The Haryana Veterinarian (June, 2025) **64(1),** 66-70 ISSN 0033-4359 (P) **Research Article**

IN VITRO ASSESSMENT OF SPERMATOLOGICAL PARAMETERS IN SEX-SORTED SEMEN OF JERSEY CROSSBRED BULL

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ABSTRACT

This study aimed to evaluate *in vitro* sperm characteristics of sex-sorted frozen semen of Jersey crossbred bull. Among various analytical techniques for the same sperm parameters, only plasma membrane integrity showed a significant difference ($p<0.01$), while no significant differences ($p>0.05$) were observed for sperm viability and acrosome integrity. Flow cytometric analysis revealed significant differences ($p<0.01$) in plasma membrane and acrosome integrity among the six sex-sorted frozen bull semen samples. Moreover, a strong correlation was found between sperm motility and semen metabolic activity, as well as between major sperm abnormalities and DNA integrity. In conclusion, the study suggests that alterations in the production of sex-sorted semen, based on *in vitro* sperm characteristics, could potentially enhance conception rates in cattle in the future.

Keywords: Jersey Bull, Sex sorted bull semen, Spermatological parameters

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Sex-sorted semen technology is gaining prominence in bovine reproduction management, addressing the need for an increased population of elite female cattle to meet growing milk demands. By shifting the sex ratio toward females, this technology expedites the production of superior females for progeny testing programs, leading to enhanced genetic gains. The gender bias introduced by sex-sorted semen contributes to the efficient production of genetically superior heifer or bull calves for herd expansion and replacement in both dairy and beef industries. Moreover, sex-sorted semen technology can be utilized to produce elite breeding bulls in regions facing a shortage of high-quality breeding bulls. High-quality sex-sorted semen with a favorable fertility index has the potential to improve farm profitability and environmental sustainability in cattle breeding compared to non-sorted conventional semen. Therefore, assessing the quality and fertility of sex-sorted semen is crucial for establishing a future herd of sex-determined heifers and cows with high reproductive potential. While routine conventional tests such as viability, motility, concentration, morphology and plasma membrane intactness provide initial assessments, they are time-consuming, lack precision, evaluate a limited number of sperm cells and are prone to technician-dependent variations. These conventional tests can serve as primary level screening, with results further validated using advanced techniques like fluorescent microscopy and flow cytometry (Arjun *et al.*, 2022). These advanced techniques aim to identify functional and structural damages in sex-sorted sperm cells.

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MATERIALS AND METHODS

This study utilized sex-sorted Jersey crossbred frozen semen straws from six different bulls obtained from Uttarakhand Livestock Development Board, Mothrowala, Dehradun. The procured semen samples underwent a primary level of *in vitro* analysis using conventional methods. Sperm characteristics, including viability, motility, morphology, metabolic activity, plasma membrane integrity and acrosomal integrity were evaluated through specific supravital staining techniques. To further validate sperm viability, frozen sex-sorted samples underwent fluorescent microscopic analysis using live and dead cell-specific fluorophores, assessing membrane intactness/ permeability. Additionally, advanced analytic techniques were employed, including flow cytometric analysis. This involved validating sperm plasma membrane integrity using membrane permeant/impermeant fluorophores, assessing acrosomal integrity with fluorescein-conjugated lectins and evaluating chromatin integrity through SCSA (Sperm Chromatin Structure Assay).

Validation of sperm *in vitro* characteristics of sex-sorted frozen semen by conventional and fluorescent microscopy methods

In this study, semen from six bulls was thawed at 37º C for 30 seconds, transferred to Eppendorf tubes, mixed thoroughly and maintained at the same temperature in a dry water bath. Sperm motility assessment followed criteria ranging from 0 to 100 in increments of 10 following guidelines by Chenoweth *et al.* (1993). Sperm viability was determined using the Eosin-Nigrosin staining method outlined by Campbell *et al.* (1956).

Evaluation of sperm morphology employed the Rose Bengal staining method described by Mondal *et al.* (2013). The Hypo-Osmotic Swelling Test (HOST) involved incubating a 100 µl semen sample in 1ml of 150 mOsm hypo-osmotic and 300 mOsm (control) iso-osmotic solutions at 37º C for 30 minutes, following the protocol by Jeyendran *et al.* (1984). Assessment of sperm metabolic activity utilized the Resazurin dye Reduction test (RRT), as described by Strzezek *et al.* (2013). Acrosomal integrity was evaluated through the Giemsa staining method, following the procedure outlined by Chowdhury *et al.* (2014). Fluorescent microscopic analysis of sperm viability, based on the intactness of the plasmalemma was carried out using the technique described by Makarevich *et al.* (2010).

Validation of sperm *in vitro* characteristics in sex-sorted frozen-thawed semen by flow cytometry

Frozen semen straws from six different bulls were thawed at 37º C for 30 seconds. The thawed samples underwent two washes with Sp-TALP medium through centrifugation at 2000 rpm for 5 minutes. Following the washes, the sperm pellet was resuspended in 200 µl of Sp-TALP medium. To assess sperm membrane integrity, the fluorochromes SYBR-14 and propidium iodide (PI) were employed, following the methodology described by Nag *et al.* (2021). Sperm acrosomal integrity was evaluated using Peanut agglutinin (PNA) conjugated with Fluorescein isothiocyanate (FITC), according to the procedure outlined by Nag *et al.* (2021). The DNA fragmentation index in spermatozoa from all bull samples was determined using the sperm chromatin structure assay, as described by Nag *et al.* (2021).

Statistical analysis

The Chi-square test was employed to assess the statistical significance between different sperm analysis techniques. Statistical analysis was conducted following the methodology outlined by Snedecor and Cochran (1989), utilizing the SPSS computer program (Version 20.0). A mean value of $P<0.05$ was considered statistically significant, $P<0.01$ was deemed highly significant and $P>0.05$ was considered non-significant.

RESULTS AND DISCUSSION

Recently, sex-sorted semen technology has become increasingly advantageous in the field of bovine breeding. This technology offers several benefits including a reduction in the number of cows required for progeny testing, the ability to produce replacement dairy heifers from a limited population of genetically superior cows and the flexibility to crossbreed dairy cows with both dairy and beef bulls. The flow cytometric sex-sorting technique stands out as the widely used, reliable, and fastest method for sorting sperm cells based on their DNA differences into X and Y sperms. This advanced technology allows for precise and efficient separation of sperm cells according to their genetic information, enabling the desired sex of the offspring to be selected with a high degree of accuracy.

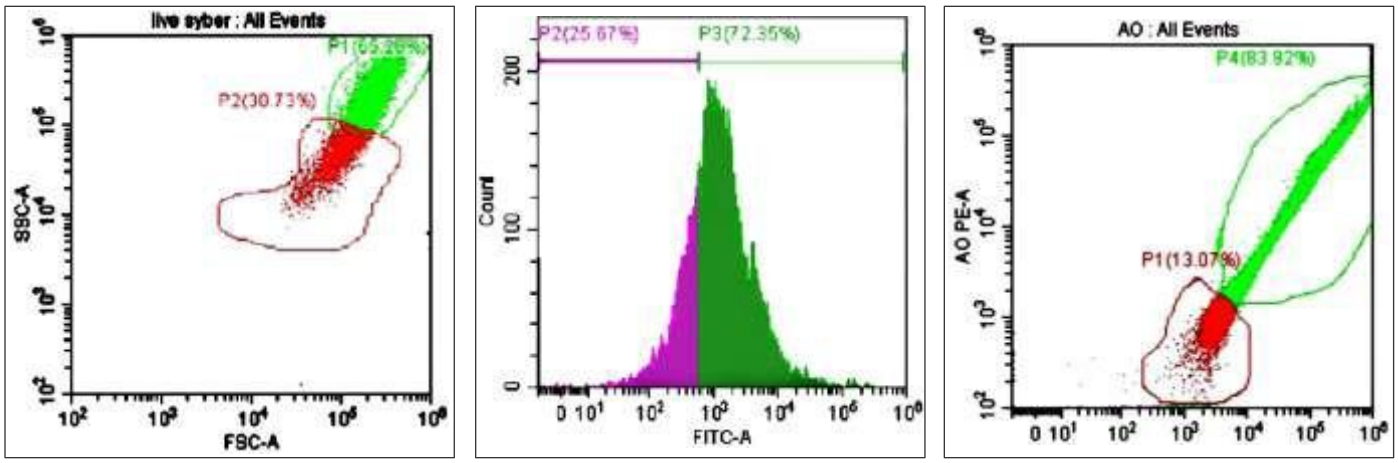
Validation of sperm characteristics in sex-sorted frozen -thawed semen by *in vitro* analysis

The total number of sex-sorted sperms was analyzed for each bull and the percentage of sperms found viable fromEosin-nigrosin and SYBR-14/PI staining techniques were recorded. Out of 200 sperms analyzed in sex-sorted frozen-thawed semen, the percentage of viable sperms in SYBR-14/PI fluorescent staining was 60.00, 63.00, 63.00, 64.00, 62.00 and 60.00 per cent for bulls 1 to 6, respectively. Chi-square test analysis revealed non-significant difference ($P > 0.05$) between the bulls. Theme an percentages of viable sperms were found to be 61.75 ±1.14 and 62 ± 0.68 per cent for sex-sorted frozen-thawed semen with Eosin-nigrosin and SYBR-14/PI staining techniques, respectively and the results did not differ significantly ($P>0.05$) between the staining methods.

The *in vitro* sperm characteristics of sex-sorted frozen -thawed bull semen were assessed using conventional methods, fluorescent microscopy and flow cytometry. In this study, the mean percentage of sex-sorted frozen-thawed sperm viability was found to be 61.75±1.14 with Eosin-Nigrosin staining and 62±0.68 with SYBR-14/PI-based fluorescent staining techniques. These results align with the findings of Underwood *et al.* (2009) and Maulana *et al.* (2019), reporting viabilities in sex-sorted semen as 64.9±1.2 and 66.67±4.44 percent, respectively. However, Kaiin and Gunawan (2017) reported lower sperm viability percentages of 48.3±6.7 and 55.9±1.4, respectively. The higher sperm viability percentage observed in the present study may be attributed to differences in sex-sorting techniques and semen processing methods, creating a favorable environment for sustaining the life span of sorted sperms through various stages of the sex-sorting process.

Plasma membrane integrity of sex-sorted frozen-thawed spermatozoa

The plasma membrane integrity of sex-sorted sperms in frozen-thawed bull semen was assessed by HOST and flow cytometric SYBR-14/PI assay. Out of 200 sperms analyzed in sex-sorted frozen-thawed semen, the percentage of membrane intact sperms in HOST was 62.00, 58.00, 61.00, 63.00, 63.00 and 59.50 per cent for bulls 1 to 6, respectively. Chi-square test analysis revealed no significant difference ($P>0.05$) between the bulls. In flow cytometric SYBR-14/PI assay for membrane integrity, 10,000 sperms were analyzed for each sex-sorted semen sample and the percentages of sperms with intact membrane were 64.52, 61.19, 62.11, 65.29, 65.26 and 60.17 per cent for bulls 1 to 6, respectively. Chi-square test analysis revealed highly



Figs.1-3. **(1)** Flow cytometric analysis of plasma membrane integrity of sex-sorted bull sperm by SYBR-14/PI assay; A: Membrane intact sperms; B: Membrane damaged sperms; **(2)** Flow cytometric analysis foracrosome integrity insex-sorted bull sperm by FITC-PNA/PI assay P3: Acrosome intact sperms; P2: Acrosome damaged sperms; **(3)** Flow cytometric analysis of chromatin integrity of sex-sorted bull sperm by SCSA, A: ds DNA; B: ss DNA

significant difference ($P < 0.01$) between the bulls (Fig. 1). Theme an percentage of membrane intact sperms was found to be 61.08 ± 0.82 and 63.09 ± 0.91 per cent for sex-sorted frozen-thawed semen with HOST and flow cytometric SYBR-14/PI assay, respectively and the results differed significantly ($P<0.01$) between the analytical techniques.

The mean percentage of membrane-intact sex-sorted frozen-thawed bull sperms in the present study with HOST and flow cytometric SYBR-14/PI assay were 61.08 ± 0.82 and 63.09 ± 0.91 per cent, respectively. The results of the present study concurred with the reports of Purdy *et al.* (2021) who reported the plasma membrane integrity in sex-sorted semen as 60.75±9.55 and 60.00 per cent, respectively. But, Rangasamy *et al.* (2017) has recorded a lower sperm membrane integrity percentage of 54.58± 0.88 per cent by flow cytometric SYBR-14/PI assay. The higher sperm membrane integrity per cent recorded in the present study might be due to the superiority of Sexed ULTRA™ method in comparison to other traditional sex-sorting methods which has maintained the sperm membrane integrity from pre-sort to the post-thaw stage after sorting and freezing. Further, the significant difference reported between these two analytical techniques and among the bulls in SYBR-14/PI flow cytometric assay in the present study might be due to the increased efficiency of flow cytometric sperm analysis, where a higher sperm number is being analyzed at a single time in a fastest manner compared to other conventional methods.

Acrosome integrity of sex-sorted frozen-thawed spermatozoa

The acrosome integrity of sex-sorted sperms was analyzed for each bull using Giemsa staining technique and flow cytometric FITC-PNA/PI assay. Out of 200 sperms analyzed in sex-sorted frozen-thawed semen, the percentage of acrosome intact sperms in Giemsa staining was 73, 72, 73, 75, 74 and 71 per cent for bulls 1 to 6, respectively. Chi-square test analysis revealed non-significant difference ($P > 0.05$) between the bulls. In flow cytometric FITC-PNA/PI assay for acrosome integrity, out of the total 9,087, 10,000, 10,000, 11,982, 10,000 and 10,000 sperms analyzed for each bull 1 to 6, the percentage of sperms with intact acrosome was 72.35, 72.33, 72.33, 75.11, 74.41 and 72.33 per cent, respectively (Fig. 2). Chi-square test analysis revealed highly significant difference ($P<0.01$) between the bulls. Theme an percentage of acrosome intact sperms in sex-sorted semen was found to be 73±0.58 and 73.14±0.52 per cent with Giemsa staining and flow cytometric FITC-PNA/PI assay, respectively and the results differed significantly ($P<0.01$) between the analytical techniques.

In the current study, the mean percentage of membrane - intact sex-sorted frozen bull sperm using HOST and flow cytometric SYBR-14/PI assay was found to be 61.08±0.82 and 63.09±0.91 percent, respectively. These results align with the findings of Purdy *et al.* (2021), reporting plasma membrane integrity in sex-sorted semen as 60.75±9.55 and 60.00 percent, respectively. However, Rangasamy *et al.* (2017) recorded a lower sperm membrane integrity percentage of 54.58±0.88. The higher sperm membrane integrity percentage observed in the present study may be attributed to the superiority of the Sexed ULTRA™ method compared to other traditional sex-sorting methods, maintaining sperm membrane integrity from pre-sort to the post-thaw stage after sorting and freezing. Additionally, the significant difference reported between the two analytical techniques and among the bulls in SYBR-14/PI flow cytometric assay in the present study may be due to the increased efficiency of flow cytometric sperm analysis. This technique allows a higher number of sperm to be analyzed at a single time in a faster manner compared to other conventional methods, contributing to the observed differences.

Correlation between sperm abnormality and chromatin integrity of sex-sorted spermatozoa

The sperms with normal morphology, major and minor abnormalities were assessed by Rose Bengal staining whereas the spermatozoa with intact chromatin were evaluated byflow cytometric SCSA. In Rose Bengal staining, the percentage of sperms with normal morphology, major and minor abnormalities were 75, 4.2 and 4.8% for bull No. 1; 73, 4.62 and 4.44% for bull No. 2; 74, 4.42 and 4.81% for bull No. 3; 78.5, 3.95 and 4.18% for bull No. 4; 76, 4.16 and 4.37% for bull No. 5 and 71, 4.65 and 4.31% for bull No. 6, respectively. In flow cytometric SCSA for chromatin integrity, the percentage of sperms with ds DNA and its respective DFI per cent were 83.02 and 15.58 % for bull No. 1, 80.66 and 17.52% for bull No. 2; 82.37 and 17.22% for bull No. 3; 83.92 and 13.47% for bull No. 4; 84.06 and 14.32% for bull No. 5 and 78.16 and 18.31% for bull No. 6, respectively (Fig. 3). The mean percentage of sperms with major abnormalities and DFI per cent of the semen were found to be 4.33 ± 0.11 and 16.07 ± 0.78 per cent, respectively and these two parameters were highly correlated with each other (P<0.01).

In the present study on sex-sorted frozen-thawed semen, the mean percentage of sperm abnormalities was found to be 8.83±0.17 percent, consistent with the reports of DeJarnette *et al.* (2011) and Yoo *et al.* (2012) who reported 8.7±1.5 and 8.9±1.1 percent, respectively. However, Thongkham *et al.* (2021) reported a higher sperm abnormality percentage, comprising 22.89±4.09, 4.27±4.09, 0.46±0.76 and 1.04±1.32 percent for abnormal tail, abnormal head, proximal droplets and distal droplets in sex-sorted bull semen. The lower percentage of abnormal sperms in the present study may be attributed to differences in sperm sex-sorting techniques, with increased cytotoxicity-induced dead or weak sperms reported in the case of IC-sexed semen (using monoclonal male-specific antibodies combined with the complement system for cytotoxicity reaction).

The mean percentages of ds DNA and DFI (DNA Fragmentation Index) in sex-sorted frozen-thawed bull semen in the present study were 82.03±0.92 and 16.07±0.78 percent, respectively. These results align with the reports of Rangasamy *et al.* (2017), who reported the mean percentages of ds DNA and DFI in sex-sorted semen as 79.96±0.94 and 20.03±0.94 percent, respectively. The negative correlation reported between ds DNA and DFI percentage in the present study supports the association of DFI with DNA damage in ejaculates or semen samples of infertile or sub fertile animals, as reported by Kumaresan *et al.* (2020). Furthermore, the strong positive correlation reported between major sperm abnormalities and DFI percentage in sex-sorted semen in the present study coincides with the findings of Enciso *et al.* (2011) and Boe Hansen *et al.* (2018), suggesting that bull spermatozoa with a higher percentage of major sperm abnormalities tend to exhibit a higher degree of sperm DNA damage.

Correlation between progressive sperm motility and metabolic activity of sex-sorted frozen bull spermatozoa

The progressive forward motility of sperms was evaluated with microscopic analysis where as the metabolic activity of the sex-sorted frozen-thawed spermatozoa was assessed by spectrophotometric analysis of RRT. On microscopic analysis of sex-sorted semen, the percentage of sperms with progressive forward motility was 60.00, 58.00, 60.00, 64.00, 62.00 and 58.00%, respectively and the OD values of spectrophotometric RRT (ratio) were 4.91, 4.85, 5.01, 5.92, 5.18 and 4.76 for bull 1 to 6, respectively. In Rose Bengal staining, normal morphology, major and minor abnormalities were 75, 4.2 and 4.8 for bull 1; 73, 4.62 and 4.44 for bull 2; 74, 4.42 and 4.81 for bull 3; 78.5, 3.95 and 4.18 for bull 4; 76, 4.16 and 4.37 for bull 5 and 71, 4.65 and 4.31 for bull 6, respectively. In flow cytometric SCSAof sex-sorted frozen bull semen for chromatin integrity, the percentage of sperms with ds DNA and its respective DFI percent were 83.02 and 15.58 for bull 1, 80.66 and 17.52 for bull 2, 82.37 and 17.22 for bull 3, 83.92 and 13.47 for bull 4; 84.06 and 14.32 for bull 5 and 78.16 and 18.31 for bull 6, respectively, which revealed a strong negative correlation among these two parameters (P<0.01).In the sex-sorted frozen bull semen, the mean percentage of sperms with major abnormalities and DFI per cent of the semen were found to be 4.33 ± 0.11 and 16.07 ± 0.78 per cent, respectively and a strong positive correlation existed between these two parameters (P<0.01).

In the present study on sex-sorted frozen-thawed semen, the mean percentage of progressively motile sperms was found to be 60.33 ± 0.95 percent. These results are consistent with the reports of Maulana and Said (2019), who reported the mean percentages of progressively motile sperms in sex-sorted bull semen as 61.3±3 and 62.01±4.30 percent, respectively. However, Missio *et al.* (2018) and Steele *et al.* (2020) reported lower percentages of progressive sperm motility at 14.95±6.15 and 27.8±6.02, respectively, in sex-sorted bull semen. The higher percentage of progressively motile sperms in the present study may be attributed to proper management practices in sex-sorted semen production, involving appropriate handling of semen during collection, processing, sorting, storage and transport.

The mean OD value of the Resazurin dye Reduction test (RRT) ratio in the present study (5.1±0.42) and the time taken for Resazurin dye reduction to pink and white color (<5 mins and ≤12 mins) with sex-sorted semen coincided qualitatively and quantitatively with the reports of Dart *et al.* (1994) and Strzezek *et al.* (2013). These studies reported the time taken for resazurin dye reduction from blue to pink and from pink to white as ≤3.5 and <15 mins,

respectively and a mean RRT ratio of metabolically active sperms as 5.71±0.17.Furthermore, the strong positive correlation reported between the metabolic activity and motility of these sex-sorted sperms in the present study reinforces their strong interdependence, contributing to the fertilizing ability of the spermatozoa, as documented by Zrimsek *et al.* (2005).

CONCLUSION

It is concluded that, the metabolic activity of sex-sorted semen is positively correlated with the sperm motility parameter and the existence of major sperm abnormalities in sex-sorted bull semen negatively correlated with its ds DNA per cent.

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