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# The Protective Impacts of $\alpha$-tocopherol Supplementation on the Semen Quality of Sapera Goat Preserved at 4°C

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## ABSTRACT

**Supplementation of vitamin E ($\alpha$-tocopherol) as an antioxidant into an extender is useful to maintain sperm quality during the process of cryopreservation by protecting the sperms from reactive oxygen species (ROS). This study aims to evaluate the protective impacts of vitamin E supplementation on the motility, viability, and reducing the abnormality of sperms in Sapera goats. The sample of semen used in the present study was obtained from fresh semen and good-quality ejaculates of the Sapera goats collected by an artificial vagina. The groups involve one controlled treatment group, which contains semen and tris egg yolk (TEY) extender without vitamin E (P0), and three treatment groups that contain semen, TEY extender, and different doses of vitamin E, i.e., 1 mg/mL (P1), 2 mg/mL (P2), and 3 mg/mL (P3), respectively. Data obtained from the experiments were analyzed using Repeated Measures Multivariate Analysis (MANOVA). The results showed that TEY extenders added with vitamin E at a dose of 1 mg/mL of vitamin E (P1) produced the highest motility and viability with the lowest abnormalities of sperm after 24$^{th}$ hour of cryopreservation. The lowest sperm motility and viability among the treatment groups was observed in the TEY extender added with vitamin E at a dose of 3 mg/mL (P3) at 96$^{th}$ hour after cryopreservation, which was almost the same as the control group without vitamin E (P0) that had the lowest motility and viability. In conclusion, the current study found that supplementation of vitamin E as an antioxidant at a dose of 1 mg/mL in TEY extender can preserve semen of the Sapera goats in refrigerator up to 96 h at 4°C and provides higher sperm motility and viability percentage, with a lower percentage of sperm abnormality.**

***Keywords: vitamin E ($\alpha$-tocopherol); extender; Sapera goat; sperm assessment; animal medicine***

## INTRODUCTION

The Sapera goat is one of the dairy goats in Indonesia, which is a crossbreed between the Saanen goat from Switzerland and the Etawah crossbreed goat (Peranakan Etawa). The crossbreed between the two goats is intended to boost both milk and meat productions with a greater carcass than the Saanen goats. The Sapera goats can produce 2 to 4 liters of milk per day during the first lactation, which is higher than the Etawah goats and the Saanen goats (Praharani *et al.*, 2016). In order to increase the national milk production of dairy goats, it is necessary to increase the population of dairy goats, such as the Sapera goats.

One of the efforts to increase the population of the Sapera goats is through the application of artificial insemination (AI), which allows a higher ratio of does to bucks compared to the natural breeding system. AI in goats has become a powerful method for breeders or farmers to obtain genetically-high quality offsprings by using superior bucks. Moreover, AI can also minimize the transmission of reproductive diseases and provide a more accurate birth of the offsprings (Cseh *et al.*, 2012; Faigl *et al.*, 2012). The success of AI using fresh semen relies on the techniques of sperm cryopreservation that semen inseminated to the female reproductive tract can reach the ovum cells (Wang *et al.*, 2015). Various cellular and molecular damages of sperm during the cryopreservation process will affect the sperm function and fertilization potential. Decreased sperm fertilization potential during semen cryopreservation can be observed and calculated from the excessive production of reactive oxygen species (ROS) that induce cryo-injury during oxidative stress (Johnston *et al.*, 2012; Kim *et al.*, 2012; Liu *et al.*, 2019). To overcome this problem, the addition of antioxidants such as vitamin E in the extenders during cryopreservation of semen is necessary (Sharafi *et al.*, 2015; Forouzanfar *et al.*, 2013a; Forouzanfar *et al.*, 2013b; Widjaya, 2017).

Antioxidants can maintain the qualities of sperms by protecting the biological membrane from damages caused by free radicals, neutralizing hydroxyl groups, superoxide, and hydrogen peroxide radicals, as well as preventing sperm agglutination

(Agarwal & Prabakaran, 2005; Breininger *et al.,* 2005). Supplementation of vitamin E into the extender will inhibit the process of lipid peroxidation reaction that helps to manage the oxidation process of phosphorylation, which is responsible for the increasing levels of Reactive Oxygen Species (ROS) in the sperm. High levels of ROS in the cells can oxidize lipids, proteins, and DNA so which can cause DNA damage, increased membrane permeability, and even reduce reproductive capacity (Noori, 2012; Paul *et al.,* 2018; Aitken, 2017). Vitamin E can function as an intracellular antioxidant that plays an important role in preventing lipid peroxidation of unsaturated fatty acids inside the cells and on the cell membrane so that it can prevent peroxidative damage that affects spermatogenesis (Spirlandeli *et al.,* 2014).

Information about the effect of antioxidant supplementation on Sapera buck's semen is limited from the previous studies. This gap in the literature drives us to study *in vitro* supplementation of Vitamin E in the semen extender to maintain the quality of Sapera buck semen.

## MATERIALS AND METHODS

### Animals

All experiments were approved by the Airlangga Health Research Ethical Clearance Committee number 011/HRECC.FODM/I. The study was conducted with three Sapera goats (2-3 years of age) with good body conditions were used and placed in hygienic and properly-ventilated sheds. The temperature inside the shed was maintained at 33-41°C (average 37°C). The semen collection and semen analysis were carried out at the PSDKU teaching farm, Airlangga University, during the period extended from January to May 2019. The animals were supplied with clean water that was available ad libitum and were fed twice a day consisting of calliandra hay and Napier grass silage as forage, tofu (soybean curd) by-product, and commercial concentrate as feed supplement according to the nutritional requirements.

### Preparations of Vitamin E and Extender

Pure Vitamin E (DL-α-Tocopherol acetate solution (10 mM), MDL Number: MFCD00006848) used in this study was obtained from Sigma (St. Louis, MO, USA) and *stored* in a refrigerator at 5°C. Prior to the addition into the semen extender, α-tocopherol was dissolved using ethanol 0.05%. Vitamin E was divided into 3 different concentrations (1 mg/mL, 2 mg/mL, and 3 mg/mL), and each concentration of vitamin E was mixed with the Tris-Egg-Yolk (TEY) extender. The extender material was made of Tris-Egg-Yolk obtained from the Singosari Artificial Insemination Center. Extender contains 1.6% Tris Aminomethane, 0.9% citric acid, 1.4% lactose, 80% distilled water, 20% egg yolk, penicillin 1000 IU/mL, streptomycin 1 mg/mL, and vitamin E (α-tocopherol) according to the aforementioned doses. Antibiotics such as penicillin and streptomycin were added to the extender to suppress bacterial growth. The equipment used for this process were cleaned and sterilized. The ratio between semen and tris-egg-yolk extender was 1:10.

### Semen Collection

Semen collections from 3 Sapera bucks were started with a physical examination of the reproductive organs of the bucks. The organs were healthy with a high libido. Semen collection was performed using an artificial vagina once a week in the morning with a total of one ejaculate. Fresh semen that was collected was evaluated macroscopically (volume, consistency, odor, color, and pH) and microscopically (sperm motility, viability, and concentration). The standard of sperm progressive motility in this study was above or equal to 70%. The sperm concentration was evaluated using a spectrophotometer.

### Experimental Design

The samples used in this study were 36 semen ejaculates collected from three Sapera bucks having good fresh semen and high libido at the ages of 2-3 years old. Tris-egg yolk extender was added to the semen, with a ratio of 1:10 (semen volume to extender). The semen mixed with TEY extender was assigned into four different groups based on the different doses of vitamin E supplementation, namely P0 (0 mg/mL), P1 (1 mg/mL), P2 (2 mg/mL), and P3 (3 mg/mL). All samples were preserved in liquid storage at 4°C for 96 hours and assessed for their parameters, namely, sperm progressive motility, percent of viability, and percent of abnormalities at 24, 48, 72, and 96 hours of storage.

### Assessment of Sperm Motility

To evaluate the motion characteristics of rewarmed sperm, computer-assisted sperm analysis (CASA, Version 5.1; Microptic, Barcelona, Spain) was used. The evaluation was performed at 37°C to facilitate the optimal movement of sperms. Calculation of sperm motility was based on the speed and direction of movement. As for the direction of movement, sperms were classified into 4 categories, i.e., P for progressive, R for reverse, O for oscillatory, and N for Necrospermia or no movement, while for the speed of movement, they were categorized with a number, i.e., 0 for no movement, 1 for slow movement, 2 for moderate movement, 3 for rapid movement, and 4 for very fast movement.

### Assessment of Sperm Viability

Sperm viability was assessed by using the Eosin-Nigrosine staining method (Najafi *et al.,* 2013). The glass slide was prepared, and then a small drop of semen (2 μL) was put on the glass slide, and eosin-nigrosin (10 μL) solution was put next to the semen drop. Subsequently, a thin preparation was made by fixation, and the preparation was dried for about 15 seconds. The sperm viability was assessed after fixation and drying of the preparation by calculating 200 sperm cells under bright field microscopy (CKX41; Olympus, Tokyo,

Japan) at 400X magnifications. Live sperms remained unstained, while dead sperms were stained, turning into a red-purple color.

### Assessment of Sperm Abnormality

Evaluation for abnormalities of sperm was conducted by using preparation used for viability testing (Felipe-Perez *et al.,* 2008). At least 200 sperms were examined under a microscope with 400x magnification. Abnormal sperm were determined based on head and tail abnormalities.

### Statistical Analysis

Data about motility, viability, and abnormal morphology of sperm were subsequently arranged in a table presented as average ± SD. Multivariate (MANOVA) on SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was employed to analyze the data. If the results indicated $p<0.05$, the subsequent analysis using Duncan's multiple range test was performed.

## RESULTS

The quality of fresh semen of Sapera bucks is presented in Table 1. The average volume of semen was 1.00 ± 0.20 mL from the macroscopic examination, with a creamy color, a goat-smelly odor, and thick consistency that were observed by tilting the tubes. The pH obtained in this study was 6.60 ± 0.54. The standard volume of semen in goats is an average of 1 ml (0.5-2 mL), the color of the semen is creamy white with a thick consistency, and pH 6.4-6.8. The initial macroscopic examination results listed in Table 1 showed the normal standards for Sapera goat semen and met the criteria for further examination.

The results of a microscopic evaluation in this study showed that the average concentration of sperms was 3876 ± 167.57 (million/mL). The average mass motility was (+++) marked by a large-wave formation of sperms. Furthermore, the sperms had progressive motility (forward movements) with an average of 82.80 ± 2.16%. The average result was 82%, with a speed of 3-4. The average sperms viability was 89.40 ± 4.33%. The results found in this study are within the normal standard of goat sperm concentrations. Sperm abnormalities in Sapera bucks sperm ranged from 3%-4.5%, with an average of 3.20 ± 0.83.

The effects of doses of vitamins E addition in the TEY extender on the sperm characteristics (progressive sperm motility, viability, and abnormal sperm (mean ± SD) of Sapera bucks following the preservation at 4 °C for 96 hours are presented in Table 2. Means of sperm motility and viability were significantly affected by the treatment of doses of tocopherol (Vitamin E) addition in the TEY extender. No significant difference in sperm abnormality was observed among the doses of tocopherol addition in the extender. However, semen diluted in TEY extender and added with vitamin E at a dose of 1 mg/ml tended to have a lower sperm abnormality. The evaluation of sperm motility showed that doses of vitamin E in the extender (0 mg/mL, 1 mg/mL, 2 mg/mL, and 3 mg/mL) ($p<0.05$) significantly affected the sperm motility. However, looking at the more detailed comparison among the doses of vitamin E, there was no significant difference in sperm motility between semen preserved in TEY extender contained vitamin E at doses of 3 and 0 mg/mL, and the highest motility was found in the semen preserved in TEY extender contained 1 mg/mL vitamin E (P1). In the semen preserved in the TEY extender added with vitamin E, the sperm motilities declined gradually when preserved at 4°C from 24 to 96 hours to 61.30 ± 3.88% and 28.78 ± 3.57%, respectively, compared to semen preserved in the TEY extender without vitamins (control) which was declined markedly to 47.99 ± 2.85% and 33.75 ± 3.57%, respectively.

In terms of sperm viability, there were significant differences in the sperm viability among the doses of vitamin E and time of preservation ($p<0.05$). In addition, there were significant decline patterns in treatments P1, P2, P3, and P0 ($p<0.05$). The highest sperm viability was found in semen preserved in TEY extender added with vitamin E at a dose of 1 mg/mL (P1) ($p<0.05$). The results revealed that there were significant differences between P1 and the other treatments ($p<0.05$). Semen, preserved in TEY extender added with vitamin E at a dose of 1 mg/mL, showed the lowest sperm abnormal-

Table 1. Ejaculates characteristics of Sapera buck semen (mean ± standard deviation)

| Evaluation | Repetition | | | | | Mean±SD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Macroscopy | | | | | | |
| Volume (mL) | 1.20 | 0.80 | 1.20 | 1.00 | 0.80 | 1.00 ± 0.20 |
| pH | 7 | 7 | 6 | 7 | 6 | 6.60 ± 0.54 |
| Consistency | Thick | Thick | Thick | Thick | Thick | Thick |
| Microscopy | | | | | | |
| Sperm motility (%) | 80 | 82 | 85 | 82 | 85 | 82.80 ± 2.16 |
| Sperm concentration (million/mL) | 3720 | 3840 | 4020 | 3720 | 4080 | 3876± 167.57 |
| Sperm viability (%) | 85 | 86 | 90 | 96 | 90 | 89.40 ± 4.33 |
| Mass movement | +++ | +++ | ++ | +++ | +++ | +++ |
| Sperm abnormalities (%) | 4 | 3 | 2 | 4 | 3 | 3.20 ± 0.83 |

Note: SD= Standard deviation. ++ = Frequent and seldom wave motion of mass sperm motility; +++ = Fast and thick wave motion of mass sperm motility

Table 2. Sperm motility, viability, and abnormality of Sapera buck treated by different vitamin E doses and time

| Vitamin E doses | Variables (%) (Mean ± SD) | | | |
|---|---|---|---|---|
| | Time | Motility | Viability | Abnormality |
| (P0) 0 mg/mL | 24 hours | 47.99 ± 2.85$^{cd}$ | 71.22 ± 6.19$^{b}$ | 5.41 ± 1.22$^{b}$ |
| | 48 hours | 47.60 ± 5.80$^{cd}$ | 62.20 ± 6.19$^{c}$ | 5.45 ± 1.04$^{b}$ |
| | 72 hours | 41.22 ± 4.61$^{d}$ | 60.32 ± 5.49$^{c}$ | 5.33 ± 1.88$^{b}$ |
| | 96 hours | 33.75 ± 3.57$^{e}$ | 49.56 ± 6.28$^{e}$ | 6.09 ± 1.19$^{b}$ |
| (P1) 1 mg/mL | 24 hours | 61.30 ± 3.88$^{a}$ | 77.85 ± 6.37$^{a}$ | 3.41 ± 1.22$^{a}$ |
| | 48 hours | 55.55 ± 6.17$^{b}$ | 70.99 ± 8.05$^{b}$ | 3.54 ± 1.66$^{a}$ |
| | 72 hours | 50.40 ± 3.93$^{c}$ | 60.52 ± 5.49$^{c}$ | 3.75 ± 1.01$^{a}$ |
| | 96 hours | 42.78 ± 3.57$^{d}$ | 54.27 ± 6.33$^{d}$ | 4.29 ± 0.16$^{ab}$ |
| (P2) 2 mg/mL | 24 hours | 49.30 ± 3.80$^{c}$ | 70.33 ± 4.33$^{b}$ | 5.01 ± 1.22$^{b}$ |
| | 48 hours | 42.40 ± 6.64$^{d}$ | 60.17 ± 9.01$^{c}$ | 5.95 ± 1.66$^{b}$ |
| | 72 hours | 39.40 ± 4.02$^{de}$ | 57.50 ± 5.22$^{cd}$ | 5.33 ± 1.09$^{b}$ |
| | 96 hours | 30.78 ± 2.99$^{e}$ | 48.20 ± 5.09$^{e}$ | 5.29 ± 1.04$^{b}$ |
| (P3) 3 mg/mL | 24 hours | 48.56 ± 2.58$^{c}$ | 71.85 ± 6.29$^{b}$ | 4.48 ± 1.22$^{ab}$ |
| | 48 hours | 49.28 ± 5.91$^{c}$ | 60.42 ± 8.67$^{c}$ | 5.22 ± 1.53$^{b}$ |
| | 72 hours | 39.40 ± 4.33$^{de}$ | 59.50 ± 6.49$^{c}$ | 5.65 ± 1.11$^{b}$ |
| | 96 hours | 28.78 ± 3.57$^{e}$ | 47.24 ± 6.00$^{e}$ | 6.22 ± 0.99$^{bc}$ |

Note: Means in the same column with different superscripts differ significantly ($p<0.05$). Control (P0)= No addition of vitamin E; P1= addition of 1mg/mL vitamin E to the extender; P2= addition of 2mg/mL vitamin E to the extender; P3= addition of 3mg/mL vitamin E to the extender, respectively.

ity. Subsequent analysis was conducted to evaluate the effect of time, i.e., at 4$^{o}$C preservation for 24, 48, 72, and 96 hours, on the motility, viability, and abnormalities of spermatozoa. The results showed a significant effect of time on motility, as was shown by the significant difference among the 24$^{th}$, 48$^{th}$, 72$^{nd}$, and 96$^{th}$ hours after freezing ($p<0.05$). Furthermore, the results of the viability analysis showed a significant difference between the 24$^{th}$ and 48$^{th}$ hours, and between the 72$^{nd}$ and 96$^{th}$ hours ($p<0.05$), and a significant decrease in patterns between the 24$^{th}$, 48$^{th}$, 72$^{th}$, and 96$^{th}$ hours ($p<0.05$).

Finally, from the two initial parameters, the motility and viability, vitamin E's addition at a dose of 1 g/mL (P1) showed the best results to Sapera bucks semen diluted with TEY on sperm quality.

## DISCUSSION

Sperm motility is powered by cellular energy production, which is resulted from oxidative phosphorylation and is transferred into the microtubules in the tail to stimulate and support tail movements (O'Connell *et al.*, 2002). There are two main pathways of energy production in the sperm, namely glycolysis and oxidative phosphorylation (Storey, 2008). The previous research shows that oxidative phosphorylation is more effective in energy production for mammalian sperm (Tafif, 2019). The process of oxidative phosphorylation occurs in the mitochondria through the electron transport chain. Sperm mitochondria are located in the central part, and energy production takes place in the gyre (Gibb & Aitken, 2016). ROS are formed during the process of oxygen reduction in oxidative phosphorylation. Although the ROS binding system exists in the electron transport chain, electron leakage by previous components can still occur (Halliwell & Gutteridge, 2015). The cooling process reduces membrane fluidity, sperm motility, and speed (Fang *et al.*, 2017). Cryopreservation also decelerates the metabolic rate in the sperm. However, a decrease in metabolic rate cannot stop cells from producing ROS (Gibb & Aitken, 2016; Prastiya *et al.*, 2019). The previous results of a study suggested that decreased sperm motility and mitochondrial function were found in refrigerated semen and mitochondrial dysfunction is a major cause of decreased sperm motility (O'Connell *et al.*, 2002; Tafif, 2019).

The positive outcomes of vitamin E supplementation in domestic animals have been widely studied, including in a sample of goats (Azawi & Hussein, 2013). Adding vitamin E to the semen extender also results in improving the male's fertility parameters (Hadi, 2016). Sperm membranes are mostly composed of Polyunsaturated Fatty Acids (PUFA) that makes the sperm is very susceptible to lipid peroxidation. Vitamin E supplementation helps in maintaining the sperm motility of cooled semen of bulls (Ratnani *et al.*, 2017). This helps in finding free radicals created from lipid peroxidation by creating two new stable radicals from one vitamin E molecule (Hajibabaei, 2016). Vitamin E has lipophilic properties (Azzi, 2007). Therefore, the percentage of lipids in the structure of sperm and lipid compounds from semen extender can influence the amount of vitamin E required for supplementation. In this study, high vitamin E concentrations showed a detrimental effect in maintaining motility, presumably because the formation of free radicals from metal reduction is facilitated by the high doses of vitamin E (Khorramabadi *et al.*, 2017). These metals are Fe and Cu (Halliwell & Gutteridge, 2015), which are contained in large quantities in the seminal plasma (Hardijanto *et al.*, 2010). In the previous studies, the effect of supplementation of vitamin E with concentrations of 1.50 mg

and 1 mg/mL in bull semen increases the percentage of sperm motility (Ullah *et al.,* 2019). These findings are similar to data reported in various species of mammals, including in bulls, Murrah buffaloes, and cattle families (Ullah *et al.,* 2019). These results indicate that vitamin E supplementation can maintain sperm motility during cryopreservation. The low antioxidant content of seminal plasma also decreases sperm motility, viability, and normal morphology (Am-in *et al.,* 2011). Vitamin E, which presents in the mammalian cell membranes and seminal plasma, cleanses ROS and consequently relieves oxidative stress (Sikka, 2004). The implementation of synthetic analogs of vitamin E also substantially protects membrane ultrastructure and improves intact plasma and mitochondrial membranes (Soares *et al.,* 2015).

Reactive oxygen is generated by leakage of the electron transport chain of the mitochondria and can also be resulted from damages in the mitochondrial structure (Halliwell & Gutteridge, 2015). ROS also impairs the mitochondria of sperm, creating multiple amounts of ROS production. They bind PUFA hydrogen and produce lipid peroxyl radicals that will bind the adjacent PUFA molecules, producing lipid peroxide and an alkyl radical. This cycle is known as a chain reaction (Hajibabaei, 2016).

An antioxidant supplement that can supply hydrogen atoms to ROS is necessary to prevent chain reactions. OH $\alpha$-tocopherol actively supplies hydrogen atoms (Herdis *et al.,* 2013). Therefore, such antioxidants can be promising to prevent oxidative damage such as lipid peroxidation (Maya *et al.,* 2012). Vitamin E is also known as a chain-breaking antioxidant because it provides its hydrogen atom and forms alpha-tocopheroxyl radicals that will react with the second free radical. Therefore, a single molecule of $\alpha$- tocopherol will consume two free radicals in the total (Hajibabaei, 2016). However, the percentage of lipids in the environment affects the solubility of vitamin E, a lipophilic antioxidant that directly affects the lipid domain. The amount of lipid content differs between semen extender and sperm architecture. Therefore, the effect of the dose of vitamin E supplementation can differ depending on the content of the extender and animal species (Azzi, 2007). According to Khorramabadi *et al.* (2019), vitamin E shows a pro-oxidant activity, so that vitamin E may be useful for eliminating complications resulting from oxidative stress. In this study, the evaluation of results produced by the addition of vitamin E at a dose of 3 mg/mL (P3) suggests that high vitamin E supplementation concentrations can bring a detrimental effect. This is likely due to the ability of vitamin E to reduce Fe and Cu (Halliwell & Gutteridge, 2015).

## CONCLUSION

In conclusion, the current study found that supplementation of vitamin E as an antioxidant at a dose of 1 mg/mL in TEY extender can preserve semen of the Sapera goats in a refrigerator up to 96 h at 4°C and provides higher sperm motility and viability percentage, and a lower sperm abnormality percentage.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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