OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Effect of Genistein Supplementation in Tris-Aminomethane Base Extender on Characteristics of Pre-Freezing and Post-Thawed Sperm Ongole Grade Bull

Chairdin Dwi Nugraha[1], Rafika Febriani Putri[1], Ari Ardiantoro[2], Wike Andre Septian[1], Ahmad Furqon[3], Kuswati Kuswati[1], Nashi Widodo[4], Suyadi Suyadi[1*]

[1]Faculty of Animal Science, University of Brawijaya, Jl. Veteran, Malang 65145 – Indonesia; [2]Laboratory of Animal Biotechnology, University of Brawijaya, Jl. Veteran, Malang 65145 – Indonesia; [3]National Research and Innovation Agency Republic of Indonesia, Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340 – Indonesia; [4]Department of Biology, Faculty Mathematics and Natural Sciences, University of Brawijaya, Jl. Veteran, Malang 65145 – Indonesia.

Abstract | Sperm undergo physiological changes during the cooling and freezing process, and especially significant changes are seen after freezing than after cooling. This research analyzed the effect of genistein supplementation in the basic diluent of tris-aminomethane on the quality of spermatozoa. Bulls aged 3–4 years used in this research. This research used an experimental method to examine the effect of genistein levels in the diluent on the characteristics of spermatozoa during pre-freezing at 4–5 ^{0}C and post-thawing (PT). The data obtained in the form of quantitative data were analyzed descriptively and analysis of variance patterns in One-Way ANOVA. 0 µM, 10 µM, 30 µM and 50 µM genistein treatment in the basic diluent tris aminomethane–egg yolk. Progressive motility post-thawing at 1 hour and 2 hours was highest at genistein concentration 30 µM ($P<0.05$). The effect of the antioxidant genistein on tris aminomethane - egg yolk diluent gave a significant effect on sperm viability in the Before Freezing (BF) phase at 1.5 hours, BF and PT at two hours ($P<0.05$). The addition of different genistein in diluent tris aminomethane - egg yolk was no significant effect ($P>0.05$) on sperm abnormality in both the BF and PT phases at 1 hours, 1.5 hours and 2 hours. The addition of genistein with a concentration of 30 µM was able to maintain better semen quality.

Keywords | Antioxidant, *Peranakan Ongole* Bull, Reactive Oxygen Species, semen diluent, semen quality

Received | January 24, 2023; **Accepted** | February 20, 2023; **Published** | June 01, 2023
***Correspondence** | Suyadi Suyadi, Faculty of Animal Science, University of Brawijaya, Jl. Veteran, Malang 65145 – Indonesia; **Email:** suyadi@ub.ac.id
Citation | Nugraha CD, Putri RF, Ardiantoro A, Septian WA, Furqon A, Kuswati K, Widodo N, Suyadi S (2023). Effect of genistein supplementation in tris-aminomethane base extender on characteristics of pre-freezing and post-thawed sperm ongole grade bull. Anim. Vet. Sci. 11(7): 1135-1143.
DOI | http://dx.doi.org/10.17582/journal.aavs/2023/11.7.1135.1143
ISSN (Online) | 2307-8316

INTRODUCTION

Ongole Grade (OG) or *Peranakan* Ongole cattle (*Bos Indicus*) are a popular breed of cattle in Indonesia, their presence is almost evenly distributed throughout in the Indonesia, starting from Sumatra, Sulawesi and Java. The highest population on the island of Java is concentrated in East Java, especially in Tuban district where there is a OG cattle breeding center, namely at UPT PT HMT Tuban in East Java . OG cattle are suitable for rearing in Indonesia because they are able to adapt well to various environmental stresses, extreme tropical climates and low quality feed. Based on the regulation of the Minister of Agriculture number: 2841/Kpts/LB.430/8/201, OG Cattle are genetic resources of Indonesian local beef cattle that need to be protected and preserved. Efforts to develop OG cattle from all aspects need to be carried out in a sustainable and systematic manner. One important aspect to be carried out is the use of reproductive technology to maintain and improve the quality of spermatozoa.

Semen high quality is the basic factor that determines the success of the Artficial Insemination (AI) program because semen has an influence on the dimensions of success for pregnant cows and contributes genetically to the offspring produced. In addition to the amount of semen that can be produced by a bull which includes the amount or volume of production per year (Suyadi et al., 2020), consistency of production throughout the year or season (Nugraha et al., 2021), the quality of semen is very important to be used as a criterion in determining the quality of semen for determine the success of livestock genetic quality improvement programs through artificial insemination (Söderquist et al., 1996; Muthiapriani et al., 2019; Novianti et al., 2020).

The processing frozen semen is a series of processes that involve physical, physiological and biochemical activities of sperm (Sharma and Sharma, 2020). Physically, there is a change in the structure and volume of the spermatozoa cells when they enter the liquid phase into the frozen phase, resulting in shrinkage or swelling according to the diluent medium and the freezing process, the linear length curve decreased by 33% after dilution-cooling and 22% after cooling freezing (Agca et al., 2002; Hussain et al., 2011). During the freezing process, spermatozoa undergo tremendous biochemical changes where the metabolic system tries to be inhibited or stopped through a very low temperature drop, so that spermatozoa cells are no longer able to carry out biochemical reactions (Agca et al., 2002; (Sharma and Sharma, 2020). As a result of the freezing process, there were many changes in the quality of spermatozoa which were generally observed in decreased motility, viability, membrane integrity, acrosome capacitation and status, and fertility.

The decrease in semen quality during the dilution, cooling and freezing process can be inhibited by the addition of antioxidants into the diluent. Antioxidants have a high potential to break the covalent bonds of lipolipic Reactive Oxygen Species (ROS) left on cell membrane lipids that bind to fatty acid side bonds (Jeong et al., 2009; Azawi and Hussein, 2013). The addition of antioxidant vitamin E can maintain the progressive motility and viability of sheep sperm before and after freezing (Pour et al., 2013). Likewise, it has been reported that administration of antioxidants can improve sperm quality of some mammals (rabbits, horses, sheep and pigs) during the freezing process (Hatamoto et al., 2006). Antioxidants are efficient agents in protecting cell membranes against ROS and lipid peroxidation in pigs (Jeong et al., 2009).

Genistein (4′,5,7-trihydroxyisoflavone) is a natural isoflavone and phytoestrogen found in soybeans and legumes, which has broad spectrum pharmacological properties such as inhibition of tyrosine and topoisomerase. In the medical field, genistein induces inhibition of cell apoptosis and excessive cell proliferation thereby inhibiting the occurrence of cancer (Mukund et al., 2017; Tuli et al., 2019). As an isoflavone compound, it has potentially important properties in cell development such as antioxidant, anti-inflammatory, anti-viral, anti-bacterial, and fat metabolism (Rassu et al., 2019; Sharifi-Rad et al., 2021). As an isoflavone compound, genistein also has activity as an antioxidant that can improve the clotting process of human sperm and prevent DNA damage due to the freezing process. The addition of 10 μM/L genistein can increase the progressive motility of spermatozoa, prevent premature capacitation (membrane lipid disorder), the formation of new ROS, chromatin condensation and DNA damage (Martinez-Soto et al., 2010).

The use of genistein in supporting metabolic processes and controlling cell activity has been widely reported in the medical field, but is still limited to the process of diluting and freezing semen in mammals, especially in the livestock sector. Besides that, the results are still very varied, both from the aspect of the concentration of genistein supplementation in the diluent and the quality of the semen produced. This research analyzed the effect of genistein supplementation in the basic diluent of trisaminomethane – egg yolk on the quality of spermatozoa after cooling and after thawing in the semen of Ongole Grade (OG) bulls.

MATERIALS AND METHODS

Research material

Semen was collected from two OG bulls at breeding center UPT PT - HMT Tuban, East Java, with healthy exterior and reproductive organs. Ambient temperature 28 °C and humidity 84%. Bulls aged 3 – 4 years, body weight 475 – 550 kg. Bull were reared in semi-open stall cages for slings, fed elephant grass and concentrate 1 kg/day. Forage feed and drinking water are provided ad libitum.

Semen collection

Five ejaculates from each bull were accommodated using an artificial vagina at 1 week intervals. The artificial vagina is prepared by filling a water tube with a temperature of 42–45°C to get hot on the thick-outer layer of 38 °C. The inside of the thick-layer is lubricated, the back end is connected to a storage tube (Elsayed et al., 2019). The semen is accommodated by a professional semen collector from the Singosari National Artificial Insemination Center (SNAIC), Malang. Semen storage is carried out between 08.00 – 09.00 AM. After being accommodated, the semen was immediately evaluated for feasibility as a research sample. The semen with minimal qualification: normal color and odor, progressive motility >70% (Ax et al., 2008), abnormality <20% (Menon et al., 2011) was only used for this research.

Diluent preparation

The basic diluent in the form of trisaminomethane (MERCK from Germany) was prepared at the Livestock Reproduction Laboratory, Faculty of Animal Science, University of Brawijaya; Genistein (Sigma Aldrich, Germany) was purchased from a chemical agent in Surabaya, and Sodium Chloride (Sigma Aldrich, Germany) was used to prepare the HOS test solution.

Stock solution of Genistein 500 µM was carried out by mixing 10 mg of genistein in powder form with 7.4 ml of Dimethyl Sulfoxide or DMSO (done in stages) in a becker glass, then adding aquadest until it reached 74 ml. Stock solutions were stored frozen until use.

Trisaminomethane – egg yolk (80:20, v/v) diluent was prepared according to the procedure of Wahjuningsih et al. (2020), the day before being used for the experiment. The mixture of trisaminomethane – egg yolk was put into a 250 ml Erlenmeyer glass, added with genistein solution according to the treatment and homogenized using a magnetic stirrer for 15 minutes, centrifuged at 1500 rpm for 30 minutes. The supernatant was transferred to another Erlenmeyer tube and stored at refrigeration temperature until used.

Research design

This research used an experimental method to examine the effect of genistein levels in the diluents on the characteristics of spermatozoa during pre-freezing at 4 – 5 ^{0}C and post-thawing. Diluents were made with compositions containing genistein according to the treatment, namely 0 µM, 10 µM, 30 µM and 50 µM genistein in the basicdiluents trisaminomethane – 20% egg yolk. Each treatment for genistein levels in the diluents was repeated 5 times. Observations on the characteristics of spermatozoa were carried out at 1 hours, 1.5 hours and 2 hours in the pre-feezing and post-thawing phases. Variables observed in fresh and frozen semen included volume, color, odor, consistency, pH, mass motility, individual motility, concentration, viability and abnormalities. Spermatozoa membrane integrity was observed when the semen was equilibrated at cold temperatures or before freezing (BF) and post thawing (PT).

Semen dilution, cooling (before freezing), freezing and thawing

Fresh semen was diluted with the prepared diluent to reach a concentration of 25 x 10^6/ml. The semen was diluted with a diluent containing genistein according to the treatment then put in a 2 ml mini-tube vial and placed on a refrigerator rack for 2 hours for the cold temperature equilibration process, this phase is called pre-freezing. During the pre-freezing phase, the quality of the spermatozoa was evaluated at 1, 1.5 and 2 hours.

The freezing procedure was carried out according to the research of Elsayed et al. (2019). For semen that was not observed at cold temperature equilibration, the semen was put into 0.25 ml straws and sealed. Straw is placed horizontally on a rack in liquid nitrogen vapor at a height of 10 cm from the surface of the liquid nitrogen for 15 minutes and then directly inserted into the liquid nitrogen.

Thawing process on frozen semen in straws was carried out at least 1 week after storage in liquid nitrogen. Thawing was done by placing the straws in warm water at 37 ˚C for 30 seconds (Pardede et al., 2022) and then evaluating each group for pre-freezing 1 hours, 1.5 hours and 2 hours.

Semen Quality Evaluation

Evaluation of semen quality was carried out on fresh semen after storage, during the cold temperature equilibration process and after thawing. Evaluation of semen quality with the effect of adding genistein in diluent was carried out on the parameters of progressive motility, viability, abnormalities and membrane integrity of spermatozoa (Mansur et al., 2018).

Membrane Integrity

Spermatozoa membrane integrity was evaluated using the Hypo Osmotic Swelling Test (HOST) technique according to previously reported procedures (Zubair et al., 2013). The HOST solution contains 1.35 g of fructose and 0.55 g of Sodium Citrate in 100 ml of distilled water with a pressure of 150 mOsml/L. A total of 0.5 ml of semen was mixed with 1 ml of HOST solution, incubated in a water bath at 37 ˚C for 30 minutes (Zubair et al., 2013). One drop of semen was smeared on an object glass, allowed to dry in the air and observed using a light microscope with a magnification of 400x. Calculations were carried out on 200 spermatozoa randomly. Spermatozoa that are able to hold hypoosmotic fluid in the cell are indicated by their intact membranes, and circular spermatozoa tails are seen (Zubair et al., 2013).

Statistical Analysis

The data obtained in the form of quantitative data were analyzed descriptively and analysis of variance patterns in One-Way ANOVA (Elsayed et al., 2019) using SPSS Version 24 software.

RESULTS

Characteristics of Fresh Semen

Characteristics of fresh semen of Ongole Grade (OG) cattle at UPT PT HMT Tuban are presented in Table 1. Table 1 shows the macroscopic and microscopic evaluation values of OG cattle semen including a volume value of 4.63 ml with a pH of 6.54. Individual motility was

Table 1: Characteristics of fresh semen of OG bull

Quality of Fresh Semen PO Bull	
Parameter	**Mean ± SD**
Macroscopic	
Volume (ml)	4.63 ± 1.92
Color	Yellowish white
Consistency	Medium
pH	6.54 ± 0.19
Microscopic	
Mass Motility	++
Individual Motility (%)	75.38 ± 9.67
Concentration (million/ml)	147.15 ± 65.75
Viability (%)	85.72 ± 5.75
Abnormality (%)	3.41 ± 0.96
Membrane Integrity (%)	63.14 ± 3.52

Table 2: Progressive motility of OG bull spermatozoa in the Before freezing and Post Thawing phases

Genistein in diluents	Sperm progresif motility (%) during cold equilibration (h)					
	1,0 h		1,5 h		2,0 h	
	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)
0 µM	59.0 ± 6.51	20.0 ± 3.53[a]	57.0 ± 5.70	17 ± 6.70	54 ± 6.51	12 ± 4.47[a]
10 µM	63.0 ± 5.70	25.0 ± 3.53[ab]	61.0 ± 7.41	17 ± 6.70	59 ± 6.51	19 ± 5.47[b]
30 µM	65.0 ± 6.12	27.0 ± 2.73[b]	64.0 ± 6.51	25 ± 7.07	63 ± 8.36	27 ± 4.47[c]
50 µM	60.0 ± 7.90	22.0 ± 7.58[ab]	57.0 ± 7.58	16 ± 8.21	56 ± 7.41	15 ± 5.00[ab]

Abbreviations: BF = before freezing (%); PT = post thawing (%)

[a-b] Values within a colum with different superscripts differ significantly at $P<0.05$

75.38%, concentration was 147.15 million/ml, viability was 85.72%, abnormality was 3.41% and membrane integrity was 63.14%.

Sperm Progressive Motility in The Before Freezing and Post Thawing Phases

Percentage of motility decreased during the shelf life of 1 hour, 1.5 hours, 2 hours both control and addition of genistein. Table 2 shows that there were significant differences in the motility of individuals post thawing at one hour and two hours (P<0.05). Genistein concentration treatment showed that the concentration of 30 µM was able to maintain the highest progressive motility in the before freezing and post thawing phases compared to other treatments. Genistein concentration of 30 µM also had the same effect on the storage time of one hour and two hours by 27%.

Sperm Viability in The Before Freezing and Post Thawing Phases

The effect of the antioxidant genistein on tris aminomethane – egg yolk diluent on spermatozoa viability gave a significant effect in the BF phase at 1.5 hours, BF and PT at two hours (P<0.05) (Table 3). In the observation of the before freezing phase, the results showed that the addition of 30 µM showed the best results, the observation of the post thawing phase also showed the same results.

Sperm Abnormalities in The Before Freezing and Post Thawing Phases

Based on the sperm abnormalities in Table 4, it shows that the addition of different genistein (0 µM, 10 µM, 30 µM and 50 µM) in diluent trisaminomethane - egg yolk had no significant effect (P>0.05) in both the BF and PT phases at 1 hours, 1.5 hours and 2 hours. The addition of genistein with a concentration of 30 µM in both the BF and PT phases showed the lowest abnormality values. The PT phases the highest abnormality value was at a concentration of 50 µM.

Membrane Integrity at Various Levels of Genistein in Diluent

The evaluation of the membrane integrity test showed that there was a decrease from the Before Freezing phase to after Post Thawing. Table 5 shows that different genistein supplementation in tris aminomethane diluents had a sig

Table 3: Sperm viability of OG bull in the pre-freezing and post-thawing phases

Genistein in diluents	Sperm viability (%) during storage (h)					
	1,0 h		1,5 h		2,0 h	
	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)
0 µM	75.63 ± 5.59	41.91 ± 7.41	72.44 ± 3.35[a]	35.91 ± 7.05	74.08 ± 4.84[ab]	30.29 ± 8.93[a]
10 µM	78.44 ± 4.72	49.01 ± 8.56	75.41 ± 5.91[ab]	38.66 ± 12.89	76.44 ± 5.29[ab]	39.27 ± 4.97[a]
30 µM	80.84 ± 3.88	51.81 ± 9.30	80.21 ± 3.43[b]	49.49 ± 13.49	81.06 ± 5.05[b]	56.85 ± 9.87[b]
50 µM	77.24 ± 6.02	46.42 ± 13.39	75.29 ± 6.48[ab]	36.43 ± 11.13	71.71 ± 6.14[a]	35.54 ± 8.71[a]

Abbreviations: BF = before freezing (%); PT = post thawing (%)

[a-b] Values within a colum with different superscripts differ significantly at $P<0.05$

Table 4: Sperm abnormalities of OG bull in the Before freezing and Post Thawing phases

Genistein in diluents	Sperm abnormality (%) during storage (h)					
	1,0 h		1,5 h		2,0 h	
	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)	BF (4 °C)	PT (30 °C)
0 µM	4.96 ± 0.75	10.66 ± 2.37	5.24 ± 0.36	10.51 ± 1.76	5.17 ± 0.65	11.70 ± 2.17
10 µM	5.54 ± 0.90	10.63 ± 1.74	5.39 ± 0.48	11.46 ± 1.82	4.90 ± 0.74	11.32 ± 2.19
30 µM	5.18 ± 0.68	9.87 ± 1.48	5.25 ± 0.75	10.27 ± 1.88	4.66 ± 0.58	10.71 ± 1.40
50 µM	4.83 ± 0.98	11.64 ± 1.65	5.43 ± 0.53	11.69 ± 1.15	5.32 ± 0.46	11.53 ± 1.51

Abbreviations: BF = before freezing (%); PT = post thawing (%)

Table 5: Average spermatozoa membrane integrity at various levels of genistein in diluent

Times	Genistein Concentration			
	0 µM	10 µM	30 µM	50 µM
BF	68.39 ± 2.82[a]	69.51 ± 2.82[a]	75.92 ± 1.19[c]	72.67 ± 1.61[b]
PT	51.76 ± 3.46[a]	56.22 ± 3.07[a]	61.17 ± 4.74[b]	55.01 ± 4.78[a]

Abbreviations: BF = before freezing (%); PT = post thawing (%)

[a-b] Values within a row with different superscripts differ significantly at $P<0.05$

nificant effect (P<0.05). The addition of genistein at a concentration of 30 µM showed the highest results in both the BF and PT phases.

DISCUSSION

Volume value in this research is classified as normal and higher than research (Susilawati et al., 2020) sperm volume of 4.58 mL. The results of research conducted by Suyadi et al. (2020) on fresh semen of Ongole Grade (OG) bull at the Singosari Artificial Inseminaton Center (SNAIC) obtained an average volume of 5.32 ± 1.93 mL and a motility of 67.43%. So it is still in the normal range for fresh semen. Garner and Hafez (2008) explained that bulls can produce semen with a volume of 5 - 8 mL in one ejaculation. The individual motility value of fresh semen in this research was above the standard criteria for good semen. Ax et al. (2008) stated that the motility value must be a minimum of 65%, so that fresh semen is suitable for processing into frozen semen. This research determined the individual motility value of 70 - 75% to test the success of diluent trisaminomethane - egg yolk with the addition of genistein. Suyadi et al. (2020) the motility of OG bull reached 67.43%.

Spermatozoa concentration is the number of spermatozoa contained in one ml of semen. The concentration of OG bull semen in this research was low (147.15 million/ml) because Bull semen with good quality has a concentration value of more than 500 million/ml (Ax et al., 2008). High semen concentrations could produce large quantities of frozen semen. Viability is the vitality of spermatozoa which can be identified by staining the cells using eosin negrosin. The viability of spermatozoa in fresh semen showed a high viability value. Garner and Hafez (2008) stated that semen is said to be of good quality, if the spermatozoa viability at least 80%. Spermatozoa abnormalities in the fresh semen from the research were in the low category. Garner and Hafez (2008) stated that good quality fresh semen had an abnormality of less than 15%. Abnormalities in the research were suspected to be included in the category of secondary abnormalities usually caused by disturbances af-

ter the spermatozoa left the seminiferous tubules, such as disturbances in the capacitation process in the epididymis, absorption disorders, abnormal secretions from accessory glands, mechanical disturbances, temperature shock. Secondary abnormalities have a normal head shape but no tail, the head does not have an acrosome, mild swelling of the tail. The integrity of the spermatozoa membranes of OG bull as a result of the research was in the normal range. Zubair et al. (2013) stated that if the spermatozoa are coiled below 40% is considered infertile. Spermatozoa with intact membranes will withstand hypoosmotic pressure inside the cells, so that their tails are coiled. Meanwhile, spermatozoa with straight tails indicate that the plasma membrane has been damaged. Tapia et al. (2012) stated the plasma membrane of the sperm is a very important structure that functions to protect the organelles in the cell.

Progressive motility of spermatozoa is an important indicator in determining the quality of spermatozoa because it has a close relationship with fertility. The motility of spermatozoa during the cooling and freezing process is largely determined by the type of diluent and the added antioxidant supplement. During the frozen semen processing, spermatozoa need to make adjustments with the diluent used so that some of the spermatozoa that cannot adjust will die. Damage to the plasma membrane of spermatozoa cells which results in a lack of energy needed by the spermatozoa so that the quality of the semen decreases. The diluent used in this research was Tris aminomethane. Tris aminomethane has the ability to protect spermatozoa during freezing by maintaining intracellular and extracellular balance so that cell metabolism processes continue and cell death is reduced. Orrego et al. (2019) added that egg yolk diluent contains low density lipoprotein which can act as a cryoprotectant.

High genistein concentrations can damage sperm motility. In this research, the addition of 50 μM genistein inhibited progressive motility through changes in sperm motility signal transduction pathways (Wong and Walker, 2013). In addition, high doses of genistein can provide a tyrosine kinase inhibitory effect. Sperm capacitation and motility are closely linked to tyrosine phosphorylation (Elsayed et al., 2019). Progressive decrease in motility value can be caused by excess Reactive Oxygen Species (ROS) which will cause oxidative stress. Oxidative stress reduces disulfide bonds between protein membranes, peroxidase reactions of membrane phospholipids and modifies the glycocalyx of spermatozoa. Oxidative stress also causes DNA damage, disrupts mitochondrial activity and decreases spermatozoa motility (Gürler et al., 2016; Peris-Frau et al., 2020). The strategy to reduce oxidative stress is to neutralize ROS and reduce ROS production. According to Kumar et al. (2019) Neutralization of ROS can be done by using antioxidants. Meanwhile, reducing ROS production can be done by reducing oxygen tension and minimizing radiation exposure, dead spermatozoa and damaged spermatozoa in the semen. Prihantoko et al. (2020) stated that the addition of genistein at concentrations of 1 μM and 2 μM resulted in higher motility, viability, and membrane integrity than control and significantly affected motility, membrane integrity, and acrosome integrity of Ongole bull semen. Elsayed et al., (2019) stated that the concentrations of genistein used in their research were 1, 5, 10, and 100 μM. The addition of 10 μM genistein to egg yolk – Trisaminomethane diluents showed better results on the motility, viability, and integrity of spermatozoa membranes after freezing and thawing. Meanwhile, the addition of 100 μM genistein tends to show a drastic decrease after 1 – 2 hours after thawing. The addition of the antioxidant genistein into egg yolk – trisaminomethan diluent was able to maintain the quality of spermatozoa. The addition of the antioxidant genistein showed a better viability value than the control treatment (0 μM). This is because genistein can theoretically prevent and inhibit the damage of spermatozoa due to the effects of free radicals and cold shock, which have been the main cause of the decline in the quality of frozen semen of OG cattle. Martinez-Soto et al. (2010) genistein is an isoflavone compound that has a direct effect on the function of mature spermatozoa by modifying the capacitation process and acrosome reaction. Genistein modifies membrane hemodialysis and causes a significant reduction in ROS (Garcia et al., 2015). On the other hand, genistein has a protective antioxidant effect on sperm DNA integrity. According to Ganai and Farooqi (2015) genistein plays a role in binding free radicals and donating hydrogen atoms from phenolic hydroxyl groups.

The post thawing phase abnormality value in this research was still within normal limits according to frozen semen standards, which was less than 20% (Garner and Hafez 2008). Spermatozoa abnormalities of more than 20% indicate the presence of infertility in the male (Garner and Hafez 2008). The presence of glycerol in the diluent for frozen semen processing helps protect the spermatozoa against temperature drops so as to reduce sperm damage due to cold shock.

The rate of slow or fast clotting in different media will affect the level of abnormal spermatozoa. Abnormalities occur due to physical changes in the living medium, both changes in osmotic pressure, and the formation of intracellular ice crystals. This can cause changes in the structure of the spermatozoa such as the shape of the spermatozoa with a bent tail or detached head. According to Kumar et al. (2016) the low viability and abnormality of frozen semen can be influenced by management or cryopreservation methods during freezing which can affect the crysurvival

of spermatozoa. Garner and Hafez (2008) these abnormalities are included in the type of secondary abnormality, because they occur during morphological development, handling time and after semen collection.

The difference in the quality of the integrity of the spermatozoa membranes of OG bull is thought to be due to the occurrence of cold shock and experiencing a critical period during freezing which results in damage to the spermatozoa membrane. According to Ratnani et al. (2017) during the cold temperature equilibration phase and the freezing process, semen was damaged by plasma membranes, acrosomes, mitochondria and spermatozoa chromatin. Research conducted by Elsayed et al. (2019) with 10 µM genistein supplementation in egg yolk tris aminomethane diluent can increase the integrity of the plasma membrane of spermatozoa tested through the Hypoosmotic Swelling (HOS) test. This increase in the integrity of the plasma membrane of spermatozoa indicates that genistein has the potential to increase the fertilization power of spermatozoa. The higher the cold stress obtained, the higher the metabolic activity of spermatozoa cells that produce lactic acid so that it affects the osmotic pressure of the solution. Increased osmotic pressure in the semen plasma can cause a decrease in the permeability of the spermatozoa membrane.

Evaluation of cell membrane integrity is generally tested using a Hypo Osmotic Swelling (HOS) Test solution to distinguish intact membranes from damaged cells (Fig. 1). In principle, the HOS Test is to see the status of the membrane, because the integrity of the membrane affects the viability of spermatozoa (Lenchniak et al., 2002).

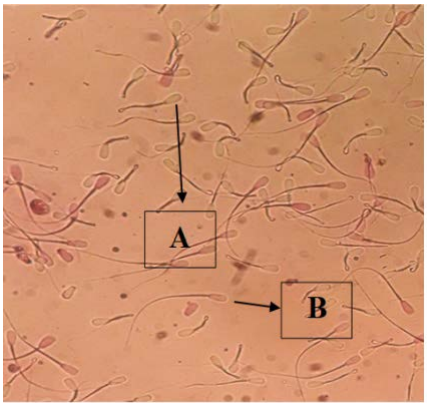

Figure 1: A coiled and straight tail of spermatozoa in HOS test

The HOS test is carried out to test the integrity of the spermatozoa cell membrane which is based on the semi-permeable nature of the spermatozoa, namely the properties that make live spermatozoa swell in hypoosmotic situations (Zubair et al., 2013). Spermatozoa with intact membranes when placed in a hypoosmotic medium will try to increase the volume of water in their bodies so that the fluid inside and outside the spermatozoa remains balanced. This effort causes a constriction of the membrane covering the tail, forcing the tail of the spermatozoa to coil. The process of bubbling begins at the end of the tail, followed by the middle and head, causing the head to bulge. A coiled or bulging tail indicates intact spermatozoa membranes or motile spermatozoa.

Increased osmotic pressure in the semen plasma can decrease the permeability of spermatozoa membranes and increase membrane damage. Damage to the membrane can cause the death of spermatozoa. The decrease in the percentage of membrane integrity is thought to occur due to the presence of toxins originating from dead spermatozoa and an increase in Reactive Oxygen Species (ROS) from the metabolism of spermatozoa. The antioxidant and anti-inflammatory properties of genistein are considered to be able to modify hemodialysis membranes so that they can reduce levels of Reactive Oxygen Species (ROS) so as to maintain better membrane integrity (Prihantoko et al., 2020).

CONCLUSION

The addition of genistein with a concentration of 30 µM was able to maintain better semen quality. The pre-freezing phase or cold temperature equilibration for 1 hour is sufficient to maintain the quality of OG cattle semen, this can save time in processing frozen semen.

ACKNOWLEDGEMENTS

Mrs. Ir. Indyah Aryani, MM. As the head of UPT PT & HMT Tuban who has provided OG bulls for semen collection. Mr. M. Zaini and Mr. A. Nuryadin who helped in the semen collection.

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest in the manuscript.

ETHICS APPROVAL

The protocol of this study was approved by the Ethics Committee of the Animal Care and Use Committee University of Brawijaya (Authorisation number: 060-KEP-UB-2022 delivered on 19th May 2020).

NOVELTY STATEMENT

Research on the addition of pure genistein in spermatozoa

diluent is rarely used and still has limited information. This study highlights the possible function of addition genistein in spermatozoa diluent to maintain spermatozoa in the post-thawing phase, so that this research can be adopted by Artificial Insemination Centers.

AUTHORS CONTRIBUTIONS

CDN collecting data, doing the research, preparing manuscript RFP collecting data AA data analysis WAS preparing manuscript AF preparing manuscript KK supervision, review the manuscript; NW supervision, review the manuscript SS conceptualization, Supervision, review the manuscript, correspondence.

REFERENCES

Agca Y., Gilmore J., Byers M., Woods E.J., Liu J., Critser J.K. (2002). Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. Biol. Reprod., 67: 1493-1501. https://doi.org/10.1095/biolreprod.102.005579

Al Mansur M. A., Alam M. G. S., Jha P. K., Rimon M. A., Naher N., Bari F. Y. (2018). Conception rate following intracervical artificial insemination using frozen semen at field level in indigenous sheep of Bangladesh. Asian J. Med. Biol. Res., 4(1): 55-62. https://doi.org/10.3329/ajmbr.v4i1.36822

Ax R. L., Dally M., Didion B. A., Lenz R. W., Love C. C., Varner D. D., Hafez B., Bellin M. E. (2008). Semen Evaluation in Farm Animal Reproduction: 7th Ed. Lippincott Williams and Wilkins, USA.

Azawi O. I., Hussein E. K. (201) Effect of vitamins C or E supplementation to tris diluent on the semen quality of Awassi rams preserved at 5 °C. Vet. Res. Forum, 201; 4(3): 157-60.

Elsayed D. H., El-Shamy A. A., Abdelrazek H. M. A., El-Badry D. A. (2019). Effect of genistein on semen quality, antioxidant capacity, caspase-3 expression and DNA integrity in cryopreserved ram spermmatozoa. Small. Rumin. Res., 177: 50-55. https://doi.org/10.1016/j.smallrumres.2019.06.009

Ganai A. A., Farooqi H. (2015). Bioactivity of genistein: A review of in vitro and in vivo studies. Biomed. Pharmacother, 76 (2015): 30–38. https://doi.org/10.1016/j.biopha.2015.10.026.

Garcia B. M., Guimaraes T., Lopes G., Rocha A., Fernandez L. G. (2015). Effect of genistein addition to equine sperm freezing extender., 66 (4): 241-248.

Garner D. L., Hafez E. S. E. (2008). Reproduction in Farm Animals. E. S. E. Hafez and B. Hafez: 7th Ed. Blackwell Publishing, USA.

Gürler H., Malama E., Heppelmann M., Calisici O., Leiding C., Kastelic J. P., Bollwein H (2016). Effects of cryopreservation on sperm viability, synthesis of Reactive Oxygen Species, and DNA damage of Bovine Sperm. J. Anim. Reprod., 86 (2): 562–571. https://doi.org/10.1016/j.theriogenology.2016.02.007.

Hatamoto L. K., Baptista Sobrinho C. A., Nichi M., Barnabe V. H., Barnabe R. C., Cortada C. N. M. (2016). Effects of dexamethasone treatment (to mimic stress) and Vitamin E oral supplementation on the spermiogram and on seminal plasma spontaneous lipid peroxidation and antioxidant enzyme activities in dogs. Theriogenology, 66 (6-7): 1610-4. https://doi.org/10.1016/j.theriogenology.2006.02.012

Hussain S. A., Lessard C., Anzar M. (2011). Quantification of damage at different stages of cryopreservation of endangered North American bison (Bison bison) semen and the effects of extender and freeze rate on post-thaw sperm quality. Anim. Reprod. Sci., 129 (3-4): 171-9. https://doi.org/10.1016/j.anireprosci.2011.12.002

Jeong Y. J., Kim M. K., Song H. J., Kang E. J., Ock S. A., Mohana Kumar B., Balasubramanian S., Rho G. J. (2009). Effect of α-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology., 58 (2): 181-189. https://doi.org/10.1016/j.cryobiol.2008.12.004

Kumar A., Prasad J. K., Srivastava N., Ghosh S. K. (2019). Strategies to minimize various stress-related freeze–thaw damages during conventional cryopreservation of mammalian spermatozoa. Biopreserv. Biobank., 17 (6): 603-612.

Kumar N., Rai B., Bhat S. A., Kharche S. D., Gangwar C., Jindal S. K., Chandra S. (2016). Effect of management system and season on semen freezability in Jakhrana Bucks. Vet. World, 9(2): 199–202.

Lenchniak D., Kedziesski A., Stainislawski D (2002). The use of HOS Test to evaluate membrane functionality of Boar Sperm capacitated in vitro. Reprod. Domest. Anim., 37 (6): 379-380.

Martinez-Soto J. C., De Dioshourcade J., Gutiérrez-Adán A., Landeras J. L., Gadea J. (2010). Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa. Asian J. Androl., 2010; 12 (3): 431-441. https://doi.org/10.1038/aja.2009.92

Menon A. G., Thundathil J. C., Wilde R., Kastelic J. P., Barkema H. W. (2011). Validating the assessment of bull sperm morphology by veterinary practitioners. Can. Vet. J. 52: 407-408.

Mukund V., Mukund D., Sharma V., Mannarapu M., Alam A. (2017). Genistein: Its role in metabolic diseases and cancer. In Critical Reviews in Oncology/Hematol., 119: 13-22. https://doi.org/10.1016/j.critrevonc.2017.09.004

Muthiapriani L., Herwijanti E., Novianti I., Furqon A., Septian W.A., Suyadi S. (2019). The estimation of semen production based on body weight and scrotal circumference on PO Bull at Singosari National Artificial Insemination Center. J. Anim. Sci., 2019; 29 (1): 1. https://doi.org/10.21776/ub.jiip.2019.029.01.09

Novianti I., Purwantara B., Herwijanti E., Nugraha C. D., Putri R. F., Furqon A., Septian W. A., Rahayu S., Nurgiartiningsih V. M. A, Suyadi S. (2020). Effect of breeds on semen characteristics of aged bulls in the Indonesian National Artificial Insemination Center. J. Anim. Sci., 30 (2): 1. https://doi.org/10.21776/ub.jiip.2020.030.02.10

Nugraha C. D., Widodo N., Kuswati K., Suyadi S. (2020). Potential semen quality rate of Ongole Crossbred Bulls at Cattle Breeding Station (UPT PT & HMT) Tuban - Indonesia. IOP Conference Series: Earth and Environmental Science, 2021; The 3rd International Conference of Animal Science and Technology, Makassar, Indonesia, 3-4 November 788 (1): 012135. https://doi.org/10.1088/1755-1315/788/1/012135

Orrego M. T., Melian S. I., Montenegro J., Cimato A. N., Cisale H., Piehl L. L. (2019). Boar sperm protein tyrosine phosphorylation in the presence of egg yolk soluble and low density lipoprotein fractions during cooling. Theriogenology,

123 (2019): 151–158.

Pardede B. P., Agil M., Karja N. W. K., Sumantri C., Supriatna I., Purwantara B (2022). *PRM1* Gene Expression and Its Protein Abundance in Frozen-Thawed Spermatozoa as Potential Fertility Markers in Breeding Bulls. Vet. Sci., 9: 111. https://doi.org/10.3390/vetsci9030111

Peris-Frau P., Soler A. J., Iniesta-Cuerda M., Martín-Maestro A., Sánchez-Ajofrín I., Medina-Chávez D. A., Garde J. J. (2021). Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. Int. J. Mol. Sci., 21 (8): 1-22.

Pour A. H., Tahmasbi A. M., Naserain A. (2013). A. The influence of vitamin E on semen characteristics of ghezel rams in during cooling and frozen process. Euro. J. Zoolog. Res., 2 (5): 94-99.

Prihantoko K. D., Yuliastuti F., Haniarti H., Kusumawati A., Widayati D. T., Budiyanto A. (2020). The Effect of genistein on the plasma membrane integrity of frozen Ongole Grade Bull semen based on skim milk-soy lecithin extender. IOP Conference Series: Earth and Environmental Science International Conference, Improving Tropical Animal Production for Food Security, South East Sulawesi, Indonesia, 22-24 November 2019, 465: 012054 https://doi.org/10.1088/1755-1315/465/1/012054.

Rassu, G., Porcu, E. P., Fancello, S., Obinu, A., Senes, N., Galleri, G., Migheli, R., Gavini, E., Giunchedi, P. Intranasal delivery of genistein-loaded nanoparticles as a potential preventive system against neurodegenerative disorders. Pharmaceutics, 2019; 11 (1): 8. https://doi.org/10.3390/pharmaceutics11010008.

Ratnani H., Ihsan M. N., Ciptadi G., Suyadi S. (2017). Effect of α-Tocopherol supplementation in the extender on the sperm quality of Maduran Bull before and after quick freezing. Int. J. Adv. Res., 5 (7): 1378–1389. https://doi.org/10.21474/ijar01/4849.

Sharifi-Rad J., Quispe C., Imran M., Rauf A., Nadeem M., Gondal T. A., Ahmad B., Atif M., Mubarak M. S., Sytar O., Zhilina O. M., Garsiya E. R., Smeriglio A., Trombetta D., Pons D. G., Martorell M., Cardoso S. M., Razis A. F. A., Sunusi U., Calina D. (2021). Genistein: An integrative overview of its mode of action, pharmacological properties, and health benefits. Oxid. Med. Cell. Longev., 3268136: 1-36. https://doi.org/10.1155/2021/3268136.

Sharma Y., Sharma M. (2020). Sperm Cryopreservation: Principles and Biology. J. Infertile. Reprod. Biol. 8 (3): 43–48. https://doi.org/10.47277/JIRB/8(3)/43.

Söderquist L., Janson L., Håård M., Einarsson S. (1996). Influence of season, age, breed and some other factors on the variation in sperm morphological abnormalities in Swedish dairy A.I. bulls. Anim. Reprod. Sci., 44 (2): 91-98. https://doi.org/10.1016/0378-4320(96)01498-4

Susilawati T., Sholikah N., Wahjuningsih S., Herwiyanti E., Kuswati, Yekti A. P. A. (2020). Relationship of Scrotal Circumference with Spermatozoa Production in Various Breed of Indonesian Local Bulls. Am. J. Anim. Vet., 15 (2): 102.107

Suyadi S., Purwantara, B., Furqon, A., Septian, W. A., Novianti, I., Nursita, I. W., Nugraha, C. D., Putri, R. F., Pratiwi, H., Herwiyati E (2020). Influences of bull age and season on sperm motility, sperm concentration, and ejaculate volume of Ongole Grade cattle in Singosari National Artificial Insemination Center. J. Indonesian. Trop. Anim. Agric., 45 (4): 261-267. https://doi.org/10.14710/jitaa.45.4.261-267.

Tapia J. A., Macias-Garcia B., Miro-Moran A., Ortega-Ferrusola C., Salido G. M., Peña F. J., Aparicio I. M. (2012). The Membrane of the Mammalian Spermatozoa: Much More Than an Inert Envelope. Reprod. Dom. Anim., m 47 (Suppl. 3),: 65–75. https://doi.org/10.1111/j.1439-0531.2012.02046.x

Tuli H. S., Tuorkey M. J., Thakral F., Sak K., Kumar M., Sharma A. K., Sharma U., Jain A., Aggarwal V., Bishayee A. (2019). Molecular mechanisms of action of genistein in cancer: Recent advances. Front. Pharmacol., 10: 1336. https://doi.org/10.3389/fphar.2019.01336.

Wahjuningsih S., Ihsan M. N., Yekti A. P. A., Tahar M. A. (2020). The effect of soybean extract supplementation to tris aminomethane–egg yolk extender on the post-thawing quality of Simmental semen. J. Anim. Sci., 30 (3): 1. https://doi.org/10.21776/ub.jiip.2020.030.03.10

Wong R. L. Y., Walker C. L. (2013). Molecular Pathways: Environmental Estrogens Activate Nongenomic Signaling to Developmentally Reprogram the Epigenome. Clin. Cancer. Res., 19(14):1-10. https://doi.org/10.1158/1078-0432.CCR-13-0021.

Zubair M., Lodhi L. A., Ahmad E (2013). Hypo osmotic swelling test as screening for evaluation of semen of bull. J. Entomol. Zool. Stud., 1: 124–128.