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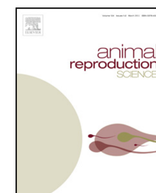

Sperm fertility and viability following 48 h of refrigeration: Evaluation of different extenders for the preservation of bull semen in liquid state

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ABSTRACT

Two experiments were conducted to compare the effectiveness of different extenders conventionally used for semen cryopreservation to maintain the viability and fertility of cooled bull semen. In Experiment 1, sperm samples obtained from 20 Nellore bulls were preserved at 5 °C for 48 h using two extenders containing 20% of egg yolk [Tris (TRIS-R) and Botu-Bov® (BB)] and another composed of 1% soy lecithin [Botu-Bov®-Lecithin (BB-L)] as substitutes for animal origin products. The samples were evaluated at 6, 24 and 48 h for plasma and acrosomal membrane integrity, quantification of thiobarbituric acid reactive substances (ng of TBARS/10^8 cells) and sperm motility parameters by computer-assisted semen analysis (CASA). In Experiment 2, pregnancy rate (P/AI) of 973 fixed-time artificially inseminated Nellore cows were compared when cows were inseminated with conventionally cryopreserved semen in TRIS-egg yolk glycerol (TRIS-C Control, $n = 253$) or semen cooled for 48 h in TRIS-R ($n = 233$), BB ($n = 247$) or BB-L ($n = 240$). Although none of the extenders used was effective on maintaining total progressive motility and cellular integrity throughout the 48-h of the refrigeration period ($P < 0.01$), BB-L conferred greater protection against oxidative stress ($P < 0.05$) than egg yolk-based medias. The P/AI for semen samples preserved in TRIS-C, TRIS-R, BB and BB-L were 39.92^a, 25.32^b, 26.32^b and 33.33^{ab}, respectively. These results demonstrate that the three conventional extenders used for semen cryopreservation do not provide the protection required to maintain bull semen fertility under refrigeration for a 48-h period, resulting in reduced pregnancy rates. However, the use of lecithin-based medium instead of egg yolk results in greater protection against lipid peroxidation, producing P/AI results comparable to those obtained using frozen semen.
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1. Introduction

Despite the importance and widespread use of frozen semen in cattle production, few advances in the development of new extenders have emerged in the past decades. A significant decrease in the integrity and functionality of spermatozoa is observed when semen is subjected to

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conventional cryopreservation protocols (Celeghini et al., 2008). Freezing is responsible for a significant decrease in sperm motility (Chaveiro et al., 2006; Thomas et al., 1998), leading to several biochemical and structural alterations that may affect different cytological compartments of the spermatozoa, causing most of the cells to die during processing (Yoshida, 2000).

The decrease in spermatozoa viability and fertility associated with cryopreservation has motivated several studies to address preserving semen in a liquid state for several species including cattle (Bucher et al., 2009; Crespilho et al., 2012a) antelope (Adeel et al., 2009), horses (Crespilho et al., 2013), goats (Purdy et al., 2010) and sheep (O'Hara et al., 2010), demonstrating promising results for sperm viability preservation. The main advantage of the cooled semen process is that it prevents damage associated with freezing, thereby ensuring greater sperm viability. This allows the insemination dose to be reduced, thereby optimizing the use of sires of high genetic merit in artificial insemination programs (Bucher et al., 2009; Verberckmoes et al., 2005).

The success of semen preservation in the liquid state requires that the reduction in sperm motility and metabolic activity caused by refrigeration be reversible (Yoshida, 2000). Considering that storage time influences the viability of cooled semen (Batellier et al., 2001), one of the main uses of cooled bull semen is in fixed-time artificial insemination (FTAI) programs. These protocols allow the insemination of a large number of animals in a short time interval (Bucher et al., 2009), making it possible to incorporate semen refrigeration technology despite the short longevity of these samples.

Thus, cooled bull semen represents a promising alternative to cryopreserved semen that may increase pregnancy rates (P/AI) and decrease the overall cost of FTAI programs. In a recent case report, the conception rate obtained after AI using the cooled semen for 24 h was 15% greater than conventional frozen-thawed bull semen, demonstrating the usefulness of cooled semen as a strategy to enhance the fertility of FTAI programs in beef cattle (Crespilho et al., 2012a).

However, because most of the studies involving cooled bull semen were conducted prior to the arrival of cattle estrous cycle synchronization protocols (Almquist and Wickersham, 1962; Blackshaw et al., 1957; Foote, 1962, 1970; O'Dell et al., 1959), the impact of cooled bull semen on the pregnancy rates in fixed time artificial insemination programs have not been addressed in the literature.

One of the main factors associated with the decrease in the motility and fertility of refrigerated sperm is the production of reactive oxygen species (ROS), which occurs as a normal consequence of sperm metabolism and results in an irreversible decrease in the quality of cooled semen (Çoian et al., 2010). Considering that spermatozoa have a limited capacity to resist oxidative stress (Nichi et al., 2006), it is essential that extenders minimize the deleterious effects of ROS.

The objective of the present study was to test the effectiveness of various extenders usually used for the cryopreservation of sperm at maintaining the viability and fertility of bull semen stored under refrigeration temperature. This study also tested the hypothesis that the use of semen subjected to refrigeration for a 48-h period may increase the P/AI ratios in fixed-time artificial insemination programs in beef cows synchronized with a progesterone and estrogen-based protocol.

2. Materials and methods

2.1. Semen collection

The methodology implemented in this study was approved (process number 228/2011) by the Ethics Committee in Animal Experimentation of the São Paulo State University, Brazil. Semen samples were obtained through electroejaculation from 20 Nellore bulls (*Bos taurus indicus*) aged 24–30 months. In Experiment 1, two ejaculates were obtained from each sire (the first intended for initial seminal quality analysis and a second sample for the experimental procedure). Minimum quality criteria for fresh semen to be included in the experimental procedure included sperm motility greater than 70%, percentage of major defects below 20%, percentage of minor defects below 20% and percentage of total defects below 30%.

Five sires were selected for Experiment 2 based on the phenotypic characteristics of the breed and seminal quality criteria used in Experiment 1, and each animal was subjected to serial seminal samplings through electroejaculation (with a 2-day interval between samplings) for 15 days to ensure a period of biological leveling and adaptation to the new routine before beginning sample collection for the artificial insemination program. To produce the insemination doses a total of 10 ejaculates (2 ejaculates from each bull) were collected, according to minimum quality criteria adopted.

2.2. Semen processing

Immediately after sample collection, the total motility and sperm vigor of the ejaculate samples were subjectively evaluated using light microscopy, and total sperm concentration was determined using a Neubauer hemocytometer chamber.

2.2.1. Experiment 1

Three extenders were used for semen refrigeration: Tris-egg yolk-fructose (TRIS; 30 g [Tris (hydroxymethyl) aminomethane], 17 g citric acid, 12.5 g fructose, 0.20 g amikacin sulfate, 2 mL Orvum Est Pastum and 20% egg yolk); Botu-Bov® (BB; Botufarma Ltda., Botucatu, São Paulo, Brazil) also containing 20% egg yolk in 1 L of final solution; and Botu-Bov-Soy Lecithin (BB-L; Botufarma Ltda., Botucatu, São Paulo, Brazil), in which the egg yolk was completely replaced by 1% lecithin, according to Crespilho et al. (2012b). All media were made in single fractions free of glycerol, and clarified by high centrifugation force (5000 × g for 1.5 h in refrigerated centrifuge).

Each semen sample was fractionated into three equal aliquots immediately after sample collection, deposited into 50 mL plastic tubes, diluted in TRIS, BB or BB-L medium and packaged in 0.5 mL French straws (IMV® Technologies, L'Aigle Cedex, France) at a final concentration of

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