



Comparative efficacy of conventional stains for evaluation of plasmalemma and acrosome integrity of buffalo spermatozoa

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The estimation of plasmalemma intactness and acrosome integrity is crucial for predicting fertility of spermatozoa (Brito *et al.* 2003). The semen quality analysis demands assessment techniques to be reliable, quick and easy to perform for routine application in semen station (Björndahl *et al.* 2003). The use of Eosin-Nigrosin (EN) and Giemsa (G) for determination of viability and acrosome integrity, respectively are the assays of choice. However, the techniques for their simultaneous evaluation should be time and labour saving. Several investigators had reported dual staining method such as Trypan blue-Giemsa (TB-G), or Eosin Nigrosin-Giemsa (EN-G) for simultaneous determination of livability and acrosome integrity/morphology (Kovács and Foote 1992, Tamuli and Watson 1994, Boccia *et al.* 2007). However, doubts remain as to accuracy of results from dual as compared to single stains. The objective of the present investigation was to compare the accuracy of single and dual staining techniques for viability, acrosome integrity or simultaneous determination of these attributes in buffalo spermatozoa.

The present study was carried out using 12 ejaculates from 3 buffalo bulls maintained at Germplasm Centre of the Institute. The animal handling and procedures in this study were performed in compliance with Institutional Animal Ethics Committee Guidelines of the institute.

All the bulls were maintained in identical feeding and management regime according to minimum standard protocol (MSP) of Government of India. The semen was collected using artificial vagina. Following collection, the semen was examined for volume, consistency, mass motility, concentration and individual progressive motility. Thereafter, each ejaculate was diluted with PBS (phosphate buffer solution) in 1:1 ratio and a drop of diluted semen was placed in 5 different slides. These slides were stained with EN, TB, G, TB-G, or EN-G, by the methods described by Mortimer (1985), Suttiyotin and Thwaites (1991),

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Watson (1975), Kovács and Foote (1992) and Tamuli and Watson (1994), respectively. Spermatozoa (200) were counted in each slide by two investigators consistently.

The values of volume, mass motility, concentration, initial progressive motility and abnormality are presented as means±SEM (Microsoft Excel 10). The Student's t-test was used for analysis of data using Graphpad Prism 6.0. A level of 5% ($P<0.05$) was considered for determining statistical significance.

The present study evaluated the staining efficiency of EN and TB probes for viability assessment, and G for acrosome integrity or EN for morphology, as compared to determination of two attributes using a combined staining protocol (EN-G and TB-G). The mean values of ejaculate volume (mL), mass motility (0 to + 5 scale), spermatozoa concentration (million/mL), initial progressive motility (%) and spermatozoa abnormality (%) were 2.58 ± 0.45 , 2.75 ± 0.30 , 1309.60 ± 115.80 , 67.5 ± 5.38 and 6.91 ± 0.70 , respectively. The mean values of results of the study were compared for any significant difference (Table 1).

Application of EN and TB stain for viability count yielded values which differed nonsignificantly. The EN is routinely used stain for viability assessment of spermatozoa (Brito *et al.* 2003). In this study, EN stain provided explicit result on viable population of spermatozoa (Fig. 1). The dye TB has ability to differentiate between live and dead sperm cells similar to EN stain. However, Suttiyotin and Thwaites (1991) reported higher concentration or greater staining time could be toxic to spermatozoa. Hence they recommended 1% concentration of TB dye with application time of 1 min to minimize the toxic effect of the stain. But recommended concentration and time yielded faintly stained spermatozoa which were difficult to discriminate between live and dead/damaged cells (Fig. 2).

Similarly, results for viability by TB-G also did not differ significantly with EN and TB; indicating that viability assay of the spermatozoa can be determined by dual staining (TB-G) without affecting results. The TB in TB-G dual stain appeared more stable and darker without any negative effect on G staining of the acrosome with brighter colors for both the viability and acrosome stains. This is in concurrence

with the observations of Sidhu *et al.* (1992), and Somfai *et al.* (2002). Further, it permitted differentiation of true acrosome reaction from degenerative acrosome loss after sperm cell death (Kovács and Foote 1992). The application of the technique is simple, useful for the simultaneous assessment of the acrosome integrity's status and viability (Fig. 3). Since the stained samples can be stored for many years it further increases the importance of TB-G stain as compared to single stain (Srivastava *et al.* 2017). In agreement, several investigators (Boccia *et al.* 2007, Serafini *et al.* 2013) reported TB-G staining protocol as a valid one for evaluation of sperm membrane integrity and viability. Though highly stained background of the TB-G stained smears may interfere with the observation of spermatozoa, it can be overcome by adjustment with duration of staining.

Contrary to this, the staining with EN-G was unsatisfactory on several counts. In the EN-G dual stained slides, the sperm cells were too lightly stained which makes the determination of live-dead unreliable. Further, intense background produced with EN makes differentiation of live from that of dead spermatozoa nearly impossible. In contrast, Tamuli and Watson (1994) found EN-G, a reliable method for determination of livability and acrosome status of spermatozoa.

The G stain was used for evaluation of acrosome integrity as a single probe, as well in combination with TB as dual stains for simultaneous assessment of viability. A non-significant difference in acrosome integrity found between

G and TB-G in this study indicated greater probability of application of dual stain. A distinct acrosome could be easily observed using the G stain (Fig. 4) which makes differentiation between acrosome intact or denuded spermatozoa effortless. This is in contrast to dull staining of the acrosome in TB-G as compared to G stain. However, as discussed earlier, the TB-G staining method permitted the assessment of livability and acrosome integrity in a single slide and hence reduced work-load and time required otherwise for preparation of separate slides for determination of two parameters. In agreement, Kovács and Foote (1992) reported similar observation in bull, boar and rabbit semen. Several investigators (Boccia *et al.* 2007, Serafini *et al.* 2013) had also reported the high efficacy of TB-G staining method and advocated to be used for semen analysis.

In conclusion, present study finds application of TB-G for evaluation of viability and acrosome integrity simultaneously as a simple, cheap and reliable method for assessment of sperm attributes in short time interval. Moreover, dual staining with TB-G is easy to perform and requires basic equipment. This technique therefore can be recommended for application in routine semen analysis.

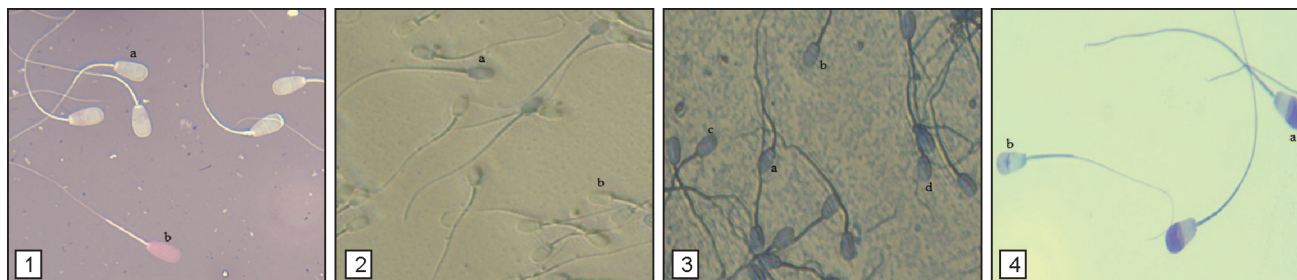
SUMMARY

The aim of the present study was to compare the efficiency of various conventional single and dual staining protocols for assessment of spermatozoa attributes. The efficiency of single stains such as Eosin-Nigrosin (EN) and

Table 1. Comparison between single and dual staining protocol for evaluation of acrosome integrity and livability of fresh buffalo bull semen

Livability (%)				Acrosome integrity (%) (Intact acrosome)						Abnormal morphology (%) Single stain	
Single stain		Dual stain		Single stain		Dual stain				EN-G	EN
EN	TB	TB-G	EN-G	G		TB-G					
				AI	AD	L-AI	L-AD	D-AI	D-AD		
82.02± 1.94	79.59± 1.32	80.50± 2.20	Obscure result	81.25± 2.79	18.75± 2.79	78.00± 2.16	2.50± 0.31	7.41± 9.41	13.00± 2.30	Obscure result	6.91± 0.70

Data are presented as means±SD. All the means for viability and acrosome integrity differs nonsignificantly. EN, Eosin Nigrosin; TB, Trypan blue; TB-G, Trypan blue-Giemsa; EN-G, Eosin Nigrosin-Giemsa; G, Giemsa; AI, Acrosome intact; AD, Acrosome denuded; L-AI, Live-acrosome intact; L-AD, Live-acrosome denuded; D-AI, Dead-acrosome intact; D-AD, dead-acrosome denuded.



Figs 1-4. 1. Buffalo spermatozoa stained with Eosin Nigrosin (a, represents live spermatozoa; b, represents dead spermatozoa); 2. Buffalo spermatozoa stained with Trypan blue stain (a, dead spermatozoa; b, live spermatozoa); 3. Buffalo spermatozoa stained with Trypan blue-Giemsa stain (a, indicates live intact-acrosome; b, live denuded-acrosome; c, dead intact-acrosome; d, dead denuded-acrosome); 4. Buffalo spermatozoa stained with Giemsa stain (a, intact acrosome; b, denuded acrosome).

Trypan blue (TB) for viability and Giemsa (G) for acrosome integrity *vis-à-vis* dual stains such as Trypan blue-Giemsa (TB-G) and Eosin Nigrosin-Giemsa (EN-G) were compared. Ejaculates (12) from 3 buffalo bulls were used for evaluation of livability and acrosome integrity using said stains. The smears stained with EN-G were difficult to visualize distinctly and provided obscure results. A nonsignificant difference was found between EN, TB and TB-G for spermatozoa viability. However, at recommended concentration, TB yielded faint images, thus making the observation unnecessarily tedious. However, staining with TB-G was found advantageous for simultaneous determination of viability and acrosome status. Moreover, the results between G and TB-G deferred nonsignificantly indicating similar effectiveness of both the stains. Overall, TB-G can be a reliable, quick and convenient method for determining viability and acrosome integrity simultaneously for routine semen evaluation.

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