**Quality of Garut Ram’s Sperm in Tris Extender Supplemented with Ethylene Diamine Tetraacetic Acid Preserved at Low Temperature (3-5°C)**

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**Abstract.** Ethylenediaminetetraacetic acid (EDTA) is a chemical substance that can bind the heavy metal and other toxic compounds so it is suitable to use for protecting sperm during preservation at 3-5°C. The research aimed to evaluate the role of EDTA to maintain the quality of Garut ram sperm in low-temperature preservation. Semen was collected by an artificial vagina. Fresh semen was evaluated and divided into 3 tubes and diluted with tris extender contain 20% egg yolk (TEY) without EDTA (T1), TEY+0.01% EDTA (T2) and TEY+0.02% EDTA (T 3). Semen was evaluated both macroscopically and microscopically before preserved in a refrigerator at 3-5°C. During storage, semen was also evaluated on daily basis (four consecutive days) that included variables relate to sperm motility (SM), sperm viability (SV), sperm membrane integrity (SMI) and sperm acrosome intact (SAI). The research design used was Completely Randomized Design with three treatments and five replications. Differences between treatments were tested with the least significant difference test. The results showed that the average of each variable gained of fresh semen was 0.88mL for volume, cream for color, thick for consistency and 7 for pH. While the mass movement of sperm was +++ and sperm concentration was 3,962 million cells/mL. The average percentages of SM, SV, abnormality, SMI, and SAI were 75.00%, 86.60%, 4.80%, 84.40%, and 84.80%, respectively. On the day 4 of storage, percentages of SM, SV, SMI, and SAI in control (T1) decreased to 48.75%, 66.25%, 64.75%, and 63.5%, respectively, but still met the requirement of sperm quality for artificial insemination purposes. Moreover, there was no significant difference between T2 (45.00%, 65.00%, 63.25% and 65.25%) and T3 (40.00%, 65.67%, 60.75% and 62.50%) of those variables measured. Finally, it was concluded that the addition of EDTA in Tris extender could maintain the quality of Garut ram sperm.

Keywords: Sperm, EDTA, garut ram, extender, Tris, preservation
1. Introduction
Garut sheep is an Indonesian crossbred sheep. It was composed of a crossbreed of Priyangan sheep (indigenous sheep in West Java), Spain-Merino sheep and African-Kaapstad sheep. Garut sheep is a prolific animal and has no breeding season. Garut ram is regularly exposed at an agility sheep contest that makes it more valuable than other local sheep. Moreover, Garut ram has a dashing posture, good libido and typical horn with a large size, sturdy, strong and circular. These all characteristics make Garut ram has a high potential to produce a good quality of semen for increasing the productivity of other local sheep through artificial insemination (AI) application.

Semen processing as chilled or frozen semen is an important step in AI procedures. The main objective of semen processing is to increase the capacity of the number of ewes that are mated by sperm ejaculate and prolong the storability of semen[2].

One of the efforts to maintain sperm quality during cryopreservation is adding some chemical compounds such as carbohydrate/sugar, vitamin, antioxidant and compound as a chelating agent (ethylene diamine tetraacetic acid/EDTA) into the extender. Sugar has the function as a source of energy and extracellular cryoprotectant so it could protect sperm during semen processing[3] [4]. Sugar has been proved to maintain the quality of frozen semen such as maltose in ram frozen semen[5], trehalose and EDTA in pampinta ram frozen semen[6][7], raffinose in spotted buffalo semen[9], and lactose in ettawa ram semen[10].

EDTA is a molecular structure which can bind heavy metals and toxic compound. Therefore, it needed to protect sperm during the preservation process. Moreover, EDTA also has a role as a cryoprotectant agent, so it can be reduced damage of sperm during cryopreservation process[6]. Adding EDTA into Tris extender can increase the quality of chilled and frozen bull semen[11]. The main objective of this research was to determine the effect of EDTA on the quality of Garut ram sperm in low-temperature preservation.

2. Methodology
The material used in this experiment was semen taken from five superior Garut ram that has bodyweight ±80kg and 4 y.o. Garut rams were placed in the individual lot and fed by 8kg/ram/day forage and 0.8kg/ram/day concentrate. Semen was collected using an artificial vagina and ejaculated semen were immediately evaluated following collection and diluted with different extender. The research design used in this research was a completely randomized design with three treatments and five replications. The treatments consisted of Tris egg yolks diluent (TEY) with no addition of EDTA (T1), TEY+0.01% EDTA (T2), and TEY+0.02% EDTA (T3). The variable measured for fresh semen were volume, color, consistency, concentration, mass movement, percentage of sperm motility (SM), percentage of sperm viability (SV), percentage of sperm membrane integrity (SMI), and percentage of sperm acrosome intact (SAI). The diluted semen was then storage at 4ºC for four days. To determine the effect of the treatment on sperm quality, evaluation of sperm quality was carried out for four consecutive days. The parameters measured for each evaluation stage consisted of the percentage of SM, percentage of SV, percentage of SMI and percentage of SAI. Data obtained were analyzed using variance analysis and differences between treatment were analyzed using Least Significant Difference.

3. Results and Discussion
The quality of semen was analyzed on two occasions, following collection (fresh semen) and after diluting with extender and storage in low temperature (3-5ºC) (chilled semen). The quality of fresh semen consisted of semen volume, color, consistency, pH, the mass motion of sperm, concentrate, sperm motility (SM), sperm viability (SV), sperm abnormality (SA), sperm membrane integrity (SMI), and sperm acrosome intact (SAI) (Tabel 1). While the quality of sperm following storage (chilled semen) was limited only on SM, SV, SMI, and SAI (Table 2).
3.1 Characteristics of Fresh Semen

Fresh semen evaluation consisted of evaluation of semen quality included volume, color, consistency, and pH of semen, and evaluation of sperm quality included mass motion, concentration, sperm motility (SM), sperm viability (SV), sperm abnormality (SA), sperm membrane integrity (SMI), and sperm acrosome intact (SAI). The results showed that those variables relate to the quality of semen and sperm in fresh semen (Table 1) were eligible for further semen preservation process.

The volume of semen obtained during collection relates to the doses of semen (number of a straw) produced, in which the lower semen gained the lower semen doses produced. The average of semen volume obtained in this research was 0.88ml. This result was consistent with the ram's semen volume in general with a range of 0.8-1.2ml [12]. Generally, semen volume was determined by several factors such as age, body size, reproductive health and the frequency of semen collection.

| Characteristics                      | Averages±SE    |
|--------------------------------------|----------------|
| Volume per ejaculation (ml)          | 0.88 ± 0.05    |
| Color                                | Cream          |
| Consistency                          | Viscous        |
| pH                                   | 7.02 ± 0.11    |
| Mass Motion                          | ± 0.00         |
| Concentration (x10^6 sperm/ml)       | 3,962 ± 874    |
| SM (%)                               | 75.00 ± 0.00   |
| SV (%)                               | 86.60 ± 0.55   |
| SA (%)                               | 4.80 ± 1.60    |
| SMI (%)                              | 84.80 ± 1.92   |
| SAI (%)                              | 84.40 ± 1.34   |

The other variables of fresh semen that were considered as important indicators that should be known in semen assessment were sperm concentration and sperm motility. The sperm concentration gained in this research was 3,962±874 million cells/mL. It was lower than previous research in which the concentration of fresh ram semen was 4,146±872 million cell/mL[17]. Whereas the average percentage of sperm motility of fresh semen gained in this research was 75.00% which was also lower than results reported by Hartanti and Karja (2014) as much as 81.25%[13], but Nalley and Arifiantini (2013)[14] reported lower result (72.92%). Fresh semen should have a motility percentage of at least 70% and sperm abnormality less than 20%[15].

3.2 Characteristics of Chilled Semen

The average of motility percentage, viability, membrane integrity and acrosome intact percentage of chilled semen during four days storage in low temperature was presented in Table 2.

| Variable  | Treatments | Time of Evaluation |
|-----------|------------|--------------------|
|           |            | Day-1             | Day-2             | Day-3             | Day-4             |
| Motility (%) | T1         | 75.00 ± 0.00      | 61.00 ± 7.42      | 53.00 ± 5.70      | 48.75 ± 2.50      |
|           | T2         | 75.00 ± 0.00      | 63.00 ± 5.70      | 52.00 ± 5.70      | 45.00 ± 4.08      |
|           | T3         | 75.00 ± 0.00      | 60.00 ± 5.00      | 53.33 ± 7.64      | 40.00 ± 5.00      |
| Viability (%) | T1        | 85.60 ± 6.19      | 75.20 ± 3.56      | 69.00 ± 7.31      | 66.25 ± 3.89      |
|           | T2        | 83.20 ± 1.79      | 75.20 ± 1.92      | 69.40 ± 4.04      | 65.00 ± 4.08      |
|           | T3        | 83.40 ± 2.61      | 76.00 ± 0.00      | 71.67 ± 1.15      | 65.67 ± 4.04      |
| Membrane  | T1        | 83.50 ± 1.73      | 74.00 ± 2.71      | 70.75 ± 2.22      | 64.75 ± 3.77      |
The result showed that semen diluted with TEY+EDTA was still proper for artificial insemination until day-4 of storage because it has a motility percentage of over 40%[18]. According to the Indonesian National Standard No. 4869.1:2008, frozen semen has to have at least 40% post thawing motility[16]. On day-4, sperm motility percentage of each treatment were 46.75%, 45% dan 40%, respectively.

In general, the addition of 0.01% (T2) and 0.02% EDTA (T3) in TEY extender could preserve the quality of sperm during 4 days of storage. However, the addition of 0.02% EDTA showed a significantly lower motility percentage than 0.01% EDTA (P<0.05). Moreover, EDTA doses used in this treatment was considered high so it tends to have a bad effect on sperm quality, although in T2 treatment showed better results compared to control treatment (T1).

The adding dissolve compound like a large number of carbohydrates into extender can increase osmotic pressure. The high osmotic pressure of extender will cause sperm swell and react to physiological conditions and even death[2]. The addition of trehalose and EDTA simultaneously in extender could repair the quality of pampinta sheep frozen semen during the cryopreservation process. It was caused by complementary influence between trehalose and EDTA but in this research, only EDTA was used without sugar[6].

The difference result was also reported in cattle and human semen that was added by 0.1g EDTA per 100mL Tris extender in chilled and frozen semen. The motility percentage, viability, and membrane integrity both chilled and frozen semen with EDTA were higher than without EDTA treatment. The addition of EDTA into human sperm extender could increase motility percentage and hence could stimulate fertility in men. Those results indicated that there was a different response of multispecies of livestock sperm to treatment.

4. Conclusion
Based on the result, it could be concluded that the addition of EDTA in Tris Egg Yolk extender could maintain the quality of chilled semen of Garut ram during four days of storage at low temperatures.

5. Acknowledgment
We would like to gratefully thank the researchers at Reproduction Laboratory Center of Agricultural Production Technology BPPT and Lesan Putra Garut Rams Farm Bogor.

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