The Effect of Genistein on the Plasma Membrane Integrity of Frozen Ongole Grade Bull Semen Based on Skim Milk – Soy Lecithin Extender

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Abstract. Beef cattle represent livestock commodities that can help support national economic stability. Ongole grade bull (PO) is one of the local cattle breeds whose population and genetic quality decrease. Various efforts have been made in improving the genetic quality and in increasing the population of the local livestock population to achieve self-sufficient status of the beef supply in Indonesia. One of the efforts is artificial insemination (IB). The study aims at examining the effect of different concentrations of genistein semen extender on the post-thawing membrane integrity of Ongole grade bull semen. It is useful in developing the genistein as a protective agent against reactive oxygen species (ROS) during cryopreservation process. It was conducted at BPBPTDK, Center for Animal Breeding Development, Animal Feed, and Veterinary Diagnostics, in the Special Region of Yogyakarta from February 7th, 2019 to May 7th, 2019. The semen of two healthy Ongole grade bulls was consecutively collected five times in the period of 3 months using artificial vagina. The semen was cryopreserved using skim milk-soy lecithin based extenders at different concentrations of genistein, which were group A (1.0 mmol genistein), B (2.0 mmol genistein) and K (without any genistein as control). The observed parameters included individual spermatozoa motility, percentage of spermatozoa viability that was observed using Eosin nigrosine staining method and spermatozoa membrane integrity that was observed using the hypo osmotic swelling test (HOST) method. The observations were made on fresh semen, extended semen, and post-thawing semen. The results of the observation were then analyzed descriptively and statistically using regression correlation test and one-way analysis of variance (ANOVA). The results of the analysis showed that the addition of Genistein at the different concentrations of 1 mmol and 2 mmol resulted in higher motility, the membrane integrity, and the acrosome integrity of the Ongole grade bull semen. The genistein concentration of 2.0 mmol gave the highest mean percentage of spermatozoa.
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motility (57.50 ± 1.00 %) after freezing. The genistein concentration of 1.0 mmol gave the highest mean percentage of spermatozoa membrane integrity and acrosome integrity (52.45 ± 0.85 % and 49.58 ± 0.98 %) after freezing.

Keywords: Ongole grade bull, genistein, cryopreservation, reactive oxygen species, membrane integrity of spermatozoa

1. Introduction

Beef cattle represent strategic livestock commodities that sustain national stability. However, national meat production has not been able to keep pace with domestic consumer demand and it triggers the increase in meat import from other countries [1]. Considering the availability of the meat, there has been an increasing trend of domestic beef production in the last five years. However, the average production is relatively stagnant. The data of Statistics Indonesia (BPS) for animal husbandry and health in 2018 showed that the mean Indonesian beef consumption in 2017 was about 3.00 kg/capita/year. The projection of the Statistics Indonesia stated that the population of the country in 2017 was 261,360,000. Therefore, the national beef demand for the beef in 2017 would be 784,000 tons, while the estimated beef production in 2017 was 532,000 tons and the consumption of the livestock product of fresh beef in 2017 was 0.469 kg/per capita/year. The figure has been increasing since 2013 [2]. Therefore, it was necessary for the government to import the beef to meet the increasing demand for it.

The government has attempted to reduce import and to improve the quality of local livestock and to increase the production of the local livestock by applying artificial insemination (IB) or cryopreservation that produced frozen semen. Cryopreservation is a technique of storing animal cells, plants, or other genetic materials (including semen) in a frozen state (-196 ℃) through the reduction of metabolic activity without any effect on organelles in the cell so that physiological, biological, and morphological functions remain [3]. The advantage of frozen semen is that it can be stored indefinitely, can be collected at any time, and can be used as needed. However, freezing process also comes up with several obstacles, including high oxidative stress during the freezing process [4].

Spermatozoa have three types of membranes, which are plasma membrane, mitochondrial membrane, and acrosome membrane. The membranes contain concentrations of polyunsaturated fatty acids and are therefore very susceptible to oxidative stress especially during freezing procedures [5]. The oxidative stress is a factor that contributes to an upsurge of cell damage caused by reactive oxygen species (ROS). Excessive ROS production in sperm will be hazardous because of its adverse impact on functional sperm count [6]. Mammalian spermatozoa membrane is susceptible to oxidation in the presence of the ROS because it is rich in unsaturated fatty acids. The lipid peroxidation chain reactions take place continuously (autocatalytically) because each reaction produces a new reactive oxygen species (ROS), which results in a new lipid peroxidation reaction that ultimately damages the entire plasma membrane of spermatozoa cells. The lipid peroxidation can cause changes in membrane function, which results in decreased sperm metabolism, sperm morphology, sperm motility and fertility [7].

Concerning with the conditions it was necessary to conduct a study in search for compounds that can be used as antioxidant agents to overcome the effects of the ROS on the cryopreservation of spermatozoa. The study is an innovation in the use of antioxidant compounds such as Genistein, phytoestrogens found in soybean plants. The phytoestrogens are chemical compounds derived from plants and able to bind and send signals to estrogen receptors. Also, they have biological activities similar to estrogen, which influence the reproductive processes in humans and animals by optimizing reproductive behaviour according to the morphology and the function of the reproductive organs. The predominant
phytoestrogens found in soybeans and their processed products are genistein isoflavones, which make up the largest portion of all soybean isoflavones, amounting to two-thirds of the total. The results of the study showed that the genistein serves several functions of inhibitor, angiogenesis, fat peroxidation, antioxidant, and anti-cancer compound [8]. The antioxidant and anti-inflammatory properties of the genistein can modify the hemodialysis membrane and cause a significant decrease in reactive oxygen species (ROS). Thus, it has direct influence on the function of adult spermatozoa cells. The effect of the genistein may support the entire reproductive process [8].

2. Materials and Methods

2.1 Materials

The main materials of this study were 20 semen samples drawn from 2 Ongole (PO) grade bulls aged 6 to 8 years, which were healthy and had normal reproductive organs. The study aimed at examining the effect of adding genistein at different concentrations on the integrity of the spermatozoa membrane in cryopreservation of Ongole grade bull (PO) frozen semen.

2.2 Chemicals

The chemicals used in the study were synthetic genistein powder (Merck, German), soy lecithin (Sigma Aldrich, USA), fructose (FHK, Japan), glycerol 10% (JT Baker, USA), fructose (FHK, Japan), aquadestillata (Ikapramindo, Indonesia), mini-glycerol straw 0.25 ml (Minitub, German), sodium citrate 3% (Merck, German), eosin (Merck, German), nigrosin (Merck, German), sodium citrate (Merck, German), glacial acetic acid (Merck, German), Nacl fisilogis (Otsuka, Japan), PBS (Thermo Fisher Scientific, USA), and acridine orange dyes (Merck, German).

2.3 Study Design

The study was conducted at BPBPTDK, Center for Animal Breeding Development, Animal Feed, and Veterinary Diagnostics, in the Special Region of Yogyakarta from February 7th, 2019 to May 7th, 2019. Once the motility, the mass movement, the concentration, the viability, and the membrane integrity of the fresh semen of the Ongole grade bulls have been examined, the semen samples were assigned to 3 groups (1 control group and 2 treatment groups). The cryopreservation process was carried out using diluents made of soy lecithin skim with the addition of genistein. The semen was drawn once a week using an artificial vagina. Observations were made on fresh semen, liquid semen, and frozen semen (post-thawing). And then, it was diluted in a soybean skim-lecithin based diluent, which was divided into two dilution stages. The composition of diluent A comprised an antibiotic buffer (skim + penicillin + streptomycin) of 13.5 ml, 1.5 ml of soy lecithin as a control and soybean skim-lecithin diluent + genistein (1.0 mM and 2.0 mM) as treatment, while the composition of diluent B comprised an antibiotic buffer (skim + penicillin + streptomycin) of 10.8 ml, soy lecithin of 1.5 ml, glycerol of 2.4 ml, glucose of 0.3 gram as a control and diluent soybean skim-lecithin + genistein (1.0 mM and 2.0 mM) as a treatment.

2.4 The Motility of Spermatozoa

The motility of spermatozoa was analyzed to find out the number of spermatozoa moving forward. The motility test started by dropping 20µL semen using a micropipette on an object glass and then covered with a cover glass. The percentage of sperm motility was assessed in the range of 0 to 100% using an estimate of five viewpoints and by comparing the number of spermatozoa moving forward. The sperm motility was evaluated using a microscope at 100x magnification, which was equipped with a heating table (37°C).
2.5 The Viability of Spermatozoa
Sperm viability test was carried out using eosin nigrosin staining method. The spermatozoa viability (%) was determined on the basis of the living spermatozoa of good and solid membrane structure that in one hand prevented the dye from entering the spermatozoa membrane. On the other hand, the dead spermatozoa had a nonfunctioning membrane structure that allowed the dye to enter the spermatozoa membrane. The viability test was started by dripping 1 drop of semen over the glass object and adding 2 drops of the eosin-nigrosin staining reagent. And then, it was stirred until the mixture was evenly distributed. Subsequently, the researcher made clear preparations on other glass objects. Fixation was done on Bunsen and the preparations were observed under a microscope at 400x magnification.

2.6 The Examination of Spermatozoa Membrane Integrity Using HOST Method
The membrane integrity of spermatozoa was analyzed using hypo osmotic swelling test (HOST) with a slight modification using a solution with a composition of 0.9 g fructose and 0.49 g Sodium citrate dissolved in distilled water until the volume was 100 ml. Two hundred of the solution was added to 20 of semen and then it was incubated at 37 °C for 45 minutes. Subsequently, smear preparation was made on an object glass and the preparation was then immersed in a methanol solution for 10 minutes, rinsed in running water, and dried. After that, at least 100 spermatozoa were examined under a microscope at 400x magnification. The spermatozoa with intact membrane were characterized by swollen tail, while sperms with damaged membrane were characterized by straight tails.

2.7 The Examination of Spermatozoa Acrosomal Integrity Using Giemsa Staining Method
The acrosomal integrity of spermatozoa was analyzed using Giemsa staining method. The staining process was started by dripping semen on an object glass. Smear preparation was made and warmed using a warming plate at the temperature of 37 °C. The preparation was fixed using methanol for 10 minutes and then rinsed in running water. It was stained using Giemsa solution with the composition of 3 ml absolute giemsa, 2 ml PBS, and 35 ml distilled water for 3 hours by immersing it in a staining jar. Subsequently, it was rinsed in running water and dried again. Two hundred cells were examined under a light microscope at 400x magnification. The spermatozoa with intact acrosome were characterized by purple head, while those with damaged acrosome were characterized by pale lavender head. Acrosomal status was assessed by dividing the number of the spermatozoa with the intact acrosome by the total number of spermatozoa and multiplying it by 100%.

2.8 Data Analysis
The data were processed using Statistical Product and Service Solution (SPSS) version 24. The results of the data processing were analyzed using the one-way analysis of variance (ANOVA) test to determine the real effect of genistein loading at different concentrations (1 mM and 2 mM) on the viability, the motility, and the integrity of spermatozoa membranes for both the liquid and frozen semen. All of the results were expressed as mean ± standard error (SE) at the significant value of P <0.05.

3. Results
Table 1. Fresh ongole grade bull sperm evaluation results

| Cattle | Macroscopic Evaluation | Microscopic Evaluation |
|--------|------------------------|------------------------|
|        | Volume (ml) | Colour | pH | Concentration (109/ml) | Motility (Group) | Motility (%) | Viability (%) | Membrane Integrity (%) |
| PO - A | 6.7 ± 1.5 | Cream | 6 | 2713 ±0.45 | +4 | 78.00 ± 0.25 | 86.56 ± 1.24 | 71.09 ± 0.68 |
| PO – B | 7.5 ± 0.7 | Cream | 6 | 2079 ±0.57 | +4 | 80.00 ± 0.75 | 84.73 ± 0.49 | 72.55 ± 0.57 |
Table 2. The effect of the cryopreservation on the Ongole grade bull spermatozoa

| Parameters                        | Fresh            | After Equilibration | After Thawing |
|----------------------------------|------------------|---------------------|---------------|
| Motility (%)                     | 79.00 ± 0.50     | 65.13 ± 1.25        | 53.63 ± 1.00  |
| Live Sperm (%)                   | 85.65 ± 0.86     | 74.69 ± 0.72        | 67.48 ± 0.94  |
| Membran Integrity HOST (%)       | 71.82 ± 0.62     | 45.31 ± 0.53        | 43.97 ± 0.58  |
| Acrosomal Integrity, Giemsa (%)  | 82.46 ± 0.58     | 58.32 ± 0.96        | 42.65 ± 0.84  |

abc Different lowercase superscripts in the same row show the significant difference (P <0.05).

Table 3. The effect of the cryopreservation and the genistein on the Ongole grade bull sperm quality at different cryopreservation stages

| Cryopreservation stage | Group         | Motility (%) | Sperm viability (%) |
|------------------------|---------------|--------------|---------------------|
| After equilibration    | Control       | 65.13 ± 1.25 | 74.69 ± 0.72        |
|                        | Treatment (genistein 1 mM) | 68.13 ± 0.75 | 74.90 ± 0.81        |
|                        | Treatment (genistein 2 mM) | 69.25 ± 0.50 | 75.09 ± 0.96        |
| After Freezing         | Control       | 53.63 ± 1.00 | 67.48 ± 0.94        |
|                        | Treatment (genistein 1mM) | 55.75 ± 0.50 | 68.39 ± 0.78        |
|                        | Treatment (genistein 2 mM) | 57.50 ± 1.00 | 68.78 ± 0.89        |

abc Different lowercase superscripts in the same column show the significant difference (P <0.05).

Table 4. The effect of the cryopreservation and the genistein on the Ongole grade bull sperm membrane integrity at different cryopreservation stages

| Cryopreservation stage | Group         | Membrane Integrity, HOST (%) | Acrosomal Integrity, Giemsa (%) |
|------------------------|---------------|-------------------------------|---------------------------------|
| After equilibration    | Control       | 45.31 ± 0.53                 | 58.32 ± 0.96                    |
|                        | Treatment (genistein 1mM) | 47.01 ± 0.91 | 68.23 ± 0.85                |
|                        | Treatment (genistein 2 mM) | 46.22 ± 0.74 | 65.76 ± 0.57                |
| After Freezing         | Control       | 43.97 ± 0.58                 | 42.65 ± 0.84                    |
|                        | Treatment (genistein 1mM) | 52.45 ± 0.85 | 49.58 ± 0.98                |
|                        | Treatment (genistein 2 mM) | 51.13 ± 0.52 | 43.96 ± 0.82                |

abc Different lowercase superscripts in the same column show the significant difference (P <0.05).

3.1 The Effect of the Cryopreservation and the Genistein on the Spermatozoa Motility

The results showed that the progressive motility (%) of the fresh semen of the Ongole grade bulls was 79.00 and it was 65.13 after equilibration, while after freezing the motility value was 53.63 (Table 2). Observations were made at the fresh semen stage, after the equilibration, and after the freezing to find out whether there were significant differences between the semen of both groups (P <0.05). At the pre-freezing stage, the progressive motility of the sperm in the two semen groups was examined at the end of the equilibration process. It was found that there were significant differences between the control and the treatment groups at the post-equilibration stage (Table 3). The percentage of the sperm motility after the equilibration process was 65.13 for the control and 68.13 for the treatment group (genistein 1 mM), and 69.25 for the treatment (genistein 2 mM). The percentage of the sperm motility at the post-thawing stage or after the freezing was 53.63 for the control and 55.75 for the treatment group (genistein 1 mM) and 57.50 for the treatment (genistein 2
mM). The percentage of the post-thawing spermatozoa motility or after the freezing in the genistein treatment group was significantly higher (P<0.05) than that in the control group (Table 3). The treatment group (genistein 2 mM) had the highest percentage of the motility both after the equilibration and after the freezing process (Table 3).

3.2 The Effect of the Cryopreservation and the Genistein on the Spermatozoa Viability
The fresh semen were observed to find out significant differences (P <0.05) between sperm viability at the post-equilibration and the post-freezing stages (Table 3). The treatment group (with the addition of 1mM and 2mM genistein) had a higher percentage of living spermatozoa than the control group at the post-equilibration stage, but the difference was not significant (P>0.05). Also, the percentage of the living spermatozoa at the post-thawing stage or after the freezing in the treatment group was higher than that of the control group although the difference was not significant (P>0.05).

![Figure 1. The evaluation of the spermatozoa under a microscope at 400x magnification using Eosin-nigrosin staining method to find out the percentage of the living and death spermatozoa. The transparent white spermatozoa were indicative of the living spermatozoa with compact cell permeability (a) and the red spermatozoa were indicative of the dead spermatozoa with poor cell permeability (b).](image)

3.3 The effect of the cryopreservation and the genistein on the integrity of the spermatozoa plasma membrane
The fresh semen at the fresh semen stage, the post-equilibration and the post-freezing stage were observed to find out significant differences between both groups (P<0.05) (Table 3). The plasma membrane integrity at the post-equilibration stage in the treatment group (with the addition of the genistein 1mM and 2mM) was significantly higher (P <0.05) than that in the control group (Table 3). Also, the plasma membrane integrity after the freezing in the treatment group was significantly higher (P<0.05) than that in the control group. In contrast to the observations of the sperm motility, the results of the observations of the plasma membrane integrity showed that the treatment group (with the addition of 1mM genistein) had the highest percentage of the plasma membrane integrity both after equilibration and after freezing (Table 3).
Figure 2. The evaluation of the spermatozoa under a microscope at 400x magnification using Hypo Osmotic Swelling Test (HOST) to find out the integrity of the spermatozoa membrane. The spermatozoa with circular tails were indicative of those with good membrane integrity (a) and the spermatozoa with straight tails were indicative of those with poor membrane integrity (b).

3.4 The effect of the cryopreservation and the genistein on the acrosomal integrity of the spermatozoa

The fresh semen at the fresh semen stage, the post-equilibration and the post-freezing stage were observed to find out significant differences between both groups (P<0.05) (Table 3). The acrosome integrity at the post-equilibration stage in the treatment group (with the addition of the genistein 1mM and 2mM) was significantly higher (P<0.05) than that in the control group (Table 4). Also, the acrosome integrity after the freezing in the treatment group was significantly higher (P<0.05) than that in the control group. In contrast to the observations of the sperm motility, the results of the observations of the acrosome integrity showed that the treatment group (with the addition of 1mM genistein) had the highest percentage of the acrosome integrity both after equilibration and after freezing (Table 3).

Figure 3. The evaluation of the spermatozoa under a microscope at 400x magnification using giemsa staining method to find out the percentage of the plasma membrane integrity. The spermatozoa with purple head were indicative of those with good plasma membrane integrity (A) and the spermatozoa with transparent white head were indicative of those with acrosomal damage (B).

4. Discussion
Previous studies show that the decrease in the motility of frozen sperm after the thawing process relates to the changes in plasma membrane stability that result in increased membrane permeability to ions [11] and increased production of reactive oxygen species or ROS [12]. Mammalian spermatozoa membranes are very susceptible to oxidation by ROS because of their high-unsaturated fatty acid content. The occurrence of lipid peroxidation can cause changes in spermatozoa membrane function, which results in decreased sperm metabolism, sperm morphology, sperm motility and sperm fertility
Sperm plasma membranes have phospholipids that contain unsaturated fatty acids so that they are very vulnerable to free radical attack. The free radicals will stimulate autocatalytic reactions that will in turn damage the double bonds that form cell membrane. It shows that the sperm membrane is the main target of the ROS, while the lipids are potential targets [13]. Almost all cellular components such as lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress [14]. Prolonged lipid peroxidation can damage the matrix structure of the lipids so that the cell membranes become unstable and hence it results in the disruption of the function of the membranes and causes the decrease in sperm membrane fluidity [15]. The damaged sperm cells because of the lipid peroxidation are characterized by the decrease in motility and fertilization capacity, damaged intracellular enzyme and damaged plasma membrane structure [16]. Excessive lipid peroxidation causes uncontrolled production of ROS and in the absence of balanced and adequate amount of antioxidants in seminal plasma it can lead to oxidative stress (OS) that is harmful to spermatozoa [17].

The results of the study showed that the genistein at the concentrations of 1 mmol and 2 mmol had significant effect on the motility, but it did not have any significant effect on the spermatozoa viability in the liquid and frozen semen samples of the Ongole grade bull. Therefore, they are consistent with previous study [18] showing that the addition of genistein 1 mmol as a protective medium for cryopreservation of human semen caused significant increase in spermatozoa motility. However, some other studies gave different results showing that the application of genistein as freezing extender at various doses (at the concentrations of 0.1 mmol, 1 mmol, 2 mmol, 4 mmol, 8 mmol) did not have any significant effect on the viability and the motility of spermatozoa [19]. The results of another study [20] that tested bull semen with the application of genistein showed that the application of the genistein at the concentrations of 0.74 mmol, 7.4 mmol, and 74 mmol did not have any statistically significant effect on spermatozoa motility (p>0.05). Still, another study [21] that examined the semen of hamsters showed that there was not any significant effect of the genistein on the motility of progressive spermatozoa that have been treated by adding 0.5 mmol genistein. Thus, based on the data it was concluded that the effects of the addition of the genistein would be different for each species.

The results of the study showed that the application of the genistein 1 mmol and 2 mmol as cryoprotectant agents had significant effect on the integrity of the spermatozoa membranes in the liquid and frozen semen samples of the Ongole grade bull. They were almost consistent with the results of another research [22] showing that the application of the genistein caused a slight increase in the value of sperm motility as compared to controls, but it did not have any significant effect on semen (p>0.05). The results of another study [23] also showed that the application of the genistein had only insignificant effect (p>0.05) on the integrity of the spermatozoa membrane of pigs, but the effect increased as compared to controls.

The findings of the study confirmed that the cryopreservation of semen had negative impact on the fertilizing ability of sperm and genistein supplementation could make sperm more susceptible to the conditions in the cryopreservation. Although the number of the animal samples of study was, it was recommended that genistein supplementation might be used as an alternative to support the cryopreservation process. However, it required further studies to get broader understanding of the mechanisms of membrane damage in the cryopreservation process, especially the mechanism of reactive oxygen species (ROS) in damaging spermatozoa membranes. The approach would be very useful in the development of cryopreservation technology in the future.

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