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Butylated hydroxytoluene can reduce oxidative stress and improve quality of frozen–thawed bull semen processed in lecithin and egg yolk based extenders

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ABSTRACT

The aims of this study were to evaluate the effects of anti-oxidant butylated hydroxytoluene (BHT), when added at different concentrations into lecithin-based Bioxcell® (BX) and two egg-yolk-based; Tris (TY) and citrate (CE) semen extenders, on post-thaw bull sperm quality and oxidative stress. A total of 30 ejaculates from three bulls were collected using an electro ejaculator. Ejaculates were extended with one of the BX, TY and CE extenders, which contained different concentrations (0.0 – control, 0.5, 1.0, 1.5, 2.0 and 3.0 mM/ml) of BHT. The extended semen samples were chilled to 4 °C, and then frozen slowly to −196 °C in 0.25 ml straws before being stored in liquid nitrogen for 2 weeks. Results showed that supplementation of BHT improved ($P < 0.05$) general motility, progressive motility, morphology, acrosome integrity, DNA integrity and malondialdehyde of sperm at 0.5 mM/ml for BX and at 1–1.5 mM/ml of BHT for TY and CE when compared with the control. However, greater concentrations of 2.0 and 3.0 mM/ml of BHT had a detrimental ($P < 0.05$) effect compared with the control with all extenders evaluated. In conclusion, BHT supplementation at lesser concentrations (0.5–1.5 mM/ml) could improve frozen–thawed bull sperm quality by reducing oxidative stress produced during the freezing–thawing procedures in either lecithin or egg-yolk based extenders.

1. Introduction

Cryopreservation is the most important tool for long-term storage of semen, reduction of reproductive diseases and control of losses to contagious diseases (Lemma, 2011). However, as important as cryopreservation is there are certain detrimental effects on sperm. The capacity of sperm to participate in the fertilization process is influenced by cryopreservation (Bailey et al., 2008; Lemma, 2011; Ozkavukcu et al., 2008; Tasdemir et al., 2013). Lessard et al. (2000) observed that sperm viability and fertility decreased by about 50% after cryopreservation. Cryopreservation exerts oxidative stress on sperm cells due to membrane lipid peroxidation during the freezing process, which raises the production of oxidants such as reactive oxygen species (ROS). The ROS are highly reactive molecules containing free oxygen radicals. The ROS are formed as a natural byproduct of normal metabolism of oxygen and have

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important roles in fertilization. The ROS concentrations increase with temperature change. With greater accumulation in semen, ROS "steal" electrons from the lipids in sperm membranes, resulting in an irreversible damage to the structure and functions of the sperm cells (Guthrie and Welch, 2012). Antioxidants such as BHT are being evaluated as an additive in semen extenders which have been found to be beneficial at optimum concentrations for sperm cell motility and viability in certain breeds such as the Holstein (Ansari et al., 2011) and Sahiwal (Asadpour et al., 2012). In addition, lipid solubility of BHT influences its preference over other antioxidants because this property gives it the ability to function as an antioxidant within and outside the sperm membrane. The BHT penetrates the sperm membrane increasing its fluidity to prevent ice-crystal formation within the cell thereby protecting the sperm (Hammerstedt et al., 1976). Butylated hydroxytoluene also scavenges ROS from surroundings of the sperm and converts these molecules into hydroperoxides (Merino et al., 2015) thereby reducing the harmful effects of ROS' on sperm cells during freeze–thaw procedures.

The benefit of using different antioxidant concentrations is the physiological effect that results from actions on the ROS. An appropriate dose of antioxidant is needed to regulate the amount of ROS. If used in greater concentrations, some important physiological mechanisms may be impaired. If used in insufficient concentrations, the actions on ROS will not be effective. However, there needs to be a sufficient amount of antioxidant to gain the greatest protection of bull sperm which varies with breed of bulls and semen extenders used. Furthermore, there is limited information on the effect of BHT on variables such as DNA damage of frozen–thawed bull sperm in a soya lecithin based extender. Also, isolated studies on the different egg-yolk extenders make it difficult to assess whether the differences in response reported with use of different egg-yolk extenders are specific or related to the experimental conditions. The authors hypothesized that the soy lecithin will require less BHT to produce maximum protection to bull sperm compared to egg-yolk in semen extender. The present study, therefore, aimed to evaluate the effects of BHT in soya lecithin-based (Bioxcell®) and egg-yolk based (Tris and Citrate) extenders on oxidative stress and semen quality variables of crossbred beef bulls when evaluated simultaneously under similar experimental conditions.

2. Materials and methods

2.1. Animals

Semen samples were collected from three healthy and sexually mature crossbred bulls; Simmental-Brangus, Brangus-Hereford and Kedah-Kelantan-Brangus at the Universiti Putra Malaysia (UPM) farm in Serdang, Selangor (2°9′18.36″ N, 101°43′49.61″ E). The bulls were 5.3 ± 0.3 years old with an average weight of 649.3 ± 9.7 kg. The body condition score (BCS) of the bulls were 6, 5 and 4, respectively, based on a scale of 1 being thin to 9 being obese (Eversole et al., 2009). All bulls were maintained under a uniform management conditions, fed freshly harvested *Brachiaria decumbens* grass, supplemented with commercial cattle concentrate and palm kernel cake (PKC) containing approximately 16% crude protein and 2.6% crude fat at a rate of 3 kg/bull/day. Bulls were also provided mineral blocks and water *ad libitum*.

2.2. Semen collection and preparation of extenders

The present study was conducted between November 2013 and March 2014. There were a total of 30 ejaculates collected at a 4 day interval every week from each bull from three bulls by electro ejaculation (Electrojac 5, USA). The samples were kept at 37 °C in a cooler box containing warm water for transportation to the Theriogenology and Cytogenetics laboratory at UPM Serdang where pre-freezing evaluations occurred. Ejaculates with more than 80% morphologically normal sperm, more than 60% general motility, and a concentration of more than 500×10^6 sperm/ml (Nagy et al., 2013) were used for the experiment. Samples were then diluted with one of the three extenders: Bioxcell® (BX), Tris-egg yolk (TY) and citrate-egg yolk (CE) extenders, and further subdivided into six groups. The respective samples were poured into dried, pre-warmed test tubes containing BHT antioxidant (prepared in ethanol) to obtain 0, 0.5, 1.0, 1.5, 2.0 and 3.0 mM/ml BHT concentrations. These mixtures were left in a water bath at 37 °C for 5 min to allow for proper uptake of BHT by sperm cells (Ijaz et al., 2009) before cooling and freezing.

Bioxcell®, a commercial semen extender (IMV, France), was diluted at a 1:4 ratio in distilled water according to the manufacturer's recommendation, while TY and CE extenders were prepared according to Bearden et al. (2004) with a penicillin–streptomycin mixture (BP2959-50) as antibiotic at 0.01 ml/ml of the extender. All the extenders were adjusted to a pH of 6.7 using a pH meter (Mettler Toledo Ltd., England). Dilute sodium hydroxide (NaOH) was used as the neutralizing agent and hydrochloric acid (HCL) for acidity to control the pH. Semen was extended to adjust the concentration of sperm to 20×10^6 cells in a 0.25 ml mini straw, then slowly chilled to 4 °C for a 2 h period. Straws were packed with the extended semen (at 4 °C working environment) and kept at the same temperature for 4 h to equilibrate. Packed straws of extended semen were frozen using a traditional vapor freezing method. The straws were placed on racks and held horizontally 4 cm above the surface of liquid nitrogen for 10 min. The racks were left to float with the straws on liquid nitrogen for 3 min before plunging them into the liquid nitrogen.

2.3. Evaluation of fresh semen quality

Sperm motility and concentration were determined by Computer Assisted Semen Analyzer (CASA) of IVOS Hamilton Thorne Biosciences, version 12.2. The CASA was programmed to capture frames at the rate 60 Hz/s at 37 °C temperature and at a video frequency of 60. The magnification factor, minimum cell size, detection contrast and cell intensity were 1.92, 5 pixel, 40, 55, respectively. The average path velocity (VAP) was 75 μm/s and straightness threshold (STR) was 80%. A diluted sample (10 μl) in 0.85% normal saline was placed on Hamilton Thorne research 2X-cell (20 μm) glass slides and loaded on the CASA for

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