BUFFALO SPERMATOZOA CLASSIFIED BASED ON PLASMALEMMA FUNCTIONAL INTEGRITY AND ACROSOMAL INTEGRITY TESTS

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ABSTRACT

The aim of this study was to assess the plasmalemma functional integrity and acrosomal integrity and their relationship with sperm functional tests in assessing buffalo semen quality. Fifty-two ejaculates from thirteen Murrah buffalo bulls were used for the study. Plasmalemma integrity and acrosomal integrity was assessed by fluorogenic and hypoosmotic swelling- Giemsa (HOS-G) test. The samples were classified into different subpopulations based on their reaction to fluorogenic staining and HOS-G test, respectively. No difference in plasmalemma integrity (%) was observed (49.11±2.53 vs. 48.59±2.48) between fluorogenic and HOS-G tests. However, the acrosomal integrity (%) assessed by HOS-G test (82.43±4.15) was significantly higher ($P<0.05$) than fluorogenic stain (68.12±3.03). The sperm subpopulation (%) positive for plasmalemma integrity and acrosomal integrity (P^+A^+) was significantly ($P<0.05$) higher in fluorogenic (49.11±2.53) than HOS-G (43.08±2.38) tests. Further, the plasmalemma functional membrane integrity (48.59±2.48 %) was significantly higher than the sperm P^+A^+ subpopulation (43.08±2.38 %). In the P^+A^+ subpopulation, significant correlation ($r= 0.529$, $P<0.05$) was observed between fluorogenic and HOS-G test. The sperm P^+A^+ subpopulation had a significant positive correlation with the progressive forward motility (r=0.733, fluorogenic; r=0.666, HOS-G), acrosomal integrity (r=0.844, fluorogenic; r=0.569, HOS-G) and sperm nuclear morphology (r=0.617, fluorogenic; r=0.752, HOS-G). The study confirmed that fluorogenic and HOS-G tests measure the functional membrane integrity. It can be concluded that the assessing of sperm P^+A^+ subpopulation by HOS-G test is easy and simple and can be recommended as a routine test to evaluate buffalo semen quality.

Key words : Buffalo bull, Semen, Plasmalemma integrity, Acrosomal integrity

INTRODUCTION

Sperm motility, plasmalemma integrity and acrosomal integrity have inconsistent correlation with fertility in cattle and human (Brito *et al.*, 2003; Aitken, 2006). The independence of different regions of sperm plasma membrane has been reported (Perez-Llano *et al.*, 2003). During fertilization, the sperm should maintain functional membrane integrity in order to travel in the female reproductive tract. Further, maintenance of optimum fertility depends on the acrosome being structurally and biochemically intact, which contains the enzymes necessary to penetrate through the outer layers of the ovum and achieve fertilization (Jeyandran *et al.*, 1984; Prathalingam *et al.*, 2006). Hence assessing the sperm subpopulation positive for functional membrane integrity and acrosomal integrity has been suggested to be important indicators of semen fertility (Perez-Llano *et al.*, 2003). Perusal of literature reveals that no study has been reported in buffalo semen analysis.

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The aim of the present work was: 1.To compare the fluorogenic and hypoosmotic swelling-Giemsa (HOS-G) tests in assessing buffalo sperm plasmalemma functional integrity. 2. To assess the subpopulation of spermatozoa classified based on functional membrane integrity and acrosomal integrity in relation to sperm functional tests in assessing semen quality.

MATERIALS AND METHODS

Fifty-two ejaculates from thirteen Murrah buffalo bulls (four ejaculates / bull) were collected using artificial vagina. The bulls were maintained under standard managemental conditions at the semen stations, Hessarghatta, Bangalore, India. The neat semen was diluted 1:1 with egg-yolk- tris buffer at 37°C and transported to the laboratory at 3-4°C within 2 hrs. In the laboratory, the samples were kept at 37°C for 10 min and then the following parameters were studied.

Sperm progressive forward motility (%) was assessed in a drop of diluted semen using phase contrast microscope (Nikon, Eclipse E200, Japan) fitted with stage warmer (Linkam DC 60, UK). The concentration was assessed using photometer. Head, mid piece and tail abnormalities were assessed in the rose bengal stained smears by counting 200 sperm under a phase contrast oil immersion and were expressed as a percentage of the cells evaluated.

Feulgen's reaction was performed for the assessment of sperm nuclear shape. Following acid hydrolysis (5N HCl for 30 min at room temperature 20-25°C), the smear was treated with Schiff's reagent (30 min at room temperature 20-25°C). The free or unbound reagent was removed by treatment with sulfate water (3X) for 2 min each. A minimum of 200 sperm was observed in a phase-contrast oil immersion. Nuclear abnormalities studied included following types: large, small, pear shaped, narrow, round, putative diploid nuclei (broader nuclear basal zone and a deeper Feulgen colouration than normal) and vacuole.

Plasmalemma integrity and acrosomal integrity

Fluorogenic stain: Plasmalemma integrity and acrosomal integrity were assessed by fluorogenic staining (Garner *et al.*, 1986). Ten micro liters of carboxy fluorescein diacetate (CFDA) stock solution (4 mg/ml of dimethyl sulfoxide, DMSO) were added and uniformly mixed into the 100µl of semen sample. After 10 min, 10 µl of propidium iodide (PI) stock solution (0.27 mg/ml in phosphate buffer) were added, the sample was mixed, and the sperm cells were collected by centrifugation at 500 X g for 5 min. Supernatant was aspirated and the resultant pellet was resuspended in 1 ml of tris buffer. After careful mixing, a drop of sperm suspension was placed onto a clean slide and covered with a cover glass. The slides were examined using an HBO 50 mercury lamp illuminated epifluorescence microscope (Nikon Eclipse 50i, Japan) equipped with CFDA filter set (excitation, 510-560nm emission, 505nm). The samples were classified into three subpopulations based on staining. First, the individual spermatozoa, presumed to be viable, retained products of the fluorescein chromophore throughout the cell were classified into plasmalemma intact (live) acrosome positive (P^+A^+). A second population of spermatozoa, presumed to be moribund, retained the green fluorophore within the acrosome and red nuclei were classified into dead acrosome positive (P^-A^+). Third populations of spermatozoa, presumed to be degenerate, possessed brightly stained red nuclei were classified into dead acrosome negative (P^-A^-). First and second subpopulations were added together to calculate acrosome positive cells.

Hypo osmotic swelling-Giemsa (HOS-G) Test: Functional membrane integrity was assessed by hypo-osmotic swelling (HOS) test. The osmolarity of the mixture of 150 milliosmoles (7.35 g of sodium citrate - tri-sodium salt and 13.51 g of fructose in 1000 ml of ultrapure water) was used as the HOS medium (Jeyendran *et al.*, 1984). The test was performed by mixing 0.1 ml of semen sample with 1.0 ml of the HOS medium. The sperm mixture was then incubated at 37°C for 30 min. After incubation, one drop of well-mixed sample was smeared on a warm glass slide and then stained with Giemsa. A minimum of 200 spermatozoa were observed in a phase-contrast oil immersion. Spermatozoa displaying different types of swelling (coiling of tail) were considered positive for the HOS test. No swelling was considered as negative. The

spermatozoa were considered acrosome intact; when a clear acrosome cap was present or acrosomal absent if the acrosome was partially loosened or lost. The samples were classified into four subpopulations based on their reaction to HOS-G test, i.e.1) spermatozoa which were HOS positive and acrosome positive (P^+A^+), 2) HOS positive and acrosome negative (P^+A^-), 3) HOS negative and acrosome positive (P^-A^+) and 4) HOS negative and acrosome negative (P^-A^-). First and second subpopulations were added together to calculate HOS positive cells, whereas first and third subpopulations were added together to calculate acrosome positive cells.

Statistical Analysis

Individual animal's data were averaged for each parameter and the values were taken for statistical analysis. Data were expressed as mean±SEM. Differences between the tests were analyzed by student test. Pearson's correlation co-efficient (r) was used to find the degree of correlation between sperm function tests. Differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

The starting quality variables (mean ±SE) of the 13 animals used in the test were: volume (4.51±0.54 ml), concentration (1552.17±1.72 million/ml), progressive forward motility (63.27±1.72 %), normal sperm nuclear morphology (86.27±5.38 %) and sperm abnormalities (head, 1.67±0.48 %; mid piece, 0.52±0.25 %; tail, 3.74±0.54 %).

No difference in plasmalemma functional membrane integrity (%) was observed (49.112.53 vs 48.59±2.48) between fluorogenic and HOS-G tests. A significant correlation ($P<0.05$) was observed in the plasmalemma ($r=0.509$) and acrosomal integrity ($r=0.565$) measured between fluorogenic and HOS-G tests. This indicates that these tests are measures of the same attributes i.e., plasmalemma functional integrity. This is similar to the observation made earlier in bull (Brito *et al.*, 2003). Though combination of fluorogenic probes such as CFDA and PI are suggested to be useful in ascertaining sperm cell functionality, it needs sophisticated instruments. Hence, the simple hypoosmotic swelling test was combined with Giemsa staining procedure to assess functional membrane as well as acrosomal integrity. The result of this test can be read with simple microscope.

In order to predict fertility of an ejaculate assays of multiple characteristics are required on individual spermatozoa to determine their physiological state, since several attributes necessary for the fertilization and some attributes are not even known (Braundmeir and Miller, 2001; Prathalingam *et al.*, 2006).

The proportion (%) of sperm P^+A^+ subpopulation assessed by HOS-G test (43.08±2.38) was significantly lesser ($P<0.05$).than the plasmalemma functional membrane integrity (48.59±2.48), but both these parameters had significant ($P<0.05$) correlation ($r=0.688$) with each other. However, the sperm P^+A^+ subpopulation was significantly ($P<0.05$) higher in fluorogenic (49.11±2.53 %) as compared to HOS-G (43.08±2.38 %) test. Since several attributes necessary for fertilization, combination of tests is essential to predict fertility especially in subfertile animals (Amann and Hammerstedt, 1993; Braundmeir and Miller, 2001). Cell cannot be considered viable unless it also possesses an intact membrane (plasmalemma and acrosome) and functioning organelles (Brito *et al.*, 2003). Since independence of the plasma membrane regions that cover the tail and acrosome, tests measuring both these attributes are essential to predict semen quality. The presence of significantly lower sperm subpopulation of P^+A^+ than plasmalemma functional membrane integrity suggests the independence of the plasma membrane regions that cover the tail and acrosome. Further the presence of P^+A^- spermatozoa with functional membrane in the tail but with a damaged acrosome and P^-A^+ spermatozoa with an intact acrosome but with a non-functional or damaged plasma membrane in the tail confirms the independence of the plasma membrane regions surrounding the sperm cell. It could be that this subpopulation of P^+A^- could correspond to live cells that have undergone the acrosome reaction during semen processing (Perez-Llano *et al.*, 2003). Further

analysis of this subpopulation might help to evaluate the tendency for capacitation in every ejaculate. In contrast the subpopulation of P^-A^+ sperm could correspond to the population of live/moribund spermatozoa that did not react to HOS test (Perez-Llano *et al*., 2003).

The sperm P^+A^+ subpopulation had significant positive correlation with the progressive forward motility (r=0.733, fluorogenic; r=0.666, HOS-G) acrosomal integrity (r=0.844, fluorogenic; r=0.569, HOS-G) and sperm nuclear morphology (r=0.617, fluorogenic; r=0.752, HOS-G). The result suggests that the sperm P^+A^+ subpopulation reflects various sperm attributes. However, the relationship between the subpopulation of P^+A^+ sperm and fertility must be studied in order to elucidate the role of this parameter to predict fertility.

The present study confirms the earlier finding of the independence of the plasma membrane regions surrounding the sperm cell. It can be concluded that the assessment of sperm P^+A^+ subpopulation by HOS-G test will be a more accurate sperm function test than HOS test, since it measures both functional membrane integrity along with acrosomal integrity. HOS-G test procedure is easy and simple, and can be useful in evaluating buffalo semen quality.

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