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Original article

Impact of green tea (*Camellia sinensis*) leaf extract in skim milk-goose egg yolk semen extender on the quality of Sapudi ram spermatozoa stored at 5°C

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ABSTRACT

Livestock production requires Sapudi rams, a breed native to Indonesia, to meet meat demand and food security. In artificial high-quality frozen semen is needed to spread Sapudi rams. To maximize the survival of spermatozoa during cryopreservation, semen should be stored in an extender. Green tea leaf extract (GTLE) and skim milk-goat egg yolk (SM-GEY) may be a good cryoprotectants due to their antioxidant properties. This study aimed to determine the effect of adding GTLE to the SM-GEY extender on the quality of Sapudi ram spermatozoa stored at 5°C. The fresh semen sample was divided into four different GTLE treatment groups, which each contained a 0.1 mL semen sample and a 25-mL extender of SM-GEY. Group T0: no GTLE added to SM-GEY; Groups T1, T2, and T3: 0.1 mL semen diluted in 25 mL SM-GEY with 0.05, 0.10, and 0.15 mg GTLE. Extended semen was then stored at 5°C, and its quality was evaluated daily for five days. The variables observed included spermatozoa motility, viability, and membrane integrity. Data were analyzed using one-way analysis of variance followed by Duncan's test using Statistical Program and Service Solution version 23. The result of this study was that adding 0.05 mg GTLE to 25 mL of SM-GEY extender significantly maintained the spermatozoa motility, viability, and plasma membrane integrity of Sapudi ram spermatozoa for three days at 5°C ($p < 0.05$). Therefore, it could be concluded that adding 0.05 mg of GTLE to the SM-GEY extender preserved Sapudi ram spermatozoa's motility, viability, and membrane integrity for three days at 5°C.

Keywords: antioxidant, green tea leaf extract, skim milk-goose egg yolk, Sapudi spermatozoa quality

INTRODUCTION

Globally, small ruminant production plays an important role for both the environment and society. Usually, small ruminants are kept using a grazing system on land that is not as productive as the land used for cattle feed (Simões *et al*., 2021). Sheep are one of the meat-producing livestock animals that have long been kept in rural areas because they are cheaper but have high economic value (Ardiansyah *et al*., 2021). The Sapudi sheep is a breed of sheep native to Indonesia which is famous for its larger tail size compared to other sheep breeds. Sapudi sheep

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had energy reserves in their tails that could be used throughout the dry season (Putri *et al*., 2021; Mudawamah *et al*., 2022). On the other hand, the large tails of Sapudi sheep also make natural mating difficult. To increase reproduction, artificial insemination is required (Hafez, 2000). Artificial insemination can increase the population and improve the genetic quality of Sapudi sheep. Artificial insemination can be conducted using liquid chilled semen or frozen semen. Different AI methods are used for semen deposition. Fresh vaginal, cervical, or laparoscopic semen deposition resulted in 50-70% pregnancy rates (Faigl *et al*., 2012). However, the only way to get acceptable pregnancy rates with frozen semen was through laparoscopic insemination (Cseh *et al*., 2012; Kumar and Naqvi, 2014). However, Sapudi ram frozen semen is not available in rural areas. Therefore, veterinarians can innovate to preserve ram semen in refrigerators to serve artificial insemination of ewes. Liquid chilled storage semen needs appropriate extenders to maintain semen quality (Gibbons *et al*., 2019). Without the addition of extenders, Sapudi ram semen only lasted 18 hours outside of male or female ram at room temperature (Puspita *et al*., 2020). Storage at a temperature of 5°C was expected to reduce the metabolism rate of spermatozoa so that when semen was inseminated, it would still survive for several days with a percentage of motile spermatozoa of more than 40% to serve artificial insemination in areas around where the ram semen was collected (Rizkallah *et al*., 2022). Semen extender is crucial to maintain spermatozoa viability in storage. The extender contained a buffer solution to control pH, maintain osmolarity, supply energy, and increase semen volume (Zhang *et al*., 2023). Extenders that can be used for ram semen are skim milk-egg yolk. Skim milk contains lecithin to protect spermatozoa against cold stress during cooling (Bustani and Baiee, 2021). Meanwhile, egg yolk contains glucose, which is used as an energy source, carbohydrates, and amino acids as a source of protein needed by spermatozoa (Bustani and Baiee, 2021). Goose eggs have a higher lipoprotein content, an expected advantage for maintaining semen quality in storage (Zhang *et al*., 2022).

Spermatozoa are susceptible to damage due to lipid peroxidation caused by cold shock. Lipid peroxidation could be reduced by adding antioxidants. One of the antioxidants that is high in flavonoids is green tea. Giving green tea leaf extract could improve the quality and quantity of spermatozoa (Roychoudhury *et al*., 2017). Adding green tea extract to the extender could neutralize ROS and prevent DNA damage caused by ROS (Alqawasmeh *et al*., 2021). Until now, there has been no publication regarding the addition of green tea (*Camellia sinensis*) leaf extract (GTLE) in skim milk-goose egg yolk (SM-GEY) on the quality of Sapudi ram spermatozoa in 5°C storage. Therefore, this study aimed to determine the best GTLE dose in the SM-GEY extender on the viability, motility, and plasma membrane integrity of Sapudi ram spermatozoa.

MATERIALS AND METHODS

Sample collection

The procedure of this study was approved by three assessment committees before the treatment of Sapudi Ram was carried out. The sample used was semen from one Sapudi ram. Semen collection using an artificial vagina was done twice a week in the morning. Before collecting semen, the ram's prepuce was washed using soap, then rinsed with warm water until clean. Each semen collection was preceded by stimulating the libido of the male ram with an ewe in estrus to obtain good quality semen. The ram could mount the ewe two to three times but intromission was prevented. In the subsequent mounting, the ram's penis was directed into an artificial vagina to accommodate the ejaculated semen. Semen collection was carried out for three weeks to obtain five replicates.

Green tea leaf extract

One thousand grams of green tea (*Camellia sinensis*) leaf powder was soaked in eight liters of 96% ethanol for 12 days. The maceration results were evaporated using a rotary evaporator

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at a temperature of 50˚C with a speed of 40 rpm for 4-5 hours until a thick extract was obtained. Freeze drying was carried out until dry and crushed using a mortar to obtain green tea leaf extract powder (Susilowati *et al*., 2021).

Skim milk-goose egg yolk extender

Goose egg shells were cleaned using 70% alcohol cotton, then broken at the blunt side to upper third using sterile tweezers. The egg yolk was separated from the egg white and placed on sterile filter paper to absorb the remaining white. Vitelline membrane was torn, and 5 mL egg yolk was mixed with ten grams of skim milk and distilled water to make a total volume of 100 mL. Mixture was stirred until homogeneous, heated for one hour at 92°C, and then cooled to room temperature. Antibiotics (1000 IU penicillin and 1 mg streptomycin) were added and stirred homogeneously (Quraini *et al*., 2022).

Fresh semen was evaluated for macroscopic and microscopic feasibility before being mixed with the extender. Macroscopic observations included volume, color, odor, viscosity, and pH. Microscopic observations included mass movement, individual motility, concentration, and spermatozoa viability (Susilowati *et al*., 2021). Only feasible ejaculate (viability and motility ≥ 70%) was used in this study. The average concentration of fresh Sapudi rams was about 1,940 million/mL which was within standard values (Hardianto *et al*., 2010). The ejaculate of each collection was diluted in extender to a final spermatozoa concentration of 400 million spermatozoa/mL and then divided into four equal volumes, which would then be used for chilled spermatozoa preservation.

Treatment for each group was as follows, group T0: 0.1 mL of semen diluted in 25 mL of SM-GEY; groups T1, T2, and T3: 0.1 mL of semen diluted in 25 mL of SM-GEY containing 0.05, 0.10 and 0.15 mg of GTLE. The diluted semen was then stored at 5°C, and its quality was assessed daily for 5 days.

Evaluation of variables

Examination of motile spermatozoa was carried out by dripping one drop of semen and one drop of physiological sodium chloride solution onto a clean glass slide, mixing gently until homogeneous, then covering with a cover glass. Spermatozoa that move forward (progressively) were counted by observation under a microscope at 400x magnification from a population of 100 spermatozoa (Hardijanto *et al.*, 2010). Spermatozoa viability was assessed based on smear preparations with eosin nigrosine staining. A drop of semen sample and eosin nigrosin dye were placed on a glass slide, smeared, and heated quickly over a flame. Viable spermatozoa were counted by observing under a microscope at 400x magnification from 100 spermatozoa. Spermatozoa with purplish red heads are dead spermatozoa, while white ones are live spermatozoa (Quraini *et al*., 2022).

Hypoosmotic swelling (HOS) test was used to determine the integrity of spermatozoa membranes. The HOS solution was made from 1.35 g fructose and 0.74 g sodium citrate dissolved in 100 mL of distilled water. A 0.1 mL semen sample was added with 0.9 mL of HOS solution, mixed and left at room temperature for 1 hour. Semen was examined under a microscope with 400x magnification on 100 spermatozoa. Spermatozoa with intact membranes showed a coiled spermatozoa tail morphology. Spermatozoa whose membranes were damaged were characterized by straight tail morphology (Susilowati *et al*., 2021).

Data analysis

Data were analyzed using one-way analysis of variance followed by Duncan's test to determine the significance of differences between treatments. Data are presented as means ± standard deviation (SD), 95% confidence interval, or percentage. Statistical analysis was done using the Statistical Program and Service Solution for Windows version 23.

RESULTS

Fresh Sapudi ram semen was studied macroscopically and microscopically for this study. The macroscopic evaluation included volume, color, odor, consistency, and pH. The

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microscopic examination included spermatozoa concentration, mass movement, and individual motility (Table 1). Spermatozoa motility, viability, and plasma membrane integrity of Sapudi ram decreased during five days of storage at 5°C.

Table 1 Macroscopic and microscopic characteristics of fresh Sapudi ram semen

volume (mL)	0.58 ± 0.08
odor	typical
color	creamy white
consistency	thick
pH	6-7
concentration (million/mm^3)	1,940.00 ± 259.98
mass movement	+++
individual motility (%)	90

Spermatozoa viability

The addition of SM-GEY to the extender increased ($p < 0.05$) the viability of spermatozoa of Sapudi rams during five days of storage in the T1 group, while the T1 group was not significantly different ($p > 0.05$) from the T2 group. The addition of GTLE starting at a dose of 0.05 mg to the SM-GEY extender resulted in the highest spermatozoa viability ($p < 0.05$) compared to spermatozoa viability in the control group (Table 2).

Table 2 Daily viability (%) of Sapudi ram spermatozoa stored at 5°C for 5 days

	day 1	day 2	day 3	day 4	day 5
T0	86.0 ± 4.2 ^{a}	82.0 ± 2.7 ^{a}	72.0 ± 7.6 ^{a}	43.0 ± 5.7 ^{a}	28.0 ± 5.7 ^{a}
T1	93.0 ± 2.7 ^{b}	88.0 ± 5.7 ^{b}	80.0 ± 5.0 ^{b}	56.0 ± 6.5 ^{b}	39.0 ± 6.5 ^{b}
T2	90.0 ± 3.5 ^{ab}	87.0 ± 2.7 ^{ab}	79.0 ± 2.2 ^{ab}	49.0 ± 4.2 ^{ab}	36.0 ± 6.5 ^{ab}
T3	86.0 ± 4.2 ^{a}	84.0 ± 4.2 ^{ab}	77.0 ± 5.7 ^{ab}	46.0 ± 6.5 ^{a}	34.0 ± 4.2 ^{ab}

T0, T1, T2, and T3: 0.1 mL of semen diluted in 25 mL of skim milk-goose egg yolk respectively without, with 0.05, 0.10, and 0.15 mg green tea (*Camellia sinensis*) leaf extract; different superscripts in the same column indicate significant differences ($p < 0.05$).

Table 3 Daily motility (%) of Sapudi ram spermatozoa stored at 5°C for 5 days

	day 1	day 2	day 3	day 4	day 5
T0	78.0 ± 2.7 ^{a}	66.0 ± 4.2 ^{a}	36.0 ± 4.2 ^{a}	5.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}
T1	86.0 ± 4.2 ^{c}	75.0 ± 3.5 ^{b}	49.0 ± 4.2 ^{c}	8.0 ± 2.7 ^{a}	2.0 ± 2.7 ^{a}
T2	83.0 ± 2.7 ^{bc}	74.0 ± 4.2 ^{b}	44.0 ± 4.2 ^{bc}	7.0 ± 2.7 ^{a}	2.0 ± 2.7 ^{a}
T3	81.0 ± 4.2 ^{ab}	70.0 ± 3.5 ^{ab}	38.0 ± 5.7 ^{ab}	6.0 ± 2.2 ^{a}	0.0 ± 0.0 ^{a}

T0, T1, T2, and T3: 0.1 mL of semen diluted in 25 mL of skim milk-goose egg yolk respectively without, with 0.05, 0.10, and 0.15 mg green tea (*Camellia sinensis*) leaf extract; different superscripts in the same column indicate significant differences ($p < 0.05$).

Table 4 Daily plasma membrane integrity (%) of Sapudi ram spermatozoa stored at 5°C for 5 days

	day 1	day 2	day 3	day 4	day 5
T0	56.0 ± 4.2 ^{a}	50.0 ± 3.5 ^{a}	47.0 ± 2.7 ^{a}	32.0 ± 5.7 ^{a}	16.0 ± 4.2 ^{a}
T1	65.0 ± 5.0 ^{b}	56.0 ± 4.2 ^{b}	52.0 ± 2.7 ^{b}	41.0 ± 6.5 ^{b}	24.0 ± 8.2 ^{b}
T2	62.0 ± 5.7 ^{ab}	54.0 ± 4.2 ^{ab}	50.0 ± 3.5 ^{ab}	38.0 ± 5.7 ^{ab}	21.0 ± 4.2 ^{ab}
T3	57.0 ± 5.7 ^{a}	52.0 ± 2.7 ^{a}	47.0 ± 2.7 ^{a}	37.0 ± 5.7 ^{ab}	20.0 ± 3.5 ^{ab}

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T0, T1, T2, and T3: 0.1 mL of semen diluted in 25 mL of skim milk-goose egg yolk respectively without, with 0.05, 0.10, and 0.15 mg green tea (*Camellia sinensis*) leaf extract; different superscripts in the same column indicate significant differences ($p < 0.05$).

Spermatozoa motility

The addition of GTLE to the SM-GEY extender increased ($p < 0.05$) the motility of spermatozoa in Sapudi rams at one to three days of storage at T1 and T2 compared to T0, both on day 1 and day 3; the highest percentage of motility was found in T1 with the addition of 0.05mg GTLE to the SM-GEY extender (Table 3). Spermatozoa motility decreased drastically until there was no significant difference ($p > 0.05$) between treatments on the fourth and fifth examination days. The 0.05 mg GTLE dose in the SM-GEY extender produced the highest spermatozoa motility ($p < 0.05$) compared to spermatozoa motility in the control group and the 0.15 mg GTLE dose (Table 3).

Spermatozoa plasma membrane integrity

The addition of SM-GEY to the extender increased ($p < 0.05$) the plasma membrane integrity of Sapudi ram spermatozoa during five days of storage at T1 compared to T0 and T3, but was not significantly different from T2. The addition of GTLE starting at a dose of 0.05 mg to the SM-GEY extender resulted in the highest spermatozoa plasma membrane integrity ($p < 0.05$) compared to spermatozoa viability in the control group (Table 4).

DISCUSSION

Macroscopic and microscopic examination of Sapudi ram semen in this study showed that they were qualified for artificial insemination (Table 1). The normal semen of Sapudi ram is milky white or creamy like other breeds of rams which are white due to the influence of the riboflavin content (Ismaya, 2014). The color of semen is considered as abnormal when it becomes red because it is mixed with blood, which indicates disease in the male genital organs. Healthy semen from a ram has a thick consistency and the semen of a good Sapudi ram has a pH range of 6-7. A pH outside this range indicated poor semen quality (Ariyanto *et al*., 2020). The concentration of Sapudi ram's fresh semen in this study was 1,940.00 ± 259.98 million/mL. This is in line with previous research, wherein Sapudi ram spermatozoa has a concentration of around 1,500–3,000 million/mL (Puspita *et al*., 2020; Retta *et al*., 2022). Fresh semen for artificial insemination must be of good quality, with a motility percentage of more than 70% (Quraini *et al*., 2022).

Motility is one of the indicators used as a requirement for semen quality for artificial insemination. With their motility, spermatozoa swim from the location of semen deposition to the site of fertilization. Spermatozoa motility only occured in live spermatozoa. Live spermatozoa can only occur if the spermatozoa plasma membrane is still intact (Tanga *et al*., 2021). In ram spermatozoa, motility energy is mainly obtained from the anaerobic glycolysis metabolic pathway. The final product of glucose metabolism is the pyruvate molecule. Under aerobic conditions, pyruvate underwent oxidation and formed acetyl-CoA, the primary substrate of the Krebs cycle (Van de Hoek *et al*., 2022). Under anaerobic conditions, pyruvate was reduced to lactic acid (Miki, 2007). Spermatozoa motility decreased rapidly when stored at room temperature due to a decreased pH of lactic acid, which comes from spermatozoa metabolism when producing energy (Reynolds *et al*., 2017). Storage at 5 °C extended the life of semen by several days (Macías *et al*., 2017). Spermatozoa metabolism slowed down when cooled, thereby reducing the rate of lactic acid formation and increasing spermatozoa shelf life. Chilled temperatures maintained semen quality during transportation and short-term storage (Di Iorio *et al*., 2014). However, storing semen at chilled temperatures (5°C) could trigger the production of free radicals which cause lipid peroxidation (Pintus and Ros-Santaella, 2021; Rizkallah *et al*., 2022).

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Semen extenders are expected to contain substances that are almost the same as the physical and chemical properties of seminal plasma so that they could provide food as an energy source for spermatozoa and maintained the quality of spermatozoa to produce high fertility (Bustani and Baiee, 2021). In this study, an SM-GEY-based extender was used. Goose egg yolk have a higher protein and fat than those of chicken yolk (Polat *et al*., 2013). Phosphocaseinate and β-lactoglobulin in skim milk were the most effective components in maintaining spermatozoa longevity during cooling (Miki, 2007). The lecithin and lipoprotein content in egg yolk could protect spermatozoa cell membranes during cooling at 5°C (Bustani and Baiee, 2021). Tris-based buffer-chicken EY could improve total motility, vitality, plasma membrane integrity, and DNA integrity when cooled slowly to 5°C (Swelum *et al*., 2022). Adding egg yolk also aimed to maintain and protect spermatozoa's integrity and lipoprotein envelope. Egg yolk could protect spermatozoa from cold shock because it contained lipoprotein and lecithin, it contained glucose, which were effectively used by spermatozoa, and the viscosity was beneficial for spermatozoa (Bustani and Baiee, 2021). Goose egg yolk had a higher LDL and lower HDL than chicken (Zhang *et al*., 2022). The higher the LDL in the extender, the more effective it is in maintaining the integrity of the spermatozoa plasma membrane (Vera-Munoz *et al*., 2011). A previous report showed that the semen of Sapudi rams in skim milk extender alone (Retta *et al*., 2022) or chicken egg yolk-skim milk (Puspita *et al*., 2020), without added antioxidants, maintained more than 40% motility for two days at 5 °C. In this study, the semen of Sapudi ram extended in SM-GEY without GTLE maintained spermatozoa motility of more than 40% for two days. This observation suggested that the function of goose egg yolk in this study was in line with the research result of Retta *et al*. (2022) which also maintained the motility of spermatozoa of Sapudi ram compared to the addition of skim milk extender alone or chicken egg yok-skim milk extender (Puspita *et al*., 2020).

Addition of GTLE as an antioxidant was expected to maintain spermatozoa motility, viability, and plasma membrane integrity when stored at 5°C. Green tea had a high antioxidant effect because it contained bioactive phenols consisting of catechins. Catechins are composed of epigalocatechin (EGC), epigalocatechinalat (EGCG), epicatechin (EC), epicatechin-gallate (ECG), and galocatechin (GC) (Khan *et al*., 2017). Catechins are secondary metabolite compounds produced naturally by plants and belong to the flavonoid group (Bernatoniene and Kopustinskiene, 2018). Supplementation with 0.10 mg/100 mL of green tea extract as a bovine semen extender can maintained spermatozoa quality as measured by motility, viability, plasma membrane integrity, necrosis percentage, and spermatozoa apoptosis (Susilowati *et al*., 2019). The addition of 0.05 mg GTLE in 25 mL of SM-GEY extender maintained the motility, viability, and plasma membrane integrity of Sapudi ram spermatozoa for three days at a storage temperature of 5°C. Green tea extract contained several substances, including alkaloids, saponins, tannins, tyrosine, glutamic acid, and carbohydrates such as cellulose, glucose, fructose, and sucrose (Tang *et al*., 2019), as well as several types of vitamins, including A, B1, B2, C, E, and K. Vitamins C and E could ward off free radicals (Chacko *et al*., 2010). The flavonoid content in green tea extract also acted as an antioxidant. Flavanoids could inhibit the formation of free radicals, inhibit oxidation reactions, and protect against reactions that damage membrane lipids (Panche *et al*., 2016). Antioxidants had two functions i.e., as a provider of hydrogen atoms, slowing the rate of autooxidation by converting lipid radicals into a more stable form (Mehta and Gowder, 2015). The higher the percentage of membrane integrity, the better the semen quality. Damage to the integrity of the plasma membrane due to lipid peroxidation, which caused an increase in unsaturated fatty acids in the plasma membrane, these fatty acids included linoleic, linolenic, and arachidonic acids (Collodel *et al*., 2020).

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The addition of 0.05 mg GTLE in 25 mL of SM-GEY extender was highly recommended because it could maintain viability, motility, and plasma membrane integrity of Sapudi ram spermatozoa for three days at a storage temperature of 5°C. However, there was no increase in motility, viability, or plasma membrane integrity in Sapudi ram spermatozoa when greater concentrations of GTLE (0.10 or 0.15 mg) were added to 25 mL of SM-GEY extender. In Kacang goats, the higher dose of GTE in skim milk-chicken egg yolk extender caused a decrease in spermatozoa quality in chilled storage (Susilowati *et al*., 2021). Adding higher levels of green tea extract could result in a more acidic pH due to the greater concentration of tannin. The higher antioxidant capacity of green tea extract affected the oxidant-antioxidant balance (Kisaoglu *et al*., 2013). Higher exposure to antioxidants (reductants) was followed by an antioxidant paradox that damaged spermatozoa quality (Halliwell, 2013; Susilowati *et al*., 2021).

CONCLUSION

These preliminary results indicate that the addition of green tea (*Camellia sinensis*) leaf extract at a dose of 0.05 mg to a combination of skim milk and goose egg yolk extender could maintain the motility, viability and membrane integrity of Sapudi sheep spermatozoa at acceptable levels. when stored at 5°C for 72 hours.

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AUTHOR'S CONTRIBUTIONS

Ardina Sahra Miranda (ASM), Tri Wahyu Suprayogi (TWS), Budi Utomo (BU), Suherni Susilowati (SS), Yeni Dhamayanti (YD).

ASM, TWS, and BU: conceived the idea, designed the mainframe of this manuscript, acquisition, analysis and interpretation of data, and manuscript drafting. SS, YD, and BU: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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