Alpha-tocopherol improves sperm quality by regulate intracellular Ca^{2+} intensity (influx/efflux) of Simmental bull cattle sperm

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Abstract

Background: The effects of α-tocopherol on intracellular Ca^{2+} intensity in semen cryopreservation by regulate intracellular Ca^{2+} intensity have not been reported yet.

Objective: The research was conducted to evaluate the effect of supplementation α-tocopherol into egg yolk skim milk extender on sperm quality and intracellular Ca^{2+} intensity.

Methods: Semen samples were collected and supplemented with respectively 0mM (P0); 0.5mM (P1); 1mM (P2); 1.5mM (P3) and 2mM (P4) α-tocopherol in extender before cryopreservation processes. Post-thawing sperm was evaluated for motility, viability, and abnormality using Phase Contrast Microscope (200x) with eosin-nigrosine staining, and intracellular Ca^{2+} intensity of the best result dose was evaluated using Confocal Laser Scan Microscope (400x) with Fluo-3 Staining.

Results: The results showed there was a significant difference ($P \leq 0.05$) in sperm motility and viability between P0; P1 with P2; P3; P4. The Motility and viability between groups P0; P1 and P3; P4 showed no significant difference ($P \geq 0.05$), while P2 with P3; P4 showed significant difference ($P \leq 0.05$). There was a significant difference ($P \leq 0.05$) in sperm abnormality of P0; P1 with P2; P3; P4. The abnormality between P0; P1 and P2; P3 showed no significant difference ($P \geq 0.05$), while P2; P3 showed a significant difference with P4 ($P \leq 0.05$). The best result in sperm quality was supplementation with 1.5mM α-tocopherol. Ca^{2+} intracellular intensity: 142.76 ± 21.8 au (P0) and 176.06 ± 61.43 au (P3).

Conclusions: It was concluded that 1.5mM α-tocopherol is the best dose to improve sperm quality by regulating intracellular Ca^{2+} intensity on Simmental bull cattle.

Introduction

Artificial insemination has been implemented to improve livestock production in agricultural practices.[1] Several factors that affect sperm motility during the artificial insemination is semen quality, including the diluter agent and the cryopreservation technique. Proper cryopreservation technique has been proven to maintain 50% motility of total spermatozoa, thus improve the chance of artificial insemination process.[2,3] Semen cryopreservation may induce lipid peroxidation that leads an excessive production of *Reactive Oxygen Species* (ROS), which reduce the motility of sperm.[4] Cryopreservation may also alter the membrane plasm permeability of spermatozoa against ion Ca^{2+}. Previous studies indicated that the increase of Ca^{2+} was related to death cell in sperm. An excessive Ca^{2+} affect the capacitation during fertilization of sperm, which leads to death cell in sperm.[5,6] Supplementation of antioxidant vitamin E in semen extender may reduce the membrane damage caused by lipid peroxidation, thus promotes a higher motility and viability of sperm as well as maintaining ion Ca^{2+} of sperm after cryopreservation.

Antioxidant vitamin E can reduce the risk of lipid peroxidation membrane damage during cryopreservation:[7] α-tocopherol is one of vitamin E antioxidant that has been frequently used in cryopreservation technique, it has been proven to increase the survivability of sperm, and the fertilization rate after cryopreservation.[8, 9] Alpha tocopherol is a substance known as an inhibitor of lipid peroxidation, which act as a scavenger of Reactive Oxygen Species (ROS) in sperm membrane.[10]

The Simmental bull cattle was chosen because this variety has been developed in Indonesia, however there were major problem such as sperm quality in frozen semen, while antioxidant supplementation hasn't been used widely in artificial insemination laboratories. Although there are several reports for beneficial effects of α-tocopherol on cryopreservation of bull spermatozoa, there is no report of cellular parameters such as Ca^{2+} intensity after freezing-thawing. Therefore, the present study was conducted to determine the potential effects of various doses of α-tocopherol in egg yolk skim milk extender for cryopreservation of bull semen. Sperm parameters, including motility, viability and abnormality morphology were also evaluated after freezing-thawing. The hypothesis of this study is that α-tocopherol can increase Simmental bull semen quality, and that regulation of Ca^{2+} intracellular can impact the cryopreservation processes.

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Materials and Methods

Semen collection

The Samples were collected from 5 Simmental bull age 2.5 - 4 years old with live weight 250-350 kg. The collection was implemented using artificial vagina method.

Experimental design

Five ejaculates from 5 Simmental bull with motility ≥75% were used in this study. Each ejaculates was extended using Egg yolk Skim Milk, semen was supplemented with respectively 0mM (P0); 0.5mM (P1); 1mM (P2); 1.5mM (P3) and 2mM (P4) of α-tocopherol. The semen was then loaded into 0.25 mL straw and refrigerated for 30 min. Before plunging into liquid nitrogen, the

straw was placed above liquid nitrogen (LN_2) surface for 5 minute. The sperm was evaluated for motility, viability and abnormality, using eosin-nigrosin staining. Ca^{2+} intensity of the best group was evaluated using FLUO-3 Staining. The evaluation was conducted after dilution at room temperature, before freezing at 5^{0}C. Post Thawing (-196^{0}C), and intracellular Ca^{2+} using Confocal Laser Scan Microscope (CLSM) 400x.

Statistical analysis

The data were analyzed with ONE-WAY ANOVA following with Duncan Multiple Range Test using SPSS 16.0 with significance level 0.05.

Results

Fresh semen quality

The average of the semen volume used in this research was 6.57±2.05 mL (Table 1). Semen Volume may vary between 5-8 mL for each collection.[11] Semen volume was affected by age of the bull and collection frequency.[12] The previous study stated that during rainy seasons the total volume of semen was reduced by 66.3% in Simmental bulls.[13] Ejaculate volume and total number of spermatozoa increased with age of bull, while sperm concentration was lower in higher age classes.[14] The age of the Simmental bulls used in this research was 2 years and 3 month. The bull's semen was collected twice a week with total semen volume around 6 ml per collection. There is a strong correlation between age and sperm quality (Volume, Motility, and Concentration) of the bull.[15] Fresh semen of Simmental bull semen was analyzed for both macroscopic and microscopic evaluation. The evaluation was conducted to provide information on sperm quality used for research purposes.[11] The data obtained are presented in Table 1.

Sperm concentration obtained in this research was 1382.6x10^6. The normal sperm concentration was >20 million/mL.[16] 1286.6-1858.6 million/mL in cattle (it is 1833.5 million /mL in Sahiwal bull[17] and 1.1- 1.2 billion/mL in Swamp Bull).[18] The mass motility of semen in this research was progressive (++). The mass motility of each samples was >+2 or ≥70%, where each samples also has varying mass motility result, these variance was affected by age, breed, nutrition, and rearing management. Genetic difference also shown a significance influence in spermatozoa concentration percentage.[19]

Sperm quality

The percentage of sperm motility in these research (Table 2) were respectively 40.5± 1.58%(P0), 42±2.58% (P1), 44.5±1.58% (P2), 48.5±2.42% (P3), 48±2.58% (P4); the viability was: 61.9±1.91% (P0), 61.6±2.41% (P1), 63.7±0.95% (P2), 67.2±0.63% (P3), 66.2±1.14% (P4). Finally, the abnormality was 17.7±1.5% (P0), 17.2±1.14% (P1), 15.1±0.99% (P2), 15.3±0.95% (P3), 13.7±1.64% (P4). The post-thawing supplementation 1mM, 1.5mM and 2mM α-tocopherol showed an improvement of sperm motility and viability, and decrease in sperm abnormality. The results showed there was significant difference (P≤0.05) in sperm motility between P0; P1 with P2; P3; P4. The motility between groups P0; P1 and P3; P4 showed no significant difference (P≥ 0.05) while P2 with P3; P4 showed significant difference (P≤0.05). Sperm viability also showed a significant difference (P≤0.05) in sperm motility between P0; P1 with P2; P3; P4. The viability between groups P0; P1 and P3; P4 showed no significant difference (P≥ 0.05) while P2 with P3; P4 showed significant difference (P≤0.05). There was a significant difference (P≤0.05) in sperm abnormality between P0, P1 and P2, P3, P4, and no significantly difference (P≥ 0.05) between P0, P1 and P2, P3, P4. The data obtained is presented in Table 2.

Calcium intracellular (Ca^{2+})

The result showed that the control group's results were 142.76±21.8 au (without supplementation of α-tocopherol) and P3 (1.5mM) were 176.06±61.43 au (best treatment of supplementation α-tocopherol). The data is presented in Table 3.

Table 1. Fresh semen quality.

No	Data	Mean ± standard deviation ($\bar{x}$ ± SD)
1	Volume	6.57 ±2.05 ml
2	Concentration	1382.6 x 10^6
3	Color	White milk
4	pH	6.62 ± 0.14
5	Mass motility	++ (progressive)
6	Individual motility (%)	(73.64±2.23)
7	Viability (%)	(93.5±2.06)
8	Abnormality (%)	(10.3±1.1)
9	Membrane integrity (%)	(79.3±1.85)

Table 2. Sperm motility, viability, and abnormality at suplementation of various dosage α-tocopherol after freezing in egg yolk skim milk extender.

Parameter	P0	P1	P2	P3	P4
Motility (%)	40.5 ± 1.58^a	42.0 ± 2.58^a	44.5 ± 1.58^b	48.5 ± 2.42^c	48.0 ± 2.58^c
Viability (%)	61.9 ± 1.91^a	61.6 ± 2.41^a	63.7 ± 0.95^b	67.2 ± 0.63^c	66.2 ± 1.14^c
Abnormality (%)	17.7 ± 1.5^a	17.2 ± 1.14^a	15.1 ± 0.99^b	15.3 ± 0.95^b	13.7 ± 1.64^c

Different notations in the same row show no significantly differences between treatments for each parameter tested (ONE-WAY ANOVA. P< 0.05. SPSS 16.0).

Table 3. Intensity of Ca^{2+} au control group *vs* P3 (the best group).

No	Control	P3 (1.5mM)
1	159.595	158.762
2	185.425	151.951
3	132.288	171.183
4	145.893	343.862
5	156.442	157.277
6	116.577	112.623
7	154.392	164.274
8	134.835	161.438
9	125.497	179.481
10	116.617	159.698
x ± SD	**142.76±21.8**	**176.06±61.43**

Discussion

The supplementation of α-tocopherol into egg-yolk milk in this study was expected increase sperm quality after cryopreservation. Control group without α-tocopherol supplementation showed a higher sperm abnormality compare with other treatment, but still in a range standard for AI (artificial insemination) <20%. Sperm motility and viability control group was lower than another, but the best treatment is 1.5mM and 2mM. Previous study found that freezing resulted significantly more non viable spermatozoa.[20] Freezing-thawing processes lead to the generation of ROS that impair sperm motility, membrane integrity, and fertilizing potential.[21] 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, excessive ROS production during cryopreservation also cause membrane damage.[22,23] The preview of viability and abnormality under phase contrast microscope with 200x magnification are shown in Figure 1 and 2.

The supplementation of α-tocopherol at 1mM; 1.5mM; 2mM improves the sperm viability during thawing process, while the control group has shown a significance decrease in sperm viability from 93.5% to 61.9% (Table 2). The decline in frozen semen viability percentage was caused by a long duration of thawing process. The best treatment of thawing was processed in 37^{0}C distilled water for 30 seconds.[24] Cryopreservation causes intracellular ice formation which results a dehydration of cell. The protein denaturation then triggered by dehydration of cell would cause a cell injury.[25] Cryopreservation may cause a molecular damage in membrane plasm, acrosome, mitochondria, influx of reactive oxygen species (ROS) level, decreased DNA integrity and motility as well as the survival of spermatozoa.[26]

ROS damage spermatozoa membrane and DNA, which affect the motility and acrosome reaction during fertilization. These would also impact the paternal gene that was associated with embryo.[27] ROS-induced lesion was the main cause of the motility reduction of spermatozoa. It affects the ATP usage inside contractile apparatus in sperm flagelum.[23]

The purpose of supplementation of α-tocopherol for cryopreservation technique in this research, consistent with finding before by Salim *et al*.[28] Simmental bull semen also susceptible with the temperature changes during thawing process due to its genetic. Post-thaw decrease in sperm quality was caused by ROS production over than antioxidant sperm capacity that supplemented, beside freezing-thawing processes, amount of sperm damage and dead in freezing-thawing processes, so that adding α-tocopherol in extender can't prevent oxidative impairment as a sperm membrane damage affected by chain reaction.[29] Several study finding supported, that extracellular antioxidants are very important for the protection of mammalian spermatozoa against oxidative stress because the little cytoplasmic at sperm morphogenesis depletes their internal store of antioxidant enzymes.[22] Vitamin E protects spermatozoa through its capacity to neutralize ROS accumulation and LPO during cryopreservation.[30] α-tocopherol plays an important role in reducing membrane damage by quenching excessive ROS production during quick freezing processes. Similarly to the previos researches' results, we found that that vitamin E could be reduced the LPO, then improved sperm motility and viability.[31]

Sperm motility has significance relationship with viability, result of this study consistent with previously that percentage of sperm viability was higher than motility. The previous study reported, that viable sperm percentage will always be higher than sperm motility.[32] The decrease sperm viability in frozen semen compared with fresh semen, caused by lipid peroxidation of oxidative stress, decreses the survival ability of sperm population.[33] The preview of viability of Simmental bull semen was shown in Figure 1.

Abnormality can potentially harm in sperm during fertilization. Sperm abnormality can be classified by its location of defect (head, mid piece, tail) and site of origin in primary, secondary, tertiary glands.[34] The results shown that the most common abnormality was found on the tail in spermatozoa. The preview of abnormality was shown on Figure 2.

There was a significance difference ($P \leq 0.05$) between P0; P1 and P3; P4, while P0, P1 with P3, P4 has no significance difference ($P \geq 0.05$) on sperm abnormality. The best treatment of α-tocopherol supplementation was 1.5mM and 2mM, while 0.5mM concentration was ineffective in decreasing sperm abnormality percentage. The low dosage of vitamin E was not effectively counter act the malicious chain reaction caused by ROS (Reactive Oxygen Species), meanwhile the high dosage of vitamin E will cause an oxidant in sperm.[35,-37]

The lowest abnormality percentage was obtained in 2mM dosage of α-tocoferol (13.7%). which was lower than control group (17.7%). The abnormality percentage standard for semen in artificial insemination is less than 20%. The higher abnormality

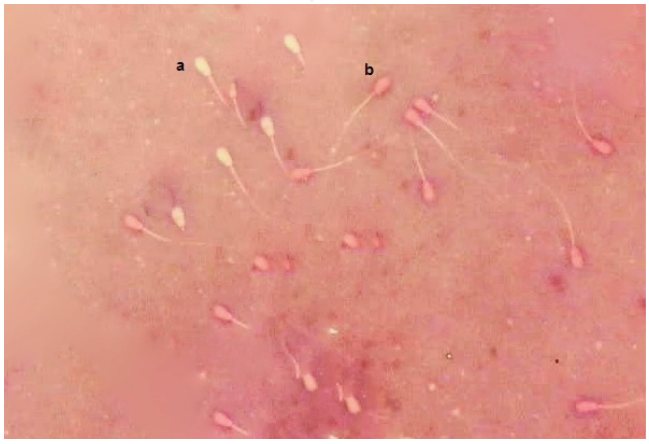

Figure 1. Sperm viability observed under Phase Contrast Microscope (200x). a) Live Sperm; b) Dead Sperm.

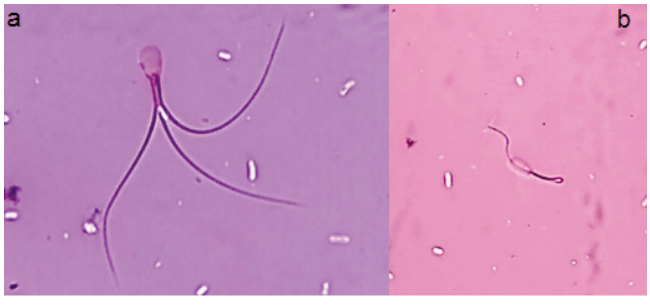

Figure 2. Sperm abnormality observed under Phase Contrast Microscope (200x): a) Tripletai; b) Distalreflex (coiled bent tail).

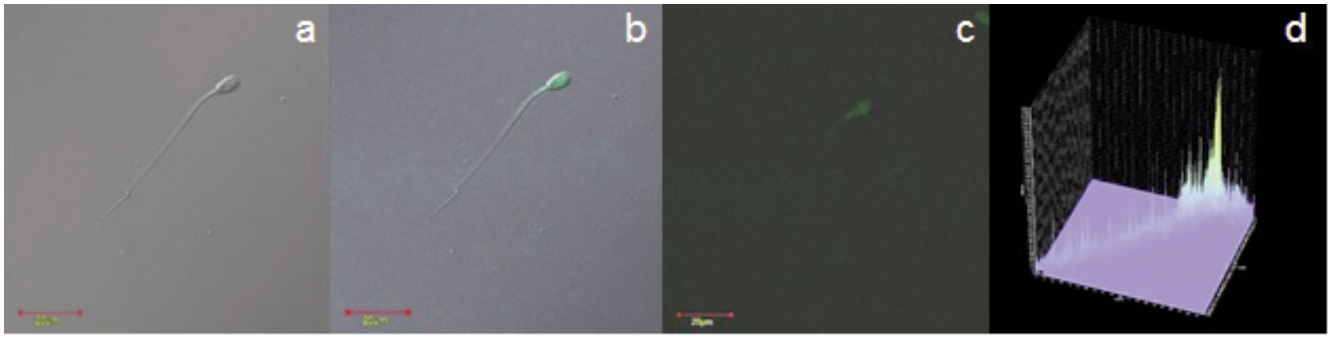

Figure 3. Ca^{2+} Intensity with Fluo-3 Staining 400XZ 2.5 P0 Group (without supplementation of α-tocopherol). a) Difference Inference Contrast; b) Super Infuse; c) Fluorescence; d) Ca^{2+} Intensity.

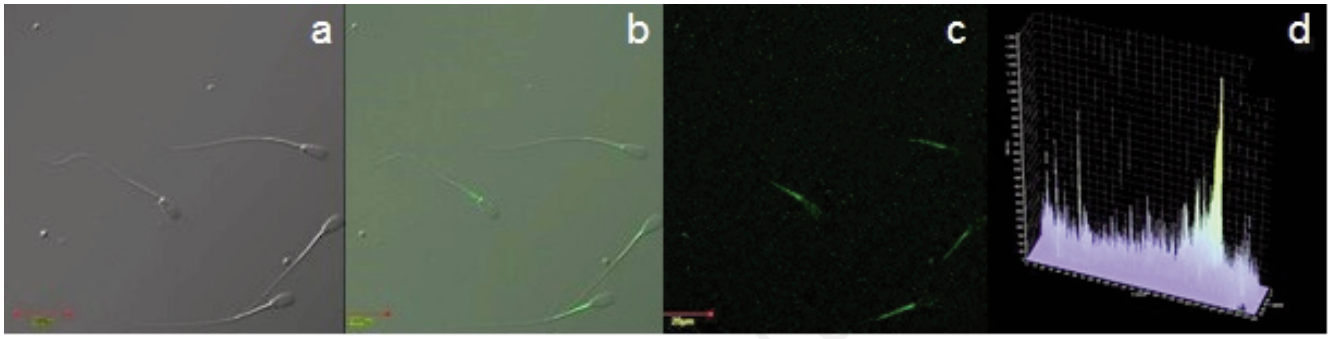

Figure 4. Ca^{2+} Intensity with Fluo-3 Staining 400XZ 2.5 Treatment P3 Group (with supplementation of α-tocopherol): a) Difference inference contrast; b) Super Infuse; c) Fluorescence; d) Ca^{2+} intensity.

percentage will result a low conception rate in cattle.[38] The previous study indicate that α-tocopherol plays a significant role on decreasing sperm membrane damage in spermatozoa that was caused by ROS during cryopreservation of the semen.[24] but the effectivity of antioxidant varies on each type dosage and species used in the process.[39]

Ca^{2+} intensity of control group was lower (142.76±21.8) au than the group with the best treatment supplementation of α-tocopherol (176.06±61.43) au with control group vs P3 motility percentage respectively 40.5% vs 48.5% and membrane integrity 54.5% vs 64.5%. It was concluded that the increase of motility and membrane integrity percentage in semen were followed by Ca^{2+} intracellular increase. The previous study found that Freezing-Thawing process cause a significant decrease ($P<0.01$) on base Ca^{2+}intraseluler.[40] Supplementation antioxidant or removing Ca^{2+} extracellular could potentially increase the motility and reduce acrosome damage during freezing-thawing process in spermatozoa.[41] The result of this research shown that control group has the lowest Ca^{2+} intracellular intensity decreases than the group with 1.5mM α-tocopherol. The Ca^{2+} intracellular intensity increase with supplementation of α-tocopherol followed with the increasing of motility in spermatozoa ranked from highest to lowest in this research were the fresh sample(73.64%), P3 (48.5%), and control group (40.5%). The preview of Ca^{2+} intensity of control and best treatment group under Confocal Laser Scanning Test (CLSM) with 400x magnification were shown in Figure 3 and Figure 4.

Conclusions

Based on the result, it was concluded that 1.5mM α-tocopherol is the best dose to improve sperm quality by regulating intracellular Ca^{2+} intensity on Simmental bull cattle.

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