Soybean Phospholipids-Based Extender as an Alternative for Bull Sperm Cryopreservation

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Abstract. This current work investigated the usage of soybean phospholipid (SP) in extenders for bull sperm cryopreservation. The sperm was obtained from Friesian Holstein (FH) bulls using an artificial vagina method. Furthermore, semen samples were pooled and diluted in a Tris egg yolk-based extender (control group; CG) or Tris extender supplemented with SP at different concentrations (G1 = 0.5%, G2 = 1%, G3 = 1.5%, G4 = 2%, G5 = 2.5%) for a final concentration of 25×10⁶ spermatozoa/0.25 mL. Subsequently, they were packed in straws (0.25 mL), cryopreserved using liquid nitrogen vapor for 10 minutes, then stored in liquid nitrogen (−196°C). As a result, control group presented significantly higher values in motility, viability and membrane integrity at the stage of dilution, equilibration and post-thawing compared to treatment group (P<0.05). After thawing (37°C/30s), no significant difference was observed between G4 and control group for all parameters observed using Computer Assisted Sperm Analysis (CASA) (P>0.05). In conclusion, the addition of 2% soybean phospholipid in Tris-based extender can be used for freezing bull semen, producing a satisfied fertility rate in post-thawing stage.

1. Introduction

Semen cryopreservation in Artificial Insemination (AI) has received significance particularly when used in breeding program. To produce cryopreserved semen with a good fertility rate, extender is employed since it enables to protect semen from cold shock, acts as energy substrate for spermatozoa [1], and prevents bacterial growth [2]. The use of egg yolk as an ingredient in the sperm extender medium has been commonly used. The egg yolk contains lecithin, a substance that exerts membrane coating properties, enabling to maintain the normal configuration of phospholipid bilayer which is the main structure of the spermatozoa membrane [3]. Nevertheless, egg yolk as a extender has the risk of microbial contamination such as bacteria and fungi [4], in which these microflora produce endotoxins able to promote detrimental effects, including reduction of spermatozoa fertility rate [5]. As an alternative candidate, soybean lecithin possesses considerable properties for replacing egg yolk as extender. According to the report [6], soybean lecithin has ingredients similar to that in egg yolks, capable of protecting semen against thermal shock during cryopreservation. Besides, soybeans are also able to reduce oxidative stress [7]. Phospholipid, a main component of the phosphate fraction, is found...
in soybeans [8]. This research was conducted to determine the best composition of the single use of soybean phospholipids in the extender media used in semen cryopreservation.

2. Materials and methods

2.1. Semen collection
Semen was collected from 3 Friesian Holstein (FH) bulls (previously kept in cage in Research Center for Biotechnology, Indonesian Institute of Science, Jawa Barat) by an artificial vagina method and carried out from November 2018 to February 2019. Fresh semen was then evaluated macroscopically and microscopically. The neat semen samples with more than 70% motility were submitted to cryopreservation.

2.2. Semen processing
The semen samples were pooled and diluted in a Tris egg yolk-based extender (2.472 g tris (hydroxymethyl) aminomethane (Merck), 1.384 g of citric acid (Merck), 1.016 g of fructose (Merck), 80 mL aquabidest (Ikapharmindo), 100 µL penicillin-streptomycin solution (Sigma), 6% glycerol (Merck) and 20 mL egg-yolk (control group; CG). For experimental groups, Tris-based extenders supplemented with soybean phospholipid (SP) (Intralipid, Sigma) at different concentrations (G1 = 0.5%, G2 = 1%, G3 = 1.5%, G4 = 2%, G5 = 2.5%) for a final concentration of 25×10⁶ spermatozoa/0.25 mL. The semen samples were packed in straws (0.25 mL) and equilibrated for 4 hours at 5°C. The freezing was carried out over liquid nitrogen vapor for 10 minutes and stored in cryobiological container (−196°C).

2.3. Sperm analysis
Fresh semen was examined macroscopically (volume, color, viscosity and pH) and microscopically (mass movement, motility, viability, abnormality and membrane integrity). Sperm concentration was estimated using photometer SDM6 (Minitube Germany). Prior to freezing, sperm quality was evaluated for motility, viability and membrane integrity at the stage of dilution and equilibration. Post-thawing evaluation (thawing in water bath at 37°C/30s) was also performed, including sperm quality in fertility using Computer Assisted Sperm Analysis (CASA).

2.3.1. Motility and CASA system
The samples were loaded into pre-heated Makler chamber, which was inserted into the CASA system (Minitube, Germany). Five aleatory fields with at least 150 sperms per field were considered. Sperm variables assessed by the CASA and considered in the present study included total sperm motility (MOT, %), progressive motility (MP, %), velocity curved line (VCL, µm/s), velocity average path (VAP, µm/s), velocity straight line (VSL, µm/s), amplitude of lateral head displacement (ALH, µm) and linearity (LIN, %).

2.3.2. Viability
Sperm cells were stained with eosin-nigrosin. Briefly, three object glasses were prepared. Raw semen (5µL) and eosin-nigrosin (10µL) were dripped on the first object glass, then mixed using a second glass object. Subsequently, it was smeared on the third glass object and fixed using bunsen. Two hundred cells from each sample were examined at a magnification of 400x under phase contrast microscope (Olympus, Japan). The cells were classified as having a high viability potential when emitting clear (uncoloured) on sperm membrane.

2.3.3. Membrane integrity
Hypo-osmotic swelling test (HOST) was employed to test sperm membrane integrity. Aliquots (5 µL) of each sample were diluted in 0.5 mL HOST solution, then incubated at 37°C for 30 minutes. Two
hundred spermatozoa were evaluated, and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was calculated.

2.3.4. Statistical analysis
Data were statistically analyzed using analysis of variance (ANOVA) in SPSS v.16.00, aiming to compare different parameters between control and supplementation replications. A significant difference between means was verified using Duncan's multiple range test at P < 0.05.

3. Results and discussion

3.1. Results
The macroscopical and microscopical evaluation of fresh semen was presented in Table 1. The results indicated desirable properties of the sample, thus they were acceptable for further steps of the experiment.

| Parameters                        | Average Value |
|-----------------------------------|---------------|
| Volume (ml)                       | 6.17 ± 1.04   |
| Color                             | milk white - cream |
| Consistency                       | medium - thickened |
| pH                                | 6.80 - 7.00   |
| Mass movement                     | ++ - +++      |
| Concentration (x10^6 cell/ml)     | 1.312,00 ± 52.33 |
| Motility (%)                      | 76.67 ± 2.89  |
| Viability (%)                     | 89.96 ± 1.80  |
| Abnormality (%)                   | 13.99 ± 2.15  |
| Membrane integrity (%)            | 80.67 ± 3.89  |

Incorporation of soybean phospholipid in dilution, equilibration and post-thawing stages resulted in lower motility (Figure 1), viability (Figure 2) and membrane integrity (Figure 3) in comparison with control group (P<0.05).

![Figure 1. Spermatozoa motility observed in dilution, equilibration and post thawing stages](image-url)
Figure 2. Spermatozoa viability observed in dilution, equilibration and post thawing stages

Figure 3. Spermatozoa membrane integrity observed in dilution, equilibration and post thawing stages

According to CASA analysis on VCL, VAP, VSL, ALH and LIN, no significant difference was detected between G4 and control group (Figure 4).
3.2. Discussion

The quality of fresh semen of FH bulls used in this study indicated a good quality and normal range based on reports from previous studies. The average volume was 5-8 ml, with milky white in color and moderate consistency suggesting the concentrations between 1,000-1,500 x10^6 cells / ml [9], pH between 6.4 - 7.8 [10], thick mass movement and fast moving, between ++ - +++ [11], minimal motility 70% [12], minimal viability 70% [13], minimal membrane integrity 60% [14] and abnormality less than 20% [15].

As mentioned above, the parameter values (motility, viability and membrane integrity) of treated samples was significantly lower than those of control group (P <0.05), which was found in all stages (dilution, equilibration and post-thawing). Among treated groups, G4 showed the highest values of motility, viability and membrane integrity in dilution and equilibrium stages. Furthermore, sperm motility was in the normal range, as also found in former study [15] which reported that after dilution, the spermatozoa motility reached at least 60%; while in equilibration stage (cooling 5°C), the motility was >55%. In terms of viability, good spermatozoa of G4 treatment was also in agreement with previous report [15], finding that the viability of good spermatozoa ranged 60-80%. This good percentage of spermatozoa membrane integrity has been reported in a study [16], which demonstrated membrane integrity of fresh semen of FH bull was 79.80 ± 4.81%. A minimum limit of membrane integrity of 60% is related to infertility [14]. In this study, G4 was found at below normal values after thawing, yielding viability of 45.6% and membrane integrity of 45.67%. This suggests that soybean phospholipid seemed to be less effective in maintaining viability and membrane integrity after freezing compared with egg yolk as a control. Lipoprotein and lecithin present in egg yolk demonstrated enabled to provide protective action on plasma membrane since presence of these components raised proportion of cholesterol and phospholipid which are meaningful in preventing cold shock [17].

Evaluation of the post-thawing spermatozoa fertilization ability based on Spermvision®CASA showed that the motility value in sample treated with soybean phospholipid was significantly lower (P
<0.05) than that in control group. Compared to egg yolk used in control, soybean phospholipid may contain lower quantity of carbohydrate as source of energy for sperm. Previous studies reported that low motility could be associated with scarcity of carbohydrate in soy phospholipids [18], as an energy source in spermatozoa metabolism [19]. In addition, CASA analysis on VCL, VAP, VSL, ALH and LIN demonstrated that there was no difference between G4 and control group. However, G4 possessed a higher fertility rate (VCL 70.45 μm/s, VAP 40.63 μm/s, VSL 32.48 μm/s, ALH 4.64 μm and LIN 64%) compared to other treatments, as well as being higher than WHO standard [20] as follows: VCL value ≥70.15 μm/s, VAP value > 25.00 μm/s, VSL value ≥25.00 μm/s, ALH value > 4.50 μm and LIN value ≥35%. Based on the pattern, spermatozoa motility was grouped into following type: hyperactive motility (VCL value ≥100µm/s, LIN value <60% and ALH value ≥5 μm), transition and non-hyperactive motility (VCL value ≥40µm/s, LIN value ≥60% and ALH value <5 μm) [9]. In this matter, spermatozoa of this work was attributed to transition motility. Fertility assessment of spermatozoa positively correlate with their ability to penetrate the oocyte pellucida zone [21].

4. Conclusion
This present work concluded that supplementation of 2% soybean phospholipid in Tris-based extender performed a satisfying result in cryopreservation of bull semen based on its fertility in the post-thawing normal range.

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