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Ultrastructure and Fertilizing Ability of *Limousin* Bull Sperm after Storage in Cep-2 Extender with and Without Egg Yolk

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Abstract: Sperm can change physiology and structure during storage in refrigerator temperature or frozen temperature that caused by cold shock or free radical. The aim of this study to evaluate ultrastructure and fertilizing ability of *Limousin* bull sperm after storage in cauda epididymal plasma-based (CEP-2) extender with or without 20% egg yolk concentration at refrigerator temperature. Semen sample collected from three *Limousin* bull were diluted with CEP-2 with 20% egg yolk and CEP-2 without egg yolk, cooled and stored at 4-5°C during eight days. Sperm ultrastructure were observed with scanning electron microscopy (SEM). Fertilizing ability of *Limousin* bull sperm were assessed on cleavage rate of embryo using *in vitro* fertilization method. The percentage data were transformed into arcsine before being analysis with ANOVA and Duncan's multiple comparison test. The result of study showed morphologically normal sperm after storage in CEP-2 with 20% egg yolk, whereas in CEP-2 without egg yolk morphologically abnormal sperm especially neck was fractured and head was destroyed. Fertilizing ability of *Limousin* bull sperm were significantly higher in CEP-2 extender with egg yolk 20% (74.29±4.95%; $p<0.05$) than without egg yolk (30.00±12.02%; $p<0.05$). Egg yolk 20% in CEP-2 extender protected ultrastructure and fertilizing ability after storage during eight days.

Key words: Ultrastructure, fertilizing ability, *Limousin* bull sperm, egg yolk, CEP-2 extender

INTRODUCTION

Liquid storage of semen at low temperature has long been practiced with the aim to facilitate artificial insemination (AI) application. Semen storage at refrigerator temperature can be alternative mainly for areas without continuous availability of liquid nitrogen. The advantages of liquid semen at refrigerator temperature is simpler storage method and lower operational cost.

Many extender have been developed for liquid storage of semen at low temperature to maintain the quality of spermatozoa and to multiply volume of semen. Verberckmoes *et al.* (2004, 2005) have developed semen extender that imitated as the condition of bovine Cauda Epididymal Plasma (CEP) and capable maintained the quality of spermatozoa for six days. In further development, CEP-2 is produced which is a improvement of CEP-1. CEP-2 extender contains fructose and citrate acid as the source of energy which is composed of ions, pH and osmolarity that is equivalent with cauda epididymal plasma of bovine but with higher concentration of Ca, Mg and P and eliminate nitrate component. CEP-2 contains sorbitol to increase osmolarity and BSA (Bovine Serum Albumin) that function as macro molecules.

Egg yolk have long been mixed with the extender of semen because considered it can give extracellular protection on the membrane of spermatozoa (Bergeron and Manjunath, 2006). This study aims to prove whether egg yolks in extender CEP-2 can give protection for the ultrastructure of membrane and whether it can maintain the ability of bovine spermatozoa to fertilize after storage at refrigerator temperature. Concentration of egg yolk that used in this study was 20%, based from beginning study, it has given best protection for motility (44.25±3.92) and viability (87.46±5.40) of *Limousin* bull spermatozoa after 8 days storage at refrigerator temperature. That was different with study of Verberckmoes *et al.* (2004), egg yolk that used in CEP-2 extender was 10%.

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MATERIALS AND METHODS

Preparation of extender: All chemical were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Kinds of chemicals to make extender CEP-2 are based on the study that developed by Verberckmoes *et al.* (2004) consisting of (in 1 liter extender): 15 mmol NaCl, 7.0 mmol KCl, 3.0 mmol $CaCl_2$ $(H_2O)_2$, 3.0 mmol $MgCl_2$ $(H_2O)_6$, 11.9 mmol $NaHCO_3$, 8.0 mmol NaH_2PO_4, 20.0 mmol KH_2PO_4, 55 mmol fructose, 1.0 g sorbitol, 2.0 g BSA, 133.7 mmol Tris, 1000 IUI penicillin; 1 gram streptomycin and 42.6 mmol citrate acid. Then the studier himself develops the method of making extender CEP-2 and egg yolk supplementation, in which all those chemicals are formed into aliquot by using deionize water sterile. Extender osmolarity was measured using electric osmolarity approximately 290-325 mOsm, pH 6-7 and sterilized using millipore membrane with 0.22 μm pore diameter. Extender was supplemented with 20% egg yolk, then precipitated for three days in refrigerator. A part which was taken for extender was part belongs to supernatant.

Collection and preparation of semen: Fresh semen was obtained from the Center of Artificial Insemination located in Singosari, Malang. The fresh semen was selected based on the quality of spermatozoa in which it satisfied the requirements of the Indonesian National Standard (SNI) for application of artificial insemination. Several provisions from the standard of SNI are the individual motility should be at least 70%; the minimum mass motility should be 2+ and the abnormality and the viability should be at least 70%. Sperm concentration was determined with accucell spectrophotometer. Spermatozoa were stored at a concentration of 25×10^6 mL^{-1} CEP-2 extender with and without egg yolk. Semen was diluted with CEP-2 extender at 37°C, then spermatozoa were stored at refrigerator in darkness.

Evaluation of sperm motility: Spermatozoa motility were assessed a drop of semen on slide warmer (37°C) under 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 on the light microscope at a magnification of 400× (Padilla and Foote, 1991; Boonkusol *et al*., 2010; Bayemi *et al*., 2010). Evaluation of motility was done by two person that observed on progress if motility that compared with backwards motility and only rotated, based method of Garner and Hafez (2008).

Ultrastructural observation of spermatozoa membrane: The sample of spermatozoa in CEP-2 extender with and without egg yolk from day 0 and day 8 were washed three times by centrifugation at 1000 rpm for 5 min using PBS (Phosphate Buffered Saline). Spermatozoa were then fixed for 2-3 h with PBS containing glutaraldehyde 2%, washed three times by centrifugation at 1000 rpm with PBS (pH 7.4) for 5 min at 4°C and post-fixed in osmic acid solution 1% for 1-2 h in 4°C (Boonkusol *et al*., 2010; Toshihiro *et al*., 1998). After washing three times with PBS, spermatozoa were dehydrated through a graded alcohol series, finally with amyl acetate absolute as preservative. Spermatozoa were placed on cover glass by width 16 mm, then dried using Critical Point Drying (CPD) and coated with gold using vacuum evaporator. Spermatozoa were observed using JSM T100 Scanning Electron Microscope (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan).

Oocyte maturation: Ovaries were collected at local abattoir and transported to the laboratory in 0.9% saline solution at 30-37°C. Oocyte maturation method were described (Boediono *et al*., 1995; Dinnyes *et al*., 2000; Cesari *et al*., 2006; Cebrian-Serrano *et al*., 2012). Cumulus oocyte complexes (COCs) and follicular fluid were aspirated from 2-10 mm diameter follicle, mixed with tissue culture medium 199 (TCM 199), then incubated in waterbath at 38°C for 10 min. During incubation, oocyte will sink to the bottom, then sediment of oocyte were washed two times with TCM 199+10% FBS, incubated at 38°C for 10 min. Oocyte with several layers of cumulus cell, morphologically bright and uniform cytoplasm were selected, then maturated in TCM 199 with supplementation using 10% Foetal Bovine Serum (FBS), 10 AU bovine Follicle Stimulating Hormone (FSH), 10 IU bovine Luteinizing Hormone (LH), 50 μg mL^{-1} penicillin G, 50 μg mL^{-1} streptomycin. Maturation was performed in groups of 20 into 50 μL droplets of each maturation medium for 26 h in incubator at 38.5°C, 5% CO_2 in humidified air.

Sperm preparation and *in vitro* Fertilization (IVF): Spermatozoa from CEP-2 extender with and without egg yolk at day 0 and day 8 refrigerator storage were thawed at 37°C in water bath 37°C for 1 min and centrifuged two times at 2000 rpm for 5 min using capacitation medium (9.98 mL EBSS medium with supplemented 10 mg mL^{-1} caffeine, 0.9 g bovine serum albumin/BSA and 10 mg mL^{-1} heparin) and observed spermatozoa motility and concentration. Mature oocyte that characterized with

respectable growth of cumulus cells were selected and washed three times in EBSS medium. Oocytes were transferred in to a new well plates with 100 μL fertilization medium (5 mL EBSS medium and supplemented with BSA 0.075 g) and final concentration of 2×10^6 were adjusted for IVF. Plates were incubated at 38.5°C, 5% CO_2 in humidified air.

***In vitro* culture:** After 6 h of spermatozoa-oocyte incubation, the oocytes were washed and cultured in TCM 199 supplemented with 10% FBS, incubated at 38.5°C in humidified air 5% of CO_2 (Boediono *et al*., 1995; Bernardi and Delouis, 1996; Gandhi *et al*., 2000). The fertilization rate were recorded on zygote ability to cleavage 2-8 cell (in%) which observed until 72 h after fertilization.

Statistical analysis: Each experiment was replicated three times, semen were collected from different individual bull. The data of the spermatozoa ultrastructure observed by using SEM (Scanning Electron Microscopy) is analyzed in qualitative-descriptive fashion. The data of the sperm ability to fertilize that observed from the zygote ability to cleavage (%) and sperm motility (%) were transformed to arcsine. One-way Analysis of Variance (ANOVA) was used to analyze significant differences between treatments ($p<0.05$). Duncan's multiple range test used to compare the means of significant difference of each treatment using SPSS software.

RESULTS

Motile spermatozoa were observed at day 0 and day 8 storage in CEP-2 extender with and without egg yolk 20%. Spermatozoa motility from *Limousin* bull after storage in CEP-2 extender with and without egg yolk showed at Table 1. The result of spermatozoa motility observation showed percentage decreased ($p<0.05$) after 8 days storage in all media.

The percentage of spermatozoa motility that stored 0 days in CEP-2 extender with egg yolk 20 supplementation were significant different with in CEP-2 without egg yolk ($p<0.05$). The percentage of spermatozoa motility in fresh semen before diluted with CEP-2 extender were 70.50±1.58. The result showed spermatozoa of *Limousin* bull that entered CEP-2 without egg yolk experienced motility change faster than CEP-2 with egg yolk. The percentage of spermatozoa motility that stored 8 days in CEP-2 extender with egg yolk 20% supplementation were higher than CEP-2 without egg yolk ($p<0.05$). The result indicated that 20% egg yolk in CEP-2 capable maintained motility of *Limousin* bull spermatozoa until storage 8 days.

Table 1: The percentage of spermatozoa motility from *Limousin* bull after storage in CEP-2 extender with and without egg yolk 20% at temperature of 4-5°C

Extender	D0	D8
CEP-2 without EY	63.50±2.43^a	15.35±1.53^a
CEP-2 with EY 20%	67.50±1.00^b	44.45±4.91^b

The different notation (a,b) on the same column showed that the treatment gave significant different result ($p<0.05$) to the motility of spermatozoa from *Limousin* bull. D0: day 0 of storage, D8: day 8 of storage, EY: egg yolk

Observation on the ultrastructure of spermatozoa from *Limousin* bull were conducted by using SEM (scanning electron microscopy). Result of the observation before and after storage for 8 days in CEP-2 extender with and without supplementation of egg yolks at refrigerator temperature were shown in Fig. 1.

Result of the observation in the beginning of storage and on day 8 after storage in extender CEP-2 containing egg yolks showed no change in the structure of the spermatozoa as it looked complete from the head area to tail. On the other hand, the spermatozoa from *Limousin* bull stored in extender CEP-2 without egg yolks experienced sizeable changed, that was, the neck area looked fractured and the membrane on the head area looked swelling. This shows that the existence of egg yolks in extender CEP-2 could gave protection to integrity of the ultrastructure of the spermatozoa during the storage period at temperature of 4-5°C.

Fertilizing ability of spermatozoa were observed on the percentage of embryo cleavage after fertilization process using *in vitro* fertilization method. Fertilization process were done when oocyte showed mature (Fig. 2).

Fertilizing ability were observed from oocyte that experienced *in vitro* fertilization with spermatozoa that stored in CEP-2 extender with and without egg yolk at day 0 and day 8 storage, then counted embryos cleavage. The percentage of embryos cleavage decreased after storage for 8 days either on extender CEP-2 with or without egg yolks; however, reduction on CEP-2 without egg yolk were bigger than with egg yolk (Fig. 3).

Percentage of fertilizing ability (embryos cleavage) from *Limousin* bull spermatozoa at day 0 storage in CEP-2 with egg yolk (76.40±4.45) were significant different ($P<0.05$). with CEP-2 without egg yolk (56.40±31.69). The percentage rate of embryos cleavage after day 8 storage at refrigerator temperature were higher ($p<0.05$) on the spermatozoa stored in extender CEP-2 with egg yolks (74.9±4.95%) than on the one without egg yolks (30.00±12.02%). The result of the analysis showed that

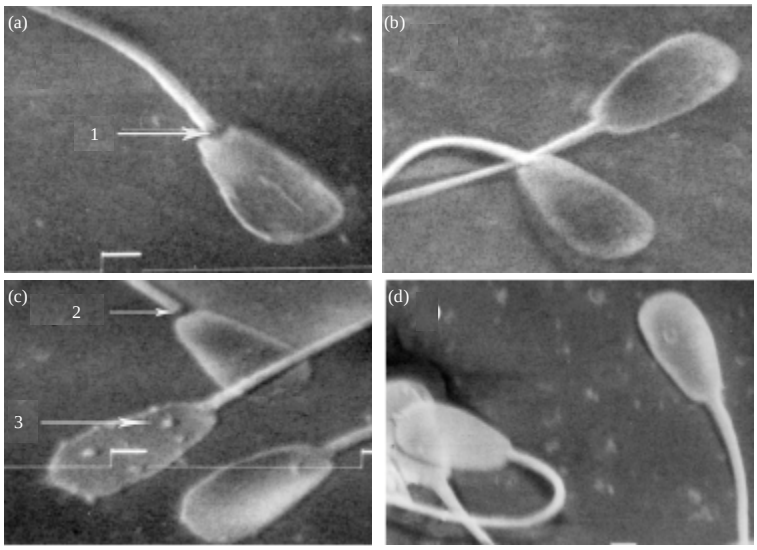

Fig. 1(a-d): The ultrastructure of spermatozoa from *Limousin* bull. (a) spermatozoa stored on day 0 in CEP-2 without egg yolk, in which the neck area looked cracked (1), (b) spermatozoa stored on day 0 in CEP-2-EY20%, the structure of the spermatozoa looked complete, (c) spermatozoa stored in extender CEP-2 without egg yolk on day 8, the neck area looked broken (2) and the membrane was swelling (3), (d) spermatozoa stored in CEP-2 EY 20% on day 8, the structure of the spermatozoa looked complete, bar = 5 μm

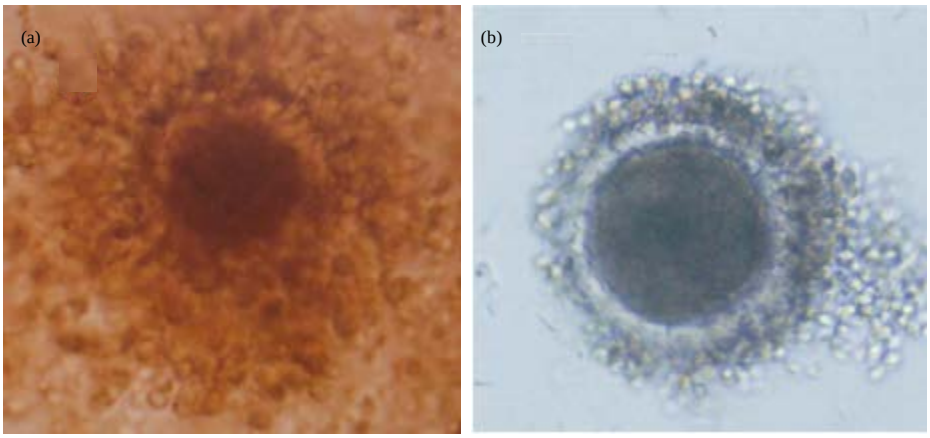

Fig. 2(a-b): Oocyte and spermatozoa of *Limousin* bull sperm for in vitro fertilization. (a) Mature of oocyte, (b) Oocyte and spermatozoa in fertilization process

supplementation of egg yolks on extender CEP-2 could maintain the fertilizing ability of *Limousin* bull spermatozoa after 8 days storage.

DISCUSSION

The quality of the spermatozoa can influence their ability to fertilize oocytes. Spermatozoa that stored in CEP-2 without egg yolk have experienced change fastly, both motility and ultrastructure that showed from beginning storage. The comparison on the motility of spermatozoa from *Limousin* bull between stored in extender CEP-2 with egg yolks and without egg yolks showed significantly different from beginning storage and after 8 days of storage. Likewise, result of the ultrastructure observation showed that the spermatozoa

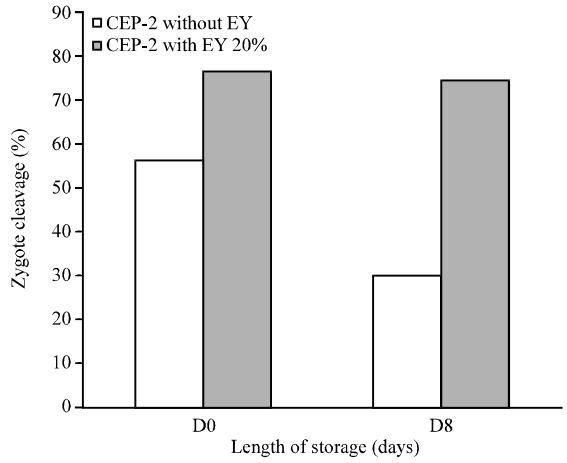

Fig. 3: *In vitro* fertilization ability (in%) of spermatozoa from *Limousin* bull after storage in extender CEP-2 with and without supplementation of egg yolk at temperature of 4-5°C. D0: day 0 of storage, D8: day 8 of storage, EY: egg yolk

have experienced ultrastructure change on beginning storage and after 8 days of storage in extender CEP-2 without egg yolk, on day 0 of storage spermatozoa experienced a damage on their neck area was cracked, whereas damage of spermatozoa bigger on day 8 of storage, their neck was broken and swelling of head membrane. Spermatozoa that entered in CEP-2 without egg yolk on beginning storage (day 0) have experienced change both motility and ultrastructure and showed to influence fertilizing ability, so that percentage of embryo cleavage big on CEP-2 without egg yolk, although after analysis of the data didn't show significantly different with CEP-2 with egg yolk.

Motility and ultrastructure of *Limousin* bull spermatozoa after stored 8 days showed change higher in CEP-2 without egg than with egg yolk, both motility and ultrastructure and showed to influence fertilizing ability, so that percentage of embryos cleavage higher in CEP-2 with egg than in CEP-2 without egg yolk. Result of the study indicated that spermatozoa can structural and functional change during storage time. Structural and functional change were caused by cold shock (Hafez, 2008; Moce *et al*., 2010) or free radical (Vishwanath and Shannon, 2000) Spermatozoa stored at low temperature can experience cold shock and reduce metabolic activity and motility caused by the penetration of sodium and calcium (White, 1993), losing of the membrane integrity caused by lipid change in membrane, release of some phospholipids and cholesterol components (Watson and Morris, 1987) and losing of some proteinase acrosin (Church and Graves, 1976), redistribution of intramembranous particles (De Leeuw *et al*., 1990). Meanwhile, the existence of ROS (reactive oxygen species) is inevitable during the storage time (Vishwanath and Shannon, 2000). The period of storage and the production of metabolism can cause increasing of ROS in extender and can influence the structure and function of spermatozoa during storage. Therefore, the extender needs lipoprotein which serve as extracellular cryoprotectan to protect the membrane of spermatozoa during storage. Existence 20% egg yolk in CEP-2 extender have protected spermatozoa quality during 8 days of storage and influenced fertilizing ability. It showed that spermatozoa quality can influence fertilizing ability. The main components that are presumably able to give protection for spermatozoa are the low density of lipoprotein (Bergeron and Manjunath, 2006; Briand-Amirat *et al*., 2007; Vera-Munoz *et al*., 2010), phospholipid (Evans and Setchell, 1978; Graham and Foote, 1987) and cholesterol (Parks *et al*., 1981).

The integrity and structure of spermatozoa membrane will be able to influence the ability of the spermatozoa to fertilize oocytes. The morphology parameter of the spermatozoa according to constitute the main parameter to predict the ability of the spermatozoa to fertilize based on ability in binding zona pellucida, while motility is the second main parameter to predict the ability of the spermatozoa to penetrate cumulus and zona pellucida (Oechninger and Franken, 2006) Spermatozoa with abnormal morphology have low ability in binding zona pellucida, that can be caused by incomplete membrane receptor that is responsible to bind the zona pellucida. The study result of Mehmood *et al*. (2009) showed that the spermatozoa with higher percentage of complete membrane and motility showed fertilizing ability higher based oocyte cleavage than spermatozoa with lower percentage of complete membrane and motility. Shibahara *et al*. (1997) used sperm quality analyzer to evaluate its fertilizing ability and their study showed that spermatozoa with high index of motility result in high index of penetration and average of fertilizing ability.

CONCLUSIONS

Ultrastructure condition of spermatozoa can influence fertilizing ability, like wise with motility. Existence of 20% egg yolk in extender CEP-2 can maintain the ultrastructure of spermatozoa membrane and motility from *Limousin* bull during 8 days storage at temperature of 4-5°C, so that fertilization ability so maintained.

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