

The addition of vitamin C in tris–egg yolk extender maintained Sapera goat semen quality in 5° C storage

Jolly Rifqi Pahlevy^{1*}, Hermin Ratnani², Tjuk Imam Restiadi², Faisal Fikri³,
Amung Logam Saputro⁴, Bodhi Agustono⁵

¹ ASA Veterinary Clinic, Raung Street 15, Kediri, East Java, Indonesia,

² Division of Veterinary Reproduction, ³ Division of Basic Veterinary Medicine,

⁴ Division of Veterinary Clinic, ⁵ Division of Animal Husbandry

Faculty of Veterinary Medicine, Universitas Airlangga

* Corresponding author, e-mail: jollypahlevy310@gmail.com

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ABSTRACT

Goats are small ruminants that are reared by the rural community for financial income and nutrition. This study was aimed to determine the effect of vitamin C addition in tris–egg yolk extender on the lifespan of Sapera goat spermatozoa stored in 5° C. Semen was collected from 1.5 years old Sapera male goat. The ejaculates were diluted in Tris-egg yolk (T-EY) extender added with 0, 0.2, 0.3, and 0.4 g vitamin C/mL extender for T0, T1, T2, and T3 groups, respectively. The spermatozoa motility, viability, and morphological abnormality were assessed along with storage at 5° C. The result showed that spermatozoa motility was the highest ($p < 0.05$) in T1 compared to other groups and qualified for AI use for up to 72 hours. In 24 hours storage, the spermatozoa viability was not significantly different ($p > 0.05$) among the groups. The spermatozoa viability in the T1 group was the highest ($p < 0.05$) among the groups along 48-96 hours of storage. The spermatozoa morphological abnormalities of the T1 group was the lowest ($p < 0.05$) compared to other treatment groups in the range of 24-72 hour storage. In the control group (T0), the less than 5% spermatozoa morphological abnormalities (qualified for artificial insemination) were only in the 24 hours storage, while those of the T1 group were up to 72 hours. It could be concluded that the addition of 0.2 g vitamin C/100 mL T-EY extender maintained the quality of Sapera goat semen for 72 hours at 5°C.

Keywords: abnormality, financial income, motility, nutrition, viability

INTRODUCTION

Goats are small ruminants that are reared by the rural community for the source of financial income and food. Sapera goat is the result of a crossing between Saanen and Etawa cross goat (Anggraeni *et al.*, 2020). Sapera goat is a dairy goat with productivity higher than that

of Saanen and Etawa cross goats (Suranindyah *et al.*, 2018). Milk production is about 740 kg per lactation period and can reach a lactation period of up to one year if the goats do not mate in the early lactation period. Sapera goats have white or pale cream fur, with black dots on the nose, ears, and mammary glands. The nose and ears are striped and black. The forehead is

broad, and the ears are medium and facing forward and downward. The nose is straight, and the face is like a triangle. The tail is thin and short, male and female goats have a pair of horns. Adult male Sapera goats weigh around 68-91 kg, while the female ones weigh around 36-63 kg. The height of the male Sapera goat is about 90 cm, and the female is 80 cm (Rusdiana *et al.*, 2015).

Artificial insemination (AI) technology is expected to play an essential role in the breeding of Sapera goats. However, the frozen semen of Sapera goat has not readily available. Alternatively, artificial insemination in Sapera goats can be conducted using liquid-chilled semen. Fresh semen is extended and stored at 5°C for several days, waiting for inseminating to estrus goat (Susilowati *et al.*, 2019). Semen extender is critical as a source of energy and a pH stabilizer to maintain the quality of semen. Fresh goat semen without extender stored at room temperature (27°C) was still feasible for AI up to 15 hours after collection (Kusumawati *et al.*, 2017). Spermatozoa motility decreases rapidly at room temperature due to lactic acid derived from the metabolism of spermatozoa as they produce energy (Reynolds *et al.*, 2017). Diluted semen in the tris-egg yolk (T-EY) based extender revealed high motility of post-thawed ram (Rostami *et al.*, 2020), goat (Sun *et al.*, 2020), dog (Schäfer-Somi *et al.*, 2021), and Sapera goat (Prastiya *et al.*, 2021) spermatozoa.

The presence of antioxidants in extenders provides their protective function; these neutralize reactive oxygen species (ROS) and reduce oxidative stress in spermatozoa (Allai *et al.*, 2018; Kumar *et al.*, 2019). Vitamin C (ascorbic acid or ascorbate) is a non-enzymatic antioxidant. The addition of antioxidants is a well-known method to improve the quality of liquid storage or cryopreserved ram spermatozoa (Azawi and Hussein, 2013). Thereby, it can be hypothesized that adding vitamin C to T-EY extenders would extend the lifespan of spermatozoa used for AI. Therefore, this study was aimed to determine the vitamin C effect in T-EY extenders on the lifespan of spermatozoa viability, spermatozoa motility, spermatozoa morphological abnormality. These results will provide valuable information for the storage and quality maintenance of Sapera goat

semen at chilled temperatures for breeding of Sapera goat.

MATERIALS AND METHODS

The study was conducted from December 2019 to January 2020. Semen was collected from Sapera goats at Bumi Kesilir Farm at Kesilir village, Banyuwangi District, East Java, Indonesia with geographical coordinates of 8°21'04.7"S 113°34'44.4"E. Laboratory works were conducted at Universitas Airlangga in Banyuwangi. The distance between Bumi Kesilir Farm to the laboratory is about 1 km, taken in two-minute travel. Semen was transported using a storage tube container inserted into a cool box. This study was excerpted from ethical approval from the Animal Care and Use Committee because semen collection using an artificial vagina did not affect the normal physiology of the male goats.

Experimental animals

Sapera male goats were reared in Bumi Kesilir Farm, Banyuwangi. Semen was collected from 1.5 years old Sapera male goat twice a week using an artificial vagina equipped with scaled glass conical tube. The collected semen was immediately brought for macroscopic (volume, colour, odour, consistency, and pH) and microscopic (mass movement, individual movement, viability, concentration, and morphological abnormality) evaluations. The ejaculates that had at least 70% spermatozoa motility and viability were used in the analysis.

Tris-egg yolk extender

All chemicals were purchased from Thermo Fisher Scientific (Melaka, Malaysia), while egg yolk was from the laboratory chicken eggs (CV. Redjo, Surabaya). Tris-egg yolk (T-EY) extender contains Tris aminomethane 1.6%, citric acid 0.9%, lactose 1.4%, distilled water 80%, egg yolk 20%, penicillin 1000IU/ml, Streptomycin 1 mg/ml (Santos *et al.*, 2021). The extender added with 0, 0.2, 0.3, and 0.4 g powder of vitamin C/mL extender for T0, T1, T2, and T3 groups, respectively.

Measurement of variables

Spermatozoa motility

Semen samples of 10 μ l, and 10 μ l of 0.9% NaCl were dripped on an object glass, homogenized, then covered with a cover glass (Brillianti *et al.*, 2021). Spermatozoa progressive (forward) motility percentage was counted under a light microscope (Nikon Eclipse E200 LED) at 400x magnification.

Spermatozoa viability and morphological abnormality

Semen sample was dropped on glass added with a drop of eosin-nigrosin, mixed homogeneously, smeared and dried over the flame quickly. The head of live spermatozoa appeared transparent or clear, while the head of dead spermatozoa appeared reddish. The percentage of live spermatozoa and morphologically (head, neck, and tail) abnormal spermatozoa were assessed for 100 spermatozoa at 400 \times magnification under a light microscope (Nikon Eclipse E200 LED) (Brillianti *et al.*, 2021).

Data analysis

The spermatozoa viability, motility, and morphological abnormality were analyzed based on treatment and storage time using one-way Multivariate Analysis of Variance (MANOVA) followed by Duncan's multiple distance test. Statistical analysis was conducted at a 95% significance level using Statistical Product and Service Solutions (SPSS, version 21; IBM Corp., Armonk, NY, USA).

RESULTS

The macroscopic and microscopic parameters of fresh semen (Table 1) were evaluated first to determine whether the ejaculates were feasible for further process. Only ejaculates with more than 70% spermatozoa motility and less than 5% morphological abnormality were subjected to this study (Susilowati *et al.*, 2019). Based on Table 1, the ejaculates qualify for storage as extended semen at 5°C.

Table 1 The results of Sapera goat semen assessment

macroscopic		microscopic	
volume (mL)	1.00 \pm 0.14	motility (%)	82.80 \pm 2.16
odour	goat specific	viability (%)	87.6 \pm 2.30
colour	creamy	abnormality (%)	3.20 \pm 0.83
pH	6.60 \pm 0.54	concentrations (million/mL)	3856 \pm 2.16
consistency	thick	spermatozoa mass movement	+++

Table 2 Effect of the addition of vitamin C in Tris-egg yolk extender on the motility (%) of Sapera goat spermatozoa chilled at 5°C for 96 hours

	24	48	72	96
T0	53.46 \pm 4.09 ^{Ab}	49.44 \pm 4.43 ^{Ab}	43.67 \pm 5.01 ^{Bb}	40.43 \pm 5.41 ^{Bb}
T1	62.25 \pm 3.51 ^{Aa}	56.21 \pm 3.89 ^{Aab}	52.44 \pm 4.17 ^{Ba}	47.26 \pm 4.63 ^{Bb}
T2	58.46 \pm 3.74 ^{Aab}	52.05 \pm 4.20 ^{Aab}	47.22 \pm 4.63 ^{Ba}	43.61 \pm 5.01 ^{Ba}
T3	55.86 \pm 3.92 ^{Ab}	47.04 \pm 4.65 ^{Bb}	46.22 \pm 4.73 ^{Ba}	41.02 \pm 5.33 ^{Ca}

different superscripts A, B in the same row; a, b in the same column were significantly different ($p < 0.05$); T0: Tris-egg yolk (T-EY) without vitamin C; T1, T2, and T3: T-EY extender with the addition of 0.2, 0.3, and 0.4 g vitamin C/mL extender, respectively.

The addition of vitamin C of 0.2 g/mL extender (T1) resulted in the highest spermatozoa motility ($p < 0.05$) compared to the other groups (Table 2). Spermatozoa motility was maintained more than 40% (qualifies to be

used for artificial insemination) for up to 96 hours of storage at 5°C, while in the control group (T0), it lasted up to 72 hours.

During 24 hours of storage at 5°C, the viability of spermatozoa was not significantly

different ($p > 0.05$) among groups. Storage of spermatozoa in the T1 group was the best ($p < 0.05$) among the other groups (Table 3).

Table 3 Effect of the addition of vitamin C in Tris–egg yolk extender on the viability (%) of Sapera goat spermatozoa chilled at 5°C for 96 hours

	24	48	72	96
T0	72.08 ± 2.77 ^A	61.81 ± 3.23 ^{Bb}	63.06 ± 3.17 ^{Bb}	56.83 ± 3.51 ^{Ca}
T1	73.67 ± 2.71 ^A	70.22 ± 2.84 ^{Aa}	69.44 ± 2.87 ^{Aa}	58.41 ± 3.41 ^{Ba}
T2	72.86 ± 2.74 ^A	64.45 ± 3.10 ^{Bb}	64.03 ± 3.12 ^{Bb}	57.42 ± 3.47 ^{Ca}
T3	71.23 ± 2.80 ^A	60.04 ± 3.32 ^{Bb}	58.83 ± 3.39 ^{Bb}	50.06 ± 3.99 ^{Cb}

different superscripts A, B, C in the same row; a, b in the same column were significantly different ($p < 0.05$); T0: Tris–egg yolk (T–EY) without vitamin C; T1, T2, and T3: T–EY extender with the addition of 0.2, 0.3, and 0.4 g vitamin C/mL extender, respectively.

Table 4 Effect of the addition of vitamin C in Tris–egg yolk extender on morphological abnormalities (%) of Sapera goat spermatozoa chilled at 5°C for 96 hours

	24	48	72	96
T0	4.02 ± 0.94 ^{Ba}	5.84 ± 0.86 ^{Aa}	6.26 ± 1.52 ^{Aa}	7.01 ± 1.61 ^{Aa}
T1	2.08 ± 0.98 ^{Bc}	3.02 ± 0.95 ^{Bb}	4.81 ± 1.03 ^{Ac}	6.81 ± 1.43 ^{Ab}
T2	3.25 ± 0.95 ^{Bb}	5.67 ± 0.89 ^{Aa}	6.60 ± 0.75 ^{Aa}	6.81 ± 0.73 ^{Ab}
T3	4.60 ± 1.08 ^{Ba}	5.06 ± 0.99 ^{Aa}	5.48 ± 0.92 ^{Ab}	6.49 ± 0.78 ^{Ab}

different superscripts A, B in the same row; a, b, c in the same column were significantly different ($p < 0.05$). T0: Tris–egg yolk (T–EY) without vitamin C; T1, T2, and T3: T–EY extender with the addition of 0.2, 0.3, and 0.4 g vitamin C/mL extender, respectively.

The spermatozoa morphological abnormalities in the 24-72 hour storage range of the T1 group was the lowest ($p < 0.05$) compared to other treatment groups. In the control group (T0), the percentage of spermatozoa morphological abnormalities was less than 5% (qualified for artificial insemination) only in 24 hours storage at 5°C. Meanwhile, in group T1 the quality of spermatozoa met the requirements for artificial insemination based on the percentage of morphological abnormalities of spermatozoa that lasted up to 72 hours of storage at 5°C (Table 4).

DISCUSSION

Volume, colour, odour, consistency, pH, spermatozoa mass movement, concentration, morphological abnormality of fresh ejaculate of Sapera goats in this study were in the same range as fresh ejaculate of Sapera goats in previous reports. Spermatozoa motility in this study ($83.25 \pm 2.36\%$) was higher than those

reported by Masyitoh *et al.* (2018) ($76.00 \pm 0.04\%$), and approximately the same with those of reported by Prastiya *et al.* (2021). Spermatozoa viability ($89.75 \pm 0.5\%$) was higher than those of reported by Masyitoh *et al.* (2018) ($82.00 \pm 0.04\%$) and approximately the same with those of reported by Prastiya *et al.* (2021). Spermatozoa motility of Sapera goat in this study was more than 70% and morphological abnormalities was less than 20%, which means that the ejaculates were qualified for frozen semen production (SNI, 2014).

Storage at 5°C extended the lifespan of semen for several days before AI use (Macías *et al.*, 2017). It was used to maintain semen quality for transportation and short-term storage (Di Iorio *et al.*, 2014). The metabolism of spermatozoa slowed when it was chilled, which decreased the rate of lactic acid formation and increased spermatozoa lifespan. Etawa goat semen in a tris extender maintained 43.5% motility for 4 days in 5°C storage (Susilowati *et al.*, 2019). There was a decrease in

spermatozoa motility and viability from $82,80 \pm 2,16$ and $87,6 \pm 2,30\%$ in fresh semen to 53.46 ± 4.09 and $72.08 \pm 2.77\%$ after 24 hours storage in T-EY extender at 5°C , respectively. While, the morphological abnormalities of spermatozoa increased from $3.20 \pm 0.83\%$ to $4.02 \pm 0.94\%$ after storage at 5°C in T-EY extender for 24 hours. Those decreases of spermatozoa motility and viability and increases of spermatozoa abnormality were consistent with the previous report (Susilowati *et al.*, 2019; Susilowati *et al.*, 2021). The decrease of temperature and duration of storage caused a reduction in the viability and motility of spermatozoa mediated to biochemical changes by osmotic disorders and cell membrane damage due to oxidative stress (Sariozkan *et al.*, 2013). Oxidative stress in the mitochondrial activity would disturb the synthesis of adenosine triphosphate, energy for spermatozoa viability, and motility (Wagner *et al.*, 2018).

Along with storage time increases, the percentage of live spermatozoa was decreased (Hahn *et al.*, 2019). In addition, temperature fundamentally affects semen motility. Tris-based extenders could preserve goat semen for 5 or 17 h at room or chilled (4°C) temperatures, respectively (Ferdinand *et al.*, 2012). Spermatozoa stored at 4°C survived longer than spermatozoa stored at 23°C (Wusiman *et al.*, 2012). The decreasing spermatozoa quality due to storage was caused by ROS accumulation, which led to the loss of membrane selectivity and permeability. The endogenous antioxidant in seminal plasma was insufficient when ROS was higher along with cooling and extended storage (Wen *et al.*, 2019).

In this study, the addition of 0.2 g vitamin C/mL T-EY extender revealed the highest spermatozoa motility and viability and lower spermatozoa with morphological abnormality (Table 2, Table 3, Table 4). This result was following the previous report that adding vitamin C to Tris diluent improved spermatozoa quality parameters and spermatozoa antioxidant capacity of Najdi goat after freezing (Mamouei *et al.*, 2021). Vitamin C also improved spermatozoa viability and motility of cryopreserved West African Dwarf goat

spermatozoa (Daramola and Adekunle, 2015). The presence of vitamin C in the T-EY extender reduced the dramatic decline in semen quality that occurred during chilled storage. High spermatozoa quality required a balance of ROS-mediated mitochondrial metabolism and endogenous antioxidants in semen plasma (Dutta *et al.*, 2019). ROS played a vital role in regulating spermatozoa functions (Wagner *et al.*, 2018). ROS also functioned in tyrosine phosphorylation, sterol oxidation, and cholesterol efflux in spermatozoa metabolism (Takeshima *et al.*, 2018). However, higher ROS caused oxidative stress, and reduced spermatozoa viability and motility (Thompson *et al.*, 2014). The antioxidants derived from the group given 0.2 g/mL vitamin C seemed to have been sufficient to offset excess ROS production. Oxidative stress occurred when there is an imbalance in oxidation and reduction reactions, which resulted in an increase in oxidants or molecules that could readily accept electrons compared with other molecules. Lipids in membranes and carbohydrates in nucleic acids could easily accept unpaired electrons (Wagner *et al.*, 2018). A higher dose of vitamin C (0.3 and 0.4 g/mL T-EY extender) revealed lower spermatozoa motility, viability, and higher spermatozoa with morphological abnormalities than those of the group added with 0.2 g/mL vitamin C. This could be due to a higher exposure to antioxidants, resulting in an antioxidant paradox (Majzoub *et al.*, 2017) which caused a lack of ROS for the physiological functioning of spermatozoa (Majzoub and Agarwal, 2018).

Spermatozoa is a critical indicator of semen quality as a requirement for AI. Goat semen can be used for artificial insemination if a minimum spermatozoa motility of 40% and a maximum morphological abnormality of 5% were met (SNI, 2014). Thus, Sapera goat semen diluted in T-EY extender alone stored at 5°C should be used within 24 hours after collection and dilution of semen. While, with the addition of vitamin C of 0.2 g/mL the quality of semen met the requirements for 72 hours storage at 5°C . This result was similar to the previous report that storage of Kacang buck semen in skim milk-egg yolk extender supplemented with green tea extract at 5°C resulted in four days (96 hours) of spermatozoa motility span. This

result was the same with those of Kacang goat semen extended in skim milk-egg yolk extender added with green tea extract (Susiowati *et al.*, 2021), and was better compared with refrigerator-preserved ram semen which reached 48 hours in tris extender (Rahman *et al.*, 2018). However, those were shorter than those of the Kacang goat spermatozoa extended in skim milk-egg yolk extender supplemented with L-Arginine which resulted in five days (120 hours) of the spermatozoa motility span at 5° C storage (Susiowati *et al.*, 2019).

This study was limited in spermatozoa motility, viability, and morphological abnormality variables. The decreasing spermatozoa quality due to storage was caused by ROS accumulation (Wen *et al.*, 2019). Therefore, future studies need to measure the total antioxidant capacity of extended semen along with storage time and spermatozoa fertility to conceive female goats.

CONCLUSION

Addition of 0.2 g vitamin C/mL to the T-EY extender maintained the quality of Sapera goat semen stored at 5°C for up to 72 hours which remained feasible for AI. The results of this study could be applied for the artificial insemination of female goats using liquid semen to improve genetic quality and increase the population of Sapera goats.

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