Ovozoa 11 No. 1, April 2022 p-ISSN 2302-6464 e-ISSN 2722-967X
Available at https://e-journal.unair.ac.id/OVZ/index

Original article

Pre-freezing at 10 cm above liquid nitrogen surface for eight minutes resulted the best Sapera goat semen quality

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Open access under CC BY – SA license, DOI: 10.20473/ovz.v11i1.2022.27-33
Received December 21 2021, Revised February 11 2022, Accepted March 10 2022
Published online April 2022

How to cite this article: Pasha ZA, Agustono B, Saputro AL. 2022. Pre-freezing on 10 cm above Liquid Nitrogen surface for eight minutes resulted the best of Sapera goat semen quality. Ovozoa: Journal of Animal Reproduction 11: 27-33.

ABSTRACT

Sapera goat is a dairy goat resulting from a cross between a Saanen buck and an Ettawah cross doe. These small ruminants are reared by rural households for poverty reduction and undernutrition prevention. Breeding of Sapera goat through artificial insemination (AI) technique was expected to accelerate the increase in population. Unfortunately, the frozen semen of Sapera buck is not available yet. This study aims to determine the effect of the distance between semen straw and liquid nitrogen surface during pre-freezing process on the quality of frozen semen of Sapera goats. Semen was obtained from 1.5 years old Sapera buck. The ejaculates were diluted in tris-egg yolk extender to obtain a concentration of 120 x 10^6 spermatozoa/mL, then equilibrated at 5°C for one hour. The extended semen was packaged in 0.5 mL French straws (60 x 10^6 spermatozoa/straw). The filled straws were pre-frozen in the cold handling cabinet (Minitube, Germany) held at 10, 11, 12, 13, 14, and 15 cm above liquid nitrogen respectively for T1, T2, T3, T4, T5, and T6 groups, each with four replicates. After pre-freezing for eight minutes the straws were immediately plunged, and stored in liquid nitrogen (-196°C) for two days, followed by quality assessment. The best post-thawed motility (41.75 ± 2.06 %), viability (49.00 ± 0.82 %) and morphological abnormalities (4.75 ± 0.96 %) were obtained from pre-freezing stage with a distance of 10 cm. It could be concluded that in tris-egg yolk extender, post-thawed spermatozoa viability and motility of Sapera buck were qualified for AI when fresh semen was pre-frozen at 10 cm distance of straws from the surface of liquid nitrogen for eight minutes.

Keywords: abnormality, motility, poverty reduction, undernutrition prevention, viability

INTRODUCTION

Sapera goat is a dairy goat resulting from a cross between a Saanen buck and an Ettawah cross (EC) doe (50% Saanen, 50% Ettawah cross), developed by the Indonesian Research Institute for Animal Production (Anggraeni *et al*., 2020). Crossbreed goats are expected to have complementary traits of a high milk production from the Saanen breed and an excellent tropical adaptation from the EC breed. Genetic improvement by crossing should be followed by selection to gather the superiority of both traits passed through to their offspring

(Anggraeni *et al*., 2020). Estimation of non-genetic and genetic factors related to growth traits is needed to develop a proper selection program and achieve a good selection response in a dairy breeding program (Kuthu *et al*., 2017; Josiane *et al*., 2020). Sapera goats have almost the same body and head sizes as EC goats, but the ears are smaller and shorter (Ariyanto *et al*., 2021). Milk production of Sapera was higher than those of the EC goats (1674.00 ± 122.77 vs 1340.00 ± 76.38 mL/head/day) (Suranindyah *et al*., 2018). These small ruminants are reared by rural households to reduce poverty and prevent malnutrition.

Artificial insemination (AI) is the technique expected to accelerate the increase in Sapera goat population. With natural mating, one ejaculate of a buck can only impregnate one doe, while AI technique allowed semen of one ejaculate to be utilized to inseminate a large number of females (Gibbons *et al*., 2019). Frozen semen could be used for AI for up to several years after semen collection and could cover large areas. Unfortunately, the frozen semen of Sapera buck is not available yet. Also, reports about Sapera goat semen freezing are rare. One of the stages in the frozen semen is the pre-freezing in which the spermatozoa is being adapted to cold temperatures before being plunged into liquid nitrogen (-196 ºC). Pre-freezing of Kacang buck straws has been conducted using liquid nitrogen vapour at a certain distance for 10 minutes (Susilowati *et al*., 2020). Best to our knowledge, there has not yet been established the distance between straws' position and the surface of liquid nitrogen during pre-freezing stage to obtain the best quality of Sapera goat semen.

Therefore, this study aimed to determine the effect of straw distance from liquid nitrogen surface during the pre-freezing process on the post-thawed spermatozoa viability, motility, and morphological abnormalities of Sapera buck semen.

MATERIALS AND METHODS

This study was conducted at Bumi Kesilir Farm, Siliragung, Banyuwangi, and semen processing and evaluation was carried out at the Laboratory of Universitas Airlangga, Banyuwangi Campus.

Experimental animal

One and a half years old Sapera buck weighing 44 kg was used in this study. Concentrate and forage was given 4 and 1 kg/day respectively, and drinking water was provided ad libitum. Semen was collected twice a week using an artificial vagina. Fresh semen was examined macroscopically for volume, pH, consistency, and colour, as well as microscopically for sperm viability, motility, and concentration. Semen with at least 70% spermatozoa motility and viability was used in the analysis (INS, 2014). Sapera buck ejaculate was subjected to this study.

Tris-egg yolk extender and semen freezing

Tris base extender consisted of 2.42 g tris (hydroxymethyl) aminomethane (Research Organics, USA), 1.28 g of citric acid (Fisher Scientific, UK), and 2.16 g fructose (Scharlau, Spain) dissolved in distilled water up to 100 mL (Riyadhi *et al*., 2017), then penicillin (1.000 IU/mL) and streptomycin (1 mg/mL) were added to the mixture. The mixture was added with egg yolk and glycerol to a final concentration of 20% and 6%, respectively (Susilowati *et al*., 2021).

The ejaculates were diluted in tris-egg yolk extender to obtain a concentration of 120 x 10^6 spermatozoa/mL, and equilibrated at 5°C for one hour. Then, the extended semen was packaged in 0.5 mL French straws (60 x 10^6 spermatozoa/straw). The filled straws were pre-frozen in the cold handling cabinet (Minitube, Germany) held at 10, 11, 12, 13, 14, and 15 cm above liquid nitrogen surface respectively for T1, T2, T3, T4, T5, and T6 groups, each with four replicates. After pre-freezing for eight minutes the straws were immediately plunged, and stored in liquid nitrogen (-196°C) for two days, followed by quality assessment (Susilowati *et al*., 2020).

Measurement of variables

Sample straws from each group were thawed in sterile water at 37°C for 30 second to evaluate post-thawed semen quality.

Spermatozoa viability and morphological abnormality

A drop of semen sample and eosin-nigrosin were mixed homogeneously, smeared, and quickly dried over the flame. The viability of the spermatozoa was examined under a light microscope (Nikon E200, Nikon Corporation, Tokyo, Japan) at 400x magnification. Live spermatozoa appeared white on the head since they did not absorb colour, while dead spermatozoa absorbed a red-purple color (Susilowati *et al*., 2020). Head, neck, and tail morphological defects were evaluated on 100 spermatozoa (Oumaima *et al*., 2018).

Motility

Semen was diluted in physiological saline (0.9% NaCl) (10 µL each), dropped on a warm glass slide, and covered with a coverslip. Progressive movement of spermatozoa was assessed under a light microscope at 400× using a computer-assisted spermatozoa analyzer (CASA, WEI-LI New Century Technical Development, China) (Susilowati *et al*., 2021).

Data analysis

Data on motility, viability and morphological abnormality were analyzed using analysis of variance, followed by Duncan's Multiple Range Test. The statistical analysis was conducted at a 95% significance level using Statistical Product and Service Solutions (SPSS) Version 23 (IBM, New York, United States).

RESULTS

The macroscopic and microscopic examination of the fresh semen of Sapera goats from this study (Table 1) were carried out to be compared with previous reports and to determine the feasibility of the semen to be processed into frozen semen. The highest post-thawed spermatozoa viability and motility among groups were obtained from the T1 group (pre-freezing with a distance of 10 cm from the straw to the surface of liquid nitrogen) ($p < 0.05$), while spermatozoa morphological abnormality (Figure 1) was not significantly different ($p > 0.05$) among groups (Table 2).

Table 1 Sapera goat's fresh semen quality

macroscopic examination		microscopic examination	
volume (mL)	1.13 ± 0.096	motility (%)	83.25 ± 2.36
smell	specific	viability (%)	89.75 ± 0.5
color	creamy	abnormality (%)	3.5 ± 1.0
pH	6,75 ± 0,50	concentration (x 10^6/mL)	3559.5 ± 166.57
consistency	thick	mass sperm movement	+++

Tabel 2 Sapera goat's post-thawed semen quality

treatments	motility	viability	abnormality
T1	41.75 ± 2.06 ^{a}	49.00 ± 0.82 ^{a}	4.75 ± 0.96
T2	38.00 ± 2.16 ^{b}	42.50 ± 2.65 ^{b}	5.25 ± 0.50
T3	37.50 ± 2.39 ^{b}	41.00 ± 3.65 ^{b}	5.75 ± 1.26
T4	36.50 ± 3.00 ^{b}	39.50 ± 4.12 ^{b}	6.00 ± 1.83
T5	35.00 ± 2.94 ^{b}	38.75 ± 2.75 ^{b}	6.00 ± 2.16
T6	34.50 ± 1.73 ^{b}	38.00 ± 2.94 ^{b}	6.50 ± 1.73

T1, T2, T3, T4, T5, T6: distance of straws from liquid nitrogen surface in the pre-freezing stage were respectively 10, 11, 12, 13, 14, 15 cm. Different superscripts in the same column indicates significant differences ($p < 0.05$).

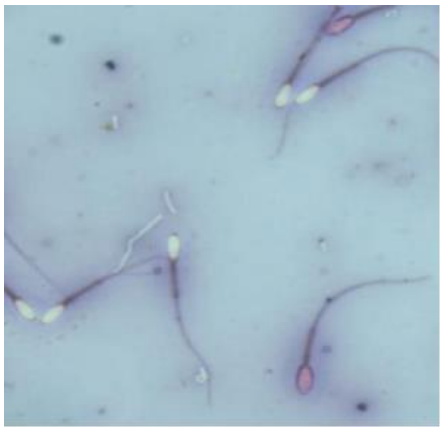

Figure 1 Viability examination of Sapera goat spermatozoa; white and transparent head of sperm indicates live sperm, red-purple colour head of sperm indicates dead sperm (Eosin-nigrosine stain; 400x magnification; Nikon microscope, Eclipse E200).

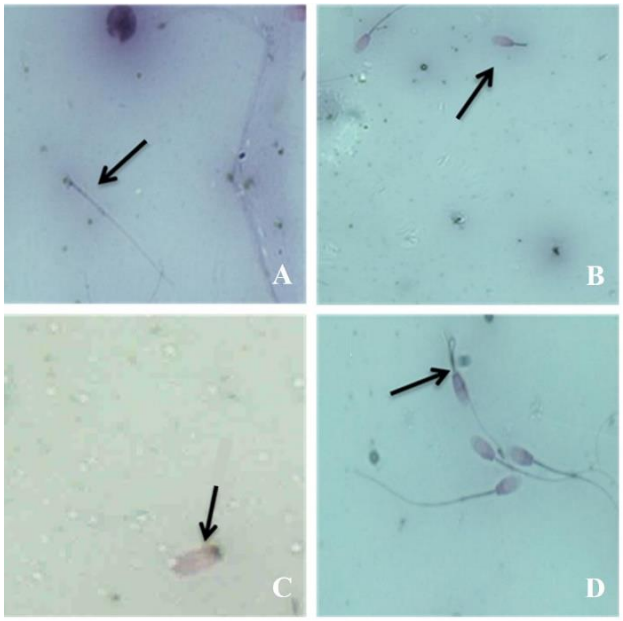

Figure 2 Spermatozoa abnormalities of Sapera Goats; A. spermatozoa with abnormal tail without a head; B. spermatozoa with broken tail; C. spermatozoa without tail, D. Spermatozoa with tangled tail; (Eosin-nigrosine staining, 100x magnification, Nikon microscope, Eclipse E200).

DISCUSSION

The volume, colour, smell, consistency, pH, 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 (83.25 ± 2.36%) was higher than those reported by Hidayati *et al*. (2018) (75.11 ± 1.11%) and in the same range as previously reported by Hanifah *et al*. (2020). Spermatozoa viability (89.75 ± 0.5%) was higher than those reported by Hidayati *et al*. (2018) (78.16 ± 1.71%) and in the same range as previous reported by Hanifah *et al*. (2020). Spermatozoa motility was more than 70% and morphologic abnormalities of Sapera buck fresh semen was less than 20%, indicating that the ejaculates were qualified for frozen semen production (INS, 2014).

There was a decrease in spermatozoa motility from 83.25 ± 2.36% in fresh semen to 41.75 ± 2.06% in post-thawed semen, and spermatozoa viability from 89.75 ± 0.5% in fresh semen to 49.00 ± 0. 82% in post-thawed semen (Table 2). Goat spermatozoa were susceptible to cold shock due to phospholipase A secreted from the bulbourethral gland (Lv *et al*., 2018), which interacted with phospholipids during semen freezing, thereby decreasing semen quality (Silva *et al*., 2019). In the freezing process, spermatozoa experienced a cold shock, osmotic and oxidative stress (Anand and Yadav, 2016). The cold shock caused a change in the ratio of polyunsaturated fatty acids (PUFA) and lowered cholesterol content, resulting in a less stable spermatozoa membrane (Van Tran *et al*., 2016). Plasma membrane integrity was essential for protecting the spermatozoa's organelles and acted as a filter for transmembrane transport; thereby, it was vital for the viability and motility of spermatozoa (Pereira *et al*., 2017). Egg yolk contained a high level of cholesterol that was essential to improve the quality of frozen semen (Anzar *et al*., 2019).

An appropriate extender was essential to maintain post-thawed semen quality (Susilowati *et al*., 2020_cryo). Extender functioned to increase the volume of semen, act as an energy source and a buffer to prevent decreases of pH due to the formation of lactic acid that was harmful to spermatozoa, and contained antibiotics to prevent bacteria. Semen extender used in this study was tris-egg yolk with 6% glycerol as cryoprotectant. Tris (hydroxymethyl) aminomethane (Tris) was a pH regulator for buffering the pH (Namula *et al*., 2019). Egg yolks contained glucose as an

energy source, lipoproteins, and lecithin, which protected spermatozoa from cold stress (Rahman *et al*., 2018). Glycerol was a dominant cryoprotectant that could cross cell membrane. Semen extenders containing egg yolk with 6% glycerol resulted in high quality post-thawed semen (Bustani and Baiee, 2021).

In this study the best quality of post-thawed Sapera buck semen based on the highest spermatozoa viability and motility was found in freezing with pre-freezing stage at a 10 cm distance of straws position to the surface of liquid nitrogen for eight minutes. The best motility (41.75 ± 2.06%) in this study met the standards of 40% spermatozoa motility (INS, 2014) along with spermatozoa viability of 49.00 ± 0.82% (Table 2). However, these results were lower than the post-thawed motility and viability from previously reported (43.51 ± 0.76; and 61.37±1.68, respectively) by Hidayati *et al*. (2018). For a high post-thawed semen quality, there were species variations in the exposure to liquid nitrogen vapour for pre-freezing stage based on time and distance of straws above the liquid nitrogen surface. The straws that contained extended semen were placed horizontally 5 cm above the surface of liquid nitrogen for 10 minutes in goat (Kozdrowski *et al*., 2007), 6 cm for 9 minutes in Limousin bull (Aini *et al*., 2014), 4 cm for 10 minutes in ram (Savvulidi *et al*., 2021), and 1 cm for 40 seconds in Madura bull (Ardiana *et al*., 2018). A longer distance between straws and liquid nitrogen surface was followed by a slower temperature decrease, which causes metabolic processes to continue and the energy used to be used up quickly. This situation could increase lactic acid as the result of spermatozoa metabolism. A higher concentration of lactic acid made the diluent more acidic and toxic to spermatozoa which eventually causes the death of spermatozoa or not motile live spermatozoa (Zhou *et al*., 2015).

Morphological abnormalities of post-thawed Sapera buck semen were less than 5%, indicating that the ejaculates were qualified for AI (INS, 2014). Morphological abnormal spermatozoa could be caused by damage to the spermatozoa plasma membrane due to high levels of free radicals (Oumaima *et al*., 2018). During freezing, semen was exposed to cold shock and atmospheric oxygen, which caused higher ROS production (Kumar *et al*., 2019). The plasma membrane's intactness was essential for spermatozoa cells as it affected the metabolism associated with motility and viability (Leahy and Gadella, 2011). During thawing, spermatozoa also underwent extreme alterations in temperature and osmolarity. Osmotic changes caused damage to the lipid membrane structure (Hezavehei *et al*., 2018). This study was limited in the distance of 10-15 cm between the straws and the surface of liquid nitrogen for the eight minutes pre-freezing stage and evaluated based on the post-thawed motility, viability, and morphologic abnormality.

CONCLUSION

In tris-egg yolk extender, post-thawed spermatozoa viability and motility of Sapera buck were qualified for AI when fresh semen was pre-frozen at 10 cm distance of straws from the surface of liquid nitrogen for eight minutes. Further study is suggested using a distance of less than 10 cm between the straw and the liquid nitrogen surface with more parameters of spermatozoa quality (motility, viability, morphologic abnormality, malondialdehyde levels, intactness plasma membrane, and total antioxidant capacity).

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