The Decreasing of Quality Liquid Semen Using Four Months Storages of Tris Aminomethan and CEP-3 Diluents

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Abstract. The purpose of this research was to evaluate the decreasing of Limmousin liquid semen quality using four months storages of Tris Aminomethan and Caudal Epididymal Plasma (CEP-3) diluents. This research was conducted at Animal Reproduction Laboratory, Faculty of Animal Science, University of Brawijaya. The material used was fresh semen of Limousin bull which was collected in artificial insemination center Singosari. The method used was experimental laboratory with two treatments and 10 replications including T0 (Tris Aminomethan + 20% Egg Yolk) and T1 (CEP-3 + 10% Egg Yolk). Data was analyzed by using t-paired test. The result showed that Tris Aminomethan + 20% Egg Yolk gave the best result in percentage of motility, viability and abnormality. The individu motility of T0 was higher (27.3±1.4) than T1 (26.8±2.4). The percentage of viability on T0 was higher (60.5±1.1) than T1 (60.0±3.0). while the percentage of abnormality was lower (13.9±0.4) than T1 (14.4±2.6). In conclusion after 4 months storage of diluents, tris Aminomethan + 20% egg yolk gave the best treatment in motility, viability and abnormality in liquid semen.

1. Introduction

Artificial insemination (AI) using frozen semen has often been applied on farm, however there are still many problems in handling frozen semen such as difficulties in obtaining liquid nitrogen. Artificial insemination technology using liquid semen used as a substitute for frozen semen was considered simpler and better [1]. The success of pregnancy AI using liquid semen with Tris Aminomethan + 20% KT diluent was better than using frozen semen [2]. Liquid semen is an alternative that can be used for AI because it is easiness, does not need liquid nitrogen, cheap and can be stored at refrigerator at 3-5ºC. Using liquid semen in 6 days storage can be applied for Artificial Insemination because it shows the results of motile spermatozoa which are higher than the standard quality by Indonesian national standart that is 40% motile spermatozoa of total concentration [3]. One of the most important factors of dilution is the diluent which can maintain the quality of semen [4]. CEP-3 and tris aminomethane are diluents that recently used for sperm dilution both in frozen semen and liquid semen.

CEP-3 diluent is modified from CEP-2 diluent by replacing the BSA (Bovine Serum Albumin) components contained in CEP-2 with egg white albumin. Egg whites albumin was able to replace BSA in CEP-2 diluents for liquid semen and maintain the quality of semen for up to 5 days storage [3]. Tris Aminomethan is can preserve the quality of semen, because it has the ingredients or substances needed by spermatozoa as a nutrient sources including fructose, lactose, rafinosa, amino acids and vitamins in...
eggs. As a result, spermatozoa can get enough energy sources. Egg yolk is an extracellular cryoprotectant that functions as a nutrient, source of energy and extra cellular protection of spermatozoa from cold shock [5]. Egg yolks are often added in diluents because they are proven to prolong the life span of spermatozoa. Egg yolks also help prevent the hypermotility and early capacitation of spermatozoa [6]. Until now there has been no research on the shelf life of diluents on the ability to maintain the quality of liquid semen so that it has an impact on prevent the decrease in spermatozoa quality. Therefore, this study aims to determine how the quality of liquid semen decreases using Tris Aminomethan and CEP-3 diluents with a storage period of 4 months.

2. Material and methods

2.1. Material
This research was conducted at the Animal Reproduction Laboratory of the Faculty of Animal Science, Brawijaya University, Malang. The diluent used were Tris Aminomethan and CEP-3 diluents with stored period of 4 months. Tris aminomethane was added with 20% of egg yolk, while the CEP-3 diluents with 10% of egg yolk and 0.4% egg white albumin before.

2.2 Methods
The method used in this study was a laboratory experimental method with 2 treatments and 10 replications. The research treatments were T0 (Tris Aminomethan + 20% Egg Yolk) and T1 (CEP-3 + 10% Egg Yolk). Variables observed were motility, viability and abnormality for three days. The data obtained were analyzed by descriptive statistics in means and deviation standart and further tested using the paired t test.

2.3 Semen dilution
Fresh semen was diluted by adding the initial volume of 1:1 diluent then stored in a cool bottom of refrigerator. The next step, the concentration of spermatozoa was observed by using haemocytometer, then the total volume of diluents was calculated in order to get a final concentration of 100 million / ml. Furthermore, the dilution was performed step by step by adding diluents in 2 stages at temperatures of 30°C and 25°C. Afterwords, the liquid semen was stored in a cool top refrigerator until it reaches 5°C. Furthermore the liquid semen was observed every day for 3 days of storages which includes motility, viability and abnormality.

2.4 Observation of individual motility
observation of spermatozoa individual motility was performed by placing one drop of spermatozoa on a glass object covered with a glass cover and observed under a 400x magnification microscope. The assessment was made subjectively to progressive spermatozoa, the score were ranging from 0 to 100% [7].

2.5 Observation of spermatozoa viability
The semen viability observation was carried out using an eosin-negrosin preparation. One dropped of semen was placed on glass object, then mix with one dropped of eosin negrosin. Afterword, A mixture of semen and eosin-negrosin was spread with another glass object to form a smear along the surface of the glass object. Then dried and observed it under microscope with 400x magnification. Living spermatozoa did not absorb color of eosin negrosin, while dead spermatozoa absorb color and became red [8].

2.6 Observation of spermatozoa abnormality
Abnormality test was observed by using sample from eosin-negrosin that was used for viability test, then continued observed under a 400x magnification. There are two kinds of spermatozoa abnormalities. i.e. primary abnormalities involving multiple heads, double tail, microcephalus, macrocephalus, coiled
3. Results and Discussion

3.1. The percentage of spermatozoa motility during storage temperature 3-5°C

The motility of spermatozoa is the most important factor that determine the ability of sperm to fertilize the oocyte. The chances of fertilization are determined by the number of progressive motile spermatozoa present in an ejaculate. The percentage of spermatozoa motility was observed every 24 hours started on the first day of storage until the third day. The pattern of decreasing spermatozoa motility percentage during storage temperatures of 3-5 °C are shown in table 1.

| Treatments/ Day of storage | T0 Motility (%) | T0 Decreasing rate (°C) | T1 Motility (%) | T1 Decreasing rate (°C) |
|----------------------------|----------------|------------------------|----------------|------------------------|
| Day 1                      | 32.50±2.64*    | 5.5*                   | 26.00±2.11*    | 6.5*                   |
| Day 2                      | 27.00±4.22*    | 4.5**                  | 22.00±5.40**   | 4**                    |

Note: * showed the significant different by T-test (P<0.05)
** showed the very significant different by T-test (P<0.01)

The results of analysis paired t test showed that on storage day 1 and day 2 there was a significant difference between T0 (80% Tris Aminomethan + 20% EY) and T1 (90% CEP-3 + 10% EY) to spermatozoa motility (P < 0.05), while there was a very significant difference on day 3 (P <0.01).

Analysis of paired t-test showed that T0 had a better ability to maintain spermatozoa motility compared to T1. The results of this study were also consistent with the research of [9] that diluents of Tris Aminomethan + 20% Egg Yolk are able to provide nutrients for the metabolism of spermatozoa and protect longer than other diluents. In addition, Tris Aminomethan has the best preservation ability because of higher egg yolk concentration in diluent. Egg yolk is an extracellular cryoprotectant that can protect spermatozoa cell membranes from cold shock during cooling at 5 °C [10].

Based on data in Table 1, the average percentage of spermatozoa motility during the study showed that T0 (80% Tris Aminomethan + 20% KT) and T1 (90% CEP-3 + 10% KT) at 3-5 °C until the 3rd day did not experience a dramatically decreasing of sperm motility which in 5.5°C and 6.5°C on the first day, while on the third day were 4.5°C and 4°C respectively. Both diluents with a shelf life of 4 months still show low decreasing of spermatozoa motility. The average decrease in individual motility every day were around 5%. This results were in good categorized according to previous research which is able to maintain individual motility> 40% until the sixth day with an average decrease in individual motility of 5% [3], [11] The longer storage of semen in cold temperatures will cause a decrease in motility. The decrease in individual motility during storage is caused by the influence of toxic substances produced by dead spermatozoa. [12] Toxic substances produced by dead spermatozoa as well as from substances contained in oxidizing diluents cause high free radicals that damage the membrane spermatozoa plasma. So that it can interfere with spermatozoa metabolism and will slowly lose motility, spermatozoa will then die.

3.2. The percentage of spermatozoa viability during storage temperature 3-5°C

The viability of spermatozoa can be observed by making a smear of spermatozoa diluted on mixtures of eosin (as a cell dye) and negrosin (as a background dye). Live spermatozoa are characterized by not absorbing color or has transparent color, while die spermatozoa will absorbing color from the eosin-negrosin solution, so that has a red color. The percentage of spermatozoa viability can be seen in Table 2.
Observation of spermatozoa abnormalities was carried out using a cold temperatures can prevent damage to the spermatozoa plasma membrane which is advantageous for storing semen at cold temperatures during storage. The longer storage will result in a decrease in pH due to an increase in lactic acid produced by spermatozoa metabolism. Damage to the spermatozoa plasma membrane will make eosin-negrosin dye into spermatozoa cells. Therefore, it will have red color when observed by using a microscope with a magnification of 400x.

The results of the paired t-test analysis showed that during cold storage there was a significant difference in the viability of spermatozoa on day 1 (P <0.05). While there was a very significant difference on day 2 and day 3 (P <0.01). Based on this, Tris Aminomethan and CEP-3 were able to maintain the viability of spermatozoa well. Tris Aminomethan + 20% Egg Yolk yielded a better percentage of spermatozoa viability compared to CEP-3 diluents + 10% Egg Yolk. This is in accordance with the previous research from Wiratri et al (2014) which in the 24 hour storage time of Tris Aminomethan + 20% Egg Yolk has a viability percentage of 42.75 ± 2.55 while CEP-2 + 10% Egg Yolk has a viability percentage of 39 ± 3.07. The ability of Tris Aminomethan added with more egg yolk does have a very good preservation and able to maintain the viability better than CEP-3 diluents. The addition of egg yolk in the diluents can prevent damage to the spermatozoa plasma membrane which is advantageous for storing semen at cold temperatures [14].

3.3. The percentage of spermatozoa abnormality during storage temperature 3-5 °C
Observation of spermatozoa abnormalities was carried out using samples with preparations by using eosin-negrosin dyes and observed using a microscope with a magnification of 400x. The calculation was performed by observing normal and abnormal spermatozoa.

The average percentage of spermatozoa abnormalities in T0 (80% Tris Aminomethan + 20% KT) was lower than the average spermatozoa abnormalities in T1 (90% CEP-3 + 10% KT). The average percentage of spermatozoa abnormalities are shown in Table 3.

Note: * showed the significant different by T-test (P<0.05)
** showed the very significant different by T-test (P<0.01)

Table 2. The spermatozoa viability in Tris Aminomethan and CEP-3 diluents

| Treatments/ Day of storage | T0          |          | T1          |          |
|----------------------------|-------------|----------|-------------|----------|
|                            | Viability (%) | Decreasing rate (%) | Viability (%) | Decreasing rate (%) |
| Day 1                      | 68.38±3.33*  | 7.99     | 67.36±2.78*  | 7.47     |
| Day 2                      | 60.39±3.77** | 7.55     | 59.89±3.76** | 7.22     |
| Day 3                      | 52.84±1.67** | 7.55     | 52.67±2.55** | 7.22     |

Based on Table 2, the percentage of viability continues to decrease during storage temperature of 3-5 °C from day 1 to day 3. The longer of storage cause the living spermatozoa will decrease in proportion and lead to decreased motility of spermatozoa. The average percentage of spermatozoa viability at T0 (80% Tris Aminomethan + 20% EY) is higher than the average spermatozoa viability at T1 (90% CEP-3 + 10% EY). [13] Decreased viability due to cold temperatures during storage. The longer storage will result in a decrease in pH due to an increase in lactic acid produced by spermatozoa metabolism. Damage to the spermatozoa plasma membrane will make eosin-negrosin dye into spermatozoa cells. Therefore, it will have red color when observed by using a microscope with a magnification of 400x.

The results of paired t-test analysis showed that during cold storage there was a significant difference in the viability of spermatozoa on day 1 (P <0.05), while there was a very significant difference on day 2 and day 3 (P <0.01). Based on this, Tris Aminomethan and CEP-3 were able to maintain the viability of spermatozoa well. Tris Aminomethan + 20% Egg Yolk yielded a better percentage of spermatozoa viability compared to CEP-3 diluents + 10% Egg Yolk. This is in accordance with the previous research from Wiratri et al (2014) which in the 24 hour storage time of Tris Aminomethan + 20% Egg Yolk has a viability percentage of 42.75 ± 2.55 while CEP-2 + 10% Egg Yolk has a viability percentage of 39 ± 3.07. The ability of Tris Aminomethan added with more egg yolk does have a very good preservation and able to maintain the viability better than CEP-3 diluents. The addition of egg yolk in the diluents can prevent damage to the spermatozoa plasma membrane which is advantageous for storing semen at cold temperatures [14].

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Table 3. The spermatozoa abnormality in tris aminomethan and CEP-3 diluents

| Treatments/ Day of storage | T0 Abnormality (%) | Decreasing rate (%) | T1 Abnormality (%) | Decreasing rate (%) |
|----------------------------|--------------------|---------------------|--------------------|---------------------|
| Day 1                      | 11.32±3.07*        | 2.01                | 12.25±2.17*        | 2.61                |
| Day 2                      | 13.33±2.45**       | 3.64**              | 14.86±2.89**       | 1.18**              |
| Day 3                      | 16.97±2.45**       | 3.64**              | 16.04±2.80**       | 1.18**              |

The average percentage of spermatozoa abnormalities during storage at cold temperatures has increased in longer time of storage because cell damage and abnormalities will increase during cold storage.
storage. Damage of spermatozoa plasma membrane will increase spermatozoa abnormalities [15]. Cell damage and abnormalities of spermatozoa during cold storage can be inhibited by the presence of egg yolk which protects and maintains the integrity of the spermatozoa membrane because there are lecithin and lipoprotein content in the yolk [9].

The analysis of paired t test showed that there were significant differences in spermatozoa abnormalities on day 1 (P <0.05) and very significant differences on day 2 and day 3 (P <0.01). T0 has a lower spermatozoa abnormality compared to T1. More egg yolk content in Tris Aminomethan which causes low spermatozoa abnormalities. The presence of lecithin and lipoprotein contained in the yolk present in both diluents will protect and maintain the integrity of the spermatozoa membrane. Therefore, the spermatozoa abnormalities did not increase dramatically. Spermatozoa abnormalities are caused by two factors, namely spermatogenesis process in testis and errors while processing the semen, such as handling of semen, semen dilution and smear preparation [9]. Both diluents are able to maintain abnormalities of no more than 20%. Semen which has an abnormality of more than 20% will result in reduced fertility [16].

4. Conclusion
In conclusion, decreasing of spermatozoa quality which include motility, viability and abnormality in liquid semen which were stored in Tris Aminomethan diluent + 20% Egg Yolk and CEP-3 + 10% Yolk four months old were low. Tris aminomethan + 20% egg yolk results a decrease in quality better than CEP-3 in motility, viability and abnormality until the third day of storage at 3-5 °C.

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