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Original article

Effect of fruit juices in skim milk extender in maintaining Sapudi ram spermatozoa quality at chilled temperature

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ABSTRACT

This study aimed to determine the effect of the addition of cherry tomato, california papaya, and honey melon juice into skim milk extender in maintaining the quality of Sapudi ram semen at chilled temperature (5°C) storage. Five replication of ejaculates were divided equally into four groups. In control group (T0) semen was diluted in skim milk extender, while in T1, T2, and T3 groups semen were diluted in skim milk extender contained 20% of cherry tomato, california papaya, and honey melon juice, respectively. The extended semen was stored at a chilled temperature, and semen quality (based on sperm motility, viability, and plasma membrane integrity) was observed daily for five days. The result showed that semen quality declined day by day during the five days of storage. Based on the minimum standard of post-thawed semen motility (40%), the spermatozoa of the control group only lasted by the second day. Meanwhile, in the groups with the addition of fruit juice motility could last up to the third day, with the highest motility ($p < 0.05$) on the addition of california papaya juice. This study concluded that the addition of 20% (v/v) california papaya juice in skim milk extender could maintain the percentage of spermatozoa motility up to three days at a chilled temperature.

Keywords: extender, fruit juice, Sapudi ram, skim milk, spermatozoa quality

INTRODUCTION

Artificial Insemination (AI) is the right technology to increase sheep population (Gibbons *et al*., 2019). AI technique can be carried out using chilled semen, when the frozen semen is difficult to obtain (Susilowati *et al*., 2021). An extender is crucial for maintaining the quality of semen because it serves as a buffer and a source of nutrition for spermatozoa. Skim milk-based extender contained lipoprotein and lecithin, which could protect spermatozoa from chilled shock during the cooling process (Bustani and Baiee, 2021). At chill temperature storage, the metabolism of spermatozoa was slower, thereby extending the lifespan (Rizkallah *et al*., 2022). However, storage at chill temperatures produced free radicals that could damage spermatozoa membranes through lipid peroxidation reactions (Asadi *et al*., 2017). Membrane damage in

spermatozoa could reduce the percentage of sperm motility and viability (Agarwal *et al*., 2014). The addition of antioxidants to an extender was expected to maintain the plasma membrane's motility, viability, and integrity (Susilowati *et al*., 2020).

Antioxidants can be obtained from juice extracted from the fruits (Hidalgo and Almajano, 2017). Fruit juices also contain carbohydrates, proteins, fats, vitamins, and minerals (Slavin and Lloyd, 2012), which could support the life needs of spermatozoa. The fruits used in this study were cherry tomato, california papaya, and honey melon. There have been no reports on the use of these fruit juices in the Sapudi ram semen extender. This study aimed at determining the effect of the addition of cherry tomato, california papaya, and honey melon juices to skim milk extender on motility, viability, and plasma membrane integrity of Sapudi ram spermatozoa stored at chill temperature (5° C).

MATERIALS AND METHODS

This study used a male and a female Sapudi sheep. Female sheep was used as a teaser at the time of semen collection. The animals were fed forage 10% of body weight and concentrate 1% of body weight per day. Drinking water was always available in the cage. Semen was collected once a week using an artificial vagina for five weeks. Sapudi ram semen that had viability and motility of more than 70%, was further processed into chilled liquid semen.

Fruit juice

Ripe cherry tomatoes (*Lycopersicon esculentum* Mill), california papaya (*Carica papaya*), and honey melon (*Cucumis melo*) juices were used in this study. Each fruit of 250 grams was thoroughly washed using distilled water, cut into pieces and then mashed using a blender without the addition of water. Fruit juices were squeezed through a fine sieve and spun at 3000 x g for 20 min. The supernatant was transferred into a sterile tube and used immediately (Zulkhair *et al*., 2016).

Skim milk extender-fruit juice

Ten grams of skim milk powder (Merck 115338) was put in a beaker glass, added with distilled water up to 100 mL, and heated in a water bath at 92-95°C for 10 minutes. The skim milk solution was allowed to cool at room temperature (20-27°C) to 32°C and then filtered. Skim milk solution was added with 1000 IU penicillin (Meiji Seika Pharma, Tokyo, Japan) and 1 mg streptomycin (Thermo Fisher Scientific, Singapore) per mL of extender, stirred until homogeneous (Susilowati *et al*., 2021). Each juice was added into the skim milk extender to a final concentration of 20% (v/v) (Zulkhair *et al*., 2016), then the pH was measured.

Treatment groups

Semen was added to the extender in a ratio of 1:10 (11 times dilution). T0 was the control group in which Sapudi ram spermatozoa was diluted in skim milk extender. T1, T2, and T3, were the treatment groups in which Sapudi ram semen were diluted in skim milk extender contained 20% (v/v) cherry tomato, california papaya, and honey melon juice respectively. The tube was closed and put in a refrigerator at 5^{o}C. Assessments of the motility, viability, and spermatozoa plasma membrane integrity were conducted daily for five days (Susilowati *et al*., 2020).

Data analysis

The data were analyzed using one-way ANOVA. As there was a significant difference, then the analysis was continued with Duncan's test with a significant level of 5% ($p < 0.05$). Statistical analysis was carried out using SPSS 23 for windows (IBM Corp., NY, USA).

RESULTS

In this study, five samples of Sapudi ram semen had creamy white color, pH 6-7, thick consistency, with mass movement +++, and individual movement of 90%. The average and range parameters of fresh semen of Sapudi ram are shown in Table 1.

Table 1 Characteristics of fresh semen of Sapudi ram collected by artificial vagina

	averages	range
volume (mL)	0.58 ± 0.08	0.5 - 0.7
concentration (x 10^6/mL)	1714.80 ± 245.43	1380 - 1944
viability (%)	93.00 ± 0.71	92.0 - 94.0
IPM (%)	61.00 ± 1.41	60 - 63

IPM: integrity of plasma membrane

The pH of the skim milk extender was 6, and the skim milk extender supplemented with 20% (v/v) cherry tomato, california papaya juice, and honey melon juice were all between 6-7. Semen quality based on motility (Table 2), viability (Table 3), and spermatozoa plasma membrane integrity (Table 4) decreased day by day during the five days of storage. Based on the minimum standard of post-thawed spermatozoa motility (40%), the spermatozoa of the control group only lasted until the second day. Meanwhile, in the groups with the addition of fruit juices spermatozoa motility could last up to the third day, with the highest motility ($p < 0.05$) on the addition of california papaya juice (Table 2).

Table 2 Motility (%) of Sapudi ram spermatozoa in chilled storage

	day-1	day-2	day-3	day-4	day-5
T0	81.00 ± 4.18 ^{a}	64.00 ± 4.18 ^{a}	37.00 ± 6.70 ^{a}	18.00 ± 5.70 ^{a}	4.00 ± 4.18 ^{a}
T1	81.00 ± 4.18 ^{a}	70.00 ± 3.53 ^{ab}	49.00 ± 5.47 ^{ab}	33.00 ± 5.70 ^{b}	10.00 ± 5.00 ^{ab}
T2	81.00 ± 4.18 ^{a}	73.00 ± 5.70 ^{b}	56.00 ± 4.18 ^{c}	38.00 ± 7.58 ^{b}	14.00 ± 4.18 ^{b}
T3	80.00 ± 5.00 ^{a}	65.00 ± 3.53 ^{a}	43.00 ± 9.08 ^{bc}	19.00 ± 9.61 ^{a}	4.00 ± 4.18 ^{a}

Different superscripts in the same column indicate significant differences ($p < 0.05$); T0: Sapudi ram semen was diluted in skim milk extender; T1-T3: Sapudi ram semen was diluted in skim milk extender contained 20% (v/v) cherry tomato, california papaya, and honey melon juice respectively; semen was diluted 11 times (1:10) in each extender; storage temperature= 5°C.

Table 3 Viability (%) of Sapudi ram spermatozoa in chilled storage

	day-1	day-2	day-3	day-4	day-5
T0	88.88 ± 1.09 ^{b}	79.80 ± 1.30 ^{a}	71.20 ± 4.60 ^{a}	43.00 ± 5.56 ^{a}	23.00 ± 3.31 ^{a}
T1	87.80 ± 1.64 ^{ab}	82.00 ± 2.44 ^{ab}	73.60 ± 4.56 ^{a}	56.40 ± 5.89 ^{b}	29.00 ± 2.64 ^{b}
T2	89.60 ± 1.14 ^{b}	84.00 ± 2.23 ^{b}	76.40 ± 4.33 ^{a}	60.60 ± 6.22 ^{b}	34.40 ± 3.36 ^{c}
T3	85.60 ± 3.64 ^{a}	80.20 ± 2.68 ^{a}	70.20 ± 9.09 ^{a}	45.40 ± 9.37 ^{a}	24.80 ± 4.14 ^{ab}

Different superscripts in the same column indicate significant differences ($p < 0.05$); T0: Sapudi ram semen was diluted in skim milk extender; T1-T3: Sapudi ram semen was diluted in skim milk extender contained 20% (v/v) cherry tomato, california papaya, and honey melon juice respectively; semen was diluted 11 times (1:10) in each extender; storage temperature= 5°C.

DISCUSSION

Sapudi ram semen diluted in skim milk extender only lasted two days to be eligible for AI. This was presumably due to the difference in pH of semen from 6-7, while the pH of the skim milk extender was 6. The decreased sperm viability during storage was due to the reduced availability of energy in the extender and the increasing concentration of lactic acid in the media (Bustani and Baiee, 2021). The increase in lactic acid leftover from cell metabolism caused the extender to become more acidic, damaging the spermatozoa plasma membrane (Kowalczyk *et al*., 2020). In addition, skim milk only contains about 3.2% (w/w) protein, about 80% of which was casein, and the remaining was whey protein (Górska-Warsewicz *et al*., 2019) which did not contain the substrates and antioxidants that fruit juices had. Antioxidants are often used to improve semen quality including vitamin E, vitamin C, and lycopene (Majzoub and Agarwal, 2018). Vitamin

C, vitamin E, and lycopene are antioxidants that can bind oxygen radicals formed during storage and prevent lipid peroxidation. Prevention of lipid peroxidation maintained spermatooa plasma membrane integrity, viability, and motility (Asadi *et al*., 2017).

Table 4 Spermatozoa intact plasma membrane (%) of Sapudi ram in chilled storage

	day-1	day-2	day-3	day-4	day-5
T0	55.20 ± 2.16 [a]	42.40 ± 2.60 [a]	34.60 ± 2.07 [a]	23.60 ± 4.09 [a]	11.80 ± 1.64 [a]
T1	55.00 ± 4.35 [a]	43.20 ± 1.78 [a]	35.80 ± 2.68 [ab]	28.60 ± 3.13 [ab]	14.20 ± 2.86 [a]
T2	56.80 ± 3.27 [a]	45.40 ± 2.60 [a]	38.60 ± 3.84 [b]	30.40 ± 4.50 [b]	17.40 ± 2.40 [b]
T3	55.20 ± 3.96 [a]	42.40 ± 2.30 [a]	35.80 ± 1.30 [ab]	24.80 ± 3.34 [a]	12.40 ± 2.07 [a]

Different superscripts in the same column indicate significant differences ($p < 0.05$); T0: Sapudi ram semen was diluted in skim milk extender; T1-T3: Sapudi ram semen was diluted in skim milk extender contained 20% (v/v) cherry tomato, california papaya, and honey melon juice respectively; semen was diluted 11 times (1:10) in each extender; storage temperature= 5°C.

With the addition of california papaya juice motility last up to the third day with the highest motility compared to the other groups. Fruit juice contains lycopene, riboflavin, tryptophan, glucose, fructose, and sucrose (Del Río-Celestino and Font, 2020). Lycopene functions as a potent antioxidant and can control free radicals more effectively than vitamin E (Imran *et al*., 2020). The addition of california papaya juice had the highest percentage of motility because california papaya has a higher vitamin C and fructose content than cherry tomatoes and honey melon (USDA, 2016). Spermatozoa need proper nutrition for their survival. Fructose is a type of sugar that is easily metabolized into energy. Fructose addition in the extender can be the primary energy source of spermatozoa in seminal plasma for metabolic processes (Pappa *et al*., 2019). Antioxidants prevent lipid peroxidation in the mitochondrial membrane, which can inhibit glycolysis and decrease sperm motility (Agarwal *et al*., 2019). Lycopene can also optimize the rate of fructolysis, thereby, the energy needs for motility and survival of spermatozoa can be fulfilled (El-Ratel, 2017).

CONCLUSION

The addition of 20% (v/v) california papaya juice in skim milk extender can maintain the quality of Sapudi ram semen for AI for up to three days at 5°C chilled storage. Further research on the percentage of california papaya juice in skim milk extender is needed to find a longer chilled storage.

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