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Sperm Motility and Viability after α-Tocopherol Dilution in Tris Aminomethane-Base Extender During Cold Storage in Bali Bull

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Abstract : This study aims to determine the effect of adding α - tocopherol in Tris Aminomethane - egg yolk extender towards sperm motility and viability of Bali bull during storage at temperature of 5°C. Semen of Bali bull was obtained from breed in the Center of Regional Artificial Insemination of Mataram. The semen was diluted with Tris egg yolk then divided into four doses treatment of α – tocopherol: 0.0; 0.2; 0.4 and 0.6 g/100 ml of diluent, respectively. Motility and viability of sperm stained by eosin-negrosin and observed with luminescent microscope. Semen which has been stored at temperature of 5°C was observed daily for 8 days. The result show that the doses of α - tocopherol in Tris aminomethane - egg yolk diluent is significantly ($P < 0.05$) give a better percentage on sperm motility and viability of Bali Bull. The best result was showed by doses of 0.4 g α – tocopherol. It preserved the sperm motility for 47 ± 5.9 and viability for 73.9 ± 3.81 until 8 days of storage at temperature of 5°C. The percentage of sperm motility and viability of Bali Bull which had been stored at temperature 5°C for 8 days is remain in a good quality to be used for Artificial Insemination (AI) of liquid semen.
Keywords: *α-tocopherol*, Bali bull, motility, viability of sperm.

Introduction

Bali bull (*Bos sondaicus*) are an original beef cattle from Indonesia that have great potential to fulfill the protein needs for Indonesian people. Bali bull was distributed throughout Indonesia, especially in the region of South Sulawesi, East Nusa Tenggara, Bali and Lombok [1]. Bali bull as one of Indonesian native germplasm has a good productivity that needs to be developed into a superior livestock. Productivity of Bali bull could be maintained with assessments on the aspects of reproduction through the semen production.

Aspects that determine the quality of semen include the volume, color, pH, consistency, motility and viability of sperm [2,3]. Motility and viability of sperm after the ejaculation strongly depends on the protective media, source of nutrient in the extender and storage temperature for liquid semen. One of the extender that comply and maintain the motility of sperm after ejaculation is tris egg yolk. Besides the extender, other treatments which also affect the motility and viability of sperm is antioxidants in semen [4,5]. Antioxidants that capable to preserve the sperm are α-tocopherol and the system of liquid semen storage.

The low motility and viability of sperm caused functional impairment of sperm, changes the organizational structure and lipid composition in the sperm membrane [6]. Functional disruption of sperm caused the abnormal percentage of motility, viability, and the sperm mortality [7]. Low storage temperature harm the sperm, both structural and functional, and lead to activated cold shock and *Reactive Oxygen Species* (ROS) [8,9].

Stress on sperm is a major effect of disfunction in sperm and inhibits the phosphorylation process [4,10]. Disturbed oxidation of phosphorylation increases the free radical in semen. Besides that, the sperm mortality due to the influence of free radicals, cause chain reaction of lipid peroxidation that damage the cell membrane of sperm [11].

Superoxide Dismutase (SOD) is an enzyme that functioned as an antioxidant defense system. However, the stored semen causes oxidative stress to the sperm easily [9]. Sperm under the stress condition formed plasma membrane that contain unsaturated fatty acids, thus cause the cell damage. The damage in plasma membrane of sperm causes abnormality on the head and tail of the sperm that affect the motility. Affected motility descends the life capacity of sperm due to the formation of free radicals [12,13]. Free radicals can be stopped by antioxidants that has ability to cut chain reaction is α tocopherol.

α tocopherol is one of the most active antioxidant in capturing peroxyl radical by providing hydrogen atoms to become a new more stable-radical (unpaired electrons). α tocopherol remain functioned as an antioxidant to cut free radical chain, despite its low concentration. α-tocopherol is a powerful and effective as an antioxidant to cut the chain that protects the lipids from free radical damage [14]. The use of 0.4 g/100 ml α tocopherol – egg yolk citrate extender to the coagulated semen of Boer goats have been carried out with a result of 60% motility [15]. Hartono [16] continued the research on the liquid semen during 18-hours of storage at temperature 5°C in maintaining motility of sperm 89.07±1.33. Therefore, we assumed to use α tocopherol in Bali bull semen after storage, which hasn't been studied yet. Based on the problems, it is necessary to assess the motility and viability of sperm in Bali bull that using Tris Aminomethane – egg yolk extender with different α *tocopherol* doses at temperature 5°C.

Materials and Method

Research of liquid semen in Bali bull conducted at the Center for Regional Artificial Insemination (*BIBD*) Banyumulek of West Nusa Tenggara (*NTB*). Samples were analyzed in Laboratory of Immunology at Mataram University and Laboratory of Faal Medical Faculty at University of Brawijaya, Malang.

Sampling

Sample of this study is fresh semen of ±3 years old Bali bull that kept in BIBD Banyumulek NTB which has 3–3.5 of Body Condition Score (BSC). Semen were taken two times per week using artificial vagina. Semen that used has requirement from Indonesian Nasional Standard to AI with a minimum criteria of pH 6-7, mass motility ≥ +2, motility and viability of ≥ 70%.

Extender treatment

The extender treatment were made of Tris – egg yolk in four doses of α-tocopherol: 0 g (P_0), 0.2 g (P_1), 0.4 g (P_2) and 0.6 g (P_3) / 100 ml of extender. Fresh semen divided into four test tube and diluted untill 20 million motile concentration of sperm per 0.5 ml. Test tubes that contain semen with doses of α-tocopherol entered into a glass beaker of clean water, and then stored in a refrigerator at temperature ± 4-5°C. Each treatments were evaluated on the aspect of motility and viability per day during eight days of storage.

Observation of sperm motility

Semen was taken with a micropipette glass to be placed on object glass, covered with glass cover. Semen preparations were placed on the slide warmer at temperature of 37°C in few minutes. The observation was conducted by using microscope with 400x magnification [17]. The assessment compared the sperm with progressive or backward motile, motion in place or spinning referred to Garner and Hafed method [18].

Observation on sperm viability used another semen smear preparation with eosin-negrosin staining. After dried, we observed the smear object with luminous microscope of 400x magnification. Live sperm was not stained, whereas dead sperm stained red [19].

Data Analysis

This study used Completely Randomized Design (CRD) factorial pattern with 20 replications. Percentage of sperm motility and viability was quantitatively analyzed with ANOVA using statistical program of CoStat for Windows (Version 6.303). Any significant differences between the treatments will be followed by further analysis of Tukey test or Least Significant Difference (LSD) [20].

Result and Discussion

Sperm Motility

Motility of sperm is essential indicators that affect the general quality of semen. Sperm motility is crucial for fertility process and measurement on the possibility of sperm to fertilize the ovum [21]. Quality of good sperm was determined by the high progressive motility whether the sperm arrive fast or not to the fertility area. The average percentage of sperm motility on the eighth days of storage is 37.1±5.0%, whereas P_2 treatment (0.4 g α-tocopherol) is 47±5.9%, which is significantly better in maintaining the motility of sperm (Table 1).

Table 1. Sperm motility in Bali bull by adding α-tocopherol in tris egg yolk extender at 5°C

Day	Control	P_1	P_2	P_3	Mean±SE
1	78.8±2.22	79.5±1.5	79.5±1.5	77.8±4.1	78.9±2.36 [a]
2	72.8±3.43	75.3±3.4	77.8±3.8	73.5±4.9	78.3±3.89 [a]
3	66.3±3.58	69.5±5.6	74.3±4.1	68.3±5.2	69.6±4.61 [b]
4	60.8±3.73	64.5±6.1	70.8±4.1	63.3±5.7	64.8±4.88 [c]
5	55.3±4.44	59.5±6.3	65.8±4.7	56.5±6.5	59.3±5.47 [d]
6	47.0±5.50	53.8±6.3	60.0±4.3	51.3±6.3	53.0±5.58 [e]
7	38.0±4.90	48.0±6.2	54.0±5.3	43.0±6.2	45.7±5.65 [f]
8	29.0±2.90	40.0±5.7	47.0±5.9	33.0±5.5	37.1±5.0 [g]
Mean±SE	56.0±3.84 [d]	61.2±5.14 [b]	67.8±4.21 [a]	58.3±5.5 [c]	

SE: Standard Error

Different superscript in rows and columns shows significant difference ($P < 0.05$) to the percentage of sperm motility.

The result showed that the control treatment without α tocopherol in tris egg yolk extender is significantly different with treatment that using α tocopherol (P_2, P_1 and P_3). P_2 treatment (0.4 g α-tocopherol) significantly gave better results ($P<0.05$) on sperm motility of Bali bull. In the first and second days of storage, the average percentage of sperm motility showed no significant differences ($P>0.05$). However, in third to eighth day of storage showed significant differences.

Significant decrease of sperm motility has not been showed in the first early storage, whereas in the next day the real decrease of motility occurred. Motility of sperm which stored longer had more decrease of motility. However, the motility until the eighth day's storage indicated adequate motility of sperm to be used in artificial insemination of liquid semen (Table 1).

The average percentage of sperm motility until the eighth day of storage between the treatments was still more than 50%. The treatment of α-tocopherol dose significantly helps in maintaining the motility of sperm in Bali bull ($P < 0.05$). This result slightly better compared to Ducha *et al*. [22] explained the average percentage of sperm motility in Limousin bull using *cauda epididymal plasma* – Egg yolk extender 20% (CEP-2-KT20%) was 44.25±3.92% in the eighth day of storage at temperature 5°C. The use of 0.4 g α tocopherol is the best dose for maintaining sperm motility of Bali bull. The use of α tocopherol in lower or higher dose from 0.4 g per 100 ml of extender result less satisfactory in maintaining the percentage of sperm motility. The motility percentage in P_2 treatment of 47 +5.9% (Fig. 1) was adequate to be used for liquid semen in artificial insemination because the sperm motility was more than 40% as stated by Rizal [23] and determined by Indonesian National Standard. It indicated that the addition of α tocopherol in tris egg yolk extender is significantly better in maintaining the percentage of sperm motility at the storage time, as in line with Sikka [24].

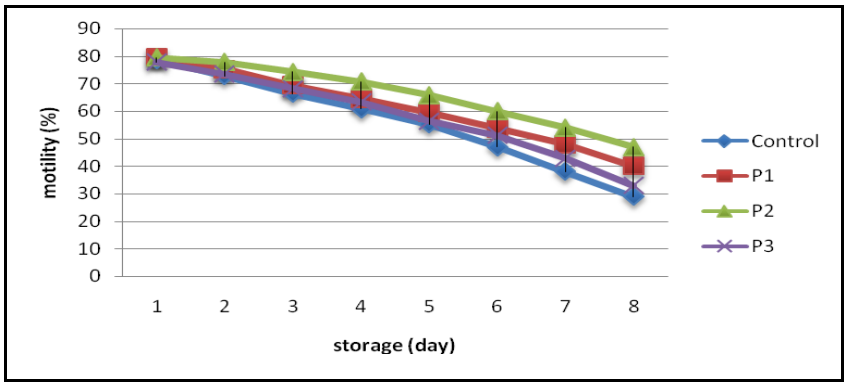

Figure 1. Sperm motility of Bali bull during storage at temperature 5°C with *α tocopherol* (P_1, P_2, and P3) and control in Tris egg yolk extender

The results of this study are equivalent to Alawiah and Hartono [15] of sperm motility in Boer goat. It stated that the α-tocopherol dose of 0.4 g per 100 ml egg yolk citrate extender is the best dose to maintain the quality of frozen semen of Boer goats with the motility of 60%. Hartono [16] continued his research on the same material but using liquid semen on the storage at temperature 5°C during 18 hours with dose of 0.4 and 0.5 g per 100 ml of extender, to maintain 89.07±1.33 and 89.46±1.12 sperm motility, respectively. Suyadi *et al.* [25] stated that there is a highly significant distinction ($P < 0.05$) between the group that use treatment α tocopherol to sperm motility of Boer goat after 1 hour of storage at temperature 5°C.

Results of ANOVA showed that interaction of α tocopherol dose and duration of storage didn't show any significant differences ($P > 0.05$) to the percentage of sperm motility. A low dose of α tocopherol was not able to stabilize free radicals in semen that stored at temperature 5°C. Conversely, excessive doses of α tocopherol in semen lead to the percentage reduction of sperm progressive motility. Assumed in giving excessive α tocopherol on the extender of semen will lead to the saturation of antioxidants; α-tocopherol is lipophilic (soluble in fat) and α-tocopherol as an exogenous chain breaker to prevent lipid peroxidation and hydrogen atom transfer.

Excessive doses of α tocopherol as an antioxidants interfere the function of ROS in the durability of semen and influence α tocopherol activity in the extender that affect the sperm motility due to apoptosis. Aside as an antioxidant, α tocopherol also functioned as pro-oxidant (less reactive) which caused by metabolic processes. The energy usage for moving caused motility reduction during storage and change the physiological characteristics of the semen in extender.

The reduction on percentage of sperm motility occurred due to the damage of sperm membrane by the osmotic pressure from the cell. As stated by Susilawati [26], membrane functioned to protect the cell. Consequently, disrupted intracellular metabolic processes of sperm, lead to the sperm mortality.

The decrease in the percentage of sperm motility during storage because the energy used by sperm derived from *glyserylphosphorylcholine*, Fructose and sarbitol that contained in the semen. Sperm can also use spare energy when the other energy are ran out [8]. Sperm movement require energy from Krebs cycle, electron transport and oxidative phosphorylation in the form of Adenosine Triphosfat (ATP) and Adenosine Mono Phosphate (AMP) which has function to change the fructose into lactic acid and CO_2 [27].

Sperm Viability

Viability of sperm is a factor that determines the success of artificial insemination, because only the survived sperm will acquire to arrive to female reproduction tract for fertilization process [28]. A high percentage of viability is expected much more survived sperm to fertilize the ovum.

The result of statistical analysis (Table 2) showed that the control treatment without α tocopherol in tris egg yolk extender is significantly different with the treatment that using α tocopherol. The treatment of α-tocopherol dose on tris egg yolk extender were significantly better maintaining sperm viability of Bali bull ($P<0.05$). P_2 treatment (0.4 g α-tocopherol) results better percentage of sperm viability significantly ($P<0.05$) than the others, i.e. 84.9±3.4%. The average percentage of sperm viability in Bali bull was consecutively P_2, P_1 and P_3 and control (Fig. 2).

Table 2. Sperm Viability of Bali bull by adding α-tocopherol in tris egg yolk extender at 5°C

Day	Control	P1	P2	P3	Mean±SE
1	91.7± 3.13	90.6± 3.38	94.4± 2.35	90.3± 3.01	91.8±2.96 [a]
2	88.9± 3.67	89.0± 3.03	91.6± 2.95	86.9± 3.51	89.1±3.29 [b]
3	85.2± 3.50	86.0± 3.52	89.3± 3.45	84.1± 4.05	86.2±3.36 [c]
4	81.7± 4.66	83.1± 3.24	87.2± 3.38	81.3± 4.05	83.3±3.83 [d]
5	78.6± 5.12	81.2± 3.40	83.8± 2.71	78.0± 4.35	80.4±3.90 [e]
6	74.7± 7.05	79.0± 4.51	81.6± 3.97	74.6± 5.16	77.5±5.17 [f]
7	68.7± 7.46	73.5± 4.10	77.5± 4.56	70.5± 6.19	72.6±5.58 [g]
8	60.5± 8.06	68.2± 4.11	73.9± 3.81	65.5± 6.75	67.0±5.68 [h]
Mean±SE	78.8±5.33 [c]	81.3±3.66 [b]	84.9±3.40 [a]	78.9±4.63 [c]	

SE: Standard Error

Different superscript in rows and columns shows significant difference (P <0.05) to the percentage of sperm viability.

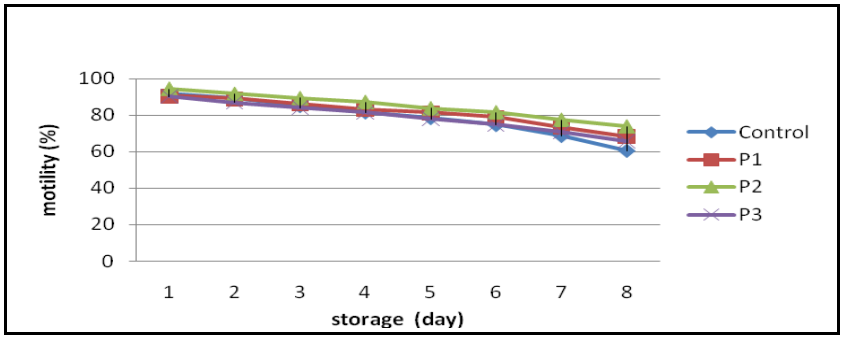

Figure 2. Sperm viability of Bali bull during storage at temperature 5°C with *α tocopherol* (P_1, P_2, and P3) and control in Tris egg yolk extender

The dose of α-tocopherol treatment on tris egg yolk extender result adequate percentage of average sperm viability; as stated by SNI for artificial insemination of at least 75% sperm was alive. It also stated similar by Said *et al.* [29] and Ax *et al.* [30] that the minimum standard of viability percentage of sperm that can be used for artificial insemination is ranged 60-75%. In this study, the average percentage of viability at dose of α-tocopherol P_1, P_2, P_3 and control was still above 75% (Table 2).

The use of 0.4 g α-tocopherol in tris egg yolk extender is the best dose in donating a hydrogen atom to the membrane of sperm. It interrupted its double bonds of the superoxide radical that result from the influence of storage duration. The result of this study was supported by Alawiah and Hartono [15] that assessed the use of 0.4 g α -tocopherol /100 ml egg yolk citrate extender of frozen semen in Boer goat. The treatment is able to maintain the viability by 60.2%. Hartono [16] continued the study on the same material using liquid semen at 18 hours of storage. It maintained the life of 89.46±1.12 sperm.

Control extender without α-tocopherol was only able to maintain the viability of 78.8±5.33%. It is lower compared to α-tocopherol P_2 by 84.9±3.4%. The high viability of sperm on α -tocopherol treatment stabilized the oxidative stress which caused by free radical. It proved that the proper dose of α-tocopherol capable to counteract the free radical, thus prevent the damage of sperm. A low or excessive doses of α tocopherol in extender of semen will decrease the percentage of sperm viability.

These results are similar to Suyadi *et al.* [25] that use 0.4 g α tocopherol on tris extender to delay the reduction of sperm viability of Boer goats. The best average sperm viability results of this study (84.9±3.4%) were slightly lower compared to Ducha *et al.* [22] that showed the average percentage of sperm viability in Limousin bull 87.46±5.40% in eighth day of storage. The difference is due to the utilization of *cauda epididymal plasma – Egg yolk* extender of 20% (CEP-2-KT20%) and different bull species.

The decrease in sperm viability occurred due to the damage of membrane which lead to osmotic

pressure to the cell. Morphologically, sperm coated by membrane called the plasmalemma or plasma membrane. This membrane composed of lipoprotein with high permeability. It protect the sperm against external threats of certain substances that would interfere the activity of cell [18,31]. If there is minor damage on the head membrane of sperm, the sperm will incapable to fertilize; although its motility doesn't impaired. Live sperm is transparant because the membrane was still properly intact as a cell protector, thus it did not get stain (Fig. 3). Whereas the dead sperm has ruptured membrane, resulted the secreted intra-cellular fluid, thus absorbed the staining [26].

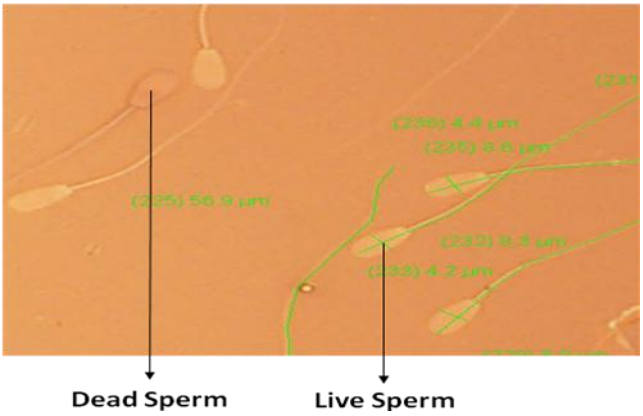

Figure 3. Sperm of Bali bull during storage at temperature 5°C with *α tocopherol* in Tris egg yolk extender

The use of tris egg yolk extender together with the treatment of α tocopherol showed better performance in maintaining the viability of sperm. The combination of α tocopherol and Tris egg yolk in a more complete nutrient composition provide important source of energy for sperm to maintain the lifespan during storage. Tris (hidroxymethyl aminomethane) functioned as salt contains [8] alkaline buffer that sustain the pH solution to be constantly stable, because pH is an essential factor that affects the quality of sperm [33].

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