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Original Article

The addition of fish Salmon Omega-3 in tris egg yolk diluents on the quality of Simmental bull frozen semen

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Abstract

The sperm plasma membrane is drastically altered by cryopreservation. Therefore, it is necessary to protect the sperm plasma membrane during the cryopreservation process. Omega-3 is a supplement that can protect plasma membrane. This study aimed to compare the quality of Simmental bull frozen semen in Tris Egg Yolk (TEY) diluents with and without omega-3. Semen samples from five Simmental bulls belonged to Lembang Artificial Insemination Center (AIC) were collected using artificial vaginas. The semen samples were evaluated immediatelly (macro and microscopically) after collection. The semen samples then divided into two tubes and diluted with TEY or TEY with Omega-3 (TEYO) diluents respectively. The semen were packed into 0.25 mL straws and equilibrated at 5 ℃ for 4 hours and froze using automatic freezing machine and stored in liquid nitrogen tank (-196 ºC) for further evaluation. The quality of frozen semen were evaluated after 24 hours of storage. The data were analyzed using independent sample T-test. The results showed that the sperm post-thawing motility in TEY and TEYO diluents were 45.17±1.98% and 47.48±3.55%, respectivelly. No significant difference between TEY and TEYO (P>0.05) was found in the sperm motility, individual score, sperm viability as well as membrane integrity. The research conclude that omega-3 supplementation in Tris egg yolk did not improve the semen quality of Simmental bull after freezing.

Keywords: Frozen semen, Tris egg yolk, Omega-3, Simmental bull, Sperm

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Introduction

Artificial Insemination (AI) technology is used to increase population and to improve the genetic quality of farm animals. The AI technology has been widely applied in Indonesia (Setiono et al., 2015), to increase cattle productivity by utilizing the semen of superior bulls (Nyuwita et al., 2015). The storage of bull semen by cryopreservation technique has many advantages, such as genetic improvement of important animals

farm species without a significant loss of fertility (Bailey et al., 2003).

One of the most favoured cattle by farmers is Simmental cattle (*Bos taurus*). Simmental is dual purpuses cattle that is as a producer of milk and meat. The quality of frozen semen is the one factor that affecting the success of AI. There is a need however to improve the reproductive efficiency of breeding with cryopreserved semen, which may involve increasing the post-thaw quality of sperm through improvements in cryopreservation diluents. The composition of diluents is an important factor affecting survival of the sperm during cryopreservation (Arifiantini and Yusuf, 2010). Tris-based diluent are commonly used for semen preservation in most farm animals (Purdy, 2006), including cattle.

Semen diluents for storing cold or cryopreserved semen have to be based on ionic or non-ionic substances that prevent changes in osmolarity and act as a buffer against changes in pH (Vishwanath and Shannon, 2000). Additionally, penetrating cryoprotectants like glycerol protect intracellular ice-crystal formation. The sugars serve concurrently as an energy source (Tsujii et al., 2006). Antibiotics are generally added to minimize the growth of microorganisms originating from the seminal plasma or by contamination (Morrel and Wallgren, 2014).

It is well accepted that Omega-3 can protect sperm cell membranes (Kaka et al., 2015; Towhidi et al., 2013). Omega-3 are polyunsaturated fatty acids (FUPAs) which mostly found in fish oil, with the highest content being found in salmon (Hull, 2011). Previous study reported that inclusion of omega-3 in diet and semen diluents improved the quality of fresh and frozen semen of ram (Nurcholis et al., 2016). However, there is limited information regarding the use of omega-3 in diluents for semen cryopreservation of cattle. Therefore, the aim of this study was to compare the quality of frozen semen of Simmental bulls in Tris yolk diluents with and without omega-3.

Material and Methods

The study was conducted at Artificial Insemination Center (AIC) Lembang, Bandung, including semen collection, evaluation of fresh semen and preparation of frozen semen, while the evaluation of frozen semen quality was done at the Reproduction Rehabilitation Unit (RRU), Faculty of Veterinary Medicine, Bogor Agricultural University (IPB University).

Diluents preparation

Unless it is mentioned all chemicals purchased from Merck Germany. The preparation of Tris diluent was followed the method of Arifiantini and Yusuf (2010), where Tris hydroxymethyl aminomethan 3.028 g and citric acid monohydrate 1.78 g and D-fructose 1.25 g were mixed and dissolved in 100 mL of aquadest. As much as 80% Tris and 20% egg yolk were homogenized and centrifuged. The supernatant (94 mL) then added with 6 mL of glycerol (Applichem, Germany) and 0.5 mg steptomycine and 500 IU of Penniciline (Meiji, Japan). The diluent than divided into two part, the first part was added with omega-3 (Salmon Fish oil; 1 mg in 50 ml diluents); TEYO and the second part without the addition of Omega-3 (TEY).

Semen collection and evaluation

Five sexually mature Simmental bulls (body weight between 900-1000 kg and 6-7 years old) were used in this study. The ejaculates were collected from bulls using an artificial vagina (Arifiantini, 2012). The collected semen samples were placed into a warm, enclosed thermos after numbered with both the bull and the ejaculate identity and then immediately transferred to the laboratory for subsequent analysis. The macroscopic evaluation includes volume, color, consistency, and pH, while the microscopic evaluation includes the motility and individual sperm score, viability, concentration, and intact plasma membranes (Arifiantini, 2012)

Frozen semen preparation

Each semen sample selected for freezing was split into two aliquots in 15 ml Falcon tubes and each aliquot was frozen using TEY and TEYO. The sample was then loaded into 0.25 ml straws (IMV, France) then organized on racks and gradually cooled at 5 ^{o}C over 4 h before freezing using an automatic freezing machine (Digitcool, IMV France). The frozen semen then stored in a liquid nitrogen tank (-1 96 ^{o}C) for further analysis, all procedure according to Lembang AIC, standard procedure.

Semen evaluation after freezing

The stored samples were thawed by placing them in a 37 ^{o}C water bath for 30 s (Baharun et al., 2017) and then evaluated for sperm motility and individual sperm score, viability, plasma membrane integrity, and the recovery rate of sperm.

Motility and individual sperm score

A drop (20 µL) of fresh or thawed semen was placed on a pre-warmed slide in and then covered with a cover slip (Arifiantini, 2012). The motility and the rate velocities of sperm were assessed with a microscope under 400x total magnification of light microscopes (Olympus CX 23). The individual sperm scores assessment was refers to the Australian Association of Cattle Veterinarians (AACV). Score 0 if there is no movement of sperm, score 1 if sperm not progressive but the tail move slowly, score 2 if the tail of sperm move slowly and progressive slowly, score 3 if progressively move with medium speed, score 4 if the sperm progressively move fast, and score 5 sperm move progressively very fast.

Sperm viability

Ten or twenty µL of fresh or thawed semen was placed on pre-warmed slide, where the homogenize mixture slides were dried for 10-15 seconds using a heating table. Sperm viability was assessed using eosin-nigrosin stain (Bark and Oko, 2000) at 400x total magnification of light microscopes (Olympus CH23) of 10 times observation under microscopes by counting around 200 sperms. The sperms that absorb color were died while the one that not absorb color are considered a live.

Plasma membrane integrity of sperm

Change in functional plasma membrane integrity was assessed using the Hypo-osmotic Swelling (HOS) Test as described by (Fonseca et al., 2005). Briefly, 50 µL of post thawed semen was added in 1000 µL/1mL HOS solution (0.9 g fructose and 0.49 g of sodium citrate into 100 mL distilled water; 150 mOsm kg^{-1} osmotic pressure) and incubated at 37 °C for 30 min. One drop (20 µL) solution was then placed in pre-warmed slide covered with cover slip to determine sperm under microscope at 400x of light microscope (Olympus CX23). The percentage of plasma membrane integrity was calculated by comparing the amount of reacted sperm (HOST positive) divided by the number of calculated (reacted and unreacted) sperm and multiplied by 100% (Nurcholis et al., 2016).

Recovery rate (RR) of sperm

Recovery Rate (RR) is the ability of sperm to recover after a cryopreservation process (Nurcholis et al., 2016). Recovery Rate was assessed by dividing sperm motility after thawing with fresh sperm motility and multiplied by 100%.

Statistical Analysis

Paired sample t-tests of SPSS 16.0 (IBM Co.) for all parameter were used to evaluate differences between diluent a post-thaw sperm quality. $P \leq 0.05$ was considered significantly different. All data are presented as mean values ± SEM.

Results

Fresh semen quality of Simmental bull

Macroscopic characteristic of fresh semen demostrated which volume and pH (mean ± SEM) were 7.6±0.93 mL and 6.42±0.4 respectively. Creamy white in color and moderate consistency. Microscopic characteristic demonstrated mass movement ++, progressive motility, viability and intact membrane (mean ± SEM) were 72 ± 1.22%, 80.60 ± 11.29%, and 68.08 ± 3.45% respectively. Sperm concentration was 1.383 million/mL. Over all, the quality of Simmental bull fresh semen were good according to Garner and Hafez (2000) and can be used for cryopreservation.

Quality of Simmental bull frozen semen after thawing

Motility and individual sperm score

There was no difference (P>0.05) between diluents on motility of Simmental bull sperm after thawing. The semen quality of Simmental bull after thawing is presented in Table 1.

Table 1: The quality of frozen thawed Simmental bull in Tris Egg yolk diluent with and without Omega-3

Variables	TEY	TEYO
Sperm motility (%)	45.17 ± 1.98	47.48 ± 3.55
Individual score (1-5)	2.70 ± 0.12	2.67 ± 0.13
Sperm viability (%)	54.67 ± 0.04	56.32 ± 0.08
Intact membrane plasma (%)	78.91 ± 0.04	72.39 ± 0.03

Description = Tris Egg yolk (TEY); Tris Egg yolk Omega-3 (TEYO)

Sperm viability

There were no differences (P>0.05) between treatments on sperm viability. The viability of Simmental bull sperm after thawing in the present study were 54.67% and 56.32% for TYE and TYEO, respectively.

Plasma membrane integrity

There were no differences between treatments on plasma membrane integrity of Simmental bull sperm after thawing. The values of membrane integrity were ranged between 72.39% to 78.92%.

Decreased quality of Simmental bull frozen semen in tris egg yolk diluents

The decrease in the quality of frozen semen of Simmental bull is presented in Table 2. The decrease values of sperm motility in both TYE and TYEO diluents were similar. The highest values were 24.52 to 26.83% during the freezing process and the lowest values were 9 to 10% during the equilibration process. The addition of omega-3 does not appear to reduce freezing damage.

Table 2: The decreased rate of sperm motility from Simmental bull frozen semen in Tris Egg yolk diluent with and without Omega-3

Sperm motility (%)	Diluent (± SE)	
	TYE	TYEO
Fresh semen to post equilibration	10 ± 4.61	9 ± 3.93
Post equilibration to post thawing	16.83 ± 3.85	15.52 ± 1.6
Fresh semen to post thawing	26.83 ± 0.76	24.52 ± 2.33

Description = TYE (Tris Egg Yolk), TEYO (Tris Egg Yolk Omega-3)

Recovery rate of sperm

Table 3: Recovery rate of sperm after freezing in Tris Egg yolk diluent with and without Omega-3

Sperm motility (%)	Diluent	
	TEY	TEYO
Fresh semen	72 ± 1.22	72 ± 1.22
After thawing	45.17 ± 1.98	47.48 ± 3.55
Recovery rate (%)	64.48 ± 2.15	66.18 ± 5.50

Description: TEY (Tris Egg yolk), TEYO (Tris Egg yolk Omega 3)

No significant differences between treatments (P> 0.05) on recovery rate of sperm observed in the present study (Table 3)

Discussion

Motility is commonly used for evaluating sperm fertility (Sukmawati et al., 2014). The motility of sperm was assessed by comparing the movement of sperm that progressively move (Arifiantini and Yusuf et al., 2006). The motility between Simmental bull sperm in Tris egg yolk and in Tris egg yolk–omega 3 diluent, after thawing were 45.17% and 47.48% respectively. It was notable that the motility of sperm after thawing in the present study was higher than reported by Sukmawati et al. (2014) using the same breed of cattle. Sukmawati et al. (2014) reported only 41.29% of sperm motility, but skimmed milk diluent was used in that study. However, motility value found in this study was lower than (49.45%) reported by Baharun et al. (2017) where Tris egg yolk was applied as diluent in pasundan bull. Pasundan bull is one of Indonesian local cattle, that well known for its high freezability.

The viability of FH bull sperm in various diluents has been found between ranges of 58.30% to 65.10% (Arifiantini and Yusuf, 2010), which were greater than the present findings, indicating the difference of breed cattle used in each different study. These values in the present study were differed from those reported by Priyanto et al. (2015) and Sukmawati et al. (2014) with the values of 67.01% and 61.72%, respectively. The main difference perhaps due to the different breed cattle were Brahman, Ongole, Simmental and Limousin. Foeh et al. (2017), states that TYEO diluents had higher levels of PUFA, which protect the membrane cells of sperm compared with TYE. Cell membranes are composed of bilayer phospholipids and proteins and unsaturated fatty acids that are easily damaged by the formation of ice crystals during cryopreservation (Foeh et al., 2017).

A decrease in the quality of frozen semen may result from the cryopreservation process. Sariozkan et al. (2009) argued that the cryopreservation process can cause permanent damage to the organelle sperm and changes in membrane and enzymatic activity that can decrease the motility and viability of sperm. The decrease values of sperm motility in both TYE and TYEO diluents were similar. The highest values were 24.52 to 26.83%, which occurred during the stage of fresh semen to after freezing, and the lowest values were 9 to 10% in the fresh semen process to after equilibration. The addition of omega-3 does not seem to be able to protect freezing damage. The decrease in total motility in this study was lower than that of Sukmawati et al. (2014) of 29.34% in skimmed diluents using the same breed of cattle and Baharun et al. (2017) reported the decreasing of 33.27% sperm

motility, with the used of Pasundan bull semen with Tris egg yolk as a diluent.

The recovery rate indicates the ability of sperm to recover after cryopreservation process (Foeh et al., 2017). The values of recovery rate of sperm in both TEY and TEYO diluents was ranged from 64.48% to 66.18%, respectively. The difference in recovery rate values is perhaps affected by the diluent type as reported by Sukmawati et al. (2014) where the semen of Simmental bull were frozen in skimmed diluent and value of the recovery rate was only 58.46%. The damage to the plasma membrane can occur in cryopreservation process resulting from the formation of ice crystals. The percentage of sperm motility decreases where damage in membrane occurs, especially in the mid piece section, thus interfering with the formation of Adenosine Tri Phosphat (ATP) by mitochondria. The more sperm damaged by freezing, the less sperm can recover after freezing (Nurcholis et al., 2016).

No significant different between treatments on the ability of sperm to recover after cryopreservation process observed in the present study. The use of omega-3 in Tris egg yolk diluent was unable to restore the damage of sperm in the process of thawing. This might be caused by the low doses of omega-3 used in the present study. Kaka et al. (2015) reported that 3, 5, 10, and 15 ng/mL of omega 3 in bull semen diluents significantly improved sperm quality post-cooling and post-thawed. Towhidi et al. (2013) also reported that 0.1, 1 and 10 ng/mL omega 3, significantly improved the in vitro characteristics of post thawed sperm. Omega-3 used in this study was 20 µg /mL. The types of omega-3 used between the previous study were different, which resulted with distinct effect. Another results reported that the effects of omega-3 on sperm freezability might be related to the proportion of docosahexaenoic acid (DHA) and docosapantaenoic acid (DPA) content on the sperm membrane (Chanapiwat et al., 2009)

On the other hand Nurcholis et al. (2016) used the same type of omega-3 with this study, found that when omega-3 was added to the diluent, the value of recovery rate of ram semen was higher (71.66%) than the control group (55.33%). This fact proved that type of omega-3 and different sperm of animals will give different result in protecting sperm membran. The outer component of a sperm cell, called the plasma membrane or plasma lemma, and the most representative lipid fraction of the sperm cell membranes are phospholipids. In an attempt to protect the sperm membrane during freezing and thawing, all researcher focusing in protecting sperm membrane and improve plasma membrane fluidity. Polyunsaturated fatty acids (PUFAs), were known to contribute to membrane fluidity and flexibility (Israelachvili et al.1980; Fleming and Yanagimachi, 1981).

Improving sperm plasma membrane fluidity can be done in two ways; through dietary modification (Robinson et al., 2006) or by supplementation of diluents. Omega-3 fatty acids, in particular DHA are important for sperm membrane integrity, sperm motility and viability, as well as cold sensitivity (Robinson et al., 2006). Omega-3 (Salmon Fish oil) is a major source of DHA and eicosapentaenoic acid (EPA), both improving plasma membrane fluidity of sperm as reported by Alizadeh et al. (2013) as well as improves sperm quality and quantity in rams and humans (Samadian et al., 2010; Safarinejad, 2011). The addition of omega-3 fatty acids to a diluents is more economical than dietary modification. Therefore, future study must be conducted to find the best concentration of Omega 3 in Tris egg yolk or other diluent to improve freezing capability of Simmental bull semen.

Conclusion

It can be concluded that addition of omega-3 to Tris egg diluent has no ability to improve the quality of frozen semen of Simmental bull. Further study is required particularly in finding the appropriate doses of omega-3 in Simmental or breed of cattle using variety of diluents.

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Contribution of Authors

Nalley WMM: Conceived Idea, Data Collection, Data Analysis, Manuscript
Meidina WSA: Data Collection, Literature Review

Kurnia A: Designed Research Methodology, Data Collection, Statistical Analysis
Arifiantini RI: Data Interpretation, Manuscript Writing, Manuscript final reading and approval

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References

Alizadeh AR, Esmaeili V, Shahverdi A and Rashidi L, 2014. Dietary fish oil can change sperm parameters and fatty acid profiles of ram sperm during oil consumption period and after removal of oil source. Cell J. 16: 289-298.

Arifiantini RI, 2012. Technique of semen collection and evaluation in Animals. IPB Press. Bogor, Indonesia

Arifiantini RI and Yusuf TL, 2010. Developing of Tris Soy Milk Diluent for Frisian Holstein bull frozen semen. Hayati J. Biol. 17:91-94.

Baharun A, Arifiantini RI and Yusuf TL, 2017. Freezing capability of pasundan bull sperm using Tris-egg yolk, Tris-soy, and andromed diluents. J. Ked. Hew. 11: 45-49.

Barth AD and Oko RJ, 1989. Abnormal morphology of bovine sperm. Iowa: Iowa State University Press

Bailey JL, Morrie A and Cormier N, 2003. Semen cryopreservation: success and persistent in farm species. Can. J. Anim. Sci. 83: 393–401.

Chanapiwat P, Kaeoket K and Tummaruk P, 2009. Effects of DHA-enriched hen egg yolk and L-cysteine supplementation on the quality of cryopreserved boar semen. Asian J. Androl. 11(5): 600-608.

Foeh NDFK, Arifiantini RI and Yusuf TL, 2017. The quality of boar frozen semen diluted in BTS and MII with different cryoprotectant supplemented with sodium dodecyl sulphate. J. Ked. Hew. 11: 6-10.

Fonseca JF, Torres CAA, Maffi li VV, Borges AM, Santos ADF, Rodrigues MT and Oliveira RFM, 2005. The Hypoosmotic swelling test in fresh goat spermatozoa. Anim. Reprod. 2: 139-144.

Fleming AD and Yanagimachi R, 1981. Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. Gamete Res. 4: 253–273.

Garner DL and Hafez ESE, 2000. Spermatozoa and seminal plasma. In: Hafez B, Hafez ESE. 2000. Reproduction in Farm Animals. 7th ed. Philadelphia (US): Lippincott Williams and Wilkins. pp. 96-109.

Hull MA, 2011. Omega-3 polyunsaturated fatty acids. Best Pract. Res. Clin. Gastroenterol. 25: 547-554.

Israelachvili N, Marcelja S and Horn RG, 1980. Physical principles of membrane organization. Q. Rev. Biophys. 13: 121–200.

Kaka A, Wahid H, Leghari RA, Memon MI, Kaka U, Mirani AH, Naeem M and Kalwar Q, 2016. Effect of in-vitro supplementation of polyunsaturated fatty acids on frozen-thawed bull sperm characteristics using Bioxcell® extender. Pure Appl. Biol. 5:399-405.

Morrell JM and Wallgren M, 2014. Alternatives to Antibiotics in Semen Extenders: A Review. Pathogens. 3: 934-946

Nurcholis, Arifiantini RI and Yamin M, 2016. Cryopreservation of garut sheep semen using Tris eggyolk supplemented with omega-3 fish oil salmon. J. Vet. 17: 309-315.

Nyuwita A, Susilawati T and Isnaini N, 2015. Quality of fresh semen and production of Simmental bull frozen semen at different ages. J. Tern. Trop. 16(1):61-68.

Priyanto L, Arifiantini RI and Yusuf TL, 2015. Detection of DNA damage in fresh and frozen semen using toluidine blue. J. Vet. 16:48-55

Purdy PH, 2006. A Review on goat Sperm Cryopreservation. Small Rum. Res. 6: 215-225.

Robinson JJ, Ashworth CJ, Rooke JA, Mitchell LM, and McEvoy TG, 2006. Nutrition and fertility in ruminant livestock. Anim. Feed Sci. Technol. 126: 259-276.

Safarinejad MR, 2011. Effect of omega-3 polyunsaturated fatty acid supplementation on semen profile and enzymatic anti-oxidant capacity of seminal plasma in infertile men with idiopathic oligoasthenoteratospermia: a double-blind, placebo-controlled, randomised study. Andrologia. 43: 38-47.

Samadian F, Towhidi A, Rezayazdi K and Bahreini M, 2010. Effects of dietary n-3 fatty acids on characteristics and lipid composition of ovine

sperm. Animal. 4:2017-2022.

Sariozkan S, Tuncer PB, Bucak MN and Ulutas PA, 2009. Influence of various antioxidants on microscopic-oxidative stress indicator and fertilizing ability of frozen-thawed bull semen. Acta Vet. Brno. 78: 463-469.

Setiono N, Suharyati S and Santosa PE. 2015. Brahman Bull's Frozen Semen Quality with Differen Doses of Cryprotectant Glycerol in Yolk Tris Sitrat Diluent. J. Ilm. Petern. Terpd. 3: 61-69.

Sukmawati E, Arifiantini RI and Purwantara B, 2014. Freezing capacity of sperm on various type of superior bulls. J.I.T.V. 19: 168-175.

Tsujii H, Ohta E, Miah Ag, Hossain S and Salma U, 2006. Effect of fructose on motility, acrosome reaction and in vitro fertilization capability of boar spermatozoa. Reprod. Med. Biol. 5: 255–261.

Towhidi AS, Zeinoaldini R, Ardebili N, Dadashpour D and Nasiri AH, 2013. Combined n-3 fatty acids and α-tocopherol supplementation improved the ovine sperm cryosurvival. Iranian . Biotech. 11: 238-243.

Vishwanath R and Shannon P, 2000. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62: 23-53.