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QUALITY OF FROZEN SHEEP SPERM USING SOYBEAN LECITHIN EXTENDER

Salmin[1,2*], Marsudi[2], Hendro Sukoco[2]
[1]Animal Husbandry Study Program, Faculty of Animal Husbandry and Fisheries, Universitas Tadulako
[2]Department of Animal Husbandry, Faculty of Animal Husbandry and Fisheries, Universitas Sulawesi Barat
Jl. Prof. Dr. Baharuddin Lopa, S.H., Baurung, Banggae Timur, Majene, West Sulawesi, Indonesia 91412
**Corresponding author*: salmin.bouato@gmail.com

ABSTRACT

The study aimed to study the impact of optimum levels of soybean lecithin in Tris extenders on the quality of frozen sheep sperm. The observed variable was the quality of the post-freezing spermatozoa, which covers the progressive motility, viability, abnormality and integrity of the sperm membrane. The results of the study showed that the level of lecithin in soybeans had a real influence (P<0,05) on all parameters of the quality of post-freezing sheep spermatozoa. The higher the level of soybean lecithin, the higher the percentage of progressive motility, viability, integrity of sperm membranes up to 3% soybean lecithin, then a decrease of 4% soybean lecithin and decreasing sperm abnormality percentages to 3% Soybeans Lecithin. The Average percentage of progressive motility (43.79%), viability (50.85%) and membrane integrity (51.65%) of post-freezing spermatozoa treated with 3% soya bean lecithin (L3) was significantly higher (P<0.05), and sperm abnormalities (13.72%) were significantly lower (P <0.05) than all treatments tried.

Keywords**:** *Membrane integrity, Motility, Frozen spermatozoa Quality, Soybean lecithin*

ABSTRAK

Penelitian ini bertujuan untuk mempelajari pengaruh kadar lesitin kacang kedelai yang optimal dalam ekstender Tris terhadap kualitas spermatozoa domba yang dibekukan. Variabel yang diamati adalah adalah kualitas spermatozoa pasca pembekuan, yang meliputi motilitas progresif, viabilitas, abnormalitas dan integritas membran spermatozoa. Hasil penelitian menunjukkan bahwa kadar lesitin kacang kedelai berpengaruh nyata (P<0,05) terhadap semua parameter kualitas spermatozoa domba pasca pembekuan. Semakin tinggi kadar lesitin kacang kedelai maka semakin meningkat pula persentase motilitas progresif, viabilitas, integritas membran spermatozoa sampai pada kadar 3% lesitin kacang kedelai, kemudian mengalami penurunan pada kadar 4% lesitin kacang kedelai dan semakin menurun persentase abnormalitas spermatozoa sampai pada kadar 3% lesitin kacang kedelai, kemudian mengalami peningkatan pada kadar 4% lesitin kacang kedelai. Rataan persentase motilitas progresif (43,79%), viabilitas (50,85%) dan integritas membran (51,65%) spermatozoa pasca pembekuan yang mendapat perlakuan 3% lesitin kacang kedelai (L3) nyata lebih tinggi (P<0,05) dan abnormalitas spermatozoa (13,72%) nyata lebih rendah (P<0,05) daripada semua perlakuan yang dicobakan.

Kata kunci: Kualitas, Lesitin kacang kedelai, Motilitas, Integritas membran, Spermatozoa beku,

INTRODUCTION

Soya lecithin is one of the agents that is potentially used as an extender component of semen. This substance is a vegetable lecithin that is essential to protect sperm membranes during the freezing process. The use of soybean lecithin as an extender components can reduce hygienic risks and be used as a substitute for egg yolks (Forouzanfar et al., 2010). Soybean lecithin plays a better role for spermatozoa than egg yolk or other ingredients during the freezing process and thawing especially reduces the risk of bacterial and mycoplasma contamination in the frozen extender. (Fukui et al., 2008).

Soya bean lecithin's mechanisms in protecting sperm membranes from cold shock are unclear, but some researchers suspect that it works simiarly to low density lipoprotein (LDL) (Zang et al., 2009) as

found in egg yolks. The correct formula in the manufacture of cement extenders enriched with soybean lecithin is currently scarce and levels vary. The results of a study on pork cement showed that the levels of soybean lecithin in extenders of 3%, 6% and 12% showed successive percentages of sperm motility of 42.3%, 44.3% and 39.8% after slow freezing (Zhang, et al., 2009). In sheep cement, soybean lecithin levels of 0.5%, 1%, 2% and 3.5% in extender have also been tested in slow freeze (de Paz et al. 2010; Forouzanfar et al, 2010; Mohsen et Al., 2009) with different results, so that so far there is no precise standard on that. However, based on some of the results of such studies, it can be drawn the provisional conclusion that too-low levels of soybean lecithin are not sufficiently effective in protecting sperm from the influence of cold shock and freezing injuries and, on the contrary, too-high levels of soya beans Lecithin will interfere with the mobility and viability of spermatozoa.

Based on the above description, to improve the quality and usability of frozen spermatozoa ready for use for Artificial Insemination (AI), research has been carried out on freezing sheep sperm using extenders enriched with soya bean lecithin cryoprotectant. The study aimed to study the impact of optimum levels of soybean lecithin in Tris extenders on the quality of frozen sheep sperm.

MATERIALS AND METHODS

Research Site

The research was carried out in 2012. Research was conducted at the Animal Physiology and Reproduction Laboratory, Gadjah Mada University. Samples maintained in Yogyakarta.

Experimental Livestock Research Material

The experimental cattle used in this study were three rams as a source of semen from two to three years old, healthy and had good reproductive status.

Research Material

The materials used in this study are Tris aminomethane, aquadestilata, citric acid, liquid nitrogen (liquid N2), glycerol aquabidestilata, streptomycin, penicillin, physiological NaCl, heyem solution, eosin Y/Negrocine, glucose, 70% alcohol, medium hypoosmotic swelling test (HOS-test), and 10% soybean lecithin.

Research Equipment

The equipment used in this study is a thermometer, ice thermos, scale tube, reaction tube, measuring glass, erlenmeyer, cup glass, blender machine (Miyako BL-101 PL), alumunium foil, mixer rod, micropipet (Transferpette®), drop pipette, optilab camera (Optilab viewer®), object glass, covering glass, Neubauer count room, artificial vagina, light microscope (Tension), refrigerator (Sanken CN®), hand tally counter, hemocytometer, analytical weighing, liquid nitrogen container, styro foam box (styro foam box), and pinset, pH meter (Sentron 501 pocket FET®, Netherlands), mini straw.

Research Procedure/Research Plan

This research was an experimental study using a completely randomized design consisting of five treatments and three replications. The five treatments are soybean lecithin levels in Tris (L) extender, consisting of :

L0 = soybean lecithin (0%) added Tris extender (95%) and added glycerol (5%).

L1 = soybeans lecithin (1%) added Tris extender (94%) and added glycerol (5%)

L2 = soya bean lecithin (2%) added Tris extender (93%) and added glycerol (5%)

L3 = soya buns lecithin (3%) added Tris extenders (92%) and added glycerol (5%)

L4 = soya nuts lecithin (4%) added Tris expanders and (91%) added glycerol (5%)

Study Variables

The variables in this study consist of independent variables and dependent variables. While the independent variable is the lecithin level of soybeans in the Tris extender. While the dependent variable is the quality of post-clotting spermatozoa (viability, progressive motility, abnormality and integrity of the spermatozoa membrane). The operational definition of each variable is as follows:

1. Sperm motility is calculated on the basis of the percentage of sperm that are progressively motile (actively moving forward). The sperm motility is the result of the division of the number of progressively motile sperm by the total sperm observed and expressed in percentage by the following formula:

$$\text{Spermatozoa's Mortality} = \frac{\text{Number of Progressive Spermatozoa 100\%}}{\text{Observed Spermatozoa}}$$

   The number of progressive motile spermatozoa is calculated by reducing the total sperm observed by the number of non-progressive non-motile sperms on ten objects observed under a microscope (Salmin, 2000; Salmin, 2002).
2. The viability or percentage of living sperm, was calculated based on the percentages of living sperms by the differential colouring of the total of observed sperms (Audia et al., 2017) and is expressed in percents with the following formula:

$$\text{Spermatozoa's Viability} = \frac{\text{Number of Progressive Spermatozoa 100\%}}{\text{Observed Spermatozoa}}$$

3. Abnormality of spermatozoa, calculated based on the percentage of abnormal sperm from the total sperm observed (Audia et al., 2017) and expressed in percentages with the following formula:

$$\text{Spermatozoa's Abnormality} = \frac{\text{Number of Progressive Spermatozoa x 100\%}}{\text{Observed Spermatozoa}}$$

4. Sperm membrane integrity, calculated on the basis of the percentage of spermatozoa having the membrane layer intact of the total sperm observed (Rahayu dan Ducha, 2022), is expressed in percentages with the following formula:

$$\text{Spermatozoa's membrane integrity} = \frac{\text{Number of Progressive Spermatozoa x 100\%}}{\text{Observed Spermatozoa}}$$

Basic Extender Configuration

The semen base extender is prepared a day before the cement shelter is done. The basic extender used in this study is a Tris-citrate-glucose solution (Salmin et al., 2022), later called the Tris extender, which consists of Tris (hydroxymethyl) aminomethane, citric acid and glucose. The process of making Tris base extender is as follows; A total of 3,634 grams of Trice (hydroxymethyl) aminomethane, 1,99 grams citrate acid and 0,50 grams glucose are dissolved with aqua distillate until a total volume of 100 ml is reached and then inserted into a clean and dry Erlenmeyer. The solution is mixed in a 1000C water irrigator for 15 minutes until homogeneous (seems clear) then cooled to room temperature and added antibiotics such as a 1,000 IU/ml penicillin extender and a 1 mg/ml streptomycin extender. Next, the solution is stored at a temperature of 3 - 50C in the refrigerator and when to be used for extender then the addition of soybean lecithin according to the rate of treatment to be tried.

Preparation of the Tris-Lecithin Extender of Soybeans

The Tris lecithin extender is prepared for the purpose of slow freezing one hour before cement storage begins. Tris base extender prepared the day before is divided into five tubes and then added soya lecithin according to the rate of treatment tried. The base extensor mixture of Tris and soybean

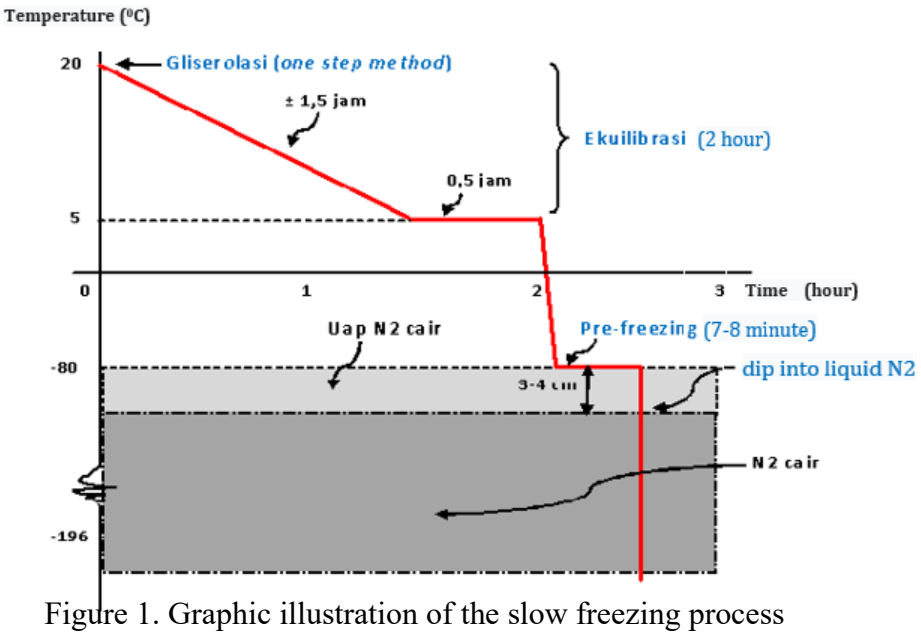

Figure 1. Graphic illustration of the slow freezing process

lecithin in each tubes is added 5% (v/v) of glycerol and then boiled to a homogeneous extender solution. Next, the extender solution is covered with aluminium foil and ready for use. The extender solution contains an antibiotic streptomycin of 1 mg/ml and penicillin of 1,000 IU/ml (Alhuur, *et al.,* 2020).

Cement Dilution and Glycerolising

Samples of semen worthy of processing are divided into five tubes of equal volume. Each of the semen samples in five tubes was then diluted with the Tris-lecithin extender of soybeans according to the treatment (L0, L1, L2, L3 and L4) to the required volume so that the sperm concentration reached 100 million cells per 0.25 ml IB dose.

The glycerolising process in this study uses the one-step method. (Evans dan Maxwell, 1987; Salamon dan Maxwell, 1995). Glycerol administration in a semen sample is only once done, that is, simultaneously with the process of dilution of semen at a temperature of 200C. The extender used for semen diluting in this study is the Tris-lecithin extender of soybeans that has been mixed with the entire volume of glycerol required.

Equilibration

Equilibration is carried out for 2 hours, i.e. since the cement has undergone a process of dilution and glycerol is used in a closed room conditioned at a temperature of 200C until during the process of temperature reduction to and at 50C in the refrigerator (Evans and Maxwell, 1987; Salamon and Maxwel, 1995). The work procedure of balancing is as follows; samples of cement in the tube that have under gathered a procedure of diluting and gyrorotation (L0, L1, L2, L3 and L4) at 200C are inserted into the glass containing water as a jacket (water jacket), then placed in the fridge until the water in the glass reaches the temperature of 50C (extended temperature decrease of approximately 1.5 hours). At this temperature, the storage of the sample in the freezer fridge is continued for 0.5 hours for the remaining balance time.

Filling and Sealing in Straw (Filling-Sealing)

The samples of cement that have undergone the process of balancing are packaged in the form of straw. The straw packaging used in this study is a mini straw with a capacity of 0.25 ml. The process of filling and sealing of liquid cement in straw

is done manually using a 1.0 ml spoit and a heated mindset. (Rizal dan Herdis, 2008).

The Freezing Process

Cement freezing in this study was carried out by slow-freezing method, i.e. using liquid nitrogen (liquid N2) in a Styrofoam box and a liquid-nitrogen container. Samples of cement in the finished straw undergoing a balancing process were initially feezed at the temperature of liquid nitrogen vapor, positioned 3 - 4 cm above the surface of the liquid Nitrogen in the Styrofoam box for 7 - 8 minutes. Immediately after pre-freezing, the straw-straw was immediately submerged in liquid nitrogen and then placed in a container at -1960C temperature. (Evans dan Maxwell, 1987). The slow freezing process is illustrated graphically in Figure 1.

Data Analysis

The data obtained had been analyzed using One Way Analysis of Variance and Duncan's Multiple Range Test using the SPSS 25 program (Nawari, 2007).

RESULTS AND DISCUSSION

Quality Spermatozoa Frozen Sheep Using Pine Bean Lesitin Extender

Quality of Spermatozoa Frozen Sheep Using Lecithin Pine Bean Extender Results of observations on the quality of spermatozoon of post-freezing sheep treated with soybean lecithin levels are listed in Table 1.

Table 1. Quality circle of sperms of sheep after freezing of various levels of lecithin of soybeans

Soya Bean lecithin rate (L)	Quality circle of sperms of sheep after freezing			
	Motility	Viability	Abnormality	Membran integrity
0% (L0)	17,76±3,64[a]	21,34±3,20[a]	17,03±2,84[a]	22,17±2,66[a]
1% (L1)	18,05±2.52[a]	22,73±2,78[b]	15,71±2,83[b]	23,65±2,83[b]
2% (L2)	30,08±2,27[b]	37,52±3,04[c]	14,57±2,94[b]	38,46±2,65[c]
3% (L3)	43,79±3,01[c]	50,85±3,49[d]	13,72±2,92[b]	51,65±3,34[d]
4% (L4)	34,88±3,11[d]	41,12±2,76[e]	15,62±2,90[b]	42,11±2,92[e]

(%) Soya bean lecithin rate (L) Quality of semen sheep post-frozen

[a,b,c,d,e] Different superscripts in the same column show a distinct difference (P<0,05).

Based on the results of the diversity analysis that the levels of lecithin in soybeans showed a distinct real influence

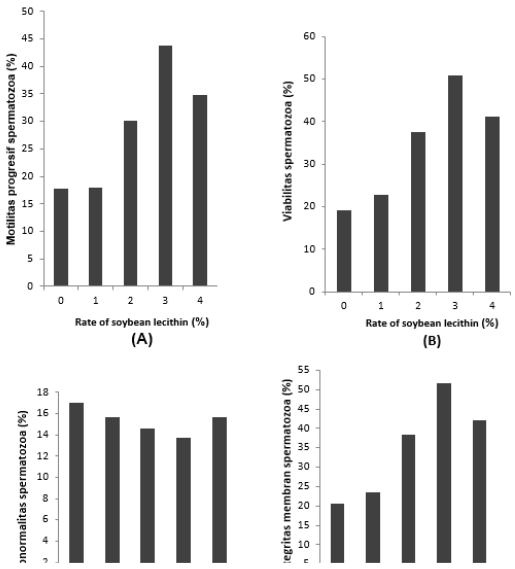

Figure 2. Influence of soybean lecithin levels on progressive motility (A), viability (B), abnormality (C) and membrane integrity (D) of lamb post-freezing spermatozoa

(P<0,05) on all the quality parameters of the post-freezing sheep spermatozoa. The higher the level of lecithin in soybeans, the higher the percentage of progressive motility, viability, and integrity of sperm membranes up to 3% of soybeans, then there is a decrease in 4% of soyabeans and the decreasing percentages of abnormalities spermatozoa up to the 3% of Soybeans and then an increase in 4% soybean lecithin. (Figure 2).

Further testing results showed that the percentage of progressive motility (43.79%), viability (50.85%) and membrane integrity (51.65%) of post-freezing spermatozoa treated with 3% soya bean lecithin (L3) were significantly higher (P<0.05), and sperm abnormalities (13.72%) were significantly lower (P <0.05) than all treatments tested. Explicatively, the results of this study are worthy to be used for use in artificial insemination activities, this is because it still contains more than 40% progressively motile spermatozoa according to the Indonesian National Standard. (Umiyasih et al., 1993; Rizal et al. 2004; National Standard Indonesia, 2005) and abnormal spermatoses not more than 15% (Ax at al., 2000).

The results in this study are supposed to be because at L3 treatment, soybean lecithin (3%) is more effective in protecting spermatozoa from the effects of slow freezing so that the progressive motility, viability, abnormality and integrity of the post-slow freeze sperm membrane remains awake. At this rate, spermatozoa are relatively avoided from the adverse effects of cryopreservation such as the occurrence of freezing injuries (Evans and Maxwell, 1987; Salamon and Maxwel, 2000) and deadly damage (freeze kill) (Bearden et al., 2004) or that reduces the viability of sperm. (Oehninger et al., 2000). As is well known, the main content of soybean lecithin is phospholipids (Shurtleff and Aoyagi, 2007) or egg-yellow-like phospholipids. (Anel et al., 2006). This compound is proven to function as a cryoprotective component to protect the integrity of the spermatozoa membrane during cryopreservation (Aurich, 2005). It is not yet known exactly how soybean lecithin works in protecting spermatozoa membranes from freezing injury/freeze kill, but some scientists think that its action is similar to LDL (low-

density lipoprotein) (Zang et al., 2009) as found in egg yolks. (Bergeron et al., 2006). In this case there are two major hypotheses developing; first, that important component of the bio-membranes of mammalian sperm cells, phospholipids play an important role in regulating the physiological functions of the biomembrane and enter the cell to lower the crystal freezing point by placing plasmalogen-plasmalogen to reduce mechanical damage to the spermatozoa's biological membranes. (Waterhouse et al., 2006). Some scientists agree with this opinion and think that soy lecithin can reduce the ratio of cholesterol or phospholipids in the sperm cell membrane by seeping into the spermatozoa membrane. Moreover, soybean lecithin phospholipids can replace some of the phospholipids of the semen membrane to maintain its structure and function.

Secondly, other experts believe that soya beans lecithin fosfolipid cannot penetrate into the spermatic membrane to alter the concentration of bio-membranes phospholipids, but it can integrate with the sperm cell membrane to form a protective film (Zhang et al., 2009) or form an interfacial layer between fatty acids and water (Anton et al. 2003), in combating the formation of lethal intracellular essence crystals and protecting the sperm membrane from damage during the mechanical and thawing process. (Zhang et al., 2009).

More specifically, there are at least three main roles that the interfacial layer plays, namely: (a) decreases the surface tension on the plasma membrane, mainly due to the presence of protein absorption on the surface of the membrane; (b) can form a mechanical defense system on the surfaces of the plasmatic membrane through the formation of a thin viscoelastic layer to prevent damage to the structures of then plasma membranes; and (c) plays a role in controlling the colloidal interaction between fatty acids and water. (Anton et al., 2003). This second hypothesis is supported by observations that microparticles of soybean lecithin are relatively larger than spermatozoa under a microscope. Therefore, they argue that it is not possible that the microparticles of soybean lecithin could enter the sperm cell membranes and this last opinion is more endorsed. (Zhang et al., 2009).

Low percentages of progressive motility, membrane integrity, viability, and high percentage of abnormalities of sheep soybean lecithin (FL4), the concentration of soybeans lecithin in the extender solution is relatively higher so that the medium becomes hypertonic. Visually, at that rate, the Tris-lecithin extender of soybeans looks a bit thicker compared to other treatments. Hypertonic extender medium can interfere with movement and harm spermatozoa. (Evans dan Maxwell, 1987). If the activity of spermatozoa movements after slow freezing continues continuously, the

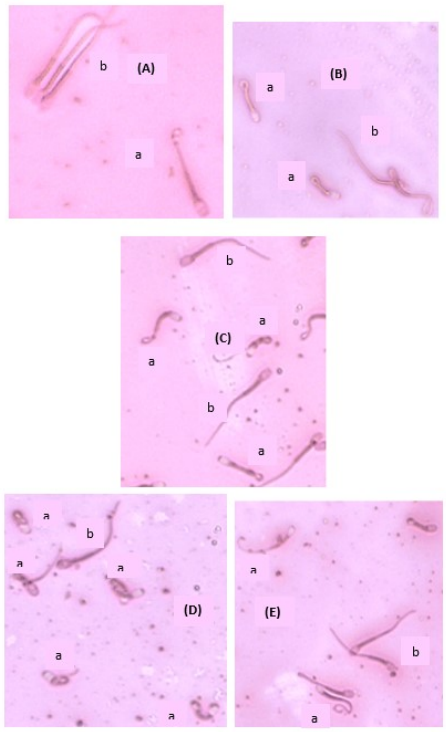

Figure 4. Preparation of the membrane integrity of the post-freezing spermatozoa from the treatment of 0% (A), 1% (B), 2% (C), 3% (D) and 4% € soya bean lecithin. The membrane of the integer spermatozoa is marked with a circular tail or a buzzing tail (a) and the membrane of the imperfect spermatozoic is marked with a straight tail (b).

condition is suspected to result in sperm exhaustion of energy and damage to the sperm morphological structure, such as tail or neck section cut off . (Bearden et al., 2004). (2) at levels below 3% of soybeans lecithin (L0, L1 and L2), soybean lecithin cryoprotect is relatively too low of the spermatozoa in other treatments are suspected to be due to; (1) at a 4% level of freezing injury or freeze kill. In this condition, the spermatozoa is suspected to have plasma membrane damage (Figure 4) which causes a disturbance of cellular bonding and the release of essential required level so it does not optimally protect sperm from the adverse effects of intracellular substances that can ultimately decrease motility and kill sperm.

The results of this study differ from previous researchers' reports on pig sperm that were cryopreserved using the soya bean lecithin extender where the quality of the sperm they obtained was lower at a rate of 3% but higher at 6% (Zang et al., 2009). This is believed to be due to species differences, where sheep sperm is likely to be more responsive to soya lecithin cryoprotectant at lower rates than pig sperm. Medeiros et al. (2002) stated that damage due to the effects of cooling and freezing on the spermatozoa membrane varies among domestic species and is influenced by several elements, namely the lipid bilayer content, the cholesterol/phospholipid ratio, the protein/phospholipid ratio and the degree of saturation of the spermatozoa membrane. hydrocarbon chain. The lower the ratio between cholesterol and unsaturated fatty acids, the more susceptible the plasma membranes are. Sheep sperm contains cholesterol of 226 mg/1000 million cells (Darrin-Bannett et al., 1973 in Rizal and Herdis, 2008) and in pig spermatozoa it may have lower levels, so Barbas and Mascarenhas (2009) categorized that pig sperms are sensitive to cold shock compared to sheep sperms.

CONCLUSION AND SUGGESTIONS

Conclusion

Soybean levels have an optimal effect in Tris extender on the quality of frozen sheep spermatozoa. The higher the level of soybean lecithin, the higher the percentage of progressive motility, viability and membrane integrity of spermatozoa. The best soy bean lecithin is obtained at a rate of 3% in the Tris extender with percentages of progressive motility, viability, abnormality and integrity of the post-freezing sperm membranes of 43.79%, 50.85%, 13.72% and 51.65%, respectively.

Suggestions

Research is needed from other aspects of the use of soybean lecithin to improve the quality of cryopreserved sheep sperm through freezing methods. Aspects that need to be studied as soon as possible may be long and methods of balance, cooling rate, ratio between soybeans lecithin and other extender components such as Tris-citrate-fructose.

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