changes associated with the cryopreservation and sorting process.

2. Methodology

2.1 Preparation of stains and buffer

- a) **Eosin-Nigrosine stain:** 2.90 g of sodium citrate dihydrate and 5.00 g of eosin was dissolved in 100 ml of double-distilled water. The solution was heated to boiling, subsequently filtered, and stored at room temperature. Subsequently, a commercially prepared nigrosine stain (HIMEDIA) was used to formulate an eosin-nigrosine staining solution in a 1:3 ratio.
- b) **Rose Bengal stain:** 3.00 g of Rose Bengal powder was dissolved in 100 ml of double-distilled water, followed by thorough mixing. Subsequently, formalin (37-41%) was added to the solution, which was then stored at room temperature.
- c) **Giemsa stock solution:** 1.00 g of Giemsa stain powder was ground with 98.00 ml of methanol. 32 ml glycerol was then added to the mixture, which was stored at 37 °C for one month. During this period, the stain was thoroughly mixed daily for several minutes and also immediately prior to use.
- d) **Giemsa working solution:** 3 mL of Giemsa stock solution was mixed with 2 mL of Sorenson's 0.1 M phosphate buffer, followed by the addition of 35 mL of double glass-distilled water. The solution was then stored at room temperature.
- e) **Sorenson's 0.1M phosphate buffer (pH 7.0):** Solution A (0.1 M Potassium dihydrogen phosphate solution): 13.609g of potassium dihydrogen phosphate (anhydrous) was dissolved in 1000ml of double glass distilled water. Solution B (0.1 M Disodium hydrogen orthophosphate solution): 14.198g of disodium hydrogen phosphate was dissolved in 1000ml of double glass distilled water. Sorenson's 0.1 M phosphate buffer was prepared by

mixing 1 mL of Solution A with 33 mL of Solution B. The pH was adjusted to 7.0, and the buffer was stored at 4 °C for further use.

2.2 Sampling

For this study, cryopreserved semen straws of Sahiwal bull were procured from the centers of Department of Animal Husbandry and Dairying, Govt. of Madhya Pradesh. Conventional cryopreserved unsorted semen straws (n = 52) and cryopreserved sex-sorted semen straws (n = 57) of Sahiwal bulls were taken and transported to the Animal Biotechnology Centre, NDVSU for further processing. All straws were carefully transported fully immersed in liquid nitrogen to ensure optimal viability.

2.3 Semen thawing

Semen straws were removed from the liquid nitrogen carefully and immersed in water bath for 60 seconds at 37 °C. The straws were then wiped to prevent contamination. After thawing, the sealed end of each straw was cut using a straw cutter, and a sterile capillary micropipette was used to extract the semen carefully.

2.4 Evaluation of spermatozoa for quality parameters

The percentage of live and dead spermatozoa were estimated by staining with Eosin-nigrosine (Blom, 1950)^[3]. The combined abnormalities were analyzed by staining with Rose Bengal stain as described before (Pervage *et al.*, 2009)^[16], and the acrosomal integrity was studied by staining the smears with Giemsa stain (Chowdhury *et al.*, 2014)^[7].

- a) **Assessment of motility (progressively motile spermatozoa %):** Percentage of progressive sperm motility (forward movement) in each semen sample was determined using phase contrast microscope, supplied with a warm stage adjusted to 37 °C. Thawed semen drops were placed on sterile glass slide and coverslip was placed over it carefully. The movement of progressively motile sperms were observed using 100X. About 200 sperms were counted across multiple fields. Formula used for the calculation: Progressive motility (%) = Number of progressive motile spermatozoa / Total number of spermatozoa observed X 100
- b) **Assessment of sperm viability (Live sperm %):** Sperm viability was assessed using eosin-nigrosine staining by evaluating live sperm and membrane integrity. A semen smear was prepared on glass slide, maintained on warm stage at 37 °C, and stained with eosin: nigrosine (1:3) solution, air-dried, and examined at 100X. About 200 sperm were counted; unstained cells were considered live, while stained cells were considered dead. The percentage of living spermatozoa was calculated using the formula below:

Percent live spermatozoa = Live spermatozoa/ Total spermatozoa counted × 100

- c) **Assessment of acrosomal integrity:** Giemsa stain was used to analyze the integrity of acrosome. Semen smear was prepared onto a sterile glass slide, subsequently dried and tempered on warming plate maintained at 37 °C, fixed in methanol for 30 min. in incubator and then placed in working solution of Giemsa for 60 min. After air drying, slides were examined at 40X and 100X magnifications. Spermatozoa with intact acrosomes had purple heads, but those with damaged acrosomes had pale

lavender heads. By dividing the total number of sperm cells by the number of acrosome-intact ones and multiplying the result by 100, the status of acrosome was determined.

- d) Assessment of combined abnormalities:** Rose Bengal was employed as a simple and effective staining agent to assess combined sperm abnormalities, with particular emphasis on visualizing sperm cell structures and identifying morphological defects such as head and tail anomalies. On a spotless glass slide, a small smear of thawed bull semen was created and left to air dry. After that, the smear was well covered with Rose Bengal solution and let to sit at room temperature for 5-7 min. The slide was then gently cleaned under running water to get rid of any remaining stain, and it was allowed to air dry. Sperm cell morphological anomalies were investigated at 40X and 100X magnification. The head, tail, and midpiece area showed a variety of anomalies.

2.5 Statistical analysis

Statistical analysis was carried out using appropriate packages of R version 4.5.2. The data was first copied to MS Excel and the variables were grouped as per Chenoweth *et al.*, 1993^[6] (Fig. 1). The categorical data was then subjected to non-parametric tests to find statistical difference in various studied traits between the cryopreserved unsorted and sex sorted semen.

Abnormalities Score		Acrosomal Integrity	
NA	0	Intact	0
Abnormal	1	Reacted	1
Individual Motility		Live Sperm %	
Up to 70	Very Good (VG)	50 Below	Poor
50–69	Good (G)	50–59	Fair / Moderate
40–50	Fair (F)	60–74	Good
30 Below	Poor (P)	75 Above	Excellent

Fig 1: Scoring criteria of various sperm quality parameters as per Chenoweth *et al.*, 1993.

3. Results

3.1 Descriptive statistics

The descriptive statistics were found out using MS Excel and its basic formula tools. The Individual motility was mostly very good in both the categories. In unsorted straws, the individual motility very good was found in 73% of spermatozoa, whereas, in sorted it was observed in 63%. In addition, the sorted group a negligible amount of 2% spermatozoa classified as poor, which was zero in unsorted group. The live sperm count percentage was majorly good in both categories with 10% excellent in unsorted group and 9% excellent type in sorted group. The acrosomal integrity was mainly intact in both (94% for unsorted and 88% for sorted groups), although sorted semen category showed 12% reacted acrosomal integrity. Abnormalities (or combined abnormalities) were very low in both the groups. The mean of abnormalities in sorted group was 0.12, whereas in unsorted group it was 0.06.

A spermatozoon exhibiting normal morphological structures having a defined oval head with clearly defined acrosome

joined to the tail by the mid-piece was considered normal (Figure 2a). Sperms showing any of such characteristics like head size variations, mid-piece deformities, broken parts, and coiled or no tail were considered abnormal (Figure 2b) (Nagy *et al.*, 2013)^[14]. Sperm cells exhibiting unstained, clear white heads were classified as viable, whereas those with partially or completely purple-stained heads were considered as non-viable sperms.

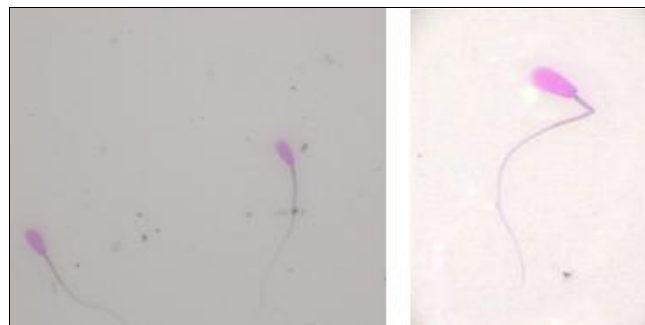


Fig 2: Sperm cells after staining with Rose Bengal stain for morphological abnormalities. (a) Normal spermatozoa (b) Deformed tail spermatozoa

3.2 Hierarchical Clustering Analysis

The hierarchical clustering dendrogram illustrated grouping among sperm quality parameters in sorted and unsorted semen. At a height of ~1.5, two main clusters emerged: one pairing acrosomal integrity and abnormalities (merged at height ~0.0), and another linking individual motility and live sperm counts (merged at ~0.5). This pattern highlights associations between structural parameters and functional viability metrics, offering visual insight into parameter interdependencies. The dendrogram showing the hierarchical clustering of the semen quality parameters is shown in Figure 3.

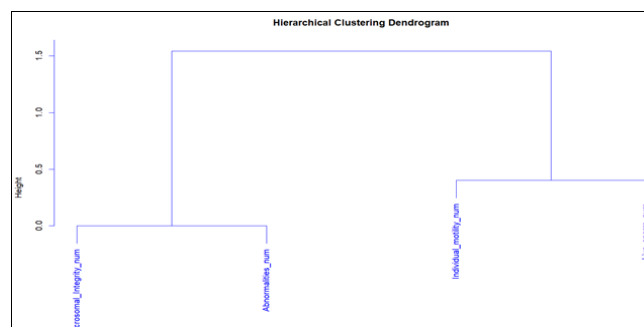


Fig 3: Dendrogram illustrating the hierarchical clustering of semen quality traits

3.3 Motility Score Distribution

The distribution of spermatozoa motility scores differed slightly between straw types. In sorted semen, 63.16% were 'Very Good' ($\geq 70\%$ progressive motility), 29.82% 'Good' (50–69%), 5.26% 'Fair' (40–49%), and 1.75% 'Poor' ($< 40\%$). In unsorted semen, 'Very Good' increased to 73.08%, with 21.15% 'Good,' 5.77% 'Fair,' and 0% 'Poor.' Median scores were 4 ('Very Good') for both, with unsorted samples showing a tighter high-score distribution. Mann-Whitney U test indicated no significant difference ($U=1337.0$, $p=0.2842$), with a small effect size ($r=0.10$). Violin plot illustrating the distribution of sperm motility scores in sorted and unsorted semen straws types is shown in Figure 4.

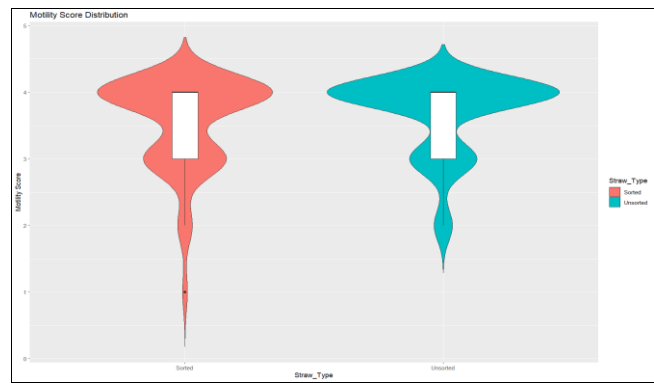


Fig 4: Distribution of sperm motility scores in sorted and unsorted semen straws

3.4 Abnormalities by straw type

Morphological abnormalities were observed in 12.28% of sorted spermatozoa (mean=0.12, SD=0.10) and 5.77% of unsorted (mean=0.06, SD=0.09), with 87.72% and 94.23% normal, respectively. The bar chart showed higher abnormality counts in sorted samples. Fisher's Exact test revealed no significant difference ($p=0.3257$). The bar plot showing the frequency distribution of normal and abnormal spermatozoa in sorted and unsorted semen straws is shown in Figure 5.

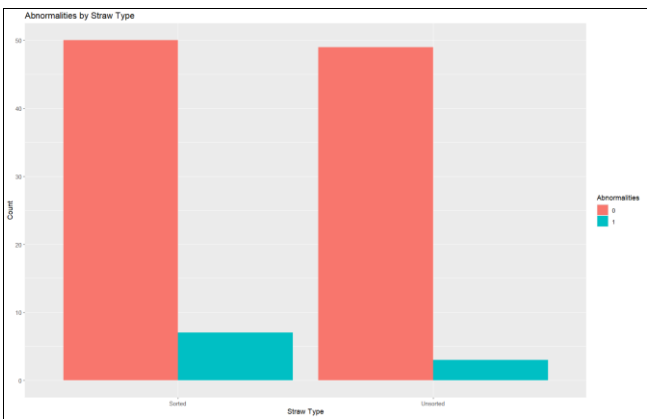


Fig 5: Comparison of sperm abnormalities between sex-sorted and unsorted semen straws

3.5 Correlation analysis of sperm quality variables

The correlation analysis showed a moderate positive relationship between individual motility and live sperm count ($r=0.597$), indicating linked viability. Both parameters were negatively correlated with acrosomal integrity and abnormalities ($r\approx-0.544$ and -0.386), suggesting improved function with fewer defects. Acrosomal integrity and abnormalities were perfectly correlated. No significant differences were observed across straw types (Fig. 6).

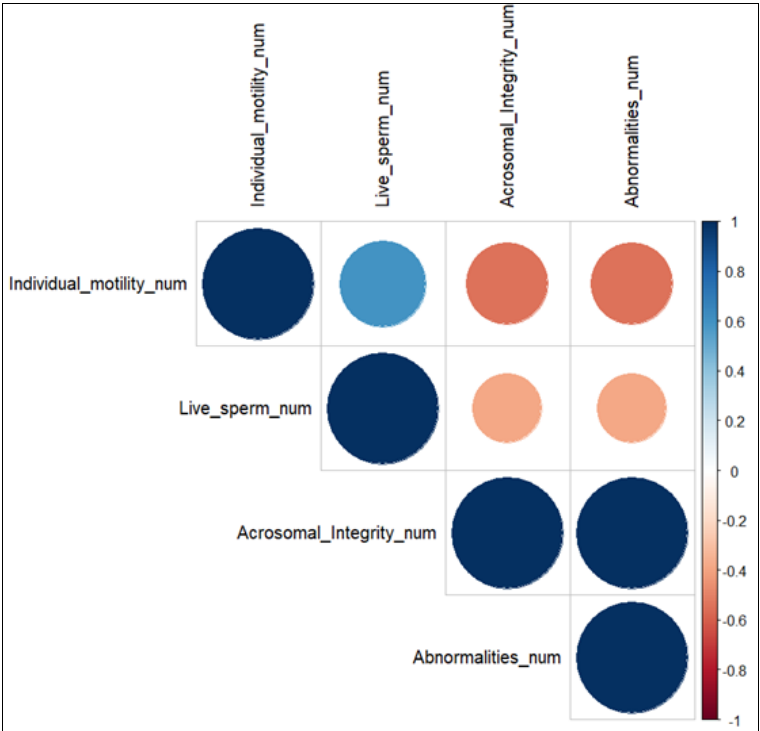


Fig 6: Correlation-Heatmap showing relationships among sperm quality parameters. (Red indicates positive correlation and purple indicates negative correlation)

3.6 Live Sperm Score Distribution

Live spermatozoa distributions showed minor variations. In sorted semen, 8.77% were 'Excellent' ($\geq 75\%$), 70.18% 'Good' (60-74%), 19.30% 'Fair' (50-59%), and 1.75% 'Poor' ($< 50\%$). Unsorted had 9.62% 'Excellent,' 75.00% 'Good,' 13.46%

'Fair,' and 1.92% 'Poor.' Medians were 3 ('Good') for both, with unsorted displaying narrower spread. Mann-Whitney U test confirmed no significant difference ($U=1397.5$, $p=0.5155$), with negligible effect size ($r=0.06$). A violin plot demonstrating the distribution pattern is shown in Figure 7.

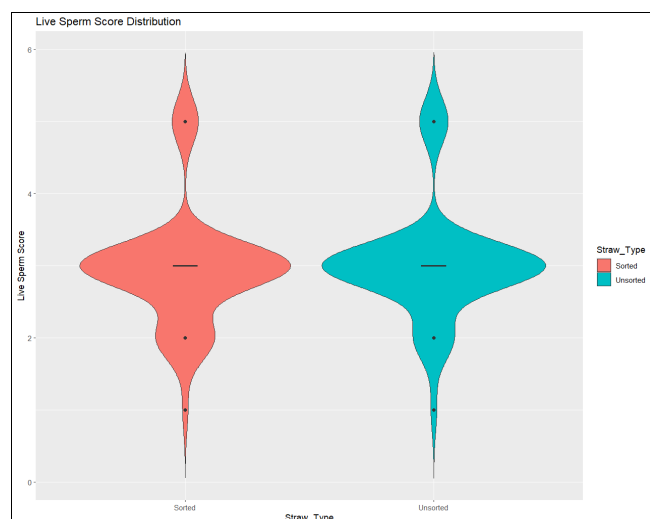


Fig 7: Violin plot illustrating the distribution of live sperm scores in sorted and unsorted semen straws.

3.7 Acrosomal Integrity by Straw Type

The comparative analysis of acrosomal integrity revealed that both sorted and unsorted semen straws contained a high proportion of spermatozoa with intact acrosome. However, a slightly higher number of sperm with compromised acrosomal integrity was observed in the sorted samples (Figure 8). Acrosomal integrity was 87.72% intact (12.28% reacted) in sorted and 94.23% intact (5.77% reacted) in unsorted. The bar chart indicated slightly higher integrity in unsorted samples. Fisher's Exact test showed no significant difference ($p=0.3257$).

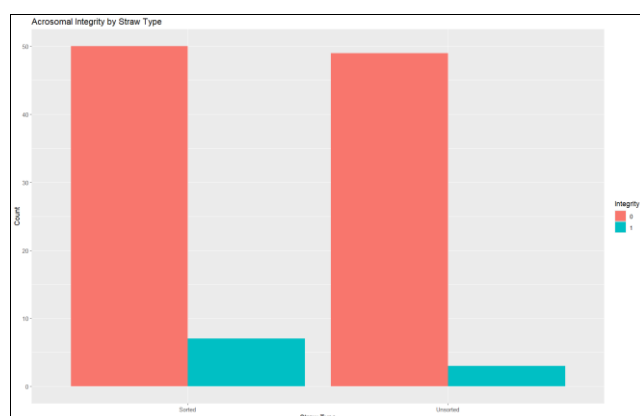


Fig 8: Comparison of acrosomal integrity between sorted and unsorted semen straws.

4. Discussion

The present study investigated the potential structural/morphological changes in both cryopreserved unsorted and cryopreserved sex-sorted spermatozoa in Sahiwal bull. The study demonstrated that sex-sorting followed by cryopreservation does not significantly compromise the post-thaw quality of Sahiwal bull spermatozoa. No statistically significant differences ($p > 0.05$) were observed between conventional unsorted and sex-sorted cryopreserved semen for key parameters including progressive motility, live sperm percentage, acrosomal integrity, and morphological abnormalities. These findings align with several recent reports. Mocé *et al.* (2006) found that X- and Y- bearing sperm responded similarly to induced acrosome reaction before and after cryopreservation, with sex-sorted frozen thawed semen showing higher live

acrosome reacted cells (23%) than unsorted controls (9%) without inducer [13]. Likewise, the present study showed comparable acrosomal integrity (87.72% intact in sorted vs 94.23% in unsorted) and no significant difference in morphological abnormalities. The apparent correlation between acrosomal integrity and abnormalities reflects their complementary nature rather than a true biological link. The slight numerical reduction in sorted samples was not significant, aligning with findings by Khalil *et al.* (2018) that cryopreservation mainly affects plasma membrane integrity, with acrosomal and chromatin damage remain within acceptable limits [10].

In our study, hierarchical clustering showed structural parameters (acrosomal integrity, abnormalities) and functional parameters (motility and live sperm) forming separate clusters, supporting findings by Gravance *et al.* (1998) [8] and Milewska *et al.* (2024) [12]. They also reported that cryopreservation can improve morphologically normal sperm by removing defective cells. Moderate positive correlation between motility and live sperm ($r = 0.597$) and negative correlations with acrosomal integrity and abnormalities ($r \approx -0.544$ and -0.386) further confirm coordinated functional-structural relationship, as noted by Prihantoko *et al.* (2020) [17] and Singh *et al.* (2024) [19]. Furthermore, in present study, the minor numerical reductions observed in sorted Sahiwal semen (decreased 'very good' motility and marginally increased abnormalities) are consistent with the subtle membrane perturbations reported by Khumran *et al.* (2015) [11] and the ultrastructural alterations described by Khalil *et al.* (2018) [10]. However, the lack of statistical significance and the negligible effect sizes suggest that these changes do not result in biologically meaningful impairment of fertilizing capacity. This observation is particularly relevant for the indigenous breeds such as Sahiwal, which exhibit inherent thermotolerance and enhanced membrane stability.

5. Conclusion

In conclusion, the present study demonstrates that the sex-sorting process does not significantly compromises the sperm structural and functional integrity. Although unsorted semen showed slightly higher motility, viability, acrosomal integrity, and lower abnormalities, these differences were statistically non-significant with minimal effect sizes. Correlation analysis revealed a positive relationship between motility and viability and negative associations with structural defects, while clustering distinguished functional and structural attributes. Despite minor reductions in sorted samples, sex-sorting combined with cryopreservation preserves acceptable sperm quality. However, a greater number of samples may be needed to decipher exact differences between both semen types.

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Ethics approval

This particular research did not involve experiments on live animals.

Conflict of Interest

No competing interests exist.

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