The effect of siam orange (*Citrus nobilis* Lour.) and pineapple (*Ananas comosus* (L.) Merr.) juices combination in extender on Garut Ram (*Ovis aries* L.) spermatozoa quality post-cryopreservation

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**Abstract.** The research aimed to find out the effect of various concentrations of siam orange (*Citrus nobilis* Lour.) and pineapple (*Ananas comosus* (L.) Merr.) juices combination (1:1) in extender on garut ram (*Ovis aries* L.) spermatozoa quality 24 hours post-cryopreservation. 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 siam orange and pineapple juices combination 0 %, 5 %, 10 %, 15 %, and 20 %. Diluted semen samples were loaded into mini straws (0.25 mL) with 50 million cells/ml dosage. Samples were equilibrated at 5 °C for two hours, then frozen and stored in liquid nitrogen. The one factor ANOVA and continued with Duncan test showed a significant differences (P<0.05) between control and sample with 10 % juices combination in the percentage of spermatozoa motility and membrane integrity, a significant difference (P<0.05) between control and sample with 5 % along with 10 % juices combination in the percentage of spermatozoa viability. Siam orange and pineapple juices combination 10% in extender was able to minimize the reduction of spermatozoa quality post-cryopreservation based on the percentage of spermatozoa motility (68.06 ± 2.34 %), viability (41.47 ± 6.64 %) and membrane integrity (46.00 ± 5.65 %).

1. **Introduction**

Garut Ram (*Ovis aries* L.) is a superior local ram of a crossbreed between local ram, Merino Ram and Kaapstad Ram, developed in Garut, West Java [1]. This ram is called superior because it has better productivity and performance compared to another local ram [2]. Male Garut Ram could be used as a semen donor to improve the quality of other local ram, through reproductive technology such as artificial insemination [3]. Artificial insemination is needed to overcome the problem of breeding in Garut Ram, such as the limited number of superior males to fertilize females [4]. Semen used in the artificial insemination could be either fresh semen or semen from cryopreservation (frozen semen) [5].

Cryopreservation is related to atmospheric oxygen, which results in increased production of Reactive Oxygen Species (ROS) on spermatozoa [6]. The collection and semen processing before loaded into the straws will cause spermatozoa to contact with excessive atmospheric oxygen, which will increase the oxidative metabolism in spermatozoa. The result of increased oxidative metabolism is increased free radical (ROS) [7]. Free radical reactivity could be removed through the scavenging activity of antioxidants [8]. The levels of antioxidants in semen will be reduced due to the process of semen dilution [9]. Therefore, ram semen extender should be added with antioxidants to reduce ROS levels in spermatozoa, so that will decrease the damage of spermatozoa membrane post-cryopreservation.
The examples of fruits that have the antioxidant potential for spermatozoa are oranges and pineapples. Siam orange fruits (Citrus nobilis) are known to contain various antioxidants, such as vitamin C, terpenoids [10], carotenoids include β-carotene and β-cryptoxanthin [11], and phenolic compounds including flavonoids [12]. Pineapple fruits are also known to contain various antioxidants, such as vitamin C and flavonoids, including anthocyanin and proanthocyanidin [13,14]. These antioxidant compounds act as scavengers for the free radicals that cause lipid peroxidation in spermatozoa.

The recent study of Daramola et al. [15] using a 5% orange and pineapple juices combination (1:1) showed that percentage of progressive motility, membrane integrity and acrosome integrity of goats spermatozoa post-cryopreservation were higher than those using only orange or pineapple juice. They suggested continuing the research with higher concentrations of fruit juices combination [15]. Based on their previous study [15] and the pre-experiment, the concentrations of siam orange and pineapple juices combination used in this research were 0%, 5%, 10%, 15% and 20% with a ratio of 1:1. The study aimed to find out the effect of various concentrations of siam orange (Citrus nobilis Lour.) and pineapple (Ananas comosus (L.) Merr.) juices combination (1:1) in extender on Garut Ram (Ovis aries L.) spermatozoa quality 24 hours post-cryopreservation.

2. Materials and methods

The experiment was carried out in the Laboratory of Animal Reproduction, Breeding and Cell Culture, Research Center for Biotechnology, Indonesian Institute of Science, Bogor. Five Garut Rams aged 2–3 years that responded well to semen collection by artificial vagina were selected. The animals were kept under intensive management and supplemented with grass and 250-g/day-pellet concentrate.

2.1. Juices preparation

The fruit juices were prepared according to the procedure by Daramola et al. [15] and El-Sheshtawy et al. [16] with some modifications. Siam oranges and pineapples were collected from Mekarsari Fruit Garden, Bogor. Siam oranges were washed and then cut into two pieces, and the seeds were removed. The fruits were squeezed to obtain a clear watery juice. Pineapples were washed, peeled, and blended using a juicer. The juices collected from each fruit were put in separate tubes and centrifuged at 3,000 rpm for 15 min. The supernatant obtained was filtered and stored at -22 °C till used.

2.2. Semen collection and initial evaluation

Semen was obtained from five mature Garut Rams using an artificial vagina collected weekly for five weeks. The semen specimens were transferred to the adjacent lab within few minutes and initially evaluated for volume, color, odor, pH, semen consistency, mass movement, motility, concentration, viability, membrane integrity, acrosome integrity, and spermatozoa abnormality. The semen specimens has the motility above 60% and 80% morphologically normal spermatozoa were introduced to freezing procedure. The semen was maintained in a holding time for 10 min at 35 °C in a water bath before dilution [16].

2.3. Semen processing

Semen specimens including in Tris-egg yolk extender with the addition of 6% glycerol. A Tris-egg yolk extender was prepared according to the procedure by Laboratory of Animal Reproduction, Breeding and Cell Culture. Siam orange and pineapple juices combination 0%, 5%, 10%, 15% and 20% at a 1:1 ratio was added to diluted semen samples. Diluted semen samples were loaded into 0.25 mL mini straws with 50 million cells/mL dosage. Semen samples cooled slowly up to 5 °C and equilibrated for 2 hours. Upon the completion of equilibration periods, the straws were located horizontally on a rack and frozen in a vapor above liquid nitrogen (LN) for 10 min and were then soaked in LN [16].

2.4. Spermatozoa quality evaluation

The evaluation was undertaken on post-cryopreservation of Garut Ram spermatozoa. Also, motility, viability, membrane integrity, acrosome integrity, and spermatozoa abnormality were evaluated for fresh semen, after dilution and 2 hours after cooling. Frozen straws were thawed at 37 °C for 10 s. The parameters investigated were subjective spermatozoa properties (motility percentage, viability percentage, membrane integrity percentage, acrosome integrity percentage, and abnormality percentage).
2.4.1. Spermatozoa motility. The motility of spermatozoa was evaluated using Computer Assisted Sperm Analysis (CASA), SpermVision according to the procedure by Laboratory of Animal Reproduction, Breeding, and Cell Culture. Spermatozoa motility approximations were conducted in five different microscopic fields for each semen sample.

2.4.2. Spermatozoa viability. The viability of spermatozoa was evaluated using eosin, 2% w/v, stained smear as described by Arifiantini [17]. A hundred spermatozoa were assessed, and the percentage of spermatozoa with unstained heads was calculated.

2.4.3. Spermatozoa membrane integrity. The membrane integrity of spermatozoa was studied by Hypoosmotic Swelling Test (HOST) as elaborated by Arifiantini [17]. A hundred spermatozoa were evaluated, and the percentage of spermatozoa with swollen tails was calculated.

2.4.4. Spermatozoa acrosome integrity. The acrosome integrity of spermatozoa was evaluated using aniline blue and crystal violet stained smear as described by Martecikova et al. [18]. A hundred spermatozoa were assessed.

2.4.5. Spermatozoa abnormality. This was evaluated using eosin, 2% w/v, stained smear as described by Arifiantini [17]. A hundred spermatozoa were evaluated and the percentage of morphologically abnormal spermatozoa with defects in the head, midpiece, and tail were calculated.

2.5. Statistical analysis
Statistical analysis data were studied using the SPSS ver.16.0 to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. A notable difference between means was determined using Duncan’s multiple range test at P<0.05.

3. Results and discussion
The parameters of fresh semen quality are presented in table 1. The evaluations of fresh semen of Garut Rams were divided into macroscopic and microscopic evaluations. Macroscopic evaluations consist of volume, color, odor, consistency, and semen pH. Microscopic evaluations consist of a mass movement, concentration, motility, viability, membrane integrity, acrosome integrity, and spermatozoa abnormality.

The parameters of spermatozoa quality in Tris-egg yolk extender supplemented with siam orange and pineapple juices combination (1:1) 24 hours post-cryopreservation are presented in table 2. The present study revealed that extender supplemented with 10% Siam orange and pineapple juices combination had higher (P<0.05) spermatozoa motility percentage [(68.06 ± 2.34) %] compared to the control group [(47.93 ± 4.37) %]. The extender supplemented with 5% and 10% Siam orange and pineapple juices combination had higher (P<0.05) spermatozoa viability percentage [(47.33 ± 7.64) % and (41.47 ± 6.64) %, respectively] compared to the control group [(27.47 ± 5.84) %]. The extender supplemented with 10% Siam orange and pineapple juices combination had higher (P<0.05) spermatozoa membrane integrity percentage [(46.00 ± 5.65) %] compared to the control group [(31.60 ± 3.68) %]. No significant differences (P>0.05) could be detected between groups in spermatozoa acrosome integrity and spermatozoa abnormality percentage.

The results of fresh semen evaluation obtained were qualified for good spermatozoa quality and could be cryopreserved (table 1). The semen samples obtained were included in normal fresh semen criteria of Garut Ram, such as spermatozoa concentration about 1,500x10^6 cell/mL [19], percentage of spermatozoa motility >60% [20], percentage of spermatozoa membrane integrity >50% [21] and percentage of spermatozoa abnormality <20% [19]. Based on the obtained characteristics of fresh semen of Garut Ram, it could be concluded that the fresh semen produced by these rams have a good quality, so it qualified to be processed into frozen semen.

The present study revealed that extender supplemented with 10% siam orange and pineapple juices combination had higher (P<0.05) spermatozoa motility, viability and membrane integrity as compared with the control group (table 2). During freezing, there is an increased production of ROS. Spermatozoa damage during freezing/cryopreservation occurs in two processes, excessive ROS production, that occurs due to the thawing process, and alteration in intracellular antioxidant defense systems, especially glutathione. Excessive ROS production may alter the structure and function of spermatozoa membrane, generally in acrosomal and mitochondria [22]. Free radical reactivity could
be removed through the scavenging activity of antioxidants [8]. Semen is known to contain endogenous antioxidants [23]. However, the antioxidative capacity of these compounds is not sufficient to counteract ROS during cryopreservation [24]. The addition of an antioxidant to the freezing and thawing extender could be useful to improve spermatozoa quality post-cryopreservation.

Siam orange and pineapple juices both contain antioxidants, such as vitamin C [10,13,14]. Also, siam orange and pineapple juices contain flavonoids [12–14]. Vitamin C and flavonoids act as scavengers of free radicals that cause lipid peroxidation in spermatozoa [25]. The mechanism of vitamin C (ascorbic acid) as a free radical scavenger is by undergoing single electron oxidation to produce an intermediate reactive agent, the ascorbyl radical [26]. The mechanism of flavonoids as free radical scavengers are by donating their hydrogen atoms to free radicals [27]. These scavenging activities of antioxidants were able to terminate the chain reaction of lipid peroxidation that causes damage of spermatozoa membrane.

Vitamin C can protect the lipoprotein sheath on spermatozoa from peroxidative damage [28]. Obtained results were similar to the results of research conducted by Rosmaidar et al. [29] using tomato juice, which both contain vitamin C as in siam orange and pineapple juices. Semen, which supplemented with natural antioxidant from the juice, had a higher percentage of spermatozoa motility compared to the control. The content of vitamin C derived from juice in semen extender was a similar effect to the results of research conducted by Rosmaidar et al. [29].

Table 1. Mean of Garut Ram fresh semen evaluation.

| Parameters       | Values               |
|------------------|----------------------|
| Volume (ml)      | 0.72 ± 0.19          |
| Colour           | white milk and cream-colored |
| Odor             | typical              |
| Consistency      | moderate–thick       |
| pH               | 6–7                  |
| Mass Movement    | (+) and (++)         |
| Concentration (x10 cell/mL) | 1.680± 424.25 |
| Motility (%)     | 74.74 ± 7.49         |
| Viability (%)    | 82.80 ± 7.25         |
| Membrane Integrity (%) | 85.73 ± 6.40 |
| Acrosome Integrity (%) | 97.80 ± 1.19 |
| Abnormality (%)  | 11.53 ± 2.76         |

Table 2. Mean of Garut Ram spermatozoa evaluation post-cryopreservation.

| Parameters       | Concentrations of Siam Orange and Pineapple Juices Combination |
|------------------|---------------------------------------------------------------|
|                  | 0 %       | 5 %       | 10 %      | 15 %      | 20 %      |
| Motility (%)     | 47.93 ± 4.37· | 53.19 ± 7.18· | 68.06 ± 2.34· | 53.18 ± 6.26· | 45.19 ± 6.38· |
| Viability (%)    | 27.47 ± 5.84· | 47.33 ± 7.64· | 41.47 ± 6.64· | 26.33 ± 5.45· | 26.13 ± 6.60· |
| Membrane Integrity (%) | 31.60 ± 3.68· | 39.73 ± 4.06· | 46.00 ± 5.65· | 39.60 ± 6.47· | 37.80 ± 8.37· |
| Acrosome Integrity (%) | 77.53 ± 6.93· | 67.07 ± 8.42· | 65.80 ± 9.10· | 65.40 ± 9.21· | 64.07 ± 7.37· |
| Abnormality (%)  | 23.40 ± 4.11· | 20.67 ± 5.22· | 20.87 ± 1.68· | 20.13 ± 2.04· | 20.40 ± 4.76· |

· means with different superscripts in the same row are significantly different (P<0.05)
4. Conclusions

Siam orange and pineapple juices combination 10 % in extender was able to minimize the reduction of Garut Ram spermatozoa quality 24 hours post-cryopreservation based on the percentage of spermatozoa motility (68.06 ± 2.34 %), viability (41.47 ± 6.64 %) and membrane integrity (46.00 ± 5.65 %).

Acknowledgments

The authors would like to thank all staffs at Laboratory of Animal Reproduction, Breeding and Cell Culture, Research Center for Biotechnology, Indonesian Institute of Science for helping us in the field and laboratory, and also providing the facilities and materials for this research.

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