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The Effects of Various High Dosage of α-Tocopherol and Ascorbic Acid in Tris Egg Yolk Extender on Post-Thawed Sperm Quality in Tropical Brahman Bulls

Langgeng Priyanto[1,2]*, Herdis[2], Santoso[2], Pradita Iustitia Sitaresmi[2], Tri Puji Priyatno[2], Rahma Isartina Anwar[2], Desiana Ade Mahari[2], Florentina Bety Indah Lupitasari[2], Maman Surachman[2], I Wayan Angga Darmawan[2], Arfan Abrar[1] and Arum Setiawan[3]

[1]Department of Animal Science, Faculty of Agriculture, Sriwijaya University, South Sumatra, 30862, Indonesia.
[2]Research Center for Animal Husbandry, National Research and Innovation Agency, Cibinong Science Center, Jalan Raya Jakarta-Bogor, Bogor, 16915, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University, South Sumatra, 30862, Indonesia

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Key words
Tocopherol, Ascorbic acid, Morphology, Sperm, Tropical bull

ABSTRACT

The primary causes of sperm quality decline during the freeze-thaw pathway are the peroxidation hazard caused by reactive oxygen species produced by the biological molecules of sperm. Alpha-tocopherol (Vitamin E) and ascorbic acid (Vitamin C) are two potent antioxidants that operate to prevent oxidation processes. The objective of this study was to analyze the effects of α- tocopherol and ascorbic acid on the motility, viability, abnormality, and plasma membrane quality of post-thawed Tropical Brahman bull sperm. Sperm samples were obtained and pooled from eight sexually mature Tropical Brahman Bull, separated into seven equal proportions, and diluted with TEY extender Semen was supplemented with 0 (C0;E0), 5 (C5;E5), 10 (C10;E10), and 15 (C15;E15) mg/mL of each α-tocopherol and ascorbic acid, respectively. Total sperm motility, viability, abnormalities, and semen membrane plasma were analyzed after thawing (%). C5 and E15 extenders resulted in higher total motility (p 0.05) than the other extenders, with the exception of C10 and C15, which were lower than that of the control. The extender E15 ($P<0.05$) exhibited the highest motility. Furthermore, compared with the other extenders, the C5 and E15 extenders led to better vitality and membrane plasma in post-thawed spermatozoa (p 0.05). Finally, compared with the other extenders, abnormalities were lower in the C5-and E10 extenders ($p<0.05$). Finally, the current findings show that C5 and E15 can increase the quality of post-thawed Brahman bull spermatozoa.

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INTRODUCTION

In general, tropical weather conditions negatively affect farm animals. In addition to the scarcity of desirable feed, the issue of small farm animal ownership by local farmers (i.e., one farmer can retain only six cattle and use agricultural waste as a source of animal feed) is a major impediment to the stagnation of the cow industry, particularly in developing nations (Agus and Widi, 2018). Cattle in the tropics tend to exhibit large seasonal fluctuations in fertility, which are related to dietary, environmental, phenotypic, and possibly photoperiodic factors. However, bull-related factors rarely contribute to seasonal fluctuations in herd fertility. This is despite the fact that bulls from tropical zones also exhibit seasonal changes in semen quality and freezing capability, the severity of which can result in economic losses and varies depending on genotype. Furthermore, tropical countries have maritime geographical characteristics, and are divided into small islands that are far away. Therefore, good management of artificial insemination using frozen semen is essential to improve the beef cattle sector's

* Corresponding author: langgengpriyanto@fp.unsri.ac.id
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ability to overcome this problem (Ratnani *et al*., 2020). In addition, an advantageous solution in the form of additional active ingredients is required to improve the sperm quality (Bustani and Baiee, 2021).

Although cryopreservation can significantly increase the time required for bull spermatozoa to be stored, it also enables them to be transported over long distances (Ugur *et al*., 2019) and solves the aforementioned issues. However, it still presents a number of difficulties, such as intracellular and extracellular frost emergence, cold shock, compound toxic effects of cryoprotectants, osmolarity concussion, lipid oxidation concussion, and apoptosis when cryopreservation and thawing are conducted (because of its biological profile, Brahman bull semen is sensitive to temperature fluctuations throughout the thawing process), which seriously damage semen quality and impair fertility (Peris-Frau *et al*., 2020). Increased plasma membrane fluidity and permeability, excessive formation of reactive oxygen species (ROS), decreased acrosome integrity (Bollwein and Bittner, 2018), diminished mitochondrial membrane potential, and decreased sperm motility have all been linked to cryopreservation. Bull spermatogenic membranes are rich in polyunsaturated fatty acids (PUFAs) and sensitive to oxygen-induced damage caused by lipid peroxidation. As a result, they are vulnerable to ROS intrusion, which reduces sperm motility and negatively affects sperm activation and acrosome reaction. This is likely because axonemal destruction and reduced sperm viability are prompted by a dramatic deterioration in intracellular ATP levels (Collodel *et al*., 2021). Antioxidants diminish, interrupt, or encourage the elimination of ROS or oxidative damage in sperm cells (Samoylenko *et al*., 2013). Natural antioxidants, such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C), prevent spermatozoa in bovine semen from impairment resulting from ROS (Ejaz *et al*., 2012). α-tocopherol is a significant chain-breaking antioxidant because it can directly suppress free radicals such as peroxyl and alkoxyl (ROO•) formed during ferrous ascorbate-induced LPO (Yimer *et al*., 2016). Ascorbic acid protects against endogenous oxidative damage by dissipating hydroxyl, superoxide, and peroxide radicals (Kaźmierczak-Barańska *et al*., 2020). But, excessive use of ascorbic acid and α-tocopherol is also known to have a negative impact on sperm quality, and until now data on the use of higher doses of vitamins has rarely been reviewed. This study aimed to determine whether adding ascorbic acid and α-tocopherol to the extender enhances the freezing ability of Brahman bull spermatozoa especially in high dosage. The influence of ascorbic acid and α-tocopherol motility, plasmalemma prospering, viability, and chromatin damage in frozen-thawed adapted tropical Brahman bull semen was, therefore, the subject of this study, which is still rare.

MATERIALS AND METHODS

Animal and semen collection

Semen samples from eight adult Brahman Tropic-adapted bulls (ages 3-6) were used in this study. These bulls were acclimated to the AI center and subjected to breeding center procedures of the Indonesian National System of Animal Health. This study was conducted between June 2022 and December 2022. The Center for Superior Animal Breeding and Forage (BPTU HPT) Sembawa (103° N, 104° E) Indonesia, provided housing for animals. All bulls appeared to be in a good condition. The animals were maintained under similar management and nutritional conditions throughout the study period. The bulls were fed a combination of forage (10% BW) and concentrate (1% BW) twice per day, with water provided *ad libitum*. Using an artificial vagina, 80 ejaculates were collected twice per week from the bulls. Bulls were confined to cows in estrus, while being handled by a person with an artificial vagina to collect ejaculates. Ejaculates were assessed and included in this study provided they satisfied the following requirements: volume of 2–10 mL; more than 80% of progressive motile sperms and less than 10% of abnormal sperm

Semen analysis

The amount of each ejaculate was measured, and sperm concentration was evaluated by diluting the sperm with 3% NaCl and counting the sperm in five squares of one chamber using a hemocytometer. Sperm motility is defined as the progressive movement of sperm cells. An experienced and knowledgeable investigator assessed the sperm motility from 0% to 100%. Sperm were placed on a heated glass slide and scoring was performed at a magnification of 200 ×. Each sample was assessed twice. The mean value was used for data analysis. Eosin-nigrosin staining was performed to assess the presence of aberrant and normal spermatozoa. Rapid dyes have been used to count spermatozoa with defective acrosomes (Kondracki *et al*., 2017). Based on the coiled and swollen tails, a hypoosmotic swelling test was performed to determine the functional integrity of sperm membranes. This was accomplished by incubating 0.1 ml of sperm with 1 ml of a 150 M hypoosmotic solution at 37 °C for 30 min. After incubation, 0.2 ml of the solution was distributed on a warm microscope slide using a coverslip. The 1000 magnification was used to examine 200 spermatozoa under bright-field microscopy (Bucak *et al*., 2012).

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Experimental design

Yolk citrate (2.9% (v/v) sodium citrate dihydrate, 100 ml aquadest mixed with egg yolk 20%, 8% (v/v) glycerol (Merck, Germany), 1.000 IU/ml Penicillin, and 1.000 mg/ml streptomycin) was used as the basic semen diluent (freezing extender). The pure Ascorbic acid and α-tocopherol used in this study was acquired from Sigma (St. Louis, MO, USA), and stored in a refrigerator at 5°C. α-Tocopherol was dissolved in 0.05% ethanol before addition to the semen extender.

The semen was dissolved to a final concentration of 50 × 10^6 cells/ml. Semen was supplemented with 0 (C0; E0), 5 mg/mL (C5; E5), 10 (C10; E10), and 15 (C15; E15) mg/mL of each ascorbic acid and α- tocopherol, respectively. Then, the semen was put into 0.25 mL straw and chilled for 30 min. For 10 min, the straw was frozen in liquid nitrogen vapor (5 cm above liquid nitrogen) and the semen was immersed in liquid nitrogen for storage. The straws were thawed separately in a water bath for 30 s after freezing for more than 6 days, for semen assessment. All the frozen sperm samples were immediately evaluated for sperm quality. The purpose of this study was to examine the effect of antioxidant supplementation on sperm quality, both before and after thawing.

Statistical analysis

All trials used at least eight repetitions for each dosage, addictive-type treatment, and control group, and the results are presented as mean values±S.D. The Kolmogorov-Smirnov test was used to analyze data normality. A general linear model procedure was used to evaluate the differences given a Gaussian distribution of the data. When the F-value was significant (P<0.05), the mean values of the percentages of motile sperm, viability, abnormality, and plasma membrane integrity were compared using Duncan's multiple range test after one-way analysis of variance (ANOVA). An independent sample t-test was used to compare the administration of ascorbic acid and α-tocopherol. Statistical product and service solutions were used for all analyses (SPSS 26 for Mac; SPSS, Chicago, USA).

RESULTS

Evaluation quality of fresh semen

Microscopic analysis revealed that the median sperm concentration in this study was 1.475 × 10^6. The average mass motility was (+++), with sperms forming massive waves. Moreover, the sperm had an average of 75.60± 4.55% progressive motility. The mean score is 76%. The average viability of sperms was 80.30±2.11%. The findings in this study were within the normal range for bull sperm concentration. Brahman bull sperm abnormalities range from 3% to 4.5%, with an average of 4.5±0.5%. Table I also display the fresh semen quality of Brahman bulls that have adapted to the tropics. When the tubes were tilted, the average volume of semen, which had a creamy colour, a bull-smelly smell, and a moderately thick consistency, was 5.00± 0.30 mL. The pH measurement was 6.80±0.35. The macroscopic and microscopic quality parameters of fresh sperm (Table I) were assessed to determine whether the ejaculates were suitable for further processing.

Table I. Mean±SD of parameter of fresh semen of Brahman bull.

Evaluation	Brahman fresh semen	Indonesian standard (SNI)
Volume (Ml)	5.00± 0.30	2.60-10.20
Colour	White	White-cream
pH	6.80± 0.35.	6.2-7.2
Consistensy	Medium	Medium
Mass motility	++	+++
Concentration (10^6)	1457	1130-2160
Motility (%)	75.60±4.55	>70
Viaility (%)	80.30± 2.11	>70
Abnormality (%)	6.80± 0.35	<10%

Impact of ascorbic acid and α-tocopherol on the cryopreservation of semen

The addition of different concentrations of ascorbic acid to the diluent significantly decreased sperm motility, viability, abnormality, and plasma membrane integrity compared to the control group (P 0.05), with the exception of the addition of 5 mg/mL ascorbic acid (C5), which improved sperm quality performance compared to the control.

Table II shows that different quantities of α-tocopherol were added to the diluents. Unlike the addition of ascorbic acid, the addition of α-tocopherol significantly increased sperm viability, acrosome integrity, plasma membrane integrity, and mitochondrial activity compared with the control group after thawing (P < 0.05). 15 mg/mL of α-tocopherol concentration produced the best results.

DISCUSSION

Bull semen quality and reproduction can fluctuate throughout the year, and this is apparent in both temperate and tropical climates, owing to factors such as genotype, environment, and linkages between the two. Moreover,

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even bulls that are thought to be very heat-tolerant, especially cattle of the Bos indicus breed, such as the Brahman bull, still undergo seasonal fluctuations in semen quality in tropical regions (Landaeta-Hernández *et al*., 2020). As shown in Table I, although ejaculates are suitable for storage as extended for frozen semen, ejaculates with more than 70% live sperm cell motility and less than 5% morphological abnormalities have been studied (Fernandez-Novo *et al*., 2021). The semen quality of these cattle was lower than that of cattle of the same climate in different breeds (Morrell *et al*., 2018). Therefore, efforts are needed to improve semen quality during dilution and cryopreservation by adding antioxidant ingredients to the diluent. Cryopreservation itself is needed, especially in countries with large areas but relatively low animal ownership capabilities, and local farmers cannot afford to keep bulls for breeding purposes, with frozen semen being a solution to the problem.

Table II. Effect of high dosage ascorbic acid and α-tocopherol in concentration on tris egg yolk (TEY) cryopreservation of Brahmand local bulls.

Concentration (mg/ml)	Sperm motility (%)	Sperm viability (%)	Membrane integrity (%)	Sperm abnormality (%)
Ascorbic acid				
Control (C0)	39.16±0.28[c]	47.26±0.65[c]	34.66±1.15[c]	5.00±0.01[b]
5 (C5)	43.33±0.24[d]	58.63±0.21[d]	49.43±0.41[d]	1.50±0.05[a]
10 (C10)	35.16±0.36[b]	44.53±0.55[b]	29.30±1.19[b]	6.30±0.22[c]
15 (C15)	24.83±0.22[a]	23.01±0.45[a]	21.83±0.29[a]	12.16±0.57[d]
α-tocopherol				
Control (E0)	38.02±1.22[ab]	45.67±3.21[a]	33.55±2.51[a]	5.21±0.28
5 (E5)	48.53±1.27[ab]	62.83±1.25[bc]	44.16±3.75[ab]	4.33±0.67
10 (E10)	52.06±0.53[c]	66.16±1.04[bc]	47.59±1.82[ab]	4.21±0.28
15 (E15)	56.05± 0.55[d]	71.51± 1.81[d]	49.54± 4.07[b]	6.57± 2.64

[a, b, c, d] different subscript means very significantly different ($p<0.001$). Control (C0; E0) = No addition of ascorbic acid and E; C5; E5= addition of 5mg/ mL ascorbic acid and α-tocopherol to the extender; C10; E10= addition of 10 mg/mL ascorbic acid and E to the extender; C15; E15= addition of 15mg/mL ascorbic acid and -tocopherol to the extender, respectively.

Cryopreservation and artificial insemination contribute to animal reproduction (Ugur *et al*., 2019). A large percentage of spermatozoa change and become infertile during cryopreservation, rendering frozen-thawed sperm inappropriate for routine employment (Sharafi *et al*., 2016). Sperm motility and viability decrease after cryopreservation. Cryopreservation aims to maintain the structural integrity, viability, motility, DNA integrity, and biological function linked to fertilization competence of as many post-thawed viable normal spermatozoa as possible. Cryopreservation diminishes sperm function and fertility. Relatively few spermatozoa survive thawing after cryopreservation, and those that have a shortened lifespan in the female reproductive system because of the damage sustained from cold shock. Cryopreservation decreases sperm motility and viability owing to a combination of cold shock, osmotic stress, and alterations in membrane fluidity and permeability (Peris-Frau *et al*., 2020).

Cryopreservation procedures have the potential to diminish the antioxidant properties of the semen (Page *et al*., 2019). In this study focused the effect of two antioxidant (ascorbic acid and α-tocopherol) on sperm quality. Ascorbic acid and α-tocopherol are responsible for the formation of collagen, proteoglycans, and intercellular matrix components. The presence of ascorbic acid and α-tocopherol in extenders may optimize sperm function by reducing cell damage generated by reactive oxidation (Lecewicz *et al*., 2018). The addition of 5 to 15 mg/ml ascorbic acid and α-tocopherol to cryopreservation conditions for tropical adapted Brahman bull spermatozoa was neither advantageous nor deleterious to sperm quality, particularly at high ascorbic acid dosages. The results showed that the addition of 10 and 15 mg/ml ascorbic acid resulted in lower sperm quality than the control, although the addition of 5 mg/ml ascorbic acid resulted in higher sperm quality. In contrast, addition of up to 15 mg/ml α-tocopherol still showed an improvement in sperm quality after thawing and was interestingly explored in greater depth, and addition alpha tocopherol gave the best performance compared to the other treatments. These recent data had similar trends mechanisms with the same addition of ascorbic acid and α-tocopherol in same range dosage in semen bull but in chilling preservation on 5°C (Hu *et al*., 2010; Page and Rosenkrans, 2019).

Ascorbic acid is a powerful antioxidant that can be diluted with water. In addition to preventing sperm hemolysis and boosting α-tocopherol recycling, ascorbic acid can quench hydroxyl, superoxide, and hydrogen peroxide radicals (Singh *et al*., 2020). Improved sperm motility and viability after cryopreservation of bull semen were achieved by the addition of ascorbic acid to the semen extender. Ascorbic acid improves semen storage and artificial insemination rates (Eidan, 2016). Ascorbic acid also reduces thawed sperm cohesiveness, thereby aiding in liquefaction (Yu *et al*., 2019). These data showed that semen addition with 5 mg/mL ascorbic acid could prevent post-thawing deterioration, which motility, viabilities and

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membrane integrity were 43.33±0.24%; 58.63±0.21%; 49.43±0.41, respectively with just 1.50± 0.05% sperm abnormality significantly higher than the control, which was 39.16± 0.28%; 47.26± 65%; 34.66± 1.15, respectively with more than 1.50±0.05% sperm abnormality (Table II) this is because hydroperoxide products, including epoxy fatty acids, alkans, alkenes, alkanates, hydroxy alkenals, and aldehydes, can be prevented from being formed by cellular oxidative chemicals due to ascorbic acid's ability to inhibit their interactions with O_2 and OH (malondialdehyde), which was also stated in a previous study that motility, viability, readings, and the proportion of sperm quality were all significantly improved when the extender was supplemented with ascorbic acid at a dose of <5 mg/mL (Hu *et al*., 2010). Higher motility and viability in this group were also attributed to the enzymes CAT and GSH, which were both triggered to higher levels. These findings also suggest that ascorbic acid protects sperm membranes by preventing membrane lipid peroxidation during freezing and thawing. The physiological generation of reactive oxygen species required for membrane modification is stimulated by low ascorbic acid levels (Ejaz *et al*., 2012). Antioxidants improved bull sperm motility the most. Oxidative stress, such as ROS, which is produced during semen metabolism, lowers sperm viability and fertility. Oxidative stress and lipid peroxidation of sperm membranes may result in high cytotoxic nitric oxide (NO) concentrations. Ascorbic acid can directly scavenge, inactivate, and repair ROS. Antioxidants reduced lipid peroxidation compared with the in that controls. Incubation increases lipid peroxidation owing to ROS-induced ATP consumption damage, which affects sperm motility and prevents membrane integrity (Bintara *et al*., 2022).

However, interesting data found that the addition of Ascorbic acid at higher doses of 10 and 15 mg/mL led to a decrease in post-thawing semen quality even the motility and viability data were lower than the control (0 mg/mL) although the addition of 10 mg/mL still gave better data than the control on the membrane integrity parameter, which was corroborated in a similar study in China, which found that addition of > 8.5 mg ascorbic acid led to frozen sperm breakage, a result similar to that reported in a previous study (Lecewicz *et al*., 2018). It could happened due to ascorbic acid is rapidly oxidised into inactive dehydroascorbate when placed in highly oxidative settings or when given in large doses (Breininger and Beconi, 2014). Furthermore, increased ascorbic acid doses could contradictorily boost ROS generation in the presence of catalyst cations such as iron as well as decreased glutathione peroxidase (GSH-Px) and GSH activity, resulting in lower motility, viability, and membrane integeration in tropically adapted Brahman bulls (Sarıkaya and Doğan, 2020). The free radical scavenging ascorbic acid might interact with oxidative stress and is involved by at least eight divergent enzymes (e.g., O_2-, OH-). Increased doses of ascorbic acid could indeed respond as a pro-oxidant in the appearance of conversion metal ions (e.g., Fe3+, Cu2+) by providing an electron that diminishes such ions to forms that can intervene with oxygen substances to frame O_2 radical and occurred elevated ROS concentration and decreased semen quality (Azawi and Hussein, 2013).

Alpha-tocopherol prevents plasma membranes from oxidative stress and is the natural primary antioxidant in sperm cells (Alahmar, 2019). Because of its solubility in lipids, α-tocopherol has the potential to act as a frontline defence against the oxidation of polyunsaturated fatty acids on membrane-bound phospholipids. Furthermore, α-tocopherol suppresses oxidative stress, prevents the cell membrane from ROS damages, and strengthens the antioxidant properties of other nutrients (Xiao *et al*., 2021). Eliminating peroxyl (ROO•), alkoxyl (RO•), and other free radicals produced during the conversion of lipid hydroperoxides in the peroxidative chain reaction is one way in which membranes can prevent lipid peroxidation reactions (Andrés *et al*., 2021). In this study, the data found that added α-tocopherol until 15 mg/mL in semen diluted significantly ($p<0.05$) increased semen quality linearly (Fig. 1A, B, C, D) this data had similar trends with previous research in different breed with α-tocopherol addition ranged from 0.1 to 40 mg/ml (Ratnani *et al*., 2020), even though addition 5 mg/ml α-tocopherol has not shown significance that is very different from the control. Alpha-tocopherol had an immediate effect on motility because of its capacity to dilute quickly on lipid from Tris-yolk extender (Haris *et al*., 2020). To protect the membrane, α-tocopherol plays a crucial role as an antioxidant. α-tocopherol may have a beneficial effect on sperm motility, membrane integrity, and membrane potential (Ugur *et al*., 2019). Also, the antioxidants in α-tocopherol added to semen extender may help protect the spermatozoa's membrane from reactive oxygen species. The addition of α-tocopherol to the sperm extender improved sperm viability, concentration, and preservation of motility during cryopreservation in liquid nitrogen containers (Chen *et al*., 2015; Haris *et al*., 2020). Another interesting data in this study was supplementation of α-tocopherol is noticeably superior to supplementation of ascorbic acid at either the same or higher doses for preserving the semen quality of bull sperm cells after thawing. The concentrations of α-tocopherol used in this study could be the highest for this purpose and still need to explore more in higher dosage of α-tocopherol which could lead toxicity or leads decreased semen quality after thawing.

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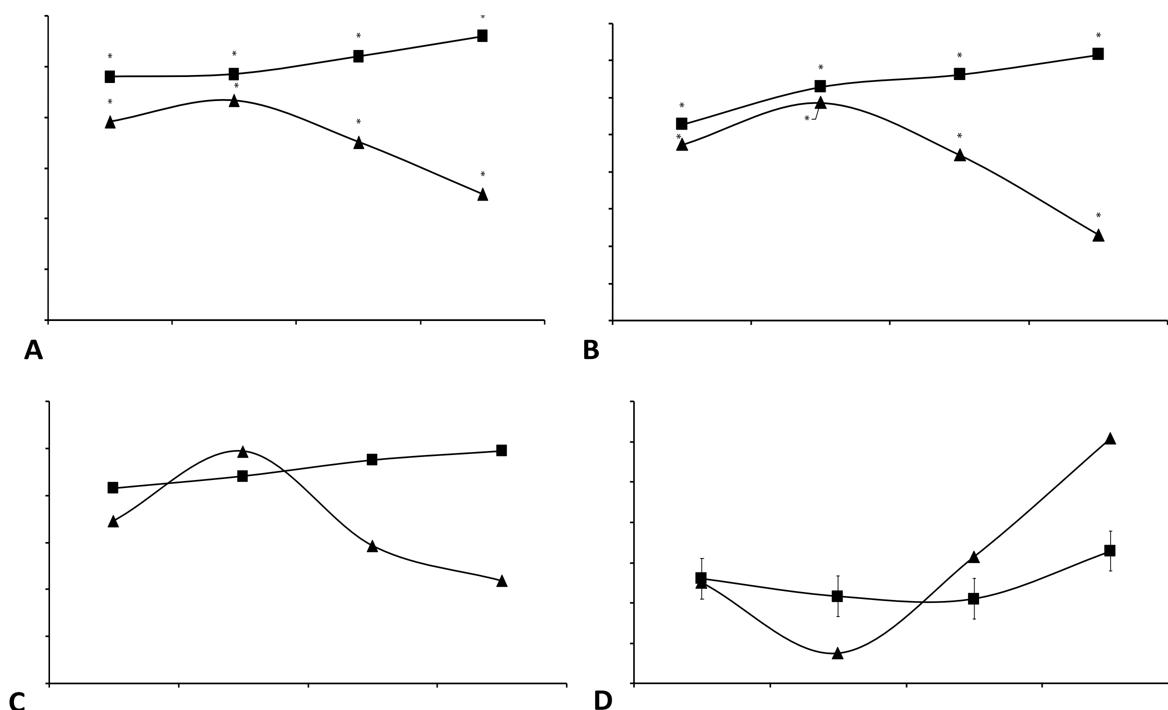

Fig. 1. Sperm quality on different dosage of antioxidant (A, B, C, D) (▲, Ascorbic acid) (■,α-tocopherol) (*P<0.05 significantly different result for the effect addition Ascorbic acid and α-tocopherol).

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Alpha-tocopherol was found to be more effective than ascorbic acid in enhancing the motility of human spermatozoa after they had been frozen. This is in agreement with findings from human semen (Askari *et al*., 1994). This is due to α-tocopherol's ability to donate a hydrogen atom from the OH group to a radical lipid peroxide, neutralising it and rendering it inert. In the other hand, Ascorbic acid just neutralises free radicals in the cytosol due to its high solubility in water; however, when the free radicals and ascorbic acid reach equilibrium stage concentration, ascorbic acid becomes saturated and has the potential to become a pro-oxidative agent. As a result of the sperm cell membrane's high lipid content, which (Shi *et al*., 2020; Yusuf *et al*., 2020) makes it susceptible to ROS-induced free radical attack, additional α-tocopherol acts as a more stable antioxidant (Yusuf *et al*., 2020). But this study also give suggestion to mix the α-tocopherol and ascorbic acid with wise dosage ascorbic acid and α-tocopherol work together to keep the number of spermatozoa stable by capturing free radicals. Alpha-tocopherol converts one electron to a radical, which then becomes a -tocopherol radical and is stabilized by ascorbic acid. Ascorbic acid, which is radical, is converted back into a stable form by antioxidant enzymes in the body via distinct mechanisms; therefore, combining ascorbic acid and -tocopherol is expected to have the greatest effect against free radical activity (Shi *et al*., 2020; Yusuf *et al*., 2020).

Table III. Varieties of abnormalities of Brahman cattle spermatozoa after thawing.

Abnormality	Ascorbic acid				α-tocopherol			
	C0	C5	C10	C15	E0	E5	E10	E15
Split	√	√	√	√	√	√	√	√
Microcephalic		√		√	√	√		
Proximal protoplasmic droplet		√				√	√	
Degenerative form	√	√	√		√	√	√	√
Folded tail	√	√	√		√	√	√	√
Bent tail	√	√	√		√	√	√	√
Bow midpiece				√			√	
Twisted tail				√	√		√	
Coiled tail	√	√	√		√	√	√	√

Abnormality sperm has primary and secondary causes. Table III showed the abnormalities types on PTW brahman sperm after addition ascorbic acid and α-tocopherol. Minor

defects in various sections of the sperm cell body, most notably the tail, lead to the secondary abnormalities seen in frozen semen. Spermatozoa are susceptible to damage from chemical reactions with oxygen and nitrogen, variances in osmotic pressure, and temperature changes that occur during semen freezing (Haris *et al*., 2020). This study found that significant data in abnormality parameter in sperm after addition of ascorbic acid, but no different found in addition of α-tocopherol. increased abnormality in addition of ascorbic acid is because high doses of ascorbic acid, which have the potential to convert to pro-oxidant, contribute to the incidence of conditions that underlie oxidative damage, the configuration of free radical fatty acid and lipoprotein hydroperoxide, concurrently exacerbating a respond between all reactive oxygen species, cell instability, reduction of membrane function and integrity (Bintara *et al*., 2022). Low dosage ascorbic acid and supplement α-tocopherol addition in sperm enhancer would give cell membrane protection from free radicals, hence decreasing the likelihood of sperm cell abnormalities and death (Kowalczyk, 2022).

CONCLUSION

Incorporation of antioxidants Certain doses of ascorbic acid and α-tocopherol can increase the quality of thawed frozen sperm; the optimal dosages are 5 mg/mL and 15 mg/mL, respectively. The addition of 15 mg/mL α-tocopherol produced the most significant improvement in post-thawing sperm quality. Also, supplementation of 10-15 mg/mL Ascorbic acid led deprimental semen quality.

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IRB approval and ethical statement

The study was registered with the ethics clearance according to the guidelines for the care and use of experimental animals of the National Research and Innovation Agency (BRIN) Indonesia with the number 065/KE.02/SK/2022.

Statement conflict and interest

The authors have declared no conflict of interest.

Online First Article

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