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The Effect of α-Tocopherol in Egg Yolk Tris Diluent on the Spermatozoa Plasma Membrane Integrity, Superoxide Dismutase (SOD) and Malondialdehyde (MDA)in Bali CattleAt 5^{O}C of Temperature

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Abstract : This study aims to examine the effect of α-tocopherol in egg yolk *Aminomethane* tris diluent on the spermatozoa plasma membrane integrity and the activity of Superoxide Dismutase (SOD), Malondialdehyde (MDA)in Bali cattle at 5^{o}C of temperature. Semen that collected by using artificial vagina of Bali cattle and kept in the region artificial insemination center (BIBD) of Mataram was evaluated and diluted by using diluent of egg yolk *Aminomethane* Tris and then divided into four parts that was given *α-tocopherol* treatment successively 0.0; 0.2; 0.4 and 0.6 g/100 ml of diluen. Semen which was given treatment stored at 5^{0}C of temperature and evaluated during eight days everyday. The observation towards the spermatozoa plasma membrane integrity used *Hypoosmotic Swelling Test* (HOST), spermatozoa membrane integrity could be observed by using luminous microscope that had 400X magnification and the examination SOD activity, MDA spermatozoa. The addition of *α-tocopherol* in egg yolk *Aminomethane Tris* diluents was better influential ($P<0.05$) to the percentage of spermatozoa plasma membrane integrity and SOD activity, MDA of Bali cattle. The best *α-tocopherol* addition was 0.4 g in maintaining the plasma membrane integrity (71.1$\pm$8.72) and SOD, MDA until eighth day storage at 5^{0}C of temperature. The percentage of spermatozoa membrane integrity in Bali cattle that was stored at 5^{0}C of temperature during eight days was still used for artificial insemination (AI).

Keywords : *α-tocopherol, Spermatozoa membrane integrity, SOD, MDA and Bali cattle.*

Introduction

Bali cattles (Bos sondaicus) are one of the races of Indonesian local cattle that have potency to fulfill the protein needs fork Indonesian people. Bali cattles are wide spreading inall of the parts in Indonesia, especially in the region of South Sulawesi, East Nusa Tenggara, Baliand Lombok[1].Bali cattles are one ofthe Indonesian native germplasm that has a high enough productivity so thatit becomes important to be expanded in

becoming a superior ranch. Productivity of Bali cattle can be maintained with the aspect's assesment of reproduction through the production and semen's preservation.

The factors that determine the quality of semen are volume, color, pH, consistency and integrity of spermatozoa membrane[2,3,4]. Spermatozoa membrane integrity after ejaculating depends more on the shield media, sources of food in diluent and temperature on semen's storage. One of diluents which can fulfill and maintain the integrity of spermatozoa plasma membrane after ejaculating is egg yolktrisaminomethane.Besides the diluent, the other treatments may affect the spermatozoa plasma membrane integrity is antioxidant and SOD, MDA in the semen[5,6,]. Antioxidants which are able to maintain the spermatozoa plasma membrane integrity is α-tocopherol and temperature storage of liquid semen.

Refrigerator that has ± 4-5^{0}C temperature is one of the ways to keep maintaining spermatozoa membrane integrity. The storage of semen at ±4-5^{0}C of temperature can be conducted by giving water to the beaker glass as a place to put the test tube containing semen after diluting. Storage of semen using water as medium can maintain life and plasma membrane integrity of spermatozoa [3,], because the water medium can maintain a stable micro-environment that is able to adapt to the drastic temperature changes that can cause *coldshoek*. Spematozoa plasma membrane integrity is wholeness of spermatozoa membrane, or a condition which shows the mechanism of membrane's physiological function to be consistent as a control towards the transport system, mortality, and viability [7]. Spermatozoa plasma membrane integrity which is low has been caused by the functional damage of spermatozoa, the changes of organization structure and the lipid's composition on spermatozoa membrane [8].

The functional damage of spermatozoa affects to the decreasement of plasma membrane integrity percentage and spermatozoa's mortality[9].The storage in low temperature can harm the spermatozoa whether in structural or functional which is the result of *Cold shock* and *reactive oxygenspecies* (ROS) happened [10,11] . Stress on spermatozoa is the main cause of spermatozoa's dysfunction and inhibit the phosphorylation process [5,12,] . Phosphorylation oxidation is being disturbed cause the increasement of free radical in semen. In the other hand, spermatozoa mortality is the result of free radical's influence that generates chain reaction of lipid peroxidation can destructive spermatozoa plasma membrane integrity [13].

Enzyme that works as an antioxidant protection system is *Superoxide dismutase* (SOD), though because the semen stored, so then spermatozoa is easy to get the oxidative depression [11]. Spermatozoa that has stress will form their plasma membrane which contains unsaturated fatty acids, so that it generates the cell damage. The damage from spermatozoa plasma membrane integrity causes abnormal in the part of head and tile that affects mortality, therefore it decreases spermatozoa's power which is caused of free radical's formation[14,15] This free radical can be stopped by the antioxidant that has capability to break chain reaction as α *tocopherol.* α *tocopherol* is one of the most active antioxidant that catches perioksil radical by giving its hydrogen atom and then become free radical that is more stable because of the unpaired electrons happened.

α *tocopherol* is able to function again as an antioxidant in breaking the free radical chain, although the concentration is low. α *tocopherol* is powerfull and effective antioxidant as an antioxidant in breaking chain that protects lipid from the free radical damage [16].

The use of α *tocopherol* 0.4 g/100 ml egg yolkcitrate diluent on the frozen semen of Boergoats that had been done by Alawiah and Hartono[17] with the motality percentage is 60.00%. Continued the same research to the liquid semen during 18-hours storage at 5^{0}C of temperature which was able to maintain spermatozoa's motility was 89.07±1.33% [18]. The study using *α tocopherol* in Bali cattle hasn't been done yet. [19] stated that the use of *αtocopherol*0.4 g/100 ml in egg yolk aminomethane tris diluents is effective to maintain SOD, MDA. The role of SOD in diluents is to catch superoxide radical in becoming $H_2O_2$and to change O_2^-in becoming H_2O_2, therefore it is important to be done the measurement of SOD's activity [20, 21].

Based on the problem above, it is important to be done the study about the spermatozoa plasma membrane integrity and the measurement of SOD's activity, Bali cattle's MDA by using egg yolk *aminomethane tris* diluents giving the addition of *αtocopherol* that is different at5^0 C of temperature.

Experimental

This research was conducted at the Regional Artificial Insemination Centres(BIBD) Banyumulek West Nusa Tenggara(NTB), the laboratory of Immunologyat Mataram University and the laboratory of Medical Faculty at Brawijaya University, Malang Indonesia. The sample used was fresh semen of Bali cattle which was kept in BIBD Banyumulek NT Babout ± 3years old by having 3-3.5 body condition score(BSC). The frequency of semen shelter was 2times a week using Artificial Vagina(AV). The criteria of semen used has requirement Indonesian national standard (INS) for artificial insemination (AI) using pH minimum6-7.5of semen, mass motility was ≥ ++and viability was ≥ 70 %.

The fresh semen which was divided into 4 parts of the test tube was diluted with egg yolk *aminomethane tris* until reached concentration of 20millions of motile spermatozoa each milliliter, then given treatment by using α-tocopherol doses was about0g(P0), 0.2g(P1), 0.4g(P2) and 0.6g(P3) /100ml of diluent. Reaction tube which containing semen with α-tocopherol doses was entered into glass beaker containing water, then stored in are frigerator at ±4-5^{0}C of temperature. Each treatment was evaluated about the plasma membrane integrity everyday for eight days of storage and the measurement of SOD's activity in the second and fifth day.

Observations of spermatozoa plasma membrane integrity used Hypoosmotic Swelling Test(HOS TES). The examination used[22],with slight modifications 0.1 ml semen was mixed9.9mlof hypoosmotic medium. Hypoosmotic medium was made to dissolve1.3g of fructose and 0.7gNa citratein 100ml of sterile distilled water(Milli-Q-Water).The mixture of semen with hypoosmotic medium was incubated in CO_2 incubator which was 5%at 38.5^{0}C of temperature during 45minutes[7]. Observation by using contrast phase microscope with 400X magnification, the spermatozoa plasma membrane integrity which was still normal(tail part was circle and plasma membrane was bulging) whereas tail was straight, its spermatozoa plasma membrane was damage [23] . The measurement of SOD's activity and MDA (µ/ml) in the second and fifth day was done at the laboratory of Medical faculty UB.

The data analysis used completely randomized design(CRD), factorial pattern with20times of repetition. The percentage of spermatozoa plasma membrane integrity that was obtained then was analyzed of variance(ANOVA) quantitatively using CoStat for windows statistical software (Version 6.303).If there wasa real difference between the treatments, it would be continued by using Turkey test or the smallest real difference test [24].

Result and Discussion

The effectiveness of α-tocopherol performance to the spermatozoa plasma membrane integrity in Bali cattle at storage 5^{0}C of temperature.

The effectiveness of α-tocopherol performance in egg yolk Aminometane Tris diluent in the refrigerator at± 4-5^{0}C of temperature has real effect (P <0.05) to the spermatozoa plasma membrane integrity in Bali cattle. The treatment of α-tocopherol dose is able to maintain the spermatozoa plasma membrane integrity at cold temperature. The integrity of spermatozoa plasma membrane which is intact is an absolute thing that must be in good spermatozoa, because spermatozoa plasma membrane plays the important role in biokemik process that occures in spermatozoa [25]. If the integrity of plasma membrane can be maintained its wholeness during the storage process at 5^{0}C of temperature, it will be maintained from the motility, viability, and the wholeness of spermatozoa's acrosome cover. The giving of E vitamine (1 mg/ml) has better effectto the percentage of spermatozoa plasma membrane integrity in cattle after sexing and cold storage [26]. The average of percentage of spermatozoa plasma membrane integrity in Bali cattle at5^{0}C of temperature is shown in the Table 1.

Table1.The Mean of percentage of spermatozoa plasma membrane integrity in Bali cattle within α-tocopherol dose to the egg yolk tris diluent at storage 5^0C of temperature.

Day	Control	P_1(0.2)	P_2(0.4)	P_3(0.6)
1	89.9±3.71[a]	91.7±5.17[a]	92.2±4.51[a]	91.7± 4.93[a]
2	86.1± 4.25[a]	88.3±5.37[a]	88.5±4.66[a]	87.8± 5.07[a]
3	82.8±5.34[a]	84.2±4.55[a]	86.7±4.97[a]	84.6± 5.17[a]
4	79.7±5.94[a]	81.6±5.38[a]	84±4.25[a]	81.9± 4.23[a]
5	75.8±7.32[a]	77.4±6.95[a]	81.2±4.86[b]	76.5± 5.36[a]
6	70.7±8.13[ac]	73.9±5.45[abc]	78.1±6.44[b]	72.5± 5.23[c]
7	63.3±8.19[a]	70.8±5.99[b]	74.8±6.48[b]	66.6± 6.5[ac]
8	50.5±10.6[a]	68.7±4.76[b]	71.1±8.72[bc]	62± 8.04[d]

Explanation: [a-b-c]The different superscript in the row and column shows real difference (P<0.05) to the percentage of spermatozoa plasma membrane integrity. The same superscript in the row and column indicates the difference that is not real (P>0.05).

The performance in addition of α-tocopherol in egg yolk tris diluent was better real effect to the spermatozoa plasma membrane integrity. The average of percentage of spermatozoa plasma membrane integrity in Bali cattle that was stored at 5 ^{0}C of temperature from the the largest to the smallest in eighth days within α-tocopherol addition in egg yolk Aminometane tris diluent was 0.4 g (71.1±8.72 %), 0.2 g (68.7±4.76%), 0.6 g (62±8.04%) and 0 g (50.5±10.6 %).The treatment of P_2(0.4 g *α tocopherol*) was better in maintaining plasma membrane status of spermatozoa that was intact and shown in the variety analysis result (Table 1). In *α tocopherol* treatment to the egg yolk tris diluents was better in maintaining the spermatozoa plasma membrane integrity which was compared with or without giving treatment of *α tocopherol* addition.

The effectiveness of *α tocopherol* performance to the spermatozoa plasma membrane integrity in Bali cattle at the 5^0Cof temperature during eight days of storage in this research is a little bit same if compared with the result of study[26], that by providing vitamine (E or C 1 mg/ml) in maintaining the percentage of spermatozoa plasma membrane integrity after cold storage by applying swim up method is 70.34 percent and percol density gradient centrifugation is 72.22 percent at 5^0Cof temperature during two days of storage. That the frozen spermatozoa plasma membrane integrity which is BIB Lembang production is 40.55 % and BIB singosari is 54.45 %. Standard score for plasma membrane integrity that can be used is 30% [27]. The low of plasma membrane integrity in the study research in two BIB locations because it didn't use antioxidant and frozen semen in strow.

The addition of antioxidant such as α-tocopherol which is optimal in semen can reduce the damage of spermatozoa plasma membrane integrity during storage. The role of α-tocopherol is the most active ingredient to catch radical perioksil by giving its hydrogen atom to become the new radical that is more stable because the unpaired electrons happened[18]. It is said that the reduction of spermatozoa motility percentage because of damage in spermatozoa plasma membrane which was caused of cell osmotic pressure. The function of plasma membrane is to protect spermatozoa, whereas the function of the damaged plasma membrane will disrupt the proccess of spermatozoa intracelular metabolism and causes the death of spermatozoa[28].

To observe the performance of α-tocopherol addition in maintaining the plasma membrane integrity in this study isby following the method that was developed by [22]. Furthermore, the spermatozoa plasma membrane integrity which is still intact in itstail is circular and its membrane is distend, while the straight tail its spermatozoa plasma membrane integrity which isn't intact get damage[23, 26].

The treatment of α-tocopherol addition inegg yolk tris diluent in maintaining spermatozoa plasma membrane integrity which is intact in Bali cattle that was stored at 5 ^{0}C of temperature is better if compared without giving α tocopherol. The level of percentage, the spermatozoa plasma membrane integrity which is intact to the treatment of α-tocopherol addition because it related to the α-tocopherol ability in catching reactive oxygen [27].Furthermore, it suggested that α tocopherol is capable to break peroxidation reaction by removing

hydrogen ions together with the electrons. Lipid peroxidation can cause the damage relation of spermatozoa plasma membrane. Spermatozoa plasma membrane which got damage caused the cessation of metabolic processes to produce energy since it came out and the limitation of enzymes in doing metabolism. Consequently ATP that is needed by spermatozoa to survive become low that will be followed with the damage of plasma membrane and spermatozoa's death.

The low percentage of spermatozoa plasma membrane integrity in the control treatment without usingα-tocopherol has correlation with the effect of antioxidants if compared with treatment which using α-tocopherol P_1, P_2 and P_3 (Table 1). The antioxidants addition such as α-tocopherol is able to inhibit damage of spermatozoa plasma membrane integrity [10, 28] . Furthermore it is said that, the use of 0.3 g / 100 α-tocopherol that has medium of spermatozoa's washing in semen of Brahman cattle after treatment is better effect in maintaining the spermatozoa plasma membrane integrity which is still intact is 83.28 ± 4.99.Opinion from [28], is almost same with the result which was obtained in this research, but in this research used egg yolk *AminometaneTris* diluents with α-tocopherol doses that 0.4 g/ 100/ml diluents in the semen of Bali cattle can maintain spermatozoa plasma membrane integrity which is intact until eighth days of storage was 71.1±8.72 %[28]. Then said by Lukmanhy [18], that the use of α-tocopherol 0.4 g/ 100/ml of diluents in the semen of Bali cattle gives the best motility and viability (47±5.9 and 73.9±3.81).

The reduction in the percentage of spermatozoa plasma membrane integrity which is intact during eight days in storage at 5°C of temperature was (63.1 ± 8.02 to 91.4 ± 4.58) and became lower together with the duration of storage. The result of this study was better if compared to the result of study conducted by [28], the plasma membrane integrity which was intact (IMPU) liquid semen of Simental cattle that was stored in the tube at 5 ^{0}C of temperature during four days was (65.03 -83.95%), while which was stored in straw package was 61.49 – 77.29% [29] . The result of study about IMPU was still within the normal range and based what said by [7], because IMPU in cattle's semen which was normal and suitable for AI was (62.6 to 82.6%). Treatment of α-tocopherol dose looked better in maintaining the spermatozoa plasma membrane integrity which was intact. The reduction of IMPU during 8 days in storage at 5^{0}C of temperature is shown in figure 1

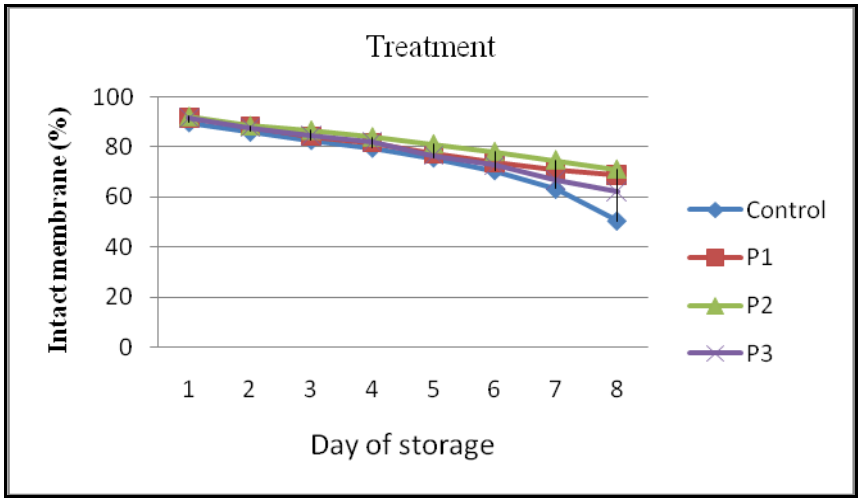


figure 1. Diagram percentage of spermatozoa IMPU in Bali cattle during eight days of storage at 5 ^{0}C of temperature with treatment of α-tocopherol (P1, P2, P3) and control in egg yolk tris diluent.

Plasma membrane for spermatozoa is needed to survive and to fertiliate ovum. The function of plasma membrane is not only to protect organs inside cell, but the more important is plasma membrane can has function as good filter in the exchange of intra- and extracellular substances. If the plasma membrane is damaged then the metabolic processes gets disturbed which is ultimately bad for spermatozoa's life [30].

Spermatozoa's membrane has function to keep integrity of membrane and form a dynamic surface between cells as well as the protection fromthe environments [31]. Spermatozoa's membrane has five main membrane areas, which is each of them relates to the primary cell component and involves with aspects of different cell functions. The head has three main areas, namely acrosome membrane, equatorial part and back part of acrosome (*post acrosomal*) which is head acrosome membrane serves to capacitation, acrosome's

reaction and ovum's penetration during the fertilization process. Membrane in ekuotorial area and back part of acrosome serves to hold the first contact and become one with ovum cell in the fertilization process. While the tail part has a different area in the central part and main part of spermatozoa's membrane. At the center of tail serves to obtain substrates for energy and dissipates motion wave as well as a major membrane has function to the spermatozoa's movement [10,30].

Morphologically, spermatozoa coated by a membrane called the plasmalemma or plasma membrane. This membrane is a compound of lipoproteins, which has high permeability and serves to protect spermatozoa against from external threats by entering of certain substances that will disturb its activity [33]. Furthermore, if there is minor damage which is difficult to detect in the plasma membrane of spermatozoa's head so the spermatozoa will not be able to fertilize although its motility is not impaired.

The Spermatozoa which its membrane is damage, has low fertilization, because the bad membrane beside can't be repaired in addition can also lead fluid of intra-cellular go out, while the liquid contains molecules (elements) that are needed when merging of spermatozoa with ovum in fertilization process [34]. Furthermore, spermatozoa's membrane also serves as a facilitator of energy transportation in ATP form throughout the all of body that was produced by enzymes in mitochondria through the Krebs cycle, whereas this energy is needed by spermatozoa in its movements.

The result of this study supported by [17], that the use of 0.4 g / 100 ml α-tocopherol in egg yolk citrate diluent in frozen semen of Boer goats are able to maintain the plasma membrane integrity from the damage, so that number of abnormal spermatozoa is low. Furthermore continued the research on the same material, but using liquid semen at 5 ^{0}C of temperature with the time duration of storage within 18 hours is to maintain spermatozoa plasma membrane integrity is 89.07 ± 1.33%. The use of dose 0.4 g / 100 ml α-tocopherol of diluent is able to maintain the highest spermatozoa plasma membrane integrity [18].

One of factors that caused the low ability of spermatozoa in fertilization process is the damage occurred on spermatozoa plasma membrane integrity during handling of semen due to peroxidation reaction by free radicals [13]. The suitable a tocopherol dose which is able to bound free radicals that was generated, so that the peroxidation process of plasma membrane does not occur so spermatozoa plasma membrane integrity can be maintained. He notes also that α-tocopherol can prevent the oxidation of unsaturated fatty acids and can protect the spermatozoa plasma membrane integrity in cattle during freezing and melting back, [16].

α-tocopherol is a main composition of the antioxidant system in mammalian cells. On the outside area, plasma membrane in spermatozoa contains carbohydrates that bounded to lipids (*glikolipida*) or proteins (*glycoprotein*). The addition of α-tocopherol in diluent as an antioxidant can maintain the spermatozoa plasma membrane integrity. Furthermore, *α-tocopherol* dose of 0.3 g/100 ml of diluent maintains spermatozoa plasma membrane integrity in PE goat before freezing was 73.34 ± 3.54 [35].

α-tocopherol is the most active ingredient to catch radical perioksil by providing a hydrogen atom to become the new radical which is more stable because the unpaired electrons happened. α-tocopherol has a phenol group which is able torelease hydrogen atom as an electron that will be caught by free radicals. According [34]α-tocopherol is one component as antioxidants system in semen. α- tocopherol as an anti-oxidant that prevents sustained chain reaction is antioxidants to break the reaction (*chain breaking antioxidant*) in system, erythrocytes and plasma. α- tocopherol in cell membrane will prevent the propagation of free radical reactions in phospholipid.

Comparation SOD's activity that contains α tocopherol with no α tocopherol in spermatozoa of Bali cattle stored at 5^{0}C(µ/ml) of temperature.

Antioxidant is compound that has high tendency to attract the most reactive electron [14]. Antioxidant divided into two major groups as enzymatic antioxidant and non enzymatic antioxidant. Enzymatic antioxidant includes *superoksida dismutase* (SOD), catalase, peroxidase gluthatione. Non enzymatic antioxidant includes E vitamine (α tocopherol), beta carotene and C vitamine or ascorbat acid [36].

The activity of free radical becomes cause of pathology condition. Free radical takes a role in reproduction process and infertility of male factor, stress oxidation attacks the fluidity of spermatozoa plasma

membrane and DNA's integrity in spermatozoa's essence The result of SOD's measurement is shown in table 2.

Table 2. SOD's activity of spermatozoa in Bali cattle after storage 2 and 5 days in increasing of α tocopherol in egg yolk tris diluents at 5^0C of temperature.

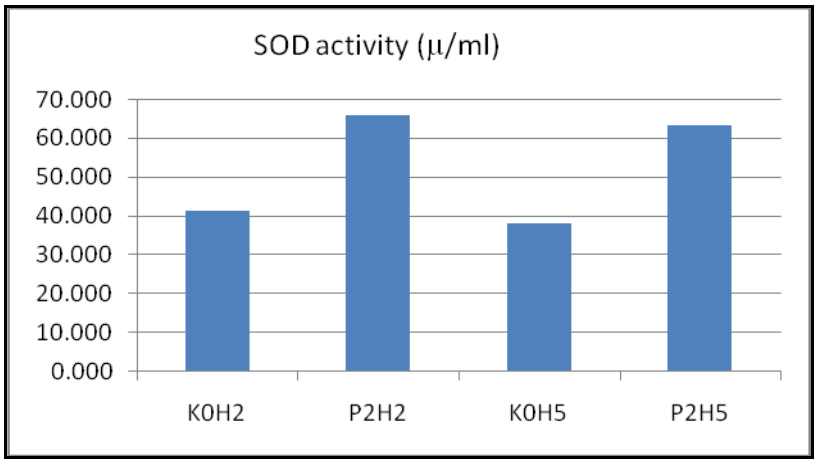
Diluent	Mean of SOD's activity (µ/ml) on	
	storage in second day	Storage in fifth day
KO =semen (noα tocopherol)	41,206 a	37,928 a
P2 = (0,4 g of α tocopherol 100 ml)+ semen	65,807 b	63,307 b

Explanation:[a-b]. Superscript which is not same with column shows treatment of α *tocopherol* is a real difference (P < 0,05).

SOD's mean in diluent of control treatment (no *α tocopherol)* in storage 2 and 5 days at 5^0C of temperature is 41,206 and 37,928 (µ/ml) compared with P_2 treatment (0,4 g α *tocopherol*) is 65,807 and 63,307 (µ/ml) is real different which is shown on table 2. This study result shows the average of SOD's activity in P_2 traetment that uses *α tocopherol*is higher than control treatment with no *α tocopherol*, as well as storage in 2 or 5 days.

Superoxide dismutase as the most important antioxidant's enzyme that is in cell as defense and takes a responsibility to the cell damage from free radical happened. Superoxide dismutase SOD is an enzyme as protection in the body cell to the free radical's attack that has function to catalyzes changes of anion superoxide (metabolic toxin of normal biological reaction that decrease oxygen) to become oxygen and hydrogen peroxide. SOD is responsible to neutralize the change of superoxide to become element of oxygen and hydrogen peroxide. This transformation called dismutase and depend on enzyme name [5]. SOD is an anti-oxidant enzyme that has role to catch radical superoxide to become H_2O_2.

SOD is an anti-oxidant enzyme that has role to catch radical superoxide to become H_2O_2or can be said that SOD has role to change O_2^-become H_2O_2. High activity of SOD means radical superoxide that is neutralized to become more [20,21], shown on figure 2.



Description: K0H2 and P2H2 = control treatment in second day and treatment of P2H2 = α *tocopherol* treatment in second day

K0H5and P2H5 = control treatment in fifth day and treatment of P2H2 = α *tocopherol* treatment in fifth day

Figure 2. Comparation of observation on SOD activity of spermatozoa in Bali cattle after storage 2 and 5 days conrol treatment with P2 (α *tocopherol)* at 5^0C of temperature.

Antioxidant is a substance that can bound compound of free radical, and consist of many kinds of components, as well as intracellular or extracellular. *α tocopherol* includes in secunder antioxidant that acts as

free radical's fastener. Antioxidant's activity in the body is a unity of cooperation system that related and influenced each other [36] . SOD's function is to accelerate dismutasion of $O_2$and keep balance between O_2's number and H_2O_2's formation [5] .

There is figure 1 above shows the use of *α tocopherol*in this study is effective to increase SOD's activity.High activity of SOD can neutralize free radical's compound in plasma seminal or in diluent. High activity of SOD as well as in storage 2 or 5 days is P2 treatment (0.4 g *α tocopherol*). SOD's content which is high indicates antioxidant that comes from good *α tocopherol*in protecting free radical's effect [16] , shown on (figure 2). High activity of SOD will be followed by activity of MDA's content which is low that shown on Table 3 and Figure 3.

MDA's activity that contains *α tocopherol* with no *α tocopherol* in spermatozoa of Bali cattle that was stored at 5^0C(ng/ml) of temperature.

MDA's content which is high will cause stress oxidative to become also high that is encountered by spermatozoa. Then, [37] stated that if free radical increase and antioxidant decrease will happen stress oxidative that followed by increasing of MDA's content. Therefore, it is important to be known the existence of free radical by doing the measurement from activity of MDA's content that shown on table 3.

Table 3. Observation from activity of MDA's content in spermatozoa of Bali cattle after storage 2 and 5 days on α *tocopherol* increasing in yolk tris diluent at 5^0C (ng/ml) of temperature.

Diluent	Mean from activity of MDA's content (ng/ml) in	
	2 days of storage	5 days of storage
KO =semen (no α *tocopherol*)	112.3 a	139.8 a
P2 = semen (0.4 g α *tocopherol* 100 ml)	97.8 b	121.5 b

Description: [a-b)]. Superscript which is not same in column shows that α *tocopherol*treatment indicates the real differences ($P < 0.05$).

The result of study of MDA's activity shows that the addition of 0.4 g α *tocopherol* dose is obtained the lowest mean in second and fifth day of storage at 5^0C of temperature was 97.8 and 121.5 (ng/ml) compared by the control treatment (no α *tocopherol)* was 112.3 and 139.8 (ng/ml) shown on table 3. It is shows that the use ofα *tocopherol* in yolk tris diluent that was stored at 5^0C of temperature is can prevent occurrence of lipid peroxidation's increasement and indicates occurrence of free radical's decreasement. Otherwise, if MDA's content will be higher, it will be also higher of stress oxidative that is encountered by spermatozoa [36]. Furthermore, it is said that free radical increase and antioxidant decrease will happen stress oxidative that is followed by the increasement of MDA's content as result increasement of lipid peroxidation. MDA's content in P_2treatment that uses *α tocopherol* has mean which is lower than control treatment without using *α tocopherol*, as well as in 2 and 5 days of storage.

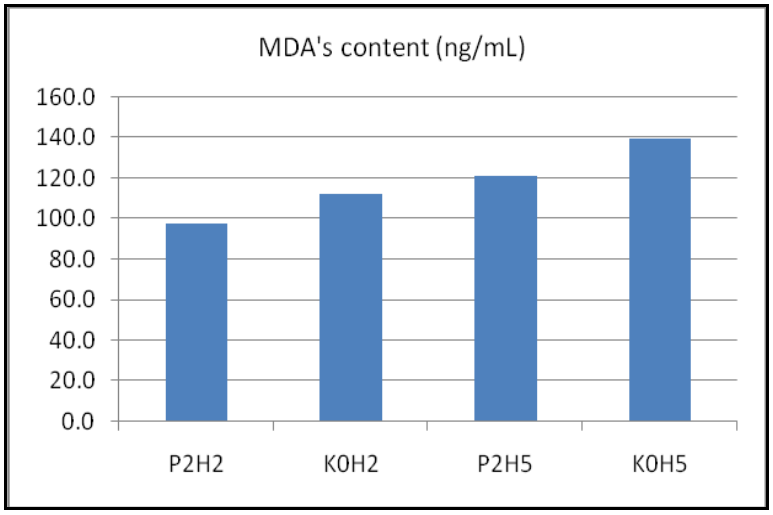
The role of *α tocopherol* can decrease free radical and neutralize ROS, which has been done by measuring the concentrate of *malondialdehyde* (MDA) (ng/ml) and *superoxide dismutase* (SOD). MDA's concentration in this study result on treatment that contains *α tocopherol* is lower if compared with control treatment without using *α tocopherol* shown on table 3 and figure 3. The role of *α tocopherol*in this study causes the SOD's content increase and then has negative effect to the concentration of MDA's content, by other words can prevent the increasement of stress oxidative and ROS that reflected in MDA's concentration which is low. Otherwise, SOD's concentration (μ/ml) shows higher (Table 2). This condition is suitable likes what was said by [37]. One of the compounds that is produced by breaker of lipid peroxidation is MDA. MDA created by the cause of OH free radical's degradation to the unsaturated fatty acid that is transportated later to become the most reactive free radical, so that measurement of MDA often used as indicator of lipid peroxidation system. [38]

The reduction of MDA's content in this study on P_2treatment by using *α tocopherol 0.4 g* dose in 2 and 5 days of storage, shows that there is correlation with the increasement of SOD's content. SOD is an extracellular enzyme that can catch free radical. The result of this study shows that the use of *α tocopherol* in

this study acts as non enzymatic antioxidant. Antioxidant is compound that can postpone, decelerate, and prevent lipid oxidation's process or free radical.

α tocopherol is a strong antioxidant that dissolve in fatty and effective as chain breaker's antioxidant that protect *unsaturated* lipid from free radical's damage [16].*α tocopherol* addition in yolk tris diluent in semen that was stored at 5^0C of temperature can protect spermatozoa from negative effect of metabolism scraps. This hypothesis is confirmed by the result of measurement of concentration test from MDA's content which is low and followed by high SOD if compared with on MDA and SOD that did not use treatment of *α tocopherol's* addition (Figure 2 and 3).

*α tocopherol*is not only to bound and neutralyze ROS, but it is also protect MDA and SOD's activity so that can maintain quality of spermatozoa at 5^0C of temperature well. MDA's content which is low indicates antioxidant that comes from *α tocopherol* that has good role in protecting from the free radical's effect [16], shown on (Figure 3).



Description :K0= control treatment and P2 treatment (α tocopherol), H2 and H5= second and fifth day

Figure3. Diagram comparation of observation in activity of MDA's content in spermatozoa of Bali cattle after storage 2 and 5 days of control treatment with P2 (*αtocopherol)* in yolk tris diluent at 5^0C of temperature.

In figure 3, shown that the use of *α tocopherol* is real difference which is better if compared without using *α tocopherol* in this study. MDA's content which is low is result of antioxidant's work in the form of *α tocopherol* that is able to neutralize free radical's compound in plasma seminal or in diluent. This study shows MDA's content which is low in treatment of *α tocopherol*, otherwise in control treatment with no *α tocopherol* as well as in 2 and 5 days of storage causes MDA's content which is high. This condition indicates that free radical's increasement occured, or in the other words antioxidant which has descreasement will happen stress oxidative that followed by MDA's content increasement. If MDA's content increase exceed of SOD's capacity, so SOD can not be able to catch again free radical that cause MDA's content as result of lipid peroxidation will increase [36]. The measurement of MDA often used as indicator of lipid peroxidation's system [6,16]. Antioxidant that decreases is followed with the increasement of MDA's content as result of lipid peroxidation's increasement. Lipid peroxidation is begun from continue's membrane of fatty acid chain to become compounds namely MDA, as well as DNA and protein also will encounter the same damage.

Conclusion

The effectivity of performance of *α tocopherol* increasing in yolk Aminomethane Tris diluent is real better in maintaining percentage of spermatozoa plasma membrane integrity in Bali cattle that was stored at 5^0C of temperature during eight days and activity of SOD, MDA. It is still good for IB with *α tocopherol* 0.4 g which is the best in maintaining percentage of spermatozoa plasma membrane integrity and SOD, MDA in Bali cattle.

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