

Effect of α -tocopherol supplementation in diluents on the motility, viability and plasma membrane integrity of Simmental bull spermatozoa after cooling

Pengaruh suplementasi α -tocopherol dalam diluen terhadap motilitas, viabilitas dan integritas plasma membran spermatozoa sapi Simmental setelah pendinginan

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ABSTRACT

Semen storage in cold temperatures might cause an increase in reactive oxygen species (ROS) production. This condition resulted in spermatozoa damage and quality decrease. This study was conducted to investigate the effects of α -tocopherol supplementation in diluents on the motility, viability, and plasma membrane integrity of Simmental bull spermatozoa after cooling. Semen samples were diluted in skim milk egg yolk supplemented with 0, 0.5, 1.0, and 1.5 mM α -tocopherol respectively for control, T1, T2, and T3. Spermatozoa were evaluated for their motility, viability, and membrane integrity in cooling temperature (5°C). The daily evaluation showed that 1.5 mM α -tocopherol was the best in maintaining motility, viability, and plasma membrane integrity, while 1.0 mM α -tocopherol was only good for maintaining viability. Therefore, it can be concluded that α -tocopherol at the concentration of 1.5 mM was an efficient antioxidant supplement for Simmental cattle semen in skim milk egg yolk diluent.

Keywords: Simmental, α -tocopherol, motility, viability, plasma membrane integrity

INTRODUCTION

Simmental is one of the beef cattle breeds included in the *Bos taurus*. There are many *Bos taurus* cattle such as Simmental, Limousine, and Angus. The Simmental has a very high level of body weight gain (Soeharsono *et al.*, 2010) an important role in fulfilling the need of meats in society, the demand weight up to 650-660 kg, and a rapid grow. Based on the above considerations, it is necessary to preserve and accelerate the increase in Simmental cattle population. An effort to accelerate the increase of Simmental cattle population can be done through the use of artificial insemination (AI) technology. The quality of semen has a great impact on the success of AI practice, which includes dilution and storage of semen.

Semen diluter has several requirements, such as providing food substances as an energy source of spermatozoa, ability to prevent cold shock, containing substances that

can stop or inhibit the activity of bacteria found in semen, act as a buffer to prevent changes in pH and includes maintaining the balance of osmotic pressure and electrolytes (Bebas *et al.*, 2016). Semen dilution is an attempt to increase the volume of semen, reduce the density of spermatozoa and maintain the survival of spermatozoa in a certain storage time limit in conditions below or above freezing temperature (Kusumaningrum *et al.*, 2002). The damage of spermatozoa cells caused by cold shock can also be minimized by the addition of antioxidants. Spermatozoa damage may be caused by many things, some of which were age factors, infection of the testicular organs, lack of protamine levels, hormonal, toxic chemicals contamination, drugs, hyperthermia of the testes, apoptosis, and especially high level of reactive oxygen species (ROS) (Priyanto *et al.*, 2015). ROS also could reduce spermatozoa motility through peroxidation of spermatozoa cell membranes, which induced a decrease in

flexibility and movement of spermatozoa tails (Tremellen, 2008).

The body itself could limit the damage caused by free radicals by equipping a set of defence systems to counteract attack of free radicals or oxidants (Singh, 1992; Jackson, 2005). The defences systems were antioxidant like micronutrients such as β -carotene, vitamin C, and vitamin E (Hariyatmi, 2004). However, in certain circumstances, when the production of free radicals or reactive oxygen compounds exceeded the body's defensive ability, oxidative stress occurred (Agarwal *et al.*, 2003). One of the vitamins that are a fat-soluble antioxidant, able to captivate free radicals, and prevent chain reactions is Vitamin E (α -tocopherol) (Suryohudoyo, 1995). Based on the background above, research examining the effect of the addition of α -tocopherol to diluter on motility, viability, and plasma membrane integrity of Simmental cattle spermatozoa in cooling temperature was conducted.

MATERIALS AND METHODS

Semen collection and evaluation

A Simmental cattle used in this study was 4-5 years old, showing high libido and healthy reproductive organs. Five ejaculates were collected using an artificial vagina and teaser bull twice a week. The collected semen was immediately evaluated macroscopically (volume, pH, viscosity, colour, and odour), and microscopically (motility, and viability).

Each ejaculate was aliquoted into four tubes, which were subjected to be mixed (1:10) with skim milk egg yolk diluent supplemented with four different concentrations of α -tocopherol (C29H50O2, Tokyo Chemical Industry). The α -tocopherol concentrations were 0, 0.5, 1.0, and 1.5 mM, respectively for control/T0, T1, T2, and T3 groups.

Treatment of semen sample

The semen samples were then cooled at 5 °C and observed for their motility, viability, plasma membrane integrity just before cooling (0 hours), and then every 24 hours until the motility was less than or equal to 40%.

Evaluation of spermatozoa motility

The progressive movement of spermatozoa was evaluated using a 1 : 1 mixture of semen and 0.9% saline on an object glass. The observation was conducted using a phase-contrast microscope with a magnification of 400 x (Susilowati *et al.*, 2010).

Evaluation of spermatozoa viability

Spermatozoa viability was evaluated utilizing eosin nigrosin staining. One drop of semen and one drop of eosin nigrosin were homogenously mixed on a clean object glass, smeared and fixed by quickly passing through the flame of a Bunsen burner several times. The fixed sample was observed using a phase-contrast microscope under 400x magnification. Dead spermatozoa are stained because of the loss of the lipid layer, whereas viable spermatozoa were unstained because of the intact lipid layer on the cell surface, which protected the spermatozoa. Therefore, the dead spermatozoa appear red or purple, whereas the viable spermatozoa remain uncoloured (Susilowati *et al.*, 2010).

Spermatozoa plasma membrane integrity

Plasma membrane integrity was evaluated using the hypoosmotic swelling (HOS) test. One hundred μ l of semen was diluted ten times in hypoosmotic solution (0.735 gr Sodium citrate, 1.351 gr fructose, and 100 ml distilled water) and incubated in a CO₂ incubator at 37 °C for one hour. After incubation sample was mixed with eosin nigrosin and smeared on an object glass. The observation was performed using a phase-contrast microscope under 400 x magnifications. In this test, spermatozoa with intact plasma membrane appeared swollen at some different part of the tail or coiled at the caudal part of the tail (Susilowati, *et al.*, 2010).

RESULTS

Macroscopic examination of five semen ejaculates showed an average volume of 7.9 ml, pH 6.6, viscous, creamy white coloured, and smell similar to cow's milk; meanwhile, microscopic examination showed the average

mass movement of +2/+3; progressive individual movements of 82%; the individual velocity of 3; concentration of $1,324 \times 10^6$ spermatozoa.mL⁻¹; viability of 92.8% and plasma membrane integrity of 63.6%.

After the feasibility test, each ejaculate was aliquoted for four treatment groups with the supplementation of 0, 0.5, 1.0, and 1.5 mM α - α -tocopherol in skim milk egg yolk diluent cooled at 5 °C. First observations were performed immediately after mixing with the

diluent just before cooling and after that daily until 72 hours after cooling, where spermatozoa motility in most of the groups was less than 40%.

Motility evaluation shows that the fastest decrease in motility was found in the T0 group, while the slightest decrease in motility was found in the T3 group (Table 1.1). A significant difference ($p < 0.05$) in motility was already seen 24 hours after cooling. Significant differences continued until 72 hours after cooling.

Table 1 Spermatozoa motility (% , means \pm SD) after cooling in skim milk egg yolk diluent with α -tocopherol supplementation

	time of observation after cooling (hours)			
	0	24	48	72
T0	81.4 \pm 3.7 ^a	59.8 \pm 3.4 ^a	42.4 \pm 7.9 ^a	24.6 \pm 2.6 ^a
T1	82.8 \pm 2.2 ^a	64.6 \pm 4.3 ^{ab}	52.6 \pm 4.3 ^b	35.8 \pm 6.7 ^b
T2	82.0 \pm 3.4 ^a	69.2 \pm 3.8 ^{bc}	55.0 \pm 6.5 ^b	39.2 \pm 4.4 ^b
T3	81.8 \pm 2.0 ^a	73.4 \pm 6.4 ^c	65.6 \pm 7.1 ^c	56.6 \pm 4.7 ^c

^{a, b, c} different superscripts in the same column indicates significant difference ($p < 0.05$); T0: control, without supplementation; T1: supplementation with 0.5 mM α -tocopherol; T2: supplementation with 1.0 mM α -tocopherol; T3: supplementation with 1.5 mM α -tocopherol; replicates: 5 (ejaculates); cooling was conducted at 5 °C.

Group T2 and T3 were the best in maintaining viability. There was no difference ($p > 0.05$) between group T2 and T3, but both groups were significantly different ($p < 0.05$)

from T0 and T1 throughout the observation time (Table 1.2). Significant differences in viability have been found 24 hours after cooling.

Table 2 Spermatozoa viability (% , means \pm SD) after cooling in skim milk egg yolk diluent with α -tocopherol supplementation

	time of observation after cooling (hours)			
	0	24	48	72
T0	93.1 \pm 2.7 ^a	75.1 \pm 4.3 ^a	53.5 \pm 4.6 ^a	32.9 \pm 3.6 ^a
T1	92.8 \pm 1.8 ^a	81.9 \pm 4.0 ^b	63.7 \pm 5.4 ^b	39.3 \pm 1.4 ^a
T2	93.5 \pm 1.8 ^a	88.3 \pm 2.9 ^c	82.3 \pm 2.4 ^c	58.8 \pm 7.5 ^b
T3	93.1 \pm 1.9 ^a	90.5 \pm 2.7 ^c	84.3 \pm 1.9 ^c	65.9 \pm 7.2 ^b

^{a, b, c} different superscripts in the same column indicates significant difference ($p < 0.05$); T0: control, without supplementation; T1: supplementation with 0.5 mM α -tocopherol; T2: supplementation with 1.0 mM α -tocopherol; T3: supplementation with 1.5 mM α -tocopherol; replicates: 5 (ejaculates); cooling was conducted at 5 °C.

Evaluation of plasma membrane integrity showed the highest maintenance of plasma membrane integrity occurred in group T3 (Table 1.3). Observation at 48 as well as 72 hours after

cooling in plasma membrane integrity showed a significant difference ($p < 0.05$) between T3 and the other groups.

Table 3 Spermatozoa plasma membrane integrity (% , means \pm SD) after cooling in skim milk egg yolk diluent with α -tocopherol supplementation

	time of observation after cooling (hours)			
	0	24	48	72
T0	63.84 \pm 7.9 ^a	43.97 \pm 8.7 ^a	20.52 \pm 6.1 ^a	8.90 \pm 2.6 ^a
T1	62.18 \pm 5.7 ^a	51.03 \pm 7.9 ^{ab}	30.15 \pm 4.7 ^b	11.90 \pm 1.5 ^a
T2	62.47 \pm 6.3 ^a	54.58 \pm 9.3 ^{ab}	31.59 \pm 4.3 ^b	18.27 \pm 2.7 ^b
T3	63.77 \pm 6.1 ^a	58.53 \pm 6.2 ^b	48.48 \pm 5.8 ^c	27.95 \pm 4.1 ^c

^{a, b, c} different superscripts in the same column indicates significant difference ($p < 0.05$); T0: control, without supplementation; T1: supplementation with 0.5 mM α -tocopherol; T2: supplementation with 1.0 mM α -tocopherol; T3: supplementation with 1.5 mM α -tocopherol; replicates: 5 (ejaculates); cooling was conducted at 5 °C.

DISCUSSION

The cooling process decreased membrane fluidity, spermatozoa motility, and velocity (Chatterjee and Gagnon, 2001). Spermatozoa membrane has a high percentage of poly unsaturated fatty acids (PUFAs). Reactive oxygen species was produced by a variety of semen components, including immotile or morphologically abnormal spermatozoa, leucocytes and morphologically normal but functionally abnormal spermatozoa. Oxidative stress (OS) has been recognized as one of the most important causes of infertility, despite the antioxidant activity of seminal plasma, epididymis, and spermatozoa, OS damaged spermatozoa function and DNA integrity. Although necessary for survival, oxygen also led to the production of free radicals. These are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. Unpaired electrons were usually highly reactive, so radicals were likely to take part in chemical reactions that damage spermatozoa plasma membrane lipids (called lipid peroxidation) (Jones and Mann, 1977). Lipid peroxidation of the spermatozoa membrane was responsible for causing perturbation of membrane structure and function (transport processes, maintenance of

ion and metabolite gradient, receptor-mediated signal transduction) (Sikka, 1996). The injury caused not only the loss of membrane integrity but also increased membrane permeability. The increase in membrane permeability was suggested to give a deleterious effect on intracellular concentration regulator for ions involved in controlling the spermatozoa movement (Kumar *et al.*, 2011). Therefore, the damage of the membrane structure reduced spermatozoa overall functions.

Mammalian spermatozoa were highly susceptible to the reactive oxygen species (ROS) stress caused by biochemical and physical modifications during the cryopreservation process as cooling process caused a sudden outburst of ROS generation (Chatterjee and Gagnon, 2001). Antioxidants such as α -tocopherol helped clear the ROS produced by the electron transport chain in the mitochondria from creating a chain reaction in the cell membrane. Alpha-tocopherol inhibited the stage of lipid peroxidation propagation through the transfer of H atoms. The reaction resulted α -tocopherol radical and non-radical products. After the formation of α -tocopherol radicals, they immediately reacted with the second free radical or each other to form a non-radical end product.

In this study, the best results were found in

diluted semen supplemented with 1.5 mM α -tocopherol. However, some studies did not show the same effect of the same dose in this study. 1.5 mM evaluation displayed an adverse effect in plasma membrane integrity maintenance (Tafif, 2019) that analogues of vitamin E had negative effects when it was added to the refrigerated medium of ram (Mata-Campuzano *et al.*, 2014) and red deer (Anel-López *et al.*, 2012) spermatozoa. This discrepancy might be related to the efficiency of antioxidants, which were affected by various factors such as the component of buffer, cryoprotectants, and incubation time.

Cryoprotectants such as egg yolk and soybean or milk have different antioxidant capacity (Alvarez-Rodríguez *et al.*, 2013) either way; the results of previous studies corresponded with the result of the present study. The supplementation α -tocopherol in egg yolk tris aminomethane diluent resulted in beneficial effect on spermatozoa motility and plasma membrane integrity in Madura bull (Ratnani *et al.*, 2017). Also α -tocopherol can be an efficient antioxidant additive in Bioxcell extender for cryopreservation of bull semen (Motemani *et al.*, 2017).

CONCLUSION

Supplementation of α -tocopherol at 1.5 mM in skim milk egg yolk diluent maintained Simmental bull spermatozoa motility, viability, and plasma membrane integrity during cooling at 5°C whereas, at 1.0 mM only spermatozoa viability were maintained.

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