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Sperm Viability and Acrosome Integrity by Conventional and Fluorescent Staining Techniques Recovered in Three Different Buffers from Sheep Epididymal Tail- A Preliminary Study

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ABSTRACT

The study was carried out to evaluate the accuracy of conventional and fluorescence based staining techniques for viability and acrosome integrity of cauda epididymal spermatozoa in sheep. Spermatozoa were recovered from fresh epididymis of testicles collected from local slaughter houses. Spermatozoa were recovered in seminal plasma (SP), Tyrode's Albumin Lactate Pyruvate (TALP) and Tris Methyl Aminomethane (TRIS) buffer. The recovered spermatozoa were evaluated immediately and stored at 4 °C for subsequent evaluation up to 72 hours. Sperm viability and acrosome integrity were assessed at 0, 24, 48 and 72 hours of recovery, using Eosin-Nigrosin and Carboxyfluorescein Diacetate (CFDA) plus Propidium Iodate (PI) for viability while Giemsa and Fluorescein Isothiocyanate-Peanut Agglutinin (FITC-PNA) plus PI for acrosome integrity. Upon general linear model statistics, the interactions viz; time x technique and buffer x time x technique were significant when TALP recovered spermatozoa were evaluated for viability and acrosome integrity, and when TRIS recovered spermatozoa were evaluated for acrosome integrity. By comparing the results at different periods, it was observed that the percentage values of both viability and acrosome integrity in SP recovered spermatozoa were significantly higher ($p<0.05$) when evaluated through conventional techniques. Compared to spermatozoa recovered in TALP and TRIS, those recovered in seminal plasma showed higher ($p<0.05$) sperm viability and acrosome integrity. Although the number of competent spermatozoa (possessing more percentage viability and acrosome integrity) were more in conventional staining methods, different classes of viable spermatozoa in all the replicates can be detected and better evaluated using fluorescent probes. In conclusion, fluorescent techniques proved more accurate and sensitive for evaluation of cauda epididymal spermatozoa in ram.

Key words: Acrosome integrity, conventional, fluorescent probes, recovery buffer, sperm

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INTRODUCTION

Interest in accurate evaluation of spermatozoa's membrane and acrosome integrity, for selection of quality samples for subsequent semen cryopreservation in ram is growing. The conventional stains like Eosin Nigrosin and Giemsa stains, used for evaluation of sperm viability and acrosome integrity of fresh and stored semen are subjective in nature. Moreover, the results estimated are based on observation of less number of spermatozoa in a smear. This raises questions of accuracy of these techniques used in the decades of literature. At the same time, accurate semen evaluation in fresh and stored samples is extremely important to assess decrease in fertility with time (Bakst *et al.,* 1991). The fluorescent probe-based semen evaluation techniques play an important role in detecting sperm damage, reflecting spermatozoa's fertility, infertility and integrity (Farah *et al.,* 2013). For the above-mentioned reasons, there is growing demand for standardization of new fluorescent techniques for sperm membrane and acrosome integrity evaluation. It is expected that detection of sperm damage can be more accurately possible by fluorescent staining techniques. Due to some shortfalls like being more laborious and costly and need standardization for clinical outcomes the applicability of these techniques are less.

More accuracy using fluorescence techniques in place of conventional ones is not fully validated. These dyes being more costly and involving centrifugation are not afforded by routine institutional and field laboratories for evaluation of semen and are supposed to incur physical damages to spermatozoa. Fluorescent techniques have been recommended in the literature for differentiation of different populations of spermatozoa in ejaculated and/or cauda recovered sperm samples. Worldwide, the goal of semen researchers remains greater accuracy, higher objectivity and repeatability of laboratory tests for fast and better spermatozoa evaluation.

Very few studies are documented regarding more accuracy and reliability of fluorescent probes over conventional semen evaluation methods. As per available literature, assessment of *in-vitro* fertility of breeding bulls has been reported as more accurate on the basis of fluorescent probe-based evaluation of membrane integrity and acrosome integrity. But, some recent studies do not agree with Shivhare *et al.* (2015), while comparing conventional and fluorescent microscopic technique to assess *in-vitro* semen quality status of Murrah buffalo males suggested that mare evaluation of mass activity, individual motility and HOST can be fairly used in absence of fluorescent method for ranking of bulls. This warrants further evaluation of accuracy of fluorescent techniques over conventional ones, especially for evaluation of membrane and acrosome integrity.

Some studies suggest fluorescent technique as more sensitive techniques for sperm viability assessment compared to conventional Eosin-Nigrosin staining, owing to higher percentage of live spermatozoa (Centola *et al.,* 1990; Tamuli and Watson, 1994; Cassinello *et al.,* 1998 and Pintado *et al.,* 2000). However, Brio *et al.* (2003), reported that conventional techniques overestimate the fluorescent techniques, as the percentage of sperm viability were found greater in conventional techniques. As far as acrosome integrity is concerned, people used FITC-PNA+PI combination probes to identify different classes of spermatozoa, categorized on the basis staining property of acrosome. Cross and Meizel (1989) have long back assumed fluorescent probes as clear edge over conventional stains due to better contrast between intact and damaged acrosome of a spermatozoa in the former technique**.** Using these assumptions, spermatozoa have been classified differently by different authors while using the same technique and methodology in different labs (although some lab-to-lab modifications are reported). Classifications have been carried out based on spermatozoa's ability to bind no, little, intermediate or altered pattern with lectin. While using FITC-PNA or FITC-PNA in combination with PI for semen evaluation in man, poultry, boar, ram and bull spermatozoa showing uniform green fluorescence of acrosome cap are designated as acrosome reacted and those bearing no stain as acrosome-intact cells (Almadaly *et al.,* 2012; Cross *et al.,* 1986, Cross and Meizel, 1989; Harayama *et al.,* 2010; Siciliano *et al.,* 2008).

Celeghini *et al. (*2007), Carvalho *et al. (*2010), Hossain *et al. (*2011) Standerholen *et al. (*2014) and Sapanidou *et al.* (2015) have done tremendous job for evaluation of functional aspects of spermatozoa. It is well established in the literature that combinations of various parameters help to accurately estimate the fertilization potential of a sperm sample (Rodríguez-Martínez, 2006; Pena, 2018). Sperm membrane and acrosome integrities determine some important sperm functional attributes like its metabolism, capacitation, binding with ovum and finally sperm acrosome reaction.

Thus, in the light of above, an experiment was conducted to predict sperm viability and acrosome integrity by conventional and fluorescent staining techniques recovered in three different buffers from sheep epididymal tail. This was performed to observe wellness of techniques for assessing spermatozoa's fertilizing ability and plasma membrane integrity. Sperm viability and acrosome integrity have been estimated mostly by conventional techniques like Giemsa and Eosin-Nigrosin. No comprehensive study

of fluorescence- based evaluation of membrane integrity and its correlation with sperm quality has been documented in sheep so far. Fluorescence microscopy using CFDA+PI and FITC-PNA+PI fluorescent probes can be a breakthrough in accurate evaluation of functional capability of spermatozoa in sheep. The present study was therefore designed to standardize and use fluorescent probes- CFDA+PI and FITC-PNA and compare/ correlate these techniques with conventional stains for evaluation of viability and acrosome integrity of spermatozoa recovered from cauda epididymis of sheep using different buffers for recovery.

Keeping the above discrepancies in the cited literature in view, the present study was designed to compare and correlate the results of conventional and new fluorescent techniques for sperm viability and acrosome integrity using sheep cauda epididymal sperms, recovered in three different buffers-seminal plasma, TALP and TRIS. The study also grouped different classes of spermatozoa to identify sperm viability and acrosome integrity assays to be adapted by semen evaluation laboratories.

MATERIALS AND METHODS

This study was carried out in the sperm biology laboratory of the Division of Animal Reproduction Gynecology and Obstetrics, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir. Testicles were collected from local slaughterhouses and transported to the laboratory at 4°C (Lone *et al.,* 2012). Epididymal tails were separated and dipped in small petri dishes, containing 3 ml of buffer- seminal plasma, TALP or TRIS, at 37 °C in an incubator. Spermatozoa were recovered to an average concentration of 2600 million per ml of buffer, by keeping tails dipped for 20 minutes. Cauda epididymis containing highly motile spermatozoa were selected for the study. The spermatozoa in all the three recovery buffers were evaluated at 0, 24, 48 and 72 hours of storage at refrigeration temperature 4-5°C Spermatozoa in each replicate and each buffer were evaluated for viability and acrosome integrity, using conventional and new fluorescence-based techniques.

Conventional staining techniques

Eosin-Nigrosin and Giemsa stains were used for viability and acrosome integrity evaluation. For viability estimation, 10 µl of semen sample was added to 10 µl of Eosin-Nigrosin stain at 37°C in 0.5ml microcentrifuge tubes and allowed to mix by gentle air inflation for 30 seconds. 5 µl of this sperm stain mixture was spread on a slide to make thin smears. The smears prepared were allowed to air dry for 10 minutes and examined immediately for calculation of live percentage by counting total 200 spermatozoa in 5-6 fields, under phase contrast microscope using 100X oil immersion objective. Stained spermatozoa were considered dead while non-stained to very light stained cells were considered live.

For Giemsa staining, thin smear was made out of 5µl of semen sample, allowed to air dry and put in Hancock's fixative for 15 minutes. The slide was washed for 20 minutes and put in Giemsa solution (prepared by mixing 3ml of Giemsa stain with 2 ml of Sorensen buffer followed by adding 35 ml of distilled water). Smears were kept overnight in Giemsa solution and examined after air drying. Intact acrosome percentage was calculated by counting 200 spermatozoa in 5-6 fields under phase contrast microscope, using 100X oil immersion objective.

Fluorescent staining techniques

Sperm viability was evaluated under dark environments using CFDA and PI combination probes and observed under fluorescent microscopy, as per the method of Harrison and Vickers (1990). For this, semen sample from each buffer was washed twice with TALP, using centrifugation @ 800g for 5 minutes at 37°C . To 40µL of suspension in the 1.5mL Eppendorf tube, 15 µL CFDA (0.5mg/mL) was added under a dark environment and incubated at 37°C for 15 minutes. Then 2µL PI (0.3mg/mL) was added to the mixture and incubated again for 5 minutes. To this mixture, 100µL of TALP was added for washing again at 800g for 5 minutes at 37°C . Supernatant was discarded and smear was prepared on a grease free slide, using 10µL of sediment. Air dried smears were examined under fluorescent microscope, connected with a Magvision software installed computer, through Magcam MU2A with 2.3 MP 1/1.19" CMOS sensor (Magnus, India). Spermatozoa were observed using fluorescence with blue (excitation wavelength of 480 nm) and green filters (excitation wavelength of 535 nm). Sperm cells appearing brilliant green emit green fluorescence under blue filter and disappearing at green filter were considered as live with intact membrane and those emitting orange to red fluorescence at blue and appearing completely or partially red at green filter were considered dead with damaged membrane (Fig.1). More than 400 sperm were counted in different fields (10-12) and viability percentage of ejaculate was determined. Further, for their long-term storage, DABCO was spread

on the dried smear as an antifoaming agent and a coverslip (22 x 40 mm) was placed.

The acrosome integrity of spermatozoa was performed under dark environment using FITC and PI combination probes and observed fluorescence microscopy, as per the method of Singh *et al* (2016). For this, semen sample was washed twice with TALP, using centrifugation @ 800g for 5 minutes at 37°C . To 40µL of suspension in the 1.5mL Eppendorf tube, 15 µL FITC-PNA (0.04mg/mL) was added under darkened conditions and incubated at 37°C for 15 minutes. Then 2µL PI (0.3mg/mL) was added to the mixture and incubated again for 5 minutes. To this mixture, 100µL of TALP was added for washing again at 800g for 5 minutes at 37°C. Supernatant was discarded and smear was prepared on a grease free slide, using 10µL of sediment. Smear after air drying was examined under fluorescent microscope. For long term storage DABCO was added on smear as an antifoaming agent. This way, spermatozoa were evaluated by observing at least 10-12 fields/400 spermatozoa for percentage of acrosome intact, acrosome reacted, dead acrosome intact and dead acrosome reacted sperms. The photographs of spermatozoa from fluorescent microscope (Olympus, Tokyo, Japan) examinations were taken using Magvision software and phase contrast microscope.

Statistical analysis

Statistical analysis was carried out with 2-way ANOVA, using the General Linear Model (IBM SPSS statistical description). The percentage values obtained at every point of time were analyzed for transformed values against each. Two techniques for each variable were compared and the interaction between technique and time as well as recovery buffer, technique and time was analyzed. Interaction was considered significant at $p<0.05$. Also, correlations between techniques for each parameter were calculated through Pearson correlation coefficient with significant correlation at $p<0.05$.

RESULTS AND DISCUSSION

The results in terms of percentage and transformed values of cauda epididymal spermatozoa are presented in tables 1a, 1b and 1c for viability and 2a, 2b and 2c for acrosome integrity. Viability differs significantly between the techniques, when spermatozoa were recovered in the TRIS buffer. However, viability significantly decreased with time, when recovery buffers were seminal plasma and TRIS. In TALP recovered spermatozoa, interaction between buffer, time and technique, as observed in statistical analysis of data, existed. As far as acrosome integrity is concerned, analyzed data reveals a significant difference in the values, between the techniques, and the values significantly decreased with the passage of time, when spermatozoa were recovered by seminal plasma. However, interaction between time and technique, buffer and technique as well as buffer x time x technique was significant.

Table 1a: Viability of epididymal spermatozoa using Eosin Nigrosin (EN) staining and Carboxy-Fluorescein Diacetate plus Propidium Iodide florescent (CFDA+PI) stain recovered in seminal plasma

Buffer	Technique	Time in hours				Overall	P value
		0	24	48	72		
Seminal plasma	EN	83.54±1.96 (9.13±0.10)	81.90±1.96 (9.04±0.14)	72.41±1.92 (8.50±0.11)	59.56±6.32 (7.71±0.40)	74.35±10.38 (8.60±0.10)	0.08
	CFDA+PI	81.88±3.14 (9.04±0.17)	74.06±4.89 (8.60±0.28)	66.16±4.05 (8.13±0.24)	63.02±5.86 (7.93±0.36)	71.28±8.60 (8.42±0.51)	
	Overall	82.71±2.58 (9.09±0.14)	77.98±5.53 (8.82±0.31)	69.29±4.45 (8.32±0.27)	61.29±5.94 (7.82±0.37)		
P Value		≤0.0001					

Values in parenthesis represent the transformed values

Table 1b: Viability of epididymal spermatozoa using EN staining and CFDA+PI stain, recovered TALP

Buffer	Technique	Time in hours				Overall
		0	24	48	72	
TALP	EN	88.41±3.61 (9.40±0.19)	83.25±2.95 (9.12±0.16)	73.98±1.42 (8.60±0.08)	67.08±3.40 (8.18±0.20)	78.18±8.91 (8.82±0.50)
	CFDA+PI	78.30±2.07 (8.84±0.11)	80.27±4.11 (8.00±0.45)	68.34±4.35 (7.45±0.34)	61.77±5.38 (6.72±0.17)	60.86±13.18 (7.75±0.84)
	Overall	83.35±6.06 (9.12±0.33)	73.74±11.37 (8.56±0.67)	64.81±10.40 (8.02±0.65)	56.18±11.97 (7.45±0.80)	

Values in parenthesis represent the transformed values; (Interaction significant, P Value =0.029)

Table 1c: Viability of epididymal spermatozoa using EN staining and CFDA+PI stain, recovered in TRIS

Buffer	Technique	Time in hours				Overall	P Value
		0	24	48	72		
TRIS	EN	88.41±3.61 (9.40±0.19)	83.25±2.95 (9.12±0.16)	73.98±1.42 (8.60±0.08)	67.08±3.40 (8.18±0.20)	79.62±7.41 (8.91±0.41)	≤0.0001
	CFDA+PI	78.30±2.07 (8.84±0.11)	80.27±4.11 (8.00±0.45)	68.34±4.35 (7.45±0.34)	61.77±5.38 (6.72±0.17)	67.59±11.55 (8.19±0.71)	
	Overall	85.09±4.62 (9.22±0.25)	77.96±5.73 (8.82±0.32)	68.31±9.62 (8.24±0.58)	63.06±9.54 (7.91±0.63)		
P Value		≤0.0001					

Values in parenthesis represent the transformed values

Table 2a: Acrosome Integrity of epididymal spermatozoa using Giemsa Staining (GS) and Fluorescein Isothiocyanate-Peanut Agglutinin plus Propidium Iodide fluorescence (FITC-PNA+PI) stain, recovered in seminal plasma

Buffer	Technique	Time in hours				Overall	P value
		0	24	48	72		
Seminal plasma	GS	92.11±2.68 (9.50±0.13)	82.17±2.49 (9.06±0.13)	74.91±1.82 (8.65±0.10)	67.38±3.18 (8.20±0.19)	79.14±9.71 (8.88±0.54)	≤0.0001
	FITC-PNA-+PI	76.25±5.73 (8.72±0.32)	70.99±4.01 (8.42±0.23)	66.13±3.75 (8.13±0.23)	64.07±5.44 (7.99±0.33)	69.36±6.49 (8.32±0.38)	
	Overall	84.18±9.43 (9.16 ±0.51)	76.58±6.72 (8.74±0.38)	70.52±5.42 (8.39±0.32)	65.72±5.42 (8.10±0.27)		
P value		≤0.0001					

Values in parenthesis represent the transformed values; (Interaction is non-significant)

Table 2b: Acrosome Integrity of epididymal spermatozoa using GS and FITC-PNA+PI stain, recovered in TALP

Buffer	Technique	Time in hours				Overall
		0	24	48	72	
TALP	GS	85.63±3.00 (9.25±0.16)	77.55±3.00 (8.80±0.13)	69.88±4.24 (8.35±0.25)	59.29±2.98 (7.69±0.19)	73.09±10.44 (8.52±0.61)
	FITC-PNA-+PI	80.94±2.06 (8.99±0.11)	74.74±2.43 (8.40±0.26)	56.18±3.28 (7.49±0.21)	46.91±3.47 (6.84±0.25)	62.19±13.28 (7.84±0.83)
	Overall	83.28±3.46 (9.12±0.18)	71.15±7.51 (8.42±0.45)	63.03±8.11 (7.92±0.51)	53.10±7.26 (7.27±0.50)	

Values in parenthesis represent the transformed values; (Interaction is significant, P value =0.03)

Table 2c: Acrosome Integrity of epididymal spermatozoa using GS and FITC-PNA+PI stain, recovered in TRIS

Buffer	Technique	Time in hours				Overall
		0	24	48	72	
TRIS	GS	88.68±2.55 (9.41±0.13)	82.87±2.25 (9.10±0.12)	77.06±1.82 (8.77±0.10)	69.87±1.03 (8.35±0.06)	74.16±10.58 (8.59±0.62)
	FITC-PNA+PI	81.50±2.99 (9.02±0.16)	73.05±2.72 (8.54±0.16)	59.56±2.93 (7.71±0.19)	56.24±9.35 (7.47±0.64)	66.34±12.72 (8.10±0.79)
	Overall	83.48±3.66 (9.13±0.24)	77.08±5.05 (8.77±0.28)	64.46±5.24 (8.23±0.46)	55.99±8.13 (7.49±0.54)	

Values in parenthesis represent the transformed values; (Interaction significant, P value =0.023)

Fluorescent microscopy based viability and acrosome integrity of the samples recovered in seminal plasma exhibited different classes of spermatozoa based on their fluorescence pattern. The results of fluorescent probe-based evaluation of cauda epididymal spermatozoa immediately after recovery indicate that viable sperm population using CFDA+PI technique consisted of membrane intact i.e., 62.67% (fluorescence as uniformly green cells) and the rest percentage consisted of 12% moribund (yellow or small red regions) and 25.33 % completely dead population (orange to uniformly red cells). Different populations of spermatozoa, based on acrosome integrity using FITC-PNA+PI probe under fluorescent microscope, consisted of 53.55% live spermatozoa with intact acrosome (appeared as colorless heads in blue filter and red in green filter), 12.38% live reacted (bright uniformly green acrosomal cap/ green boundary of apical portion with colorless to slightly green posterior head region), 16.96 % dead acrosome intact (completely red acrosome with no green tinge in apical region or apical border) and 17.11% dead acrosome reacted (red acrosome with bright green apical portion). When similar observations were recorded for samples recovered in TALP and TRIS, the percentages of viable, moribund and completely dead populations were 61.33, 14.24 and 24.43%, respectively for TALP and 61.73, 13.33 and 24.94%, respectively for TRIS. The live percentage population values showed significant decrease at 24, 48 and 72 hours of storage at refrigeration ($p<0.05$). The similar trend was observed in the data generated for all times in the conventional techniques of semen evaluation. The figures 1, 2, 3 and 4 represent all the classes of spermatozoa observed in the staining techniques.

The correlations between the conventional and fluorescent techniques for both the parameters were positive and statistically significant ($p<0.05$). The various correlations are presented in table 3.In the present study we used CFDA and PI combination probes to assess viability of sheep cauda epididymal spermatozoa. Using seminal plasma as recovery buffer, we observed that live spermatozoa percentage was not significantly different between the techniques during the entire storage period. However, there was a significant decrease in the percentage with the passage of time. Normally it has been observed that a higher percentage of live sperm populations are observed in conventional stains. For viability by Eosin-Nigrosin staining method (Hancock, 1951), spermatozoa were categorized as stained (dead) and very light to unstained (Live) in different fields to estimate live percentage in each replicate. However, use of CFDA + PI has already been reported to identify live, membrane damaged/ dead, and a transitional population (Bayyari *et al.,* 1990).

Fig. 1: Epididymal tail sperm viability using Eosine-Nigrocine staining

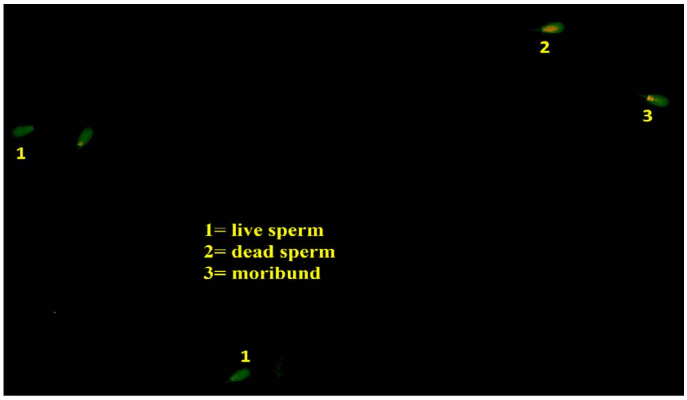

Fig. 2: Epididymal tail sperm viability using CFDA + PI fluorescent probe

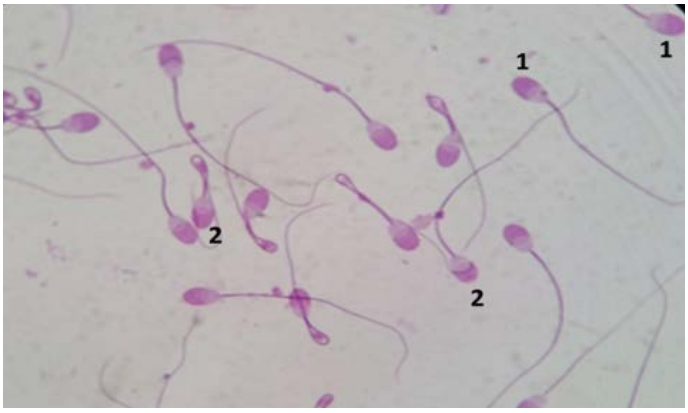

Fig. 3: Epididymal tail sperm viability using Giemsa staining
1- Intact acrosome; 2- Damaged acrosome

Fig. 4: Epididymal tail sperm viability using FITC-PNA + PI fluorescent probe

Table 3: Intra and Inter correlation between fluorescence and conventional techniques for acrosome integrity and viability of sheep cauda epididymal spermatozoa

Recovery buffer	Correlation between	r value	Significance
Seminal plasma	AI (Fluorescent and conventional)	0.40	p<0.05
	V (Fluorescent and conventional)	0.64	
	AI and V (Fluorescent)	0.50	p=0.092
	AI and V (Conventional)	0.46	
TALP	AI (Fluorescent and conventional)	0.38	p<0.05
	V (Fluorescent and conventional)	0.65	
	AI and V (Fluorescent)	0.71	p=0.12
	AI and V (Conventional)	0.80	
TRIS	AI (Fluorescent and conventional)	0.52	p<0.05
	V (Fluorescent and conventional)	0.67	
	AI and V (Fluorescent)	0.73	p=0.21
	AI and V (Conventional)	0.63	

In general, in all the three recovery buffers, we observed a significantly lower viable sperm percentage in fluorescence based methods. This lower percentage of cells can easily be attributed to inclusion of lightly stained cells in the conventional stain in the live population pool, but these cells might not have retained fluorescent stain and were thus included in the dead population in the latter technique. The other reason for less percentage could be attributed to the impact of centrifugal force in the fluorescence technique. Washing by centrifugation has been associated with sperm membrane damage. Moreover, PI used in this technique, after entering into membrane damaged cells, binds to nucleic acids- DNA and RNA by intercalating between the bases. Binding with nucleic acids, its fluorescence is enhanced 20- to 30-fold and emits red fluorescence (Arndt and Jovin, 1989). This can better differentiate the dead population.

While using CFDA+PI combination probes (Garner *et al.,* 1986; Harrison and Vickers, 1990), live sperm, dead sperm, moribund and spermatozoa with damaged membrane were categorized based on varying degrees of green and red staining. The spermatozoa that retained the CFDA green fluorescence were considered live with intact membrane, while those which did not retain the green fluorescence and stained red with PI were considered dead populations with damaged membrane (All the sperm populations are shown in figure 1 and 2). Sperm viability stains have been used in combination with some traditional dead cell stains such as PI and ethidium dimers (EthD-1 and EthD-2), for identifying dead or moribund spermatozoa (Garner *et al.,* 1997).

Retention of dye which later emits green fluorescence at blue filter and adjustments in the fluorescence (increase/decrease control knob) is correlated with mechanism described long back by Garner *et al.,* 1986 wherein authors describe that CFDA enters plasma membrane and undergoes an enzymatic reaction inside the cell (hydrolyzed by an esterase to release free 6-Carboxyfluorescein). 6-Carboxyfluorescein acts as an impermeable fluorescent probe, retained within the cells and emits green fluorescence. Emission of green fluorescence using fluorescent microscopy could be well distinguished between 405-800 nm ultraviolet and 800-1200 nm infrared, using differ-

ent optical filters depending on wavelength range, which depends on the dye utilized (Lichtman and Conchello, 2005). Moreover, propidium iodide has been shown to stain nuclear chromatin as red in ram spermatozoa (Yaniz *et al.,* 2013). The percentage population of membrane intact live and membrane damaged/compromised dead spermatozoa obtained in the present study at all time periods and in each replicate were in agreement with Brito *et al.* (2003); Santos *et al.,* 2015, wherein the authors well identified two sperm populations based on fluorescence patterns. The authors published one class of sperms with only PI red staining over the head and a second class constituted by those sperms bearing a clear dual staining pattern over the head. In the same study, varying degrees of Green fluorescence over the midpiece has been associated with retention of CFDA within intact mitochondria. Furthermore, background staining, as always associated with CFDA+PI stained samples, was related to the presence of extracellular esterase. The same authors suggested that in dual -stained sperm population, PI stain spreads from the sperm neck region in the direction of the apical region, owing to more susceptibility of sperm neck to damage than other portions of the membrane. However, some other sperm populations showing intermediate staining patterns like orange or yellow with homogenous and heterogeneous patterns have not been classified accurately so far.

Giemsa staining for acrosome evaluation (Wells and Awa, 1970) was used and intact acrosome was identified as purple color with straight posterior boundary whereas loose or damaged acrosome was distinguished by pale color, swollen head and separated head. The technique was already proven quick, simple and superior over Trypan blue (Jankovicova *et al.,* 2008) and Congo red (Kovacs and Foote, 1992). At the same time, studies suggested fluorescent dye staining technique as more accurate and efficient in classifying acrosomes, based on its integrity. These techniques are becoming widely used ones to assess fresh, stored, and cryopreserved semen samples. But semen evaluation in ram with dual fluorescent stain (FITC-PNA +PI) has been performed by only a few recent studies (Santos *et al.,* 2015) which warrant its further validation. Moreover, such protocols are under continuous modifications in the worldwide laboratories. Also, similar studies have not been extensively carried out for assessment of spermatozoa recovered from epididymal tails of dead or slaughtered animals, particularly those of high genetic merit.

The sperm populations observed in the present study, using Giemsa and FITC-PNA+PI staining, are shown in figure 3 and 4. The present study used FITC-PNA +PI dual fluorescent stain to evaluate and identify different classes of spermatozoa based on acrosome status of ram spermatozoa stored for a period of 72 hours following recovery from cauda epididymis. Based on fluorescence pattern of acrosomal cap we identified different populations of spermatozoa and classified them as: Class 1 spermatozoa included live non reacted/intact acrosome population where the sperm head as well as acrosomal cap did not stain; class 2 included live reacted acrosome population where sperm head did not stain red with PI but acrosomal cap fluorescence uniformly green; Class 3 included dead reacted populations where sperm head stained red with green fluorescence on cap and class 4 included dead with intact acrosome population where sperm head stained red without any green fluorescence over its cap. The techniques of acrosome integrity, so far used in the cited studies, have utilized FITC-RCA 11, FITC-ConA, FITC-PNA and FITC-PSA (Cross *et al.,* 1986). Using all these probes along with nuclear stains like Hoechst 33258 (Cross *et al.,* 1986) and PI (Almadaly *et al.,* 2012; Harayama *et al.,* 2010; Santos *et al.,* 2015 and Siciliano *et al.,* 2008), different authors have categorized spermatozoa as live intact, dead reacted and dead intact acrosome entities. Alvarez *et al. (*2012) have classified human spermatozoa as FITC-PNA negative and PI positive as non-viable or dead sperm with intact acrosome.

Higher percentage values in conventional Giemsa stain in the present study contrasts with the past findings (Cross and Meizel, 1989; Centola *et al.,* 1990; Tamuli and Watson, 1994; Cassinello *et al.,* 1998 and Pintado *et al.,* 2000) which suggest that 1) Giemsa binding only with outer acrosomal membrane leaving acrosome-reacted spermatozoa unidentified and 2) Lectins binds glycoconjugates of acrosomal matrix and sugar moiety α-mannoside present in acrosome. This results in a clear edge of these stains over conventional stains for better contrast between intact and damaged acrosome of a spermatozoa. However, differences in the ability of these stains to stain spermatozoa have not been addressed till date.

Controversial assertions have been given in literature in classifying spermatozoa based on the pattern of green fluorescence on acrosomes by lectins. We tried to validate the techniques of viability and acrosome integrity for ram semen in our lab so as to evaluate and compare these with the routinely used cytochemical techniques. Uniformly green live cell populations and bright green caps on acrosomes in the reacted population of spermatozoa in the present study are in agreement with Brito *et al.* (2003) for viability and Santos *et al.* (2015) for acrosome integrity.

CFDA+PI-stained samples in the present study revealed three distinct populations. 1) Spermatozoa fluoresced uniformly green/bright green were assigned CFDA positive and PI negative and classified as the live popula-

tion with intact membrane, 2) light green with partially red attained or red dots at nuclear regions were assigned intermediate type of fluorescence cells and classified as non-viable/moribund population and 3) no green fluorescence and red fluorescence of head nuclear regions, assigned CFDA negative and PI positive and classified as completely dead sperms.

FITC-PNA+PI-stained samples in our experiment revealed presence of four distinct populations of spermatozoa. 1) spermatozoa exhibiting uniformly light green fluorescence or no fluorescence at acrosome were assigned FITC-PNA and PI negative and classified as live population with intact acrosomes, 2) light green fluorescence or no fluorescence on head/acrosome but bright green and distinct cap over apical portion were assigned FITC-PNA positive and PI negative and classified as live acrosome reacted population, 3) red fluorescence at head/acrosome without any green spot on apical portion were assigned negative for both FITC-PNA and PI and classified as dead with intact acrosome population and 4) red fluorescence at head/acrosome with a bright green apical portion were assigned FITC-PNA as well as PI positive and classified as dead with reacted acrosome cells. Few sperms in the present study exhibited green dots over the border of the apical region. Since this population was very low in all the replicates and were excluded from generated data.

It is well established that PNA and PSA bind specifically to glycosyl moieties released out of acrosome membrane disruptions. These lectins along with FITC display specific labeling of the acrosomal regions. Differential lectin binding abilities of spermatozoa have been reported in the available literature from 1980s till date and classification of acrosome reactions by fluorescent techniques have been diversely presented by different authors worldwide. Some important findings published in this regard will be discussed in this manuscript. Researchers ascertain that most commonly used lectins-PNA and PSA, when used along with FITC display specific labeling of the acrosomal regions. Some sperm show little or no lectin binding ability while some others show intermediate and altered patterns of lectin binding. This lectin binding is correlated in many studies with the reacted acrosomes and appears as a green cap over the acrosomal region. But many other studies display figures of acrosome reacted sperms: a shaving uniform green fluorescence on the entire head or no fluorescence on head. This remains controversial between published studies in reputed journals. Moreover, this lectin binding depends on the degree of fusion between plasma membrane and outer acrosomal membranes over the anterior sperm head. These fusions release some residual acrosomal contents-glycoconjugates- sugar Galactosyl β-1,3 N-acetyl galactosamine in acrosomal membranes of the acrosomal matrix or outer acrosomal membrane (Cross *et al.,* 1986; Mortimer *et al.,* 1986), having affinity for binding lectins. Same characteristic property of lectins has been used as a probe to visualize acrosomal integrity by Kishida *et al. (*2015), Almadaly *et al. (*2012), and Harayama *et al. (*2010). Some studies are of the opinion that the PNA lectin in this probe is specific for terminal β-galactose moieties which are exposed at acrosome reaction. Lectin binds specifically to this moiety in only acrosome-reacted sperm (Lewis *et al.,* 2012) and live sperms can thus be quantified through green fluorescence and also this has no connection with species specific acrosome morphologies (Hossain *et al.,* 2011).

A remarkable study in ram spermatozoa evaluation by Santos *et al. (*2015) classified sperms in a similar staining and fluorescence pattern using FITC-PSA and PI probes as 1) FITC-PSA as well as PI negative as the population with Intact plasma membrane and intact acrosome, 2) FITC-PSA positive and PI negative as Intact plasma membrane and altered acrosome, 3) FITC-PSA negative and PI positive as Altered plasma membrane and intact acrosome and 4) FITC-PSA as well as PI positive as altered plasma membrane and altered acrosome. Some other remarkable studies in this direction include those by Almadaly *et al. (*2012), Alvarez *et al.* (2012), Cross *et al.* (1986) and Harayama *et al.* (2010). In a recent study, Rajabi-Toustani *et al.* (2019) evaluated acrosomal status of a bull sample and categorized sperms with acrosomal caps fluorescing uniformly green as normal/intact acrosome, non-uniformly green capped as slightly disordered but intact acrosome, bright fluorescent capped as severely disordered acrosome, fluorescence on one side of acrosomal cap as a severely deformed acrosome and at the same time acrosome with almost no fluorescence was also classified as non-damaged acrosome Based on comprehensive review of studies we found neither conventional nor fluorescence based techniques able to classify sperms for accurate evaluation of semen. This puts on record uncertainty between the assays. This remains an important issue faced by researchers for identifying a more accurate evaluation technique for acrosome integrity, especially species wise evaluation. Thus, an ideal assay for acrosome status evaluation needs to be established for all species. Further comparison of conventional staining with new fluorescent stains have not been comprehensively studied to verify reliability of each so as to suggest a more economical and accurate technique for evaluation. Also, involvement of centrifugation leading to variation in the results of viability and acrosome integrity of sperm. Some authors reported centrifugation modifying both the viability and acrosome reaction assay results using fluorescent probes (Cross *et at.,* 1986). However, later Cross and

Meizel in 1989 reported no effect of washing by centrifugation using FITC-PSA. For viability evaluation, Brito *et al.* (2003) reported Eosin-Nigrosin to overestimate CFDA+PI stain. The authors found more percentage of cells viable in conventional staining. Cross *et al.* (1989) while reviewing literature suggested fluorescent techniques using FITC-PSA, FITC- PNA coupled with PI as more accurate acrosome integrity evaluation techniques which has later been proved by a number of studies in bovine (Kishida *et al.,* 2015), ovine (Santos *et al.,* 2015) and swine (Siciliano *et al.,* 2008). This is also already explained by the fact that the anterior head region of mammalian spermatozoa bears receptors for almost 12 lectins (Tamuli and Watson, 1994).

Our data suggested that the results between the techniques were positively correlated; indicating similar reliability of the techniques as far as sperm membrane integrity evaluation is concerned. Very sparse literature regarding this comparison is available. Our results are consistent with a comparison study carried out by Panmei *et al.* (2015) for ranking of bulls on the basis of sperm acrosome integrity and viability evaluation. The authors reported conventional and fluorescent based evaluation tests bare similar value in ranking of the bulls. The authors showed positive and statistically significant correlation ($p<0.01$) between acrosome integrity by FITC-PNA and conventional technique. Moreover, membrane integrity evaluation using SYBR 14-PI and acrosome integrity evaluation by FITC-PNA were both highly correlated with percent live spermatozoa. Nevertheless, Giwercman *et al.* (2003) and Sills *et al.* (2004) report negative correlation between DNA damage and DNA fragmentation index and other conventional semen parameters, such as motility, morphology, and concentration in humans. Percentage of live with intact membrane spermatozoa observed in the present study using CFDA-PI was higher than reported by Vijetha (2011) (52.6%) and Singh (2014) (67) in bull neutered semen. Also, using FITC-PNA+PI, we observed a higher percentage of live acrosome reacted spermatozoa in cauda epididymal semen of sheep compared to that reported by Sandeep (2010) in bull neutered semen.

Compared to Tris and TALP, seminal plasma recovered samples showed higher viability and acrosomal integrity in either technique which could be attributed to greater chances of spermatozoa to thrive in seminal plasma, owing to the natural environment offered to the recovered spermatozoa (Singh *et al.,* 2024). The enzyme system and antioxidant reservoir in seminal plasma could help the spermatozoa to thrive better in this buffer. This is absolutely a new finding which needs further evaluation. Thus, seminal plasma was a more efficient recovery buffer which is consistent with a recent study by Singh *et al.* (2024), wherein the authors report a good attempt for recovery of epididymal spermatozoa in seminal plasma. Moreover, the classification of sperm on the basis of identifying intact and damaged/reacted/compromised sperms more accurately was attempted.

CONCLUSION

Although sperm quality evaluation using fluorescent techniques in ram epididymal samples is more accurate, conventional criteria for plasma membrane and acrosome integrities may not be underestimated. The fluorescent techniques prove more sensitive to differentiate various populations of spermatozoa in a heterogenous semen sample.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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