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The effect of pineapple [*Ananas comosus* (L.) Merr.] juice in extender on Garut ram (*Ovis aries* L.) spermatozoa quality postcryopreservation

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Abstract. This study was intended to determine the effect of various concentrations of pineapple [*Ananas comosus* (L.) Merr.] juice in extender on garut ram (*Ovis aries* L.) spermatozoa quality 24 h postcryopreservation. Semen samples were collected from five garut rams once a week using an artificial vagina. The samples were diluted in Tris-egg yolk extender with pineapple juice at concentrations of 0 % (KK), 5 % (KP 1), 10 % (KP 2), 15 % (KP 3), and 20 % (KP 4). The diluted semen samples were loaded into mini straws (0.25 mL) with 50 million cells/mL dosage. Samples were equilibrated at 5 °C for 2 h, and then frozen and stored in liquid nitrogen. Parameters evaluated were motility, viability, membrane integrity, acrosome integrity, and morphological abnormality. One-way ANOVA followed by Duncan's test showed significant differences ($P < 0.05$) between KK and KP 3 in the percentage of motile spermatozoa and membrane integrity. Pineapple juice at 15 % could minimize the reduction of spermatozoa quality based on the percentage of motile spermatozoa (52.19 ± 5.32 %) and membrane integrity (38.52 ± 4.85 %).

Keywords: Cryopreservation, Garut ram, pineapple juice, spermatozoa

1. Introduction

Garut ram (*Ovis aries* L.) is a breed found in Garut, West Java, Indonesia [1]. This indigenous ram is prolific and has a higher body weight than other local rams. Moreover, garut rams have been bred for traditional ram-fighting contests, so they have high economic value [2]. Garut rams can be used as semen donors to develop other local rams using reproductive technology [3]. Therefore, there is a need to perform semen cryopreservation to provide high-quality semen of garut rams for further use.

However, semen cryopreservation lowers the quality of spermatozoa and affects their capacity for fertilization [4]. Lipid peroxidation caused by reactive oxygen species (ROS) results in cellular damage to spermatozoa, which is a reaction involving an autocatalytic process [5-7]. Mammalian sperm cells are susceptible to lipid peroxidation because of their high level of unsaturated fatty acids [8]. Antioxidants protect the cells by neutralizing the excess of free radicals [9]. Fruits contain constituents exhibiting antioxidant activity, such as vitamins, flavonoids, and other phenolic metabolites [10]. It was reported that the administration of pineapple [*Ananas comosus* (L.) Merr.]



juice at 10 % in semen extender improved the quality of spermatozoa of West African Dwarf goats after cryopreservation [11]. Against this background, the aim of the present study was to investigate the effect of adding different concentrations of pineapple juice to semen extender on the quality of sperm of garut ram after their cryopreservation.

2. Materials and method

2.1. Pineapple processing

The pineapples were bought in Kebun Buah Mekarsari, Indonesia. They were peeled, washed, cut into pieces, and the juice was extracted by a juicer. The juice was centrifuged at 3000 rpm for 15 min and stored at -25 °C until use.

2.2. Semen collection

Five mature rams reared at the Dombing Cluster, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), were included in this study. Semen samples from these five rams were collected using an artificial vagina at weekly intervals for 5 weeks. The samples were transferred to a lab and initially evaluated for volume, color, odor, consistency, pH, mass activity, concentration, sperm motility, viability, membrane integrity, acrosome integrity and abnormality. The semen was stored in a water bath at 37 °C before dilution.

2.3. Semen processing

A control extender (Tris-citric acid-egg yolk-fructose) was prepared for the control group. Extenders with 5 %, 10 %, 15 % and 20 % pineapple juice in Tris-citric acid-egg yolk-fructose solution were prepared for the treatment groups. Semen samples were diluted in each extender, loaded into 0.25 straws, and equilibrated at 5 °C for 2 h. Subsequently, the straws were placed above liquid nitrogen in the vaporous phase for 10 min and were then immersed in liquid nitrogen for 24 h.

2.4. Semen quality assessment

Some frozen straws were thawed at 37 °C for 10 s. The parameters studied were sperm motility, viability, membrane integrity, acrosome integrity and morphological abnormality.

2.4.1. Sperm motility (%). Sperm motility was assessed using Sperm Vision software. A 5 L semen sample was diluted in 95 L of NaCl. A 10 L diluted semen sample was placed on a heated microscope slide and assessed. For each sample, five microscopic fields were examined to observe progressively motile spermatozoa; the mean of these five evaluations was recorded as the final progressive motility score.

2.4.2. Sperm viability and abnormality (%). Sperm viability and abnormality were evaluated using 2 % eosin smears under a microscope (200x magnification). For the parameter of viability, 100 spermatozoa were assessed and the numbers of live (unstained) and dead (stained) cells were counted. For the abnormality parameter, 100 spermatozoa were counted for normal and morphologically abnormal cells.

2.4.3. Sperm membrane integrity (%). Sperm membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) [12], under a microscope (200× magnification). A total of 100 spermatozoa were assessed and those in a swollen state were counted, as characterized by a swollen tail, indicating an intact plasma membrane.

2.4.4. Sperm acrosome integrity (%). Sperm acrosome integrity was evaluated using 5 % aniline blue and 0.5 % crystal violet staining [13], under a microscope (200× magnification). A total of

100 spermatozoa were assessed and those with intact (stained) and non-intact acrosome membrane (unstained) were counted.

2.5. Statistical analysis

Data analysis was performed using the program SPSS 16.0. Analysis of variance (ANOVA) was performed to analyze the statistical significance of differences in parameters between control and treatment groups. The significance of differences between means was calculated using Duncan's multiple range test, with a significance threshold of $P < 0.05$.

3. Results and discussion

The present study revealed that supplementation with 15 % pineapple juice increased post-thaw motility (52.19 ± 5.32 %) and membrane integrity (38.52 ± 4.85 %), with the values being significantly ($P < 0.05$) higher than those of the control groups. No significant differences were detected between control and treatment groups for viability, acrosome integrity, and abnormality parameters (table 1).

Supplementation with pineapple juice improved the quality of garut ram spermatozoa after cryopreservation. The supplementation with 15 % pineapple juice in semen extender improved the motility and membrane integrity of spermatozoa. Pineapple contains several components that have antioxidant activity, such as vitamin C and phenolic compounds [14]. Vitamin C is oxidized in two one-electron steps: the first involves the oxidation of ascorbic acid to ascorbyl radical, and then the second progresses to dehydroascorbic [15]. Supplementation with vitamin C was reported to confer a protective effect on rat spermatozoa subjected to lead treatment to induce the production of ROS [16]. Moreover, Aurich et al. [17] reported that supplementation with ascorbic acid improved motility and membrane integrity of stallion spermatozoa during storage. Flavonoids prevent cellular damage by several mechanisms, including the direct scavenging of free radicals. These compounds are oxidized by radicals, resulting in more stable, less reactive radicals [18].

Cryopreservation decreases the quality of spermatozoa, which may be due to the production of excess ROS and cold shock. Free radicals cause oxidative damage to the membrane of spermatozoa [19]. Chen et al. [20] proved that the induction of hydroxyl radicals decreased the motility of spermatozoa and increased the concentration of malondialdehyde. This molecule is the product of lipid peroxidation and is used as a quantitative index of damage to the membrane of spermatozoa by lipid peroxidation [20]. Lipid peroxidation results in increased membrane permeability and the loss of the ability of cells to regulate intracellular ions. This may affect the motility of spermatozoa [19]. The loss of cell motility that cannot be recovered after incubation at a warm temperature is a sign of cold shock [21].

The percentages of motile spermatozoa after cryopreservation for all groups are higher than the percentages of spermatozoa membrane integrity. This showed the existence of damaged but motile cells [22, 23]. Valcárcel et al. [23] reported that this group of spermatozoa would be immotile after incubation at 37 °C for h, while the membrane integrity could be maintained during incubation. All of the groups also exhibited higher acrosome integrity percentages than the percentages of membrane integrity. Valcárcel et al. [24] showed that the plasma membrane of spermatozoa is more susceptible to cryodamage than the acrosome membrane. Ponglowhapan et al. [25] stated that damage to the plasma membrane occurred earlier and was more pronounced than damage to the acrosome. This could be explained by the plasma membrane and acrosome membrane having different compositions [26].

The mean percentages of motility and membrane integrity declined at a pineapple juice concentration of 20 %. This might have been associated with the increase of acidity of the extender. Arienti et al. [27] reported that the motility of human spermatozoa decreased with increasing acidity of the medium. Another factor that may contribute to this is the bromelain enzyme.

Table 1. Mean (\pm SD) parameters (%) of ram spermatozoa cryopreserved with Tris-egg yolk extenders supplemented with different concentrations of pineapple juice.

Parameter	0 %	5 %	10 %	15 %	20 %
Motility	39.52 \pm 7.30 ^{ab}	43.72 \pm 7.26 ^{ab}	47.33 \pm 4.11 ^{bc}	52.19 \pm 5.32 ^c	36.48 \pm 5.44 ^a
Viability	11.97 \pm 1.62 ^a	11.98 \pm 1.30 ^a	8.53 \pm 1.17 ^a	6.76 \pm 2.35 ^a	7.07 \pm 1.76 ^a
Membrane Integrity	22.57 \pm 6.24 ^a	27.73 \pm 9.19 ^a	29.76 \pm 6.78 ^{ab}	38.52 \pm 4.85 ^b	27.97 \pm 4.23 ^a
Acrosome Integrity	75.45 \pm 2.51 ^a	60.44 \pm 11.84 ^a	58.34 \pm 13.21 ^a	62.65 \pm 8.83 ^a	60.48 \pm 13.51 ^a
Abnormality	17.67 \pm 4.31 ^a	17.20 \pm 4.29 ^a	14.53 \pm 4.07 ^a	14.29 \pm 3.03 ^a	15.42 \pm 2.75 ^a

^{a,b,c} Values within the same row with different subscripts differ significantly ($P < 0.05$);

SD = Standard Deviation

Bromelain is a protein-digesting enzyme found in pineapples [28]. It is expected that the high acidity and bromelain enzyme concentration in the 20 % group were causes of the damage to the membrane of spermatozoa.

The viability, acrosome integrity, and abnormality parameters showed no significant difference from those in the control group ($P > 0.05$). All the treatment groups had a normal rate of abnormality, namely, less than 20 %. Suthutvoravut and Kamyarat reported that the dilution of human semen in pineapple juice (pH = 3) showed no significant effect on the morphology of spermatozoa [28].

4. Conclusion

It is concluded that the supplementation of semen extender with pineapple juice at a concentration of 15 % improved sperm motility and membrane integrity. No significant differences were detected between control and treatment groups for viability, acrosome integrity, and abnormality parameters.

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