The relationship of physical and chemical conditions of CEP diluent with egg yolk addition to bull spermatozoa quality before and after storage at temperature of 4-5°C

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Abstract. Storage of semen requires diluent to dilute semen and maintain sperm quality. One of the diluent for bull semen was CEP. The purpose of this study was to assess the association of bull spermatozoa quality with the physical and chemical conditions of CEP diluents with the addition of egg yolk before and after the storage process. The study used Limousin bull with 5 replications. The quality of spermatozoa included motility and viability. Physical and chemical conditions included the pH and osmolarity of the diluent. The motility of spermatozoa was observed under a light microscope with 200 X magnification at 37°C by two people. The viability of spermatozoa was observed under a light microscope with 400 X magnification with nigrosine eosin staining. Data were analyzed with ANOVA and continued Duncan’s test. Dilution pH was measured using pH indicator paper ranging from 6-8. The osmolarity of the diluent was measured by electrical osmolarity. The results showed that the addition of egg yolk in the CEP diluent decreased the pH and increased osmolartitas, but the quality of spermatozoa can be kept up to 8 days of storage. The conclusion in this study was the addition of egg yolk in the CEP diluent provided physical and chemical conditions that can maintain the quality of spermatozoa during storage at a temperature of 4-5°C.

Keywords: CEP diluent, bull spermatozoa quality, egg yolk, pH, osmolarity

1. INTRODUCTION

Storage of spermatozoa at low temperatures can affect the quality of spermatozoa, thus affecting the success of artificial insemination (1, 2, 3). Spermatozoa can decrease motility, viability, the integrity of the plasma membrane, the integrity of DNA, thereby decreasing the fertility of spermatozoa (3, 4). The decrease in spermatozoa quality can be caused by cold shock and free radical presence. Spermatozoa plasma membrane is highly susceptible to cold shock and the presence of free radicals. This is due to the high content of polyunsaturated fatty acids (PUFAs) in the plasma membrane makes spermatozoa sensitive to damage caused by cold shock and peroxidation by free radicals that affect motility, metabolism, ultrastructure and fertility (5, 6, 7).

In the process of spermatozoa storage, there is the addition of diluent media into the semen. The diluent is a medium containing both organic and inorganic material added in fresh semen from both animal and human and aims to increase the volume of semen and to provide protection against spermatozoa in order to maintain the quality of spermatozoa. The diluent contains buffers to maintain...
pH and osmolarity, macromolecules to protect spermatozoa from cold shock, energy sources and antibiotics to suppress bacterial growth (8, 9).

The physical and chemical conditions in the diluent can affect the quality of spermatozoa. Changes in pH may decrease sperm motility(10). The level of osmolarity in the diluent can also affect the motility and viability of spermatozoa (11, 12).

CEP diluent is diluent that developed based on physical and chemical conditions such as cow epididymis, which is expected to provide suitable environmental conditions for bull spermatozoa during storage at low temperature. This diluent was originally developed by, and Ducha et al. Have modified the method of making, kinds of antibiotics and yolk concentrations (7). This study aims to examine the pH state and the osmolarity of CEP diluents, with and without the addition of egg yolk in a CEP diluent to the quality of spermatozoa after storage at a temperature of 4-5°C.

2. MATERIAL AND METHOD

2.1 Making CEP Extender

CEP extender contained NaCl 15 mmol/l; KCl 7.0 mmol/l; CaCl2(H2O)2 3.0 mmol/l; MgCl2(H2O) 63.0 mmol/l; NaHCO3 11.9 mmol/l; NaH2PO4 8.0 mmol/l; KH2PO4 20.0 mmol/l; fructose 55 mmol/l; sorbitol 1.0 gr/l; BSA 2.0 gr/l; Tris 133.7 mmol/l; penicillin 1000 IUI; streptomycin 1 gr; and citrate acid 42.6 mmol/l (Bioworld, USA). All chemicals were made in the form of aliquots, and sterilized using a millipore membrane, followed by the addition of 20% egg yolks. The pH measurement was performed using pH indicator paper. Measurement of osmolarity was done by electrical osmolarity.

2.2 Preparation of Semen

Fresh semen was obtained from Limousin bull obtained from Artificial Insemination (AI) center, Singosari, Indonesia. Fresh semen collection used artificial vaginal method. Fresh semen tested the quality of spermatozoa in advance, including motility and viability.

2.3 Observation of Sperm motility

Spermatozoa motility was assessed a drop of semen on a slide warmer (37°C) under a light microscope for the percentage of progressive motility. Spermatozoa in CEP-2 extender with and without egg yolk at day 0 and day 8 refrigerator storage) were taken using stick glass and placed on object glass, covered with cover glass and placed on the slide warmer at 37°C, then observed under a light microscope at a magnification of 200X. Evaluation of motility was done by two-person that observed on progress if motility that compared with backward motility and only rotated.

2.4 Observation of Sperm Viability

Observation of sperm viability used eosin nigrosin staining. Spermatozoa in CEP-2 extender with and without egg yolk at day 0 and day 8 refrigerator storage) were taken using stick glass and placed on object glass, covered with cover glass and placed on the slide warmer at 37°C, then observed under the light microscope at a magnification of 400x. Non-viable sperm had purple or dark-pink heads and viable sperm had white or faintly-pink heads.

3. RESULT AND DISCUSSION

3.1 Result

The pH measurement of the diluent is necessary, since pH may affect the viability and motility of spermatozoa(13). Average and deviation standard of pH measurement at the beginning of storage and on the last day of storage can be seen in Table 1.
Table 1. pH of diluent CEP at the beginning of storage and after storage 8 days on temperature of 4-5°C

| Various of treatment | Average ± deviation standard of pH |
|----------------------|-----------------------------------|
|                      | Day-0                              | Day-8                              |
| CEP                  | 6.8 ± 0.00                         | 6.8 ± 0.00                         |
| CEP + semen          | 6.5 ± 0.12                         | 6.6 ± 0.00                         |
| CEP + 20% egg yolk   | 6.6 ± 0.00                         | 6.6 ± 0.00                         |
| CEP + 20% egg yolk + semen | 6.4 ± 0.00 | 6.4 ± 0.00 |

Based on the results of pH measurements in Table 1, it was seen that the CEP diluent pH without egg yolk as well as with the addition of egg yolk was still within the normal range for spermatozoa. pH range normally for fresh semen was 6.4-6.8(14). The addition of egg yolk and mixing with semen decreased pH of CEP dilution pH, which was 6.8 to 6.4. The decrease in pH may occur because the presence of the main component of yolk was triacylglycerol(14, 15).

Triacylglycerol is a source of energy and is also a form of deposits of fatty acids. The process of triacylglycerol hydrolysis by lipases produces glycerol, fatty acids, and H⁺ ions. The presence of fatty acids and H⁺ ions will decrease the pH of the environment inside and outside the cell. This decrease caused the CEP diluent conditions to be closer to the pH conditions of the cauda epididymis(16) which states that the pH of cauda oxidized epididymis was low, ie 6.4.

Development of CEP diluent based on the ionic composition of epididymis plasma with certain osmolarity, therefore measurement of diluent osmolarity before addition of egg yolk and after egg yolk addition to know whether there was difference osmolarity at CEP diluent after mixed with egg yolk. Average and deviation standard of the diluent osmolarity can be seen in Table 2.

Table 2. The osmolarity of CEP diluent with and without egg yolk supplementation at the beginning of storage and after storage 8 days on temperature of 4-5°C

| Various of diluent | Average ± deviation standard of osmolarity (mosm) |
|-------------------|-----------------------------------------------|
|                   | Day-0                              | Day-8                              |
| CEP               | 290.00 ± 0.00                         | 290.00 ± 0.00                       |
| CEP + semen       | 380.00 ± 10.00                       | 286.00 ± 6.93                       |
| CEP + 20% egg yolk| 1395.33 ± 4.51                       | 1395.33 ± 4.51                      |
| CEP + 20% egg yolk + semen | 1106.67 ± 10.16 | 893.00 ± 2.00 |

The osmolarity of the CEP diluent was 290 mosm, of which the osmolarity was considerably high according to the osmolarity state in the plasma of the cauda epididymis ie above 250 mosm / kg of water(17). The osmolarity of cauda plasma epididymis in cattle was 339 mosm / kg of water, which was due to the presence of ions such as Na, K, Ca, Cl, Mg, P, bicarbonate(16), and the
The presence of macromolecules such as glucose, cholesterol and lactate, as well as the presence of several amino acid molecules (17). The addition of yolk to the CEP diluent increased the osmolarity from 290.00 mosm to 1106.67 mosm.

The comparison of osmolarity between the CEP diluent with and without egg yolk was significant difference, the addition of the egg yolk caused a high increase in osmolarity in the CEP diluent, this may be due to the presence of macromolecules present in egg yolk. Results research of Oyolede et al. showed that the yolk component is 50-65% liquid, composed of lipids and proteins, with a ratio of 2:1. Lipid in local chicken egg yolk is 66.7%, while for broiler chicken 70, 5% (15), other researchers mentioned that lipid in chicken egg yolk is 62.4-65.2% (18). The main components of yolk lipid are LDL, triacylglycerol, then followed by various phospholipids, and the least components are cholesterol (14, 15).

Observation of spermatozoa quality included motility and viability. The result of spermatozoa motility can be seen in Table 3.

Table 3. The average of spermatozoa motility percentage and deviation standard in CEP diluent with and without egg yolk before and after storage at temperature of 4-5°C

| Various of diluent | Average and deviation standard of spermatozoa motility percentage before and after storage |
|-------------------|------------------------------------------------------------------------------------------|
|                   | Day-0                                           | Day-8                                           |
| CEP               | 63.75 ± 2.43                                   | 0.35 ± 0.58                                     |
| CEP + egg yolk 20%| 67.50 ± 0.00                                   | 44.25 ± 3.92                                    |

Note: The different notation (a, b) on the same column showed that the treatment gave a significantly different result (P≤0.05) to the motility of spermatozoa from Limousin bull.

Based on Table 3 showed there was a significant difference in the motility of the Limousin bull spermatozoa on CEP diluent without egg yolk compared with egg yolk. A very large motility difference was seen after eight days of storage. Dilution condition and storage duration may affect spermatozoa viability. The observation results of spermatozoa viability can be seen in Table 4.

Table 4. The average of spermatozoa viability percentage and deviation standard in CEP diluent with and without egg yolk before and after storage at temperature of 4-5°C

| Various of diluent | Average and deviation standard of spermatozoa viability percentage before and after storage |
|-------------------|------------------------------------------------------------------------------------------|
|                   | Day-0                                           | Day-8                                           |
| CEP               | 88.89 ± 4.82                                  | 19.79 ± 11.62                                   |
CEP + 20% egg yolk  |  95.43<sup>b</sup> ± 1.49  |  87.46<sup>b</sup> ± 5.40  

Note: The different notation (a,b) on the same column showed that the treatment gave a significantly different result (P<0.05) to the viability of spermatozoa from Limousin bull.

The observation result of spermatozoa viability in Table 4 showed a significant difference between diluent with and without egg yolk. A very big difference was seen after 8 days of storage.

Comparison of spermatozoa quality in CEP diluent with and without egg yolk showed a significant difference. The pH state of the CEP diluent with the egg yolk was slightly lower than without egg yolk. The most striking difference was the osmolarity of the CEP diluent without egg yolks compared with egg yolk. Osmolarity in diluent with egg yolk supplementation was very high, exceeding the normal osmolarity of plasma seminal fluid. The high osmolarity in the CEP diluent with egg yolk addition was due to the presence of large molecules (macromolecules) in the egg yolk, but the molecule did not enter the cell, just outside, so it did not affect the osmolarity of spermatozoa. The existence of these macromolecules proved to give positive effect for the spermatozoa, which was able to provide spermatozoa protection during the storage process.

The osmolarity of the epididymal ducts from the head to the cauda has increased by more than 250 mosm, caused by organic macromolecules such as the presence of several proteins, lipoproteins, and lipids. The macromolecule is assumed to play an important role in protecting spermatozoa and epididymal epithelia from osmotic pressure stress(17), similar to this role that exists in the kidneys, also protects spermatozoa from ROS effects during storage of spermatozoa in cauda epididymis(19). Organic macromolecules are also assumed to play a role in regulating water movement through the epithelial cell membrane and spermatozoa cell membrane. High levels of the presence of organic macromolecules in the cauda epididymis in some species, have an important role in storing spermatozoa for a long time, such as in bats that store spermatozoa in cauda epididymis for 6 months with high osmolarity ie greater than 1000 mosm / kg water(17).

4. CONCLUSION

Physical and chemical conditions in the form of pH and osmolarity in CEP diluents with the addition of egg yolk were able to provide a good environment for spermatozoa so that the quality of spermatozoa can be maintained during storage at a temperature of 4-5°C.

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