

Comparison of different poultry egg yolks-citrate extender with green tea (*Camellia sinensis*) extract addition on Sapudi ram spermatozoa quality in chilled temperature storage

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ABSTRACT

Preservation of ram semen has an impact on the exposure of artificial conditions to the spermatozoa which was followed by overproduction of reactive oxygen species (ROS) and resulted in functional damages of spermatozoa. This study aimed to determine the effect of the combination of chicken, quail, and duck egg yolks with green tea extract (GTE) on the quality of Sapudi ram spermatozoa at 5°C storage. Ejaculates of Sapudi ram with more than 70% spermatozoa motility were divided into four groups. Semen were extended 1 : 10 in citrated-chicken egg yolk extender (control group, T0), and citrated yolk of chicken (T1), quail (T2), and duck (T3) egg added with 0.05 mg GTE per 100 mL of extender. Extended semen was kept in a tube, which was put in a beaker containing distilled water and stored in a refrigerator (5°C). Spermatozoa motility, viability, and plasma membrane integrity (PMI) were evaluated daily for five days. The results showed that GTE in duck egg yolk citrate extender could maintain the highest spermatozoa motility, viability, and PMI for five days ($p < 0.05$). It could be concluded that the duck egg yolk citrate extender with the addition of GTE (*Camellia sinensis*) was beneficial for maintaining the semen of Sapudi ram spermatozoa at chilled temperatures. Further study is needed to use the extender for freezing Sapudi ram semen.

Keywords: egg yolk, green tea extract, Sapudi ram, spermatozoa quality

INTRODUCTION

Artificial insemination (AI) is common in animal husbandry and breeding programs (Parkinson and Morrell, 2019). Semen quality is an essential factor determining the success of the AI program (Oliveira *et al.*, 2012). AI can be conducted using fresh semen, chilled stored semen, or frozen semen (Al-Bulushi *et al.*,

2019). Preservation of semen resulted in an exposure to artificial conditions to spermatozoa which was followed by overproduction of reactive oxygen species (ROS) and subsequently caused functional damages of spermatozoa (Sabeti *et al.*, 2016; Kameni *et al.*, 2021). Free radicals can be inhibited by the addition of antioxidants derived from GTE. Green tea (*Camellia sinensis*) contains

flavonoids, the largest group of polyphenols that are effective as antioxidants. The content of polyphenols, especially catechins in green tea, can suppress the production of ROS. Green tea is a tea product with the highest catechin content among other teas (Musial *et al.*, 2020). Therefore, GTE could be used as an ingredient of choice and added to the extender as an antioxidant to counteract free radicals (Susilowati *et al.*, 2021).

Egg yolk-based extender is often used for semen preservation. Lipoproteins and lecithin in egg yolk played a role in maintaining the integrity of the spermatozoa plasma membrane (Ondřej *et al.*, 2019) and PMI was crucial in the fertilization process that determines the success of AI (Wurlina *et al.*, 2020). A previous study reported that chickens, quail, and duck's egg yolks were helpful as an extender in Pelung rooster semen (Widiastuti *et al.*, 2018). The effect of chicken, quail, and duck egg yolks in combination with GTE (*Camellia sinensis*) for Sapudi ram semen has never been studied. Spermatozoa motility is a primary indicator of semen quality (Chakraborty and Saha, 2022), while spermatozoa quality was related to PMI (Shan *et al.*, 2021). Therefore, this study aimed to determine the effect of chickens, quails, and ducks' egg yolk combined with GTE to maintain Sapudi ram spermatozoa quality in 5°C storage.

MATERIALS AND METHODS

This study used one Sapudi ram, reared at the Faculty of Veterinary Medicine, Universitas Airlangga. Green tea (*Camellia sinensis*) extract was made at the Laboratory of Veterinary Pharmacology, while semen quality was evaluated at the Laboratory of Artificial Insemination, Faculty of Veterinary Medicine, Universitas Airlangga. This study was conducted from September to October 2020. The ejaculate of Sapudi ram used in this study had a characteristic odor, white-beige color, pH of 6-7, thick consistency, with a mass movement of ++++. The range and averages of semen sample parameters were volume of 0.7 - 0.9 (0.8 ± 0.1) mL, concentration of 1680 - 2520 (2078.40 ± 332.71) (million/mL), spermatozoa motility of 85 - 90 (87.60 ±

2.51)%, viability of 94 - 96 (95.00 ± 0.71)%, and PMI of 69 - 77 (73.60 ± 2.97)%.

Green tea extraction

Dried green tea leaves were ground using a grinding machine. For maceration procedure, ground green tea leaf was then soaked in 96% ethanol solvent for three days, covered with aluminum foil.

The soaked green tea powder was filtered and squeezed using filter paper. Filtrate was then evaporated in a rotary evaporator at a temperature of 50°C at 45 rpm to obtain a dense concentration extract with moisture content of 4-5%. Furthermore, GTE was freeze-dried to obtain a dry and powdered extract.

Egg yolk citrate extender

Sodium citrate 2.9 grams and sulfanilamide 0.3 grams were extended in 100 mL of distilled water. Fresh eggshells (chicken, quail, and duck) were sterilized using 70% alcohol soaked cotton. Eggs were then broken at the blunt end using sterile tweezers, and all egg whites were slowly removed. The egg yolks are transferred to filter paper to remove any remaining egg white. The egg yolk was added with sodium citrate solution in the ratio of 1 : 4. Egg yolk citrate extender was added with 1000 IU penicillin (Meiji Seika Pharma, Tokyo, Japan) /mL extender and 1 mg streptomycin (Thermo Fisher Scientific, Singapore)/mL extender and stirred until homogeneous (Susilowati *et al.*, 2021).

Treatment groups

In the control group (T0), 0.1 mL of Sapudi ram semen was diluted in 1 mL of citrated chicken egg yolk. Meanwhile, in groups, T1, T2, and T3, 0.1 mL of Sapudi ram semen was diluted in 1 mL of citrated egg yolk of chickens, quail eggs, and duck eggs containing 0.05 mg GTE per 100 mL extender (Khoirunnisa *et al.*, 2019). Extended semen was contained in a tube, which was placed in a beaker glass containing aqua dest and stored in a refrigerator (5°C). Evaluation of spermatozoa motility, viability, and PMI (Susilowati *et al.*, 2018) was conducted daily in each group for five days.

Data analysis

The data obtained were statistically analyzed using Analysis of Variance (ANOVA) followed by Duncan's Multiple Distance Test with a significance level of 5%. Statistical analysis was conducted using SPSS 23 software for Windows (IBM Corp, Chicago, USA).

RESULTS

The control group met the requirements to use AI up to three days. Meanwhile, those of the T1 group were four days, and the T2 and T3 groups were five days (Table 1). The highest sperm viability and PMI at five days of storage were found in the T3 group (Table 2, Table 3).

Table 1 Spermatozoa motility (% , means ± SD) of Sapudi ram in poultry egg yolk-citrate extenders for five days chilled storage (5°C)

	day 1	day 2	day 3	day 4	day 5
T0	84.00 ± 2.24 ^a	71.00 ± 2.24 ^a	55.00 ± 5.00 ^a	38.00 ± 4.47 ^a	25.00 ± 3.54 ^a
T1	87.00 ± 2.74 ^{ab}	78.00 ± 4.47 ^b	68.00 ± 4.47 ^b	53.00 ± 4.47 ^b	37.00 ± 2.74 ^b
T2	88.00 ± 2.74 ^b	82.00 ± 2.74 ^{bc}	71.00 ± 4.18 ^{bc}	58.00 ± 4.47 ^{bc}	41.00 ± 4.18 ^{bc}
T3	88.00 ± 2.74 ^b	83.00 ± 2.74 ^c	74.00 ± 2.24 ^c	63.00 ± 5.70 ^c	43.00 ± 2.74 ^c

Different superscripts in the same column showed significant differences (p <0.05); T0: Sapudi ram semen was diluted in citrate-chicken egg yolk extender; T1, T2, and T3: Sapudi ram semen was respectively diluted in citrate-chicken, quail, and duck egg yolk extender containing 0.05 mg green tea extract per 100 mL of the extender; in all group semen was diluted 11 times; replicate= 5

Table 2 Spermatozoa viability (% , means ± SD) of Sapudi ram in poultry egg yolk-citrate extenders for five days chilled storage (5°C)

	day 1	day 2	day 3	day 4	day 5
T0	90.40 ± 1.52 ^a	79.60 ± 3.44 ^a	69.40 ± 2.41 ^a	59.00 ± 6.33 ^a	45.60 ± 7.23 ^a
T1	93.00 ± 1.23 ^b	86.20 ± 1.92 ^b	77.00 ± 3.61 ^b	68.60 ± 4.16 ^b	58.20 ± 4.03 ^b
T2	91.80 ± 2.17 ^{ab}	87.40 ± 1.95 ^b	77.60 ± 2.88 ^b	70.00 ± 4.30 ^b	59.20 ± 3.35 ^b
T3	92.80 ± 1.64 ^b	89.40 ± 1.52 ^b	82.00 ± 1.58 ^c	73.80 ± 4.15 ^b	63.80 ± 3.56 ^b

Different superscripts in the same column showed significant differences (p <0.05); T0: Sapudi ram semen was diluted in citrate-chicken egg yolk extender; T1, T2, and T3: Sapudi ram semen was respectively diluted in citrate-chicken, quail, and duck egg yolk extender containing 0.05 mg green tea extract per 100 mL of the extender; in all group semen was diluted 11 times; replicate= 5

Table 3 Spermatozoa plasma membrane integrity (% , means ± SD) of Sapudi ram in poultry egg yolk-citrate extenders for five days chilled storage (5°C)

	day 1	day 2	day 3	day 4	day 5
T0	60.20 ± 2.78 ^a	52.60 ± 6.27 ^a	42.20 ± 4.38 ^a	31.80 ± 3.90 ^a	25.20 ± 4.66 ^a
T1	67.80 ± 4.32 ^b	58.80 ± 6.22 ^{ab}	48.00 ± 4.85 ^b	38.80 ± 3.96 ^b	31.80 ± 4.97 ^{bc}
T2	66.80 ± 3.96 ^b	58.60 ± 3.36 ^{ab}	48.60 ± 2.30 ^b	39.60 ± 5.98 ^b	29.20 ± 4.38 ^{ab}
T3	70.60 ± 3.21 ^b	63.20 ± 2.39 ^b	54.20 ± 2.28 ^c	44.20 ± 3.56 ^b	35.60 ± 3.65 ^c

Different superscripts in the same column showed significant differences (p <0.05); T0: Sapudi ram semen was diluted in citrate-chicken egg yolk extender; T1, T2, and T3: Sapudi ram semen was respectively diluted in citrate-chicken, quail, and duck egg yolk extender containing 0.05 mg green tea extract per 100 mL of the extender; in all group semen was diluted 11 times; replicate= 5

DISCUSSION

Motility is an essential indicator of semen quality (Suprayogi and Susilowati, 2018). Sapudi ram semen in duck egg yolk-citrate extender added with 0.05 mg GTE per 100 mL of the extender was able to maintain spermatozoa motility of more than 40% for five days in chilled storage at 5°C. This corresponded to El-Shamaa *et al.* (2012) report that duck egg yolk could maintain spermatozoa motility better than chicken egg yolk when used as an extender for buffalo semen. The carbohydrate content in duck egg yolks is higher than in chicken and quail egg yolks (Liu *et al.*, 2018). Duck egg yolk could maintain the motility of spermatozoa better when compared to chicken and quail egg yolk because higher carbohydrate content served as a source for ATP synthesis (Peña *et al.*, 2021).

Lipoprotein and lecithin in egg yolk could maintain integrity and stabilized the plasma membrane. Thereby, the extender has the ability to maintain the motility of spermatozoa (Ondřej *et al.*, 2019). The higher lipoprotein and lecithin content in the extender was followed by a better ability to maintain the integrity of the plasma membrane of spermatozoa. Low-Density Lipoprotein (LDL) content in duck egg yolks is also higher than those in chicken egg yolks and quail egg yolks. (Widiastuti *et al.*, 2018).

The cholesterol content in duck egg yolks was higher than those of chicken egg yolks and quail egg yolks (Aziz *et al.*, 2012). The cholesterol content in egg yolk was the most effective agent to protect spermatozoa when stored at low temperatures. Higher cholesterol content in the egg yolk would be followed by its ability to protect spermatozoa cells. This was due to cholesterol ability to maintain the integrity of the plasma membrane, where the integrity of the plasma membrane was indispensable for spermatozoa to survive (Anand *et al.*, 2017). The cholesterol in egg yolk was a lipid component of spermatozoa membranes which was very important for spermatozoa in maintaining membrane fluidity. The higher the cholesterol content in the egg yolk, the better the membrane's fluidity or, the more flexible it is. The lower cholesterol

content in the extender will cause the spermatozoa membrane to be more easily damaged (Behera *et al.*, 2015). The integrity of the plasma membrane is needed for normal metabolic processes and is related to the spermatozoa motility and survival of spermatozoa (Wurlina *et al.*, 2020). In a hypoosmotic solution, spermatozoa with a membrane with good function, there will be swelling of the plasma membrane and bending of the tail (Teja *et al.*, 2018).

CONCLUSION

The extender of duck egg yolk citrate supplemented with green tea extract (*Camellia sinensis*) maintained spermatozoa quality of Sapudi ram at 5°C for five days.

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