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Research Article

Effect of Fish Oil Addition to the Skim Milk-egg Yolk Extender on the Quality of Frozen-thawed Bali Bull Spermatozoa [1]

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Abstract

This study was conducted to investigate the effect of fish oil addition to the semen extender on post-thaw quality of Bali bull spermatozoa. Fish oil was added at the doses of 0 (control), 50, 100, 150 and 200 mg/100 mL to the skim milk-egg yolk extender. Supplementation of fish oil at the dose of 100 mg/mL significantly (P<0.05) increased both live sperm rate and motility, but its dose of 200 mg/mL caused a significant (P<0.05) decrease in the live sperm rate when compared with the control group. A significant (P<0.05) increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added. In conclusion, addition of 100 mg fish oil to 100 mL of skim milk-egg yolk extender could be beneficial for the improvement of the quality of Bali bull spermatozoa after freeze-thawing.

Keywords: *Bull semen, Fish oil, Cryopreservation, Live sperm, Motility*

Süt Tozu-Yumurta Sarısı Sulandırıcısına Balık Yağı İlavesinin Dondurulmuş-Çözdürülmüş Bali Boğa Spermatozoonlarının Kalitesi Üzerine Etkisi

Özet

Bu çalışma sperma sulandırıcısına katılan balık yağının Bali boğa spermatozoonlarının çözdürme sonrası kalitesi üzerine etkisini araştırmak amacıyla yapıldı. Süt tozu-yumurta sarısı sulandırıcısına 0 (kontrol), 50, 100, 150 ve 200 mg/100 mL dozunda balık yağı ilave edildi. 100 mg/100 mL balık yağı ilavesi kontrol grubu ile karşılaştırıldığında hem canlı sperm oranını hem de motiliteyi önemli derecede (P<0.05) artırdı, ancak 200 mg/100 mL'lik doz canlı sperm oranında önemli (P<0.05) bir azalmaya neden oldu. 50 mg/100 mL'lik doz hariç balık yağının tüm dozları ilave edildiğinde kontrol grubuna kıyasla dondurulmuş-çözdürülmüş spermatozoonların anormallik oranında önemli (P<0.05) bir artış gözlendi. Sonuç olarak, süt tozu-yumurta sarısı sulandırıcısının 100 mL'sine 100 mg balık yağı ilavesi Bali boğa spermatozoonlarının dondurma-çözdürme sonrası kalitesinin artırılması için faydalı olabilir.

Anahtar sözcükler: *Boğa sperması, Balık yağı, Kriyoprezervasyon, Canlı sperm, Motilite*

INTRODUCTION

Artificial insemination (AI) is an important tool for genetic improvement and has effectively been used in several livestock species including cattle, sheep and goats. One of the factors affecting the success of AI is the semen cryopreservation. The good post-thawed semen quality depends on the composition of the cryopreservation media. The process of semen cryopreservation is a stage of procedure that causes several forms of spermatozoa damages. One of the most significant components in semen processing is the ingredients of extender that provide protection to the sperm against cold shock [1]. The injuries in post-thawed spermatozoa are generally associated with osmotic changes, membrane alteration, inter-and intra-cellular ice crystal formation during cryopreservation process [2,3]. Therefore, cryoprotectants are supplemented to the semen extender to reduce damaging effects of cryopreservation processes [4]. Considerable evidences suggest that lipidic composition of the sperm membrane is

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responsible for the detrimental effect of cryopreservation [5]. Before semen freezing, percent docosahexaenoic acid was greater in fatty acid treatment than that in the group without fatty acid and reduced significantly in both groups after thawing [6].

Fish oil contains omega 3 fatty acid and is a major source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [7]. However, it has been reported [8] that fish oil contains 1970 mg saturated fatty acid (SFA), 35.5% poly-unsaturated fatty acid (PUFA), 180 mg eicosapentaenoic acid (EPA) and 120 mg docosahexaenoic acid (DHA). Many researchers have assessed the effect of fish oil addition on semen quality parameters in domestic animals. They have reported that fish oil caused increments in sperm motility and reductions in abnormal sperm in boars [9], improved fertility in turkeys [10] as well as increased total of sperm count in rams [11]. On the other hand, the addition of fish oil as a source of omega-3 fatty acids to diet of boars has been demonstrated to possess no significant improvement effect on the quality of frozen-thawed sperm [12]. Similarly, incorporation of DHA as an omega-3 fatty acid in the diet has been found to improve significantly the motility, live sperm and normal morphology of fresh semen [13,14]. Addition of DHA from fish oil to the egg yolk extender was found to be effective for increase the progressive motility and membrane integrity in post-thawed boar semen [15]. Although there are many evidences about the influence of DHA enriched extenders on freezing of boar semen, however, there are limited numbers of reports about the effect of fish oil and/or DHA enriched extender on the quality of frozen-thawed bull semen [16,17]. The objective of this study was to evaluate the effect of fish oil addition to the extender on live sperm rate, motility and abnormality of post-thawed Bali bull spermatozoa.

MATERIAL and METHODS

Semen Collection

Semen was collected by artificial vagina from four Bali bulls raised in Center Insemination Banjarbaru, province of south Kalimantan-Indonesia. All the bulls of at least 4-6 years of age and average weight of 625-650 kg were raised under a similar grazing system (various kinds of grass) and supplemented with concentrat at the rate of 4.5 kg/head/day. Two ejaculates were obtained from each bulls every week for one month. Immediately after semen collection, the ejaculates were immersed in the warm water bath at 37°C until their assessments in the laboratory. Then, semen parameters were assessed based on the macroscopic and microscopic characteristics. Macroscopic evaluations included volume, pH and color. Microscopic evaluations included live sperm rate, motility and abnormality. The main extender consisted of 10% skim milk, 5% egg-yolk, 1% glucose, 8% glycerol, Streptomicine (1 mg/mL) and Penicilin (1000 IU/mL).

This study was designed to compare the effect different concentrations of fish oil *(liquid)* with doses 0 (control), 50 mg, 100 mg, 150 mg and 200 mg in the 100 mL skim milk-egg yolk extender. The cryoprotective extender for the control group was the same as that for the treatment groups except that it was not supplemented with fish oil [(natural, 1000 mg) containing Omega-3 Marine; Triglycerides (300 mg) as EPA (180 mg) and DHA (120 mg) from Nature's Care (Manufacture Pty, Ltd. Minna Close Belrose, Australia)].

Freezing, Thawing and Evaluation

Semen was diluted to obtain a final concentration of 25 x 10^6 sperm/mL. Diluted semen was packed into 0.25 ml straws (Biovet, France). After sealing, straws were placed horizontally on a cold rack (5°C) and lowered into nitrogen vapors (-50°C), 4 cm above the surface of liquid nitrogen. After 3 min, when the temperature reached -120°C, the frozen straws were transferred into goblets of appropriate size and transferred into a liquid nitrogen tank. After two weeks of storage, the straws per treatment were randomly chosen, thawed in water at 37°C for 30 sec and evaluated for post-thawed semen quality including live sperm rate, motility and abnormality.

To evaluate sperm motility, a small droplet (≈10 µL) of post-thawed semen was placed in the center of a pre-warmed slide and covered with cover slip. It was transferred to a heated microscope stage at 37°C and subjectively calculated by phase contrast microscopy (400× magnification). For assessment of live sperm percentage, a modification of the eosin-nigrosin procedure described by Evans and Maxwell [18] was used. 10 µL of post-thawed semen was mixed with 30 µL of eosin-nigrosin stain. A thin smear was prepared and 200 spermatozoa was observed under a microscope (400 x). The same eosin-nigrosin stained slides were also used to define sperm abnormality for which 200 spermatozoa were examined for defects associated with tail region, mid-piece and sperm head.

Statistical Analysis

The data are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) with tukey's multiple comparison test was applied to determine differences among the treatments using SPSS statistical software (version 16.0). Differences with P value 5% were considered to be statistically significant.

RESULTS

The effect of addition of different doses of fish oil on post-thawed live sperm rate, motility and abnormality is shown in *Table 1* and *Fig. 1*. Addition of 100 mg fish oil to the 100 mL extender significantly ($P<0.05$) increased both live sperm rate and motility when compared to the control and other treatments. However, the treatment with 200 mg fish oil caused significant ($P<0.05$) reduction in live sperm

Table 1. *Post-thawed sperm parameters including different dose fish oil Bali bulls*

Treatments	Sperm parameters		
	Live Sperm Rate (%)	Motility (%)	Abnormality (%)
Control	54.64±2.41^{b}	42.50±2.50^{ab}	18.83±23.06^{a}
50 mg/100 mL	57.32±2.15^{bc}	43.33±1.44^{b}	21.17±50.49^{ab}
100 mg/100 mL	60.36±1.76^{c}	46.67±1.42^{c}	23.66±17.01^{b}
150 mg/100 mL	53.34±2.25^{b}	43.33±1.37^{b}	22.67±48.05^{b}
200 mg/100 mL	47.30±2.02^{a}	40.01±0.21^{a}	22.33±09.21^{b}

^{a,b,c} *Values in the same column with different superscripts indicate significant difference at P<0.05, (n= 24)*

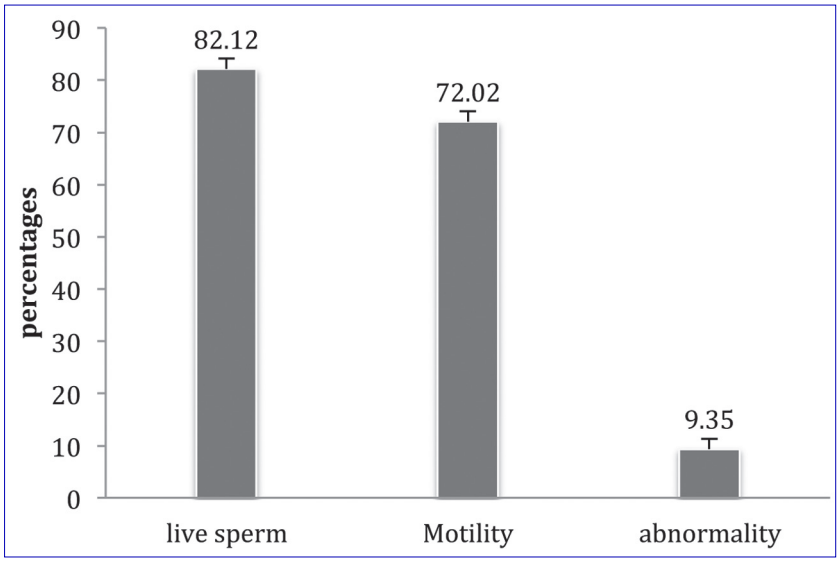

Fig 1. Caracteristic fresh semen of Bali bulls spermatozoa (Mean±SEM) (n= 24)

rate in comparison with the control and other treatments. In addition, 200 mg fish oil supplementation also caused significant (P<0.05) reduction when compared with the other treatment groups but not control group. A significant (P<0.05) increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added.

DISCUSSION

In the present study was evaluated the effects of fish oil supplementation on post-thaw semen quality in Bali bull spermatozoa. We hypothesized that fish oil supplement can enhanced semen quality in terms of live sperm rate and motility but not on sperm abnormality. The results obtained from this study are generally in agreement with the findings of previous studies conducted in other species such as turkeys [10], pigs [8] and rams [10], in which fish oil supplement improved semen quality. Meanwhile, reactive oxygen species (ROS) produced from cryopreservation can also induce acrosome reaction and spermatozoa damage [19]. The toxicity effects of ROS eventually results in protein ionization and inactivation, peroxidation of lipids, in particular polyunsaturated fatty acids, and DNA damage which destabilize spermatozoa plasma membrane [20,21].

Live sperm rate and motility of semen are important factors because spermatozoa must travel from the vagina and uterus if semen is deposited by natural mating and AI, respectively. Cryopreservation, freezing and thawing processes may induce spermatozoa damages especially to the plasma membrane and organelles [22,23]. The determination of live sperm rate and motility is important for the evaluation of post-thaw quality of semen. In the present study, it was observed that addition of 100 mg fish oil to the 100 mL extender significantly increased both live sperm rate and motility, but treatment with 200 mg fish oil caused significant reduction in live sperm rate in comparison with the control and other treatments. In addition, 200 mg fish oil supplementation also caused significant (P<0.05) reduction when compared with the other treatment groups but not control group. This situation is shown that the dose of 100 mg fish oil provides an ideal concentration in diluent so as to provide comfort to the spermatozoa, thus preventing damage to the process freezing, cryopreservation and thawing. These results are confirmed by previous reports by Abdi-Benemar et al.[24], who revealed that all frozen-thawed sperm characteristics are significantly enhanced with a surge in DHA levels from fish oil. One of the components of fish oil is PUFA with long chains and has been found in the spermatozoa of various species including ram, man and bull. These fatty acids improve the fluidity of the sperm plasma membrane which is then responsible for increased resistance of the sperm to cold conditions [25]. On the other hand, DHA can be presented as a cryoprotectant that will be able to penetrate the plasma membrane of the sperm and thus act intracellularly. Therefore, modifications in fatty acid composition of surrounding diluents with fish oil supplement can cause changes in the fluidity and elasticity of the sperm plasma membrane by incorporation its fatty acids in the lipids of sperm [26]. In the present study, 150 and 200 mg fish oil supplementations were observed to decrease the percentages of live sperm and motility. This result are

supported by the data previously reported by Kandelousi et al.[27], who stated that higher levels of omega-6 fatty acids result in decreased sperm concentration, motility, and altered morphology.

In the present study, a significant increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added. Majority of sperm abnormalities detected in this research were minor or secondary defects like simple coiled or bent tails that usually encounter in response to change in temperature, but not the defects of major type such as acrosome defect, defects associated through the sperm head and mid-piece, which are irreversible and genetically related [28].

In conclusion, the results indicate that addition of 100 mg fish oil to 100 mL of skim milk-egg yolk extender could be beneficial for the improvement of the post-thaw quality of Bali bull spermatozoa including live sperm and motility. Therefore, fish oil addition at the dose of 100 mg per 100 mL skim milk-egg yolk extender can be recommended to bovine semen production centers.

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