

MOTILITY AND ABNORMALITY OF SHEEP SPERMATOZOA THAT IS BEING FROZEN USING SOYBEAN LECITHIN (SOYBEAN LECITHIN)

Salmin¹, Marsudi², Hendro Sukoco³, Deka Uli Fahrodi⁴

¹Animal Husbandry Department, Faculty of Animal Husbandry and Fisheries, Universitas Tadulako

^{2,3,4}Animal Husbandry Department, Faculty of Animal Husbandry and Fisheries, Universitas Sulawesi Barat

e-mail: salmin.bouato@gmail.com¹, marsudi@unsulbar.ac.id², hendrosukoco@unsulbar.ac.id³, dekaulifahrodi@unsulbar.ac.id⁴

*Correspondence: salmin.bouato@gmail.com

Submitted: 25 November 2021, Revised: 10 December 2021, Accepted: 20 December 2021

Abstract. The specific purpose of this study was to determine the effect of optimal levels of soybean lecithin in sperm extenders on motility and abnormalities of sheep spermatozoa after undergoing the freezing process. This research was conducted experimentally in a laboratory with a completely randomized design (CRD) with five treatments and three replications. The five treatments tested were soybean lecithin levels in Tris (L) diluent, consisting of: L0 = 0% soybean lecithin + 95% Tris diluent + 5% glycerol; L1 = 1% soy lecithin + 94% Tris diluent + 5% glycerol; L2 = 2% soy lecithin + 93% Tris diluent + 5% glycerol; L3 = 3% soy lecithin + 92% Tris diluent + 5% glycerol; L4 = 4% soy lecithin + 91% Tris diluent + 5% glycerol. The independent variable in this study was the level of soy lecithin in the Tris diluent. The dependent variable is the progressive motility and abnormalities of post-clotting spermatozoa. Soybean lecithin levels had a significantly different effect ($P \leq 0.05$) on the percentage of progressive motility and spermatozoa abnormalities of post-freezing sheep. The level of 3% soya bean lecithin is the best level in maintaining the quality of post-freezing sheep spermatozoa.

Keywords: abnormalities; spermatozoa; sheep; soybean lecithin.

INTRODUCTION

The conventional method that has been used for sperm freezing or cryopreservation is slow freezing, but during the slow freezing process the sperm undergoes cold shock, freezing injury and the formation of ice crystals ([Arrebola, González, Torres, & Abecia, 2013](#)); ([Silva, Cajueiro, Silva, Soares, & Guerra, 2012](#)); ([O'Hara et al., 2010](#)) inside and outside spermatozoa cells. Cold shock and/or freezing injury can result in decreased permeability of spermatozoa membranes to water and solutions and damage the acrosomal membrane ([Jie Liu et al., 2016](#)), folding the middle section and tail towards the head so that motility or sperm movement appears to be circular or backwards. Intracellular ice crystals can damage the cell wall and structure of spermatozoa while extracellular ice crystals can increase the salt concentration which causes cell damage or abnormalities of spermatozoa ([Forouzanfar, Abid, Hosseini, Hajian, & Esfahani, 2013](#)).

Motility and abnormalities have a positive correlation with fertility, in which the higher the number of motile spermatozoa and the lower the number of abnormal spermatozoa, the higher the fertility. This correlation gradually decreases after the freezing and thawing processes, because during these processes there may be damage to cell function or the exhaustion of energy. A drastic decrease occurs after the sperm has cooled down and/or frozen due to cold shock, which is characterized by the folding of the middle section and tail towards the head so that the sperm movement appears circular or backwards.

The problem of sperm freezing or cryopreservation in the form of cold shock or freezing injury can be partially solved by the use of a protective agent (cryoprotective agent) in the sperm extender ([Zhou, Wu, Shi, & Zheng, 2010](#)). Protective materials that are popular and universally used for freezing or cryopreservation of sperm to date include glycerol and lecithin ([Kannaki, Shanmugam, & Verma, 2011](#)); ([Layek, Mohanty, Kumaresan, & Parks, 2016](#)); ([Rickard, Pool, Druart, & de Graaf, 2019](#)). The main source of lecithin that has been used for a long time is egg yolk, but the use of this material as a source of lecithin to prevent the effects of cold shock carries the risk of contamination by microorganisms that harm spermatozoa and the female reproductive tract ([Muro et al., 2016](#)), such as bacteria ([Moran, Guzman, Ropars, & Illmer, 2010](#)); especially *Salmonella typhimurium*.

One of the potential protective materials developed to protect spermatozoa from the adverse effects of freezing or cryopreservation is soy lecithin. This material is a vegetable lecithin which contains phospholipids which are very important to protect spermatozoa membranes in the cryopreservation process. Naturally, the lecithin content in soybeans is 1.48 – 3.08%. The main components of soy lecithin are phospholipids consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositols and glycolipids ([Saurabh, Sharma, & Gautam, 2018](#)). Hygienically, soybean lecithin was not found to contain any micro-organisms that could harm spermatozoa or female

reproductive tract ([Tian et al.](#), 2019).

Based on this description, it is deemed necessary to conduct research on motility and abnormality of sheep spermatozoa being frozen using soy lecithin.

METHODS

Time and Location

This research was carried out at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Husbandry and Fisheries, Tadulako University from July to November 2021.

Experimental Livestocks

Types of experimental livestock used in this study are male sheep. The number of male sheep as a source of sperm are three sheep, 2-3 years old, in good health and have good reproductive characteristics.

Research Materials

The materials used for this research were 10% soybean lecithin (CENTROL 3 flub, certificate number: TSC 04020, USA), Tris (hydroxymethyl) aminomethane, citric acid, glucose, glycerol, aquadestilata, aquabidestilata, penicillin, streptomycin, intermediate hypoosmotic swelling test (HOS test), physiological NaCl, hayem's solution, eosin Y/Negrosine, 70% alcohol and liquid nitrogen ([Zadoks et al.](#), 2014).

Research Equipments

The equipments used in this study were artificial vagina, thermometer, ice flask, scale tube, test tube, measuring cup, erlenmeyer, beaker, blender machine ([Rui Liu et al.](#), 2021) aluminum foil, stirring rod, micropipette (Transferpette®), dropper, lig

ht microscope (Tension), optilab camera (Optilabpro viewer®), object glass, cover glass, haemocytometer, Neubauer counting chamber, pH meter ([Kyte, Kleyn, Scoggins, & Bridgen](#), 2013), refrigerator (Sanken CN®), hand tallycounter (Laboratory Dc Counter: DBC-9. K Gemini Ind. Corp.®, USA), analytical balance, liquid nitrogen container (Taylor Wharton), styrofoam box (styrofoam box), tweezers, and mini straws.

Research design

This research was conducted in a laboratory experimental approach with a completely randomized design with five treatments and three replications. The five treatments tested were the levels of soy lecithin in the Tris (L) diluent, consisting of: L0 = 0% soy lecithin + 95% Tris diluent + 5% glycerol
L1 = 1% soy lecithin + 94% Tris diluent + 5% glycerol
L2 = 2% soy lecithin + 93% Tris diluent + 5% glycerol
L3 = 3% soy lecithin + 92% Tris diluent + 5% glycerol
L4 = 4% soy lecithin + 91% Tris diluent + 5% glycerol

Research variable

The independent variable in this study was the level of soy lecithin in the Tris diluent. The dependent variable is the progressive motility and abnormalities of post-clotting spermatozoa.

RESULTS AND DISCUSSION

The results of observations on the motility and abnormalities of spermatozoa

in post-freezing sheep treated with soy lecithin levels in the Tris extender are listed

in Table 1.

Table 1. The mean of progressive motility and abnormality of post-freezing sheep spermatozoa from various levels of soybean lecithin in Tris extenders (%).

Lecithin Levels Soybeans (L)	Progressive Motility	Abnormality
0% (L0)	16,79±3,64 ^a	17,03±2,84 ^a
1% (L1)	17,55±2,52 ^a	15,71±2,83 ^b
2% (L2)	34,18±2,27 ^b	14,57±2,94 ^b
3% (L3)	43,79±3,01 ^c	13,72±2,92 ^b
4% (L4)	34,24±3,11 ^d	15,62±2,90 ^b

Note. : a,b,c,d, Different superscripts in the same column showed significant differences ($P \leq 0.05$).

Based on the results of analysis of diversity, the levels of soya bean lecithin in the Tris extender showed a significantly different effect ($P \leq 0.05$) on the motility and abnormalities of post-freezing sheep spermatozoa. The higher the levels of soy lecithin in the Tris extender, the more progressive motility percentages increased to 3% of soy lecithin levels, then decreased to 4% of soy lecithin levels and the percentage of spermatozoa abnormalities decreased to 3% soy lecithin level, then the levels increased to 4% of soybean lecithin (Figure 1).

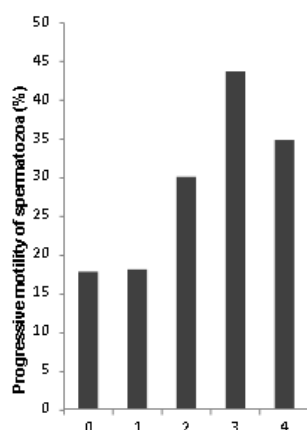


Figure 1. Soybean lecithin levels (%)

(A)

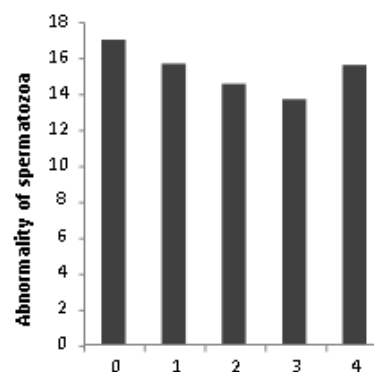


Figure 2. Soybean lecithin levels (%)

(B)

Effect of soybean lecithin levels on progressive motility (A) and abnormalities (B) of post-slow freezing sheep spermatozoa.

Further test results showed that the percentage of progressive motility (43.79%) of post-freezing spermatozoa treated with 3% soybean lecithin (FL3) was significantly higher ($P \leq 0.05$) and spermatozoa abnormalities percentage (13.72%) was significantly lower ($P \leq 0.05$) than all the treatments that were tried. Applicatively, the results of this study are suitable for artificial insemination because they contain more than 40% of progressive motile

spermatozoa according to the Indonesian National Standard ([Martin et al.](#), 2014); ([Hoesni](#), 2017) and abnormal spermatozoa was not more than 15%.

The results in this study was presumably achieved because in the FL3 treatment, 3% of soy lecithin levels were more effective in protecting spermatozoa from the effects of freezing so that progressive motility and morphological normality of post-freezing spermatozoa were maintained. At this level, spermatozoa are relatively spared from the adverse effects of cryopreservation in the form of freezing injury ([Hoesni](#), 2016) and freeze kills ([Kharche et al.](#), 2013) or those that reduce the spermatozoa's ability to survive ([Arrebola et al.](#), 2013). As it is known before, the main content of soy lecithin is phospholipids ([Collares, Bongalhardo, Deschamps, & Moreira](#), 2018) or egg yolk-like phospholipids ([Choudhary, Pardhi, & Bhoyar](#), 2013). These phospholipids have been identified as cryoprotective components that protect the integrity of spermatozoa membranes during cryopreservation ([Fermini et al.](#), 2016). The working mechanism of soya bean lecithin in protecting spermatozoa membranes from freezing injury or freeze kill is not known for certain, but some researchers suspect that the mechanism is similar to that of low density lipoprotein (LDL) as found in egg yolks. In this case there are two main hypotheses that arise; First, as an important component of the bio-membrane of mammalian sperm cells, phospholipids play an important role in regulating the physiological function of the bio-membrane and enter the cell to lower the

freezing point of crystals by placing plasmalogens to reduce mechanical damage to the bio-membrane of spermatozoa. Some experts who agree with this opinion believe that soy lecithin can reduce the cholesterol/phospholipid ratio of sperm cell membranes by seeping into the spermatozoa membranes. In addition, soya bean lecithin phospholipids can replace some of the phospholipids of sperm cell membranes to maintain their structure and function. Second, other experts believe that the phospholipid soy lecithin cannot enter the sperm cell membrane to change the bio-membrane phospholipid concentration, but it can integrate with the sperm cell membrane to form a protective film. or form an interfacial layer between the fatty acids and water ([Gallier, Gordon, & Singh](#), 2012) to counter the formation of lethal intracellular ice crystals and protect sperm cell membranes from mechanical damage during freezing and thawing ([Zhang, Hu, Li, Jiang, & Zhang](#), 2009). In more detail, there are at least three main roles carried out by the interfacial layer, namely (a) lowering the surface tensions on the plasma membrane, especially those caused by the absorption of proteins on the membrane surface (b) forming a mechanical defense system on the surface of the plasma membrane through the formation of a thin layer that is viscoelastic so as to prevent damage to the plasma membrane structure and (c) playing a role in controlling colloidal interactions between fatty acids and water. This second hypothesis is also supported by the result of observation showing that soybean lecithin microparticles are relatively larger

than spermatozoa under a microscope. Therefore, they argued that it was impossible for soy lecithin microparticles to enter the sperm cell membrane and this latter opinion was the one agreed the most.

The low percentage of progressive motility and the high percentage of sheep spermatozoa abnormalities in other treatments were considered to be caused by; (1) at the concentration of 4% soybean lecithin (FL4), the concentration of soybean lecithin in the extender solution was relatively higher so that the medium became hypertonic. Visually, at this level, the soya bean Tris-lecithin extender was slightly thicker than the other treatments. Hypertonic extender medium can interfere with locomotion and harm spermatozoa. If the movement activity of spermatozoa after freezing continues under these conditions, it is suspected that it will result in the spermatozoa running out of energy and cause damages to the morphological structure of the spermatozoa in the form of a severed tail or neck (Figure 2). Spermatozoa that run out of energy in turn will reduce motility and viability and even death of spermatozoa. (2) at levels below 3% soya bean lecithin (L0, L1 and L2), cryoprotectant soy lecithin is relatively too low than the required level so that it does not optimally protect spermatozoa from adverse freezing effects in the form of freezing injury or freeze kill.

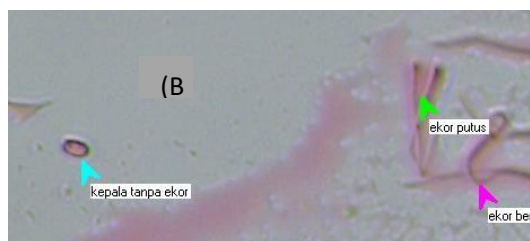
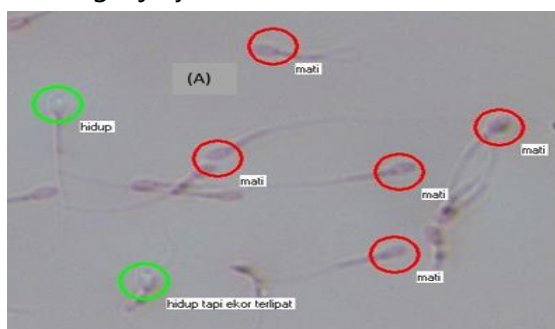


Figure 2. Sperm preparation of spermatozoa viability (A) and spermatozoa abnormalities in the form of severed tail and neck (B) after freezing from 4% soybean lecithin treatment (L4)

The results of this study are different from previous reports by researchers on pig spermatozoa cryopreserved using a soybean lecithin extender where the quality of the spermatozoa they obtained was lower at 3% soy lecithin levels but higher at 6% levels. This is thought to be caused by species differences, in which sheep spermatozoa may be more responsive to cryoprotectant lecithin from soybeans at lower levels than pig spermatozoa. Stated that damage due to cooling and freezing effects on spermatozoa membranes varied between domestic species and was influenced by several elements, namely the cholesterol/phospholipid ratio, lipid bilayer content, degree of chain saturation hydrocarbons and protein/phospholipid ratio. This opinion is supported by which states that susceptibility to cold shock is related to the ratio between cholesterol and phospholipids. The lower the ratio between cholesterol and unsaturated fatty acids, the more vulnerable the plasma membrane is. Spermatozoa with higher cholesterol levels and lower levels of unsaturated fatty acids will have a more compact plasma membrane structure and tend to be more resistant to cold shock.

Sheep spermatozoa contain cholesterol 226 mg/1000 million cells and in pig spermatozoa the levels may be lower, so categorize that pig spermatozoa are very sensitive to cold shock compared to sheep spermatozoa.

CONCLUSIONS

Based on the the results and discussion above, it can be concluded that the results of the study are as follows: 1) Levels of soybean lecithin in the Tris extender showed a significantly different effect ($P \leq 0.05$) on the progressive motility and abnormality of post-freezing sheep spermatozoa. 2) The higher the level of soy lecithin in the Tris extender, the more the percentage of progressive motility increased to 3% of soy lecithin, then decreased to 4% of soy lecithin and the percentage of spermatozoa abnormalities decreased to 3% of pea lecithin. soybeans, then increased levels of 4% soy lecithin. 3) The best progressive motility and abnormalities of post-freezing spermatozoa were obtained in the L3 treatment, namely 3% soybean lecithin + 92% Tris extender + 5% glycerol, with results of 43.79% and 13.72%, respectively.

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