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Technique to simultaneously evaluate ram sperm morphology, acrosome and membrane integrity

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Abstract

Several laboratory methods to evaluate ram sperm quality have been developed. The combination of different fluorescent probes is suitable to simultaneously evaluate different sperm characteristics but the need for a fluorescent microscope restricts its use. The aim of the present study was to evaluate the efficacy of Hypoosmotic Trypan Blue Giemsa (HTBG) staining to simultaneously detect sperm morphological abnormalities, plasma and acrosomal membrane integrity using phase contrast and fluorescence microscopy as the gold standards. Samples from twelve fresh ejaculates from three rams (4 ejaculates/ram) were used in the study. Sperm cells were evaluated using HTBG, phase contrast (PC) and fluorescent (FLUO) techniques. HTBG was more effective in detecting sperm defects when compared to PC ($P < 0.05$). No significant differences ($P > 0.05$) were observed between HTBG and FLUO in the assessment of plasmatic membrane and acrosome integrity. High correlation between HTBG and FLUO techniques was observed when assessing plasma membrane and acrosome ($R = 0.97$ and 0.96, respectively). In conclusion, the HTBG staining is suitable to assess ram sperm morphology, plasma and acrossomal membrane integrity simultaneously.

Keywords: fluorescence, giemsa, hypoosmotic swelling test, ovine, sperm, trypan blue.

Introduction

Several laboratory methods have been used to evaluate the quality of fresh or frozen/thawed semen (Cross and Meizel, 1989). In spite of the advances made within the last few years, correlations between spermatic parameters and *in vivo* fertility indexes are variable (Crespilho *et al.,* 2009). Sukardi *et al*. (1997) were pioneers in combining the fluorophore fluorescein isothiocyanate-conjugated *Pisum Sativum Agglutinin* and propidium iodate (FITC-PSA and PI) to evaluate acrosome integrity and viability in ram semen. Simultaneous evaluation of plasma and acrosome membrane integrity by using fluorescent probes with lectin/Hoechst (Valcárcel *et al*., 1997) as well as mitochondrial function with PI + FITC-PSA + JC-1 improves precision of sperm analysis, allowing to more accurately estimate the percentage of sperm cells with fertilization potential within a semen sample (Celeghine *et al.,* 2010). The combination of different fluorescent probes is suitable to simultaneously evaluate different sperm characteristics, but the need for a fluorescent microscope restricts its use.

A simple alternative to evaluate sperm plasma membrane activity is the hypoosmotic swelling (HOS) test, which is widely used in sperm from several species (Oberst *et al.,* 2003; Siqueira *et al*., 2007). In this regard, the higher the number of cells that maintain plasma membrane integrity, which is extremely important during many physiological events that occur during fertilization, the higher the sperm quality (Nie and Wezel, 2001; Oberst *et al.,* 2003). To perform the technique, sperm is subjected to a hypoosmotic solution that results in sperm tails swelling. The swelling of sperm membranes induces bending and coiling of the sperm tail, which is easily observed using a phase-contrast microscope. Swollen sperm or HOS-positive (responsive to HOS) cells have an intact plasma membrane (Jeyendran *et al.,* 1984). Siqueira *et al*. (2007) demonstrated a negative correlation between HOS and acrosome reaction tests in bovine semen, indicating that the higher number of cells positive for HOS is correlated with a lower percentage of acrosome-reacted cells, indicating plasma and acrosome membrane integrity. The fructose-citrate solution 100 mOsmol/l is designated to evaluate sperm plasma membrane functional integrity in ram and buck fresh semen in order to recognise the percentage of reactive cells (Salgueiro *et al*., 2003; Moura *et al*., 2010).

Silva *et al*. (2003) used a double Trypan Blue Giemsa (TBG) staining to evaluate membrane and acrosome integrity in bovine sperm, observing an intermediate positive correlation with oocyte cleavage *in vitro*. TBG staining was shown to be efficient to evaluate canine sperm integrity when correlated to the thermo-resistance test and fertility rates after insemination of bitches in natural estrus (Santos *et al*.,

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2007). Furthermore, Zúccari *et al.* (2008) found a high correlation between TBG and FITC-PNA/PI techniques for the assessment of live bovine sperm cells.

Based on the aforementioned facts, we hypothesized that a technique combining HOS and TBG would be suitable to detect sperm morphological abnormalities and to assess plasma and acrosomal membrane integrity simultaneously. Our aim was to compare a simple and practical technique, that could be conducted using simple equipment, such as a bright field microscope, with phase contrast and fluorescence microscopy, that require more expensive equipments or reagents.

Materials and Methods

Twelve fresh semen samples collected from three rams (once a week during 4 weeks) by using an artificial vagina during the breeding season (kindly provided by the owner), were used to evaluate sperm morphology, sperm membrane integrity and acrosomal status. To perform semen evaluations, the following methods were used: humid chamber (phase contrast-PC), supravital staining HTBG and fluorescence (PI and FITC-PSA). After semen collection, an aliquot of 20 µl was diluted in 1 ml of formol-citrate solution (2.94%). One 5 µl drop of each sample was placed in a slide, covered with a coverslip, sealed with nail polish and analysed under a phase contrast microscope. A total of 200 cells were counted in each slide. The sperm were classified according to the "Manual de Andrologia do Colégio Brasileiro de Reprodução Animal" (Colégio Brasileiro de Reprodução Animal - CBRA, 1998). Ejaculates were classified as high (>70%), intermediate (50-70%) or low (<50%) quality according to the percentage of normal cells.

Supravital staining HTBG was performed according to the methods described by Santos *et al*. (2007). Briefly, a 20 µl aliquot of semen was added to 20 µl of Trypan Blue solution 0.2% (0.5 ml bi-distilled water + 0.5 ml Trypan Blue 0.4%; Sigma T8154, USA) with an osmolality of 120 mOsm/l (Osmomat®030, Gonotec, Berlin, Germany) in a 1.5 ml microtube and incubated in a water bath at 37ºC for 60 min. Afterwards, 1 ml of bi-distilled water was added to each vial and the samples were centrifuged (700 g, 5 min) to remove excessive staining solution. The supernatant was discarded and the sample was resuspended in 0.5 ml bi-distilled water. Three smears were prepared, quickly dried in an air flow and fixed in methanol for 5 min. After drying, smears were stained with Giemsa 10% overnight. Afterwards, slides were washed in bi-distilled water and dried in a heated plate. In each slide, 200 sperm cells were counted under 1000X magnification and the percentage of cells with coiled tail (positive endosmosis) was recorded as proposed by Revell and Mrode (1994). The calculation of the number of HOS-reactive spermatozoa was performed using the formula cited by Melo and Henry (1999), where: HOS (%) = (% sperm tail alterations after HOS) - (% sperm tail alterations before HOS). Sperm tail alterations before HOS were evaluated through phase contrast microscopy. Sperm were classified according to Table 1.

Table 1.Ram sperm classified according to the staining pattern post-association of Trypan Blue (TB), Giemsa and response to the hypoosmotic solution (HO).

Sperm	TB*	Giemsa**	HO
Intact membrane, intact acrosome and coiled tail	-	+	+
Intact membrane, altered acrosome and coiled tail	-	-	+
Altered membrane, intact acrosome and non-coiled tail	+	+	-
Altered membrane, altered acrosome and non-coiled tail	+	-	-

Trypan Blue*(+): blue nuclei; Giemsa**(+): pink acrosome.

The two-color fluorescence staining using fluorescein isothiocyanate-conjugated *Pisumsativum* agglutinin and propidium iodate (FITC-PSA+PI) was performed according to the methods described by Celeghini *et al*. (2010). One 150 µl semen aliquot diluted in tyrode's albumin lactate pyruvate (TALP) sperm medium (Bavister *et al*., 1983; 25 x 10^6 sperm/ml) was placed in a 1.5 ml microtube and warmed (37°C). Then it was added to 3 µl of PI 0.5 mg/ml and 50 µl of FITC-PSA (100 µg/ml). The sample was incubated for 8 min in a dark chamber at 37°C. An 8µl sample was placed on the slide, covered with a coverslip and immediately evaluated under epifluorescence microscope (Leica, DMI4000B, Wetzlar, Germany), with the following filters: L5-BP (480/40, RKP 505, BP 527/30); N2.1-BP (515 - 560 RKP 580 LP 590). In each slide, 200 sperm cells were counted under 1000X magnification (using immersion oil) and classified according to the intensity of each probe (Table 2).

Data were tested for normal distribution and submitted to arcsine transformation when necessary. Data from rams, subunits within rams (ejaculates) and techniques (treatments) were submitted to analysis of variance (ANOVA). The percentage of normal cells, cells with intact membrane and cells with intact acrosome was compared between techniques. Plasma and acrosome integrity (dependent variable) results obtained with the different staining methods (HTBG and FLUO = independent variables) were submitted to Pearson correlation analysis. All analysis were performed using the SAS program, 1998 and the level of significance was set at $P < 0.05$.

Table 2. Ram sperm classified according to staining with fluorescent probes fluorescein isothiocyanate-conjugated *Pisum Sativum Agglutinin* (FITC-PSA) and propidium iodate (PI).

Sperm	PI*	FITC-PSA**
Intact plasma membrane and intact acrosome	-	-
Intact plasma membrane and altered acrosome	-	+
Altered plasma membrane and intact acrosome	+	-
Altered plasma membrane and altered acrosome	+	+

PI*(+): red nuclei; FITC-PSA**(+): yellow-green acrosome.

Results

Initially, the percentage of intact (normal) sperm cells was assessed using different techniques. No significant differences were observed between HTBG and FLUO (Table 3) regardless of the semen parameters (ejaculates with high, intermediate or low quality).

Regarding membrane and acrosome integrity, no significant differences were observed between FLUO and HTBG techniques (60.83 ± 12.18 *vs*. 61.50 ± 12.19 and 59.25 ± 12.32 *vs*. 59.33 ± 11.64, for plasma membrane and acrosome, respectively; Fig. 1B and 1C, respectively). When evaluating sperm morphology excluding plasma membrane alterations, PC detected more morphologically normal cells ($P < 0.03$) compared to the HTBG. Thus, PC was less effective in detecting sperm defects (Fig. 1A).

Figure 1D shows the sensibility of different techniques to detect sperm integrity. No significant differences were observed between HTBG and FLUO but both techniques differed from PC ($P < 0.02$), the PC being less effective in detecting sperm defects. Pearson correlation was applied to test the correlation between FLUO and HTBG, and the following equations were obtained: $Y=0.95+0.97X$ and $R = 0.97$ for percentage of sperm with intact plasma membrane (Fig.1E) and $Y=-1.53+1.02X$ and $R = 0.96$ (Fig.1F) for percentage of sperm with intact acrosome. These correlations demonstrated that the techniques have a similar power to detect viable spermatozoa.

The pattern of ram sperm cells stained with hypoosmotic Trypan Blue Giemsa and fluorescent probes (FITC-PSA and PI) and those submitted to phase contrast (PC) are shown in Fig. 2.

Discussion

Several techniques are used to evaluate ram sperm morphology, with phase contrast being the most commonly used during breeding soundness evaluation. Other techniques such as HOS, supravital staining and fluorescent probes that aim to evaluate plasma membrane and/or acrosome have been developed. Nevertheless, the correlation between sperm parameters and *in vivo* fertility remain variable (Crespilho *et al*., 2009).

The association of HOS and the double staining HTBG used in the present study to evaluate ram sperm morphology (major and minor defects), plasma and acrosome membranes, was proven to be suitable to detect sperm alterations. HOS is currently used to assess sperm from several domestic species, by detection of the plasma membrane activity. In the present study, it was observed that HOS-reactive sperm did not show Trypan Blue stained nuclei, demonstrating intact plasma membrane. However, HOS-negative sperm had Trypan Blue stained nuclei. These results are in accordance with previous studies where it was demonstrated that the higher the number of cells that maintain plasma membrane integrity the higher the semen quality (Jeyendran *et al*., 1984; Nie and Wenzel 2001; Oberst *et al*., 2003).

Membrane integrity is crucial to sperm fertility. In the present study, the percentage of cells with intact plasmatic and acrosomal membranes detected using FLUO and HTBG techniques did not differ. Celeghini *et al*. (2010) observed 86.79 ± 4.92 and 93.33 ± 2.89 sperm with intact plasma and acrosome membrane, respectively, in fresh ram semen. The discrepancy between previous reports and this studycan be explained by the fact that in the present study ejaculates from different standards were evaluated (Table 3).

The efficiency of FLUO and HTBG were used to evaluate plasma and acrosome membrane integrity in sperm from ejaculates of different standards. To reveal the correlation between FLUO and HTBG techniques, Pearson correlation was applied and the following equations were obtained: $Y=0.95+0.97X$ ($R=0.97$) for plasma membrane and $Y=-1.53+1.02X$ ($R=0.96$) for acrosome (Fig.1E and 1F), revealing technical similarities for detection of ram sperm lesions. This correlation is in agreement with previous results from Zúccari *et al*. (2008), which observed a high intensity correlation ($R = 0.69$; $p < 0.05$) between TBG and FITC-PNA/PI when evaluating bovine live sperm.

HTBG was proven to be efficient to evaluate sperm morphology, plasma and acrosome membrane integrity in fresh ram sperm. The afore described results corroborate with those from Silva *et al*. (2003), who, using TBG, obtained a positive correlation between acrosome membrane integrity from bovine sperm cells obtained from ejaculate or epididymis. Santos *et al*. (2007), using TBG to evaluate dog sperm viability, demonstrated that the technique is proficient when compared to the thermo-resistance test and fertility rates after insemination of bitches in natural estrus.

In the present study it was observed that HTBG could be used to assess other sperm alterations (major and minor defects) being superior to phase contrast. Furthermore, HTBG allows for the evaluation of plasma and acrosome membrane from ram sperm, being as

efficient as FLUO (Fig. 1D) with the following advantages: it is inexpensive, easy to perform, and does not depend on costly equipment, as the slides are evaluated under a simple microscope. If stored properly, the slides can be kept for long periods of time. In addition, all required reagents are easily accessible and can be kept at room temperature, allowing for use under both laboratory and field conditions. By contrast, the FLUO technique depends on a fluorescence microscope and the components (fluorophores) should be stored at 5°C or frozen and slide evaluation must be performed immediately after confection.

In conclusion, the results of this study support the association of the hypoosmotic swelling test and the double staining Trypan Blue Giemsa techniques since they can effectively evaluate ram sperm morphology and membrane and acrosome integrity simultaneously. The presented protocol can be used proficiently and comparably to more sophisticated, expensive and time-consuming techniques, such as fluorescent probes.

Table 3. Intact sperm (mean ± standard deviation) in fresh semen from three rams with different semen standards evaluated by phase contrast (PC), Hypoosmotic Trypan Blue Giemsa staining (HTBG) and Fluorescent probes (FLUO).

Technique	Ejaculates	Semen standard (mean ± standard deviation)		
		High	Intermediate	Low
PC	4	$(90.0 \pm 1.9)^{a,A}$	$(85.0 \pm 1.9)^{b,A}$	$(76.2 \pm 1.9)^{c,A}$
HTBG	4	$(75.0 \pm 2.9)^{a,B}$	$(60.2 \pm 2.9)^{b,B}$	$(49.2 \pm 2.9)^{c,B}$
FLUO	4	$(72.0 \pm 3.2)^{a,B}$	$(59.2 \pm 3.2)^{b,B}$	$(46.5 \pm 3.2)^{c,B}$

Different superscripts in the same row (^{a,b,c}) and column (^{A,B}) represent significant differences by Tukey's test ($P < 0.05$). Ejaculates were classified as high (>70%), intermediate (50-70%) or low (<50%) quality according to the percentage of normal cells.

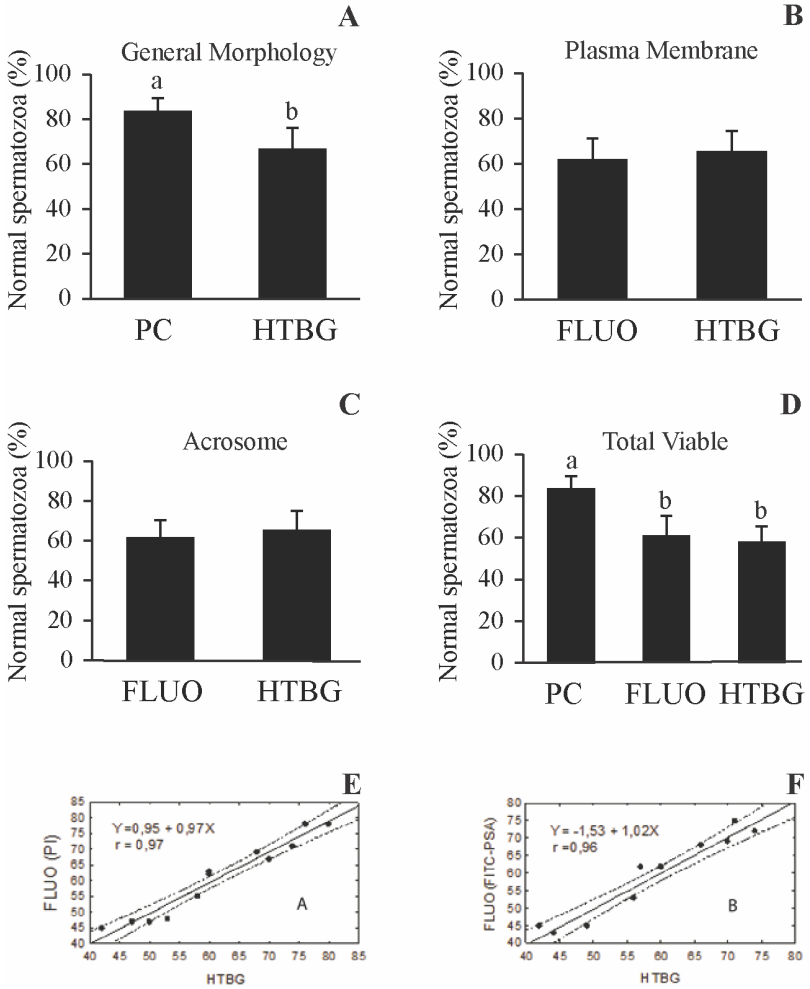

Figure 1. Viable sperm using the following techniques: phase contrast (PC), Hypoosmotic Trypan Blue Giemsa (HTBG) and Fluorescence (FLUO). Figures E and F show correlations between fluorescent techniques (FLUO PI, FLUO FITC-PSA) and Hypoosmotic Trypan Blue Giemsa (HTBG). E = spermatozoa with intact plasma membrane; F = sperm with intact acrosome.

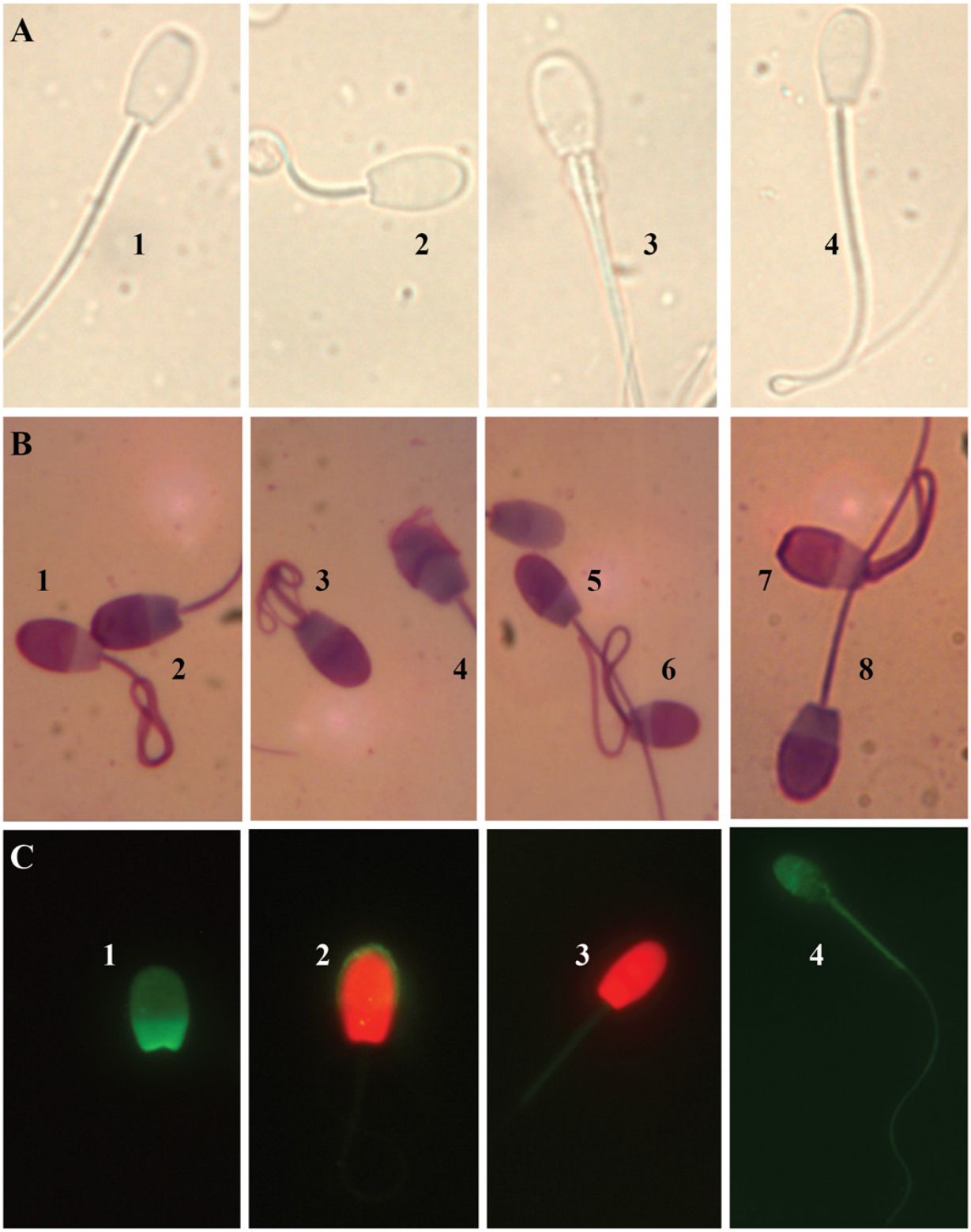

Figure 2. Ram sperm cells analysed using microscopy **A**: Phase contrast (1000X) 1 altered acrosome, 2 coiled tail, 3 altered middle piece and 4 strongly bent tail**. B**: Hypoosmotic Trypan Blue Giemsa under light microscopy (1000X) 1 intact plasma membrane (PM) and acrosome (AC) HOS-responsive, 2,4 altered PM, intact AC and tail HOS-negative, 3 Intact PM, altered AC and HOS-responsive, 5,6 Altered PM and AC and HOS-negative, 7 Altered PM and AC absence, 8 Altered PM and AC and HOS-negative. **C**: Fluorescent probes red PI and green FITC-PSA (1000X) 1 Intact PM and AC, 2 Altered PM and intact AC, 3 Altered PM and AC, 4 Intact PM and altered AC.

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