Semen evaluation of Murrah buffalo bulls using sperm functional tests

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ABSTRACT: The aim of this experiment was to evaluate membrane integrity, vitality, and mitochondrial cytochemical activity, in frozen semen samples of buffalo bulls and compare those functions with the routine semen evaluation and field fertility. Twenty one frozen semen batches from 2 buffalo bulls were used for AI. For the semen evaluation, after thawing, an aliquot was evaluated for motility and vigor. An aliquot of each batch was used to evaluate the cytochemical activity using the 3-3’ diamino benzidine. Samples were scored in four classes according to the degree of midpiece staining, being class I showing midpiece totally stained, indicating full mitochondrial activity, and class IV showing no staining of the midpiece, indicating no mitochondrial activity. Two other aliquots were used for the hypo-osmotic swelling test (HOST) and the eosin nigrosin staining (VIT), to evaluate membrane integrity and vitality, respectively. Correlations were found between pregnancy rate and vitality and class II and III of the DAB staining (r=0.53, r=0.39, and r=0.38, respectively; p=0.05). No correlation was found for pregnancy rate and motility or vigor. Results indicate that functional tests may be an alternative to better predict the fertility of buffalo frozen semen samples.

Key words: Sperm, Buffaloes, Functional tests, Fertility, Semen evaluation.

INTRODUCTION - For the last decade, buffalo world population has increased 7.8% (Food and Agriculture Organization - FAO, 2005). In order to allow an improvement of productivity followed by the multiplication of animals with high genetic potential, the use of artificial insemination (AI) is essential (Baruselli et al., 2005). Semen evaluation is extremely important in order to predict the fertility of a given sperm sample, especially when using AI with cryopreserved semen, in which an ejaculate may provide hundreds of straws. Conventional semen analysis techniques are known to show limitations because they are unable to detect some sperm functional impairments, which are responsible for a decreased fertility (Aitken et al, 1984; Aitken, 2006). The use of techniques aiming to evaluate sperm functionality, such as sperm plasma membrane integrity, mitochondrial activity, and DNA fragmentation, can be extremely helpful on predicting the fertility of a given sperm sample.
The objective of the present study was to evaluate cryopreserved semen of buffalo bulls using functional tests and correlate those data with field fertility under an artificial insemination program.

MATERIAL AND METHODS - For this experiment, 21 cryopreserved semen batches, from two buffalo bulls were used for AI in different farms from the region of Vale do Ribeira, São Paulo State, Brazil. Each batch was used for at least 50 inseminations. Each batch was also evaluated for mitochondrial activity, plasma membrane integrity, and vitality. Cytochemical activity was measured according to the method described and standardized by Hrudka (1987). A 50 µL semen aliquot was incubated with 250 µL of an incubation medium containing 3-3' Diaminobenzidine (DAB) at 1mg/mL of D-PBS at 37ºC, in a dark room, for 1 hour. Following incubation, the samples were smeared onto a microscope slide, fixed with a 10% solution of formaldehyde and air-dried. The slides were evaluated under a phase contrast microscope x 1000. Two hundred spermatic cells were evaluated and classified according to the grades of staining as proposed by Hrudka (1997), in classes I (mid-piece totally stained), II (most of the mid-piece stained), III (mid-piece partially stained) and IV (no staining of the midpiece). The hypoosmotic swelling test (HOST) was performed according technique proposed by Jeyendran et al. (1984). Two hundred µL of thawed semen was added to 200 µL of both iso (300 mOsm; 7.35 g of sodium citrate, 13.51 g of fructose and 500mL of bi-distilled water) and hypoosmotic mediums (100 mOsm; prepared by diluting the isosmotic solution). The solution was then homogenized and incubated for 36 m at 37ºC. After the incubation, 5 µL of formalin solution (10%) was added in order to stop reaction and fixate the spermatic cells. The samples were evaluated using wet mount under phase contrast microscope (Leitz Dialux 20, 1000x), with the results of percentage of sperm showing intact membrane calculated as the difference between the swollen sperm found on the hypoosmotic and the isoosmotic mediums (200 cells counted in each medium). Eosin nigrosin staining for vitality was performed according to the well-known technique firstly described by Blom (1950). Statistical analysis was performed using the SAS System for Windows v.8 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND CONCLUSIONS - Pregnancy rate was correlated with vitality and class II and III of the DAB staining (r=0.53, r=-0.39, and r=-0.38, respectively; p<0.05), indicating that samples showing high percentage of live sperm and low percentage of sperm with an impaired mitochondrial potential showed decrease pregnancy rates after AI. Those results suggest that an impaired mitochondrial potential, but no absence of this potential (Class 4), may have lead to a higher susceptibility of sperm to damages. This could be explained by the attack of reactive oxygen species (ROS). Several reports in human indicate that a low sperm mitochondrial potential is related to a higher ROS production (Wang et al., 2003; Schulze-Osthoff at al., 1992; Gil-Guzman at al., 2001). However, further studies, already in progress by our group, are necessary to confirm this hypothesis. No correlations were found between pregnancy rates and motility or vigor. However, vitality was correlated with vigor (r=0.35, p=0.05). This confirms our hypothesis that some routine semen evaluation techniques do not correlate with fertility. According to recent studies, this may be due to the physiological heterogeneity of sperm (Lewis, 2007), as well as to the inability of routine semen evaluation techniques to access sperm functional competence (Petrunkina et al., 2007).
Membrane integrity evaluated by the HOST was correlated with mitochondrial potential class I and motility ($r=0.41$ and $r=0.30$, respectively; $p<0.05$). These results may indicate that whether plasma membrane integrity is important on maintaining a high mitochondrial potential, or sperm showing a lower mitochondrial potential would induce damages to the sperm membrane. According to Wang et al. (2003), the lower mitochondrial potential in ejaculated sperm occurs due to incomplete apoptosis during spermatogenesis, which would induce higher ROS production. Our hypothesis to explain the correlation between HOST and mitochondrial potential is that cryopreservation induced damages to the mitochondria, which would induce oxidative damages to the osmotic regulation mechanism of the sperm membrane. Furthermore, this would impair sperm motility. Surprisingly, no correlation was found between vitality evaluated by eosin nigrosin and membrane integrity evaluated by the HOST ($r=0.15$, $p>0.05$). Furthermore, while HOST detected 76.9% of impaired membrane sperm, eosin nigrosin was only able to detect 4.1% of damaged membrane sperm. This may indicate differences on the mechanisms in which both tests evaluate membrane integrity. While eosin nigrosin detects membrane disruptions, HOST detects sperm osmotic regulation ability. Therefore, our results indicate that even when presenting intact membrane, sperm may show impaired osmotic regulation, extremely important to resist against different environments (e.g., female genital tract; Petrunkina et al., 2007).

Results showed that the use of tests that evaluate the different functions of the cells (e.g., membrane integrity, mitochondrial potential) may provide a better predictability of the potential fertility of a given semen sample.

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