Effect of Extender Medium and Cooling Rate on the Quality of Frozen Semen Post-*Thawing* at Bali Bull *(Bos sondaicus)*

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ABSTRACT

This study was aimed to analyze effect of extender medium and cooling rate on the quality of frozen semen post-*thawing* at Bali bull *(Bos sondaicus).* The experiment was conducted in a completely ramdomized design factorial of 6 threatment (2x3) and 3 replicates. Data were statistically analyzed using analysis of variance (ANOVA) and Duncan test was employed to assess statistical differences between treatment. The first factors was extender such as Tris citrat egg yolk (TCEY) and Tris citrat soy milk (TCSM). The second factors was the *cooling rate* such as: 15°C/min, 10°C/min and 5°C/min. Variables measured include: sperm motility, live sperm, sperm abnormality, and membrane integrity of spermatozoa Bali bull. The results of this study on the treatment of extender medium TCEY (A1) shows the best results with the sperm motility of 43.15 ± 4.67%, live sperm 53.78 ± 3.79%, sperm abnormality 18.89 ± 1,07% and membrane integrity 35.44 ± 3.01%. As well as the results of research on the treatment with *cooling rate* of 10°C /min (B2) showed the best results with sperm motility of 44.35 ± 5.28%, live sperm 57.17 ± 1.18%, sperm abnormality of 17.84 ± 0.23 % and membrane integrity 36.83 ± 2.12%. The interaction between extender medium and *cooling rate* was founded significant different (P>0.05). Results of analysis of variance showed that there was a highly significant difference (P> 0.01) between the two extender medium and three *cooling rate* on post - thawed of motility, live sperm and membrane integrity and had no significant (P <0.05) on the sperm abnormality, It can be concluded that the extender medium Tris-citrat egg yolk (TCEY) with *cooling rate* of 10°C / min resulted the best in sperm motility, live sperm, and membrane integrity spermatozoa.

Keywords: Frozen semen, Bali bull, *Cooling rate*, Extender medium.

INTRODUCTION

Bali bull was one of the local cattle be maintained in Bali. Bali bull have enormous potential to provide meat people's nutrition and plays an important role in improving people's income, but Bali bull population has decreased, due to the shortage of superior males, the purity of Bali bull to extinction because of uncontrolled mating system through artificial insemination or mating with nation another cattle (Mardiansyah *et al.,* 2016). Low producti-vity of Bali bull due to the difficulty of superior males, and maintenance are still traditional and not get a touch of reproductive technology (Feradis, 2010). So that the *calving rate* becomes longer, it was necessary to increase the Bali bull population by improving the management of maintenance, use of technology, and control of livestock expenses (Anton *et al.,* 2005). Artificial Insemination (AI) was a globally accepted method of breeding cattle and was also effective for increasing population and for genetic improvement (Susilawati, 2011). Cryopreservation semen was associated with the reduction in spermatozoa viability and fertilizing ability. The quality of of frozen semen was one of the limiting factors to the

success of the AI program in cattle (Ahmad *et al.,* 2014). According to Susilawati (2011) that the semen freezing problem revolves around the influence of *cold shock* on the frozen cells and the formation of ice crystals. This weakness could be partially overcome by using protective substances in the diluent and a gradual decrease in temperature (Jessie *et al.,* 2016). The shields are used is glycerol. Glycerol during the freezing efficiency is strongly influenced by the equilibration time. Factors that affect sperm life durability: extender medium and *cooling rate*. Toelihere, (1993) that the motility and acrosome integrity of spermatozoa frozen at temperature of 16°C with reduced rates of egg yolk citrate medium was significantly higher at 50.17%. In general, the problem revolves around the influence of sperm freezing *cold shock* and intracellular changes during freezing (Udin *et al.,* 2013).

MATERIALS AND METHODS

Semen Collection. This research was carried out from 3 ejaculates of 2 Bali bulls as local cattle in Bali. Bali bull shelter semen was collected using an artificial vagina. Fresh semen evaluation**.** Fresh semen was evaluated macroscopic and microscopic to determine the quality for further processing. Macroscopic evaluation included; volume, color, odor, pH, color and consistency. While microscopic include a concentration, motility, live, abnormalities and membrane integrity of spermatozoa (Hafez, 2008).

Extender medium**.** Soon after the shelter, semen should be treated with caution to prevent cold shock, contamination with water, urine, chemicals, excessive shaking or vibration, or exposure to air or direct sunlight (Feradis, 2010)**.** Gliserolisasi. A total of 6% was spent on diluents then added the blanks with as much as 6% glycerol into thinner and mixed with semen. Glycerolization process required considerable time diluent solution with gliserol, so that any evaluation of frozen semen no visible crystalline clusters. Printing,

Filling and Sealing straw. Printing particulars on empty straw to provide arrangements in writing in order to distinguish straw. Straw differentiated by color per temperature. For temperature 15°C/min, 10°C/min and 5°C/min with each having a straw color red, yellow and blue. Filling and sealing were the process of filling the semen that had been in diluting and clamping of straw by using automatic filling and sealing mechine. This process was done in cool top.

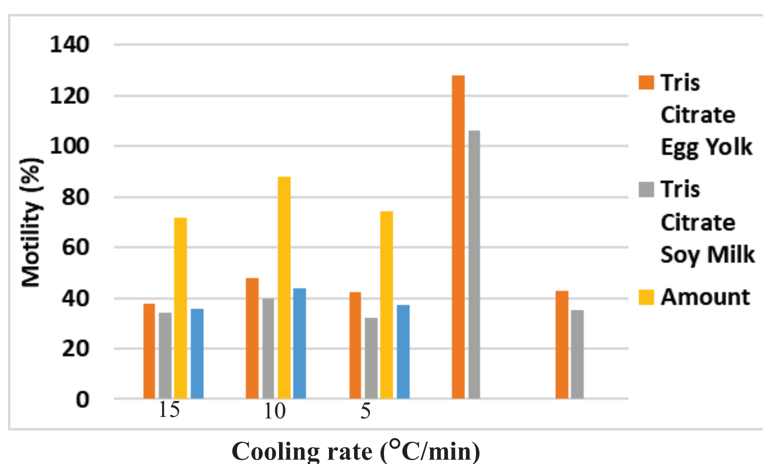
Equilibration and cooling. Equilibration time was period which needed by spermatozoa before freezing to adjust the diluent so that when freezing death exaggerated of spermatozoa could be prevented. Cooling semen which has been in dilute temperatures approached the 0°C was an adaptation period to reduce the metabolism of spermatozoa. Freezing semen**.** Using a freezing cooling rate equipment ice cube from 4 to -120°C CR temperature 15°C/min: 8 minutes 30 seconds CR 10°C/min: 12 minutes 4 seconds CR 5°C/min: 24 minutes 8 seconds. Before placing into liquid N_2, semen placed on the surface of the liquid N_2 temperature ± -110°C for 9 minutes. After the semen could be frozen by placing semen in liquid N_2 and stored in the container.

Post thawing semen evaluation. Motility, live sperm, abnormality and membrane integrity of spermatozoa were assessed at every day after thawing. Sperm motility estimated by phase constrast microscope (CASA), one drop of thawed semen was placed on warm (37°C) stage and spermatozoa with progressive motility counted in 200 spermatozoa. Live sperm was assessed by eosin Y- nigrosin staining (Swelum et al, 2011). To detection of membrane integrity, one drop of each treatment added to 8 drop of hypoosmotic solution and after incubation (45 minutes at 37°C) one drop of mixture was placed on microscope and covered with cover glass. Spermatozoa with swelling tail was evaluated as those having kept their membrane integrity.

Statistical Analysis. The experiment was conducted in a completely ramdomized design factorial of 6 threatment (2x3) and 3 replicates. Data were statistically analyzed using analysis of variance (ANOVA) and Duncan test was employed to assess statistical differences between treatment. The first factors was extender such as Tris Citrat Egg Yolk (TCEY) and Tris Citrat Soy Milk (TCSM). The second factors was the *cooling rate* such as: 15°C/min, 10°C/min and 5°C/min.

RESULTS AND DISCUSSION

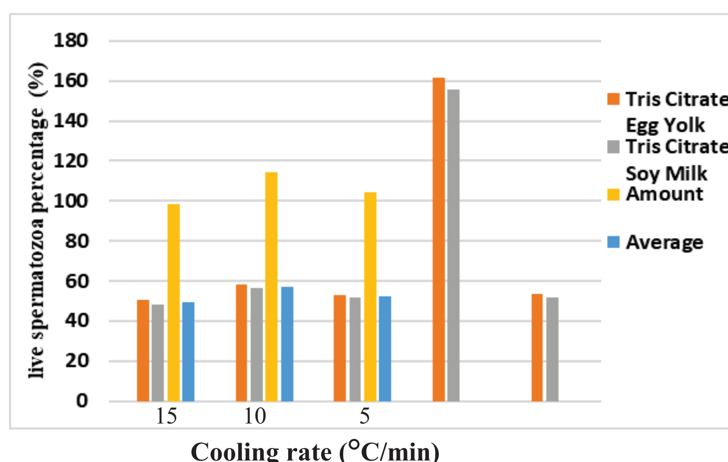
The highest post – thawed sperm motility was observed in Tris-Citrat Egg Yolk (42,67± 5.17%) and the lowest was in Tris-Citrat Soy Milk (35,33± 4,16%). The highest sperm motility was observed in TCEY with *cooling rate* 10^0C (44,00±5,66 %) and the lowest was in cooling rate 15^0C (35,84±2,60%). The sperm motility in TCEY at cooling rate 5^0C was in (37,17±7,30%) respectively (figure.1)



Cooling rate (°C/min)

Figure1. Post – Thawed Motility of Spermatozoa

Result of live spermatozoa percentage are shown in figure 2. Results of analysis of variance showed that there were highly significant differences in interaction ($P < 0.01$) between the tris-citrate egg yolk and tris-citrate soy milk to the percentage of live spermatozoa post-thawing of frozen semen. The percentage of live spermatozoa was higher in tris-citrate egg yolk than extender medium tris-citrate soy milk.



Cooling rate (°C/min)

Figure 2. Post – Thawed Percentage of Live Sperm

Results of analysis of variance, showed no significant interaction (P<0.05) between the cooling rate and extender medium for sperm abnormality. Speed cooling rate of 10^0C/min yield a lower average value of abnorma-lities in the amount of 17.84 ± 0.23%, compared to treatment reduced rates of freezing temperature 15^0C/min and 5^0C/min, each of which is equal to 19.84 ± 0,23%, and 19.12 ± 0.23%. Further-more, the lower the average abnormal spermatozoa achieved at the level of treatment extender medium TCEY by 18.89 ± 1.07% compared to the level of treatment of TCSM that is equal to 19.00 ± 1.00% higher than TCEY

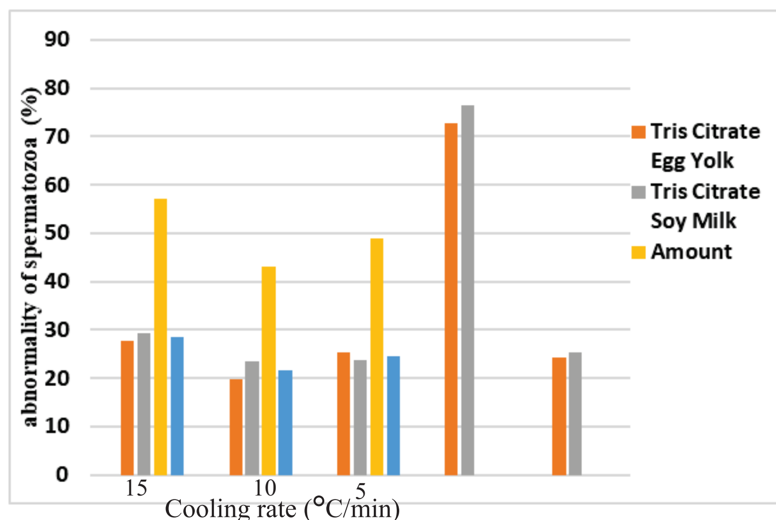


Figure 3. Post – Thawed abnormality of spermatozoa

Results of analysis of variance, showed that there were highly significant differences interaction (P<0.01) between the two treatments. Results of analysis of variance with factors A showed highly significant effect (P<0.01) extender medium TCEY 35.44 ± 3.01% yield plasma membrane intact higher than the extender medium TCSM 33.44 ± 2.45%. Results of analysis of variance with factor B showed no significant effect (P<0.05) to membran integrity spermatozoa after thawing of frozen semen in Bali bull.

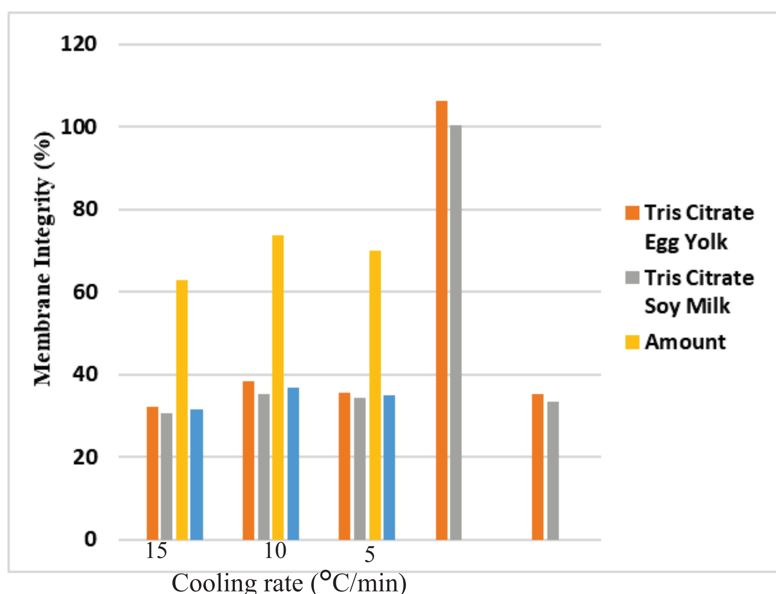


Figure 4. Post – Thawed Membrane Integrity

Statement explaining that the average sperm motility for each factor A, namely: 42.67 ± 5.17% and 35.33 ± 4.16%. In using treatment TCEY (A1) is the highest in comparison with TCSM (A2) who obtained lower motility compared to A1. This is caused due to the use of extender TCEY is generally used as the main component in the extender for freezing semen catle in the estimate has a good buffer capacity and low toxicity at high concentrations (Feradis, 2010). *Cooling rate* on the results of this study showed that the average each is 35.84 ± 2.60%, 44.00 ± 5.66%, and 37.17 ± 7.30%. Among the average was seen that the *cooling rate* of 10°C/min (B2) get the highest motility compared with two other temperatures, *cooling rate* of 15°C/min (B1) and 5°C/min (B3). Results of analysis of variance of the factor A showed highly significant effect (P<0.01) against the percentage live of spermatozoa after freezing, extender medium TCEY percentage live of spermatozoa produce relatively higher at 53, 78 ± 3.79% compared with extender medium TCSM 52.00 ± 4.17%. . In this result post – thawed motility was similar to obtained by Leite *et al.,* (2010) reported that 26.85 in Tris extender. Results of analysis of variance of the factor B shows the effect of significantly different (P <0.05) against the percentage of live spermatozoa after freezing, the pace of *cooling rate* 10°C/min resulted in averaging 57.17 ± 1, 18% indicates the percentage of live spermatozoa were relatively high in comparison with *cooling rate* 15°C/min and 5°C/min, each of which produces a row is averaging 49.36 ± 1.89% and 52.17 ± 0.71% (Arifiantini *et al.,* 2005). The level of *cooling rate* 10°C/min highly significant (P<0.01) lower than 15°C/min and 5°C/min. Based on the Duncan's Multiple Range Test (DMRT) plasma membrane intact spermatozoa at different levels of *cooling rate* 10°C/min was significantly higher (P<0.01) compared to the *cooling rate* 15°C/min and 5°C/min. Furthermore, the plasma membrane intact spermatozoa at the level of the extender medium TCEY treatment was significantly higher (P<0.01) compared with treatment extender medium TCSM. The present study demonstrated that the type of semen extender medium and *cooling rate* 10°C/min on membrane integrity (Hafez,2008)

CONCLUSIONS

Based on the results and discussion, it can be concluded that the decrease in cooling rate (15^0C/min, 10^0C/min, 5^0C/min) and extender medium (TCEY and TCSM) effect on motility, live sperm, and membrane integrity, but did not affect sperm abnormality . There is an interaction between treatment *cooling rate* with extender medium. *Cooling rate* of 10^0C/min and extender medium TCEY provide excellent response with the average percentage of each is motility 44.00 ± 5.66% and 42.67 ± 5.17%, live spermatozoa 57.17 ± 1.18% and 53.78 ± 3.79%, and membrane integrity 36.83 ± 2.12% and 35.44 ± 3.01% for frozen semen quality post thawing in Bali bull *(Bos sondaicus).*

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