Animal Reproduction Science 170 (2016) 108–113

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effect of various concentrations of butylated hydroxyanisole and butylated hydroxytoluene on freezing capacity of Turkman stallion sperm

Afshin Seifi-Jamadi[a], Hamid Kohram[a,b,*], Ahmad Zareh-Shahne[a], Parvaneh Dehghanizadeh[a], Ejaz Ahmad[c]

[a] Department of Animal Science, University College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University, Ahvaz, Iran
[c] Department of Clinical Sciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form 14 April 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
Horse semen
BHA
BHT
Lipid peroxidation
Freezing
Sperm motility

ABSTRACT

The present study aimed to determine the effect of different concentrations of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on post-thaw stallion sperm quality. The ejaculates collected from four healthy mature Turkmen stallions were pooled and divided into eight aliquots. The samples were diluted with extenders containing different concentrations (0.5, 1 or 2 mM/mL) of BHA or BHT. The positive control (PC) samples were diluted with extender containing 0.5% ethanol (v/v) whereas; the negative control (NC) samples were diluted with basic extender only. Semen samples were frozen according to a standard protocol. After thawing of samples, sperm motility, viability, membrane integrity, total abnormality and lipid peroxidation were assessed. The greatest ($P < 0.05$) values for total sperm motility, viability and plasma membrane functionality and least values for malonedialdehyde (MDA) concentration were observed in samples supplemented either with 1 mM BHT or 2 mM BHA. However, the progressive motility was greater ($P < 0.05$) only in samples treated with 2 mM BHA. In conclusion, the use of 1 mM BHT or 2 mM BHA in extender improves the freezing capacity of stallion sperm by reducing oxidative stress during freeze-thaw process.

1. Introduction

Cryopreservation of mammalian sperm has become a useful technique to propagate the genetics of males with greater genetic value worldwide. However, the oxidative stress during cryopreservation process is the potential cause of cell death and poor fertility after artificial insemination with frozen-thawed semen in domestic animals. Although, moderate amounts of ROS are essential for natural processes such as sperm maturation, capacitation and acrosome reaction (Taylor, 2001), excessive production of ROS during cryopreservation induces oxidative stress which lead to the cell death. The ROS are synthesized as a by-product of mitochondrial oxidative phosphorylation. Subsequently these free radicals damage the mitochondrial membrane and this damage results in additional production of free radicals. The sperm plasma membrane is the primary site where ROS interacts owing to the presence of greater than typical amounts of polyunsaturated fatty acids (PUFA) (Gilgun-Sherki et al., 2003; Baumber et al., 2005; Loomis and Squires, 2005; Aitken et al., 2012; Alvarenga et al., 2012). Previous studies indicate amounts of ROS

* Corresponding author at: Department of Animal Science, Faculty College of Agriculture and Natural Resources, University of Tehran, P.O. Box 31587, Karaj 77871, Iran.
E-mail address: Hamid_Kohram@yahoo.com (H. Kohram).

http://dx.doi.org/10.1016/j.anireprosci.2016.04.010
0378-4320/

are negatively correlated with sperm motility, viability and morphology of frozen thawed sperm in men (Agarwal et al., 2014), bulls (Pankaj et al., 2009), goat bucks (Memon et al., 2012) and stallions (Baumber et al., 2000; Ball, 2008; Morillo-Rodríguez et al., 2012).

Antioxidants form a defense system by removing O_2, binding to metal ions needed for ROS generation, (Gilgun-Sherki et al., 2001, 2003) scavenging ROS or the precursors (Da Silva et al., 2010), and by repairing membranes (Sies, 1994). The seminal plasma is a natural reservoir of antioxidants, however, in case of managing stallion semen it is generally removed before cryopreservation. Hence, the sperm become more susceptible to oxidative stress due to elimination of existing endogenous antioxidants (Loomis and Squires, 2005; Ball, 2008; Alvarenga et al., 2012). Therefore, the supplementation of antioxidants to the extender is beneficial because it reduces ROS production and provides protection against cold shock during cryopreservation of sperm.

The BHT and BHA are synthetic analogues of vitamin E which function by reducing oxygen radicals and interrupting the development of oxidation processes. These substances are being widely used as antioxidants and preservatives to prevent oxidative deterioration of fatty acids in food industry, cosmetics and pharmaceuticals (Jeong et al., 2005; Sebranek et al., 2005; Henrotte et al., 2010). The BHT has been used extensively as a non-enzymatic antioxidant to eliminate ROS and improve sperm quality after freezing and thawing of bull (Shoae and Zamiri, 2008; Ansari et al., 2011), goat buck (Khalifa et al., 2008; Naijian et al., 2013), ram (Farshad et al., 2010), boar (Roca et al., 2004) and dog (Neagu et al., 2010) semen. Similarly the addition of BHA in extender improves motility of ram sperm during storage at 15 °C for 1 day (Upreti et al., 1997). Moreover, the presence of BHA has proved beneficial to decrease ROS-induced DNA damage in the cells treated with estrogenic compounds (Cemeli and Anderson, 2011). Therefore, it was hypothesized that the addition of BHT and BHA in cryopreservation medium might improve the freezing capacity of stallion sperm. The present study was, therefore, conducted to evaluate the effect of different concentrations of BHT and BHA antioxidants on sperm motility, velocity variables, membrane functionality, morphological abnormalities and MDA concentration of Turkmen stallion sperm after freezing and thawing.

2. Materials and methods

2.1. Chemicals and reagents

Eosin, nigrosin and glycerol were obtained from Merck & Co. Inc., penicillin from the SHAFA FARMED industrial Co. (Karaj, Iran) and all of other reagents including HEPES, Citric acid and glucose monohydrate were obtained from Sigma-Aldrich Company (St. Louis, MO, USA).

2.2. Semen collection and processing

A total of 24 ejaculates (six ejaculate per stallion) were collected from four healthy fertile Turkmen stallion (8–10 years old) twice a week during breading season (July and August 2014) by using an artificial vagina (Missouri model, IMV, France). Immediately after collection, the ejaculates were filtered to eliminate the gel fraction and were then immersed in a water bath (32–35 °C) until primary evaluations were made. Only the semen samples with at least 80% of total sperm motility and 10% or less of sperm morphological abnormalities were used for further processing. Sperm concentration was determined with a Neuobauer chamber (World Health Organization, 2010). The gel free semen were then diluted with pre-warmed (to 37 °C) buffer (1:1) and centrifuged to eliminate the semen plasma ($600 \times g$ for 10 min). Almost all of the supernatant (>90%) was removed and the sperm rich pellets were pooled to reduce individual (stallion) differences.

2.3. Extender composition

The basic extender (350 mOsm and pH = 7 to 7.1) used in this study was composed of HEPES (10 g/L), Citric acid (6.5 g/L), glucose monohydrate (50 g/L), penicillin (10 IU/mL), egg yolk (5% v/v) and glycerol (4% v/v). The extender was used for primary evaluation and centrifugation was without glycerol and egg yolk.

2.4. Freezing protocol

Pooled sperm samples were extended to a final concentration of 200×10^6 sperm/mL and divided to eight equal aliquots. The aliquots were diluted with extenders supplemented with different concentrations (0.5, 1 or 2 mM/mL) of BHT or BHA. The positive control samples were diluted with basic extender (without antioxidants) containing ethanol 0.5% (v/v) whereas, negative control samples were diluted with basic extender only. All the samples were cooled to 4 °C, equilibrated for 150 min and then loaded in 0.5 mL French straws (IMV, France). The straws were placed in liquid nitrogen vapors for 10 min and then plunged into liquid nitrogen (−196 °C) and stored until evaluation.

2.5. Thawing and post thaw evaluation

Three frozen straws per each treatment were used to evaluate the post thaw sperm quality. The thawing was conducted in water bath at 37 °C for 30 s. The sperm quality was evaluated immediately after thawing.

2.6. Sperm motility and velocity variables

Motility and velocity variables of sperm were evaluated by Computer assisted semen analyzer (CASA) software (VideoTesT®, Sperm 3.1, Russia) equipped with a phase contrast microscopy system (LABOMED® Lx400, USA). The system variables for CASA were 50 frames per second, minimum contrast was 30, minimum cell size was five pixels, average path velocity (VAP) cutoff value was 30 μm/s, VAP cutoff value for progressive cells was 70 μm/s, straightness was 80%, straight line velocity (VSL) cutoff value was 0 μm/s and amplitude of lateral head displacement (ALH) cutoff value for progressive cells was 0.3 μm. A 10 μL sample of sperm was placed on a pre-warmed glass slide and