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Research Article

EVALUATION OF FROZEN SEMEN BY ACROSOMAL INTEGRITY AND SPERM CONCENTRATION - TWO VITAL QUALITY PARAMETERS OF MALE FERTILITY IN BOVINES

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ABSTRACT: Acrosomal integrity and sperm concentration are two important parameters to assess the quality of frozen semen doses which in terms validates the fertilizing capacity and conception rate. The present study was undertaken to evaluate acrosomal integrity by Giemsa's stain and sperm concentration of FSS using improved neubauer chamber in Exotic pure Jersey, Crossbred Jersey, Indigenous Gir cattle and Indigenous Murrah buffalo prior to the field use. The overall values of Giemsa's stain were observed as 73.74±0.31, 18.65±0.33 and 7.79±0.25 percent for Intact Acrosome, Partially Damaged Acrosome and Fully Damaged Acrosome, respectively. Overall values of sperm concentration were 21.98±0.28 million per straw. The study indicated that there was no significant difference (P<0.05) among the breeds and the values mostly correlates with the guideline of Minimum Standard Protocol for Production of bovine semen, 2012 of Govt. of India.

KEY WORDS: Acrosomal Integrity, Sperm Concentration, Giemsa's staining, Active volume.

INTRODUCTION

There is ever increasing demand of frozen semen straws from different parts of the country for superior quality germplasm. It is also known that a more accurate estimation of fertility of the ejaculate is achieved by increasing the number of tests performed in the laboratory. In animals, capacitation and the subsequent acrosome reaction of spermatozoa are the essentials steps before fertilization and formation of zygote. Acrosomal evaluation, two methods (double staining and Hoechst/FITC-PSA) are commonly used but produces contradictory result (Risopatron *et al.,* 2001, Kohn *et al.,* 1997). The determination of acrosomal integrity of spermatozoa by single Giemsa's staining technique is an essential tool to evaluate fertilizing capacity of the semen.thistechnique is very simple, rapid and allows examining the results after many years (Wells and Awa 1970). It binds over an outer acrosomal membrane but does not bind to the acrosome-reacted spermatozoa. Giemsa's is

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superior over single staining with Trypan blue (Jankovicova *et al.,* 2008) and Congo red (Kovacs and Foote 1992).

Sperm counting is another essential step in the evaluation of male fertility as the success of artificial insemination depends on the optimum number of spermatozoa in the frozen semen doses. The World Health Organization (WHO 1992) recommends the use of a haemocytometer for determining sperm concentration in semen. Photometric estimation is most common method of estimating sperm concentration during processing of semen. The validation of photometric sperm concentration is generally achieved by improved Neubauer chamber haemocytometer (Mahmoud *et al.*, 1997). The present study was undertaken to judge the semen quality with two vital quality tests *i.e.* acrosomal integrity and sperm concentration of frozen semen straw in Exotic pure Jersey, Crossbred Jersey, Indigenous Gir cattle and indigenous Murrah buffalo.

MATERIALS AND METHOD

Production 0.25 ml French mini FSS:

The semen straws were prepared from the Exotic pure Jersey, Crossbred Jersey, Indigenous Gir cattle and Indigenous Murrah buffalo bulls maintained at Frozen Semen Bull Station, Salboni, West Bengal. Twenty samples of 0.25 ml FSS of different days production over a period of two month were taken into the study. The concentration of neat semen was checked in photometer (Accucell, IMV, France) to keep the final concentration of 20 million when packed in automated filing and sealing machine (IS-4 machine). Further the straws were kept in LN_2 for quality test by Giemsa's stain for acrosomal integrity and sperm concentration by improved Neubauer chamber.

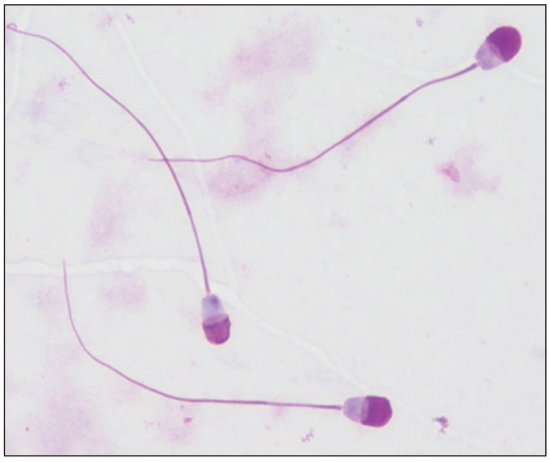

Fig.1. Intact Acrosome of Murrah Buffalo.

Acrosomal integrity Test by Giemsa's stain:

The stock Giemsa's stain was prepared as per manufacturers' protocol. Firstly, 0.77 gm Giemsa's powder (MERCK) was measured in weighing balance and saturated for long time (2-3 hrs) with methanol and glycerol mixture pre-warmed at 40^0C. The methanol-glycerol mixture was prepared by measuring 75 ml absolute alcohol and 25 ml of glycerol (98% pure). After proper mixing, the stain was filtered with 0.22 µm sterile Millex (Millipore). The solution was kept at 37^0C in an incubator for 7 days in amber color bottle for maturation with intermittent shaking. The stock solution was ready to assess the acrosomal structure of spermatozoa.

The staining procedure was followed the protocol of Watson, 1975. Firstly, FSS were thawed in 37^0C for 30 sec and content was poured into pre-warmed sugar tube at 37^0C. 3 µl of Sorenson Phosphate Buffer (SPB) was put into clean grease free slide, on that 30 µl of frozen semen was mixed and a smear was drawn

Table 1: Acrosomal Integrity values.

Particulars	Pure Jersey (N=20)	Cross-bred Jersey (N=20)	Indigenous Gir (N=20)	Murrah Buffalo (N=20)	Overall (N=80)
Intact Acrosome (%)	73.05 ±	75.15 ±0.71* 0.59	73.05 ± 0.48	73.70 ± 0.61	73.74 ± 0.31
Partially Damaged Acrosome (%)	18.60 ±	17.25 ± 0.67 0.57	20.00 ± 0.75*	18.75 ± 0.54	18.65 ±0.33
Fully Damaged Acrosome (%)	8.40 ±0.58*	7.70 ± 0.48	7.55 ± 0.48	7.50 ± 0.50	7.79 ± 0.25

Values depicted as Mean ±SE, * Indicates highest value among the breeds, N= No. of observations

Table 2 : Straw Volume values.

Particulars	Yellow for pure Jersey (N=20)	Transparent for Cross-bred Jersey (N=20)	Orange for Indigenous Gir (N=20)	Grey for Murrah buffalo (N=20)	Overall (N=80)
Packaged volume (ul)	211.90 ± 0.84	212.07 ± 1.04	229.48 ±1.54*	211.49 ± 0.61	216.23 ±1.00
Active volume (ul)	207.57 ± 0.70	206.41 ±0.85	218.83 ±1.00*	205.66 ± 0.49	209.62 ± 0.72
Wastage volume (ul)	4.33 ±0.44	5.63 ± 0.71	10.65 ±1.59*	5.82 ± 0.41	6.62 ±0.5

Values depicted as Mean ±SE, * Indicates highest value among the breeds, N= No. of observations

and air dried. The slides were put into 5% formaldehyde solution for fixing at 37^0C for 30 min (Campbell *et al.,* 1960). The slides were removed from the solution, washed in running tap water and air dried for further processing. The working solution of Giemsa was prepared mixing Giemsa's stock-3 ml, SPS-2 ml and Milli-Q water- 45 ml in a cup linger and warmed at 37^0C for 30 min. The smeared slides of spermatozoa was put into the working solution and kept at 37^0C for 2 hrs. The slides were removed from the stain and washed in running tap water and finally air dried. The counting of intact, partially damaged and fully damaged acrosome was carried out in oil immersion Olympus microscope (BX51) at1000X (10X100) magnification.

Table 3: Sperm Count in frozen semen doses.

Particulars	Pure Jersey (N=20)	Cross-bred Jersey (N=20)	Indigenous Gir (N=20)	Murrah Buffalo (N=20)	Overall (N=80)
No. of spermatozoa as per obtained active volume (million/FSD)	21.80 ± 0.57	21.57 ± 0.56	22.96 ± 0.56*	21.60 ± 0.55	21.98 ± 0.28
No. of spermatozoa as per MSP given active volume (million/FSD)	22.05 ± 0.56	21.95 ± 0.60	22.05 ± 0.56	22.05 ± 0.60	22.02 ± 0.28

Values depicted as Mean ±SE, * Indicates highest value among the breeds, N= No. of observations.

Sperm Concentration in 0.25 ml French Mini Straw:

The sperm concentration was achieved using Improved Neubauer chambers (China). The solution of 0.2% eosin yellow and 0.9% sodium chloride in 100 ml milli-Q water was used for this quality test of sperm. Firstly, the thawed-FSS was poured into sugar tube at 37^0C. Then two times dilution of sperm with eosin solution were made to give final dilution 1:1000. Then 20 µl of diluted semen was charged on a neubauer chamber on both sides with a cover slip on it. Then the chamber was kept in a Petri dish in wet environment for 10 min for settling down of the sperm. The total 400 small WBC counting squares were counted for presence of sperm. Finally, total no of sperm present was calculated with mathematical formula in the following ways;

Sperm concentration/ 0.25 ml straws = AXBX10X 10^6

Where, A = Total no of sperm in upper/lower chamber, B =Active volume of the semen in straw and 10 is the Multiplication factor.

In Minimum Standard Protocol for Production of Bovine Semen (MSP) of Govt. of India the active volume of semen straw (0.25 ml) is given as 0.21 ml. A study was also conducted to find out the active volume by the following method.

Firstly, the blank straws of different color of different breeds *i.e.* orange (Gir), transparent (CBJ), yellow (PJ) and grey (Buffalo) were weighted in Weighing balance (Sartorius, Germany). The filled straws with semen of different breeds were also weighted. The difference between filled and empty straws was calculated and packaged volume was determined by multiplying with specific gravity of Tris-citrate-egg-yolk dilutor (specific gravity 1.04 gm/dl). Secondly, the active volume in FSS was calculated. The semen from FSS was discarded with the help of A.I. gun (French Mini) cutting the laboratory seal. Then the empty-straw having factory plug and cut portion of laboratory seal was weighted breed wise. The weight difference between the filled straw and empty-straw was calculated and usable volume/

active volume was determined multiplying with specific gravity of dilutor (1.04).

The data were analysed using a computer programme, SPSS 16.0 ®.

RESULT AND DISCUSSION

The aim of the study was focused to evaluate Frozen Semen by acrosomal integrity and sperm concentration test to supply good quality of FSS for field artificial insemination. Firstly, the morphology of acrosome was tested by Giemsa's staining methods in all four breeds (Fig. 1). Results revealed that there was no significant difference among breeds. The average intact acrosome, partially damaged acrosome and fully damaged acrosome was 73.74%, 18.65% and 7.79%, respectively (Table 1). Highest intact acrosome value was obtained in Crossbred Jersey bulls. The results were quite satisfactory in terms of guidelines fixed in MSP of Govt. of India and such good morphology of acrosome of spermatozoa indicated good fertility of the spermatozoa as well as the efficacy of this simple but accurate method of Giemsa's staining for all four breeds under study. The acrosomal reaction was validated to the fertility using FITC-PSA technique in bovines (Jancovicova *et al.*, 2006). The FITC-PSA labeling technique is less time consuming, yolk environment is not limiting factor, but requires fluorescent microscopy, sperm morphology not cleared, and signal fades with time and also have strong poisoning effect. Evaluation of buffalo semen was graded into acrosome-intact live (AIL), acrosome-intact dead (AID), acrosome-lost live (ALL) and acrosome-lost dead (ALD) with double staining procedure Trypan Blue/Giemsa's stain (Boccia *et al.*, 2007). The almost same result of acrosome reaction was achieved by double stains (37.98%) versus Hoechst/ FITC-PSA staining (39.33%) in frozen semen (Jancovicova *et al.*, 2008). It can be concluded that Giemsa's stain could be used in semen station for rapid screening of large number of frozen semen doses.

Further, the active volume of FSS in all breeds was determined using mathematical formulas. Overall active volume was obtained as 0.2096±0.72 ml (Table 2). In case of FSS of Gir breed, the highest packaged volume as well as highest wastage- volume was obtained in orange straws (0.2295 ml and 0.0107 ml respectively). Then the sperm concentration in FSS was calculated in all types of breeds using obtained active volume and the overall concentration per frozen semen straw was 21.98 and 22.02 million, respectively (Table 3). In all the cases, recognizable spermatozoa, including loose heads were counted; other germinal line cells and free tails were ignored. Lineweaver *et al.* (1970) reported that when 1 ml of fresh or frozen semen containing millions of live spermatozoa was placed in mid-cervix in heifers, 4 hours later, 469 or 191 numbers of spermatozoa were found in the oviducts and 1559 or 733 numbers of spermatozoa were found in uterus, respectively. We found that overall sperm concentration of 21.98±0.28 million per dose with effective semen volume of 0.209±0.72 ml. It was also found that 6.62±0.53 ul of semen was absorbed into the factory plug, reducing the active volume discharged into the uterus during A.I. The active volume could be further increased by using next generation filling sealing machine (like MX5 of IMV, France) where, only the dilutor will be used to soak the factory plug resulting in zero absorption of sperm. Though many researchers calculated sperm concentration with

sophisticated techniques *i.e.* flow cytometry (Takumi *et al.,* 2002) and Computer Assisted Semen Analyzer (CASA) (Coetzee *et al.,* 2001), these instruments also have problems in reproducibility and accuracy, and there is variation within and between the laboratories (Vantman *et al.,* 1988, Keel *et al.,* 2000). The sperm counting by improved Neubauer chamber is simple, less cost involvement and also hardly takes 20-25 min to calculate sperm concentration per sample. We also found that there was no significant difference ($P<0.05$) in sperm concentration among all the four breeds of this semen station.

Therefore, it can be concluded that both acrosomal integrity by Giemsa's stain and sperm concentration using improved neubauer chamber method are most effective semen analysis techniques to validate male fertility at semen station level. However, further investigation requires verifying the semen quality breed wise with calf born data obtained from the field.

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