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THE PRODUCTION OF FREEZE-DRIED EGG YOLK POWDER AND ITS EFFECT ON THE QUALITY OF GARUT RAM LIQUID SEMEN

Oriza Savitri Ariantie[1,2], Amrozi Amrozi[3], Tuty Laswardi Yusuf[3], Nurul Taufiqu Rochman[4], and Bambang Purwantara[3]*

[1]Reproductive Biology Study Program, Graduate *School* IPB University, Bogor, Indonesia
[2]Seameo Biotrop (Southeast Asian Regional Centre for Tropical Biology), Bogor, Indonesia
[3]Clinic Reproduction and Pathology Department, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia
[4]Metallurgical and Materials Research Center, Indonesian Institute of Sciences, Tangerang Selatan, Indonesia
*Corresponding author: purwantara@ipb.ac.id

ABSTRACT

This research aims to evaluate the protective potency of freeze-dried egg yolk powder on Garut ram sperm during the liquid semen preservation process. Semen with good sperm quality was divided into three groups and diluted using the following Tris diluents: fresh egg yolk-Tris (FEY-Tris), commercial egg yolk powder-Tris (CEY-Tris), and freeze-dried egg yolk powder-Tris (DEY-Tris). Semen that had been diluted was observed every 12 hours until sperm progressive motility was 50%. Sperm quality was tested for progressive motility percentages, viability, intact plasma membrane (IPM), and intact acrosome (IA). Fresh egg yolk had a particle size of 14460.00±330.76 nm, with polydispersity index value (PI) and zeta potential (ZP) being 1.00±0.11 and -9.22±0.30 mV, respectively. The particle size of commercial egg yolk powder was 877.90±168.86 nm with PI value 0.34±0.04 and ZP-28.7±1.24 mV. The particle size of freeze-dried egg yolk powder was 1296.00±86.73 nm, with PI value 0.70±0.04 and ZP -34.5±0.64 mV. Progressive motility percentage showed that DEY-Tris diluent managed to survive to 51.11±6.06% for 168 hours, compared to FEY-Tris (51.25±6.74%) which survived for 156 hours, although no longer than CEY-Tris diluent (53.47±6.33%) which survive for 180 hours (P<0.05). Higher sperm motility was also supported by viability percentage, IPM, and IA, which were all higher (P<0.05). In conclusion, freeze dried egg yolk powder mixed with Tris buffer was able to maintain sperm quality during the liquid semen preservation process.

Key words: commercial egg yolk powder, freeze-dried egg yolk powder, Garut ram, liquid semen, particle size

ABSTRAK

Penelitian ini bertujuan mengetahui kemampuan bubuk kuning telur kering beku terhadap perlindungan spermatozoa domba Garut selama proses preservasi semen cair. Semen dengan kualitas spermatozoa yang baik dibagi menjadi tiga bagian yang diencerkan dengan pengencer Tris, yaitu: Tris-kuning telur segar (Tris-KTS), Tris-bubuk kuning telur komersial (Tris-KTK), dan Tris-bubuk kuning telur kering beku (Tris-KTB). Semen yang telah diencerkan, diamati setiap 12 jam hingga motilitas progresif spermatozoa 50%. Kualitas spermatozoa yang diuji adalah persentase motilitas progresif, viabilitas, membran plasma utuh (MPU), dan tudung akrosom utuh (TAU). Kuning telur segar memiliki ukuran partikel sebesar 14460,00±330,76 nm, dengan nilai polydispersity index (PI) dan zeta potential (ZP) masing-masing 1,00±0,11 dan -9,22±0,30 mV. Ukuran partikel bubuk kuning telur komersial adalah 877,90±168,86 nm, dengan nilai PI 0,34±0,04 dan ZP -28,7±1,24 mV. Ukuran partikel bubuk kuning telur kering beku adalah 1296,00±86,73 nm, dengan nilai PI 0,70±0,04 dan ZP-34,5±0,64 mV. Persentase motilitas progresif dalam pengencer Tris-KTB dapat bertahan sampai 51,11±6,06% selama 168 jam lebih baik dibandingkan dengan Tris-KTS (51,25±6,74%) selama 156 jam, meskipun tidak lebih lama dari Tris-KTK (53,47±6,33%) selama 180 jam (P<0,05). Motilitas spermatozoa yang tinggi diimbangi pula dengan persentase viabilitas, MPU, dan TAU yang tinggi (P<0,05). Disimpulkan bahwa bubuk kuning telur kering beku yang dicampurkan dengan buffer Tris mampu mempertahankan kualitas spermatozoa selama proses preservasi semen cair.

Kata kunci: bubuk kuning telur kering beku, bubuk kuning telur komersial, domba garut, semen cair, ukuran partikel

INTRODUCTION

Egg yolk-Tris diluent is a semen diluent that is widely used in some countries, including Indonesia. Freeze-dried egg yolk powder has been used to dilute the semen of sheep, goats, and cows (Ansari *et al.*, 2010; Alcay *et al.*, 2015; Alcay *et al.*, 2016; García *et al.*, 2018). Egg yolk is a cryoprotective agent with a collection of fat and proteins with a size of 0.8-10 µm (Stádník *et al.*, 2015; Villarreal *et al.*, 2018). The components of egg yolk that are responsible for the cryoprotectant effect are lecithin, phospholipid, and lipoprotein fraction (Vishwanath and Shannon, 2000; Aboagla and Terada, 2004). Egg yolk as a semen diluent functions as an energy source, a buffer for osmotic pressure, and an extracellular cryoprotectant which can cover the plasma membrane to protect sperm from cold stress and maintain plasma membrane stability during the semen preservation process (Moussa *et al.*, 2002; Amirat *et al.*, 2004; Khalifa and El-Saidy, 2006; Ricker *et al.*, 2006).

Fresh egg yolk is processed into powdered egg yolk by freeze-drying it or by lyophilization, which is a method of drying biological products that involves freezing and dehydration (Alcay *et al.*, 2016). The advantages of this method are that it is able to maintain the quality of the drying results for a long time, is more practical so that it is ready to use without having to manually crack the fresh eggs for the yolk, is available at all times, and is very suitable for protein biomolecules, like the egg yolk (Alcay *et al.*, 2015). This method provides a solution that produces a dry egg yolk product that is stable both in quality and structure, so that it does not change the aroma, color, and other organoleptic elements. It also does not shrink or change shape in the structure of the material, and has high rehydration power with an excellent drying result that is very hollow and is also lyophile so it can return

to almost the same physiological, organoleptic, and physical properties before drying (Alcay *et al.,* 2016; García *et al.,* 2018).

Preservation of liquid semen that is stored for long periods of time can result in physical and functional changes of spermatozoa which can reduce motility, viability, plasma membrane integrity, and acrosome integrity (Riha *et al.,* 2006; Chelucci *et al.,* 2015). Using egg yolk powder can be an alternative to fresh egg yolk as a semen diluent in the liquid semen preservation process. In Indonesia, the information regarding the production and use of freeze-dried egg yolk powder as a semen diluent is not yet known. Therefore, this study was conducted with the aim of making, proving, and comparing the potential of egg yolk powder with fresh egg yolk and commercial egg yolk powder on the preservation of liquid Garut ram semen. The benefit of this research is to find an alternative to fresh egg yolk as a semen diluent for Garut ram semen by utilizing freeze-dried egg yolk powder.

MATERIALS AND METHODS

Experimental Animals

The animals used as a source of semen were six male rams in the age group of 3-6 years old with a body weight range of 35-55 kg. Males with good semen quality were identified by the following criteria: motility of more than 75%, a concentration of more than $2500x10^6$ cells per mL, and abnormalities less than 15% (Yodmingkwan *et al.,* 2016). The feed given was fresh elephant grass at 20% of body weight and concentrated in the form of pellets containing 16% protein and bran containing 12.5% protein each as much as 2% of body weight. The rams were given this feed twice a day and were watered *ad libitum*. The use of experimental animals and all handling procedures in the research had received approval from the Commission for the Supervision of Welfare and Use of Animal Research, Bogor Agricultural University, Number 118-2018 IPB.

Research Procedure

The production of freeze-dried egg yolk powder and its effect on the quality of liquid semen consisted of several procedures: making freeze-dried egg yolk powder, analyzing particle size, polydispersity index (PI), and zeta potential (ZP) of fresh egg yolk (FEY), freeze-dried egg yolk powder (DEY powder), and commercial egg yolk powder (CEY powder), measuring zeta potential (ZP) of Garut ram sperm cells, CEY powder and CEY powder morphology, preparing diluent media, collecting semen and evaluating fresh semen, and preserving the liquid semen.

Producing Freeze-Dried Egg Yolk Powder

The egg yolk used for the freeze-dried egg yolk was egg yolk from Lohmann Brown chicken eggs with an age range of 54-84 weeks (Global Buwana Farm, Ciampea, Bogor). The criteria for the eggs used are eggs from brood stock that are not inseminated by males; that are also fresh, oval, dark brown, thick, not cracked or dirty, and with little to no red spots on the eggshells. When the shell was broken down the yolk had to be still intact and the yolk light in color (not pale).

The yolk was separated from the egg white by placing it on filter paper and discarding the egg white. The yolk membrane was then broken down, and placed in a 500 mL measuring cup. The yolk was then frozen at 20° C for 24 hours. The yolk drying process was carried out using a freeze dryer (Christ®, Gamma 1-20) at a temperature of -20° C and a pressure of 1.030-0.630 millibars for 144 hours, and then crushed using a mortar before being filtered with a stainless-steel sieve to separate the fine and coarse egg yolk powder. Soft egg yolk samples were stored at -20° C.

Analysis of Particle Size, Polydispersity Index and Zeta Potential of Fresh Egg Yolk, Freeze Dried Egg Yolk Powder, and Commercial Egg Yolk Powder

Each 1.04 g of DEY powder and CEY powder were dissolved in 10 mL of aquadest. FEY, CEY powder, and DEY powder were taken at upwards of 45 µL each and mixed with 20 mL of aquadest, before being put into each disposable sizing cuvette for particle measurement and PI value. For ZP measurement, the samples were put into clear disposable zeta cell. Measurements were made using the Malvern Zetasizer Nano Series Nano-ZS (Malvern, UK) in dynamic light scattering (DLS) mode for measurement of particles and PI values, and in electrophoretic light scatting (ELS) mode for ZP measurements (Bhattacharjee, 2016). Each measurement corresponded to twelve autocorrelation functions which were repeated three times.

Zeta Potential Measurement of Garut Ram Sperm Cells

The sperm cell surfaces of the Garut rams were measured using the Malvern Zetasizer Nano Series Nano-ZS (Malvern, UK). The semen diluent medium (1:100) (sperm cells: deionized water) was put into a 1 mL cuvette, and then placed into a zetasizer for measurements (Magdanz *et al.,* 2019). Measurements were made three times and yielded a 100% mean zeta potential of -42.5±7.26 mV (Figure 1).

DEY powder and CEY powder was sprinkled on the surface of the double-type carbon that coated the specimen holder, and then they were coated with gold with a sputter current of 20 mA for 60 seconds using a Quorum Q150R ES sputter coater (Quorum Technologies Ltd, UK). DEY powder and CEY powder that had been coated on the specimen holder were installed in during the stage. They were each inserted in the chamber with the imaging using SEM ZEISS Type EVOMA10 (Carls ZEISS, USA). SEM was operated with standard operating parameters using a secondary electron detector, and a working distance of 10.0 mm and an electron high tension (EHT) of 16.00 kV.

Preparation of Diluent Media

The diluent medium used was a Tris buffer diluent containing 2.98 g of Tris hydroxymethyl

aminomethane, 1.65 g of citric acid monohydrate, 2 g of fructose, and 100 mL of milli-Q water (Ariantie *et al.*, 2013). The Tris buffer diluent was added with FEY, CEY powder, and DEY powder each up to as much as 20% (Guerrero 2002; Alcay *et al.*, 2016; Anand *et al.*, 2017) as presented in Table 1. FEY-Tris, CEY-Tris, and DEY-Tris diluent were stirred with a speed of 500 rpm. The solution was centrifuged for 30 minutes at a speed of 3000 rpm, and each diluent was added with penicillin and streptomycin antibiotics, each with a dose of 1000 IU/mL and 1 mg/mL, respectively.

Semen Collection and Fresh Semen Evaluation

The semen was collected by using an artificial vagina once a week with six reservoirs, each consisting of two ejaculates (two reservoirs in one holding period). The collected semen was immediately stored in a water bath (32° C) during evaluation and immediately processed into liquid semen. Fresh semen evaluation was carried out both macroscopically and microscopically. The macroscopic evaluation of the semen includes volume, pH, consistency, and color. The volume was determined by observing the measuring pipette. The degree of acidity (pH) was assessed using pH indicator paper 6.4-7.2. Consistency was assessed by tilting the tube with viscous and moderate assessment criteria. The color was visually assessed and differentiated into cream and milky white. Meanwhile, microscopic evaluation of spermatozoa quality included mass movement, progressive motility, individual movement, concentration, viability, abnormal morphology, intact plasma membrane (IPM) and intact acrosomal (IA).

The mass movement was carried out by putting 5 µL of fresh semen on a microscope slide and observing it using a microscope with 100x magnification. The assessment was carried out by looking at the thickness of the mass of the spermatozoa and the speed of the spermatozoa waves migrating (David *et al.*, 2015).

The motility of the spermatozoa and individual movements were observed under a microscope at 400x magnification with 10 µL of semen being added to 80 µL of saline solution. The percentage of spermatozoa motility was estimated from 5 fields of view by comparing the number of spermatozoa moving forward with the movements of other spermatozoa (dead spermatozoa, moving in place, turning, and moving backward) (David *et al.*, 2015).

The concentration of total spermatozoa was counted using Neubauer's counting chamber (Marienfeld, Lauda-Konigshofen, Germany) (O'Brien *et al.*, 2019). The semen was diluted using a formolsaline solution in a ratio of 1:500 (2 µL semen : 998 µL formolsaline) and homogenized in a 1.5 mL microtube. The composition of the formolsaline solution was 6.19 g of disodium hydrogen phosphate dihydrate, 3.54 g of potassium dihydrogen phosphate, 5.41 g of sodium chloride, 125 mL of 37% formaldehyde solution, and 875 mL of Milli-Q water (Nagorsen and Peterson, 1980; Munson, 2000). The total concentration of spermatozoa was observed under a microscope at 400x magnification.

The viability and abnormality of spermatozoa was tested using eosin-nigrosin staining (Barth and Oko, 1989). 5 µL of semen was added with 50 µL of eosin

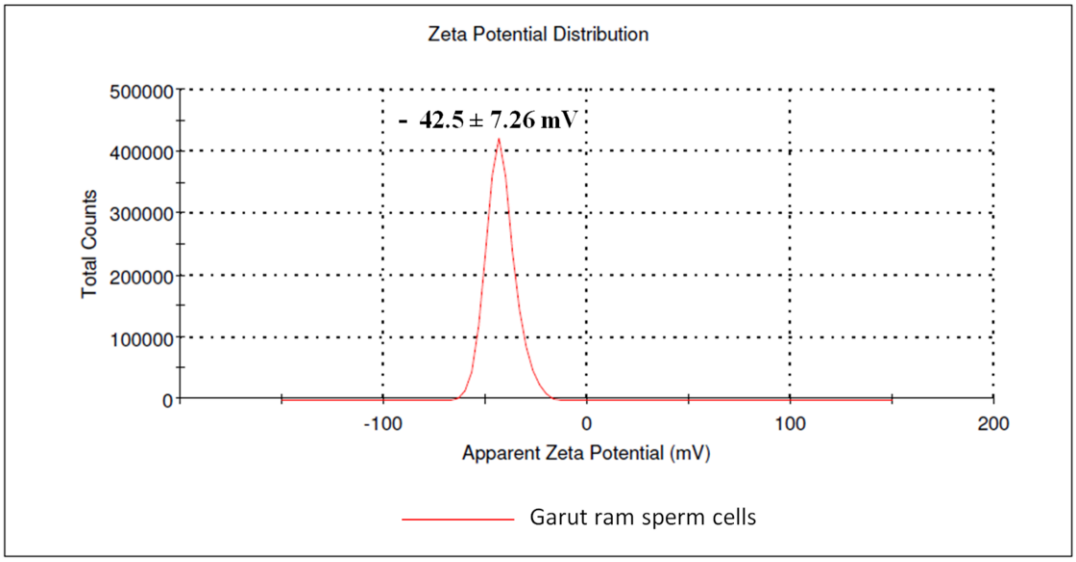

Figure 1. Zeta potential distribution of Garut ram sperm cells with a value of -42.5±7.26 mV

Table 1. Composition of liquid semen diluent

Material	FEY-Tris	CEY-Tris	DEY-Tris
Tris buffer (mL)	20	22.39	22.39
FEY (mL)	5		
DEY/CEY powder (g)		2.61	2.61
Total (mL)	25	25	25
Antibiotics: Penicillin (IU/mL)	1000	1000	1000
Streptomycin (mg/mL)	1	1	1

FEY-Tris= Fresh egg yolk-Tris; CEY-Tris= Commercial egg yolk powder-Tris; DEY-Tris=: Freeze-dried egg yolk powder-Tris

nigrosin dye, then homogenized on a glass object and made into a peripheral smear and dried on a heating table. The composition of the eosin-nigrosin staining was 20 g nigrosin, 1.5 g sodium citrate, 3.3 g eosin yellow, and was dissolved in 300 mL milli-Q water. The calculations were carried out in 10 fields of view or with a minimum number of spermatozoa with 200 sperm cells using a light microscope with a magnification of 400x. The viability assessment was carried out by looking at the dead spermatozoa that absorbed the red color, and the living ones that did not absorb the color (transparent). Meanwhile, abnormalities were assessed by looking at primary, secondary, and tertiary abnormalities.

IPM spermatozoa was carried out by dissolving 10 µL of semen in 1000 µL of hypoosmotic swelling test (HOS-test) solution before being incubated at 37° C for 30 minutes. The composition of the HOS solution followed the method by Jeyendran *et al.* (1984), with 7.5 g sodium citrate and 13.52 g fructose dissolved in 1000 mL milli-Q water. The osmolarity of the HOS solution was 150 mOsm. The spermatozoa were calculated in 10 fields of view or with a minimum number of spermatozoa with 200 sperm cells at a microscope magnification of 400x. The IPM was characterized by a coiled spermatozoa tail, while the damaged one was characterized by a straight tail.

IA spermatozoa was done by diluting the semen using formolsaline solution ,which functions to fix the spermatozoa, and was made into a peripheral smear and dried on a bunsen and stained with 5% (v/v) aniline blue and 0.5% (v/v) crystal violet for 15 seconds (Martecikova *et al.*, 2010). Spermatozoa that still have intact acrosomal were marked with 1/2 to 2/3 of the anterior part of the head darker than the posterior part. Meanwhile, incomplete spermatozoa were seen without any boundaries between the anterior and the posterior parts (Petkov *et al.*, 2007). Spermatozoa were calculated in 10 fields of view or with a minimum number of spermatozoa with 200 spermatozoa cells using a CKX41 phase-contrast microscope (Olympus, Tokyo, Japan) at 400x magnification.

Liquid Semen Preservation

Fresh semen from the two ejaculates was mixed into one, then divided into three parts and diluted with FEY-Tris, CEY-Tris, and DEY-Tris diluents (Table 1). The diluted semen was inserted into a 0.2 mL centrifuge tube with a dilution dose of $50x10^6$ spermatozoa per mL. The diluted semen was packed in a tube with a water jacket cooling system, and then stored in a refrigerator (3-5° C) and observed every 12 hours until the motility of spermatozoa was 50% (Ariantie *et al.*, 2014). The quality of the spermatozoa tested was measured by the percentage of progressive motility, viability, IPM and IA.

Data Analysis

The data was analyzed using comparative descriptive analysis and analysis of variance (ANOVA). If a significant difference was found between treatments, the Duncan test was then performed.

RESULTS AND DISCUSSION

Particle Measurement

The results of the FEY particle size measurement were 14460.00±330.76 nm as presented in Table 2. Table 2 shows that the three groups of FEY particle size distribution were not readable on the particle measuring instrument because the accurate size range read on the particle meter ranged from 10-10000 nm. FEY which went through a freeze-drying process for 144 hours at a temperature of -20° C and a pressure of 1.030-0.630 millibars was dried until FEY became a DEY powder. DEY powder was measured using a particle measuring instrument resulting in a size of 1296.00±86.73 nm (Table 2). Based on Table 2, there are three groups of DEY powder particle sizes, 74.3% DEY powder particles with a size of 749.2 nm and a concentration of 74.3% and the smaller size (191.9 nm) with a concentration of 19.4%. In addition, CEY powder particle size measurements were also carried out and resulted in a size of 877.90±168.86 nm, of which 82.6% of CEY powder particles were 446.7 nm in size and 12.3% had a size of 126.4 nm (Table 2). The uniformity of particles assessed based on the PI value showed that the FEY particles, DEY powder and CEY powder were 1.00±0.11, 0.70±0.04, and 0.34±0.04, respectively (Table 2).

The level of particle stability was indicated by the ZP value and the ZP values of the FEY particles, CEY powder and DEY powder were -9.22±0.30 mV, -28.7±1.24 mV and -34.5±0.64 mV, respectively (Figure 2).

The morphology of all CEY powder particles and DEY powder particles were spherical and polymorphic, respectively (Figure 3). CEY powder is uniformly spherical, which is also evidenced by the PI value of 0.34±0.04 and ZP -28.7±1.24 mV, which means that the CEY powder has a fairly good uniformity (medium monodipersion) and has a stable charge. DEY powder is in the form of polymorph, proven by the PI value of 0.70±0.04, which means that the DEY powder particles are high in polydispersion, but have a particle charge of -34.5±0.64 mV which means that they are very stable. If the particle morphology of CEY powder and DEY powder were related to particle size using a particle measuring instrument, the size in the particle morphology image was greater than the size on the particle measuring instrument. This is because when taking the image, the area that was taken was an area that had a large particle size.

Fresh egg yolk with a size of 14460.00±330.76 nm (Table 2) was processed into freeze-dried egg yolk powder. The fresh egg yolk freeze-drying process lasted for 144 hours at a temperature of -20° C and a pressure of 1.030-0.630 millibars. In addition to produce freeze-dried egg yolk, the process could reduce the particle size of fresh egg yolk with a size of 1296.00±86.73 nm, with a ZP value of -34.5±0.64 mV which means it is very stable. The freeze-dried egg yolk powder will be dispersed after diluted with distilled water. This monodisperse dilution will induce

more free movement of the freeze-dried egg yolk. The size of the freeze-dried egg yolk could not be reduced further by commercial milling equipment because of the hygroscopic nature of the egg yolk. Because egg yolk absorbs water molecules, during the process of reducing the size of the egg yolk powder it had a tendency to stick to the commercial milling tool (Figure 4). However, the freeze-dried egg yolk powder can be used as a semen diluent and was tested in comparison with fresh egg yolk and commercial egg yolk powder. The particle size of commercial egg yolk powder was measured and had a size of 877.90±168.86 nm.

The shrinkage of freeze-dried egg yolk powder is estimated to only last as long as the yolk becomes freeze-dried egg yolk powder, and the size of the frozen egg yolk powder is estimated to return to its initial size

Table 2. Distribution of sizes of FEY, CEY powder and DEY powder

Sample	Z-average (d.nm)	PI	Peak 1 mean (d.nm)	Peak 2 mean (d.nm)	Peak 3 mean (d.nm)	Peak 1 area%	Peak 2 area%	Peak 3 area%
FEY	14460.00±330.76	1.000±0.11	0.000	0.000	0.000	0.0	0.0	0.0
CEY	877.90±168.86	0.34±0.04	446.7	126.4	0.7650	82.6	12.3	5.1
DEY	1296.00±86.73	0.70±0.04	749.2	191.9	0.8963	74.3	19.4	6.3

FEY= Fresh egg yolk; CEY= Commercial egg yolk powder; DEY= Freeze-dried egg yolk powder

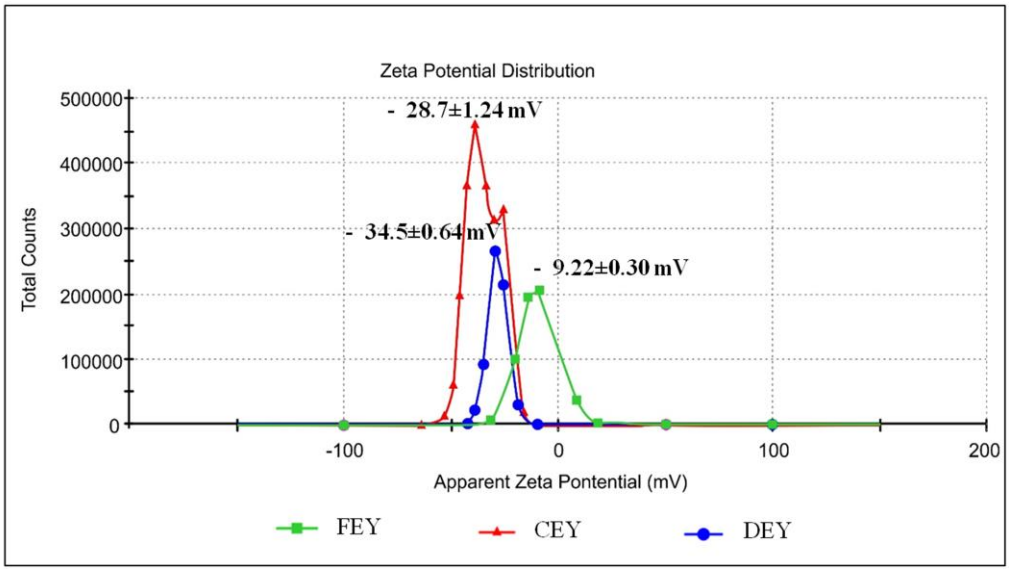

Figure 2. Distribution of sizes of FEY, CEY powder, and DEY powder

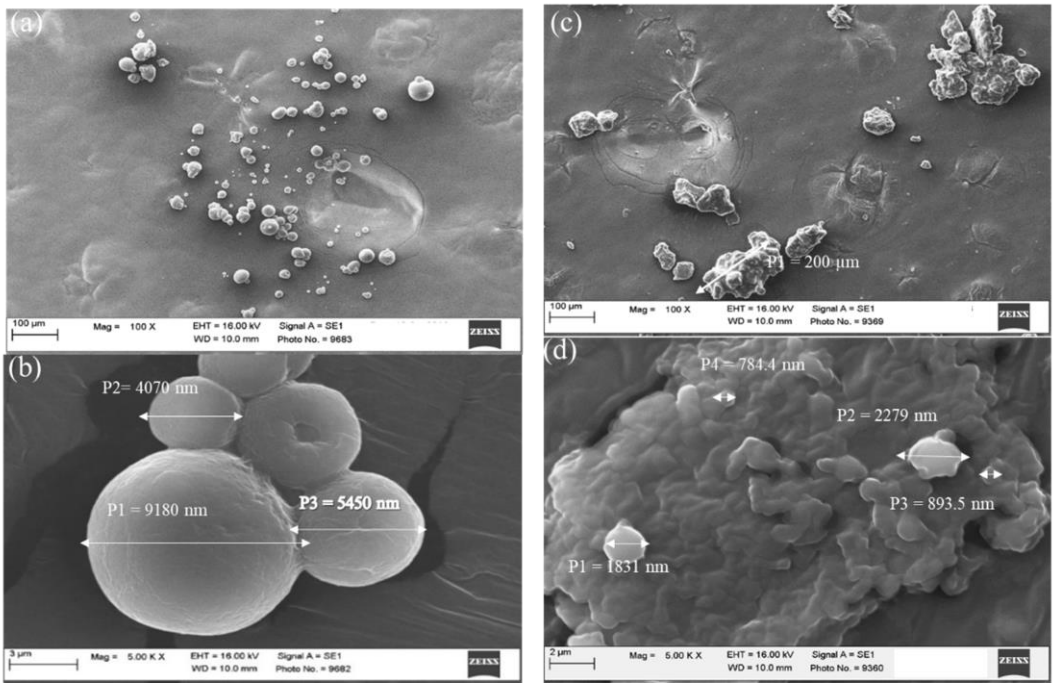

Figure 3. Scanning electron microscope (SEM) shows the morphology of commercial egg yolk powder (A= 100x, b= 5000x) and freeze-dried egg yolk powder (c= 100x, d= 5000x)

before the yolk is processed into egg yolk powder. This is based on the statement from Alcay *et al.* (2016) and García *et al.* (2018) which states that the freeze-drying process can cause shrinkage of the size of a particle but the freeze-drying product has a high rehydration element with a very hollow and lyophile drying result. This is so that it can return to almost the same physiological, organoleptic and physical properties before drying.

Fresh Semen and Liquid Semen Quality

Macroscopic and microscopic examination results of fresh semen from Garut ram in this study was semen that had a volume of 1.50±0.18 mL, pH 6.4, with a cream-color with moderate to thick consistency. It also tended to have thick mass waves and rapidly changed places, with a percentage of progressive motility of 79.31±1.93% with fast and very fast individual movement. Spermatozoa concentration was 3096.53±571.91 x 106 cells per mL, and the percentage of viability and abnormality was 88.12±3.40% and 7.23±3.23%, respectively. The percentage of IPM was 86.67±3.13% and IA was 84.80±2.52%.

The results of liquid semen storage at 3-5° C showed that the percentage of progressive motility of spermatozoa in FEY-Tris diluent could last up to 51.25±6.74% for 156 hours, which was lower than DEY-Tris (55.14±6.29%) and CEY-Tris (60.14±4.89%) ($P<0.05$). The percentage of progressive motility in DEY-Tris diluent was up to 51.11±6.06% for 168 hours, which was higher than FEY-Tris (46.53±6.83%) ($P>0.05$), but lower than CEY-Tris (56.25±5.57%) ($P<0.05$). The percentage of progressive motility in CEY-Tris diluent was up to 53.47±6.33% for 180 hours, higher than FEY-Tris (42.36±8.08%) and DEY-Tris (47.78±7.57%) ($P <0.05$) (Figure 5). Progressive motility examination of spermatozoa was supported by examination of viability, IPM and IA.

The percentage of viability of spermatozoa in FEY-Tris diluent could last up to 61.37±5.80% for 156 hours, which was lower and not significantly different from DEY-Tris (64.72±5.54%) ($P>0.05$), but significantly different from FEY-Tris (71.56±5.01%) ($P<0.05$). The percentage of viability of spermatozoa in

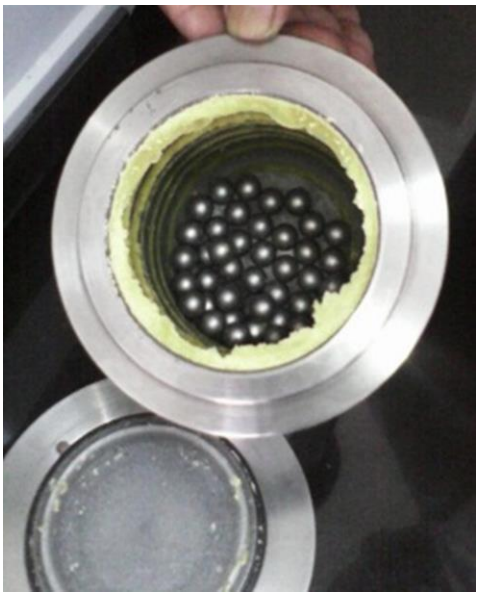

Figure 4. The crushing process to reduce the size of freeze-dried egg yolk powder

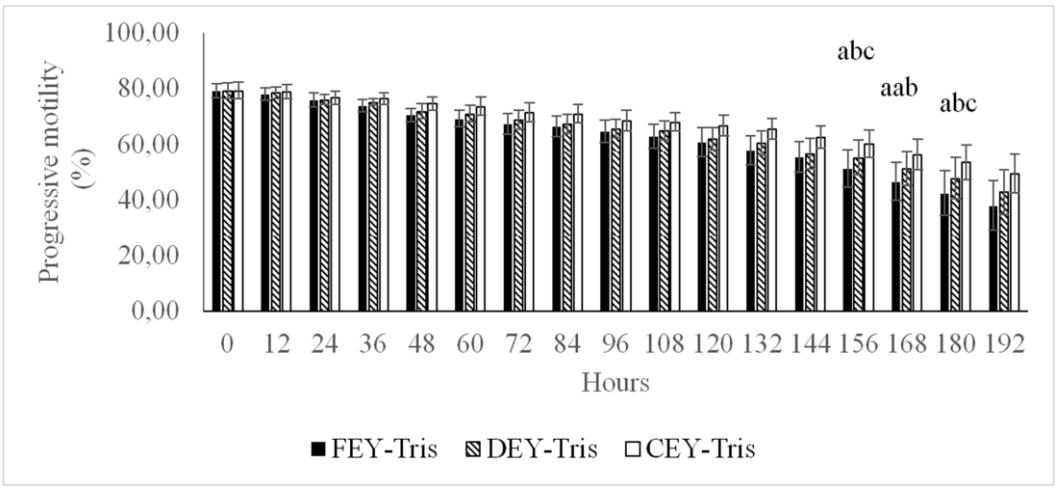

Figure 5. The percentages of progressive motility from the preservation of liquid Garut ram semen. [a, b, c]Superscripts at the same observation time showed a significant difference ($P<0.05$) in the groups between treatments

DEY-Tris diluent could last up to 61.96±6.11% for 168 hours, higher than FEY-Tris (57.15±7.06%) (P>0.05), but lower than FEY-Tris (69.16±5.15%) (P<0.05). The percentage of viability in CEY-Tris diluent was up to 66.00±4.78% for 180 hours, which was higher than FEY-Tris (53.14±8.48%) and DEY-Tris (59.15±6.02%) (P<0.05) (Figure 6).

The percentage of IPM in FEY-Tris diluent could last up to 59.43±5.89% for 156 hours, lower than DEY-Tris (63.05±5.62%) and CEY-Tris (69.58±4.33%) (P<0.05). The percentage of IPM in DEY-Tris diluent could last up to 60.21±6.32% for 168 hours, higher than FEY-Tris (55.62±6.92%), but lower than FEY-Tris (67.67±4.47%) (P<0.05). The percentage of IPM in CEY-Tris diluent could last up to 65.03±4.16% for 180 hours, which was higher compared to FEY-Tris (52.05±8.02%) and DEY-Tris (57.04±6.68%) (P<0.05) (Figure 7).

The percentage of IA in FEY-Tris diluent could last up to 58.27±5.69% for 156 hours, which was lower compared to DEY-Tris (61.87±5.50%) and CEY-Tris (67.82±4.06%) (P<0.05). The percentage of IA in DEY-Tris diluent could last up to 58.91±6.35% for 168 hours, higher than FEY-Tris (54.18 ± 7.19%), but lower than CEY-Tris (66.14 ± 4.38%) (P<0.05). The percentage of IA in CEY-Tris diluent could last up to 63.69±4.20% for 180 hours, higher than FEY-Tris (50.58±8.59%) and DEY-Tris (55.67±6.81%) (P<0.05) (Figure 8).

The average difference between progressive motility and viability of spermatozoa was 11.17%, and the difference between the percentage of IPM and IA and the percentage of progressive motility of spermatozoa up to 50% at the same hour was 11.17%, 9.28%, and 8.35% respectively (Table 3). The difference in the mean percentage of viability, IPM, and IA was higher than the percentage of motility because of the number of living spermatozoa, having intact plasma membranes and acrosome, but not necessarily all progressive motile.

The concentration of FEY, DEY, and CEY mixed with Tris diluent was 20% each (Guerrero, 2002; Alcay *et al*., 2016; Anand *et al.*, 2017). The quality of liquid semen using CEY-Tris diluent was longer in maintaining the progressive motility of spermatozoa with a percentage of 53.47±6.33% for 180 hours compared to DEY-Tris and FEY-Tris diluents with the percentage of progressive motility of 51.11±6.06% and

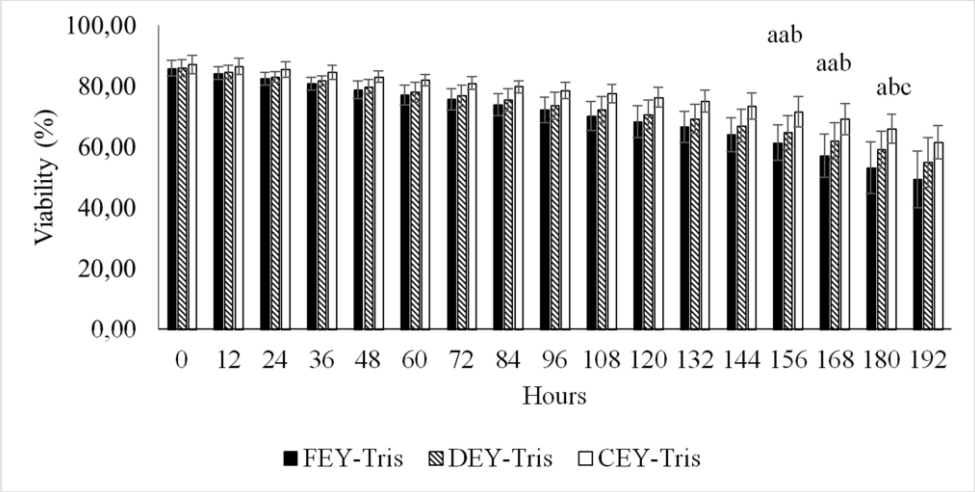

Figure 6. The percentages of viability from the preservation of liquid Garut ram semen. [a, b, c]Superscripts at the same observation time showed a significant difference (P<0.05) in the groups between treatments

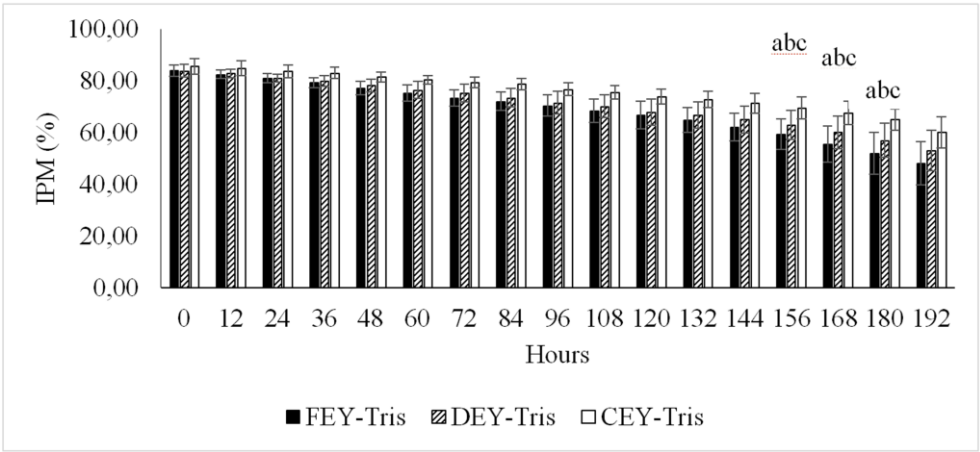

Figure 7. The percentages of IPM from the preservation of liquid Garut ram semen. [a, b, c]Superscripts at the same observation time showed a significant difference (P<0.05) in the groups between treatments

51.25±6.74%, respectively for 168 hours and 156 hours (Figure 5). The percentage of progressive motility of spermatozoa in the older CEY-Tris diluent was also supported by the higher percentage of viability, IPM and IA compared to the DEY-Tris and FEY-Tris diluents (Figure 6-8).

The percentage of spermatozoa quality using CEY-Tris diluent was higher because the commercial powder egg yolk added to the Tris diluent was smaller (877.90±168.86 nm) compared to freeze-dried egg yolk powder (1296.00±86.73 nm) and fresh egg yolk (14460.00±330.76 nm) (Table 2). The smaller size is thought to have less movement resistance, which makes it easier for spermatozoa to move. In addition, small particle sizes have large surface areas (Khan *et al.*, 2019). Ober and Gupta (2011) further explained that the surface area and the percentage of molecules for particle sizes of 100 nm, 1000 nm, 10000 nm are 1.26 x 105 nm2 and 2.97%; 1.26 x 107 nm2 and 0.30%; and 1.26 x 109 and 0.03%. The particle surface area of commercial egg yolk powder that is larger than fresh egg yolk and freeze-dried powder is thought to be more optimal in protecting spermatozoa cell membrane during the preservation process of liquid semen.

Freeze-dried egg yolk powder and commercial egg yolk powder had a particle size distribution marked with a PI value of 0.70±0.04 and 0.34±0.04, respectively, which means that the two powdered egg yolks have moderate levels of polydispersion (Ponnuraj *et al.*, 2015). The PI value of fresh egg yolk was 1.00±0.11, which means that it has a very high level of polydispersion (Ponnuraj *et al.*, 2015). Ponnuraj *et al.* (2015) further described that the range of PI values is divided into 4: 1) 0-0.05 (high monodispersion), 2) 0.05-0.08 (moderate monodispersion), 3) 0.08-0.7 (moderate polydispersion), and 4) >0.7 (high polydispersion). Based on the PI value, it shows that freeze-dried egg yolk powder and commercial egg yolk powder have a slightly better uniformity levels than fresh egg yolk, although based on the literature it has a moderate polydispersion level.

Freeze-dried egg yolk powder had a particle charge marked with a ZP value of -34.5±0.64 mV which means it is very stable, and commercial egg yolk powder had a charge value of -28.7±1.24 mV which means that it is also quite stable, while fresh egg yolk had a charge value of -9.22±0.30 mV which means it is very unstable (Bhattacharjee, 2016). Further explained by Bhattacharjee (2016), the ZP value is classified into 4 parts: 1) very unstable (0-10 mV), 2) quite stable (10-20 mV), 3) stable (20-30 mV)), 4) very stable (>30 mV). ZP values greater than +30 mV or less than -30

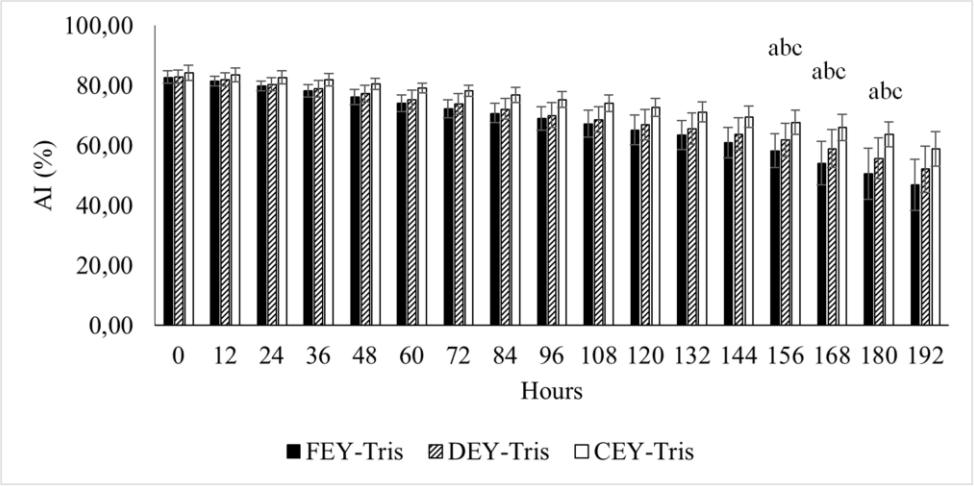

Figure 8. The percentage of IA resulted from the preservation of liquid Garut ram semen. [a, b, c]Superscripts at the same observation time showed a significant difference (P<0.05) in the groups between treatments

Table 3. The difference between the percentage difference in viability, intact plasma membrane, and intact acrosome with spermatozoa motility up to 50% at the same hour

Treatment	FEY-Tris	CEY-Tris	DEY-Tris	Difference in average (%)
Progressive motility and viability (%)				
Progressive motility	51.25	53.47	51.11	
Viability	61.37	66.00	61.96	
Difference (%)	10.12	12.53	10.85	11.17
Progressive motility and IPM (%)				
Progressive motility	51.25	53.47	51.11	
IPM	59.43	65.03	60.21	
Difference (%)	8.18	11.56	8.10	9.28
Progressive motility and IA (%)				
Progressive motility	51.25	53.47	51.11	
IA	58.27	63.69	58.91	
Difference (%)	7.02	10.22	7.80	8.35

IPM= Intact plasma membrane, IA= Intact acrosome, FEY-Tris= Fresh egg yolk-Tris, CEY-Tris= Commercial egg yolk powder-Tris, DEY-Tris= Freeze-dried egg yolk powder-Tris

mV will produce a stable particle. If the particles have a large negative or positive ZP value, the particles will repel each other and there will be no aggregation. On the other hand, if the particles have a small ZP value there is no force to prevent the particles from attracting each other and aggregation will occur (Yedurkar *et al.,* 2016).

The overall surface of the Garut ram spermatozoa cells was -42.5±7.26 mV. Meanwhile, the surface of bovine spermatozoa cells has a ZP value of -13.00 to -27.7 mV (Magdanz *et al.,* 2019). Magdanz *et al*. 2019 explained that even though the overall cell load of spermatozoa is negative, it is possible that some areas are positively charged on the surface of the spermatozoa cell membrane due to the loss of integration of negatively charged glycoproteins. Spermatozoa cells from the epididymis and ejaculation will cause a dynamic change in the surface charge of the spermatozoa from negative to positive (Simon *et al.,* 2016). Simon *et al.* (2016) explained that changes in the surface charge of spermatozoa occur due to the accumulation of components in the form of proteins and glycoproteins from the epididymis which gives the sperm a negative charge. The glycosylation of sialic acid residues forms a negative layer of 20-60 nm thick around the sperm which gives a negative charge of -16 to -20 mV (Yudin *et al.,* 2005; Chan *et al.,* 2006). Spermatozoa become positively charged when they lose glycoproteins attached to the plasma membrane (Simon *et al.,* 2016). Loss of glycoproteins occurs during apoptosis when the integrity of the membrane is lost due to translocation of phosphatidylserine from the inside to the outside of the plasma membrane and affects the fluidity of the plasma membrane (Mourdjeva *et al.,* 2005).

Egg yolk components that are involved in protecting spermatozoa during the preservation process of liquid semen include phospholipids, low-density lipoproteins (LDL), and cholesterol (Moussa *et al.,* 2002; Aboagla and Terada, 2004; Bergeron and Manjunath, 2006; Mocé *et al.,* 2010). These components are believed to be able to associate with the plasma membrane to form a protective layer on the surface and are able to replace plasma membrane phospholipids and maintain plasma membrane integrity (Kampschmidt *et al.,* 1953; Quinn *et al.,* 1980; Graham and Foote, 1987; Moussa *et al.,* 2002; Hu *et al.,* 2011). The use of fresh egg yolk can be replaced with freeze-dried egg yolk powder and commercial egg yolk powder because the two egg yolk powders have been shown to maintain the quality of spermatozoa during the liquid semen preservation process.

CONCLUSION

Freeze-dried egg yolk powder-Tris has the potential to maintain spermatozoa quality (up to a percentage of spermatozoa motility of 50%) longer (168 hours) than fresh egg yolk-Tris (156 hours). Commercial egg yolk powder-Tris can maintain the quality of liquid semen for 180 hours longer than freeze-dried egg yolk powder-Tris and fresh egg yolk-Tris.

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