Short Communication

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QUANTIFICATION OF X SPERM BY RAMAN SPECTROSCOPY IN PERCOLL DENSITY GRADIENT CENTRIFUGED BUFFALO SEMEN

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ABSTRACT

The present study was conducted to observe effect of percoll density gradient centrifugation of buffalo bull semen on quantity of X sperms. Ejaculates were collected by artificial vagina method. Semen with mass motility >+3 and progressive motility >70 % were selected for experiment. X sperm Enrichment of semen was done by discontinuous percoll density gradient centrifugation and three groups were formed ie Group 1 (3 layer 70%, 50% and 30%) Group 2 (7 layer 70%, 60%, 50%, 40%, 30%, 20% and 10%) Group 3 (7 layer 80%, 70%, 60%, 50%, 40%, 30% and 20%). Centrifugation of semen of three groups and control (fresh semen without gradients) was done. After centrifugation, the supernatant part was removed and the pellet of each group was used for X sperm enrichment assessment by Raman spectroscopy. Results revealed that X sperm enrichment was higher in the pellets of Group 2 followed Group 3, Group 1 and Control as Raman peaks on DNA specific bands corresponds to more number of x sperm were higher respectively.

Keywords: *Bubalus bubalis*, buffaloes, Raman spectroscopy, percoll, centrifugation, PBS

INTRODUCTION

Sexed semen relates to more than 90% births of desired sex offspring compared to nearly 50% using conventional semen (DeJarnette *et al*., 2009; Norman *et al*., 2010, Sharma and Sharma, 2016). Not even a single method of sperm sorting is 100% efficient though lots of methods are tried. Flow cytometric enrichment of X sperm has nearly 90% accuracy (Garner *et al*., 1983; Morrell *et al*., 1988; Johnson, 2000) but fertility of semen is reduced (Cran *et al*., 1993, 1994; Merton *et al*., 1997; Lu *et al*., 1999; Xu *et al*., 2009; Schenk *et al*., 1999; Maxwell *et al*., 2003; Bodmer *et al*., 2005) and more chances of abnormal embryos production (Maxwell *et al*., 2004).

Percoll is frequently used to enrich and isolate cell populations. Depending upon the differences in the sedimentation density, high sedimentation density of X spermatozoa than Y spermatozoa enables settling of X sperm in the

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bottom portion of column and Y spermatozoa remains at the top of column. Percoll density gradient centrifugation could enrich the X-bearing viable sperm up to 70%, at lower cost (Hossepian de Lima, 2007; Chaudhary *et al*., 2023) without damaging the sperm membrane (Oliveira *et al*., 2011; Chaudhary *et al*., 2023) and acrosome (Resende *et al*., 2010, Chaudhary *et al*., 2022) compared to flowcytometric separation of X and Y sperms (Sharma *et al*., 2018). Percoll gradient centrifuged enrichment of X sperm in association with *in-vitro* production of embryo resulted in more female calves birth (72.3%) compared to the male (48.2%). Significant sex ratio deviation toward female gender was also observed at parturition (Hossepian *et al*., 2015). Similarly, Resende *et al*. (2011) reported the 13% increase in birth of female calves. Semen was centrifuged twice by discontinuous percoll density gradient (3 layers) method and AI with enriched semen resulted in birth of female fetuses increased significantly ($P<0.05$) to 66.66% compared to 46.15% with nonsexed semen when scanned by ultrasonography (Bhat, 2017). In buffalo also, there is an obvious requirement of sex sorting method other than flowcytometry (Sharma *et al*., 2022) to shift the sex ratio towards the female calves. Buffalo semen fertility varies with season (Sharma *et al*., 2018) Hence, present study was designed to enrich the X sperms in buffalo semen by percoll density gradient centrifugation and assess the enrichment by Raman spectroscopy.

MATERIALS AND METHODS

Experiment was conducted at Semen Production Center, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. A buffalo bull aged 7 years weighing 450 to 500 kg reared at the Semen Production Center was used for study. Semen samples were collected twice a week by AV method. Ejaculatory volume >3 ml, sperm mass motility >+3 and progressive motility >70% were selected for further processing.

A 90% percoll stock solution having the density of 1.123 g/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 g/L Gentamicin Sulfate and 6mM HEPES (Hydroxy Ethyl Piperazine Ethane Sulfonic acid) buffer (Sigma-Aldrich, India). (Hossapian de Lima *et al*., 2015). Eighty 80, 70, 60, 50, 40, 30, 20 and 10% of percoll was prepared by mixing 90% stock solution with DMEM (1X), 0.3% bovine serum albumin (Sigma-Aldrich, India), 0.01 g/L Gentamicin Sulfate and 6 mM HEPES (Hossapian Lima *et al*., 2015).

X sperm Enrichment of semen was done by discontinuous percoll density gradient centrifugation and three groups were formed ie Group 1 (3 layer 70%, 50% and 30%) Group 2 (7 layer 70%, 60%, 50%, 40%, 30%, 20% and 10%) Group 3 (7 layer 80%, 70%, 60%, 50%, 40%, 30% and 20%). Then 1 ml semen was layered on the top of the conical centrifugation tube containing 1 ml of each percoll gradient and without percoll (control) and was centrifuged for 20 minutes at 750 rcf at 24ºC. Obtained pellets of all groups samples were diluted in PBS medium, processed to get same volume (400 µl) and number of sperms ($2x10^6$) in each sample and sent to UGC-DAE Consortium for Scientific Research Center, Indore for Raman spectroscopy. Raman spectroscopy was performed under laser light (25 w power) with exposure time of 30 seconds in each group at 473 nm wavelength and 100X magnification with wave numbers varied from 500 to 1800 cm-[1]. Before loading, samples were gently shaken, 4 µl of semen from eppendorf tube was taken with the help of micropipette and it was loaded in chamber to get Raman spectra.

Raman bands of wave numbers 500 to 1800 cm^{-1}were observed. These bands were mapped to identify the DNA rich region. Statistical analysis of the spectrum of various was done to find out the best gradient of percoll density for X and Y sperm separation of buffalo semen.

RESULTS AND DISCUSSIONS

The Raman analysis is an efficient diagnostic tool for the structure and the composition determination of biomolecules. Therefore, the spectroscopic characteristics of X sperm enriched buffalo bull semen were observed. To study the effect of percoll density gradient separation on X sperm enhancement in buffalo bull semen the Raman spectroscopy of samples was performed. Nucleic acid and DNA are the importantmost important part of any biomolecules. Normally, the nucleic acids and DNA observed in the region of (700 to -1600 cm^{-1}) in Raman spectrum. Raman bands assigned to nucleic acid and DNA are observed around 723 cm^{-1}, 795 cm^{-1}, and 1581 cm^{-1} (1494-1650 cm^{-1}) (De Luca *et al*., 2014).

The Raman spectrum revealed specifica specific band corresponding to proteins and lipids at 1094 cm^{-1}. Figure 1 shows the Raman spectrum for Group 1I, Group 2II, and Group 3III. Analysis of Raman spectrum of different groups suggested that the Raman bands are almost same in all the cases. However, the Raman intensity varied in all the groups.

Intensities at DNA specific peak was compared as presented in Table 1 and Figure 2. There was significant ($P<0.05$) difference between intensities at DNA specific wave numbers in all the groups.

From the above mentionedabove-mentioned Raman analysis it was clearly observed that the intensities for DNA specific wave numbers were highest for 7 layer 70% percoll pellet followed by 3 layer 70% percoll pellet, control and 7 layer 80%.

In bovine, X-sperm enriched semen was recovered at 75% gradient of Percoll® (Promthep, 2016). And the DNA difference between X and Y sperm, results in higher density of X buffalo sperm, is 3.6 (Johnson, 2000; Lu *et al*., 2006) which is lesser than X bull spermatozoa iei.e., 3.8 (Garner *et al*., 1983; Garner, 2001, 2006; Johnson and welch, 1999). This might be a probable reason for better X-sperms separation at lesser gradient i.e.i.e., <75%. (Johnson, 2000) and alsoand reduced intensities at DNA specific band relates to lesser X sperm enrichment in pellets of 7 layers percoll gradient (80% to- 10%) centrifugation of buffalo semen. Additionally, buffalo spermatozoa isare more susceptible to variations like temperature and viscosity (Raizada *et al*., 1990; Andrabi *et al*., 2008).

The different intensities at DNA specific wave numbers between X-chromosome bearing or Y chromosome-bearing sperm reflect the relative change in the DNA content (De Luca *et al*., 2014). The increased intensity attributed to higher DNA concentration corresponds to more numbers of X sperms in pellets of 70% 7 layers compared to pellets of other semen samples obtained after percoll density gradient centrifugation.

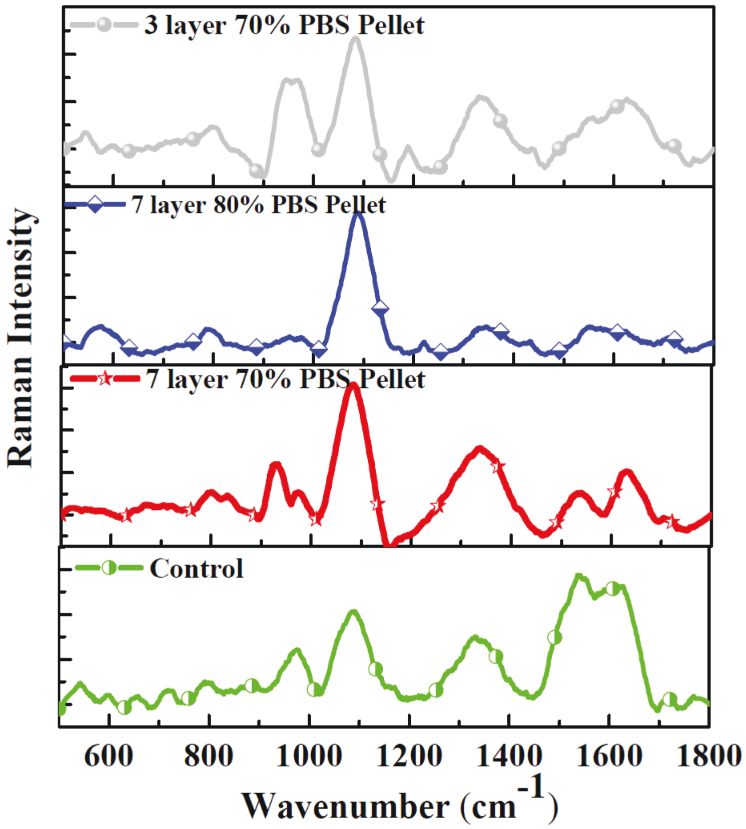

Figure 1. Raman spectra of X sperm enriched PBS diluted buffalo semen.

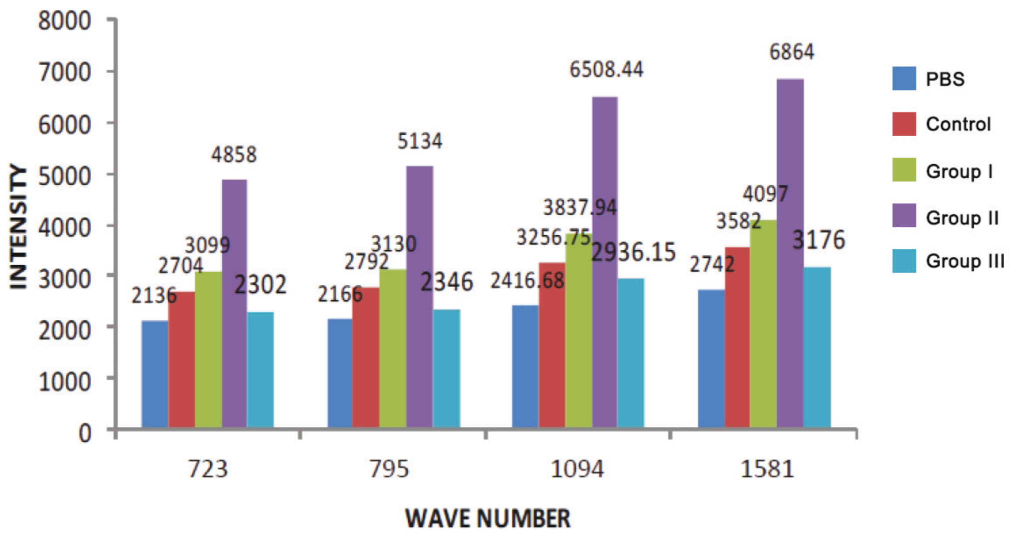

Figure 2. Effect of X sperm enrichment by percoll density gradient method on Raman spectra of PBS diluted buffalo semen with respect to DNA specific wave numbers.

Table 1. Effect of X sperm enrichment by percoll density gradient method on Raman peaks of PBS diluted buffalo semen at DNA specific wave numbers (mean±SE, n=6).

Wave number (cm^{-1})	Control group	Group 1	Group 2	Group 3
723	2704±1.10	3099±2.14	4858±0.65	2302±0.54
795	2792±0.57	3130±2.07	5134±0.22	2346±1.05
1094	3256.75±1.11	3837.94±0.56	6508.44±0.37	2936.15±0.55
1581	3582±0.32	4097±0.83	6864±1.05	3176±0.89

CONCLUSION

Percoll density gradient method specifically 7 layers of 10 to 70% percoll density gradient centrifugation could be used enhance the X - sperms in buffalo semen. Though, further experiments to observe its effect on sex ratio and conception rate after AI with enriched semen, are needed. Raman approach could be a promising method to assess the quantity of X sperms in enriched buffalo semen on the basis of difference in the intensities at DNA specific wave numbers.

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