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Canthaxanthin enhances the quality of cryopreserved goat spermatozoa

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Abstract

The excessive production of reactive oxygen species during the freezing and thawing process triggers lipid peroxidation in the sperm membrane, resulting in oxidative harm and a decline in semen quality. The aim of this study was to examine the impact of different concentrations of canthaxanthin on the quality of goat spermatozoa throughout the process of cryopreservation. Semen was collected from each goat using an artificial vagina for a continuous duration of 3 weeks. The semen was divided into five equal portions and mixed with an extender having different concentrations of canthaxanthin: 0.00 (control), 5.00, 15.00, 25.00, and 35.00 µM. The computer-assisted semen analyzer was used to determine the sperm motility and kinetic parameters. The findings indicated that the inclusion of canthaxanthin at a concentration of 5.00 µM resulted in a minimal enhancement in sperm kinematic factors such as overall motility, progressive motility, fast progressive motility, curve-line velocity, distance curve line, amplitude of lateral head displacement, beat-cross frequency, wobble, linearity, and straightness. Nevertheless, these enhancements did not demonstrate statistical significance compared to the control, 15.00, 25.00, and 35.00 µM. The introduction of 5.00 µM of canthaxanthin resulted in a significant rise in straight-line velocity, average path velocity and head activity compared to the control group. Hence, the addition of 5.00 µM of canthaxanthin could be employed to improve the cryopreservation of goat spermatozoa.

Introduction

Semen cryopreservation can lead to structural damage and reduced sperm functionality due to cellular oxidative stress, cryoprotectant toxicity and osmotic pressure imbalances.[1,2] The sperm cell membrane primarily consists of substantial amounts of polyunsaturated fatty acids, which are crucial for maintaining plasma membrane properties such as domain fluidity, elasticity, and differential permeability.[3-5] Nevertheless, this high polyunsaturated fatty acid content also causes spermatozoa susceptible to lipid peroxidation (LPO) due to the high vulnerability of polyunsaturated fatty acids to oxidative attack by reactive oxygen species (ROS).[6,7] During freezing and thawing, excessive ROS formation induces LPO in the sperm membrane, resulting in oxidative damage, reduced semen quality, and diminished fertilization potential.[8,9] Goat sperm exhibits distinct characteristics, including a higher concentration of cell membrane lipids, which renders it more vulnerable to oxidative damage during cryopreservation.[10] This increases its susceptibility to cold shock and LPO.[11,12] Additionally, a high concentration of lipids might enhance the membrane permeability to water, resulting in osmotic stress and alterations in cell volume during the freezing and thawing processes.[13] Thus, to mitigate these adverse effects, enhancing the anti-oxidation capacity of sperm is considered a promising strategy. Research has demonstrated that the addition of exogenous antioxidants to the extender is a successful strategy for improving the quality of semen cryopreservation.[2,4,8,14-17] Canthaxanthin, also known as β, β-carotene 4,4' dione, is a carotenoid found in various organisms such as plants, algae, fungus, bacteria, crustaceans, fish, and birds. Additionally, it can also be synthesized in the laboratory by through microbial, particularly by using Escherichia coli.[18,19] This compound

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is well-documented for its antioxidant properties, including its ability to remove ROS and inhibit lipid phosphorylation.[20] Several investigations have shown evidence for the protective properties of canthaxanthin as an antioxidant in cell cultures.[18,21] Research examining the impact of canthaxanthin on human sperm revealed significant improvements in parameters including motility, viability, morphology, acrosome reaction, chromatin packaging, and DNA integrity during the freeze-thaw process.[22] Addition of 1.00 μM of canthaxanthin enhances acrosome integrity, chromatin packing (toluidine blue), and DNA denaturation and fragmentation.[23] Furthermore, studies on Santa Ines goats also evaluated the impact of various concentrations of canthaxanthin (0.00, 0.10, 1.00, 10.00, and 25.00 mM) as a supplement in Tris-egg yolk extender on the semen quality after thawing and incubation at 37.00 ˚C. Results indicated that the addition of 10.00 and 25.00 mM of canthaxanthin during the freezing process of goat semen maintains sperm motility after thawing and incubation at 37.00 ˚C for 2 hr.[4] Despite these findings, limited research has explored the anti-oxidant potential of canthaxanthin in goat semen extender. Therefore, here we evaluated the effect of different concentrations of canthaxanthin in Tris-egg yolk extenders on goat semen quality during cryopreservation and pregnancy rate.

Materials and Methods

Animal management and semen collection. This study used five goats aged 2 to 4 years. Semen was collected weekly from each goat, with two ejaculations every week using an artificial vagina during a period of 3 consecutive weeks.[24] After collecting, the semen was transported to a laboratory and maintained at 35.00 ˚C in a water bath. The ejaculates from each goat were assessed for motility percentage with a minimum motility of 75.00% set as the threshold for fresh semen analysis. The care and use of animals in this study were regulated by Center of Animal Use and Care Committee at University of Phayao, Thailand. The committee scrupulously followed its guidelines, as indicated by approval No. UP-AE 63–01-04-0011.

Preparation of extenders and processing of semen. The cryopreservation of goat semen was conducted using a Tris-base (HiMedia, Mumbai, India) extender solution containing fresh egg yolk at a concentration of up to 20.00%. The Tris-base extender solution comprised 150 mM tris-(hydroxymethyl)-aminomethane, 47.50 mM citric acid, 14.00 mM glucose, 6.00% (v/v) glycerol, 2,000 IU mL^{-1} benzyl penicillin, and 1.00 mg mL^{-1} streptomycin sulfate. The samples were divided into five equal fractions and combined with extenders containing 0.00 (control), 5.00, 15.00, 25.00, and 35.00 μM of canthaxanthin (Cayman Chemical Co., Ann Arbor, USA), respectively.[25] Afterward, the semen samples were diluted and divided into 0.25 mL straws. The straws were quickly cooled to 4.00 ˚C in 90 min and maintained at that temperature for an extra 90 min.[26] The semen straws were placed 5.00 cm above liquid nitrogen for 15 min before being submerged in liquid nitrogen for storage at –196.00 ˚C.

Assessment of sperm movement characteristics. Three semen straws from each freezing group were thawed at 37.00 ˚C for 30 sec in a water bath. Following thawing, 3.00 μL of semen from each straw was pipetted and placed onto pre-heated slides maintained at 37.00 ˚C. The motility and kinetic characteristics of the semen were assessed using Computer-Assisted Sperm Analysis with the Androvision® (version 1.0.0.9; Minitübe GmbH, Tiefenbach, Germany).[27] The following parameters were measured: Total motility, progressive motility, progressive fast motility, curve-line velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), distance curve line (DCL), distance straight line (DSL), distance average path (DAP), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), head activity (HAC), wobble (WOB), linearity (LIN), and straightness (STR).

Female management and artificial insemination. The study utilized 39 female Thai native × Boer crossbred goats, aged between 1 and 3 years. Animals were selected based on the following characteristic including, body condition ratings ranging from 2.50 to 3.50, vaccination records (peste des petits ruminants and foot-and-mouth disease), parasite control documentation (latest albendazole treatment: 14.20 ± 3.50 days prior to the trial) and confirmed seronegative status for caprine arthritis-encephalitis and brucellosis. The feeding regimen (forage and concentrate composition), housing environment (barn design, stocking density, and ventilation), and daily husbandry practices including routine health monitoring and biosecurity measures were also controlled. The initiation of estrus was assessed utilizing sexually active goats. The initiation of estrus (0 hr) was marked by the goat receptivity for mounting by the buck. A total number of 39 female goats were subjected to cervical insemination on two occasions. All does were inseminated at precisely 24 and 36 hr using frozen-thawed straw semen (0.25 mL) containing 200 × 10^6 spermatozoa *per* 0.25 mL straw. Pregnancy diagnosis was conducted 42 days post-insemination using trans-abdominal ultrasonography with HS-1600V (Honda Electronics, Toyohashi, Japan).[28] The birth details were documented to verify that they originated from insemination.

Statistical analysis. The data were statistically analyzed using the General Linear Model technique and analysis of variance (ANOVA) in R Software (R Foundation for Statistical Computing, Vienna, Austria). The data were reported as the mean ± SEM, and *p*-values less than 0.05 were deemed statistically significant using Duncan's multiple-range test for post hoc pairwise comparisons.

Results

Canthaxanthin supplementation in semen extender enhanced the quality of post-thawed sperm in goats. To enhance the quality of goat sperm after thawing, canthaxanthin supplementation was incorporated into the Tris-egg yolk extender. The group supplemented with 5.00 µM of canthaxanthin showed a slight increase in total motility, progressive motility and parameters such as VCL, DCL, ALH, WOB, LIN, and STR. However, these increases were not statistically significant compared to the control group and the groups supplemented with 15.00, 25.00, and 35.00 µM of canthaxanthin ($p \geq 0.05$). The group supplemented with 5.00 µM of canthaxanthin showed a marked enhancement in specific parameters such as VSL, VAP, and HAC relative to the control group, while DSL and DAP also presented elevated values at 5.00 µM, exhibiting partial significance with the groups supplemented with 35.00 µM of canthaxanthin. However, these differences were not significantly different compared to the other supplemented groups (Table 1).

Efficiency of thawed goat spermatozoa in artificial insemination and pregnancy rate. To evaluate the effect of 5.00 µM canthaxanthin supplementation in the extender on semen cryopreservation efficiency during artificial insemination in goats, pregnancy outcomes were analyzed. Ultrasound findings indicated that eight out of 20 animals (40.00%) in the control group and nine out of 19 animals (47.36%) in the 5.00 µM canthaxanthin-treated semen group were pregnant the odds ratio of 1.35 (95.00% CI: 0.38 - 4.80). Although the pregnancy rate was higher in the 5.00 µM canthaxanthin-treated group compared to the control group, the difference was not statistically significant ($p > 0.05$).

Discussion

Oxidative damage during sperm freezing is primarily caused by the excessive production of ROS, leading to significant changes in several sperm characteristics. Oxidative stress diminishes sperm quality by initiating a cascade of events including reduce intracellular adenosine triphosphate levels, release apoptotic agents (such as procaspase, cytochrome C, and apoptosis inducers) from damaged mitochondria into the cytosol, disruption of biochemical pathways, impairment of enzyme function and increased membrane permeability.[23,29] This process triggers apoptosis and compromises the flexibility and stability of the sperm membrane, leading to reduced fecundity due to the oxidation of unsaturated fatty acid phospholipids.[30,31] Canthaxanthin is a powerful keto-carotenoid recognized for its diverse antioxidant properties and significant coloring effects.[20] It exhibits free radical scavenging and antioxidant properties such as the induction of catalase and superoxide dismutase.[21,24] Canthaxanthin was integrated into exosomes using sonication which boosted total antioxidant capacity and reduced ROS levels, sperm DNA fragmentation, and sperm death.[32,33] In this study, we identified canthaxanthin as a potential cryoprotectant for goat semen intending to examine its protective effects during sperm freezing and to determine the optimal dosage. Our results demonstrated that the incorporation of 5.00 µM canthaxanthin into the semen extender during cryopreservation enhanced the motility of goat sperm post-thawing.[34] The performance indicated that VSL, VAP, and HAC achieved values of 27.65 ± 2.32, 35.96 ± 2.65, and 0.26 ± 0.02, respectively, after thawing but when the concentration of canthaxanthin was overly elevated, the protective effect on semen was not

Table 1. Effects of different percentages of canthaxanthin supplementation in Tris-base extender on quality of cryopreserved goat semen.

Parameters	Control	5.00 µM	15.00 µM	25.00 µM	35.00 µM
Total motility	55.96 ± 3.26	62.07 ± 4.08	60.27 ± 4.32	57.63 ±3.17	56.93 ± 2.70
Progressive motility	37.46 ± 2.76	45.67 ± 4.50	43.76 ± 4.10	40.01 ± 3.00	37.71 ± 2.49
Progressive fast motility	15.75 ± 1.28	20.22 ± 2.50	20.43 ± 2.33	16.75 ± 1.77	15.83 ± 1.40
Immotile	44.04 ±3.26	37.93 ± 4.08	39.73 ± 4.32	42.37 ± 3.17	43.07 ± 2.70
VCL	57.41 ± 3.07	67.99 ± 5.66	66.94 ± 5.23	59.53 ±3.90	58.15 ± 3.13
VSL	22.14 ± 1.28^{b}	27.65 ± 2.32^{a}	26.31 ± 1.85^{ab}	23.36 ± 1.27^{ab}	23.09 ± 1.03^{ab}
VAP	28.67 ± 1.66^{b}	35.96 ± 2.65^{a}	33.93 ± 2.30^{ab}	30.64 ± 1.70^{ab}	30.36 ± 1.38^{ab}
DCL	14.77 ± 0.77	16.52 ± 1.25	15.89 ± 1.26	14.78 ± 0.89	14.10 ± 0.84
DSL	5.02 ± 0.28^{ab}	5.79 ± 0.48^{a}	5.28 ± 0.39^{ab}	4.80 ± 0.29^{ab}	4.48 ± 0.28^{b}
DAP	7.02 ± 0.36^{ab}	7.98 ± 0.57^{a}	7.45 ± 0.48^{ab}	6.90 ± 0.38^{ab}	6.56 ± 0.38^{b}
ALH	0.72 ± 0.04	0.80 ± 0.06	0.78 ± 0.05	0.73 ± 0.05	0.70 ± 0.04
BCF	6.65 ± 0.37	7.27 ± 0.62	7.35 ± 0.53	6.31 ± 0.39	6.21 ± 0.41
HAC	0.21 ± 0.01^{b}	0.26 ± 0.02^{a}	0.24 ± 0.02^{ab}	0.23 ± 0.01^{ab}	0.22 ± 0.01^{ab}
WOB	0.52 ± 0.01	0.54 ± 0.01	0.52 ± 0.01	0.53 ± 0.01	0.53 ± 0.01
LIN	0.40 ± 0.01	0.43 ± 0.01	0.40 ± 0.01	0.41 ± 0.02	0.41 ± 0.01
STR	0.77 ± 0.01	0.78 ± 0.01	0.77 ± 0.01	0.76 ± 0.01	0.76 ± 0.01

VCL: Curve-line velocity; VSL: Straight-line velocity; VAP: Average path velocity; DCL: Distance curve line; DSL: Distance straight line; DAP: Distance average path; ALH: Amplitude of lateral head displacement; BCF: Beat-cross frequency; HAC: Head activity; WOB: Wobble (VAP/VCL); LIN: Linearity (VSL/VCL); STR: Straightness (VSL/VAP).

[ab] Different superscripts within a row means significant difference ($p < 0.05$).

good, which was manifested by the decreasing trend of VSL, VAP and HAC parameters.[35] Therefore, we proposed that 5.00 µM of canthaxanthin might be the optimal concentration for supplementation. Alteration of the body anti-oxidant balance leads to ROS over-generation, causing LPO in the aggregated sperm plasma membrane, hence, compromising its integrity. Notably, the addition of canthaxanthin at concentrations of 15.00, 25.00, and 35.00 µM have resulted in a small reduction in the kinetic parameters of VSL, VAP, and HAC in sperm cells post-thawing.[36] Previous studies indicated that the addition of 10.00 and 25.00 µM canthaxanthin in ram semen extenders effectively safeguarded ovine sperm against kinetic changes after a 2-hr incubation at 37.00 ˚C post-thawing during cryopreservation. The impact of various concentrations of canthaxanthin, specifically 0.00, 0.10, 1.00, 10.00, and 25.00 µM, on human sperm parameters during the freeze-thaw process has been investigated.[23] The results demonstrated that 25.00 µM canthaxanthin significantly improved progressive and total motility, vitality, normal morphology, chromatin packaging and acrosome integrity, and decreased DNA denaturation and fragmentation. The effects of canthaxanthin on DNA denaturation, chromatin structure and DNA fragmentation are crucial for understanding its possible impact on genomic stability and cellular systems.[37] Ten micromolar canthaxanthin dramatically enhanced overall motility, vitality, normal morphology, chromatin packaging, acrosome integrity and DNA denaturation and fragmentation. In the 1.00 µM group, significant differences were observed mainly in the enhancement of acrosome integrity, chromatin packaging, and DNA denaturation and fragmentation.[23]

Furthermore, a 5.00 µM canthaxanthin supplement in the extender was used in artificial insemination for determining the pregnancy rate. The pregnancy rate was increased from 40.00% in the control group to 47.36% in the canthaxanthin-supplemented group, reflecting a 7.36% improvement. However, the *p*-value (0.647) suggested that this difference lacked statistical significance and miht be attributable to random variation. The efficacy of semen extenders and subsequent artificial insemination outcomes may be significantly affected by internal (biological) factors.[38] Cryopreservation, crucial for assisted reproductive technologies and the preservation of genetic resources, relies mainly on cryoprotective agents to mitigate the detrimental effects of ice crystal formation and osmotic stress during freezing and thawing procedures.[39] Variability in sperm resistance to cryodamage including differences in membrane lipid composition and endogenous anti-oxidant concentrations may lead to different post-thaw survival rates even among sperm from the same ejaculate.[40] Endogenous seminal proteins and enzymes may affect exosome absorption or canthaxanthin bioavailability, hence, altering sample consistency.[32] External factors such as uterine pH, immunological reactions and cervical mucus viscosity may influence sperm motility and viability following insemination.[41,42]

The current research showed that a 5.00 µM concentration of canthaxanthin added to semen extender enhanced sperm parameters including VSL, VAP, and HAC, post-cryopreservation and is applicable in goat artificial insemination. Further research is essential to elucidate the chemical mechanism by which canthaxanthin functions as a potent anti-oxidant affecting sperm parameters.

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Conflict of interest

The authors stated that there are no conflicts of interest related to the research.

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