The effect of water clover (*Marsilea crenata*) extract addition in egg yolk and skim milk extender on frozen goat semen quality

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Abstract. Frozen semen has a longer storage time, but the effect of cold shock during processing reduces the semen quality. Antioxidants in water clover extract are expected to be able to maintain the quality of frozen semen. The study aimed to evaluate the effect of addition of water clover extract (WCE) in the base semen dilution (egg yolk-skim milk) to the quality of post-thawing semen. The material used in this study was goat semen. There were four treatments: T0 as control (base dilution + 0% WCE), T1 (base dilution + 1% WCE), T2 (base dilution + 3% WCE), and T3 (base dilution + 5% WCE), with ten replications each. This study used a randomized completed design, ANOVA, and continued with Duncan's Multiple Range Test. The results showed that the addition of water clover extract to egg yolk skim extender had a significant effect (P < 0.05) on the motility of individual spermatozoa, viability and membrane integrity, but didn't have a significant effect (P > 0.05) on sperm abnormalities. The research concludes that the addition of 3% water clover extract in egg yolk skim milk extender was the best concentration to maintain the quality of frozen goat semen.

1. Introduction

Artificial Insemination (AI) is a reproductive technology which can improve the genetic quality and livestock populations. The use of frozen semen for AI has advantages as it can be stored for a longer period in liquid nitrogen and can be distributed to various regions. The low quality of frozen semen is one of the factors causing low pregnancy rates. The addition of appropriate diluents into the semen can maintain the quality of spermatozoa because during the dilution, freezing, and storage processes at low temperatures the spermatozoa will be damaged [1]. The pregnancy rate of artificial insemination using frozen semen in goats varies from 7 to 79% [2].

The quality of diluents will affect the quality of semen after thawing. In general, diluent for cryopreservation of sperm includes a non-penetrating cryoprotectant, a penetrating cryoprotectant, a buffer, one or more sugars, organic acid and antibiotics [3–4]. Skim milk used as an extender is often combined with egg yolk. Egg yolks have lipoprotein and lecithin which function is to protect spermatozoa from cold shock. Previous research revealed that goat semen diluted with egg yolk and skim milk produced better spermatozoa survival than coconut yolk, but did not provide good fertility results. Reduced fertility is thought to be related to the lack of ability of skim milk extender to prevent the damage of spermatozoa membrane. This damage is caused by the presence of free radicals produced by the metabolic process of spermatozoa, which continue to be active during cold storage. Free radicals that react with oxygen will produce reactive oxygen species (ROS) so that it will produce...
lipid peroxidation, and if there is excessive production of free radicals on the spermatozoa, it will cause oxidative stress.

Water clover extract contains flavonoid bioactive components. Previous research showed that water clover extract has antioxidant activity. The antioxidant content of water clover extract is expected to inhibit damage to spermatozoa due to free radicals. Through the ability of flavonoids as antioxidants in semen extender is expected to be able to optimize the quality of semen from the effects of freezing. The present research aimed to evaluate the effect of water clover extract addition in egg yolk skim milk extenders on frozen goat semen quality.

2. Materials and methods

2.1. Semen collection and treatments

Semen was collected from a healthy buck, aged 2.5 to 3 years old with a body weight of 48 kg twice a week for 5 weeks using an artificial vagina after stimulating with an estrus doe. Standard quality of sample semen was individual motility ≥ 70% and sperm abnormality ≤ 10 %.

Fresh semen was diluted in egg yolk skim milk as a base extender with the ratio of 1:10 (v/v). The sample of semen was divided into four treatments, as follow: T0 (Egg Yolk Skim Milk + 0% WCE), T 1 (Egg Yolk Skim Milk + 1% WCE), T 2 (Egg Yolk Skim Milk + 3% WCE), and T 3 (Egg Yolk Skim Milk + 5% WCE).

Procedure of WCE based on Sokunbi et al. [5].

2.2. Evaluation of semen quality and freezing-thawing process

Sperm motility, viability, plasma membrane integrity, and abnormality were observed before freezing and post-thawing. Sperm motility was assessed under a light microscope with 400x magnification based on the percentage of progressive sperm motility [6]. The sperm viability and abnormality were evaluated using an eosin-nigrosine staining procedure [7–8]. The hypo-osmotic swelling test evaluated the functional membrane integrity. Freezing and thawing process according to Wahjuningsih et al. [9].

2.3. Statistical analysis

This study used Completely randomized Design with ten replications of each treatment. The data on semen quality traits were analyzed using analysis of variance and then Duncan multiple range test to determine differences among the treatments. Statistical significance was set at P<0.05.

3. Results and discussions

It was observed that the sperm motility, viability, plasma membrane integrity, and abnormality before freezing and post-thawing in all of Water Clover Extract (WCE) concentration was decreased compared to the fresh semen. The study used mangosteen peel extract supplementation on the Tris aminomethane base diluent carried out by Isnaini [10] also found that there was a decrease in the quality of semen post-thawing compared to before freezing and fresh semen. The sperm motility and viability before freezing and post-thawing in 0%, 1% and 5% supplementation of WCE were statistically lower than 3% WCE.

Decreasing semen quality after the freezing process is caused by spermatozoa experiencing cold shock due to a very drastic decrease in temperature when frozen [11–12–13]. Decreasing the percentage of plasma membrane integrity in post-thawing is caused by plasma membranes consisting of unsaturated fatty acids, which are very easy to experience lipid peroxidation. Antioxidants from water clover extract into skim milk-egg yolk diluents can maintain the integrity of plasma membranes. Antioxidants will be subjected to free radical attacks so that these compounds can break or prevent the work of free radicals and prevent chain lipid peroxidation reactions that can damage the cell's plasma membrane. The integrity of the plasma membrane is essential because the damaged plasma membrane will affect the metabolic processes associated with the motility and viability of the spermatozoa [14].
Table 1. Effects of water clover extract (WCE) supplementation in egg yolk skim milk extender on semen quality before freezing

| Parameters (%)         | Fresh semen | Semen quality before freezing (%) |
|------------------------|-------------|-----------------------------------|
|                        |             | 0% WCE  | 1% WCE  | 3% WCE  | 5% WCE  |
| Sperm motility         | 80.5 ± 3.68d| 61.5 ± 6.67a | 66 ± 5.77b | 69 ± 3.33c | 67 ± 4.16b |
| Sperm viability        | 88.4 ± 5.63c| 78.86 ± 1.03a | 78.12 ± 2.63a | 82.09 ±2.01b | 78.94 ± 3.40b |
| Sperm abnormality      | 8.2 ± 1.02a | 8.27 ± 1.24a | 8.39 ± 1.22a | 8.82 ± 1.54a | 8.46 ± 1.48a |
| Plasma membrane integrity | 85.2 ± 5.45d | 64.17 ± 2.06a | 65.73 ± 2.88a | 77.10 ±3.41c | 70.06 ± 3.99b |

a,b,c,d Different superscripts within rows indicate significant differences at p<0.05

Table 2. Effects of water clover extract (WCE) supplementation in egg yolk skim milk extender on semen quality post thawing

| Parameters (%)         | Fresh semen | Semen quality post thawing (%) |
|------------------------|-------------|--------------------------------|
|                        |             | 0% WCE  | 1% WCE  | 3% WCE  | 5% WCE  |
| Sperm motility         | 80.5 ± 3.68d| 49.5 ± 4.25a | 50 ± 4.08a | 58.5 ± 4.74a | 52.5 ± 5.50a |
| Sperm viability        | 88.4 ± 5.63c| 66.93 ± 3.40a | 66.33 ± 3.34a | 77.17 ±2.11c | 71.70±1.62a |
| Sperm abnormality      | 8.2 ± 1.02a | 8.38 ± 0.58a | 8.6 ± 1.95a | 8.15 ± 1.31a | 8.34 ± 1.11a |
| Plasma membrane integrity | 85.2 ± 5.45d | 55.65 ± 3.87a | 56.02 ± 2.82a | 68.15 ±2.08a | 59.04±1.65b |

Different superscripts within rows indicate significant differences at p<0.05

The results showed that 3% WCE supplementation in egg yolk skim-based extended was the best to improve semen quality at before freezing and post-thawing stage compared to other concentrations. The antioxidant content of the water clover extract can inhibit spermatozoa damage due to free radicals. Free radicals cause lipid peroxidation reactions that can damage the lipid matrix structure [15]. Lipid peroxidation reaction processes will change the structure of spermatozoa, especially the acrosome, loss of motility, rapid metabolic changes, and coating of intracellular components [16]. The antioxidants in the water clover extract which added in the diluter must be optimum concentration so that it can work optimally to protect the spermatozoa due to the reaction of lipid peroxidation, excessive antioxidant administration will be harmful on sperm quality. In table 1 and table 2, we can see that if water clover extract supplementation more than 3%, the quality of semen will decrease. The amount of antioxidant concentration added can affect the rate of oxidation, but too high a concentration of antioxidant activity becomes prooxidant [9].

4. Conclusion
The addition of 3% water clover extract in the base extender of egg yolk skim milk produces the best quality of frozen semen in maintaining the quality of frozen goat semen.

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