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Effect of Extender and Cooling Rate on the Quality of Frozen Thawed Semen of Bali Bull (*Bos sondaicus*)

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ABSTRACT

The purpose of this study was to observe the effect of extender and cooling rate on the quality of frozen thawed semen of Bali bull (*Bos sondaicus*). The experiment was conducted at Laboratory of Animal Biotechnology, Faculty of Animal Husbandry, Andalas University. A completely randomized design of factorial 2 x 3, 2 different extenders and 3 cooling rates with 3 bulls as the replicate. Data were analyzed using analysis of variance (ANOVA). The first factors was extender such as tris citrate egg yolk (TCEY) and tris citrate soy milk (TCSM). The second factors was the cooling rate such as: 15ºC/min, 10ºC/min and 5ºC/min. Variables were: motility, viability, abnormality, and membrane integrity. The results of treatment of TCEY extender on the average of motility 42.67±5.17%, viability of 53.78±3.79%, abnormality of 18.89±1.07%, and membrane integrity of 35.44±3.01%. The effect of cooling rate of 10°C /min has the highest semen motility of 44.35±5.28%, viability of 57.17±1.18%, abnormality of 17.84 ± 0.23 % and membrane integrity of 36.83±2.12%. The interaction between extender and cooling rate was founded significant different (P<0.05). Otherwise of extender and cooling rate on post-thawed of motility, viability and membrane integrity, had no difference (P>0.05) on semen abnormality. It can be concluded that tris-citrate egg yolk (TCEY) extender with cooling rate of 10ºC / min were the best semen motility, viability, and membrane integrity of spermatozoa.

Keywords: Bali bull, Cooling rate, Extender, Frozen semen

Introduction

Bali bull was one of the local cattle germplasm be maintained in Bali, Indonesia. Bali bull have enormous potential to provide meat people's nutrition and plays an important role in improving people's income, because it has good reproducibility and the proliferation of relatively faster compared to other cattle race. The condition of the population of Bali bull was currently experiencing a decline, due to the shortage of superior bulls, so it is feared purity Bali bull will be disrupted because of the mating system that is not controlled by artificial insemination (AI) using straw breeding other bull or natural mating with the breed other bulls (Mardiansyah *et al*.., 2016). The AI activities in farmers mostly use frozen semen originating from breed Limousin and Simental bulls (Aji *et al*.., 2017).

Increasing population and the genetic quality of Bali bull needs to be pursued using AI to improve the genetic quality of livestock (Ismaya, 2014). The AI is the inclusion of activities and delivery of semen into the female reproductive tract by using a special tool (insemination gun). The AI function by utilizing one ejaculation can produce acceptor many females were inseminated with semen cryopreservation way of doing or freezing (Susilawati, 2013). The AI was one of the most applicable reproductive technology and has been widely popular in the community, especially in cattle (Anton *et al*.., 2005).

Frozen semen quality was one of the limiting factors to the success of the AI program in cattle (Ahmad *et al*.., 2014). In general, semen freezing problem revolves around the influence of cold shock of the cells are frozen and the formation of ice crystals on changes in intracellular (Susilawati, 2013; Udin *et al*.., 2013). This drawback can be overcome by using protective substances in the extender and cooling rate. The protector used is glycerol. The effectiveness of glycerol is strongly influenced by the equilibration time (Umar and Maharani, 2005).

The factors affecting the survival of spermatozoa was reduced rates of extender and cooling rate were used. Tris citrate egg yolk (TCEY) and tris citrate soy milk (TCSM) was an

extender that was used on bull semen. Each of these extender has different performance (Susilawati, 2013). The factor of cooling rate also do differently was 15ºC/min, 10ºC/min, and 5ºC/minute, from equilibration temperature 5ºC until frozen at 120ºC- (-196ºC). The cooling rate was done in order to avoid cold shock to suddenly frozen cells (Susilawati, 2013). Park and Pursel (1993) state that the motility and acrosome integrity of spermatozoa frozen at 16ºC cooling rate with a real TCEY media was higher by 50.17%.

Materials and Methods

The experiment was conducted in December 12 to February 31, 2015. Research and semen collection at the Bali bull was done of Animal Biotechnology Laboratory, Faculty of Animal Husbandry, University of Andalas.

Material

Materials used in this study was the fresh semen Bali bull as much as 3 heads to the age of 3-4 years and body weight ranging between 500-650 kg. The feed given to Bali bulls in the form of 60% forage and 40% concentrate. Feeding was done on the morning of forage (35-40 kg) + corn + concentrations (16-18% protein), while in the afternoon was given concentrate + sprouts (1 kg/day) + hay (2 kg/day) + mineral (0.5 gr selenium). Tools for measuring the cooling rate were *IceCube* (14 M-A, automatic freezer with Windows® tablet, 230V).

Methods

Semen collection. This research was carried out from 3 ejaculates of 2 Bali bulls. Bali bull shelter semen was collected using an artificial vagina. Fresh semen was evaluated macroscopic and microscopic to determine the quality for further processing (Hendri *et al.*., 2004). Extender semen. Soon after the collection semen, semen sample was divided into two parts, one part was an extender with TCEY and TCSM. Extender with the single-stage method was done by inserting an extender through the wall of glass flask containing the semen slowly until all the extender homogeneously mixed. Mixing between fresh semen and extender using a water bath with a temperature of 37ºC (Feradis, 2010).

Gliserolisasi. A total of 6% was spent on extender then added the blanks with as much as 6% glycerol into thinner and mixed with semen. The glycerolization process required the considerable time extender solution with gliserol so that any evaluation of frozen semen no visible crystalline clusters (Swelum *et al.*., 2011).

Equilibration process was performed after the semen sample was mixed with a extender respectively TCEY, TCSM and lasted for 2 hours in the refrigerator at a temperature of 5ºC. The cooling process is done by inserting a measuring cup sealed and placed in glass beaker containing water with a temperature of 37ºC and then placed in a cooler. Cooling was done gradually with the degree of cooling rate as distinguished from the cooling rate of 15ºC/min, 10ºC/min and 5ºC/min.

The process of decline in semen freezing temperature (cooling rate) was done using a tool *IceCube* (14 M-A, automatic freezer with Windows® tablet, 230V) with a temperature range of 4ºC - (- 120ºC). Equilibration time for ± 3 hours straw immediately inserted into the *IceCube* tool that has been set from the speed of 15ºC/min, 10ºC/min and 5ºC/min. Straw first with a temperature of 15ºC/minute was added to the tool *IceCube* takes 8 minutes, 30 seconds, the second temperature 10ºC/min for 12 minutes, 4 seconds, and the temperature was third 5ºC / min for 24 minutes, 8 seconds (Park and Pursel, 1993).

Freezing semen and semen evaluation. Semen freezing process was process of cooling rate before storing in liquid N_2. Semen was placed on the surface of the Liquid N_2 ± -110ºC temperature for 9 minutes before it was added to liquid N_2 (Ismaya, 2014). Semen can be frozen by placing them in liquid N_2 and stored in the container. The evaluation was conducted using frozen semen thawing for 15-30 seconds in the water bath at 37ºC.

Variables measured

Semen motility estimated by phase constrast microscope CASA (Computer-assisted sperm analysis) (Ismaya, 2014). Viability semen performed by using dye eosin staining technique by counting 200 spermatozoa of life and death. Spermatozoa life becomes colorless, while spermatozoa die to absorb color. Abnormalities of semen were calculated based on the primary and secondary. The percentage of membrane integrity can be performed using a solution HOST (Susilawati, 2013).

Statistical Analysis.

The experiment was conducted in a completely randomized design factorial of 2x3 and 3 replicates. The first factors were extenders such as tris citrate egg yolk (TCEY) and tris citrate soy milk (TCSM). The second factors were the cooling rate such as: 15ºC/min, 10ºC/min and 5ºC/min.

Result and Discussion

Characteristic of Bali's fresh semen

Examination of the quality of spermatozoa was carried out macroscopic and microscopic (Table 1). Bali bull semen volume assessment with an average of 7.94 ± 0.61 ml. These results were consistent with Hafez (2008) that the bull semen volume in the range of 5-10 ml per ejaculation. Semen color was cream. In general, Bali bull semen color was white turbid, milky white, cream. Ismaya (2014) states that bull semen good color (normally) is milky or creamy whitish. These colors were normal (Arifiantini *et al.*., 2005). Bali bull semen consistency was the consistency being. The pH value and the smell of

Bali bull semen were 7 and has a distinctive odor semen (Toelihere, 1993).

Based on microscopic examination found a mass movement +++, in a concentration of 2.400 x 106/ml. The mean percentage of motility and viability of 79.37 ± 3.02% and 80.67 ± 1.89%. In general, the results obtained have been able to continue the process until the freezing stage, as it has produced semen motility accordance with the standards or still within the normal ranges 70-85% (Belala *et al.*., 2016).

Evaluation of quality *mean of Bali bulls' frozen semen*

Based on Figure 1, the average motility of the semen in a extender respectively: 42,67±5,17% dan 35,33±4,16%. Treatment using an extender TCEY the highest compared with the gain TCSM lower motility. This is because the use of extender TCEY as a major component in an extender for freezing semen bull has a good buffer capacity and low toxicity at high concentrations (Campbell *et al.*., 2003; Feradis, 2010). Thun *et al.*. (2002) state that the processing of bull semen with extender TCEY produces the best motility of the frozen semen quality and fertility. The results also showed that the pace of decline freezing temperatures showed the average motility respectively 35.84 ± 2.60%, 44.00 ± 5.66%, and 37.17 ± 7.30%. Reduced rates of freezing temperatures 10ºC/min obtain the highest motility than 2 drops in freezing temperatures the others, 15ºC / min and 5ºC / min. This is because the level of semen motility can be maintained at optimum speed reduction freezing temperatures are in the range of 10ºC / min ie for 12 minutes, 4 seconds (Park and Pursel, 1993).

Based on Figure 2, the mean viability of semen showed a significantly different effect (P<0.05) frozen semen of Bali bull (*Bos sondaicus*).

The TCEY extender produce semen viability higher at 53.78 ± 3.79% compared with 52.00 ± TCSM only 4.17%. Feradis (2010) states that the addition of citrate in egg yolk useful for spermatozoa, preservation and durability (viability) spermatozoa. The results of cooling rate also show the effect of significantly different (P<0.05) on the viability of the semen. The cooling rate of 10ºC/min resulted in averaging 57.17 ± 1.18% higher than 15ºC / min and 5ºC / min.

Table 1. Characteristic of Bali's fresh semen

No	Characteristics	Bulls			Mean
		I	II	III	
1.	Macroscopic				
	Volume	8 ml	7,3 ml	8,5 ml	7,94±0,61 ml
	Color	cream	cream	cream	cream
	Consistency	medium	medium	medium	medium
	pH	7	7	7	7
	Odor	normal	normal	normal	normal
2.	Macroscopic				
	Mass movement	+++	+++	+++	+++
	Concentration (10^6)	2000	2.800	2.400	2.400±40,00
	Viability (%)	78,50	82,00	81,50	80,67±1,89
	Motility (%)	75,90	81,50	80,70	79,37±3,02
	Abnormality (%)	12,50	13,50	13,10	13,03±0,50
	Membrane Integrity (%)	81,00	83,50	82,50	82,30±1,26

Note: +++ Good result.

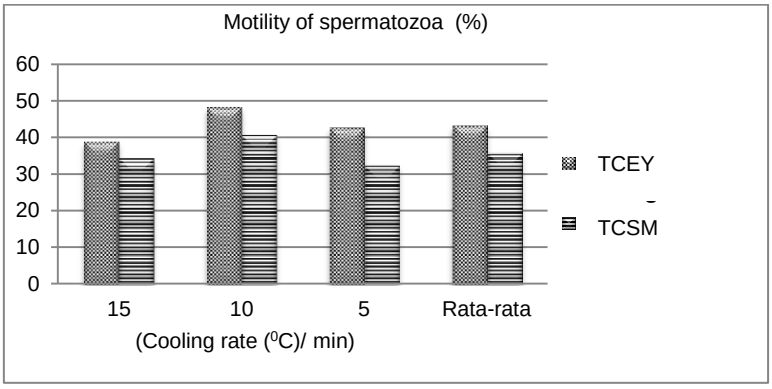

Figure 1. Motility mean of Bali bulls' frozen semen.

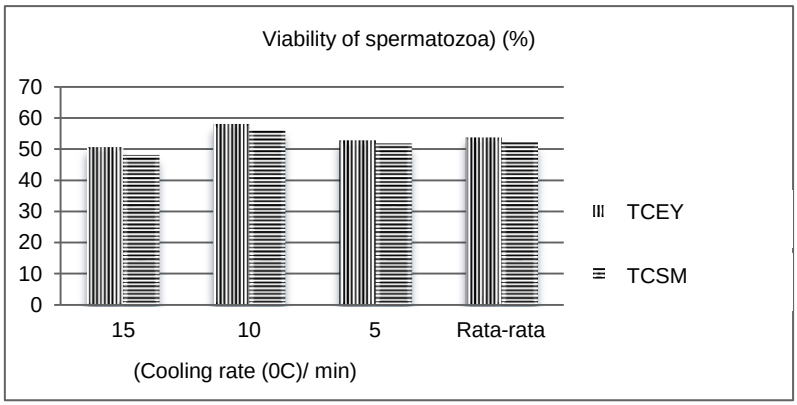

Figure 2. Viability mean of Bali bulls' frozen semen.

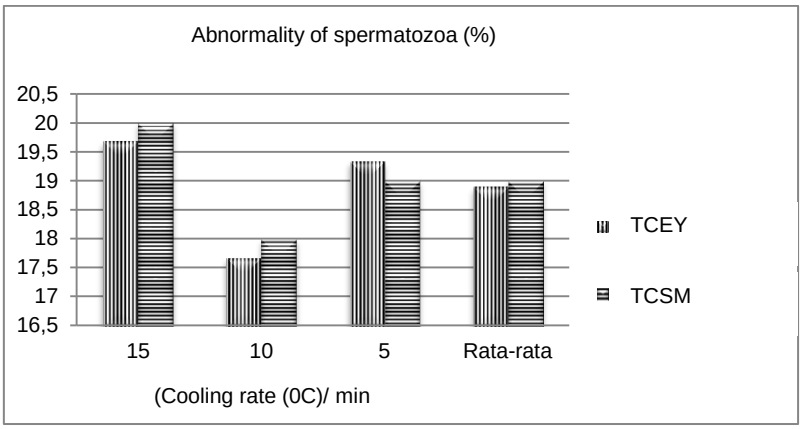

Figure 3. Abnormality mean of Bali bulls' frozen semen.

The lowest average abnormality of semen in TCEY thinners were 18.89 ± 1.07% compared to TCSM of 19.00 ± 1.00% (Figure 3). The results also showed that the cooling rate of 10ºC/min resulted in a lower average abnormality in the amount of 17.84 ± 0.23%, compared to treatment of 15ºC/min and 5ºC/min, 19.84 ± 0.23%, and 19.12 ± 0.23% respectively. The shape of the secondary abnormality were found in this study, such as the tail roll, a broken neck, the head and neck broke. Secondary abnormality caused by the treatment when making preparations pillowcase (Susilawati, 2013).

Results of analysis of variance on the extender showed significantly different effect (P<0.05; Figure 4) on the membrane integrity, with the extender TCEY 35.44 ± 3.01% higher membrane integrity compared with 33.44 ± 2.45 TCSM %.

This occurs because the mechanism of the formation of ice crystals in the extender TCEY occur more slowly with the crystal size fine, and can prevent dehydration process semen cells during the freezing process takes place, so that physical damage or chemical semen cells can be reduced (Susilawati, 2013). Results of analysis of variance at reduced rates also show the influence of cooling rate significantly different (P<0.05) on semen membrane integrity. Statistical analysis showed a significant interaction (P<0.05) between the extender with reduced rates of cooling rate on motility, viability and membrane integrity of spermatozoa after thawing Bali bull, but not significant (P> 0.05) on semen abnormality.

Conclusions

The extender of (TCEY and TCSM) and cooling rate (15ºC/min, 10ºC/min, 5ºC/min) have an effect on motility, viability and semen membrane integrity, but no significant effect on semen abnormality. There is no interaction between the two factors of the Bali bull frozen semen. TCEY extender and cooling rate of 10ºC/min provides excellent response with the average motility of 44.35 ± 5.28% and 43.15 ± 4.67%, viability of 57.17 ± 1.18% and 53.78 ± 3.79%, and the integrity of the membrane of 36.83 ± 2.12% and 35.44 ± 3.01% of the post-thawing frozen semen quality of Bali bull (*Bos sondaicus*).

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