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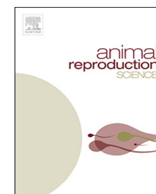

Does lipid peroxidation and oxidative DNA damage differ in cryopreserved semen samples from young, adult and aged Nellore bulls?

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A R T I C L E I N F O

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A B S T R A C T

The aims of this study were to evaluate cryopreserved semen of Nellore bulls of different ages and verify whether sperm quality declines with advancing age and whether lipid peroxidation and DNA damage are involved in this process. For this purpose, 40 Nellore bulls were divided into three age groups: Young, aged 1.8–2 years (n = 9); Adult, aged 3.5–7.0 years (n = 19); and Seniors, aged 8.0–14.3 years (n = 12). Three ejaculates were collected from each bull, cryopreserved and evaluated for various parameters including membrane integrity, mitochondrial potential (FITC-PSA and JC1), lipid peroxidation (C-11BODIPY 581 / 591) and oxidative DNA damage (8OHdG) using flow cytometry. The thawed semen of senior bulls was characterized by a low percentage of motile sperm (33.7 ± 6.1%), higher damage to the plasma and acrosomal membrane (37.5 ± 9.8%), and low mitochondrial potential (29.1 ± 13.8%), as well as higher percentages of peroxidated cells (53.6 ± 12.2%) and DNA damage (44.1 ± 11.0%; $P < 0.05$). Lipid peroxidation was negatively correlated with motility ($r = -0.35$, $P < 0.0002$), average mitochondrial potential ($r = -0.42$; $P < 0.0001$) and showed a positive correlation with membrane injury and oxidative DNA damage (r = 039; P = 0.0003). Young bulls presented superior thawed sperm quality, possibly due to greater resistance to oxidative stress and, consequently, to cryopreservation. In conclusion, the sperm quality of bull semen declines with advancing age and is strongly associated with increased oxidative damage to both the plasma membrane and DNA.

1. Introduction

The effects of age on semen parameters have been intensely discussed in prepubertal to adult bulls, with the average studied age

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ranging from 1 to 7 years (Brito et al., 2004; Brito et al., 2002; Hallap et al., 2006, 2004). Despite many studies regarding the sexual maturity of young bulls, research evaluating the impact of advancing age on bull fertility, especially in senior animals, are scarce. The few existing studies indicate changes in spermatogenesis (Kumi-Diaka et al., 1981), lipid balance and production of antioxidants (Kelso et al., 1997). Much of the work done in the bovine seeks to relate sperm quality at different ages with other factors such as environmental, nutritional, breed differences and seasons of the year (Bhakat et al., 2011; Brito et al., 2002; Nichi et al., 2006).

Studies using mice as the experimental model indicate that increased DNA damage with aging is associated with increased susceptibility to oxidative stress (Zubkova et al., 2005; Zubkova and Robaire, 2006). This is because aging leads to the reduction of important antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are responsible for protecting the sperm nucleus from free-radical attack, and some of the affected enzymes, such as peroxiredoxin, are fundamental for DNA stability (Weir and Robaire, 2006; Ozkosem et al., 2015). Recent studies also indicated that the germ cells of senior males show a reduced DNA repair mechanism and present inadequate response toward oxidative insults compared to young male germ cells (Paul et al., 2011; Selvaratnam et al., 2015).

Despite such evidence, most of the studies in bovines have aimed to evaluate the effects of ROS on sperm quality and function in cryopreservation protocols (Bilodeau et al., 2000; Gürler et al., 2015; Mostek et al., 2017). Few studies have evaluated the relationship between age and oxidative stress in bovine semen (Kelso et al., 1997), and no work has determined whether the age of the bull influences the oxidative damage in cryopreserved semen. This question is of fundamental importance in the search for prevention methods and alternative therapies that can optimize the sperm quality of high-genetic merit bulls with advanced age in seminal cryopreservation programs. *Bos indicus* cattle show late sexual maturation compared to *Bos taurus* (Nogueira, 2004) cattle and need more time to confirm genetic selection in progeny testing. In Brazil, commercialization of the semen of bulls with advanced age is a reality. Therefore, the objective of this study was to evaluate the quality of thawed semen from Nellore bulls of different age groups and test the hypothesis that the sperm quality of senior bulls declines because of increased oxidative damage.

2. Materials and methods

2.1. Selection of animals

A total of 40 healthy Nellore bulls from a sperm cryopreservation center located in the southeastern region of Brazil (21° 04′52″ S and 48°02′24″ W) was used in this study and kept in a native pasture (*Cynodon plectostachyus*) with a balanced-energy diet. Three ejaculates were collected from each bull using the artificial vagina method within a twice-a-week collection schedule. Samples were cryopreserved (Tris-egg yolk, 7% glycerol in 250 μL straws) according to the artificial insemination center standards and stored in liquid nitrogen until evaluation. Bulls were always handled by the same persons. Two technicians were involved in semen assessment, one of them for analysis pre-freezing and post-thawing and the other for cooling, packaging and freezing technique. The animals were divided into three groups according to age: (i) the Young group, aged from 1.8 to 2 years (n = 9); the Adult group, aged between 3.5 and 7 years (n = 19); and the Senior group, aged from 8 to 14.3 years (n = 12). This classification was based on the study by Kumi-Diaka et al. (1981), who, through histological analysis of testicles, concluded that reproductive senility in bulls begins at 9 years of age. Bulls between 2 and 3 years are classified as young, and adults are considered mature between 5 and 6 years of age (Kelso et al., 1997).

2.2. Microscopic evaluations and flow cytometry

Microscopic evaluations were performed using an Olympus BX61 microscope (Olympus, Tokyo, Japan) equipped with a light field, phase contrast, differential interference contrast (DIC) and epifluorescence.

Flow cytometry analysis was performed with an Attune® instrument (Applied Biosystems by Life Technologies, Grand Island, NY, USA) equipped with 488 nm and 405 nm argon-ion lasers with the following emission filters: BL 1 530/30 nm “bandpass” (BP), BL 2 575/24 nm BP, BL 3 640 nm “long pass” (LP), VL 1 450/40 nm BP, VL 2 522/30 nm BP, and VL 3 603/48 nm BP.

2.3. Motility, vigor and sperm morphology

The straws were thawed at 35 °C for 20 s, and 5 μL of semen was poured on a pre-warmed plate (37 °C), covered with a cover slip and visualized at 200X magnification. Sperm was evaluated for progressive motility (%) and vigor (0 to 5), determined by visual estimation (CBRA, 2013). Vigor was analyzed using a scale from 0 (absence of any movement) to 5 (strong, vigorous forward movement) (CBRA, 2013).

To assess sperm abnormalities, 200 cells were counted using DIC (1000x). Sperm alterations were classified into major defects (i.e., primary acrosome defects, proximal droplets, abnormal loose heads, abnormal head contour, abnormal midpiece, nuclear vacuoles, double forms, and dag defects) and minor defects (abnormal head size, decapitated sperm head, coiled tails with cytoplasmic droplets, distal cytoplasmic droplets, abaxial midpiece) (Blom, 1973).

2.4. Evaluation of plasma membrane and acrosome integrities

For simultaneous evaluation of plasma membrane and acrosome integrities, one straw of each sample was thawed (35 °C / 20 s), and the semen was diluted to 2×10^6 sperm in 200 μL of PBS (phosphate buffered saline: NaCl 10 g/L; KCL 0.25 g/L; NaH2PO4 1.4 g/

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