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Effects of astaxanthin supplementation on the sperm quality and antioxidant capacity of ram semen during liquid storage

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ABSTRACT

The aim of this study was to evaluate the effects of astaxanthin (AST) supplementation of semen extender on the quality of ram semen during storage at 4 °C for periods up to 72 h. Semen samples were obtained from five Small-tail Han rams by use of artificial vagina during normal reproduction season (August to October in 2014), and diluted with glucose-egg yolk buffer supplemented with 0 (control), 0.5, 1, 2, or 4 μM AST. The results showed that supplementation of the extender with 2 μM and 4 μM of AST increased sperm vitality and plasma membrane integrity ($P < 0.05$), and significantly reduced malondialdehyde and reactive oxygen species levels during the 72 h storage period ($P < 0.05$). Acrosome integrity did not differ among treatments. After 72 h of storage in extender, samples were analyzed with computer-assisted semen analysis. The motility of sperm in the 2 μM and 4 μM AST treatment groups increased significantly ($P < 0.05$) compared to the control treatment, while curvilinear velocity, progressive velocity, and path velocity were not affected. In conclusion, the present study demonstrated that supplementing semen extender with AST can improve ram semen preservation via protecting plasma membrane integrity.

1. Introduction

Sperm suffer from a decrease in motility and membrane integrity as semen cools, leading to shorter sperm survival time in the female reproductive tract, reduced fertility, and increased embryonic loss (Kasimanickam et al., 2007). Since oxidative stress from defective antioxidant defense may be responsible for decreasing quality during sperm preservation (Bucak et al., 2007), we sought a manner to protect against this stress during storage.

Sperm are vulnerable to oxidative stress due to proportionately high levels of polyunsaturated fatty acids within their membranes (Almbro et al., 2011). The most significant effect of reactive oxygen species (ROS) on spermatozoa occurs following the ionophore-induced acrosome reaction and subsequent production of singlet oxygen species, leading to lipoperoxidation (LPO) (Griveau et al., 1995). Currently, the majority of studies on the effects of LPO assess the production of malondialdehyde (MDA), a product of lipoperoxidation. In addition, at maturation these unique cells lose most of their cytoplasm and undergo chromosomal condensation, reducing their endogenous antioxidant ability and increasing their dependence upon the extracellular environment.

Some progress has been made in antioxidant treatment of stored semen. *In vitro* studies have shown that

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exogenous antioxidant treatment improves the vitality and motility of sperm from bulls (Sariozkan et al., 2014), rams (Asadpour et al., 2012) and boar (Luno et al., 2014) during liquid storage. Recently, the red carotenoid pigment astaxanthin (AST), a potent antioxidant present in some marine organisms (Lai et al., 2004), has been utilized in numerous studies (Fassett et al., 2008; Kuroki et al., 2013). AST readily penetrates biological membranes, and is known to protect fatty acids and biological membranes from lipid peroxidation with minimal negative side effects (Goto et al., 2001; Dona et al., 2013). The antioxidant activity of AST is reported to be approximately ten-fold higher than that of other carotenoids, and about a hundred-fold greater than that of tocopherol. However, the effects of AST on ram semen have not been reported previously.

The aim of this study was to investigate the antioxidant capacity and potentially protective effects of AST on ram sperm vitality, acrosomal integrity, plasma membrane integrity, and kinematics during liquid storage at 4 °C for periods of up to 72 h.

2. Materials and methods

2.1. Animals and diet

All procedures involving animals were conducted under the approval of the Chinese Academy of Science Animal Care and Use Committee. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Five Small-tailed Han breeding rams were used, each 2.5–3.0 years old and weighing approximately 60–70 kg. All animals were fed the same diet according to National Research Council (NRC) recommendations of 60:40 forage:concentrate *ad libitum* and had free access to fresh water.

2.2. Collection and spermatological examination of semen

Ejaculates were collected using an artificial vagina twice a week for 10 weeks in the autumn of 2014. A total of 50 second ejaculates (the first ejaculates being discarded) from the 5 rams were obtained. Ejaculates with a volume of 0.7–2.0 mL, a sperm concentration of $>2.5 \times 10^9$/mL, and motility of over >80% were pooled.

2.3. Semen dilution, cooling, and preservation

The extender contained 80% (V:V) basal solution (3.00% glucose, 3.00% sodium citrate, penicillin (10 IU), streptomycin (10 IU)) in distilled water and 20% (V:V) egg yolk (fresh). To make experimental extenders, AST was added to final concentrations of 0.5, 1, 2, and 4 μM. Extender without AST supplementation was used as a control.

Every ejaculate was thoroughly mixed, divided into 5 aliquots, and incubated at 37 °C for 30 min. After dilution to a sperm concentration of 1.0×10^9 cells/mL, aliquots were placed at 4 °C for storage. Semen samples were assessed for sperm motility, acrosomal and plasma membrane integrity and antioxidant capacity (MDA and ROS levels) at 0, 24, 48 and 72 h of storage time. Kinematic parameters were analyzed by computer-assisted sperm analysis (CASA) after 72 h of storage.

2.4. Sperm vitality assessment

Nigrosineosin stain dilution was dissolved 1.40% eosin-Y (Beyotime, Jiangsu, China), 8.72% nigrosin (Beyotime, Jiangsu, China), and 2.53% sodium citrate in distilled water. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

2.5. Acrosome integrity detection

To assess sperm acrosomal integrity, 100 μL of semen sample was fixed in 500 μL of 1% formal citrate (2.79% tri-sodium citrate dihydrate, 0.37% formaldehyde in distilled water). Acrosomes of 200 spermatozoa were examined under oil immersion at 1000× magnification. The integrity of each acrosome was determined by the presence of a normal apical ridge (Andrabi, 2009).

2.6. Hypo-osmotic swelling test (HOST)

In brief, 50 μL of semen was incubated with 300 μL of hypo-osmotic solution (2.04% fructose and 0.71% sodium citrate in distilled water) at 37 °C for 60 min. Then, a 100 μL drop of the mixture was spread on a warm slide with a cover slip and 200 sperm were evaluated under 1000× magnification using a light microscope. The morphology of sperm (normal tails vs. swollen or coiled tails) was recorded (Buckett et al., 1997).

2.7. The detection of sperm lipid peroxidation

The content of MDA in semen samples was measured using Lipid Peroxidation MDA Assay Kit (Beyotime, China). Briefly, 100 μL of sample or standard was combined with 200 μL of thiobarbituric acid (TBA) working solution, boiled for 15 min, and cooled to 25 °C. The OD was determined by a Microplate Reader (Imark, Bio-Rad, USA) at 532 nm and 450 nm. The content of MDA of each sample was determined using the standard curve, and expressed as μmol MDA/10^9 sperm.

2.8. The detection of total ROS

The intracellular ROS content of the spermatozoa was determined using the Reactive Oxygen Species Assay-Kit (Beyotime, China). Briefly, the samples were diluted to 20×10^6 cells/mL, and the probe (6-carboxy-2′-7′-dichlorodihydrofluorescein diacetate, DCFH-DA) was added to a final concentration of 10 μM. Following incubation, sperm cells were washed with PBS and the OD value was determined by a Microplate Reader (Imark, Bio-Rad, USA) at 488 nm and 525 nm. The ROS concentrations were determined using a standard curve and expressed as μmol/mL.

2.9. Analysis of sperm kinematics using CASA

The kinematics of each sperm sample was assayed at the end of the 72 h storage period, using a computer-aided sperm analysis system (CASA, Minitube, Germany). The following sperm characteristics were analyzed: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), progressive velocity (VSL, μm/s), and path velocity (VAP, μm/s).

2.10. Statistical analyses

Values are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using SPASS18.0 (SPSS Inc, Chicago, IL, USA) for Windows. The effects of AST supplementation on sperm motility parameters, plasma membrane integrity, acrosome status, MDA and ROS level were analyzed by analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. The effects of AST on sperm quality

3.1.1. Vitality

The vitality of sperm treated with 2 μM and 4 μM AST trended upwards compared to the control group at 48 h of storage at 4 °C, although without statistical significance ($P > 0.05$). After 72 h at 4 °C, significant increases were observed in ejaculates treated with 2 μM and 4 μM AST in vitality (74.17% ± 2.60, 73.83% ± 2.29) compared to the control group (65.49% ± 3.03) ($P < 0.05$) (Fig. 1A).