The effect of siam orange juice (*Citrus nobilis* Lour.) 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 Siam Orange juice in extender on Garut Ram spermatozoa quality 24 hours post-cryopreservation. Semen was collected from five rams once a week using an artificial vagina. Semen sample was diluted in Tris-egg yolk-based extender containing 0 % (KK), 5 % (KP 1), 10 % (KP 2), 15 % (KP 3) and 20 % (KP 4) Siam orange juice. Semen was packed in 0.25 ml straw with a final concentration of 50×10^6 spermatozoa/mL. Sperm was equilibrated at 5° C for two hours then frozen and stored in liquid nitrogen (LN) tube. Sperm parameters, including motility, viability, membrane integrity, acrosome integrity, and abnormality, were assessed. One-way ANOVA test results showed significant differences between treatment groups on the percentage of motility, viability, and membrane integrity (p <0.05). Duncan test results indicated that KP 2 had significant differences in all treatment groups. Therefore, orange juice at 10 % concentration (KP 2) was the best concentration that was able to minimize the reduction of ram spermatozoa quality post-cryopreservation based on motility [(58.68 ± 0.68) %], viability [(59.73 ± 6.47) %], and membrane integrity [(54.87 ± 5.58) %].

1. **Introduction**

Garut Ram is a local Indonesian ram, which is the result of crossing local ram (fat-tailed sheep), Merino Ram (Australia), and Kaapstad Ram (South Africa) [1]. This ram has several advantages such as high productivity, no mating season, and the body weight is larger than other local rams [2, 3]. The superior phenotype of this ram may be used to improve the genetic quality of other local rams through reproductive technology [3]. Artificial insemination (AI) with frozen semen is one of the ways that can be used [4]. AI with frozen semen is more effective and efficient [5]. The superior ram can be a sperm donor for many ewes [6]. So, the superior genetics of the parents can be inherited to their offspring [6].

Semen cryopreservation is a semen storage technique in liquid nitrogen for extended period of times [7]. Cryopreservation can decrease the quality of spermatozoa, such as spermatozoa cell membrane damage. The process of cryopreservation triggers an oxidative stress and the formation of reactive oxygen species (ROS) [8]. ROS molecules will attack polyunsaturated fatty acids (PUFA), which are the main constituents of cell membranes [9]. Ram spermatozoa cell membranes contain unsaturated fatty acid and saturated fatty acid composition with a ratio of 3: 1 [10]. Therefore, efficient antioxidants need to be added to prevent spermatozoa cell membranes damage due to lipid peroxidation [8].

Semen has endogenous antioxidants in seminal plasma. The ejaculated semen contains antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GP), and catalase (CAT). The antioxidant concentrations may be reduced due to dilution during the cryopreservation process [11].
Siam Orange fruit (*Citrus nobilis* Lour.) is one of the fruits that contain antioxidant compounds such as flavonoids, carotenoids, and citric acid [12]. These antioxidant compounds are worked by breaking the chain-oxidation reaction of free radicals or by scavenging the free radical [13]. The research of the protective effects of orange juice (*Citrus sinensis*) at 2.5%, 5%, 7.5%, and 10% concentration in Tris-egg yolk-extender on dwarf goat spermatozoa post-cryopreservation has been done [14]. Hence, this research aims to evaluate the effect of Siam Orange juice (*Citrus nobilis* Lour.) in Tris-egg yolk-base extender on the quality of Garut Ram (*Ovis aries* L.) post-cryopreservation at 5%, 10%, 15%, and 20% concentration.

2. Materials and methods

2.1. The collection of and initial evaluation

Semen was obtained from five Garut Rams less than 2–3 years old with 40–45 body weight once a week using artificial vagina. Garut Ram reared at Dombing cluster, Research Centre for Biotechnology, Indonesian Institute of Science, Cibinong, Bogor, Jawa Barat. The semen samples were transferred to the adjacent lab within few seconds and initially evaluated for volume (in a tube) and sperm motility. The fresh semen samples with more than 70% motility were admitted to freezing procedure.

2.2. Siam Orange juice processing

Siam Orange fruits were obtained from Mekarsari Fruit Garden, Cileungsi, Bogor, West Java. Siam orange juice was made by cutting the orange into two parts and then squeezed using a manual orange squeezer. Next, the orange juice was transferred into a 15 mL tube. The orange juice was centrifuged at 3000 rpm for 15 min then the supernatant was taken. The juice was transferred into a sterile bottle and stored in the refrigerator at 5°C for 24 hours. Then, the juice was filtered using a 125 mm filter membrane and stored in a 15 mL sterile tube. The orange juice was stored in the freezer at -22°C.

2.3. Semen processing

A tris-citric-acid-egg yolk-fructose [TCYF] extender was prepared. TCYF extender compositions were 2.472 g tris (hydroxymethyl)-aminomethane, 1.384 g of citric acid, 1.016 g of fructose, 80 mL aquabides, 100 µL Penicillin-Streptomycin solution, and 20 mL egg-yolk. TCYF/OJ (orange juice enriched extender [OJEE]) [v/v] (0.25/4.75 (5%), 0.5/4.5 (10%), 0.75/4.25 (15%), 1.0/4.0 (20%)) were prepared in a tube then were added with 6% glycerol (v/v) [15]. Semen samples were diluted in TCYF (control, 0% OJEE) and the initial concentrations of OJEE to confirm the 50 million motile spermatozoa/mL [16]. Semen was packed into 0.25 mL straw with filler and sealer machine then equilibrated at 5°C for 2 hours. After the period of equilibration, the straws were placed horizontally on a rack and frozen in a vapour 4 cm above liquid nitrogen (LN) for 10 minutes then were dipped in LN.

2.4. The assessment of semen quality

The assessment was conducted after 24 hours freeze thawing of ram spermatozoa. Raw semen was evaluated macroscopically (volume, colour, smell, pH, and viscosity) and microscopically (mass movement, motility, abnormality, membrane integrity, and acrosome integrity) as well. Frozen straws were thawed at 37°C/10 s. The observed parameters were focused on semen properties (motility %, viability %, abnormality % membrane integrity (hypo-osmotic swelling test (HOST)) %, and acrosome integrity %).

2.4.1. Motility. The motility of spermatozoa was assessed with computer-assisted sperm analysis (CASA). 2 mL raw semen samples were diluted in 398 mL of physiological NaCl, and 5 mL of diluted semen samples were diluted in 95 mL of physiologic NaCl. Then, the sample was stored on a thermal block at 37°C. The 10µL-diluted semen was dripped on a glass object, covered with a cover glass then placed on a microscope with 200 times magnification. CASA was operated. Then, the average progressive motility of the five fields can be known.

2.4.2. Viability and abnormality. This was evaluated using eosin-nigrosin stained for raw semen and 2% eosin for diluted semen. Three object glasses were prepared, followed by the addition of 2 µL of raw semen and 6 µL of post-cryopreservation on the first object glass then mixed using a second
Table 1. Raw semen evaluation.

| Parameters                  | Values                  |
|----------------------------|-------------------------|
| Volume (ml)                | 0.7 ± 0.28              |
| Colour                     | cream                   |
| Smell                      | semen odour             |
| Viscosity                  | medium–thickened        |
| pH                         | 7                       |
| Mass Movement              | (+) -- (+++)            |
| Concentration (x10 cell/mL)| 1.925 ± 1.84            |
| Motility (%)               | 78.40 ± 10.64           |
| Viability (%)              | 83.67 ± 8.73            |
| Membrane Integrity (%)     | 80.07 ± 6.78            |
| Acrosome Integrity (%)     | 98.93 ± 0.86            |
| Abnormality (%)            | 7.02 ± 1.12             |

object glass. 10 μL of diluent semen and 10 μL of 2 % eosin were dripped on the first object glass then mixed using second object glass. Then, it was smeared on the third glass object and fixed using Bunsen. One hundred spermatozoa were assessed and the percentage of uncoloured membrane for viability and abnormal head or tail for abnormality.

2.4.3. Integrity of the membrane. The membrane integrity of the sperm was investigated by the hypo-osmotic swelling test (HOST). 5 mL raw semen was mixed with a 500 mL HOST solution. 5 mL diluent semen was mixed into a 95 mL HOST solution then incubated at 37 °C for 30 minutes. One hundred spermatozoa were evaluated, and the spermatozoa percentage with curled tails (swollen/intact plasma membrane) was measured.

2.4.4. Acrosome integrity. Acrosome integrity was assessed with 5 % aniline blue and 0.5 % crystal violet stained. 10 μL semen was dripped on the glass object. Then, it was fixed using bunsen and stored in a 3.9 % formalin solution for 24 hours. Next, it was stained by 5 % aniline blue then 0.5 % crystal violet for 15 seconds. Spermatozoa with acrosome intact were dark on the apical head of sperm and spermatozoa without acrosome intact was on the apical part of the sperm head. One hundred spermatozoa were evaluated and the spermatozoa percentages with acrosome intact were measured.

2.5. Statistical analysis
Statistical analysis data was assessed by the SPSS computerized program version 16.00, the analysis of variance (ANOVA) for the various factors between control and additives replications was conducted. A notable difference between means was measured using Duncan’s multiple range tests at P < 0.05.

3. Results and discussion
The result of raw semen evaluation was performed macroscopically and microscopically (table 1). Concerning to frozen-thawed semen, the present study revealed that supplementation of extender with 10 % orange juice increased the post-thaw motility [(58.68 ± 0.69) %], viability [(59.73 ± 6.47) %], membrane integrity [(54.87 ± 5.58) %] and these values are significantly (P < 0.05) higher than the control group. The addition of 5 % orange juice increased the motility [(49.76 ± 5.17) %], this value was notably (P < 0.05) higher than the control group (table 2). No significant (P > 0.05) differences could be detected between groups in acrosome integrity and total sperm abnormality.

The average of semen color was cream. This color was normal semen color. Arifiantini [17] showed that reddish-white semen color indicated a wound in urethral or greenish-white indicated a certain bacteria. Ram semen had a distinctive smell of semen. Arifiantini [17] showed that the strong smell was not expected because it was associated with bacterial content in the semen. The average volume was 0.7 ± 0.28 ml. This volume was within the normal range. Arifiantini [17] showed that the average volume of ram semen was 0.5–2 mL. The viscosity was medium-thickened. The viscosity was
within the normal range. Arifiantini [17] showed that ram semen viscosity was in the medium-thickness. The average acidity (pH) level was 7. The degree of acidity (pH) was in normal condition. Susilawati [9] showed that the degree of acidity (pH) of normal ram was 6.9–7.3.

The average concentration of raw semen was 1,926 ± 184 million cells/mL. The concentration was in normal condition. Mattner and Voglmayr [18] research showed that the average concentration of ram semen was 1800 million cells/mL. The mass movement was (+)–(++) Positive 1 (+) showed thin and slow waves movement. Positive (++) showed heavy waves but slowly movement or medium waves but quickly movement [17]. The average of motility, viability, and membrane integrity were 78.40 ± 10.64 %, 83.67 ± 8.73 %, and 80.07 ± 6.78 %. The percentage of motility, viability, and membrane integrity of spermatozoa were in normal condition. Herdis and Darmawan [19] showed that percentage of motility and viability were 70.13 ± 8.76 %, and 84.36 ± 6.84 %. The average of acrosome integrity was 98.93 ± 0.86 %. The acrosome integrity was in normal condition. The percentage of average abnormalities obtained in the study was 7.20 ± 1.12 %. Rizal [20] showed that the percentage of abnormality was 6.20 ± 1.17 %.

The spermatozoa quality decreased when subjected to cryopreservation. Cryopreservation was a very vulnerable process. Spermatozoa would be damaged during the process of freezing and thawing [9]. The freezing and thawing process caused spermatozoa membrane damage. Susilawati [9] showed that semen cryopreservation process leads to the decrease of spermatozoa viability, change in spermatozoa function, change in lipid composition, and change in the arrangement of spermatozoa plasma membranes. The damages were caused by cold shock and reactive oxygen species (ROS). The cold shock occurred due to freezing at extreme temperatures. It could be reduced by adding the intracellular cryoprotectants [9]. The increase in ROS occurred due to the activity of spermatozoa metabolism and long semen processing [21]. In addition, an increase in ROS would also decrease the intracellular antioxidants [9]. The thawing process was a critical period because the temperature drastically increases. The drastic increase in temperature caused high spermatozoa metabolic activity. This resulted in high free radical production as a result of spermatozoa metabolism [22].

The supplementation of orange juice in Tris-egg yolk extender gave a positive effect on the quality of ram spermatozoa post-cryopreservation. This showed by supplementation of the extender with 10 % orange juice increased the post-thaw motility; viability, membrane integrity, and these values are significantly (P < 0.05) higher than the control group. The addition of 5 % orange juice increased the motility, and this value was significantly (P < 0.05) higher than the control group. Siam orange juice contained antioxidant compounds such as vitamin C and flavonoids. Vitamin C or ascorbic acid was a water-soluble free radical scavenger. Vitamin C turned into ascorbic radicals by donating electrons to free radicals, so the lipid peroxidation chain reaction ended [23]. Flavonoids acted as scavengers by donating the hydrogen atoms from hydroxyl groups (–OH) to free radicals and forming phytoxyt flavonoid (FIO) radicals, so the free radical compounds become more stable [24].

4. Conclusions
The results showed that the semen that was added with 5 %, 10 %, and 15 % orange juice had a higher percentage of motility, viability, and membrane integrity than the control group. However, the semen was added with 20 % orange juice is the same with the control. This might be caused by the pH of 20 % orange juice. The pH was 6.06, where the value is lower than pH ram semen. The pH of ram semen was 6.2–7.0 [18]. So, many spermatozoa were dead. Duncan test results indicated that KP 2 had

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Table 2. Semen evaluation post-cryopreservation.

| Parameters          | Treatment groups |
|---------------------|------------------|
|                     | KK 0% | KP1 5% | KP2 10% | KP3 15% | KP4 20% |
| Motility (%)        | 35.09 ± 3.12    | 49.76 ± 5.17 | 58.68 ± 0.69 | 40.02 ± 5.90 | 35.37 ± 1.98 |
| Viability (%)       | 43.20 ± 6.50    | 51.33 ± 5.43 | 59.73 ± 6.47 | 46.27 ± 5.58 | 43.80 ± 6.19 |
| Membrane Integrity% | 35.80 ± 2.42    | 48.07 ± 6.74 | 54.87 ± 5.58 | 42.73 ± 3.75 | 36.60 ± 1.79 |
| Acrosome Integrity% | 88.33 ± 3.27    | 92.73 ± 3.93 | 90.33 ± 3.51 | 89.40 ± 5.46 | 90.47 ± 3.55 |
| Abnormality (%)     | 20.40 ± 3.68    | 18.07 ± 4.99 | 18.13 ± 6.40 | 20.13 ± 7.16 | 20.20 ± 7.39 |

**Note:** means with different superscripts in the same row are significantly different (P<0.05)

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Within the normal range. Arifiantini [17] showed that ram semen viscosity was in the medium-thickness. The average acidity (pH) level was 7. The degree of acidity (pH) was in normal condition. Susilawati [9] showed that the degree of acidity (pH) of normal ram was 6.9–7.3.

The average concentration of raw semen was 1,926 ± 184 million cells/mL. The concentration was in normal condition. Mattner and Voglmayr [18] research showed that the average concentration of ram semen was 1,800 million cells/mL. The mass movement was (+)–(++) Positive 1 (+) showed thin and slow waves movement. Positive (++) showed heavy waves but slowly movement or medium waves but quickly movement [17]. The average of motility, viability, and membrane integrity were 78.40 ± 10.64 %, 83.67 ± 8.73 %, and 80.07 ± 6.78 %. The percentage of motility, viability, and membrane integrity of spermatozoa were in normal condition. Herdis and Darmawan [19] showed that percentage of motility and viability were 74.17 %, 87 %, and 84.83 %. The average of acrosome integrity was 98.93 ± 0.86 %. The acrosome integrity was in normal condition. The percentage of average abnormalities obtained in the study was 7.20 ± 1.12 %. Rizal [20] showed that the percentage of abnormality was 6.20 ± 1.17 %.

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significant differences in all treatment groups. Therefore, orange juice at 10% concentration (KP 2) was the best concentration that was able to minimize the reduction of ram spermatozoa quality post-cryopreservation based on motility \( [(58.68 \pm 0.68) \%] \), viability \( [(59.73 \pm 6.47) \%] \), and membrane integrity \( [(54.87 \pm 5.58) \%] \). This indicated that the 10% orange juice concentration (KP 2) was the best concentration that can be used to minimize the reduction of Garut Ram spermatozoa quality post-cryopreservation.

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**References**

[1] Basrizal 2007 *Morphological and Frequency Stages of Spermatogenesis in Garut Ram* (Bogor: Institut Pertanian Bogor) Undergraduate Thesis, p 12

[2] Hastono H, Inounu N and Hidayati H 2001 *Proc. Seminar Teknologi Peternakan dan Veteriner* (Bogor: Puslitbang Peternakan) pp 106–12

[3] Rizal M and Herdis 2005 *Hayati J. Biosci.* 12 61–6

[4] Medeiros C M, Forell F, Oliveira A T and Rodrigues J L 2002 *Theriogenology* 57 327–44

[5] Salomon S and Maxwell W M 2000 *Anim. Reprod. Sci.* 62 77–111

[6] Gunawan A and Noor R R 2006 *Media Peternakan* 29 7–15

[7] Alamo D, Batista M, González F, Rodríguez N, Cruz G, Cabrera F and Gracia A 2005 *Theriogenology* 63 72–82

[8] Bucak M N, Ateşşahin A, Varişli O, Yüce A, Tekin N and Akçay A 2007 *Theriogenology* 67 1060–7

[9] Susilawati T 2011 *Spermatology* (Malang: Universitas Brawijaya Press) p 176

[10] Holt W V and North R D J. *Reprod. Fertil.* 73 285–94

[11] Silva S V, Soares A T, Batista A M, Almeida F C, Nunes J F, Peixoto C A and Guerra M M P 2011 *Reprod. Dom. Anim.* 46 874–81

[12] Dharmawan J 2008 Characterization of Volatile Compounds in Selected Citrus Fruits from Asia (Singapore: National University of Singapore) Ph.D. Thesis, p 6

[13] Sayuti K and Yenrina R 2015 *Antioksidan Alami dan Sintetik* (Padang: Andalas University Press) p 16–69

[14] Daramola J O et al. 2016 *Anim. Reprod.* 13 7–13

[15] El-Sheshawey R I, El-Sisy G A and El-Nattat W S 2016 *Asian Pac. J. Reprod.* 5 335–9

[16] Badan Standarisisasi Nasional 2014 SNI 3869.3: 2014 *Semen Beku - Bagian 3: Kambing dan Domba* (Jakarta: Badan Standarisisasi Nasional) ICS 65.020.30, p 2

[17] Arifiantini R I 2012 *Teknik Koleksi dan Evaluasi Semen pada Hewan* (Bogor: IPB Press).

[18] Mattner P E and Voglmayr J K 1962 *Aust. J. Exp. Agric.* 2 78–81

[19] Herdis and Angga I W 2012 *Indonesian Journal of Science and Technology* 14 197–202

[20] Rizal M 2006 *Jurnal Pengembangan Peternakan Tropis (JPPT)* 31 224–31

[21] Bailey J, Morrier A and Cormier N 2003 *Can. J. Anim. Sci.* 83 393–401

[22] Rizal M and Herdis 2010 *Wartoza* 20 139–45

[23] Nims S B and Pal D 2015 *RSC Adv.* 5 27986–28006

[24] Kumar S and Pandey A K 2013 *The Scientific World Journal* 2013 ID162750