Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/4849
DOI URL: http://dx.doi.org/10.21474/IJAR01/4849
ISSN NO. 2320-5407

RESEARCH ARTICLE

EFFECT OF α-TOCOPHEROL SUPPLEMENTATION IN THE EXTENDER ON THE SPERM QUALITY OF MADURAN BULL BEFORE AND AFTER QUICK FREEZING.

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Manuscript Info

Manuscript History

Received: 14 May 2017
Final Accepted: 16 June 2017
Published: July 2017

Key words:-
Sperm quality, Quick Freezing, α-Tocopherol, Madura Bull Cattle

Abstract

Processing semen needs appropriate technology to produce high quality of sperm for successful artificial insemination (AI) program. Quick freezing in this study is alternatif method of sperm cryopreservation processes that can be used to produce frozen semen in a large number, besides not required an expensive equipment, it is not time consuming with shorter time compared than ussual method of frozen semen. The present study was conducted to evaluate the effect usage quick freezing method and suplementation alpha tocopherol to egg yolk tris-aminomethan extender on sperm motility and plasma membrane integrity. Quick freezing of semen performed with diluted of fresh semen in extender and suplemented with 0.0 mM (P0); 0.5 mM (P1); 1 mM (P2) and 1.5mM (P3) alpha tocopherol, respectively, then loaded into 0.25 ml straw and referigerated for 30 min. Before plunging into liquid nitrogen (LN2), the straw containing sperm was placed 5 cm above liquid nitrogen surface for equilibration to nitrogen vapour temperature for 5 min before direct plunged into liquid nitrogen. The sperm was evaluated for motility and membrane integrity by hypo-osmotic swelling test (HOST) before freezing (5^0C) and after freezing. The results showed that sperm motility before freezing were 54.00 ± 3.94%, 56.00 ± 3.94%, 61.00 ± 2.11% and 56.00 ± 5.16% (P>0.05) for Control, P1, P2 and P3 groups, respectively. Plasma membrane integrity (PMI) were 58.80 ± 1.87%, 60.10 ± 3.78%, 67.00 ± 2.04%, 60.05 ± 3.28% for the respective groups (P>0.05) (P3). After quick freezing respectively, sperm motility were 32.00 ± 4.22% (control), 34.00 ± 5.16% (P1), 42.00 ± 4.22% (P2), 36.00 ± 5.16% (P3); and plasma membrane integrity were 51.05 ± 1.30% (control), 52.75 ± 4.01% (P1), 60.50 ± 2.02% (P2), 52.45 ± 1.10% (P3). Sperm motility and plasma membrane integrity before quick freezing no significantly (p≥ 0.05) improved at all treatment, while after quick freezing P2 (1mM) significantly (p≤0.p05) improved. In conclusion, semen quick freezing with suplemntation α-tocopherol to egg yolk tris aminomethan extender resulted beneficial effect on sperm motility and plasma membrane in Madurans bull cattle.

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Introduction:-

Artificial insemination (AI) has been widely used in cattle industry, successfully of these program necessary an appropriate protocol, such as progress of improvement of semen collection, analysis, cryopreservation and processing (**Hossain et al., 2011**). The main factor in semen cryopreservation was a semen quality (**Walsh et al., 2011**), which is the percentage of motile spermatozoa as an indicator of plasma membrane integrity and metabolic activity of semen (**Johnson et al. 2000, Estienne et al. 2007, Kaeoket et al. 2010)**. It has been known that the freezing-thawing process remain between 40%–50% of the spermatozoa donot survive (**Watson, 2000**).

Several study about rapid freezing have found, that sperm survival was compared between controlled cooling and ultra-rapid freezing or vitrification, butno difference in maintaining the sperm DNA integrity of teratozoospermic samples in post-thaw samples (**Schuster et al., 2003; Kalludi et al., 2011**). Rapid freezing procedure by using liquid nitrogen vapour freezing method in Chinese Taihang black goat caused a significant reduction in sperm motility and increasing abnormalities (**Shi et al., 2014**). Rapid freezing was a good alternative for cryopreservation technique, since the slow cooling technique involves the use of expensive instrument and is a time consuming protocol (**Kalludi et al., 2011**).

Cryopreservation induces the formation of ROS in animals (**Bilodeau et al., 2000; Kim et al., 2010**), caused by high amount of polyunsaturated fatty acids (PUFA) (**Wassall and Stillwell, 2009**). ROS in low concentration play an important role in sperm physiological processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes necessary for fertilization. Freezing and thawing induce the detrimental effects on sperm ultrastructural, biochemical and functional damage (**Watson, 2000**), resulting in a reduction of motility, membrane integrity and fertilizing ability (**Purdy, 2006**).

Natural and synthetic antioxidant systems have been known as a defense functioning mechanism against lipid peroxidation (LPO) in semen (**Shoae and Zamiri, 2008; Jayaganthan et al., 2013**). Extracellular antioxidants are very important for the protection of mammalian spermatozoa against oxidative stress (**Jeong *et al*.,2009**). Vitamin E or α-tocopherol is a primary component of sperm antioxidant system (Surai et al.,1998), and one of the main membrane protectors against ROS (**Akiyama, 1999**) andcould be added in feed, directly to semen extender, or to insemination catheters (**Petruska et al., 2014**).The addition to semen extenders of suitable antioxidants is suggested to reduce oxidative damage during freeze-thawing of bull spermatozoa (**Ansari et al., 2011**),vitamin E is one of the major membrane protecting against ROS and LPO attack, addition of vitamin E to the semen extender increased sperm resistance to lipid peroxidation **(Cerolini et al., 2000)**

Based on the principle of rapid freezing or quick freezing is not time consuming nor does it require expensive equipment. In the present study, would be performed a new protocol to observe the influence of various doses α-tocopherol suplemented into egg-yolk Tris-aminomethane extender to sperm quality (motility and plasma membrane integrity) usage quick freezing method

Material and Method:-

Semen samples:-

Semen sample were collected from 3 Madurans bull with sperm motility ≥ 70%, at National Artificial Insemination Center (BBIB), Singosari, Malang, Indonesia.

Semen Collection:-

Semen collection was carried out two times per week using an artificial vagina, where as on each collection two ejaculates were taken at an interval of 20-30 minute between successive ejaculates and each ejaculates was preceded by a period of sexual preparation consisting of at least two false mounts separated by about a one minute restraint.

Semen Cryopreservation:-

One aliquot of 1 ml of fresh semen was separated and diluted by Egg-yolk Tris Aminomethane (BBIB Singosari), glycerol 7%, penicilline 1.000 IU/ml, streptomycin 100 mg Sample were allowed to liquefy for 30 min at room temperatur adding diluent and suplemented with α-tocopherol (Sigma- Alderich). Sample were divided into 4 group : 0 mM (control), 0.5 mM (P1), 1 mM (P2) and 1.5 mM (P3), cooling at 5^0C , adding glycerol, sealing/chilling into 0,25 ml straw and prefreezing with nitrogen vapour, than plunged into liquid nitrogen and then storage into nitrogen

container. Sperm motility and plasma membrane integrity was observe soon, after cooling (before freezing) and post thawing (after Freezing).

Quick Freezing:-
Straw were subjected to static cooling at 5^0C and then placed horizontal above tray and equilibrated with nitrogen vapour at height 5 cm upper liquid nitrogen surface for 5 min (quick freezing) before being plunged into liquid nitrogen (-196^0C) and finally storage in the container that contain liquid nitrogen.

Semen thawing :-
Thawing process was carried out after >10 min after storage into container that contain liquid nitrogen by placed straw into a water bath at 37°C for 30 sec. Frozen straws were thawed at 37 ˚C for 30 s in a water bath for microscopic evaluation (Ashrafi et al. 2011).

Semen evaluation:-
Semen sample with ≥ 70% motility were used in the study, each ejaculate was evaluated first for volume and mass motility, sperm concentration was count using spectrofotometer, mass motility rates from 3 to 4, and less than 20% morphological abnormalities. Semen analysis (progressive motility and plasma membrane integrity) were performed to observe semen quality.

Progressive Motility:-
Lasley (1942), reported that the estimate of mass motility is not very precise, progressive individual motility is one of the major criterions of semen quality. Initial progressive motility rating was scored using 200X magnifications with a phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan). Percent progressive motility (0-100%) was measured at five representative areas of the slide. The average of the five scores for each category was recorded. If the difference between two consecutive counts exceeded 10 percent, two new counts were performed. The suspension was kept for one minute at room temperature (27°C).

Plasma Membrane Integrity:-
An important property of the sperm cell membrane is its ability to permit selective transport of molecules. Hypo-osmotic swelling test (HOST is very important to analyze the functional integrity of the sperm membrane because these characteristics are crucial for the viability and fertilizing ability of spermatozoa (**Jeyendran et al., 1984**). Hypo-osmotic swelling test for sperm membrane integrity was assessed using the hypo-osmotic swelling test according to the methods described by **Correa and Zavos (1994**). Hypo-osmotic solution (Sodium citrate- 0.735 g; Fructose- 1.351 g; Millipore water- 100 ml and Osmolality- 150 mOsm kg-1) was mixed with 0.1 ml of semen and incubated at 37°C for one hour. Following incubation, a drop of well-mixed solution was placed on a clean dry glass slide and covered with a cover-slip. Sperm tail curling was recorded as an effect of swelling due to influx of water. A total of about 200 spermatozoa were counted in different fields with 40× objectives under Microscope phase contrast. The total proportion of swollen spermatozoa was calculated by dividing the number of reacted cells by the total spermatozoa counted in the same area and multiplying the figure by 100.

Statistical Analysis:-
Post -thawing motility and membran integrity spermatozoa at height 5 cm were examined by one-way analysis of variance (**Snedecor and Cochran, 1967**).(SPSS All data (Motility and PMI) were analysed by SPSS package computerised software, version 16.0 for Windows, Inc. Chicago, IL, USA), Prior to the analysis, proportionality data (motility and HOST) were transformed using percent (**Snedecor and Cochran, 1994**) with adjustment to allow for zero values. Comparison between different treatment groups was done by Fisher's Least Significant Difference (LSD) test. The differences at $P \leq 0.05$ were considered to be statistically significant.

Results and Discussion:-

Motility before freezing:-
The present study were evaluated the influence of quick freezing semen and suplementation α-tocopherol in extender to sperm quality and reactive oxygen species in of Maduran bull cattlehas been present in 30 min storage at 5^0C from 70% to (54.00 ± 3.94) % (P0), it was caused by ROS production over the basal antioxidant capacity of sperm seminal plasma, so that required exogenous antioxidant suplementation.The percentage of sperm motility in these research (Tabel 1) were respectively, 54 ± 3.94 (P0), 56 ± 3.94 (P1), 61 ± 2.11 (P2), 56 ± 5.16 (P3).The result

was indicatedsignificantly difference (P<0.05) beween P0 with three others groups (P1, P2, P3), butnon significantly difference among others.

Tabel 1:- Sperm cell motility and plasma membrane integrity (PMI) in Egg-Yolk Tris-aminomethan extenders suplemented with α-tocopherol before freezing.

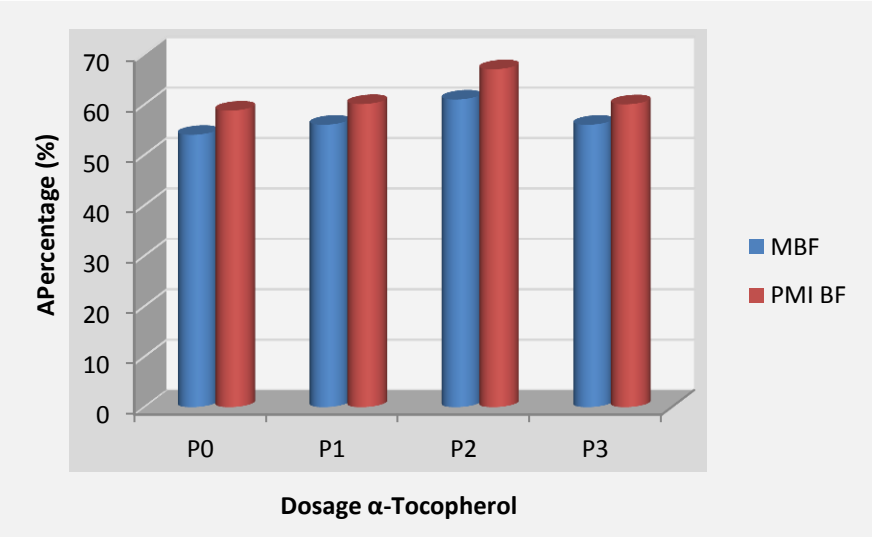
	P0 0.0 mM	P1 (0.5 mM)	P2 (1.0 mM)	P3 (1.5 mM)
Motilitas (%) x̄ ± sd	54.00 ± 3.94[a]	56.00± 3.94[ab]	61.00 ± 2.11[ab]	56.00 ± 5.16[ab]
PMI (%) x̄ ± sd	58.50± 1.87[a]	60.10± 3.28[a]	67.00 ± 2.04[b]	60.05 ±3.28[a]

Different notations in the same row show differences between treatments for each parameter tested (One- Way ANOVA, P< 0.05, SPSS 16.0).

Control group, without suplementaton with α-tocopherol there was reduced in sperm motility in refrigerator storage (at 5^0C) for 30 min. Similar with finding by **Verberckmoes *et al*. (2005)**, there is a significant decrease in sperm motility and the fertility of bovine spermatozoa during storage. Significantly decreasewas observed in the conception rates of cows inseminated with bovine semen cooled for 48 h compared with conventionally cryopreserved (**Crespilho *et al*., 2009**), and maintenance of bovine semen in liquid form under refrigeration has a maximum efficiency in viability when stored for up to 24 h in conventional egg yolk-based extenders (**Crespilho *et al*., 2012**).

Suplementation with 0.5, 1 and 1.5 mM α-tocopherol in this study was significantly improved in sperm motilty compared than control, may be at refrigerator (5^0C) storage the atioxidant have required in anticipation ROS generation, due to decreasing capacity of antioxodant plasma seminalis during dilution and decreased in metabolism

The previous study in bovine found, that during cool storage semen cause significant decreased in sperm motility (**Verberckmoes *et al*., 2005**) and fertility. Sperm cryopreservation cause elevating ROS production following by decreasing antioxidant levels, induce the detrimental effects of sperm ultrastructural, biochemical and functional damage, resulting in a reduction of motility, membrane integrity and fertilizing ability (**Chatterjee and Gagnon, 2001; Bilodeau et al., 2000; Purdy, 2006** and **Watson, 2000**), in control group without suplementation α-tocopherol during cooling (before freezing) indicated that there are no membrane protection and inadequate cytoplasmic defenses at these process, consistent with earlier finding that high concentrations of the ROS cause sperm pathology (ATP depletion) leading to insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability(**Bansal and Bilaspuri, 2011**), while the levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules.



The supplementation of water-soluble-α-tocopherol analogue (Trolox), significantly improved the post-thawed human semen quality, especially progressive motility before freezing (**Minaei et al., 2012**).Similar with the result of these study, resulted significant improve in sperm motility before freezing at suplementation 1mM α-tocopherol. This was caused, although difference of height and lenght of time during equilibration (in liquid nitrogen vapor 10 cm above the surface of liquid nitrogen at -80^{0}C for 15 minutes, while in these study 5 cm above the surface of liquid nitrogen for 5 minutes) before plunged into liquid nitrogen (-196^{0}C) for storage, resulted the same effect in improving sperm motility.

During cryopreservation, one of the harmful effects is ROS production (**Breininger et al., 2005**). Oxidative stress on spermatozoa, when imbalance between the amount of ROS produced and scavenger would cause sperm damage, A shift in the levels of ROS towards pro-oxidants in semen can induce an oxidative stress on spermatozoa. In order to scavenge ROS and reduce their destructive action under normal physiological conditions, a complex antioxidant system is present in sperm and seminal plasma (**Minaei et al., 2012**).

Previous studies in equine semen concluded that the addition of vitamin E at concentrations of 1.0, 2.0 and 4.0 mM did not significantly alter the maintenance of sperm motility during storage at 5^{0}C (**Ball et al., 2001**). In contrast with our findings 0.5 , 1 and 1.5 mM α-tocopherol significantly improve sperm motility. But similar with previus finding in boar, thatα-tocopherol suplementationto semen diluents increased cell viability, through its prevention of an oxidativereduction in the levels of the major PUFA (**Cerolini et al., 2000**).Suplemantation an antioxidant α-tocopherol with a PUFA could improve freezing ability of goat semen via changing the lipid composition of sperm cel (**Ansari et al., 2012**). Perhaps phospholipids of the spermmembrane exhibited different phase transition temperatures, inducing the transition to the gel phase in othermolecules, which in turn influenced its diffusion coefficient (**Maia et al., 2009**) and fusion capacity of the membrane (**Holt and North, 1986**). It seems that optimum level of α-tocopherol may depend on different factors such as kind of cryopreservation, components of extender, and even freezing procedure, species specific, consentration antioxidant.

Among the antioxidants, especially α-tocopherol is a chain breaking antioxidant, can break chain reaction by ROS in membrane lipids. The principal chain-breaking antioxidant vitamin E is present within the cell membrane. It neutralizes H2O2 and protects the plasma membrane from lipid peroxidation (**Kessopoulou et al, 1995**).The result of present study consistent with previous study, that α-tocopherol plays an important role in reducing membrane damage caused by excessive ROS production during cryopreservation. Based on the highest in improved sperm motility before freezing, α-tocopherol at 1mM is said to be the exact doses in protection sperm membrane plasma against ROS attack during cooling processes. In line with the present study, the suplementation at level 1mM of α-tocopherol during cooling to the extender had a positive effect on improved sperm motility in boar (**Cerolini et al., 2000; Pena et al., 2003; Pena et al., 2004**),by inhibition lipid peroxidation (**Pena et al.,2003**), at thisconcentration α-tocopherol have a protective effect against oxidative damage and counteract the effect of ROS affected by dilution and cooling. Otherwise, decrease of motility at control group before freezing in the present study not, similar with in boar semen cryopreservation, that the 5^{0}C cooled showed no reductions in sperm motility (**Kim et al., 2011**).

Increased ROS levels also have been associated with reduction in the sperm motility (**Lenzi et al., 1993; Agarwal et al., 1994; Armstrong et al.,1999**).Could be explain by many hypotheses, that a high content PUFA in sperm membran, rendering them highly susceptible to oxygen-induced damage. Subsequently, a rapid loss of intracellular adenosine tri-phosphate (ATP) by LPO causes axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects, all of which contribute to decreased sperm motility (**Sikka et al., 1996; Bansal and Bilaspuri, 2007, 2010; Gharagozloo and Aitken, 2011**).

Plasma membrane integrity before freezing:-

The percentage of PMI before freezing in our study were control (58.80 ± 1.87) P1(60.10 ± 3.78), P2 (67.00± 2.04), P3 (60.05 ± 3.28). There were significantly difference (P≤ 0.05) between P2 and three others grous (P0, P1, P3), while among three others non significanly difference (P≥0.05). Suplementation with 1 mM α-tocopherol is the best result.

The decease of plasma membran integrity, respectively P0, P1, P3, probably due to dose dependent, spermatozoa are very susceptible to cold shock when cooled below 15^{0}C (**Watson, 2000**). As temperature declines, thereis a modification ultrastructure, and biochemical components resulted in reduction in the membrane integrity, (**Johnson**

et al., 2000). Whereas the sperm plasma membrane is the primary site of cold-induced damage (**Bailey et al., 2008**), moreover membrane stress associated with phase changes in lipids and an altered functional state of the membrane (**Watson, 2000**).

Tariq et al, (2015), described that α-tocopherol plays an important role in reducing membrane damage caused by excessive ROS production.So that,suplementation α-tocopherol in present study,could maintained sperm plasma membran integrity before freezing or at cooling step, because ROS had been exists from cooling at 5^0C.Biologically vitamin E, stabilize plasma membrane spermatozoa (**Surai et al., 2000**).

Motility after freezing:-
The percentage of sperm motility after quick freezing at a dosage 1 mM α-tocopherol or P2 (42 ± 4.22) significantly difference (P≤0.05) with three others group control (32 ± 4.22), P1 (34 ± 5.16) and P3 (36 ± 5.16), while among three others group no significantly difference (P≥0.05).

Tabel 2:- Sperm cell motility and plasma membran integrity in Egg-Yolk Tris-aminomethan extenders suplemented with α-tocopherol after freezing.

	P0 0 mM α-T	P1 (0,5 mM)	P2 (1 mM)	P3 (1,5 mM)
Motilitas (%) $\bar{x}$ ± sd	32.00 ± 4.22[a]	34.00 ± 5.16[a]	42.00 ± 4.22[b]	36.00 ± 5.16[a]
PMI (%) $\bar{x}$ ± sd	51.05 ± 1.30[a]	52.75 ± 4.01[a]	60.50 ± 2.02[b]	52.45 ± 1.10[a]

Different notations in the same row show differences between treatments for each parameter tested (One- Way ANOVA, P< 0.05, SPSS 16.0).

The quick freezing technique using liquid nitrogen vapour for a few minutebefore plunged into liquid in this study, as showed a decrease in sperm motility at control group.Several study before, about rapid freezing without supplementation antioxidant to the extender has been reported.**Minaei et al. (2012)**found,that normally there were a balance between ROS producing and scavenging activities, when this equilibrium is impaired, and ROS would be induced oxidative stress, causedthesperm membran damage, followed by reduced sperm motility. **Agha-Rahimi et al.(2014)**reported**,** that sperm parameters including motility, viability and morphology, declined in rapid freezing technique. In contrast with finding by**Schuster et al.(2003)** and **Kalludi et al.(2011**), that a sperm survivals was compared between controlled cooling and ultra-rapid freezing or vitrification. But a sperm DNA integrity was not found in post-thaw(**Schuster et al.,2003 and Kalludi et al., 2011; Agha-Rahimi et al.,2014**).**Kim et al. (2012**) found, that the rapid freezing of canine sperm without cooling equilibration improved sperm motility, viability, and plasma membrane integrity, and even freezing for more than 2 min in LN_2 vapor increased acrosome-membrane integrity. These difference results, was might be caused by differences in distance and duration of equilibration, besides the difference in extender medium, antioxidant supplemented and species. While the quick freezing method in our study, performed by prefreezing at a distance 5 cm above the surface of Liquid nitrogen for 30 minute before plunged into liquid nitrogen container, using egg-yolk Tris-aminomethan based extender, supplemented with various dosage α-tocopherol, in purpose to balance decreased of antioxidant during preservation processes.

Supplementation of α-tocopherol at dosage 1 mM could prevented oxidative damage by ROS generation and and thus improved sperm motility. It was predicted by mechanisms loss of antioxidant capacity (**Aitken and Sawyer,2003; Hsu et al., 1998**). Similar results have indicated that loss of motility is correlated with lipid peroxidation (**Minaei *et al*., 2012; Maia *et al*., 2010**). In our study, we have identified that the addition of antioxidant resulted in better levels of sperm motility, indicating that anti-oxidative mechanisms contribute to prevent the sperm-membrane damage. Also, a positive correlation found between the percent motility of the spermatozoa and antioxidant activity with dose-dependent.

Plasma membrane integrity after Freezing:-
The percentage of PMI after freezing at a dosage of α-tocopherol1 mM (42 ± 4.22) significantly difference (P≤0.05) with three others group respectively, control, 0.5 mM, 1.5mMwere 51.05 ± 1.3, 52.75 ± 4.01, 52.45 ± 1.10, while among three others group non significantly difference (P≥0.05).

Control group without suplementation α-tocopherol, indicate the lower PMI compared than three others. Consistent with reported by previous study, that cryopreservation have a detrimental effect on motility, membrane integrity and mitochondria, deformity spermatozoa (Shi et al., 2014).Afterfreezing-thawing process intracellular antioxidant capacity in sperm decrease(Tuncer et al., 2010).Storage at 5°C could affected in membrane structure, cause sensitive to ROS at the time sperm contacts with Oxygen (Sankai et al., 2001; Kardivel, et al., 2009).

Cryopreservation processes,with the decrease in temperature, causes oxidative stress on sperm membranes,results in irreversible damage to the sperm organelles and changes in enzymatic activity, related to reduction in sperm motility, functional membrane integrity and fertilizing ability (**Bucak et al., 2009a,b**).Excessive generation of ROS during cryopreservation of mammalian semen could reduce the viability and fertilization capacity of the spermatozoa (**Bilodeau et al., 2000**).ROS molecules can cause damage to sperm motility, plasma membrane, acrosomal and DNA integrity (**Aitken et al., 1998; Bilodeau et al., 2001; Lenzi et al., 2002; Kumar et al., 2011**).

The membrane damage may be manifested by the change in its lipid composition as lipid modification due to cryopreservation (**Cerolini *et al*., 2001; Chakrabarty *et al*., 2007; Zaniboni and Cerolini, 2009**), which is responsible for the fluidity of membrane bilayer (**Sanocka and Kurpisz, 2004**).Chilling/cryopreservation of buffalo semen resulted in decreased antioxidant activity and higher ROS molecules, decrease in motility, plasma membrane integrity and Viability (**El-Sissy et al., 2007; Anzar et al., 2010; Kumar et al., 2011**).The major factor in insemination outcome with frozen-thawed semen is the addition of cryoprotectants and spermatozoal damage due to internal ice crystals**; El-Harairy et al., 2011**). The lower sperm PMI in control group could be caused by LPO during quick freezing (dilution, cooling, freezing, thawing). As we have known, thatsperm plasma membrane with high contain of PUFA and lack a significant cytoplasmic antioxidants cause susceptible to LPO (**Lenzi et al., 2002; Bucak et al., 2007**). Lipid peroxidation cause decrease in fluidity and integrity plasma membrane (**Bansal and Bilaspuri, 2010**), Changes in the sperm plasma membrane during cryopreservation include lipid phase transition, resulting in a spatial redistribution of the membrane components, modification of membrane domains and microdomains, and consequently reaggregation of membrane proteins (**Muller et al., 2008**), with subsequent loss of membrane integrity, impaired cell function and decreased sperm motility (**Lenzi et al., 2002; Bucak et al., 2007**).

Suplemention 0.5 and 1.5 mM α-tocopherol, also showed lower in PMI, it was caused by an increase dose of α-tocopherol is pro oxidant, rather than antioxidant, while in contrast at lower dose ineffective (**Tariq et al., 2015**).

The best result of suplementation of α-tocopherolwas dosage 1 mM as well as sperm motility at the same dose, which is at the optimal level prove to maitain membrane function. Important role of this antioxidant was reduced membrane damage caused by excessive ROS production during quick freezing, by break the covalent links betweenfatty acid side chains in membrane lipids, resulted an increase of sperm motility and membrane integrity.It has been known that α-tocopherol as chain breaking antioxidants and In many trials it had been shown that vitamin E is is one of the major membrane protectants against ROS and lipid membrane peroxidation (**Moreira da Silva et al., 2010**). Vitamin E is a chainbreaking and not a scavenging antioxidant, non-significantly in elimination amount of the ROS (**Dad et al., 2006**), results in the neutralizing of lipid radicals (**Chow, 1991**), protection to membrane components without influencing ROS generation (**Sharma and Argawal, 1996)**.

It was prove in our research, that ROS level decrease with suplementation 1mM α-tocopherol compared with control, and finally PMI increase. Alpha-tocopherol was an efficient antioxidant for reducing the oxidative stress caused by cryopreservation, as well as decreasing lipid peroxidation on equine spermatozoa (**Franco et al., 2013**).**Pena et al.(2003)**, found that inhibition lipid peroxidation and the influence depending on the concentration of the antioxidant.

Tabel 3:- Sperm cell motility and plasma membran integrity before and after freezing in Egg-Yolk Tris-aminomethan extenders suplemented with α-tocopherol

Dosage α-Tocopherol	Motility		PMI	
	BF	AF	BF	AF
P0 (control)	54.00 ± 3.94 [a]	32.00 ± 4.22[a]	58.80± 1.87[a]	51.05 ± 1.30[a]
P1 (0.5 mM)	56.00± 3.94[a]	34.00 ± 5.16[a]	60.10± 3.28[a]	52.75 ± 4.01[a]
P2 (1 mM)	61.00 ± 2.11 [ab]	42.00 ± 4.22[b]	67.00 ± 2.04[b]	60.50 ± 2.02[b]
P3(1.5 mM)	56.00 ± 5.16[ab]	36.00 ± 5.16[a]	60.05 ±3.28[a]	52.45 ± 1.10[a]

Different notations in the samecollum show significantly differences (One- Way ANOVA, P< 0.05, SPSS 16.0).

Castro et al. (2016) found, that rapid freezing in Holstein bulls by using a cryopreservation machine Cryogen® (Neovet, Uberaba, MG, Brazil) during the cooling and freezing process resulted sperm cell damage in membranes plasma, acrosome, mitochondria and even chromatin damages, without recovery after 2 hs of incubation. During this process, the critical moment is when sperm are subjected to freezing temperatures.

Freezing–thawing processes lead to the generation of ROS that impair sperm motility, membrane integrity, and fertilizing potential (**Hu et al., 2010**). The success of cryopreservation depends not only on preserving the motility of the spermatozoa but also on maintaining their metabolic function (**Watson, 2000**). The major factor affecting in outcome of insemination with frozen–thawed semen is the addition of cryoprotectants and spermatozoa damage by internal ice crystals formation, due to the increase in solute concentration in the extender or interaction of both factors (**El-Harairy et al., 2011**)

The inhibitory effects of ROS on motility point to a mitochondrial-independent mechanism. The reductionin motility point to a mitochondrial-independent mechanism, where as a ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum (**Guthrie and Welch, 2012**).

Based on the statement above, It was proved that rapid freezing with usage of LN2 vapor for a few minute resulted decrease in sperm motility and membrane integrity, and α-tocopherol plays an important role in reducing membrane damage by quenching excessive ROS production during Quick freezing processes. Similar with the research before, that vitamin E or α-tocopherol could reduced the LPO, then improved sperm motility , viability (Amrit *et al*., 2008) and plasma membran integrity (**Cerolini et al., 2000; Pena et al., 2003; Pena et al., 2004**).

The suplementation 1 mM (P2) α-tocopherol give a protective effect against oxidative damage and counteract ROS generation,that caused by diluent and cooling. Addition of α-tocopherol in extender was proved in maintain plasma membrane integrity of frozen semen of bucks (Wahyuningsih and Achadiah, 2012). This antioxidantbreak the covalent links betweenfatty acid side chains in membrane lipids, and finally to increase sperm motility and membrane integrity. **Pena et al.(2003**), found that inhibition lipid peroxidation and the influence depending on the concentration of the antioxidant.

Suplementation 0.5 mM (P1); 1.5 mM (P3) no significantly ($P \geq 0.05$) difference. In improved motility as low as control. Dosage 0.5 mM α-tocopherol in this study is low dose, have low protection to sperm plasma membran, while at 1.5 mMtoo high dose role as oxidant that damage to sperm membrane. Sperm motility no significanly increase after quick freezing with suplemented 0.5 and 1.5mM doses, it has been reported that an increase dose of α-tocopherol is pro oxidant, rather than antioxidant, while in contrast less effective (**Tariq et al., 2015**).

The influnced of suplementation 1 mM α-tocopherol to semen extender against sperm motilty and PMI before and after freezing were higher than control, 0.5 mM, and 1.5 mM (Fig 3). Vitamin E provides biological stability to the spermatozoal plasma membrane (**Surai et al., 2000**). Added vitamin E to the chicken semen extender during in vitrostorage of semen has beneficial effects on sperm motility, viability rates and lower morphological defects due to reduction of ROS (**Tabatabaei et al., 2011**).

Conclusion:-

The suplementation dosage 1mM α-tocopherol to egg yolk tris-aminomethan extender resulted beneficial effect on sperm motility and plasma membrane integrity before and after quick freezing in Maduran bull cattle.

Acknowledgements:-

We thank to The Directorate General of High Education; The Dean of Faculty Animal Husbandry, Brawijaya University, Malang City; The Dean of Faculty Veterinary Medicine, Airlangga University, Surabaya City; West Java, Indonesia.This Research was supported by BPDN Goverment Scholarship Program for Post Gradutae Study and Research of the Indonesia Republic.

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