

SEX ENRICHMENT OF CROSSBRED BULL SEMEN USING CONVENTIONAL METHODSS. RANGASAMY*, S. BALASUBRAMANIAN¹, G. DHINAKAR RAJ², A. PALANISAMMI³, D. REENA⁴,
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Received: 21.08.2023, Accepted: 14.10.2023

ABSTRACT

The sex selection of spermatozoa has been compromised due to higher cost of flow cytometric sex sorted semen doses to poor dairy farmers. The sex enrichment of semen using percoll density gradient and swim up methods were analyzed by flow cytometry. Flow cytometric analysis of sperm separated by swim up method showed the percentages of X and Y sperm to be 34.24±1.16 and 65.76±1.16, respectively and sperm separated by percoll density gradient method showed the percentages of X and Y sperm to be 65.06±1.11 and 34.94±1.11, respectively. Conventional methods of sex sorting such as swim up and percoll density gradient helped to separate around 65.00% of Y and X sperm each.

Keywords: Bull semen, Conventional methods, Percoll density gradient method, Sex Enrichment, Swim up method

How to cite: Rangasamy, S., Balasubramanian, S., Dhinakar Raj, G., Palanisammi, A., Reena, D., Gopikrishnan, D. and Chitra, R. (2024). Sex enrichment of crossbred bull semen using conventional methods. *Haryana Vet.* 63(SI): 89-91.

Alternative sperm sexing methods could therefore be of relevance for the benefit of small dairy farmers both in biological and economic terms (Mota *et al.*, 2013). Sperm sexing techniques were based on size and weight (Bhattacharya *et al.*, 1996), electrical surface charge (Shirai *et al.*, 1974), immunological properties (Sang *et al.*, 2011) and swimming ability (Madrid-Bury *et al.*, 2003) of X and Y chromosome bearing sperm. These techniques include differential separation through percoll (Machado *et al.*, 2009), albumin gradients (Machado *et al.*, 2009) and modified swim up (Azizzeddin *et al.*, 2014).

MATERIALS AND METHODS**Sex enrichment of Y sperm by swim up method**

Semen samples collected from crossbred bulls were centrifuged and washed with Sp TALP medium and further subjected to swim up method as described by Parrish *et al.* (1986). Briefly, one ml of semen was diluted with 5 ml of pre-equilibrated Sp TALP by centrifugation at 1500 rpm for 5 min at room temperature (RT). The supernatant was removed and fresh Sp TALP was added and the above procedure was repeated twice. Finally, 200 µl of the sperm pellet was layered under 1 ml of Sp TALP medium in three sugar tubes and incubated for swim up at 38.5° C in 5 per cent CO₂ in air for 1 h. At the end of incubation, the superficial layer of 0.5-0.8 ml of the medium containing the motile sperm fraction was removed from each tube and pooled in a 15 ml centrifuge tube and washed with 10 ml Sp TALP by centrifugation at 1500 rpm for 5 min at RT. The final sperm pellet was subjected to flow cytometry for determining the percentage of X and Y sperm by DNA staining method.

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Sex enrichment of X sperm by percoll density gradient method

Motile spermatozoa were obtained by the Percoll® density gradient method as described by Rosenkrans *et al.* (1993). Briefly, 100 per cent percoll solution was mixed with 10X salt solution (NaCl, 2.889 g; KCl, 0.238 g; KH₂PO₄, 0.116 g; CaCl₂, 0.112 g; HEPES, 0.163 g; 50 ml of milli Q water) to form 90 per cent (v/v) percoll solution. A 45 per cent (v/v) percoll solution was prepared from this by adding an equal volume of HEPES-TALP. The gradient was formed by pipetting 1.5 ml of 90 per cent percoll solution into a 15 ml conical tube overlaid with 1.5 ml of 45 per cent percoll solution. On to the top of the 45 per cent gradient, 400 µl of semen was layered and centrifuged at 2000 rpm at RT. After removing the supernatant, the pellet was washed with 10 ml of HEPES-TALP by centrifugation at 1500 rpm for 10 min and re-suspended in Sp TALP and subjected to flow cytometry for determining the percentage of X sperm by DNA staining method.

RESULTS AND DISCUSSION

The mean (± SE) of flow cytometric analysis of X and Y sperm population of fresh crossbred bull semen by swim up and percoll density gradient methods is presented in Table 1 and Figs. 1 and 2.

Flow cytometric analysis of 2,27,869 sperm separated by swim up method showed the percentages of X and Y sperms to be 34.24±1.16 (81,755) and 65.76±1.16 (1,46,114), respectively. Flow cytometric analysis of 1,34,173 sperm separated by percoll density gradient method showed the percentages of X and Y sperms to be 65.06 ± 1.11 (87,575) and 34.94±1.11 (46,598), respectively. The

Table 1. Flow cytometric analysis of X and Y sperm populations (Mean ± SE) of fresh crossbred bull semen by swim up and percoll density gradient methods

Spermseparation method	Number of replicates	Number of sperm cells analyzed	Sperm population (%) (Mean ± SE)	
			X sperm	Y sperm
Swim up	6	2,27,869	34.24±1.16 ^a (81,755)	65.76±1.16 ^a (1,46,114)
Percoll density gradient	6	1,34,173	65.06±1.11 ^b (87,575)	34.94±1.11 ^b (46,598) ²

Values within parenthesis indicate sperm number

Values bearing different superscripts within columns differ significantly (P < 0.01)

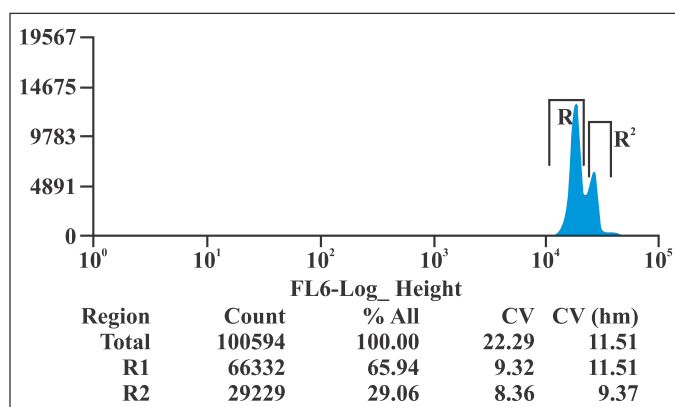


Fig. 1. Histogram showing sex sorting of swim up separated sperm (R¹ gate representing Y sperms and R² representing X sperms)

increased percentage of Y sperm with swim up method and X sperm with percoll density gradient method showed the sex enrichment effect of the sperm separation methods.

Azizzeddin *et al.* (2014) reported that the modified swim up method was capable of separating X and Y chromosome bearing sperm, as X bearing sperm had more DNA content than Y bearing sperm which resulted in faster migration velocity of Y than the X bearing sperm (Yan *et al.*, 2006). Swim up selected the viable sperm (Lucio *et al.*, 2012) with higher motility (Henkel and Schill, 2003); normal morphology (Younglai *et al.*, 2001) and was effective in increased percentage of recovery of motile sperm (Ren *et al.*, 2004) which led to fertilization success (Rurangawa *et al.*, 2004). Modified swim up technique has previously been reported to yield sperm population which resulted in a high percentage of male births (Check *et al.*, 1989).

Ericsson (1994) reported that swim up recovery and quality in final fraction was affected by many factors including temperature, preparation of TALP solution, the isolated tube dimensions and the number of spermatozoa layered per tube. It might be speculated that X chromosome bearing sperm, owing to their higher density (due to size of the sperm head; Cui and Matthews, 1993) move out from semen sample in higher percentage (Hafez, 1991) and travel to upper nearby (1ml) layers compared to distant (4-5 ml) travel of Y chromosome bearing sperm in long narrow glass tube. It is also relevant to mention that Y

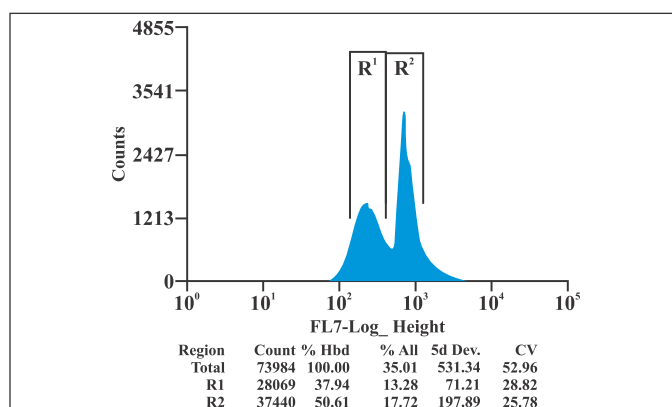


Fig. 2. Histogram showing sex sorting of percoll density gradient separated sperm (R¹ gate representing Y sperms and R² representing X sperms)

chromosome bearing spermatozoa have higher forward velocity than X chromosome bearing spermatozoa (Ericsson *et al.*, 1973). Few contradictory studies are also available where no difference of X and Y chromosome bearing sperm proportion of supernatant was reported after swim-up in human (Madrid-Bury, 2003 and Yan *et al.*, 2006).

In the present study, the sperm separation by percoll density gradient method showed higher percentage of X over Y sperm. Resende *et al.* (2010) reported that the sexual deviation in favor of females using thawed semen centrifugation in percoll density gradient was 59.6 per cent. Blottner *et al.* (1993) demonstrated a deviation of 90 per cent in favour of females for *in vitro* produced embryos. In literature, results of fresh sperm sexing using percoll density gradient method; 55.7 per cent (Kobayashi *et al.*, 2004) to 74.3 per cent (Hossepian de Lima *et al.*, 2011) was in favour of females.

Flow cytometric analysis of 2,27,869 sperm separated by swim up method showed the percentages of X and Y sperm to be 34.24±1.16 (81,755) and 65.76±1.16 (1,46,114), respectively. Azizzeddin *et al.* (2014) reported that the modified swim up method was capable of separating X and Y chromosome bearing sperm, as X bearing sperm had more DNA content than Y bearing sperm which resulted in faster migration velocity of Y than the X bearing sperm (Yan *et al.*, 2006).

Swim up selected the viable sperm (Lucio *et al.*, 2012) with higher motility (Henkel and Schill, 2003); normal morphology (Younglai *et al.*, 2001) and was effective in increased percentage of recovery of motile sperm (Ren *et al.*, 2004) which led to fertilization success (Rurangawa *et al.*, 2004). Modified swim up technique has previously been reported to yield sperm population which resulted in a high percentage of male births (Check *et al.*, 1989). Ericsson (1994) reported that swim up recovery and quality in final fraction was affected by many factors including temperature, preparation of TALP solution, the isolated tube dimensions and the number of spermatozoa layered per tube.

It might be speculated that X chromosome bearing sperm, owing to their higher density (due to size of the sperm head; Cui and Matthews, 1993) move out from semen sample in higher percentage (Hafez, 1991) and travel to upper nearby (1 ml) layers compared to distant (4-5 ml) travel of Y chromosome bearing sperm in long narrow glass tube.

It is also relevant to mention that Y chromosome bearing spermatozoa have higher forward velocity than X chromosome bearing spermatozoa (Ericsson *et al.*, 1973). Few contradictory studies are also available where no difference of X and Y chromosome bearing sperm proportion of supernatant was reported after swim-up in human (Madrid-Bury, 2003; Yan *et al.*, 2006 and Cesari *et al.*, 2006). The results of the program are encouraging the conventional methods of sex sorting such as swim up and percoll density gradient helped to separate around 65.00 per cent of Y and X sperms each.

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