The effect of soybean extract supplementation to tris aminomethane–egg yolk extender on the post-thawing quality of Simmental semen

Sri Wahjuningsih^{*1)}, Muhammad Nur Ihsan¹⁾, Aulia Puspita Anugra Yekti¹⁾, Muhammad Agus Tahar¹⁾

¹⁾ Faculty of Animal Science, Brawijaya University, Jl. Veteran, Ketawanggede, Kec. Lowokwaru, Kota Malang, Jawa Timur, Indonesia, 65145

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ABSTRACT: This research aimed to evaluate the effect of soybean extract (*Glycine max* (L.) Merr.) supplementation to tris aminomethane-egg yolk extender on the post-thawing quality of Simmental bull frozen semen. The fresh semen was collected twice a week from 3 Simmental bulls aged 3 to 3.5 years by using artificial vagina. The semen was then selected for sperm motility and abnormality. Semen that had >70% motility and <15% abnormality, was used for the research. The research was conducted in a completely randomized design with 4 treatments and 30 replications. The research treatments include 0% (T0), 7.5% (T1), 10% (T2), and 12.5% (T3) soybean extract supplementation to tris aminomethane-egg yolk extender. The observed variables include spermatozoa motility, viability, abnormality, and plasma membrane integrity. The collected data were analyzed with ANOVA and followed with Duncan's Multiple Range Test to determine mean differences. The results showed that soybean extract supplementation gave significant effect ($P < 0.05$) on the post-thawed quality of Simmental semen. The research concludes that 10% (v/v) soybean extract supplementation to tris aminomethane egg-yolk extender was effective to maintain sperm motility, viability, and plasma membrane integrity of the post-thawed Simmental semen.

Keywords: Bull; Soybean extract; Frozen semen; Motility; Viability; Plasma membrane integrity

*Corresponding Author: yuning@ub.ac.id

INTRODUCTION

Artificial insemination (AI) has been known as an effective method for cattle farm breeding management, both on industrial and small-scale level, due to its practicality and high success rate. However, the successful insemination highly depends on the post-thawed semen quality, which found to be a problem commonly found on the small-scale cattle farm, considering the limited capital and facilities. One of the approaches that can be used to tackle this problem is to apply an effective and efficient semen extender which could maintain frozen semen quality prior to be used for insemination. Tris aminomethane- egg yolk extender is one of the widely used semen extenders, which had shown the ability to maintain semen pH and shelf life of bull semen during cryopreservation (-196°C). Tris contained fructose and citrate which could prevent pH alteration due to lactic acid production by spermatozoa, while also maintain osmotic pressure and electrolytes stability, aside from providing energy. Moreover, egg yolk contains lipoproteins and lecithin that would act as extracellular cryoprotectant.

Research has shown that cryopreservation could decrease spermatozoa qualities due to the experienced cold shock (Wahjuningsih, *et al.*, 2019). Rahayu *et al.* (2014) showed that the damaged spermatozoa plasma membrane contributes to the higher mortality, in which could be prevented with lecithin supplementation. Soybean is one of natural sources for lecithin and the soybean extract supplementation is believed to be able to protect spermatozoa from cold shock. Soybean contained 1.49% to 3.08% lecithin, higher than peanut which is around 1.11%. Lecithin has the membrane coating properties by maintaining the phospholipid bilayer configuration of the spermatozoa (Rezki *et al.*, 2016). The phospholipid of the egg yolk and lecithin of the soybean would not penetrate and alter spermatozoa plasma membrane, but instead formed a protective

layer against extracellular damage (Ariantje *et al.*, 2013). The progressive sperm motility, viability and plasma membrane integrity evaluation has been then used as general indicator for semen quality. In this research, the supplementation of soybean (*Glycine max* (L.) Merr.) extract to tris aminomethane-egg yolk extender were observed to determine its effect toward post-thawed Simmental bull semen qualities.

MATERIALS AND METHODS

Semen Collection

The semen used in this research was obtained from 3 Simmental bulls at the age of 3 to 3.5 years, weighed at 665 to 672 kg, and reared intensively at Artificial Insemination Center, Ungaran, Indonesia. The semen was collected twice a week using artificial vagina, and the collected semen was selected based on semen motility (>70%), viability (>70%), mass motility (2+), and abnormality (<15%) to be used for the research.

Tris Aminomethane-egg Yolk and Soybean Extender

The production of tris aminomethane-egg yolk was done by following the method described by Wahjuningsih *et al.* (2019). As much as 2 g of soybean was added with 100 ml distilled water and heated in hot plate magnetic stirrer (100°C, 6 speed) for 15 minutes. The produced supernatant was then used for the research.

Macroscopic Observation

The macroscopic observation of fresh semen qualities included semen volume, color, and pH. The direct observation was done to measure the semen volume, colour, smell, while semen pH was observed by using pH meter.

Microscopic Observation

The microscopic observation included spermatozoa concentration, mass motility, individual motility, viability, plasma membrane integrity and abnormality. The mass motility was observed by following the method described by Arifiantini (2012), while individual motility was observed

using microscope at 400x magnification (Lukman *et al.*, 2014). The semen viability and abnormality were observed by using eosin-negrosin staining observation. The spermatozoa were observed under 400x magnification and stained spermatozoa indicate dead cell (Ducha *et al.*, 2012). The membrane integrity was observed by placing diluting 50 μ L semen with 400 μ L hypo-osmotic solution in an eppendorf tube, and followed with 30 min incubation at 37°C. The spermatozoa membrane cell integrity was then observed under microscope with 400x magnification (Karunakaran *et al.*, 2017).

Experimental Design and Data Analysis

The research was conducted in a completely randomized design with 4 treatments and 30 replications. The research

treatments were soybean extract supplementation at 0% (T0); 7.5% (T1); 10% (T2); and 12.5% (T3) to the base semen extender (80% Tris aminomethane and 20% egg yolk). The data were then analyzed using analysis of variance (ANOVA) and followed with Duncan's Multiple Range Test to determine mean differences among treatments.

RESULT AND DISCUSSION

Fresh Simmental Bull Semen Qualities

Prior to be added with semen extender, the fresh Simmental bull semen qualities were evaluated through macroscopic and microscopic observations. The results of fresh semen evaluation is presented in Table 1.

Table 1. Fresh Simmental bull semen observation

Parameters	Mean $\pm$ SD
Volume (mL)	7.21 $\pm$ 0.42
Color	Milky white
pH	6.59 $\pm$ 0.20
Concentration (10^6 cell/mL)	1459.80 $\pm$ 123.22
Mass sperm motility	+++
Individual sperm motility (%)	80.75 $\pm$ 5.50
Viability (%)	86.75 $\pm$ 6.53
Plasma membrane integrity (%)	84.35 $\pm$ 6.75
Abnormality (%)	6.81 $\pm$ 0.85

The semen volume is known to be affected by several factors, i.e., bull age, physical condition, environment, and technical skill of the inseminator. Ismaya (2014) further elucidates that age would affect testicle size, which increased the seminiferous tubules along with increased age and thus resulted with higher semen production until reached certain age.

The average Simmental bull semen pH in this research was 6.59 $\pm$ 0.20 and categorized as normal. The mass and individual spermatozoa motility of fresh semen in this research were categorized as normal and fulfilled the requirements for

cryopreservation. Sperm motility requirements according to SNI standard is at 70% for individual motility and for mass motility was ++. The spermatozoa viability found in this research was higher compared to the finding by Effendi *et al.* (2015), which shown spermatozoa viability of 83.64 $\pm$ 7.85%.

Before Freezing and Post-thawed Sperm Qualities

The progressive sperm motility, viability, plasm membrane integrity in this research was observed both on pre-freezing and post-thawed, and the results is presented in Table 2

Table 2. Mean motility, viability, plasma membrane integrity of bull semen with different soybean extract supplementation levels to tris-egg yolk extender

Parameter	T0	T1	T2	T3
Motility				
Before freezing (%)	63.40 ± 4.21 ^a	63.62 ± 4.331 ^a	65.00 ± 2.42 ^b	63.55 ± 3.51 ^a
Post-thawed (%)	52.21 ± 0.00 ^a	52.32 ± 0.00 ^{ab}	55.30 ± 2.42 ^c	52.50 ± 2.64 ^b
Viability				
Before freezing (%)	70.24 ± 6.69 ^a	70.96 ± 6.44 ^b	72.43 ± 6.18 ^c	70.28 ± 7.51 ^b
Post-thawed (%)	60.23 ± 5.31 ^a	60.22 ± 4.12 ^a	62.12 ± 4.58 ^c	61.12 ± 4.40 ^b
Plasma membrane integrity				
Before freezing (%)	69.16 ± 5.39 ^a	69.34 ± 6.03 ^b	71.82 ± 7.37 ^c	69.98 ± 6.91 ^b
Post-thawed (%)	60.14 ± 6.21 ^a	60.82 ± .12 ^{ab}	64.32 ± 6.15 ^c	61.31 ± 6.25 ^b

Description: Different superscripts on the same row indicate significant differences (P<0.05)

The finding of this research showed that the supplementation of soybean extract to Tris extender at various concentration levels could maintain progressive sperm motility, with the best value was found at 10% (T2) soybean extract concentration level. Furthermore, the overall post-thawed progressive motility of Simmental semen in this research was at 52.21% to 55.30%, which has fulfilled the Indonesian national standard that was set at >40% as written in SNI 4869-1:2017 regulation.

The decreased post-thawed progressive motility compared to the fresh semen was due to the cold shock experienced by the spermatozoa. The cold shock would be able to damage the spermatozoa, thus a protection toward cold shock should be done for successful insemination. The supplementation of soybean extract in this research has shown the protective effect toward cold shock, which can be seen from higher progressive motility of all research treatments compared to the control. The results were due to the low-density lipoprotein content in soybean extract, which act as protective agent against cold shock.

The anti cold shock compounds are mainly found on egg yolk (animal-based lecithin) and soybean (plant-based lecithin). The addition of lecithin on semen extender would form a coating which would maintain phospholipid bilayer structure of the

spermatozoa (Rezki *et al.*, 2016), thus preventing cell damage caused by the cold shock. Moreover, research also found that the phospholipid compounds from the egg yolk and lecithin from soybean extract would form a protective coating against extracellular cold shock or other physical damage during freezing to thawing (Ariantje *et al.*, 2013).

The sperm viability is also one of the important indicators to determine semen quality. The viability observation in this research showed that soybean extract supplementation to Tris extender gave significant effect (P<0.05) both on pre-freezing and post-thawed. The decreased viability of frozen semen on semen cryopreservation was caused by extreme temperature changes, which then disrupted plasma membrane of the spermatozoa (Priyanto *et al.*, 2015). Plasma membrane act as protective agent for the spermatozoa towards external damage, and the disrupted plasma membrane indicate damaged spermatozoa head and would be stained during staining observation.

During cold shock, post-thawed viability of the spermatozoa would be decreased as reactive oxygen species (ROS) would be produced due to the cooling rate. Moreover, electrolytes concentration would be increased and dilute lipoprotein compounds on the spermatozoa cell wall during thawing, resulted with alteration on

cell membrane permeability and followed with dead spermatozoa cell (Hoesni, 2016). The supplementation of soybean extract at 10% (v/v) to Tris aminomethane-egg yolk extender in this research showed the best ability to maintain spermatozoa viability. Noviansyah *et al.* (2017) elucidate that soybean could protect plasmic membrane of the spermatozoa during cryopreservation due to the phosphatidicholin compound that would act as coating to maintain lipid bilayer configuration of the spermatozoa.

Other research also showed that the supplementation of soybean extract to citrate extender could increase spermatozoa viability and prevent necrosis of Sapudi goat semen (Immelda *et al.*, 2019). Zhang *et al.* (2009) added that the addition of 6% soybean lecithin to the semen extender showed the best spermatozoa motility and plasma membrane integrity in frozen-thawed boar spermatozoa.

The phospholipids in soybean lecithin may be replaced the phospholipids of the sperm membrane and maintain the structure and function of spermatozoa. Research by Tarig *et al.* (2017) showed that 1.5% soybean lecithin supplementation to 2% VCO Tris-based extender improve the bull semen qualities, even though there was no improvement to the post-thawed semen qualities.

The analysis of variance showed that the supplementation of soybean extract to the Tris extender had significant effect ($P < 0.05$) to maintain plasma membrane integrity of post-thawed Simmental semen. The research also found that the best post-thawed membrane integrity was at 10% (T2) soybean extract supplementation. This indicates that 10% soybean extract supplementation to Tris extender was optimum to maintain plasma membrane integrity of frozen Simmental semen. Mondal *et al.* (2010) reported that the cryopreservation could reduce spermatozoa membrane integrity up to 25%.

The finding in this research is higher compared to Ariantie *et al.* (2013) that showed 54.14% membrane integrity with

the utilization of glycerol and dimethylformamida as cryoprotectant. The plasma membrane integrity was reduced during cryopreservation due to the lipid peroxidation which damage the membrane structure. Moreover, the thawing of frozen semen alters protein activity and membrane permeability. One of the main factors which disrupt plasma membrane cell integrity is the formation of ice crystals during cryopreservation (Carreira *et al.*, 2013). Semen cryopreservation, which was done at -196°C would induce cold shock to the spermatozoa.

The effort to suppress the plasma membrane damage could be done by providing extracellular cryoprotectants, such as lecithin and lipoproteins. The supplementation of soybean extract to semen extender is aimed to provide lecithin which protect the plasma membrane from cold shock. The similar properties were also found on low density lipoproteins from egg yolk. Other research also showed that soybean extract supplementation to semen extender could provide cryoprotection for the spermatozoa, thus maintain its membrane integrity (Pamungkas and Krisnan, 2017).

Reactive oxygen species (ROS) also known to affect spermatozoa membrane integrity during cryopreservation. The ROS would be formed during cryopreservation, and at high concentration level, the ROS would cause extreme oxidative stress. The oxidative reaction then alters polyunsaturated fatty acids on spermatozoa membrane and damage the cell. In order to prevent this oxidative reaction, research by Lone *et al.* (2019) showed that antioxidants can be added to the semen extender.

This elucidate the better plasma membrane integrity of frozen semen with soybean extract supplementation of this research. Wahjuningsih *et al.* (2019) added that antioxidant compounds could prevent lipid peroxide chain reactions by the free radicals which damage plasma membrane integrity.

The natural antioxidants from fenolic compounds are categorized as secondary metabolites produced by plant. Moreover, soybean also contains isoflavon, genistein and daidzein (Asih, 2009) and lecithin, a phospholipid compounds that could prevent damage to the spermatozoa plasma membrane during extension, cooling, and cryopreservation (Layek *et al.*, 2016). In this research, it is found that 10% (v/v) soybean extract supplementation to tris-egg yolk extender showed the best result on maintaining post-thawed Simmental bull semen quality, whether on spermatozoa motility, viability, plasma membrane integrity.

The supplementation more than 10% (v/v) showed negative result. The result was due to the excess antioxidant supplementation from the soybean extract, which could reduce spermatozoa progressive motility, viability, and plasma membrane integrity as well. Excessive antioxidant supplementation could damage the plasma membrane by altering the osmolality of semen extender. Thus, a sufficient antioxidant supplementation level should be taken into consideration so that semen quality could be maintained by inhibiting ROS activity (Partyka *et al.*, 2012) without altering the osmolality of semen extender.

CONCLUSIONS

The supplementation of soybean (*Glycine max* (L.) Merr.) to Tris aminomethane-egg yolk extender showed significant effect on the post-thawed quality of Simmental bull semen. The soybean extract supplementation at 10% (v/v) showed the best ability to maintain post-thawed semen quality.

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