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# Relationship among damaged chromatin, motility and viability in cryopreserved spermatozoa from Brahman bulls¤

## *Relación entre la alteración de la cromatina, movilidad y viabilidad en espermatozoides criopreservados de toros Brahman*

## *Relação entre a alteração da cromatina, motilidade e viabilidade dos espermaotozoides criopreservados de touros Brahman*

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***Summary***

***The aim of this study was to determine the percentage of sperm with damaged chromatic measure with toluidine blue stain and it´s relationship with motility and viability in criopreserverd semen from Brahman bulls. Three ejaculates from six Brahman bulls were used. Immediately after thawing, sperms were stained with toluidine blue to establish chromatin integrity (sperms with normal chromatin were light blue or green while sperms with damaged chromatin were dark blue or violet). Sperms were also stained with eosin-nigrosin to determine viability (live sperms were unstained while dead sperms were pink). Motility was measured under light microscope. Effects of bull, ejaculate, and the interaction between variables were assessed. The percentage of live sperms was 50.02 ( ± 14.13%). The mean motility was 33.88 (± 12.43%), while the percentage of sperms with damaged chromatin was 4.17 ( ± 2.96%). Viability was positively correlated with motility (r=0.77217, p=0.0002), and negatively correlated with damaged chromatin sperms (r= -0.43104, p=0.0087). Motility percentage was negatively correlated with the percentage of sperms with damaged chromatin (r=-0.48337, p=0.0421). In conclusion, cryopreserved semen of Brahman bulls presented a low level of chromatin damage, and this trait was negatively correlated with sperm motility and viability.***

**Key words:** *brahman bulls, cryopreservation, sperm chromatin, toluidine blue stain.*

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***Resumen***

***El objetivo del presente trabajo fue determinar el porcentaje de espermatozoides con cromatina dañada medida con la tinción de azul de toluidina, y su relación con la motilidad y la vitalidad del semen criopreservado de toros Brahma. Para ello, se utilizó semen de tres eyaculados de seis toros Brahman, el cual una vez descongelado se procedió a teñir con azul de toluidina para determinar la integridad de la cromatina (espermatozoides con cromatina normal teñidos de azul o verde claro; espermatozoides con cromatina anormal teñidos de azul oscuro o violeta), también se tiñeron con eosina-nigrosina para determinar la viabilidad (espermatozoides vivos permanecen blancos; espermatozoides muertos se tiñen de rosado) y se estimó la motilidad espermática mediante microscopía óptica. Se evidenciaron las diferencias en todos los parámetros evaluados debidas al efecto toro y al eyaculado, así como a la interacción entre estas dos variables. El porcentaje de espermatozoides vivos fue de 50.02 ± 14.13% y la motilidad espermática promedió un 33.88 ± 12.43%, mientras que el porcentaje de espermatozoides con cromatina dañada fue de 4.17 ± 2.96%. El porcentaje de espermatozoides vivos se correlacionó positivamente con la motilidad (r=0.77217, p=0.0002), y negativamente con el porcentaje de espermatozoides con cromatina dañada (r= -0.43104, p=0.0087), mientras que el porcentaje de motilidad se correlacionó negativamente con el porcentaje de espermatozoides con cromatina anormal (r= -0.48337, p=0.0421). En conclusión, el semen criopreservado de toros Brahman presenta un bajo nivel de espermatozoides con daño en la cromatina, lo cual se correlaciona negativamente con la motilidad y la vitalidad espermática.***

**Palabras clave:** *criopreservación, cromatina espermática, tinción azul de toluidina, toros brahman.*

***Resumo***

***O objectivo deste estudo foi determinar a percentagem de espermatozóides com cromatina danificada, determinada pela coloração com azul de toluidina e sua relação com a viabilidade e a mobilidade do esperma cripreservado de touros Brahman. Para isso, foram utilizados três ejaculados de sêmen de seis touros Brahman, que uma vez descongelado foram coradas com azul de toluidina para determinar a integridade da cromatina (espermatozóides com cromatina normal coloream de azul ou verde; cromatina de espermatozóides con cromatina danificada, coloream de azul escuro ou violeta). Também foram corados com eosina nigrosina para determinar a viabilidade (espermatozóides vivos permanecem brancos e os mortos de cor rosa) e a motilidade espermática foi estimada por microscopia de luz. Foram encontradas diferenças significativas em todos os parâmetros, devido ao efeito de touro e o ejaculado, bem como a interacção entre essas duas variáveis. A percentagem de espermatozóides vivos foi de 50.02 ± 14.13% e motilidade espermática média de 33.88 ± 12.43%, enquanto a percentagem de espermatozóides com cromatina danificada foi de 4.17 ± 2.96%. A percentagem de espermatozóides vivos foi positivamente correlacionada com a motilidade (r=0.77217, p=0.0002) e negativamente com a porcentagem de espermatozóides com cromatina danificada (r = -0.43104, p= 0.0087), enquanto que a percentagem de motilidade correlacionou negativamente com a percentagem de espermatozóides com cromatina danificada (r = -0.48337, p=0.0421). Em conclusão, o sêmen de touros Brahman criopreservados tem um baixo nível de dano da cromatina, que está correlacionada negativamente com a motilidade e a vitalidade do esperma.***

**Palabras-chave:** *criopreservação cromtina espermatica, coloração de azul de toluidina, touros.*

## Introduction

Sperm quality is a very important issue to reach optimal reproductive efficiency in herds and *in vitro* embryo production systems, because sperm can affect embryo development during the early stages after fertilization (Eid *et al*., 1994; Hansen, 2002). Sperm DNA composition and structure differs from somatic cells DNA. The DNA in sperm is disposed in chromatin, which is formed by DNA and basic proteins called protamines. The cysteine residues in protamines form disulfide bonds which compact the DNA six times more than in somatic cells. These bonds protect the genetic material from stressing agents such as reactive oxygen species and high temperatures during the transit through the male and female reproductive tract (Evenson *et al*., 2002; Kosower *et al*., 1992; Oliva, 2006; Zini *et al*., 2001).

Along with classical parameters of sperm quality evaluation (Gillan *et al.,* 2008; Januskauskas *et al*., 2001), chromatin integrity is currently considered a determinant part of sperm quality. In bulls, the level of sperm with damaged chromatin has been negatively correlated with motility and viability (Januskauskas *et al.,* 2003; Kasimanickam *et al.,* 2006; Khalifa *et al.,* 2008). Sperm chromatin integrity has been shown to affect the reproductive potential in several studies (Ballachey *et al*., 1987; Dobrinski *et al*., 1994; Madrid-Bury *et al*., 2005).

Several factors have shown to affect chromatin integrity. There exists ample evidence on the possibility of cryopreservation negatively affecting sperm DNA integrity. However, its precise underlying mechanism is still much debated. A recent study reports that cryopreservation-induced injury to sperm DNA is mediated primarily through oxidative stress rather than apoptosis (Thomson *et al.,* 2009). Semen cryopreservation induces sperm DNA fragmentation, which is comparatively higher in bulls with poor semen quality (Mukhopadhyay *et al.,* 2011).

Techniques such as Sperm Chromatin Structure Assay (SCSA; Ballachey *et al*., 1987; Bochenek *et al*., 2001; Charles-Ostermeier *et al*., 2001; Januskauskas *et al*., 2001; Januskauskas *et al*., 2003; Hallap *et al*., 2005; Madrid-Bury *et al*., 2005; Waterhouse *et al*., 2006), Terminal Deoxynucleotidyl Transferase mediated dUTP nick end labelling (TUNEL; Fatehi *et al*., 2006; Waterhouse *et al*., 2006), Acridine Orange (AO; Khalifa *et al*., 2008; Celeghini *et al*., 2008), and Comet assay (Slowínska *et al*., 2008; Urrego *et al*., 2008) have all been used to evaluate sperm chromatin integrity in fresh and thawed semen from bulls. These techniques are expensive and time consuming, which limits their application in research laboratories. Therefore the use of alternative methods to evaluate sperm chromatin integrity during the routine sperm quality evaluation could give more information on this important parameter and the factors affecting the reproductive potential of bulls.

Toluidine Blue (TB) is a nuclear dye used to evaluate sperm chromatin integrity by detecting the absence or rupture of disulfide bonds. Thus, dark stained nuclei indicate altered sperm chromatin. This stain has been used to evaluate sperm chromatin integrity in several species, such as bulls, horses, rabbits, buffaloes and humans (Mello, 1982; Beletti and Mello, 1996; Mello and Beletti, 2002; Beletti and Mello, 2004; Erenpreisa *et al*., 2003; Beletti *et al*., 2005; Sardoy *et al*., 2008; Zúccari *et al*., 2008). In humans, TB stain showed high correlation with SCSA, TUNEL and AO (Erenpreisa *et al*., 2003; Erenpreiss *et al*., 2004); while in rabbits, it showed a high correlation with Feulgen reaction (Beletti and Mello, 2004), validating TB as a technique suitable for studying sperm chromatin integrity. However, most studies published so far were conducted using *Bos taurus taurus* semen and scarce information has been published about levels of sperm with damaged chromatin and its relationship with another parameters of sperm quality in *Bos taurus indicus* bulls, especially in Brahman bulls. Therefore, the aim of this study was to determine the percentage of sperm with damaged chromatin measured with toluidine blue stain and its relationship with viability and motility in cryopreserved semen of Brahman bulls.

## Materials and methods

### *Semen samples*

Three ejaculates from six Brahman bulls were used. Bulls were located at the VIATECA Artificial Insemination Center in Machiques County, Zulia State, Venezuela. Ejaculates were collected using an artificial vagina. After routine evaluation, only ejaculates that reached at least the minimum quality standards established by the Center for Brahman bulls were processed (concentration: $850x10^6$ sperm/mL; individual progressive motility: 60%). Semen was diluted in two stages to reach a final concentration of ~$30x10^6$ motile sperm/mL. After dilution and equilibration at 5 °C, semen was loaded into 0.5 mL straws, which were frozen in liquid nitrogen vapours 4 cm above N surface for 10 min, and then plunged into the liquid nitrogen. Semen extender was prepared with skim milk, egg yolk medium (20%), and glycerol (7%).

*Motility and viability in thawed semen*

Immediately after straw thawing in a water bath at 37 °C for 30 seconds, motility was estimated under light microscope at x 400 magnification in bright field illumination. Sperm viability was measured with eosin-nigrosin stain (Tamuli and Watson, 1994), mixing 15 µL of semen and 10 µL of stain for 30 seconds. Spermatozoa were smeared onto a pre-warmed glass slide and air dried. The percentage of viable (unstained) sperm was determined by smear observation under light microscope at x 1000 magnification. These analyses were conducted by one technician.

*Chromatin integrity in thawed semen*

Sperm chromatin integrity was assessed with toluidine blue stain (Agarwal and Said, 2004). Air dried semen smears were fixed in an ethanol:acetic acid (3:1 v/v) solution for 30 minutes and then smears were hydrolysed for 5 minutes in 0.1 N HCl, washed in distilled water, air dried and stained with a 0.05% toluidine blue (pH 4) in McIlvain buffer for 10 minutes (Agarwal and Said, 2004). Smears were observed under a light microscope at x 1000 magnification. Light blue or green sperm were considered as having normal chromatin, whereas dark blue or violet sperm were considered as having damaged chromatin. This analysis was conducted by one technician.

*Statistical analysis*

At least 200 cells were counted in duplicate for each smear in both eosin-nigrosin and TB. Statistical data analysis was conduected using Statistical Analysis System for Windows, software 8.2 (*SAS Inst. Inc*.; Cary, NC. USA). Percentages of viability and sperm with normal chromatin integrity were transformed through the square arcsine method to obtain a normal distribution. Analysis of variance was conducted with the General Lineal Model (GLM procedure) and results are shown as means ± SD. Bull and ejaculate effects on viability, motility and percentage of sperm with abnormal chromatin integrity were evaluated. A Spearman's correlation test was used to establish correlation among the measured parameters.

## Results

The analysis of variance showed that percentages of viability, motility and sperm with damaged chromatin were significantly affected by bull, ejaculate and the interaction between both variables. Results per bull are shown in Table 1. Percentage of live sperm averaged 50.02 ± 14.13. Motility ranked from 23% to 56%, with a mean of 33.88 ± 12.43%, while the level of sperm with damaged chromatin (Fig 1) was 4.17 ± 2.96%. Spearman's correlation test revealed a positive correlation between percentage of live sperm and motility (r=0.77217, p=0.0002), whereas the percentage of damaged chromatin had a negative correlation with viability (r=-0.43104, p=0.0087) and motility (r=-0.48337, p=0.0421).

**Table 1.** Percentages of viability, motility and damaged chromatin sperm in cryopreserved semen of Brahman bulls (means ± SD).

| Bull | Live sperm | Motility | Damaged chromatin |
|---|---|---|---|
| 1 | 31.58 ± 10.50 $^{a}$ | 23.33 ± 5.77 $^{a}$ | 4.33 ± 1.34 $^{a}$ |
| 2 | 65.91 ± 5.70 $^{b}$ | 56.66 ± 5.77 $^{b}$ | 2.33 ± 0.70 $^{b}$ |
| 3 | 47.40 ± 3.09 $^{c}$ | 25.00 ± 5.00 $^{a}$ | 7.11 ± 2.61 $^{c}$ |
| 4 | 52.58 ± 16.84 $^{d}$ | 30.00 ± 5.00 $^{ad}$ | 4.88 ± 4.88 $^{a}$ |
| 5 | 46.16 ± 9.23 $^{c}$ | 31.66 ± 7.63 $^{ad}$ | 4.77 ± 1.66 $^{a}$ |
| 6 | 56.50 ± 9.02 $^{d}$ | 36.66 ± 5.77 $^{d}$ | 1.61 ± 0.74 $^{b}$ |

Values with different superscript in the same column differ significantly ($p<0.05$).

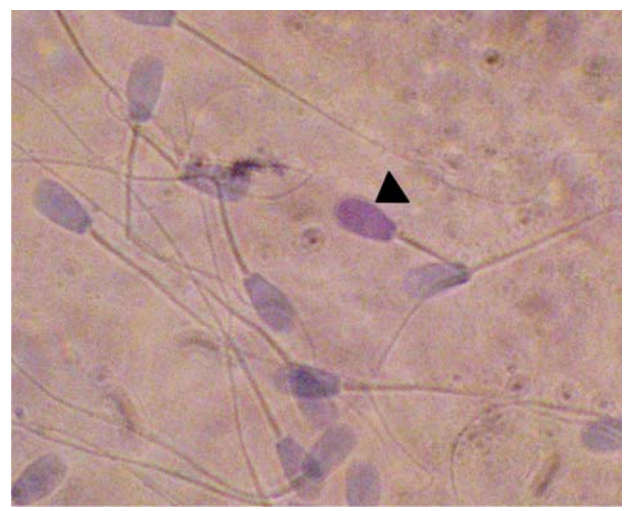

**Figure 1.** Sperm stained with toluidine blue. The arrow head points to a dark violet sperm which is assumed as having damaged chromatin, while the light blue sperms preserve chromatin integrity.

## Discussion

In the present study, the percentage of cryopreserved sperm with damaged chromatin using TB stain and its relationship with viability and motility were determined. Little information is available about this subject in bulls under tropical conditions. Recently, Zúccari *et al*. (2008), using TB stain, showed that cryopreserved Nellore semen had 3.15 ± 1.74% of sperm with damaged chromatin, a result lower than the observed in the present study. Some studies suggest that the presence of sperm with damaged chromatin in cryopreserved semen could be a consequence of the cryopreservation process (Khalifa *et al*., 2008; Slowínska *et al*., 2008), but the effect of cryopreservation on sperm DNA integrity, even though significant, seems to be low when measured through the comet assay (Slowínska *et al*., 2008).

The level of damaged chromatin was affected significantly by bull effect, and this is in agreement with Gillan et al. (2008) and Khalifa *et al.* (2008). Evaluation of sperm chromatin integrity alone (Madrid-Bury *et al*., 2005) or in combination with other parameters (Gillan *et al.,* 2008) could explain fertility variations between bulls to select superior bulls. More sperm with damaged chromatin were observed in poorly fertile bulls (Beletti and Mello, 1996; Vieytes *et al*., 2008). In previous studies, a negative correlation between sperm chromatin integrity and other parameters of sperm quality was observed (Januskauskas *et al.,* 2003; Kasimanickam *et al.,* 2006; Khalifa *et al.,* 2008). The correlation coefficient observed in the present study suggests that sperm chromatin integrity, viability and motility could be associated but are not concomitant, indicating that live or motile sperm could or not carry damaged chromatin. Thus sperm chromatin damage could be used independently to evaluate sperm quality. Additionally, the results could explain the negative association between sperm chromatin damage and fertility observed in several studies (Ballachey *et al*., 1987; Dobrinski *et al*., 1994; Madrid-Bury *et al*., 2005; Fatehi *et al*., 2006; García-Macías *et al*., 2008).

In this study, TB was used to identify sperm with damaged chromatin. Dye variations of stained sperm on the slide diminish the repeatability when compared with other methods for evaluating sperm chromatin. Therefore, studies comparing TB stain with other methods for evaluating sperm chromatin integrity are necessary. In humans, TB showed high correlation coefficients with SCSA, TUNEL and AO (Erenpreisa *et al*., 2003; Erenpreiss *et al*., 2004). However, in the case of bull semen, there is a lack of studies comparing these techniques. In cryopreserved semen of buffaloes (*Bubalus bubalis*), Melo and Beletti (2002) observed a moderate correlation between TB and acridine orange (r=0.38). Additionally, TB was considered more sensitive and less subjective in identifying sperm with abnormal chromatin than acridine orange, although both methods had similar stability and reliability. Additionaly, the TB stain is a practical methodology to study sperm morphometric features and to identify nuclear regions susceptible to chromatin alterations (Beletti *et al*., 2004; Beletti *et al*., 2005).

In conclusion, cryopreserved semen from Brahman bulls has low level of sperm with damaged chromatin, measured with the TB stain. Damaged chromatin is negatively correlated with viability and motility and could be used to estimate sperm quality. Further studies to compare toluidine blue stain with other methods are needed.

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