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Incubation of spermatozoa with Anandamide prior to cryopreservation reduces cryocapacitation and improves post-thaw sperm quality in the water buffalo (*Bubalus bubalis*)

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ABSTRACT

Anandamide (AEA), an endocannabinoid, has been shown to reduce capacitation and acrosomal exocytosis in human spermatozoa. Because buffalo spermatozoa are highly susceptible to cryopreservation induced damage, AEA was assessed as to whether it could protect spermatozoa from cryo-damage. Six ejaculates from six Murrah buffalo bulls (total 36 ejaculates) were utilized for the study. Each ejaculate was divided into four aliquots; spermatozoa in Aliquot 1 were extended in Tris-Citrate-Egg Yolk and frozen as per the standard protocol. Spermatozoa in Aliquots 2, 3 and 4 were incubated with AEA at 1 nM, 1 μM and 10 μM, respectively in Tris-Citrate extender for 15 min at 37 °C before cryopreservation. Cryopreserved spermatozoa were thawed at 37 °C for 30 s before assessment of sperm motility, membrane integrity, capacitation, acrosome reaction, mitochondrial membrane potential (MMP) and lipid peroxidation status. The proportion of motile and membrane intact spermatozoa were greater ($P < 0.05$) with use of 1 μM AEA incorporated group compared with other groups. The proportion of un-capacitated and acrosome intact spermatozoa was greater ($P < 0.05$) with use of 1 or 10 μM of AEA compared with the other groups. When compared to the control group, use of 1 μM AEA resulted in a greater proportion of spermatozoa with high MMP ($P < 0.05$). There was no significant difference in the lipid peroxidation status of spermatozoa among any of the four groups. It was inferred that the protective role of AEA during cryopreservation of buffalo spermatozoa was dose dependent and incubation of spermatozoa with AEA at 1 μM concentration prior to cryopreservation reduced cryo-capacitation and improved post-thaw sperm quality in buffalo.

1. Introduction

Cryopreservation leads to lethal and sub-lethal damage to spermatozoa that include osmotic stress and intra-cellular ice crystal formation, which ultimately reduces the motility, functions and fertilizing potential (Cormier et al., 1997; Watson, 2000; Yeste, 2016). At least 50% of the spermatozoa are rendered immotile during the process of freezing and thawing (Watson, 1995). In addition, it has been reported that premature capacitation-like changes occur in a considerable proportion of the surviving spermatozoa leading to decreased fertility with frozen semen (Watson, 1995; Bailey et al., 2000). Several additives have been tested for

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their ability to prevent cryodamage to spermatozoa, including antioxidants, cholesterol loaded methyl β-cyclodextrin, seminal plasma proteins and oviductal proteins (Bailey et al., 2000; Kumaresan et al., 2006; Kumaresan et al., 2012; Srivastava et al., 2013; Blanch et al., 2014; Tariq et al., 2015) but with limited success.

N-Arachidonoylethanolamide (Anandamide, AEA) is an endogenously synthesized bioactive lipid mediator derived from esters, amides and ethers of long chain polyunsaturated fatty acids (Munro et al., 1993). The AEA, its receptors and synthesizing and degrading enzymes together form the endocannabinoid system (ECS) (Paria, 2002; Wang et al., 2004; Maccarrone, 2005; Schuel and Burkman, 2005). In ejaculated spermatozoa, AEA has been reported to control sperm energy homeostasis, by exerting a lipogenic effect on lipid metabolism leading to accumulation of energy substrates, thus saving energy for final fertilization events (Rossato et al., 2005; Rossato, 2008). In bulls, both AEA and met-AEA (a non-hydrolyzing analogue of AEA), when used at micromolarconcentrations, induced a persistent decrease in sperm motility (Gervasi et al., 2009) and inhibited heparin-induced sperm capacitation (Schuel et al., 1999). The AEA, at micromolar concentrations, inhibits mitochondrial membrane potential and oxygen consumption in spermatozoa, thus interfering with mitochondrial electron transport (Sarafian et al., 2003; Athanasiou et al., 2007). Such an inhibitory role was, however, not observed when used at nanomolar concentrations.

An endocannabinoid gradient is present in the epididymis of mice; greater concentrations of endocannabinoids have been detected in mouse spermatozoa isolated from the caput of the epididymis, where these cells are functionally immotile, while lesser concentrations have been found in spermatozoa isolated from the cauda where these cells acquire motility (Ricci et al., 2007; Cobellis et al., 2010). Further, AEA is also present in the seminal plasma and may function as a decapacitating factor (Schuel et al., 2002). Because endogenous/exogenous AEA suppressed sperm motility and protected spermatozoa from pre-mature capacitation, it was hypothesized in the present research that incorporation of AEA would protect spermatozoa from cryo-damage during cryopreservation. In support of this hypothesis, a recent study conducted on ram spermatozoa indicated that met-AEA at 1 mg/mL concentration protected ram sperm against cryo-capacitation and acrosome reaction (Talebiyan et al., 2015). Because the buffalo spermatozoa are highly prone to cryo-damage (Singh et al., 2016) resulting in a reduction in quality and fertility of the semen sample, the present study was undertaken to access the protective effects of AEA on cryopreservation induced damage to buffalo spermatozoa. Further, different concentrations of AEA were also evaluated to ascertain the effect on post-thaw sperm quality.

2. Materials and methods

The present study was conducted at Artificial Breeding and Research Centre and Theriogenology Laboratory, Animal Reproduction, Gynaecology& Obstetrics, National Dairy Research Institute (NDRI), Karnal, Haryana, India. All the experimental procedures and protocols were duly approved by the Institute's Animal Ethics Committee.

2.1. Experimental animals

Murrah breeding buffalo bulls ($n = 6$; age 4–6 years of age) used under a progeny testing program were utilized for the study. All the experimental bulls were maintained using uniform management conditions. Vaccination, de-worming, regular check-up for communicable diseases and other herd-health programs were followed as per the farm schedule, and the bulls were free from major infectious diseases. Six ejaculates from each bull (total 36 ejaculates) were utilized for the study.

Ejaculates were collected using artificial vaginas (IMV, L'Aigle, France) at weekly intervals. Standard semen characteristics such as mass activity and progressive motility were evaluated and only those ejaculates having ≥ +3 mass activity and ≥70% progressive motility was considered for further processing and cryopreservation. Semen was divided into fouraliquots-A1, A2, A3 and A4. Spermatozoa in Aliquot A1 were cryopreserved as per the standard protocol (control). Briefly, semen sample was extended in Tris Egg yolk-citrate extender (hydroxymethyl aminomethane − 274 mM, citric acid- 87 mM, glucose- 43 mM, Glycerol- 6.4%, Egg yolk- 20%, benzyl-penicillin-10^6 IU/L, streptomycin- 1 g/L) in the ratio of 1:10. Diluted semen was placed in 0.25 mL French straws, sealed with polyvinyl alcohol and equilibrated at 4 °C for 4 h before exposing to liquid nitrogen vapor for 10 min followed by plunging the straws into liquid nitrogen. Spermatozoa in Aliquots A2, A3 and A4 were incubated with AEA at 1 nM, 1 μM and 10 μM, respectively in Tris-citratebuffer for 15 min at 37 °C and then cryopreserved as indicated previously in this Materials and Methods.

Frozen semen straws prepared from each aliquot were utilized for sperm function assay. Before evaluation, semen was thawed at 37 °C for 30 s. All assays were performed in duplicate, and at least 200 spermatozoa were examined in each smear. The following assays were conducted with frozen-thawed buffalo spermatozoa.

2.2. Sperm motility

Post-thaw motility was assessed soon after thawing. A drop of semen was placed on a pre-warmed slide, covered with a cover slip and examined under a microscope fitted with a stage warmer. Analysis of sperm motility and sperm functional attributes (blind evaluation of the samples) was conducted by a single experienced person to avoid individual variations.

2.3. Sperm membrane integrity

Carboxyfluorescein diacetate − propidium iodide (CFDA-PI) staining was performed according to method described by Singh et al. (2016) with minor modifications. Frozen-thawed spermatozoa were washed twice in sperm-Tyrode's albumin lactate pyruvate (TALP − 100 mMNacl, 3.1 mMKCl, 25 mM $NaHCO_3$, 0.29 mM NaH_2PO_4, 21.6 mM $C_3H_5NaO_3$, 2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM