Original Research

Evaluation of Acrosomal Integrity and Viability in Bull Spermatozoa: Comparison of Cytochemical and Fluorescent Techniques

Alok Kumar[1, 2*], Atul Saxena[2] and Mukul Anand[3]

College of Veterinary Science & Animal Husbandry, DUVASU Mathura, UP, INDIA

[1]Institute of Paraveterinary Sciences, DUVASU Mathura, U.P, INDIA
[2]Department of Veterinary Gynaecology and Obstetrics
[3]Department of Veterinary Physiology

***Corresponding author:** dr.alokshukla.vet@gmail.com

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Abstract

This experiment was aimed to compare two well-known methods for assessment of acrosomal integrity and another two methods for assessment of viability in bull spermatozoa during cryopreservation. For this purpose, ejaculates were collected from four Hariana bulls using artificial vagina at biweekly interval. The semen samples which possesses more than 70% progressive motility and above 500 million/ml spermatozoa concentration was subsequently subjected to processing for liquid nitrogen (LN_2) vapour freezing. Semen samples were extended in Tris-egg yolk-glycerol extender and further subjected to two staining methods, cytochemical by Giemsa staining and fluorescent method by fluorescein isothiocyanate - pisum sativum agglutinin (FITC-PSA) at two stages i.e. pre freeze and post thaw. In the same way to assess the viability eosin-nigrosin staining is compared with fluorescent method i.e. Annexin-V/PI method. Results showed that fluorescent staining were more sensitive as compare to cytochemical method for spermatozoa of Hariana bulls.

Key words: Acrosomal Integrity, Annexin-V/PI, Bull, Giemsa, FITC-PSA, Spermatozoa

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Introduction

In process of evaluation of fertility in a male, or in the assessment of methods of semen preservation, most important parameters are the analysis of sperm viability along with acrosomal integrity. However successful fertilization and zygote formation largely depends upon capacitation and subsequent acrosomal reaction in mammalian spermatozoa including bull. Thus semen used for insemination must contain spermatozoa with intact acrosomal membrane (Jankovicova *et al*., 2008). Acrosomal integrity of spermatozoa is required for

post thaw motility and acrosome reaction to occur at appropriate for successful fertilization (Thomas *et al*., 1997; Singh *et al*., 2017). Acrosome is membrane bound structure present over the nucleus of spermatozoa containing enzymes which plays crucial role in sperm penetration through zona pelucida (ZP) (Yanagimachi, 1994). Moreover binding of sperm to ZP triggers acrosome reaction leading to fusion of outer acrosome membrane with plasma membrane for subsequent vesiculation. Therefore, intact acrosome is pre requisite for fertilization (Rodriguez-Martinez *et al*., 1997).

Earlier acrosomal integrity was evaluated in vitro using phase-contrast microscopy, differential interference-contrast microscopy or light microscopy for unstained and stained samples (Rodriguez-Martinez, 2006). However recently fluorescent microscopy based staining techniques have been developed such as chlortetracycline staining (CTC) (Fraser *et al*., 1995; Hossain *et al*., 2010), paramagnetic beads (Ohashi *et al*., 1994), quinacrine (Amin *et al*., 1996), FITC-PSA (Samardzija *et al*., 2006) and FITC-concanavalin A (FITCConA) (Holden *et al*., 1991; Fukushima *et al*., 2005). The evaluation of acrosomal integrity of spermatozoa by single Giemsa's staining technique is very simple, quick and one of the primitive and reliable methods (Wells and Awa, 1970) in which intact acrosome appears purple and damaged as pale-lavender. Giemsa staining is superior over other single staining i.e. Trypan blue (Jankovicova *et al*., 2008) and Congo red (Kovacs and Foote, 1992).

Recently lectins i.e. pisum sativum agglutinin (PSA) conjugated with Fluorescein isothiocyanate (FITC) emerged as popular method for identification of acrosome integrity moreover it is a sophisticated method which need epifluorescent microscope. Therefore present study was aimed to compare two reliable techniques (Giemsa and FITC-PSA) for evaluating acrosomal integrity in term of their accuracy. Several methods have been used to distinguish between viable and non-viable cells, such as the eosin- nigrosin stain (Hancock, 1951), and the fluorescent probes carboxyfluorescein diacetate, propidium iodide (Garner *et al*., 1986; Harrison and Vickers, 1990). Eosin and Nigrosin staining is based on principle that live cells are impermeable to eosin therefore remains unstained which appears white against black background provided by nigrosin whereas dead spermatozoa stains pink as eosin enters nonfunctional plasma membrane of spermatozoa. PI is membrane impermeable and generally excluded from viable cells. PI is commonly used for identifying dead spermatozoa in semen sample. PI binds to DNA by intercalating between the bases. PI also binds with RNA and once it binds with nucleic acid its fluorescence is enhanced 20- to 30-fold and emits red fluorescence.

Materials and Methods

Semen Collection and Initial Evaluation

Semen was collected biweekly from each bull with the help of artificial vagina (AV) and analyzed for routine semen attributes. The ejaculates containing higher than 70% progressively motile spermatozoa were

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selected for further processing. Overall 28 ejaculates (seven ejaculates from each bull, n=7) were extended with Tris egg yolk glycerol extender to obtain the final concentration of 80 million/ml.

Cryopreservation

Extended semen cooled up to 4^0C followed by four hours equilibration and subsequently subjected to freezing up to -140^0C (From 4°C to -10 °C @ 10 °C /min, From -10°C to -100°C @ 40°C/min, From -100°C-140°C @ 20°C/min) in a programmable biological cell freezer (IMV, France). Finally straws ware plunged into liquid nitrogen (LN_2) to reach desired temperature to -196^0C.

Experiment 1: Evaluation of Acrosomal Integrity of Spermatozoa

Evaluation of Acrosomal Integrity by Giemsa Staining

Evaluation of acrosomal integrity was performed by Giemsa staining as described by Chowdhury *et al*. (2014) and Sachan *et al*. (2017) with slight modification, in that washing of glass slides at post incubation stage were carried out by using gentle stream of triple distilled water.

Evaluation of Acrosomal Integrity by FITC-PSA Assay

Acrosomal integrity of the bull spermatozoa was evaluated by using FITC –PSA under epifluorescent microscope with phase contrast (Nikon 140 Eclipse TE 2000-S, Japan) as described by Kumar *et al*. (2018).

Experiment 2: Evaluation of Viability of Spermatozoa

Evaluation of Viability by Eosin – Nigrosin method of Staining

A method described by Bloom (1950) and Hancock (1951) was followed. Around 200 sperms were assessed. Sperms that were colorless (unstained) were classified as live and those that showed any pink colouration were classified as dead. Per cent viable spermatozoa were calculated by using simple mathematical formulae.

Evaluation of Viability by Annexin-V/PI Assay

Phosphatidylserine translocation precedes loss of membrane integrity which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes. Therefore staining with Annexin-V is typically used in conjugation with vital dye such as propidium iodine (PI). Viable cells with intact membrane exclude PI, whereas membranes with damaged or dead cells are permeable to PI. This specific stain PI emits red fluorescence when bound to nucleus (DNA) of damaged plasma membrane of a cell (Arruda *et al*., 2003).

An Annexin V-FITC Apoptosis detection kit (catalog no- APOAF Sigma-Aldrich USA) was used to detect the viability in spermatozoa as per manufacturer's specification with slight modification. Post thawed, spermatozoa were washed twice with calcium and magnesium free Dulbecco's Phosphate Buffered Saline

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(DPBS) and centrifuged at 500 × g, 10 minutes at 25^{0}C). Sperm pellet re-suspended in 100 µl Annexin V binding buffer. (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) and concentration adjusted to approx 1×10^6 spermatozoa/ml, later on supplemented with 5µl of Annexin V-FITC conjugate and 10 µl of PI solution in a ependorf tube. Incubation carried out at room temperature for 15 minutes in a dark place. (Intire procedure i.e. washing, staining, and incubation carried out at room temperature). 10µL spermatozoa suspension placed over clean grease free slide covered with glass cover slip and observed under phase contrast microscope (40 X) Nikon 140 eclipse TE 2000-S (Nikon, Japan) with epifluorescence using dual filter, at least 200 spermatozoa per slide with different fluorescent pattern were observed.

Table 1: Different fluorescent patterns observed under epifluorescent microscope and their results

S. No	Pattern Observed	Results
1	Annexin-V-negative/PI-negative (AN-/PI-)	Viable spermatozoa
2	Annexin-V-positive/PI-negative (AN+/PI-)	Viable spermatozoa with impaired but integer plasma membranes
3	Annexin-V-negative/PI-positive (AN-/PI+)	Avital spermatozoa
4	Annexin-V-positive/PI-positive (AN+/PI+)	Dead spermatozoa

Pattern 3 and 4 (PI positive) were added and substituted with 200 spermatozoa followed by calculation of percentage of viable spermatozoa by simple mathematical formulae. Semen samples were evaluated at post equilibration (pre freeze) and post-thaw stages for acrosomal integrity and viability. The frozen semen was thawed at 37^{o}C for 45 seconds in thawing unit (IMV, France).

Statistical Analysis

The data were analyzed using SPSS V 23 (IBM Corp) statistical software and expressed as mean and standard error of mean (Mean ± SEM). The means were compared by employing paired T test.

Results and Discussion

The result of Experiment 1, acrosomal integrity by Giemsa and FITC assay has been depicted in Table 2 and 3. The results of evaluation at pre freeze and post thaw stage including individual bull and overall value showed significantly ($p<0.05$) different percentage of spermatozoa with intact acrosome in Giemsa staining and fluorescent method using FITC-PSA. The overall result of FITC-PSA assay showed 6.36% higher number of spermatozoa with intact acrosome as compared to Giemsa staining in pre-freeze stage while 7.75% higher at post thaw stage. In the same way result of Experiment 2. Evaluation of viability of spermatozoa by eosin-nigrosin and Annexin-PI method including individual and overall value showed significant difference ($p<0.05$) between aforesaid two methods. Overall result indicates 2.18% higher number of viable spermatozoa at pre-freeze stage and 1.86% higher number at post thaw stage, using fluorescent staining method.

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Table 2: Evaluation of acrosomal integrity by two staining methods at pre freeze stage (Mean ± SEM)

Pre freeze Stage of Evaluation (Acrosomal Integrity %)		
Bull	**Giemsa Staining**	**FITC-PSA Staining**
Bull no – 623 (n=7)	$63.14^{a} \pm 4.03$	$68.42^{b} \pm 3.51$
Bull no – 580 (n=7)	$49.14^{a} \pm 3.66$	$51.57^{b} \pm 3.29$
Bull no – 533 (n=7)	$57.42^{a} \pm 3.25$	$66.00^{b} \pm 3.36$
Bull no – 531 (n=7)	$51.42^{a} \pm 3.31$	$61.14^{b} \pm 4.81$
Overall (n = 28)	$55.42^{a} \pm 1.97$	$61.78^{b} \pm 2.17$

Means bearing different superscripts in rows differ significantly ($p<0.05$)

Table 3: Evaluation of acrosomal integrity by two staining methods at post thaw stage (Mean ± SEM)

Post thaw Stage of Evaluation (Acrosomal Integrity %)		
Bull	**Giemsa Staining**	**FITC-PSA Staining**
Bull no – 623 (n=7)	$47.71^{a} \pm 2.67$	$55.14^{b} \pm 3.27$
Bull no - 580 (n=7)	$40.28^{a} \pm 2.06$	$44.71^{b} \pm 2.67$
Bull no – 533 (n=7)	$45.42^{a} \pm 2.14$	$54.71^{b} \pm 2.59$
Bull no – 531 (n=7)	$49.42^{a} \pm 3.25$	$59.57^{b} \pm 2.92$
Overall (n = 28)	$45.78^{a} \pm 1.38$	$53.53^{b} \pm 1.71$

Means bearing different superscripts in rows differ significantly ($p<0.05$)

Table 4: Evaluation of viability of spermatozoa by two staining methods at pre freeze stages (Mean ± SEM)

Pre freeze Stage of Evaluation (Viability %)		
Bull	**Eosin - ve Staining**	**PI -ve Staining**
Bull no – 623 (n=7)	$67.14^{a} \pm 3.05$	$68.28^{b} \pm 3.66$
Bull no - 580 (n=7)	$52.28^{a} \pm 2.67$	$55.42^{b} \pm 2.89$
Bull no – 533 (n=7)	$67.57^{a} \pm 1.52$	$70.71^{b} \pm 1.94$
Bull no – 531 (n=7)	$62.85^{a} \pm 3.96$	$64.14^{b} \pm 4.41$
Overall (n = 28)	$62.46^{a} \pm 1.82$	$64.64^{b} \pm 1.93$

Means bearing different superscripts in rows differ significantly ($p<0.05$)

Table 5: Evaluation of viability of spermatozoa by two staining methods at post thaw stages (Mean ± SEM)

Post thaw Stage of Evaluation (Viability %)		
Bull	**Eosin - ve Staining**	**PI -ve Staining**
Bull no – 623 (n=7)	$53.57^{a} \pm 2.02$	$55.28^{b} \pm 1.71$
Bull no - 580 (n=7)	$45.28^{a} \pm 3.01$	$48.85^{b} \pm 2.72$
Bull no – 533 (n=7)	$55.42^{a} \pm 1.73$	$56.71^{b} \pm 1.60$
Bull no – 531 (n=7)	$59.14^{a} \pm 3.34$	$60.00^{b} \pm 3.46$
Overall (n = 28)	$53.35^{a} \pm 1.57$	$55.21^{b} \pm 1.41$

Means bearing different superscripts in rows differ significantly ($p<0.05$)

The purpose of Experiment 1 was to compare two most commonly used methods of evaluating acrosomal integrity of spermatozoa such as cytochemical method - Giemsa staining and fluorescent technique using FITC-PSA. Giemsa staining is better over other cytochemical staining methods thus routinely used. (Jankovicova *et al*., 2008; Kovacs and Foote, 1992). However in recent time fluorescent staining using lectins such as PSA conjugated with FITC became more popular. FITC-PSA labeled staining technique is rapid and presence of yolk is not a limiting factor for this technique but it need advanced microscopy i.e.

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epifluorescent microscope at the same time sperm morphology remains unclear, and fluorescence fades with time (Chowdhury *et al*., 2014).

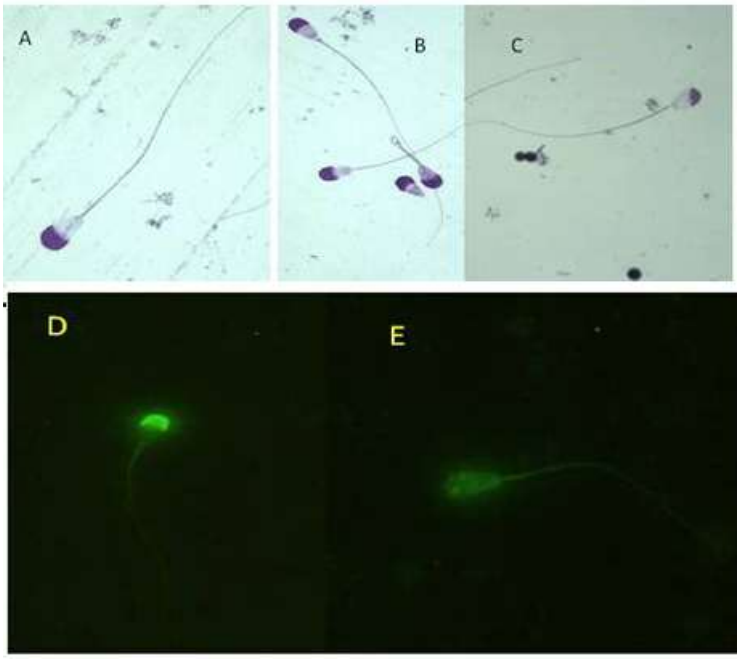

Fig. 1: Photomicrograph in showing (A) intact acrosome, (B & C) various degree of acrosomal damage as shown by Giemsa staining whereas (D) intact and (E) damaged acrosome observed under fluorescent staining using FITC-PSA.

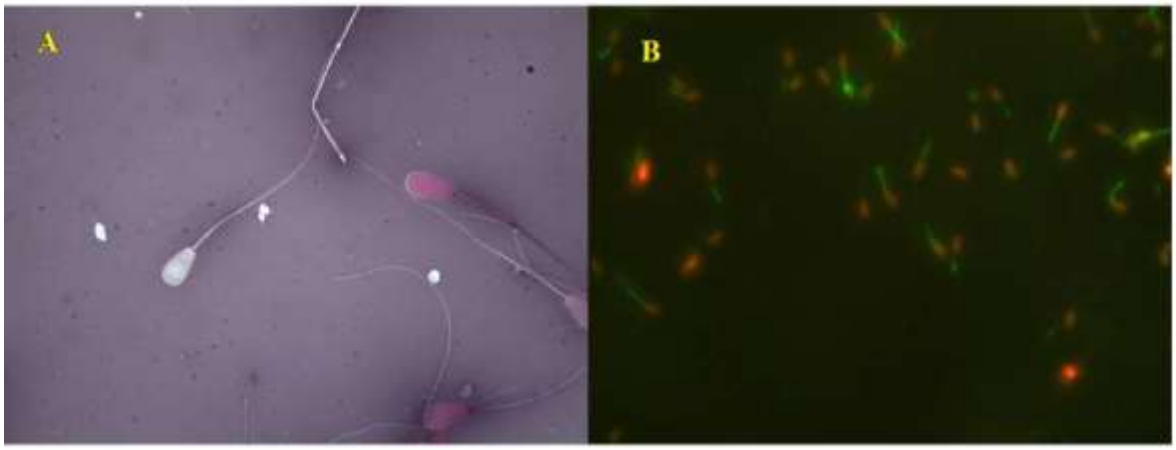

Fig. 2: Photomicrograph showing (A) viable spermatozoa unstained (white) and dead as pink stained spermatozoa whereas (B) showing dead spermatozoa with red (fluorescence) stained nucleus (head piece)

The number of spermatozoa with intact acrosome as detected by FITC-PSA was more than those identified by Giemsa staining in both stages of evaluation i.e. pre-freeze and post-thaw. Therefore, our result revealed that the fluorescent probes are more sensitive indicators of spermatozoa damage than phase-contrast assessment of acrosomal status using any cytochemical method including Giemsa staining (Harrison and

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Vicker, 1990; Boccia *et al.*, 2007; Ferlin *et al.*, 1992). The difference between these two methods can be attributed to their principle of staining acrosome region. Giemsa binds with outer acrosomal membrane but does not bind to the acrosome-reacted spermatozoa (Chowdhury *et al*., 2014). Intact acrosome appears with straight posterior boundary whereas loose or damaged acrosome seems like an arch (Hancock, 1951). PSA binds with glycoconjugates of acrosomal matrix (Cross and Maizel, 1989). It has similitude for terminal α-D glucosyl and α-D mannosyl residue of glycoprotein and binds with sugar moiety α-mannoside present in acrosome as described by Cross and Maizel (1989). Therefore use of fluorescent probes indicates clear edge over conventional stains as fluorescent probes provide better contrast between intact and damaged acrosome of a spermatozoa (Cross and Meizel, 1989).

In continuation to above, Experiment 2 compares another two most widely used methods for evaluation of viability of spermatozoa i.e. Eosin-Nigrosin and Annexin/PI method. In which later stands better due to higher percentage of viable spermatozoa observed using fluorescent stain at pre freeze and post thaw stages as compare to eosin, which is in agreement with previous similar studies (Centola *et al.*, 1990; Tamuli and Watson, 1994; Cassinello *et al.*, 1998 and Pintado *et al.*, 2000). It is not well understood why there are differences in the ability of these stains to stain spermatozoa. However, Harrison and Vickers, (1990) explained that higher no of cells stained by PI could be due to longer incubation period and artifact which later incorporated in fluorescent probe but in our study we avoided this problem by applying shorter incubation period (15 minutes) but still reported difference between two methods. In spite of fact that Eosin-Nigrosin method is yielding lower values in term of viability, still it is well adopted method due to its simple, rapid economical procedure which requires phase contrast microscopy. Furthermore, entire procedure can be carried out in a basic biological lab even in front of a light source whereas fluorescent probe need advance molecular laboratory with epifluorescent microscopy moreover major part of procedure have to be performed in dark.

Conclusion

Result of both experiments express the higher sensitivity of fluorescent method over conventional methods. However, it is a well-known fact that cytochemical methods are very economical, rapid and easy to perform in a semen lab with basic equipment i.e. light/contrast microscope moreover fluorescent method need expensive reagent along with advanced microscope i.e. epifluorescent microscope. More sensitive fluorescent probes can be the choice for researchers working in semen biology labs.

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