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Effect of Percoll Density Gradient Centrifugation on Semen Quality of X- Sperm Enriched Crossbred Bull Semen

Deeksha Chaudhary[1], Kamal Devlal[2] and Mridula Sharma[1*]

[1*]Department of Veterinary Gynaecology and Obstetrics, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

[2]School of Science, Uttarakhand Open University, Haldwani (Nainital) 263139 Uttarakhand

ABSTRACT

The present study was conducted to observe the effect of percoll density gradient method on X-sperm enrichment and quality of semen. It was performed at Semen Production Centre, CVASc, GBPUAT, Pantnagar, Uttarakhand. Ejaculates (n=6) were collected by AV method from Crossbred bulls. X-sperm enrichment was done by percoll density gradient method i.e. 7 layers (70-10%). One ml semen was placed over the gradient in conical centrifuge tube. Centrifugation was done at 750g (22-24°C) for 15 min. The pH of percoll density gradient centrifuged (supernatant) decreased significantly (P<0.05) in Crossbred semen. Progressive motility of percoll density gradient centrifuged (supernatant) decreased significantly (P<0.05) in Crossbred cattle semen. Live spermatozoa (%) of enriched semen (pellet) were increased in Crossbred cattle semen. Acrosome integrity was not affected in all the groups in Crossbred bull. HOST responsive sperm were not affected in all the groups of Crossbred cattle semen. Thus, percoll density gradient centrifugation (7 layer 70%) did not affect the semen quality and fertility of semen hence it may be used to increase birth of female calves using A.I.

Key words: Percoll density gradient, Semen, X-enrichment, Qualitative assessment, Crossbred cattle.

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INTRODUCTION

To get more profit from dairy industry and livestock farmers, enhancement of female calf birth ratio is needed. Female calves are considered as future replacement stock of a dairy farm. Therefore, it becomes important to increase the female calf number with high reproductive potential. Sex sorted semen or embryo sexing technique can be used to increase the number of desired sex calf. There are several methods of embryo sexing (Seidel, 1999) with high accuracy, but these require biopsy of embryos, damage the embryos resulting in lower pregnancy rates. This technology is time consuming and thus, used much less than originally anticipated. This procedure identifies sex of embryos,

*Corresponding author.
E-mail address: sharmavetmridula@gmail.com (Mridula Sharma)
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but does not pre-determine sex, so half of the embryos are not of the desired sex (George and Seidel, 2003).

Sperm sexing raises great interest due to extensive application in animal production (Sharma and Sharma, 2016). New separation techniques with better accuracy, better fertility and low costs are necessary. Numerous ineffective efforts were made in the past to separate spermatozoa but failed to be used on a wider scale (Cran and Johnson, 1996).

Among all the techniques and methods used so far only flow cytometry with 90% accuracy separates two populations of sperm (X and Y-bearing) is used commercially (Garner, 2006). However, it is difficult for farmers to adopt this technique because of many disadvantages such as equipment costs (about US$ 300,000 per machine), damage to sperm during sexing (Seidel, 2003), low conception rates and altered mRNA expression of embryos (Mortan *et al*., 2007). Therefore, there is urgent need of an alternative method of semen sorting which can have good accuracy, lower cost of production, better fertility of semen and conception rates.

Bovine fresh semen in discontinuous Percoll density gradients used and observed 62 to 70% in X-sperm enrichment (Hossepian *et al.,* 2003) with lower cost and without damages to the sperm acrosome (Resende *et al*., 2010) and plasma membrane (Oliveira *et al*., 2011).

MATERIALS AND METHOD

Preparation of Percoll stock solution (90%)

A 90% percoll stock solution with density of 1.123 gm/ml was prepared by adding 9 parts (v/v) of 100% percoll (Sigma-Aldrich, India) with 1 part (v/v) of Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, India), 0.01 gm/L, Gentamycin Sulfate and 6mM HEPES (Hydroxy Ethyl Piperazine Ethane Sulfonic acid) buffer (Sigma-Aldrich, India). The pH of 90% stock solution of percoll was 7.4 and osmolarity was 280-320 mOsm/kg H_2O (Hossepian *et al.,* 2015).

Preparation of different gradients

Seven gradients of percoll were prepared i.e, 70, 60, 50, 40, 30, 20 and 10% by mixing above prepared 90% stock solution with DMEM (1X), 0.3% BSA (bovine serum albumin) (Sigma-Aldrich, India), 0.01 gm/L Gentamicin Sulfate and 6 mM HEPES (Hossepian *et al.,* 2015).

Separation of two populations of semen

One ml of each gradient i.e., 80, 70, 60, 50, 40, 30, 20 and 10% percoll solutions were taken in a 15 ml conical centrifugation tube with densest at bottom i.e. for Group II 70%, for Group III 80% and lighter at top i.e. for Group II 10% and for Group III 20%. In each group, six ejaculates (n=6) were taken.

Then 1ml of semen was layered on the top of conical centrifugation tube containing 1ml of each percoll gradient. The centrifugation of tube containing semen and percoll gradient was done for 15 minutes at 750 x g (2955 RPM) at 22-24°C.

Conversion formula for x g (RCF) into RPM;

$$g = (1.118 \times 10^{-5})\ R\ S^2$$

Where;

g is the relative centrifugal force
R is the radius of the rotor in centimeters
S is the speed of the centrifuge in revolutions per minute

Quality Assessment of X-enriched Semen

Semen was evaluated in fresh (Control), after centrifugation (Group I) and supernatant after centrifugation in percoll density gradient (Group II) and pellet after centrifugation in percoll density gradient (Group III). Ejaculates (n=6) were selected on the basis of mass activity as well as individual motility. The ejaculates with mass motility of <+3 and a progressive motility of <70% were selected for our experiment (Salisbury, 1978).

The pH, mass motility, progressive motility, live spermatozoa %, abnormal spermatozoa %, HOST % and intact acrosome % was evaluated to assess the quality of enriched semen.

RESULTS AND DISCUSSION

The pH of X-enriched semen was 6.73±0.03, 6.69±0.1, 6.4±0.12 and 6.79±0.06 in control, group I, II and III respectively. The progressive motility (%) of X-enriched semen was 85.83±1.54, 80.00±1.82, 78.50±0.80 and 88.67±1.44 in control, group I, II and III respectively. The live spermatozoa (%) of X- enriched semen were 92.33±3.33, 89.50±3.33, 85.33±4.51 and 95.83±0.99 in control, group I, II and III respectively. The abnormal spermatozoa (%) of X-enriched semen were 5.9400±1.27, 6.8167±1.25, 7.8667±0.83 and 3.5533±1.42 in control, group I, II and III respectively. The HOST (%) of X-enriched semen were 55.1667±1.45, 57.5±0.76, 54.6667±0.87 and 62.3333±9.74 in control, group I, II and III respectively. (Table-1)

Table 1: Qualitative Assessment of X-enriched crossbred bull Semen

Parameters	Control	Group I	Group II	Group III
pH	6.73±0.03[a]	6.69±0.1[ab]	6.4±0.12[b]	6.79±0.06[a]
Progressive motility	85.83±1.54[a]	80.00±1.82[b]	78.50±0.80[b]	88.67±1.44[a]
Live spermatozoa %	92.33±3.33[ab]	89.50±3.33[ab]	85.33±4.51[a]	95.83±0.99[b]
Abnormal spermatozoa %	5.9400±1.27[ab]	6.8167±1.25[ab]	7.8667±0.83[a]	3.5533±1.42[b]
HOST %	55.1667±1.45[a]	57.5±0.76[a]	54.6667±0.87[a]	62.3333±9.74[a]

Means bearing different superscripts in rows differ significantly (P>0.05)

Table 2: Acrosomal integrity test of X-sperm enriched crossbred bull Semen

Parameters	Control	Group I	Group II	Group III
Fully intact	88.91±2.11[a]	86.77±2.13[a]	85.95±1.99[a]	91.26±2.59[a]
Partially damaged	4.53±1.53[a]	6.13±1.63[a]	7.02±1.36[a]	3.55±1.39[a]
Fully damaged	6.55±0.74[a]	7.10±0.74[a]	7.03±0.88[a]	5.18±9.67[a]

Means bearing different superscripts in rows differ significantly (P>0.05)

The intact acrosome spermatozoa % of X-enriched semen was 88.91±2.11, 86.77±2.13, 85.95±1.99 and 91.26±2.59 in control, group I, II and III respectively. The partial damaged spermatozoa (%) of X-enriched semen was 4.53±1.53, 6.13±1.63, 7.02±1.36 and 3.55±1.39 in control, group I, II and III respectively. The fully damaged spermatozoa (%) of X-enriched semen was 6.55±0.74, 7.10±0.74, 7.03±0.88 and 5.18±9.67 in control, group I, II and III respectively. (Table-2)

The pH of X-enriched semen, pellet increased non significantly and of supernatant decreased significantly after centrifugation in percoll density gradient and respectively compared to fresh semen of Crossbred bull semen and also decreased non significantly in centrifuge. In our study, sperm enrichment by percoll density gradient method didn't affect pH of semen but changes in pH during sorting process by flow cytometry was observed which further decreased the semen quality (Chaudhary *et al.,* 2022; Singh, 2017).

The progressive motility of X-enriched semen, pellet increased non significantly and of supernatant decreased significantly after centrifugation in percoll density gradient and respectively compared to fresh semen of Crossbred bull semen and also decreased significantly in centrifuge. Similar to our observation, many workers reported increased progressive motility after centrifugation in percoll density gradient. Increase in motility after centrifugation (78%) compared to before centrifugation (64%) was recorded (Resende et al., 2010). Also, 20% increase in total sperm motility was observed in bull (Lucio et al., 2008), buffalo bull (Rawat, 2020) and goat. Whereas, no change in progressive motility was observed (Resende et al., 2010). Contrary to our results, lower motility in sexed semen was observed as compared to non-sexed semen (Hossepian et al., 2015).

In flow cytometry, motility of sperms gets highly decreased compared to non-sexed semen (Sharma and Sharma, 2016; Sharma *et al.,* 2018). Hence present method of X sperm enrichment is more beneficial in terms of progressive motility.

The live spermatozoa (%) of X-enriched semen, pellet increased non-significantly and of supernatant decreased non significantly after centrifugation in percoll density gradient and respectively compared to fresh semen of Crossbred bull semen and also decreased non significantly in centrifuge. In our study, number of live spermatozoa (%) were increased in percoll density gradient centrifuged semen (pellet). Similarly, decreased dead spermatozoa compared to untreated semen was observed in bull (Lucio *et al.,* 2008) and buffalo bull (Rawat and Sharma, 2020). Also, beneficial effect of percoll density gradient was reported on viability of spermatozoa (Bhat and Sharma., 2020). However, viability of semen was not affected after centrifugation in earlier experiments. Chaudhary *et al.* (2022) observed reduction in viability of spermatozoa after centrifugation in percoll (66±3.7%) compared to before centrifugation (87±2.0%). Additionally, decreased sperm viability was observed in sexed semen when centrifuged in percoll gradient medium (Hossepian *et al*., 2015, Sharma, 2018). Live sperm percentage was 74.7-86.6% in fresh semen after collection and also 83.5% in fresh semen of HF bull. The average live sperm percentage in fresh semen of Holstein bull ranged from 70%-90% Singh *et al.* (2017).

The abnormal spermatozoa (%) X-enriched semen, pellet decreased significantly and of supernatant increased non significantly after centrifugation in percoll density gradient and respectively compared to fresh semen of Crossbred bull semen and also increased non significantly in centrifuge. Decrease in sperm abnormalities was also

observed in buffalo compared to control after centrifugation in percoll density gradient (Rawat, 2020)**.**

The HOST spermatozoa (%) X-enriched semen, pellet increased non-significantly after centrifugation in percoll density gradient compared to fresh semen in Crossbred bull's semen and increased non significantly in centrifuge. Similarly, Oliveira *et al*. (2011) also reported enhancement of sperms with intact membrane after centrifugation in percoll gradient medium in bull, goat and buffalo bull (Rawat and Sharma, 2020). Whereas, Malik & coworkers (2011) reported significant (P<0.05) decrease in percent spermatozoa with intact membrane (HOST reactive) (Singh, 2017). Very less no. of sperm cells with intact membrane (HOST reactive) were present in sexed semen with flow cytometry compared to non-sexed semen (Bhat and Sharma, 2020). Flow cytometry method of sexing highly increased the percentage of spermatozoa with damaged plasma membrane compared to non-sexed semen (Chaudhary *et al.,* 2022, Sharma, 2022).

The intact acrosome spermatozoa (%) X-enriched, pellet increased non-significantly and of supernatant decreased non significantly after centrifugation in percoll density gradient and respectively compared to fresh semen in Crossbred bull's semen. The partially damaged spermatozoa decreased in pellet and increased in supernatant non significantly after centrifugation in percoll density gradient. In agreement to our study, increased percentage of live spermatozoa with intact acrosome after centrifugation through percoll density gradient was observed (Lucio *et al*., 2008; Rawat and Sharma, 2020). Resende *et al.* (2010) reported no damage in acrosomal integrity when semen is centrifuged through percoll medium for enrichment of X-spermatozoa. Centrifugation in percoll medium had no effect on normal sperm morphology before and after centrifugation. Compared to other methods of sexed semen, flow cytometry compromises the acrosomal integrity of spermatozoa (Sharma and Sharma, 2016) which affect the sperm fertility and conception rate.

CONCLUSIONS

In conclusion, by percoll density gradient method (7 layers, 70% to 10%) seminal parameters were not affected. These findings suggest that in general enriched semen could be used to increase the birth rate of female calves following artificial insemination. In the future, it will be important to explore the potential use of enriched semen for artificial insemination to assess the sex of the foetus and its fertility in terms of conception rates in different species of livestock.

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