INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
23–0343/2023/30–5–353–358
DOI: 10.17957/IJAB/15.2095
http://www.fspublishers.org

***Full Length Article***

# First Study of Aqueous Soybean (*Glycine max*) Extract Nanoparticles as a Substitute of Egg Yolk on Motility and Kinetic Parameters Spermatozoa of Frozen Semen

**Dian Ratnawati[1,2*†], Gatot Ciptadi[2†], Sri Rahayu[3†] and Trinil Susilawati[2†]**
[1]*Research Center for Animal Husbandry, National Research and Innovation Agency of the Republic of Indonesia (BRIN), Bogor, Indonesia*
[2]*Department of Animal Reproduction and Breeding, Faculty of Animal Science, Universitas Brawijaya, Indonesia*
[3]*Department of Biology, Faculty of Mathematic and Natural Science, Universitas Brawijaya, Malang, Indonesia*
[*]For correspondence: dian.sapo.2019@gmail.com
[†]Contributed equally to this work and are co-first authors
*Received 04 September 2023; Accepted 14 September 2023; Published 05 October 2023*

## Abstract

Nanotechnology could improve the solubility and stability of the particles, increase the surface area and effectiveness of the particles. The aims study was to examine the effectiveness of using aqueous soybean extract (ASE) nanoparticle as a substitute for egg yolk on the motility and kinetic parameters of frozen semen spermatozoa. The study was conducted at Beef Cattle Research Station, using OC bulls with progressive motility of fresh semen >70%. The basic diluents used was CEP-3(m) and aqueous soybean extract (nanoparticles) as a substitute for egg yolks. The research design was an experimental design, with 6 treatments and 5 repetitions, namely: P1 (control) = CEP-3(m) + EY 10%; P2 = CEP-3(m) + ASE 40%, P3 = CEP-3(m) + ASE 50%; P4 = CEP-3(m) + ASE 60%; P5= CEP-3(m) + ASE 70%, P6 = CEP-3(m) + ASE 80%. Parameters measured were fresh semen and motility of frozen semen spermatozoa (M, PM, VCL, VAP, VSL, LIN, STR, WOB, BCF, ALH, H) at 5$^{o}$C, before freezing (BF) and post thawing (PT). Data were analyzed using ANOVA with SPSS 16. Nanoparticles of aqueous soybean extract 50% and 60% in CEP-3 (m) based diluent were most effective in supporting the motility and its kinetic parameters of frozen semen spermatozoa, (PM, VSL, VAP, LIN and STR) and of low ALH and hyperactivity values. The pattern of movement of spermatozoa is regular, progressive and non-rotating, with good speedy. Further study of this diluent formula is needed with various parameters of spermatozoa quality. © 2023 Friends Science Publishers

**Keywords:** Aqueous soybean extract; Frozen semen; Motility

## Introduction

The process of semen cryopreservation causes a lot of damage to spermatozoa, so that it can reduce their fertility. The role of cryoprotectant is very important in preventing cold shock of spermatozoa during cryopreservation (Kasimanickam *et al.* 2011; Singh *et al.* 2012). The lecithin content in egg yolk is very commonly used as an extracellular cryoprotectant. However, it turns out that there are several negative effects that can be caused by egg yolk, including: antigenic, cytotoxic, bacterial endotoxin reduces spermatozoa fertility, potential for bacterial contamination; potential debris/ vacuole; non-standard composition; and can change the structure and integrity of the spermatozoa membrane (Bousseau *et al.* 1998; Layek *et al.* 2016; Toker *et al.* 2016; Mafolo *et al.* 2020; Toker and Alcay 2022).

Utilization of planted based of lecithin is very possible as an alternative to the use of egg yolks. The results of previous studies stated that lecithin in soybeans can replace the role of egg yolk lecithin as an extracellular cryoprotectant (Alvarez-Rodriguez *et al.* 2017; Pamungkas and Krisnan 2017; Sun *et al.* 2020). The use of commercial soy lecithin has been widely applied as a component in spermatozoa diluent. The results of research on the use of soybean as a raw material as a substitute for egg yolk have also been carried out on cattle and sheep with varying results ( Sugiarto *et al.* 2014; Coester *et al.* 2019; Immelda *et al.* 2019). Various methods of soybean extraction can be done including mechanically and chemically. The soybean extraction method determines the composition of the resulting extract and its ability to protect spermatozoa during frozen storage (Layek *et al.* 2016). Studies on the use of total soybean extract (whole soybean extract) in semen diluent are still limited. This is also related to the standard soybean extraction procedure for semen diluent which is not yet available. The results of previous studies showed that aqueous soybean extract at level 30% could support the quality of liquid semen until day 3–5 (Ratnawati *et al.* 2023). Nevertheless, the study

**To cite this paper:** Ratnawati D, G Ciptadi, S Rahayu, T Susilawati (2023). First study of aqueous soybean (*Glycine max*) extract nanoparticles as a substitute of egg yolk on motility and kinetic parameters spermatozoa of frozen semen. *Intl J Agric Biol* 30:353–358

of the usage of aqueous soybean extract as egg yolks replacer for frozen semen have not been conducted before.

There are obstacles in the utilization of soybean as a substitute for egg yolks (extracellular cryoprotectant), including its low solubility. One solution in this case is to reduce the particle size to be a nano size (0.1–<1000 nm), or better known as nanotechnology (Biswas *et al.* 2014; Lankalapalli *et al.* 2014). The advantages of using nanotechnology include; improve the solubility and stability of the particles, increase the surface area, increase the effectiveness of the particles. The cellular size of the nanoparticles allows for increased binding, absorption and reactivation. The resulting integration of cellular processes and physiological pathways is better, without disturbing the normal cell biological systems (Saadeldin *et al.* 2020). The results of previous research by (Nadri *et al.* 2019) showed that the use of soy lecithin nanoparticles (commercial) in semen diluents can increase the resistance to freezing (cryo-survival) of goat spermatozoa. Soy lecithin nanoparticle size (commercial) allows for increased interaction, reduced cold shock and increased antioxidant capacity during the freeze-thawing process (Sun *et al.* 2021).

The aim of this study was to examine the effectiveness of using aqueous soybean extract nanoparticle size as a substitute for egg yolk on the motility and kinetic parameters of frozen semen sperm of OC cattle.

## Materials and Methods

### Experimental details and treatment

**Experimental material:** The research activity was carried out at the Beef Cattle Research Institute. Duration of research was 2 months (March–April 2022). Material was used OC bulls aged 4–5 years with progressive motility of fresh semen >70%. The semen collection method used an artificial vagina, with a teaser using a estrous cow.

### Treatments

The research design used a completely randomized design (CRD) with 6 treatments and 5 repetitions. Treatment were given: T1 = CEP-3(m) + EY 10% (as a control); T2 = CEP-3(m) + ASE 40%; T3 = CEP-3(m) + ASE 50%; T4 = CEP-3(m) + ASE 60%; T5 = CEP-3(m) + ASE 70%; and T6 = CEP-3(m) + ASE 80%.

### Preparation of aqueous soybean extract nanoparticle

Soybean as much as 100 mg was cleaned and soaked in water for 6–9 h. Discard the soaking water and put the soybeans into warm water at 65°C for 10 min, then drained. Grinded the soybeans with 200 mL of distilled water and strained. Filtration results were centrifuged at 1008 *g*. Taken the top clear layer using a micropipette. The aqueous soybean extract obtained was mixed with CEP-3(m) as a based diluent according to the level of treatment. Aqueous soybean extract particles were measured using a PSA (Particle Size Analyzer) Horiba SZ-100, and potential zeta analysis.

The aqueous soybean extract obtained had a particle size of 223.1±113.0 nm with a polydispersity index (PI) of 0.981 (Fig. 1). The results of the zeta analysis of the potential of aqueous soybean extract showed a value of -31.9.

### Preparation of diluents CEP-3 (m)

Preparation of the basic diluent CEP-3(m) was started by mixing the components of the basic diluent of CEP-3(m), there were: KCl 0.05 mg; NaCl 0.09 mg; $MgCl_2(H_2O)_6$ 0.08 mg; $CaCl_2(H_2O)_2$ 0.04 mg; $NaH_2PO_4$ 0.11 mg; $NaHCO_3$ 0.1 mg; $KH_2PO_4$ 0.27 mg; Fructose 0.27 mg; citric acid 0.82 mg; Tris 1.62 mg; streptomycin 0.01 mg; penicillin 0.01 mg and 100 mL sterile distilled water. Stirred all the ingredients until homogeneous. Evaluated pH and made sure the pH around of 7 (neutral).

### Collecting fresh semen and observation of frozen semen quality

Semen collection activities was carried out twice a week. Fresh semen obtained was analyzed, including several parameters (pH, mass movement, concentration, motility, viability and abnormalities of spermatozoa). Fresh semen with progressive motility > 70%, processed into frozen semen and observed its motility at three observation points (5°C, before freezing and post thawing).

The initial stage of the motility examination is thawing the straw at 37°C for 30 sec. The mixture was placed into Eppendorf, tube then taking 3 $\mu$L of the mixture and placed on a glass object. Then put the glass object on the stage warmer, which was conditioned at 37°C and covered it with a cover glass. All the samples were observed using SCA (Microptics v. 2.1, Barcelona, Spain) at a magnification of 20, 10 and 5x fields.

### Statistically analysis

Parameters measured were fresh semen (pH, mass movement, concentration, motility, viability and abnormalities of spermatozoa) and motility of frozen semen spermatozoa (motility/M, progressive motility/PM, velocity curve linear/VCL, velocity average pathway/VAP, velocity straight linear/VSL, linearity/LIN, straightness/STR, wobble/WOB, beat cross frequency/BCF, amplitude lateral head/ALH, hyperactivity/H) at 5◦C, before freezing (BF) and post thawing (PT). Data were statistically treated for ANOVA using SPSS 16.

## Results

### Fresh semen quality

The fresh semen quality of OC cattle was shown in Table 1. The progressive motility requirement for fresh semen to be

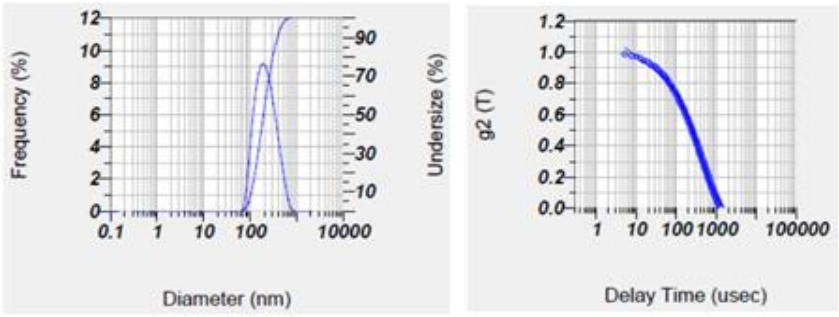


**Fig. 1:** The analysis results of aqueous soybean extract particle size with a particle size analyzer (PSA)

**Table 1:** Fresh Semen Quality

| No | Parameter quality of Fresh Semen | | | |
|---|---|---|---|---|
| 1 | Volume (mL) | 6.7 | ± | 1.6 |
| 2 | pH | 6.4 | ± | 0.0 |
| 3 | Colour | Creamy | | |
| 4 | Consistency | Thick | | |
| 5 | Concentration (million/mL) | 2191.9 | ± | 643.6 |
| 6 | Mass movement | 2.3 | ± | 0.8 |
| 7 | Motility | | | |
| | a. Progresif motility (%) | 85.6 | ± | 4.7 |
| | b. Motility (%) | 97.6 | ± | 3.4 |
| | c. VCL (µm/s) | 73.5 | ± | 4.5 |
| | d. VSL (µm/s) | 41.4 | ± | 9.3 |
| | e. VAP (µm/s) | 58.2 | ± | 8.4 |
| | f. LIN (%) | 56.1 | ± | 10.4 |
| | g. STR (%) | 70.5 | ± | 6.9 |
| | h. WOB (%) | 78.9 | ± | 7.7 |
| | i. ALH (µm) | 2.3 | ± | 0.3 |
| | j. BCF (Hz) | 7.7 | ± | 1.5 |
| | k. H (%) | 9.7 | ± | 4.7 |
| 8 | Viability (%) | 87.8 | ± | 5.4 |
| 9 | Abnormality (%) | 3.4 | ± | 3.0 |

processed into frozen semen must be >70%. Based on Table 1, it was known that the quality of the fresh semen has fullfilled the requirements for frozen semen. The standard requirements (quality semen) for bulls including; motility >50%, spermatozoa abnormalities <20%, concentration >1000 million/mL (Hafez and Hafez 2000). The fresh semen was processed into frozen semen and observed for its motility at 5°C, before freezing and post thawing.

**Motility and its kinetic parameter of frozen semen**

The observation of motility and its kinetic parameters of frozen semen was shown in the Fig. 2. Values of PM and M at $5^0$C and BF did not show many differences, nevertheless it was known at PT that CEP-3(m) and ASE 80% sharply decreased. Likewise, motility at CEP-3(m) + ASE 80% also showed a decrease at PT. Progressive spermatozoa motility in CEP-3 (m)+ EY 10% and CEP-3(m) + ASE (50–60%) was higher than the other diluents. Spermatozoa motility in CEP-3(m) + ASE 80% was significantly lower than other diluents.

Velocity at $5^0$C and BF also did not show much changed. At PT, it was found that the VCL value of diluent CEP-3(m) + EY 10% was significantly higher than that of diluent CEP-3(m) + ASE (40–80%). The VSL value of spermatozoa in CEP-3(m) + ASE 50% diluent was higher than other diluents. The VAP values of spermatozoa in CEP-3(m)+ EY 10% and CEP-3(m) + ASE (40–50%) diluents were higher than other diluents.

The LIN, STR and WOB values at PT are higher than at $5^0$C and BF. At PT, the LIN, STR, and WOB spermatozoa values in CEP-3(m) + EY 10% were significantly lower than the other diluents. The ALH and H values increase when BF and decrease when PT, meanwhile BCF values increase at BF and PT. During PT, the ALH and H values of spermatozoa in CEP-3(m) + EY 10% were higher than CEP-3(m) + ASE (40–80%).

## Discussion

Spermatozoa motility is a parameter of spermatozoa quality, which is needed by spermatozoa in reaching the zona pellucida. Motile spermatozoa indicate that the spermatozoa are alive and have an intact membrane. Damaged cell membranes can disrupt the metabolism of spermatozoa so that ATP is not produced and unable to move, which ends in death. Some of these considerations indicate the importance of spermatozoa motility as an initial parameter to determine the quality of spermatozoa. Diluents is one of the factors that determine the motility of spermatozoa. The components in the diluent are expected to support the life of spermatozoa and maintain good quality during cryopreservation (Ratnawati *et al.* 2017, 2018; Luna-Orozco *et al.* 2019). The use of aqueous soybean extract (nanoparticles) as a substitute for egg yolk in its role as an extracellular cryoprotectant against spermatozoa motility is shown in Fig. 2.

Assessment of spermatozoa motility using CASA produces an objective, accurate, fast, efficient assessment and provide a detailed description of spermatozoa motility (Contri *et al.* 2010). The PM and M of frozen semen spermatozoa decreased gradually at $5^0$C, BF and PT. Spermatozoa motility during PTM was very low, especially in CEP-3(m) + ASE 80% diluent. Spermatozoa motility during PTM between controls with CEP-3(m) + ASE (50–60%) showed the highest results compared to other formulas. This shows that the diluent formula can minimize damage due to cryopreservation (cryodamage) and maintain the quality of

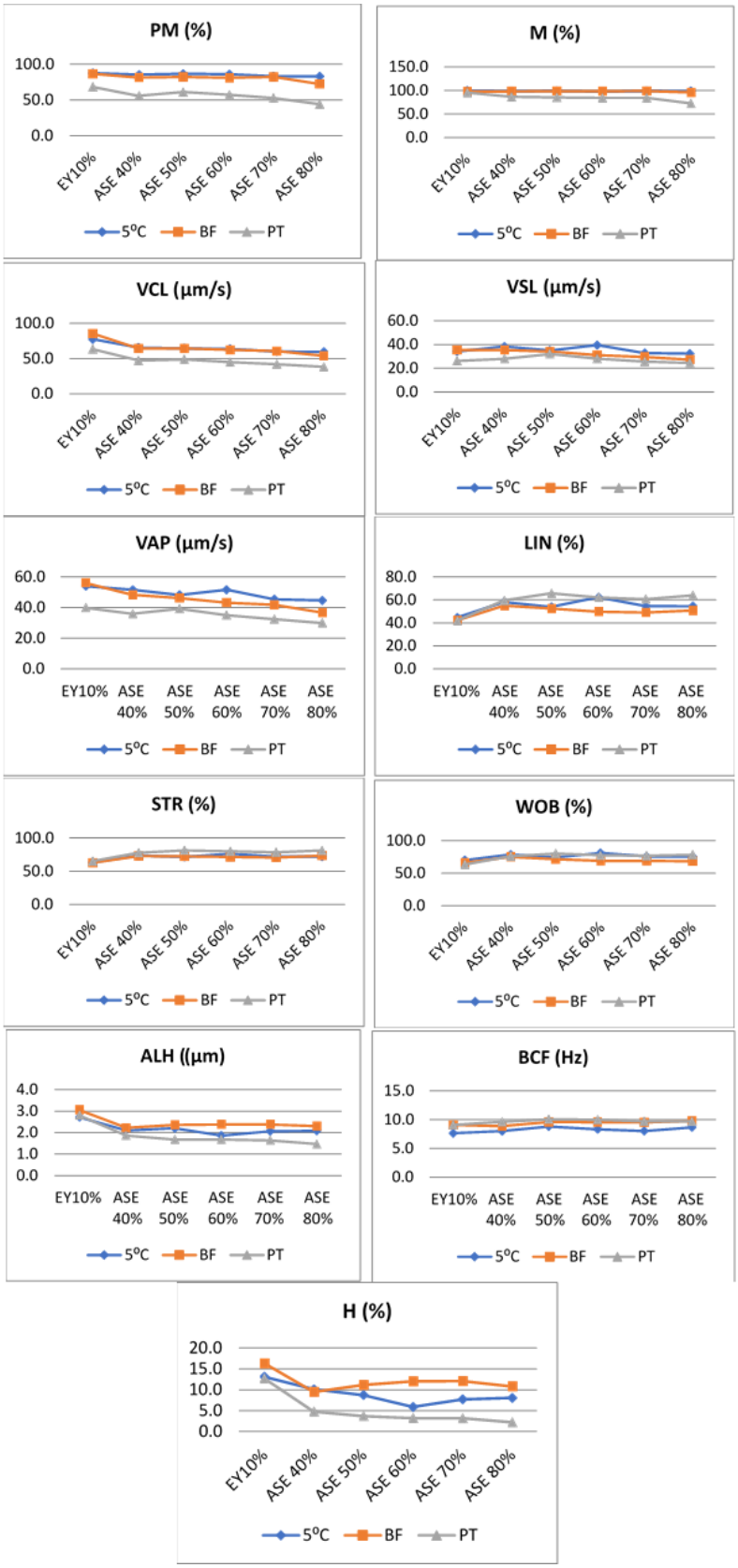


**Fig. 2:** The Motility and its kinetic parameter spermatozoa of frozen semen at three points of observation

spermatozoa after cryopreservation (Luna-Orozco *et al.* 2019). Cryodamage occurs due to various factors including; cool shock, physical, chemical, osmotic changes and the presence of ROS production which causes damage to spermatozoa so that the quality decreases after freezing. The CEP-3(m) + ASE 40% formula is not enough to protect spermatozoa from cryodamage and maintain the quality of frozen semen. Likewise, formula CEP-3(m) + 80% ASE that the composition of ASE was too high causes increasing viscosity thereby inhibiting spermatozoa motility. This may also be due to the high lecithin component as an extracellular cryoprotectant, which tends to be toxic to spermatozoa.

The motility character of spermatozoa in CEP-3(m) and 10% EY showed higher movement speed (VCL and VAP) than other diluents. The VCL value indicated the vigor or strength of spermatozoa movement. However, if a high VCL is followed by high ALH and low LIN, it tends to result in hyperactive spermatozoa movement. The ALH and spermatozoa hyperactivity values in the control diluent also showed higher values than the other diluents. A high hyperactivity value also indicates a surplus of energy sources or can also be a sign of spermatozoa capacitation. This is also supported by the poor swimming pattern of spermatozoa, which is characterized by lower LIN, STR and WOB values than spermatozoa in CEP-3(m) and ASE diluent (40–80%).

Characteristics of spermatozoa motility in CEP-3(m) and ASE (40–80%) diluents showed lower spermatozoa motility than CEP-3(m) and 10% EY diluent. However, the swimming pattern of spermatozoa was better as indicated by the higher LIN, STR and WOB values than diluent CEP-3(m) and 10% EY. The lower VCL and ALH values than spermatozoa in CEP-3(m) and 10% EY indicated that spermatozoa did not tend to produce hyperactive movement patterns (low hyperactivity value). Hyperactive spermatozoa are needed when the fertilization process runs naturally (in vivo). However, spermatozoa that are hyperactive during cryopreservation showed a poor sperm quality because hyperactive spermatozoa will quickly run out of energy to move, then die before entering the female reproductive tract.

The composition of egg yolk and ASE is different, resulting in different motility characters. The composition of egg yolk, including: low density lipoprotein (LDL), lipids, triglycerides, phospholipids, cholesterol and protein (Anton 2013). Some of the functions of egg yolk in diluents include being an extracellular cryoprotectant, a source of nutrition, buffering osmotic pressure and maintaining plasma membrane stability, and can replace damaged plasma membrane phospholipids during the freezing process (Fraser *et al.* 2014). Meanwhile, the composition of aqueous soybean extract were lecithin, fatty acids, glucose, amino acids, flavonoids, isoflavones and vitamin E. Lecithin functions as an extracellular cryoprotectant that protects spermatozoa from cold shock through the mechanism of coating the spermatozoa membrane with various lecithin fractions (phosphatidylcholine, phosphatidyletanolamine, phosphatidylinositol, phosphatidic acid, fatty acids) (Estiasih *et al.* 2013; Pamungkas and Krisnan 2017). The extracellular cryoprotectant function was also strengthened by the composition of glucose in ASE. Fatty acids function to maintained the stability of the spermatozoa membrane. Amino acids, flavonoids, isoflavones and vitamin E contained in ASE can function as antioxidants thereby helping to minimize the negative effects of reactive oxygen species (ROS) thereby reducing lipid peroxidation in the spermatozoa membrane.

Aqueous soybean extract nanoparticle was obtained through a combination of 2 methods, namely top-down and bottom-up (Möschwitzer 2010). Aqueous soybean extract nanoparticle has a size of 223.1±113.0 nm so it is categorized as a nanoparticle. Composition aqueous soybean extract (nanoparticles) allows to interact better with the spermatozoa cell membrane in protecting against damage due to cryopreservation. The characteristics of particles with sizes smaller than 500 nm are better than those with sizes larger than 500 nm (Tiyaboonchai 2013). The size of the aqueous soybean extract nanoparticles increases the number of filler atoms (filler) and the surface area of the nanoparticles resulting in a stronger bond between the aqueous soybean extract nanoparticles as fillers and the nanoparticle matrix (spermatozoa cell membrane) (Khan *et al.* 2019). This condition causes an increase in the protective effect of aqueous soybean extract on spermatozoa cell membranes from damage during cryopreservation. Smaller particle sizes can minimize obstacles to the movement of spermatozoa. This aqueous soybean extract exhibited a polydispersity index (PI) of 0.981 (high polydispersity), *i.e.* indicating a wide and non-uniform particle size distribution caused by particle aggregation. The zeta potential value of aqueous soybean extract was -31.9 mV, reflecting the surface charge of the nanoparticles and indicating a high level of stability of the nanoparticles (very stable). A large negative or positive ZP value means that the particles will repel each other and no aggregation (sediment) will occurred (Yedurkar *et al.* 2016). This is also seen in CASA, the debris was very small and at a small amount, so that spermatozoa moved more freely and CASA can assess movement of spermatozoa more optimally.

## Conclusion

Aqueous soybean extract nanoparticle at level 50% and 60% in CEP-3 (m) based diluent are most effective in supporting the motility and its kinetic parameters of frozen semen spermatozoa, including progressive motility, high VSL, VAP, LIN and STR, as well as low ALH and hyperactivity values. The pattern of movement of spermatozoa is regular, progressive and non-rotating, with good speedy. Further study of this diluent formula is needed with various parameters of spermatozoa quality.

## Acknowledgements

Our gratitude goes to The Ministry of Agriculture of

Indonesia through the Agency of Agricultural Research and Development for funding this study.

## Author Contributions

DR: Designed the study, collected data, analyzed data, reviewed the final version of the manuscript. GC, SR, TS: Designed the study, supervised of the research and reviewed the final version of the manuscript. All authors have read, reviewed, and approved the final manuscript.

## Conflict of Interest

The authors state that there is no conflict of interest with any party regarding the material written in this study.

## Data Availability

All the data in this study will be available on the corresponding author.

## Ethics Approval

Experiments of this research has been approved by the animal ethics committee of the Agricultural Research and Development Agency (Balitbangtan/Lolitsapi/Rm/01/2022).

## References

Alvarez-Rodriguez M, A Vicente-Carrillo, H Rodriguez-Martinez (2017). Exogenous individual lecithin-phospholipids (phosphatidylcholine and phosphatidylglycerol) cannot prevent the oxidative stress imposed by cryopreservation of boar sperm. *J Vet Med Surg* 1:11

Anton M (2013). Egg yolk: Structures, functionalities and processes. *J Sci Food Agric* 93:2871–2880

Biswas AK, MR Islam, ZS Choudhury, A Mostafa, MF Kadir (2014). Nanotechnology based approaches in cancer therapeutics. *Adv Nat Sci: Nanosci Nanotechnol* 5:043001

Bousseau S, J Brillard, B Marquant-Le Guienne, B Guerin, A Camus, M Lechat (1998). Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* 50:699–706

Coester J, A Sulaiman, M Riza (2019). Viability of limousin spermatozoa preserved with tris diluent and various concentrations of soybean extract. *J Trop Anim Sci Technol* 6:175–180

Contri A, C Valorz, M Faustini, L Wegher, A Carluccio (2010). Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. *Theriogenology* 74:424–435

Estiasih T, K Ahmadi, E Ginting, D Kurniawati (2013). Yield optimization of lecithin extraction of anjasmoro variety soybean oil by water degumming. *J Teknologi dan Industri Pangan* 24:97–97

Fraser L, E Jasiewicz, W Kordan (2014). Supplementation of different concentrations of Orvus Es Paste (OEP) to ostrich egg yolk lipoprotein extender improves post-thaw boar semen quality. *Polish J Vet Sci* 17:225–230

Hafez E, B Hafez (2000). *Semen evaluation in reproduction in farm animals.* Lea and Febiger, Philadelphia, Pennsylnania, USA

Immelda KH, S Susilowati, IS Yudaniayanti (2019). Pengaruh bahan pengencer sari kacang kedelai (*Glycine max*) terhadap viabilitas dan nekrosis spermatozoa domba Sapudi. *Ovozoa: J Anim Reprod* 8:36–42

Kasimanickam R, V Kasimanickam, A Tibary, K Pelzer (2011). Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4 C. *Small Ruminant Res* 99:208–213

Khan I, K Saeed, I Khan (2019). Nanoparticles: Properties, applications and toxicities. *Arab J Chem* 12:908–931

Lankalapalli S, KC Routhu, S Ojha, VVK Tenneti (2014). Nanoparticulate drug delivery systems: Promising approaches for drug delivery. *J Drug Delivery Ther* 2011–14:72–85

Layek S, T Mohanty, A Kumaresan, J Parks (2016). Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Anim Reprod Sci* 172:1–9

Luna-Orozco J, M González-Ramos, G Calderón-Leyva, L Gaytán-Alemán, F Arellano-Rodríguez, O Ángel-García, F Véliz-Deras (2019). Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation. *Iran J Vet Res* 20:126–130

Mafolo K, C Pilane, T Chitura, T Nedambale (2020). Use of phosphatidylcholine in Tris-based extender with or without egg yolk to freeze Bapedi ram semen. *S Afr J Anim Sci* 50:389–396

Möschwitzer J (2010). Particle size reduction technologies in the pharmaceutical development process. *Amer Pharm Rev* 2010:54–59

Nadri T, A Towhidi, S Zeinoaldini, F Martínez-Pastor, M Mousavi, R Noei, M Tar, AM Sangcheshmeh (2019). Lecithin nanoparticles enhance the cryosurvival of caprine sperm. *Theriogenology* 133:38–44

Pamungkas FA, R Krisnan (2017). Utilization of soybeans as the extender change of egg yolk for cryopreservation of animal spermatozoa. *J Penelitian dan Pengembangan Pertanian* 36:21–27

Ratnawati D, N Isnaini, T Susilawati (2017). Pemanfaatan casa dalam observasi motilitas spermatozoa semen cair Sapi Madura dalam pengencer berbeda. *J Ilmu-Ilmu Peternakan Univ Brawijaya* 27:80–95

Ratnawati D, N Isnaini, T Susilawati (2018). Character motility of liquid semen on Ongole crossbreed (PO), Bali and Madura bulls with different diluents at cold storage. *Asian J Microbiol Biotech Environ Sci* 20:21–28

Ratnawati D, K Kuswati, APA Yekti, G Ciptadi, S Rahayu, T Susilawati (2023). Effect of modified CEP-3 diluents with aqueous soybean extract on liquid semen quality in Ongole crossbred bull. *Vet World* 16:1075–1083

Saadeldin IM, WA Khalil, MG Alharbi, SH Lee (2020). The current trends in using nanoparticles, liposomes, and exosomes for semen cryopreservation. *Animals* 10:2281

Singh A, V Singh, B Narwade, T Mohanty, S Atreja (2012). Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5ºC) in egg yolk-and soya milk–based extenders. *Reprod Domestic Anim* 47:596–600

Sugiarto N, T Susilawati, S Wahyuningsih (2014). Kualitas semen cair sapi limousin selama pendinginan menggunakan pengencer CEP-2 dengan Penambahan berbagai konsentrasi sari kedelai. *Ternak Tropika J Trop Anim Prod* 15:51–58

Sun L, W Fan, C Wu, S Zhang, J Dai, D Zhang (2020). Effect of substituting different concentrations of soybean lecithin and egg yolk in tris-based extender on goat semen cryopreservation. *Cryobiology* 92:146–150

Sun L, M He, C Wu, S Zhang, J Dai, D Zhang (2021). Beneficial influence of soybean lecithin nanoparticles on rooster frozen–thawed semen quality and fertility. *Animals* 11:1769

Tiyaboonchai W (2013). Chitosan nanoparticles: A promising system for drug delivery. *Naresuan Univ J Sci Technol* 11:51–66

Toker M, S Alcay (2022). Comprehensive effects of fetal calf serum in soybean-lecithin based goat semen cryopreservation extenders and impacts on incubation resilience. *Kafkas Univ Vet Fakultesi Dergisi* 28:455–460

Toker MB, S Alcay, E Gokce, B Ustuner (2016). Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. *Cryobiology* 72:205–209

Yedurkar S, C Maurya, P Mahanwar (2016). Biosynthesis of zinc oxide nanoparticles using ixora coccinea leaf extract—a green approach. *Open J Synthesis Theory Appl* 5:1–14