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Effect of Coconut Water with Egg Yolk Combination and Storage Length on Spermatozoa Abnormality and Membrane Integrity of Bali Bull

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Abstract *— This study aimed to investigate the effect of coconut water with egg yolk combination in the extender and storage length on liquid spermatozoa abnormality and membrane integrity of Bali bull. Method used in this study was experiment in a completely randomized factorial design with 2 factors. The first factor was the level of coconut water and egg yolk which consisted of 5 treatments, including P0 (control) = Tris amino methane, P1 = 85% coconut water + 15% egg yolk, P2 = 80% coconut water + 20% egg yolk, P3 = 75% coconut water + 25% egg yolk, P4 = 70% coconut water + 30% egg yolk. The second factor was the length of storage at low temperature which consisted of 3 treatments, including 1 day, 2 days and 3 days. All treatments were replicated 10 times. Variables observed were spermatozoa abnormality and membrane integrity. Results showed that the abnormality value of spermatozoa in P0 after 3 days of storage was 12.54%. This value was lower (P<0.05) than in P1, P2, P3 and P4 groups with 16.52%, 15.62%, 14.66%, and 13.65% of abnormality value, respectively. Membrane integrity value of spermatozoa which was diluted in P0 treatment after 3 days of storage was 66.23%, which was significantly higher (P<0.05) compared to those diluted in P1 56.93%, P2 59.23%, and P3 61.73%, but not significantly different compared to P4 64.51%. The conclusion of this study is that the use of the extender with the ratio of 70% coconut water and 30% egg yolk could maintain the spermatozoa abnormality and membrane integrity of Bali bull.*

Keywords *— Coconut water, egg yolk, storage length, spermatozoa abnormality and membrane integrity, Bali bull.*

I. INTRODUCTION

Increasing the productivity of Bali cattle needs to be supported by the availability of reproductive technology, especially which is associated with the efficiency and management of reproduction, in order to improve and maintain the fertility. Improvement of the fertility which easily applied is by controlling estrus and insemination time through artificial insemination technology by using chilled semen or frozen semen. The success of the artificial insemination program not only depends on the quality and quantity of the semen ejaculated by the bulls but also on the ability to increase semen volume and maintain semen quality for longer time after ejaculation. Therefore, more female cattle can be inseminated.

Egg yolk contains lipoprotein and lecithin which will maintain and protect the spermatozoa from the integration of the lipoprotein sheath and also protect from cold shock. Coconut water could be used to fulfill the requirement of simple carbohydrates as a source of energy in the extender, because it contains carbon element in the form of simple carbohydrates such as glucose, sucrose, and fructose. Nitrogen elements in the form of protein, composed of amino acids such as aline, arginine, alanine, and serine [1]. The ideal extender should be able to demonstrate the ability to minimize the degradation rate of spermatozoa quality, so it could extend the storage length after the dilution process. Not all extender exhibit the same ability to maintain the quality of spermatozoa. So it is necessary to know whether there is any effect of the semen extender and storage time on the quality of liquid semen of Bali bull.

II. MATERIAL AND METHOD

Material used in this study was fresh semen of Bali bull which was reared in Singosari National Artificial Insemination Center. The semen was collected from 5 Bali bulls aged at 3 to 5 years old.

Experimental Design

Method used in this study was experiment in a completely randomized factorial design with 2 factors. The first factor was coconut water and egg yolk extender ratio which consisted of 5 treatments, including P0 (control) = Tris amino methane, P1 = 85% coconut water + 15% egg yolk, P2 = 80% coconut water + 20% egg yolk, P3 = 75% coconut water + 25% egg yolk, P4 = 70% coconut water + 30% egg yolk. The second factor was the length of storage at low temperature which consisted of 3 treatments, including 1 day, 2 days and 3 days. The semen used in this study had motility ranged from 50 to 55%.

Preparation of the Extender

Firstly, coconut water (green coconut) was filtered by using filter paper, and then placed into the Erlenmeyer. After that, the pH of the coconut water was measured by using litmus paper. In this study, the coconut water had pH of 6.4, so it should be neutralize before being used as extender. Coconut water was then inactivated by using thermo-string with temperature of 170^{o}C, while the temperature of the water in the control remains stable at 56^{o}C, for 20 minutes. After inactivation, 500 ml of coconut water was added with 0.6 g of $NaHCO_3$, then homogenized for 10 minutes. After these processes, the pH of coconut water became 7.0. Coconut water was then poured into the measuring cup according to the treatment. The laying hen eggs were prepared and the yolk was separated from the albumen by using filter paper. Egg

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yolk was then added to coconut water according to the treatment. After that, it was added with 1,000 IU of penicillin and 1 mg of streptomycin per ml of the extender.

Evaluation of Spermatozoa Abnormality

Abnormality showed the anomaly of the spermatozoa morphology which could reduce the spermatozoa fertility. Spermatozoa abnormality was calculated by formula :

$$\frac{the\ number\ of\ abnormal\ spermatozoa}{the\ total\ number\ of\ observed\ spermatozoa} x100\%$$

Evaluation of Spermatozoa Membrane Integrity

Membrane integrity was tested by using Hypo Osmotic Swelling Test (HOST) according to [2]. The composition of the HOST solution was 1 ml of a 150 m osmol hypo osmotic solution (consisting of 7.35 grams of sodium citrate. 2H2O, 13:52 grams of fructose dissolved in 1000 ml of aquadest), added with 0.1 ml of semen and then incubated at 37°C for 30 minutes. Ten µl of semen sample was then dropped on an object glass and then closed with cover glass. The sample was then observed under light microscope with 400 times of magnification. Spermatozoa which had a circular tail was categorized as intact plasma membrane, while spermatozoa which had a straight tail was categorized as damaged plasma membrane. The total number of observed spermatozoa was 200 spermatozoa.

Sperm membrane integrity was calculated by using formula =

$$\frac{\text{the number of spermatozoa whic has a circular tail}}{\text{the total number of observed spermatozoa}} \text{x100\%}$$

Independent and Dependent Variables

Independent variables were the different ratio of coconut water and egg yolk extender and storage length. Dependent variables were spermatozoa abnormality and membrane integrity of Bali bull.

Data Analysis

Data were tabulated and analyzed by using analysis of variance in SPSS Program for Windows Ver. 23.0. Data with significant effect was then further analyzed by using Duncan's Multiple Range Test (DMRT) to know the differences between each treatment.

III. Results and Discussion

The Characteristics of Fresh Semen of Bali Bull

Results of the macroscopic and microscopic evaluation of fresh semen of Bali bull is shown in table I.

TABLE I. Average value of fresh semen analysis.

Variables	Average
Volume (ml)	6.1±0.61
Color	Yellowish white
Odor	Specific
pH	6.5±0.31
Mass motility	++
Individual motility (%)	55.00±1.58
Viability (%)	75.38±3.07
Abnormality (%)	8.66±0.52
Membrane integrity (%)	74.37±3.34
Concentration (106)/ml	830.4±81.38

Source: Processed primary data (2018)

Spermatozoa Abnormality

Spermatozoa abnormality is deformity of spermatozoa from normal structure that can be caused by several factors, such as environment, genetic or combination of both [3]. The relationship between spermatozoa abnormality and fertility is influenced by several factors, both of external and internal factors. The presence of the external factor that increases spermatozoa abnormality should be controlled and handled. The external factor that can affect the abnormalities of spermatozoa including semen collection technique.

Spermatozoa abnormality can occur at the time of spermatozoa formation and during semen handling (both of during and after semen collection). Spermatozoa abnormality can be produced by the failure of spermatogenesis or spermiogenesis process due to genetic factors, disease, and unsuitable environmental condition. It can also be caused by improper semen handling. Effect of extender treatment and storage length on spermatozoa abnormality of Bali bull is shown in table II.

TABLE II. Spermatozoa abnormality of Bali bull.

Storage length (days)	Treatment	Spermatozoa abnormality (%)
1	P0	9.96±0.71[a]
	P1	14.27±0.82[e]
	P2	13.20±0.83[d]
	P3	12.22±0.87[c]
	P4	11.19±0.87[b]
2	P0	11.35±0.82[a]
	P1	15.47±0.77[e]
	P2	14.49±0.74[d]
	P3	13.42±0.79[c]
	P4	12.44±0.89[b]
3	P0	12.54±0.46[a]
	P1	16.63±0.48[e]
	P2	15.63±0.50[d]
	P3	14.66±0.57[c]
	P4	13.65±0.51[b]

Notes: P0 (control) = Tris amino methane, P1 = 85% coconut water + 15% egg yolk, P2 = 80% coconut water + 20% egg yolk, P3 = 75% coconut water + 25% egg yolk, P4 = 70% coconut water + 30% egg yolk, [a-h]Different superscript within column showed a significant different (P<0.05)

Table II showed that the average abnormality percentage in P0 (control) at 1 day of storage was 9.96%. The highest abnormality percentage was recorded in P1 at 3 days of storage that was 16.63%. Generally, the average of spermatozoa abnormality of Bali bull during this study was still within the normal range. Bovine semen which had morphology with both of primary and secondary abnormalities less than 20% was categorized as normal spermatozoa cells [4].

Duncan's multiple-range test showed that the level of extender and storage length had a significant effect (P<0.05) on the spermatozoa abnormality at 2 days of storage. P0 (control) treatment had spermatozoa abnormality of 11.35%, which significantly lower (P<0.05) than P1 with the value of 15.47 %, P2 with the value of 14.49%, P3 with the value of 13.42%, and P4 with the value of 12.44%.

At 3 days of storage, P0 (control) had spermatozoa abnormality of 12.54%, which significantly lower (P<0.05)

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than P1 with the value of 16.63%, P2 with the value of 15.63%, P3 with the value of 14.66%, and P4 with the value of 13.65%. Although P4 had higher abnormality value compared to P0, but P4 had the lower abnormality value compared to P1, P2, and P3. So, it could be stated that the use of P4 was the most promising extender compared than the other treatments. The combination of young coconut water and egg yolk extender at a certain level can maintain spermatozoa survival and percentage of abnormality value [5]. Young coconut water and egg yolks extender can prevent hypotonic osmotic pressure on cell membranes that cause abnormality in spermatozoa cells [6].

Storage length could increase the spermatozoa abnormality of Bali bull. This result may be due to the environmental effect during storage which could increase the number of abnormal spermatozoa cells. [7] Stated that the high or low percentage of sperm abnormality was affected by the storage length, the physiological condition of the extender, and the male factor during the semen collection which is associated with the fertility of the livestock itself. The influence of environmental factors during storage and physical changes of living media can increase the percentage of spermatozoa abnormality [6]. [8] Explained that cooling treatment and storage duration could increase hypotonic osmotic pressure on cell membranes that cause swollen of spermatozoa neck, spermatozoa shrinkage, and a circular tail.

Percentage of Membrane Integrity of Spermatozoa Cell

Sperm which has a circular tail was categorized as intact plasma membrane, while sperm which has a straight tail was categorized as damaged plasma membrane. The total number of observed spermatozoa was 200 spermatozoa. Average value of membrane integrity of spermatozoa cell of Bali bull after treated with different extender and storage length is shown in table III.

TABLE III. Spermatozoa membrane integrity of Bali bull.

Storage length (days)	Treatment	Spermatozoa membrane integrity (%)
1	P0	71.00 ± 3.66^{a}
	P1	62.12 ± 4.49^{d}
	P2	64.69 ± 4.67^{cd}
	P3	66.80 ± 4.25^{bc}
	P4	69.23 ± 3.83^{ab}
2	P0	68.37 ± 3.64^{a}
	P1	59.62 ± 4.20^{d}
	P2	62.29 ± 4.25^{cd}
	P3	64.46 ± 3.96^{bc}
	P4	66.87 ± 3.73^{ab}
3	P0	66.23 ± 3.47^{a}
	P1	56.93 ± 3.79^{d}
	P2	59.23 ± 3.49^{cd}
	P3	61.73 ± 3.95^{bc}
	P4	64.51 ± 3.53^{ab}

Notes: P0 (control) = Tris amino methane, P1 = 85% coconut water + 15% egg yolk, P2 = 80% coconut water + 20% egg yolk, P3 = 75% coconut water + 25% egg yolk, P4 = 70% coconut water + 30% egg yolk, $^{a-f}$Different superscript within column showed a significant different (P<0.05)

The lowest membrane integrity was recorded in P1 at 3 days of storage with the value of 56.93%. The highest membrane integrity was obtained in P0 (control) at 1 day of storage with the value of 71.00%. In this study, the number of spermatozoa cell with circular tail more than the number of spermatozoa cell with straight tail. Spermatozoa which had intact plasma membrane integrity was characterized by a swelling in the head followed by a spinning tail with a bright color, whereas the spermatozoa which had defective membrane was marked with no swelling in the head and straight tail [9].

Results showed that the extender level and storage length showed no significant interaction (P>0.05). Spermatozoa membrane integrity in P0 (control) at 2 days of storage was 68.37%, which was significantly higher (P<0.05) than P1 (59.62%), P2 (62.29%), and P3 (64.46%), but there was no significant effect (P>0.05) compared to P4 (66.87%). At 3 days of storage, spermatozoa membrane integrity in P0 (control) was 66.23%, which was also significantly higher (P<0.05) than P1 (56.93%), P2 (59.23%), and P3 (61.73%), but not significantly different (P>0.05) compared to P4 (64.51%). This finding indicated that Tris aminomethane could be substituted by the extender containing 70% coconut water with 30% egg yolk because both extenders had almost the same membrane integrity value.

The extender treatment showed a very significant difference on the spermatozoa membrane integrity. The treatment of 70% coconut water with 30% egg yolk showed the best result as the extender of spermatozoa of Bali bull. The use of egg yolk extender could maintain the quality of spermatozoa cell membranes because they contain lecithin compounds. [6] Stated that the content of lipoprotein and lecithin in egg yolk served to maintain and protect the integrity of bovine spermatozoa cell membranes. Lecithin could bond the plasma membrane (enveloped the plasma membrane), so lecithin could maintain the stability of the membrane [10]. In another study, [11] showed that a component which affects soy lecithin was low density lipoprotein (LDL), which was similar with egg yolk, which may protect the integrity of membrane phospholipids.

IV. CONCLUSION

According to the results above, it could be concluded that the combination of coconut water and egg yolk at 5°C of storage did not affect spermatozoa membrane integrity. This is because of the combination of 70% coconut water with 30% egg yolk is the best combination of the extender and could be used as an alternative of spermatozoa extender media of Bali bull.

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