Effect of water clover (Marsile crenata) extract within tris-fructose citric glycerol extender on frozen semen quality of boer goat

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Abstract. The consistency of the semen extender used is one of the deciding factors in the quality of the frozen semen. Water clover extract (WCE) was supposed to retain the semen content during freeze storage with the antioxidant compounds. The objective of this analysis was to determine the impact on the consistency of post-thawed Boer Goat Semen of the water clover extract supplement. The Boer semen was collected from 3 Boer bucks aged at 3 to 3.5 years Boer bucks which weighed at 40 to 42 kg. The semen was obtained by artificial vagina two days a week and the semen used for this research should have at least 80 percent individual motility and feasibility and maximum anomalies of 10%. The semen was added with WCE at different concentration levels (0, 2, 4 and 6%) by using Tris-fructose citric glycerol diluents as the base extender. The study was done in a randomized way and the results were evaluated using a variance analysis followed by a multiple range test from Duncan. The results showed that a WCE supplementation with Tris-fructose citric glycerol diluent as the base extender had a significant (\(P<.05\)) effect on motility, viability and integrity of plasma membrane but not on abnormalities (\(P>0.05\)). The research shows that the best way to preserve frozen boer goat semen consistency was to add 4% WCE in tris fructose citric glycerol.

1. Introduction
Artificial insemination was developed to improve species genetical and population quality. The application of artificial insemination on does can be employed by using fresh, liquid or frozen semen. The use of previously frozen semen for artificial insemination has its own advantages when compared to fresh semen. Freeze-thawed goat semen can be utilized for artificial insemination to improve livestock populations [1]. Cryopreservation of goat sperm cells extends the reproductive life of a buck, even though the percentage of post-thawing motility remains low with varying results [2,3]. However, half of the sperm will die during cryopreservation and those who survive are normally of low fertility. One of the factors which affect the quality of frozen semen after thawing is the semen expansion unit. The semen diluent would maintain quality, including the motility, viability and fertility. The materials used in semen diluents should be able to provide nutrients, protection against cold shock, buffer capacity, regulations of osmotic balance, and inhibition of the growth of microorganisms and cryoprotectants for the spermatozoa [4].

The flavonoids in water clover plants possess antioxidant activities, while antioxidant compounds would maintain the quality of frozen semen. This condition thus enhances the capacity for semen expansion of water clover plants. This study aims to determine the consistency of Boer Goat semen post-thawed by using Tris-fructose citric glycerol as a diluent, supplemented with leaf water clover extract (WCE).
2. Materials and methods

2.1. Semen collection
The semen was obtained from three Boer bucks aged 3 to 3.5 years, boer bucks weighing 40 to 42 kg. Semen collections were carried out with an artificial vagina twice a week for 10 weeks. For its individual motility, viability and anomalies, the semen was then observed. The semen with at least 80 per cent individual motility and viability were then used for testing with a maximum abnormality of 10 per cent.

2.2. Preparation of diluent and water clover extract
Fresh semen was diluted by using Tris-fructose citric glycerol (TCG), while the base diluent as control was made by following to Qureshi et al. [5] with slight modification as follows: 2.4 g Tris, 1 g fructose, 1.4 g citric acid, 1.4% glycerol (v/v), 100 mg/ml streptomycin and 100 μg/ml penicillin G. Fresh semen was diluted with diluent at the ratio of 1:10 (v/v). Tris-fructose citric glycerol diluent was then supplemented with either 0, 2, 4 or 6% WCE, respectively. The extraction process of WCE was based on previous research with slight modifications [6].

2.3. Freezing and thawing procedures
A 0.25 mL French mini-straw (IVM, France) was filled with the diluted semen, cooled to 5°C for 4 h to meet the balancing time, put 4 cm higher than liquid nitrogen for 10 minutes before frozen to 196°C with liquid nitrogen. The semen was stored for 4 weeks in liquid nitrogen containers before post-thawing evaluation. The thawing was performed by submerging frozen semen in 37°C water for 30 seconds.

2.4. Assessment of sperm quality
Progressive sperm motility in each semen sample was evaluated by using a phase contrast microscope (Olympus) with 400X magnification [7]. Evaluation of spermatozoa viability was performed as follows: a smear from diluted semen was deposited on a glass slide and stained by dual staining eosin-nigrosine [8]. A total of 200 sperm were examined in each sample at 400X under a light microscope (Olympus). Number of dead spermatozoa (i.e. red) were counted. Sperm abnormalities were evaluated according to previous research [9]. Plasma membrane integrity was measured by hyposomotic swelling test [10].

2.5. Data analysis
Statistical analysis was performed by using SPSS 13.0 for Windows. Comparison of semen quality for these four treatments were evaluated by using analysis of variance with significance was set at p<0.05.

3. Results and discussion
Post-thawed semen quality measurements, which include individual motility, viability, plasma membrane integrity and abnormalities were presented in Table 1. The results indicate that cryopreservation suppressed the sperm cell motility. A decrease in cryopreserved sperm cell motility was due to ultrastructural, biochemical and functional changes of cryopreserved sperm cells. These changes were caused by freezing temperature and thawing process [11].

The fresh semen quality was shown to be decreased after freezing. The decreased quality was likely due to the cold shock experienced by the spermatozoa caused by a very drastic decrease in temperature when frozen [12]. The Boer semen that supplemented with 4% WCE (T2) with TCG as base diluent showed higher motility, viability and membrane integrity (P<0.05) as compared to T0, T1 and T3. One problem within the cryopreservation process of semen involves destruction of the spermatozoa plasma membrane due to formation of lipid peroxidation. The lipid peroxidation was occurred due to the unsaturated fatty acids in spermatozoa membrane that are susceptible to peroxidation damage [13].
Table 1. Effects of different water clover extract concentration on post-thawed semen quality

| Variable (%)                  | Fresh Semen | Post thawed semen | SEM | P value |
|-------------------------------|-------------|-------------------|-----|---------|
|                               |             | T0 (0% WCE) | T1 (2% WCE) | T2 (4% WCE) | T3 (6% WCE) |       |
| Sperm motility                | 80.5        | 45.5     | 45.0     | 55.5     | 45.5     | 1.97   | <0.001 |
| Sperm viability               | 87.6        | 45.6     | 46.7     | 63.4     | 46.8     | 1.98   | <0.001 |
| Sperm abnormality             | 7.49        | 7.82     | 7.94     | 7.92     | 7.15     | 0.12   | <0.001 |
| Plasma membrane integrity     | 85.80       | 64.5     | 64.7     | 78.4     | 66.1     | 1.67   | <0.001 |

Different superscripts within rows indicate significant differences at p<0.05

Efforts to minimize such damage to these membranes during the cryopreservation can be done by adding antioxidants to diluent. The antioxidant supplementation with the addition of 4% water clover extract in this study are shown to be effective in protecting spermatozoa from lipid peroxidation. In this study, the sperm quality shown to be increased along with the higher WCE concentration up until 4% and lower quality was shown at 6% WCE concentration. Other researches also showed similar results, and showed that excessive antioxidant concentrations in semen can reduce spermatozoa progressive motility, viability and plasma membrane integrity [14, 15,16]. Research by Bildeau et al. [17] has elucidate that high antioxidants concentration alter extender osmolality, which leads to plasma membrane damage. On the other hand, insufficient antioxidant supplementation would not be able to effectively prevent reactive oxygen species that induce peroxidation which affect sperm functions and viability [18].

4. Conclusion
Water clover extract supplementation within Tris-fructose citric glycerol diluent positively affected post-thawed semen quality. Supplementation with 4% WCE was the optimal concentration in terms of maintaining the quality of frozen semen.

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