Sperm Longevity of Garut Ram Frozen Semen in Tris-Egg Yolk Extender

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ABSTRACT

The longevity of sperm after freezing and thawing influenced by the extender. The aim of this study was to evaluate the quality of post thawing motility and longevity of Garut ram sperm in Tris-egg yolk (TEY) extender. Twelve Garut rams were used as a source of semen. Semen collection was conducted once a week using an artificial vagina. Immediately after collection the semen was evaluated macro and microscopically. Semen with sperm motility >70% and sperm concentration of >2,000×10⁶ cells ml⁻¹, was individually divided into three tubes. Each tube diluted with TEY, tris with omega three egg yolk (TEYO), and tris with egg yolk supplemented with omega three gel (TEYOS). Diluted semen were packed into 0.25 ml mini straw and equilibrate at 5°C for 4 hours. The straw was then frozen on liquid nitrogen for 10 minutes and stored in liquid nitrogen tank until further evaluation. The present study demonstrated that no differences were found in the post thawing motility in all extenders as well as in sperm recovery rate. Post-thawing quality in TEY, TEYO, and TEYOS were 56.00±8.94, 55.00±9.35, and 59.00±5.48%, respectively. Longevity of sperm in TEYO at 4th hour incubated at 37°C was significantly lower (P<0.05) than TEY or TEYOS. In conclusion, TEY, TEYO, and TEYOS extender can be used for Garut frozen semen.

Key Words: Longevity, Ram Semen, Tris-Egg Yolk, Omega 3

INTRODUCTION

Sheep population in Indonesia was 16,509.33 in 2015 (Ditjen PKH 2015). Demand for lamb is increasing from year to year. This increasing demand should be compensated by increasing the population of the animals. Artificial insemination (AI) is a reproductive technology that can increase livestock populations. Breeding with AI has several advantages including: (1) Solve problem to animals that cannot mate due to anatomical or pathological abnormalities; (2) Increase the number of female in imprisoned per ejaculate; (3) Facilitate the genetic improvement of livestock population; (4) Facilitate the control of diseases transmitted through natural mating; (5) Select the quality of semen that will be used; and (6) Store superior genes for conservation with cryopreservation techniques (England & Millar 2008).

Ram frozen semen were currently being produced at the Lembang Artificial Insemination Center (AIC). The AIC produces sheep frozen semen referring to the Indonesian National Standard (SNI) No 4869.3.2014 in 2014 for frozen semen of sheep and goats which has a motility of 40% with an individual movement (score) of at least two. In terms of post-thawing quality evaluation, besides using the SNI standard quality, AIC has added a test called water incubator test. The test was carried out by placing post...
thawed frozen semen at 37°C for 4th hours. Sperm which shows motility of more than 10%, are considered feasible to be inseminated, can be stored for distribution.

After cryopreservation, sperm fertility depends on the endurance of the sperm after thawing before being inseminated. The endurance of sperm after thawing was influenced by the type of diluent. The diluent used at Lembang AIC for the production of ram frozen semen is the AndroMed® commercial diluent (Minitube Germany). These commercial diluents used soybeans as a source of lecithin (Achlis 2011). Semen diluents for ram can also be home made recipe, for example, egg yolk tris diluents (Arifiantini & Yusuf 2006).

Chicken egg yolks were currently available in market, as regular egg and omega-3 (chicken eggs whose feed has been added to omega 3). Eggs that contain Omega 3 has been shown to strengthen the sperm plasma membrane, therefore protects the sperm during chilling (Nalley & Arifiantini 2011). Considering that sperm resistance is very important in the process of cryopreservation of semen and the quality of frozen semen is influenced by the type of diluent, research on the quality of frozen semen and the longevity (sperm motility within a certain time) of sperm after thawing on ram semen is important. The aim of this study was to compare the quality and longevity of sperm in Garut ram frozen semen diluted with tris egg yolk diluent added with omega 3.

MATERIAL AND METHODS

Animals

Twelve sexual mature Garut ram and one female were used as a teaser. The rams were kept individually in a 1.5×0.75 m cage. Each ram was given 5-7 kg of grass and 250 g of concentrate daily and ad libitum water.

Preparation of semen diluents

Tris buffer composition was adopted from Kulaksiz et al. (2012), consist of 3.63 of Tris (hydroxymethyl) aminomethane, 1.99 g of citric acid and 0.5 g of glucose in 75 ml of distilled water, 20 ml of egg yolks and 5% glycerol. The diluent composition of frozen semen consisted of various types of egg yolk, namely Tris regular egg yolks (TEY), Tris egg yolks omega-3 (TEYO), and Tris omega-3 gel egg yolks (TEYOS) as show at Table 1.

Table 1. Composition of frozen sheep semen extender

<table>
<thead>
<tr>
<th>Component</th>
<th>TEY</th>
<th>TEYO</th>
<th>TEYOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer tris (%)</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Regular egg yolk (%)</td>
<td>20</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Omega-3 egg yolk (%)</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Omega-3 (wild salmon oil) (g)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Glycerol (%)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Penicilline (IU/ml)</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Streptomycin (mg/ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

TEY: Tris regular egg yolk; TEYO: Tris egg yolk contains omega-3; TEYOS: Tris regular egg yolk + omega-3 (wild salmon oil)
Semen collection and evaluation

Semen collection was done by using an artificial vagina, once a week in the morning. Sheep were used as male libido anglers (teasers). The semen that was obtained, immediately taken into the laboratory for analysis. Semen collection and analysis techniques were carried out referring to Arifiantini (2012). The semen evaluation was done macroscopically including measurement of volume, color, consistency and pH of semen. Semen volume was measured using a 1 mL pipette with a 0.1 ml scale. The color of semen was observed visually. The consistency of semen was observed by tilting the tube containing semen, then returning it to its original position. The value consists of watery, moderate, thick and very thick. Acidity (pH) of semen was measured using pH indicator paper. The results obtained are matched to the color indicator.

Microscopic evaluation of semen includes sperm motility consisting of mass movements and individual movements/motility, concentration of sperm, sperm life (viability), and morphology of sperm (normality and abnormalities of sperm). Mass movements were carried out to see the movements of the sperm that move together. One drop of semen was placed on one object glass and then covered with a glass cover. Mass movements were assessed using a binocular microscope (Olympus CH 20) with a magnification of 100 times. The value of the mass movement was +++ (very good), ++ (good), and + (moderate).

Individual motions or motility of sperm is to assess sperm individually, either speed or comparison between those who were progressively active and other sperm motions (Arifiantini 2012). The motility of sperm is assessed by mixing one drop of semen with eight drops of physiological solution and homogenized. One drop of the solution mixture was placed on a glass object and covered with a glass cover. Motility assessment using a microscope with 400 times magnification in five fields of view. Its value was expressed in percent. The concentration of sperm was calculated using the Neubauer counting chamber. Calculation was done by diluting semen with formol saline 500 × (1 μl of fresh semen and 499 μl formol saline) in the microtube then homogenized. The homogeneous mixture was inserted into the Neubauer counting chamber. The concentration of sperm was calculated by multiplying the number of sperm from five boxes of 25×10^6.

The viability of sperm (live and dead sperm ratio) was observed using eosin-nigrosin staining with a ratio of 1:10. A mixture of eosin-nigrosin solution and semen was smeared into a preparation for drying. Sperm ratio that lives and dies using microscope magnification 400× from 10 fields of view or minimum cell count of 200. Sperm that lives do not absorb color and dead sperm will absorb color.

\[
\text{Live sperm} = \frac{\text{Live sperm}}{\text{Total sperm}} \times 100\%
\]

The morphology of sperm was carried out using eosin nigrosin staining as in the calculation of viability. Sperm was assessed based on normal forms and sperm abnormalities that occur in the head and tail.

\[
\text{Abnormal sperm} = \frac{\text{Abnormal sperm}}{\text{Total sperm}} \times 100\%
\]
Semen processing

Semen has sperm motility of >70% with a concentration of >2,000 × 10⁶ cells/ml divided into three tubes. Each tube was diluted with TEY diluent, TEYO, or TEYOS. Dilution formula as follows:

\[
\text{Total vol} = \frac{\text{Semen vol} \times \text{Sperm concentration} \times \text{Motility} \times \text{Vol of straw (0.25 ml)}}{\text{Insemination dose (50 × 10⁶)}}
\]

The semen that has been diluted, then packed into a 0.25 ml mini straw (Minitub Germany), stored at 5°C for four hours for equilibration process. Freezing were done by placing a freezing rack, 5 cm above the surface of liquid nitrogen (-130°C) placed in a styrofoam box for 10 minutes. The frozen semen was then stored in a liquid nitrogen container (-196°C) until further evaluation.

Post-thawing semen quality evaluation

Frozen semen was tested after 24 hours of storage. The semen was thawed at 37°C for 30 seconds, the contents of the straw were inserted in the microtube. Post-thawing motility (PTM), was done by placing the semen that has been thawed on an object glass and then covered with a cover glass. The motility of sperm was evaluated under a 400X magnification microscope. Value is expressed in percentage.

Sperm longevity after thawing

Frozen semen which has been suspended in the microtube was incubated in a 37°C water bath. Longevity of the sperm was tested by assessing the motility of the sperm from the same sample every hour until the motility of the sperm reaches 0%. The microscope magnification and the method of assessment are the same as those for the PTM test.

Calculation of recovery rate (RR)

The success of the freezing process was also assessed from the number of sperm that recovered after the freezing called recovery rate (RR). The RR value refers to Garner & Hafez (2000) as follows:

\[
\text{RR} = \frac{\text{Percentage of sperm motile after thawing}}{\text{Percentage of sperm motile of fresh thawing}} \times 100\%
\]

Data analysis

Data were analyzed using Analysis of Variance (ANOVA). If a significant difference is found, continue with the Duncan test using SPSS 20. Presented as means ±SD.

RESULTS AND DISCUSSION

Quality of fresh semen Garut ram

Macroscopic evaluation of fresh semen of Garut ram showed a volume of semen 0.76±0.30 ml, creamy, thick consistency with pH of 6.30±0.30. These results were in the
normal range of ram fresh semen according to Garner & Hafez (2000). Fresh semen of ram according to Garner & Hafez (2000) was creamy with thick consistency. The results of microscopic evaluation showed sperm mass movements ranging from ++ to ++++, sperm concentration of 35150±99.26×10^6 cells ml^-1. The sperm motility of 74.00±5.48% with the number of sperm living 83.00%. The number of abnormal sperm of garut ram in the study was very low at only 3.89%.

The results of this fresh semen were good, macroscopically or microscopically according to the quality range of ram semen. Semen which has high sperm concentration and motility with normal sperma morphology were suitable to be processed into frozen semen (Roca et al. 2006).

**Quality of frozen semen Garut sheep**

The quality of frozen semen extender used for freezing of Garut semen did not show any differences in quality after thawing (P>0.05). Frozen semen of Garut sheep showed excellent sperm motility with values between 59.17±4.92 to 62.50±2.78% (Table 2). The result of this study was high compared to other researchers with a value between 42.5±14.4% (Gustari 2003) up to 53.00±2.74% (Herdis et al. 2003).

The quality of Garut ram frozen semen in this study showed that the appropriate value even exceeds the quality requirements by SNI 4869.3: 2014 for frozen semen of goats and sheep in 2014.

**Table 2. Sperm motility after thawing of Garut ram semen with three types of extender**

<table>
<thead>
<tr>
<th>Extender</th>
<th>Sperm motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh semen</td>
</tr>
<tr>
<td>TEY</td>
<td>74.00±5.48</td>
</tr>
<tr>
<td>TEO</td>
<td>-</td>
</tr>
<tr>
<td>TEO</td>
<td>-</td>
</tr>
</tbody>
</table>

Sperm motility after thawing values in all three extenders have a good value. According to Amirat et al. (2007) egg yolks were added to the diluent, functioning to maintain and protect the integrity of sperm cell lipoprotein membrane. The extender that was contained in omega-3 salmon oil was better than eggs that contained omega-3 in protecting the sperm plasma membrane from cold stress during the cryopreservation process.

Longevity or survival is the ability of sperm to survive at a certain temperature (Arifiantini et al. 2005). The motility of sperm decreases in all treatments during storage in a water bath. This shows that the increase in time has a relationship to a decrease in sperm motility. Evaluation of sperm longevity at Lembang AIC requires that at the 4th hour the sperm still has a minimum motility of 10%. Referring to the AIC at 4th, the sperm in TEO and TEY both showed a value of >10% (Table 3), although statistically there were no differences. Frozen semen in TEO and TEY extender, both are suitable for distribution and use for AI.
Table 3. Longevity of ram sperm in tris egg yolk extender after thawing

<table>
<thead>
<tr>
<th>Hours of storage</th>
<th>Extender (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEY</td>
</tr>
<tr>
<td>0</td>
<td>60.00±0.00a</td>
</tr>
<tr>
<td>1</td>
<td>50.00±6.33ab</td>
</tr>
<tr>
<td>2</td>
<td>43.33±7.53bc</td>
</tr>
<tr>
<td>3</td>
<td>31.67±10.33c</td>
</tr>
<tr>
<td>4</td>
<td>11.67±17.22d</td>
</tr>
<tr>
<td>5</td>
<td>7.50±13.69d</td>
</tr>
</tbody>
</table>

Different small letters that follow the numbers on the same row show significant differences (P<0.05)

Another indicator of the success of freezing semen is the recovery rate (RR) value. The sperm RR values in all three diluents were not different (P>0.05). RR values for TEY extender were 81.08±15.02%, TEYO 79.96±0.16%, and TEYOS 84.46±3.76% (Table 4). These results indicate that sperm from all extender have an excellent sperm recovery ability after freezing.

Table 4. Recovery rate (RR) of Garut ram sperm in various extenders

<table>
<thead>
<tr>
<th>Extender</th>
<th>Post-thawing motility (PTM, %)</th>
<th>Recovery rate (RR, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEY</td>
<td>60.00±0.00a</td>
<td>81.08±15.02a</td>
</tr>
<tr>
<td>TEO</td>
<td>59.17±4.92a</td>
<td>79.96±0.16a</td>
</tr>
<tr>
<td>TEYOS</td>
<td>62.50±2.74a</td>
<td>84.46±3.76a</td>
</tr>
</tbody>
</table>

Different small letters that follow the numbers on the same row show significant differences (P<0.05)

Decreasing motility in the cryopreservation process is very common. Quality degradation occurs due to the freezing process starting from dilution using cryoprotectant which is hyper tonic, extreme temperature changes starting from freezing until after thawing (post-thawing). This situation shows that during the freezing and thawing process there is a reduction in the quality of sperm which is characterized by a decrease in the percentage of living sperm, the percentage of intact acrosome and the percentage of intact plasma membrane sperm (Herdis 2005).

Based on PTM values and RR values, all semen extender can protect sperm during freezing and thawing. The three extenders can be used to freeze garut ram semen with frozen semen quality according to even exceeding the SNI standard for frozen semen of goat sheep. Longevity of sperm in the three extenders also did not differ (P>0.05), but based on motility values, sperm in TEY and TEOY passed the water incubator test due to value of sperm motility of more than 10% in the 4th hour after thawing.

Omega-3 according to Towhid et al. (2013), contains polyunsaturated fatty acids (PUFA) and Eicosa Pentenoic Acid (EPA) and Docosahexaenoic Acid (DHA) as plasma membrane protectors in vitro. Their study proved PUFA and DHA are needed to maintain the sperm plasma membrane during cryopreservation. Cryopreservation process causes the formation of ice crystals which can reduce the quality of sperm. The content of PUFA
and DHA contained in omega-3 salmon oil is better when compared to omega-3 eggs in increasing the protection of sperm plasma membranes from cold stress during cryopreservation (Nurcholis et al. 2016). This study proves that the administration of omega-3 does not improve the quality of Garut ram frozen semen.

Omega-3 of wild salmon added to the tris egg yolk extender, contain 10 IU of vitamin E and 50 mg of lecithin. Vitamin E functions as an antioxidant to ward off free radicals or lipid peroxidation by donating hydrogen into a reaction that is able to convert peroxyl radicals (the results of lipid peroxidation) to less reactive tocopherol radicals, so as not to damage the fatty acid chain (Winarsi 2007). The content of 50 mg of lecithin in omega 3 is expected to strengthen the work of lecithin in regular eggs, therefore showed longevity of 13.33±16.63% after 4th hours.

CONCLUSION

Addition of Omega 3 in Tris-egg yolk extender of Garut ram frozen semen did not improve post-thawed semen quality. Longevity of sperm in Tris regular egg yolk added with salmon omega-3 slight higher than tris regular egg yolk or tris egg yolk omega-3. The fertility test of frozen semen is needed and an economic value of the addition of omega-3 salmon to the egg yolk extender is necessary done.

REFERENCES


DISCUSSION

How was the result of artificial insemination for goat compared to artificial insemination for cattle?

Answer

The problem of AI ram: (1) We need optimum time for mating; (2) Ram sperm is 25 million cell; (3) After thawing, ram sperm is more sensitive than cattle sperm; (4) Need nutrition; (5) The service is very small; (6) TEY regular yolk, omega 3; (7) Absolutely room quality control standard national, incubator; and (8) Sperm using both extenders still survived after 4 hours.