Effect of Addition of Different Egg Yolks in Basic Tris-Soya Diluent on Quality, Membrane Integrity of Senduro Goat Sperm, and Free Radicals during Storage at Temperature of 4-5º C

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Abstract. Semen diluents need macromolecules to protect sperm from the impact of temperature during storage and the presence of free radicals. The purpose of this study was to examine the effect of adding yolk from different types of eggs to the quality and integrity of the sperm membrane. Fresh semen was diluted with basic tris-soya diluent which was added with different types of chicken egg yolk, such as local chicken eggs and leghorn chicken eggs. As a control group, fresh semen was diluted with basic tris-soya diluent without the addition of egg yolk. The addition of egg yolk was 20% of the diluent volume. Quality observations include motility and viability of sperm. Sperm motility was observed using a microscope with 200 X magnification. The sperm viability was observed using the eosin and negrosin staining method, then it observed under a microscope with 200 X magnification. The integrity of sperm membrane was observed using the HOST method, and then observed under a microscope with 200 X magnification. Free radicals then observed with MDA test. The results showed that the motility, viability and membrane integrity of sperm only lasted until the 12 hour of storage, with the greatest motility, viability and membrane integrity obtained in diluents with the addition of leghorn chicken egg yolk. The smallest MDA test was 2.72, which obtained from diluents with the addition of leghorn chicken egg yolk. Based on the results of the study it can be concluded that the leghorn chicken egg yolk has the best ability to maintain the quality of Senduro Goat sperm during 4-5ºC storage temperature.

1. Introduction
The application of Artificial Insemination (AI) technology is very important in Indonesia as an effort to increase the productivity of meat and milk. One of the livestock that is widely developed by Indonesian people is the Senduro Goat. Senduro goats are loved by many breeders, because they have two advantages, they can grow quickly with good quality meat/ carcass, so they can be used as meat-producing cattle. In addition, female goat can produce milk in large quantities and good quality.

The development of AI technology is inseparable from the sperm storage technology. The long-standing semen storing method is storage at low temperatures, both at 4-5ºC in the refrigerator and at freezing temperatures in liquid nitrogen [1]. Development of sperm storage technology is based on the principle that cells will experience low metabolism at low temperatures that was used to adapt in their environment, so that they can extend their life [2]. Therefore the development of sperm storage
technology is mainly storage at low temperatures. The usual way to store sperm is stored at 4-5°C (in the refrigerator) and minus temperature (freezing).

Storing semen at low temperatures can reduce the quality of sperm. The cooling process has induced the reorganization of intramembranous particles in the plasma membrane of cow and goat sperm [3]. Gaczarzewicz et al. [4] reported that there has been a decrease in the integrity of plasma membranes and acrosomes, as well as the motility of spermatozoa during the dilution and storing of goat semen liquid. Therefore, on the storage process of sperm we need diluent media that can maintain the sperm quality during it storage. In diluents, there are some ingredients that protect sperm from the outside of the membrane (macromolecules) and materials that can protect sperm from the inside (intracellularly, for example glicerol). Results showed that macromolecules could protect sperm during storage [5], Ducha et al. [6], Ducha et al. [7], Ducha et al. [8]. Then, this study aim was to examine the effect of adding egg yolks from different types of chicken, as macromolecular material in semen diluent during 4-5° C storage temperature.

2. Material and Method

2.1 Process of Making Basic Tris-Soya Diluent with Egg Yolk Supplementation

The ingredients for making tris base diluents were: 2.96 gr tris base (Bioworld, USA); 1.65 gr of citric acid (Bioworld, USA); 2.00 gr Fructose (Bioworld, USA); 100,000 IU penicillin (Meiji, Japan), 0.1 gr streptomycin (Meiji, Japan), and 100 ml sterile distilled water (Milli-Q-water). The tris base extender was then added to the egg yolk from leghorn chickens and local chickens at 20% of the tris base volume. For the control group using a tris base diluent without the addition of egg yolk.

2.2 Semen Preparation and Dilution Process

Fresh semen of the Senduro Goat was obtained from the Technical Implementation Unit, Animal Husbandry Department, and East Java Province, Indonesia. Semen was collected using the artificial vaginal method. The fresh semen then evaluated macroscopically and microscopically before the dilution process, and the concentration was calculated using an "accucell" spectrophotometer. The next step was the dilution process with a concentration of 25 x 106 / ml.

2.3 Observation of Semen Quality

Sperm Motility
Spermatozoa motility was observed under a light microscope at 200x magnification. Fresh semen and treatment during storage every 6 hours were taken using an ose needle, then it dropped on an object glass and placed on a slide warmer at 37 ° C on the microscope object table. The observation of sperm motility was carried out by two observers.

Sperm Viability
Method of observation for sperm viability uses eosin-negrosin staining. The advantages to this stain were that permanent slides can be made and the nigrosin provides a dark background for easier recognition of the non-stained, viable cells. Non-viable sperm have red or dark-pink heads and viable sperm have white or faintly-pink heads.

2.4 Integrity of Sperm Membrane
Membrane integrity was identified by Hipoosmotic Swelling Test (HOST), using a solution with low osmotic pressure (Fonseca, et al, 2005). This test was performed by incubating 100 µl semen (control and treatment) with 1 ml of 125 mOsm/l hipoosmotic (0.31 g Natrium Sitrat dan 0.565 g Fruktosa in 50 ml Aquades) at 37°C for 30 min. . After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm were evaluated (magnification 400×) using light microscopy. Sperm with swollen or coiled tails were recorded.
2.5 MDA Test

Seminal MDA levels were analyzed according to Rao, et al (1989). MDA was assessed using the thiobarbituric acid method. Briefly, semen samples were centrifuged for 7 min at 2000 g, and then 100 μl of seminal plasma (supernatants) was added in 900 μl of distilled water into glass tube. To each tube, 500 μl of thiobarbituric acid reagent (0.67 g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g NaOH and 100 ml glacial acetic acid added) was added and then heated for 1 h in a boiling water bath (all samples run as duplicates). After cooling temperature, each tube was centrifuged for 10 min at 4,000g and the supernatant absorbance of these was read on a spectrophotometer at 534 nm.

2.6 Data Analysis

Data of Sperm motility, viability, membrane integrity was in the form of a percentage. First of all, data were transformed using the archsin transformation. The results of the next transformation were tested for normality. The normality test results if the data indicate normal-distributed, then ANOVA test was conducted, and then followed by Duncan's test to see differences between treatments.

3. RESULTS & DISCUSSION

The fresh semen which was diluted, then it stored at 4-5 ° C temperature. During the storage process, the sperm quality was observed every 6, 12 and 24 hours after storage. Parameters of sperm quality were including: motility, viability and membrane integrity. In addition, the presence of free radicals was also observed based on MDA test.

3.1. Sperm Motility

The average data of the Senduro Goat sperm motility can be seen in Table 1.

| TREATMENT/REPETITION | The Average of Motility Percentage at Hour Of | 6 | 12 | 24 |
|----------------------|---------------------------------------------|---|----|----|
| Tris                 | 30,83 ± 5,46<sup>a</sup>                    | 0,00 ± 0,00<sup>b</sup> | 0,00 ± 0,00<sup>c</sup> |
| Tris + leghorn chicken egg yolk | 58,33 ± 3,02<sup>a</sup>                  | 5,00 ± 0,00<sup>a</sup>   | 0,00 ± 0,00<sup>b</sup> |
| Tris + local chicken egg yolk     | 9,17 ± 1,47<sup>d</sup>                      | 0,00 ± 0,00<sup>b</sup>   | 0,00 ± 0,00<sup>a</sup> |

Based on the Table 1, the observation results showed that there was a change in sperm motility in all treatments and showed significant differences during storage. A quite drastic change was in the basic tris-soya diluent supplemented with egg yolk from localchicken. The Senduro Goat sperm motility was only able to survive at 6 o'clock on all diluents.

3.2. Sperm Viability

The observation result of the Senduro Goat sperm viability during storage in the basic tris-soya diluent supplemented with different types of egg yolk can be seen in Table 2.
Tabel 2. Data of Senduro Goat Sperm Viability in Basic Tris-Soya Diluent Supplemented with Different Types of Egg Yolk at 4-5°C Storage Temperature

| Treatment/ Repetition | The Average of Viability Percentage At Hour of - |  |
|-----------------------|-----------------------------------------------|--|
|                       | 6     | 12   | 24   |   |
| Tris                  | 47.12 ± 3.64<sup>c</sup> | 9.00 ± 5.07<sup>ab</sup> | 0.00 ± 0.00<sup>a</sup> |   |
| Tris + leghorn chicken egg yolk | 65.45 ± 1.67<sup>b</sup> | 18.34 ± 1.18<sup>a</sup> | 0.00 ± 0.00<sup>a</sup> |   |
| Tris + local chicken egg yolk | 16.33 ± 3.36<sup>d</sup> | 8.39 ± 1.34<sup>b</sup> | 0.00 ± 0.00<sup>a</sup> |   |

Based on Table 2, results showed that the viability of Senduro Goat sperm has decreased in various treatments. A drastic reduction occurred in tris diluent with local chicken egg yolk supplementation. Duncan's test results showed a significant difference in mean viability between all treatments. The best viability was in the tris-base with leghorn egg yolk.

3.3. Membrane Integrity

Another parameter which observed was the integrity of the sperm membrane. Data of the integrity of sperm membrane during storage in the basic tris supplemented with egg yolk diluent can be seen in Table 3.

Tabel 3. Data of Senduro Goat Sperm Membrane Integrity in Basic Tris-Soya Diluent Supplemented with Different Types of Egg Yolk at 4-5°C Storage Temperature

| Treatment/ Repetition | The Average of Membrane Integrity Percentage At Hour of - |  |
|-----------------------|--------------------------------------------------------|--|
|                       | 6     | 12   | 24   |   |
| Tris                  | 45.34 ± 3.64<sup>b</sup> | 11.62 ± 5.07<sup>ab</sup> | 0.00 ± 0.00<sup>a</sup> |   |
| Tris + leghorn chicken egg yolk | 74.34 ± 1.67<sup>a</sup> | 18.65 ± 1.18<sup>a</sup> | 0.00 ± 0.00<sup>a</sup> |   |
| Tris + local chicken egg yolk | 15.95 ± 3.36<sup>c</sup> | 12.64 ± 1.34<sup>b</sup> | 0.00 ± 0.00<sup>a</sup> |   |

Based on Table 3, results showed that the membrane integrity of Senduro Goat sperm has decreased in various treatments. A drastic reduction occurred in tris diluent with leghorn chicken egg yolk supplementation, but it the best membrane integrity was in the tris-base with leghorn chicken egg.

3.4. MDA Test (Free Radicals Indicator)

MDA test results can be seen in Table 4.

Tabel 4. The Existence of Free Radicals (MDA)

| Diluent Types                     | MDA Concentration at First Storage | MDA Concentration at Last Storage |
|-----------------------------------|------------------------------------|----------------------------------|
| Tris                              | 2.249                              | 3.701                            |
| Tris + leghorn chicken egg yolk   | 0.686                              | 2.272                            |
| Tris + local chicken egg yolk     | 1.595                              | 3.445                            |

The observation results showed that the greatest MDA concentration was obtained in tris diluent only, without the addition of BSA or egg yolk.
In diluents, it is necessary to add other ingredients that can be used as extracellular protectors during storage process [8]. Egg yolk is often added in diluents because it has been shown to prolong the life span of cattle sperm [9]. It provides a membrane infrastructure and increase the membrane fluidity, which it can increase fertilization ability [10], change the lipid transition phase during temperature changes so that it can reduce sensitivity to cold temperatures [11]. Based on research, data showed different results on the sperm quality from various types of egg yolk of different animals. The motility, viability and integrity of the spermatozoa membrane were only able to survive the sixth hour storage, whereas in the 12th hour storage all diluents were very low, even the motility was zero in the tris base extender without egg yolk and with the addition of local yolk. These results were very different from the results of previous studies [5, 6]. which were applied to cow semen using CEP diluent.

The condition of the goat semen showed distinctiveness with other animal semen. This was due to the fact that there was a-phospholipase enzyme in goat plasma called the Egg Yolk Coagulating Enzyme (EYCE) which was secreted by the bulbouretral gland (cowper gland) and was toxic to sperm. This enzyme can coagulate a medium containing egg yolk due to hydrolysis of the yolk lecithin into lysol esitin and fatty acids which catalyzed by those enzyme [12]. Susceptibility of the plasma membrane to damage was due to the high ratio of unsaturated fatty acids in phospholipids. Prolonged lipid peroxidation will damage the structure of the lipid matrix which caused instability in the membrane and changes in the concentration of the lipid matrix structure [Ducha, 2018]. The toxicity of EYCE depended on the pH, temperature, concentration of semen plasma, semen production season, and the content of egg yolk [13, 14]. Efforts to minimize the occurrence of toxicity to spermatozoa was to reduce the presence of seminal plasma, ie by means of centrifugation [14, 15]. The results of the study of Ferreira et al [15] showed that the quality of storage of spermatozoa in goats was better in semen that was removed from the seminal plasma.

4. CONCLUSION

Based on the results of the study it can be concluded that the addition of egg yolk from leghorn chickens and local chickens in the tris base diluent has not been able to provide optimal protection against the quality of Spermatozoa Senduro Goats during storage at 4-5⁰C.

5. ACKNOWLEDGEMENTS

The research was funded by PNBP Surabaya State University (Unesa) in 2019. The researcher thanked the Unesa Rector, Chairperson of Unesa LPPM for giving the researchers the opportunity to carry out the research, and to the Head of the Indonesian AI Center in Singsosari and the Head of the Livestock Service Office of East Java Province has permitted research to be carried out.

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