Short Communication

# *IN VITRO* VALIDATION OF SEX SORTED BULL SEMEN PRODUCED USING FLOW CYTOMETRIC METHOD

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## ABSTRACT

*A commercially procured flow cytometrically sorted semen was reanalyzed to validate the effect of flow cytometry on structural and functional viability of spermatozoa. Flow cytometric analysis of commercially procured sex sorted frozen thawed bull semen showed the percentage of X sperms to be 95.31± 0.21 and Y sperms to be 4.69 ± 0.27, respectively. As expected sexed sperm had lower post thaw motility than the non-sexed sperm. However, no significant differences in the percentage of sperms with intact acrosome and expression of ROS were observed. Interestingly, DNA fragmentation index was statistically lower for the commercially procured sorted than for unsorted frozen thawed bull semen.*

**Key words:** Bull, Sex sorted semen and flow cytometry

Received : 10.07.2023 Revised : 01.11.2023 Accepted : 01.11.2023

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## INTRODUCTION

Flow cytometric sperm sorting is considered as the most successful sperm sexing technique with a purity of 85 to 95 per cent of the sorted sperm (Duarte *et al*., 2007). The reduction in sperm quality in the sex sorted commercially procured semen could have been caused by exposure to Hoechst 33342 stain, the laser light or exposure of the droplets to electrical charges (Garner, 2006). According to Smith (1993), the effect of exposure to dye and then the laser might reduce the mitochondrial activity in bovine oocytes. This

type of damage could explain the decreased motility of sexed sperm, since mitochondria produce ATP as an energy source for sperm. Another physical characteristic expected to be affected by the process of sexing was integrity of the membrane. But no such changes were noted in the sorted sperm, indicating that the mechanical stress during sorting was probably less.

## MATERIALS AND METHODS

Commercially procured sorted frozen Jersey crossbred bull semen straws were validated by flow cytometry analysis of X and Y sperm population per straw, live and dead percentage, acrosomal integrity and sperm chromatin structure assay (SCSA) and reactive oxygen species (ROS) / oxidative stress (OS) with unsorted Jersey crossbred bull semen. In each group five straws were used for entire analysis.

### Evaluation of sperm viability

Sperm viability was determined using Propidium Iodide (PI) method as outlined by Graham *et al.* (1990). Flow cytometry analysis was performed by using Beckman Coulter (MoFlo$^{TM}$XDP) fitted with a bevelled tip and the results were analyzed using density plots.

### Evaluation of acrosomal integrity

Acrosomal integrity of the bull sperm was evaluated by the method as mentioned by Graham *et al.* (1990) using fluorescein isothiocyanate (FITC) labelled peanut agglutinin (PNA) and assayed by flow cytometry to assess the percentage of cells without an intact acrosome (i.e. exhibiting an acrosome reaction; AR). The percentage of the acrosome reacted sperms was analysed from the density plot obtained.

### Sperm chromatin structure assay (SCSA)

Sperm chromatin structure assay (SCSA) was evaluated by Acridine orange (AO) as described by Agarwal and Said, (2009). The results were analysed and interpreted based on the percentage of DNA fragmentation or DNA fragmentation index (DFI). Calculation of DFI and Interpretation: DFI = Red / Red + green; High DFI > 75 per cent indicates high DNA damage (Sterile bull); Moderate DFI of 25-75 per cent indicates moderate DNA damage (Sub fertile bull); Low DFI < 20-25 per cent indicates less DNA damage (Fertile bull).

### Evaluation of reactive oxygen species (ROS) / oxidative stress (OS)

Reactive oxygen species / oxidative stress in sperm was assessed by using Cell Rox ® Deep Red reagent fluorescent probe (2.5 mM, Life Technologies, New York, USA) as per manufacturer's instructions. The percentage of ROS / OS was assessed using the density plot.

### Statistical analysis

The data collected were statistically analysed using Kreskal - Wallis test. The statistical programme used was SPSS version

15.0 and comparison of sorted and unsorted sperms were analysed by t-test and fresh and frozen semen evaluations of crossbred bulls was done by Chi-square test.

## RESULTS AND DISCUSSION

### Flow cytometric analysis of X and Y sperm population in sorted frozen thawed Jersey crossbred bull semen

In six replicates, out of 21,93,789 sperms analyzed, the mean per cent of X sperms was 95.31± 0.21 (20,93,421) and Y sperms was 4.69 ± 0.27 (1,00,368), respectively. Flow cytometric sperm sorting is considered as the most successful sperm sexing technique with a purity of 85 to 95 per cent of the sorted sperm (Duarte *et al*., 2007). In the present study too, reanalysis of the sorted sperm proved the purity of the sorting technique. The results of the present study was in agreement with Biswas *et al.* (2013) who reported that the average purity (per cent) in terms of calf born percentage by the sorted X sperm was higher than the purity of sorted Y sperm. They also reported that the specific reason for poor purity of sorted Y sperm was not known but they attributed it to a particular bull effect which had poor motility and viability of sperm, thus, contributing to decrease in overall purity.

### Post thaw viability percentage of unsorted and sorted Jersey crossbred bull semen

In six replicates, out of 8,58,777 unsorted crossbred frozen thawed bull sperms analyzed, the mean per cent of live sperms was 58.81 ± 2.10 (4,97,188). Out of 5,15,373 commercially procured sorted frozen thawed bull sperms the mean per cent of live sperms was 48.62 ± 0.29 (2,51,059). The mean per cent of post thaw viable sperms was significantly higher ($P < 0.01$) for unsorted than for the commercially procured sorted frozen thawed semen.

### Acrosomal integrity of unsorted and sorted frozen thawed Jersey crossbred bull semen

The mean per cent of viable sperms for the unsorted frozen thawed crossbred bull sperms subjected to acrosomal integrity studies was 58.32 ± 1.94 of which 50.75 ± 1.54 and 7.57 ± 0.87 per cent, respectively had intact and damaged acrosomes. The mean per cent of viable sperms for the commercially procured sorted frozen thawed bull sperms subjected to acrosomal integrity studies was 57.65 ± 2.30 of which 51.17 ± 1.92 and 6.48 ± 0.93 per cent, respectively had intact and damaged acrosomes. Statistical analysis showed no significant difference in acrosomal integrity of viable sperms of unsorted crossbred frozen thawed and commercially procured sorted frozen thawed bull sperms (t-value 0.170).

### Expression of sperm chromatin structure assay in unsorted and sorted frozen thawed Jersey crossbred bull semen

Of the 2,91,469 unsorted crossbred frozen thawed bull sperms analyzed, the mean percentage of sperms expressing

double standard DNA was 81.85 ± 0.35 (2,39,281) and the mean per cent of DNA fragmentation index (DFI) was 17.95 ± 0.38. Of the 24,58,875 commercially procured sorted frozen thawed bull sperms analysed, the mean percentage of sperms expressing double standard DNA was 98.07 ± 0.33 (24,14,050) and the mean per cent of DFI was 1.95 ± 0.33. The mean per cent of DFI was significantly lower for the commercially procured sorted frozen thawed sperms when compared to the unsorted crossbred frozen thawed sperms (t-value 31.417).

**Expression of reactive oxygen species (ROS) in unsorted and sorted frozen thawed Jersey crossbred bull semen**

Of the 2,15,742 unsorted frozen thawed crossbred bull sperms and 2,22,307 commercially procured sorted frozen thawed bull sperms analysed, the expression of ROS was 25. 16 ±1.15 (54,125) and 28.67 ± 1.44 (63,748) per cent, respectively. The expression of ROS in unsorted crossbred and commercially procured sorted frozen thawed bull sperms did not differ significantly (t-value 1.899).

The reduction in sperm quality in the sex sorted commercially procured semen could have been caused by exposure to Hoechst 33342 stain, the laser light or exposure of the droplets to electrical charges (Garner, 2006). According to Smith (1993), the effect of exposure to dye and then the laser might reduce the mitochondrial activity in bovine oocytes. Although there was some indication that exposure of sperm to UV radiation during sexing by flow cytometry affected chromatin integrity (Bordignon and Smith, 1999 and Boe-Hansen *et al*., 2005), there was no such effect seen with the sex sorted sperms in the present study.

In fact, sorted sperms had significantly higher expression of double stranded DNA and significantly lower DFI when compared to unsorted sperms. Perhaps bovine sperms were somewhat less sensitive to UV exposure than sperm from other mammals, due to their greater content of protamine in the DNA (Gimes, 1986), which augmented chromatin stability. In that regard, DNA which was more tightly packed could account for the apparent lack of damage in the sexed-sperm chromatin. However, there is always the possibility that the deleterious effects of exposure to UV could be manifested only at later stages of development (i.e. after blastocyst formation).

In the present study, the expression of ROS for commercially procured sorted sperms was higher than the unsorted sperms, but the difference was statistically not significant. The increased ROS expression in commercially procured sex sorted sperm could be attributed to reduced seminal plasma content by excessive extension and washing of the sperm suspension. Since, much of the antioxidant capacity relied on seminal plasma (Ball, 2008), the increased oxidative status of sorted

spermatozoa might result from increased production of ROS and from decrease in the antioxidant capacity of the medium. Several steps in sorting processes might lead to ROS production, but their negative effect on lipid peroxidation could be minimized by media supplementation with antioxidative substances (Maxwell and Stojanov, 1996 and Pena *et al.,* 2003).

## CONCLUSION

Flow cytometry sorting affected the viability of sorted semen with no detrimental effects on other parameters such as acrosomal integrity, sperm chromatin structure assay (SCSA) and reactive oxygen species (ROS) / oxidative stress (OS).

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