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THE EFFECT OF SKIM MILK ADDITION IN CEP-2 DILUENT ON MOTILITY AND VIABILITY OF LIMOUSIN BULL SPERM DURING STORAGE AT REFRIGERATOR

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Abstract

Sperm storage need diluent for maintenance sperm quality. The aim of this research was to study the effect of skim milk in CEP-2 diluent on motility and viability of Limousin bull sperm. The research used Randomized Block Design with five times from five different bulls. Sperm was stored during five days. Sperm motility was observed on light microscopy (x200) at 37ºC temperature by two person. Sperm viability was oberserved with eosin negrosin staining at light microscopy (x400). The result of the research showed skim milk in CEP-2 diluent effected to motility and viability of Limousin bull sperm during storage at 4-5°C. Best skim milk concentration was 15% in protected motility and viability of Limousin bull sperm after storage at 4-5ºC.

Key words : CEP-2, skim milk, motility, viability, Limousin bull sperm

INTRODUCTION

Semen stored at low temperature need extender to maintain the quality of spermatozoa and to multiply the volume of semen. In Europe storage of bovine semen at temperature of 4-5^0 C uses tri-gliserol as basic extender (De Leeuw et al., 1992), while in New Zealand they are stored in extender of caprogen that can maintain the quality of spermatozoa for 3 days (Vishwanath and Shannon. 1997; Verbeckmoes *et al*., 2004). Verbeckmoes *et al*. (2004, 2005) have developed extender of bovine semen for storage at temperature of 4-5^0 C that emulate the conditions in CEP and can maintain the quality of spermatozoa for 6 days. In further development, CEP-2 is produced which is a refinement of CEP-1. Extender CEP-2 contain fructose and citrate acid as a source of energy with composition of ions, pH and osmolarity that is equivalent with CEP-1 but with more higher concentration of Ca, Mg and P than CEP-1. Also CEP-2 contain sorbitol to increase osmolarity that is equivalent with CEP-1 and contain BSA (Bovine Serum Albumin) that serve as macro molecules (Verbeckmoes *et al*., 2004, 2005). Ducha *et al*. (2012) has modified component and method to make CEP-2 and can maintain the quality of spermatozoa that suitable for AI standart until 8 days.

During in the storage, spermatozoa may experience structural and functional changes. Sperm stored at low temperature can experience cold shock which in turn may reduce activities of metabolism and mortality caused by penetration of sodium and calcium (White G. 1993), lose of membrane integrity caused by lipid phase change and release of some phospholipids and cholesterol components (Watson and Morris, 1987), and lose of some

proteinase acrosine (Church and Graves 1976). Meanwhile, process of storage can't avoid the existence of ROS (reactive oxygen species) (Vishwanath and Shannon, 2000). Period of storage and product of metabolism can be cause of increasing of ROS in extender, and may influence structure and function of spermatozoa during storage. Therefore, extender need macro molecules which serve as extracellular cryoprotectan to protect membrane of spermatozoa during storage.

Skim milk have mixed in extender of semen to protect on membrane of spermatozoa during storage. This research aims to prove whether skim milk in extender CEP-2 can give protection for motility and viability *Limousin* bull sperm.

RESEARCH METHOD

Design of Research

This research uses design of random group with three repetitions from different individual bull that breed in Artificial Insemination (AI) Center with homogeneous condition. Semen is mixed with only CEP-2 and CEP-2 with skim milk, and stored at refrigerator temperature (3-5ºC). Motility and viability of sperm was observed every day during storage.

Preparation of extender

Chemicals to make extender CEP-2 is based on the research that have been developed by Verbeckmoes et al. (2004, 2005) with antibiotic and making method different (Ducha et al., 2012) consisting of NaCl 15 mmol/lt; KCl 7.0 mmol/lt; $CaCl_2(H_2O)_2$ 3.0 mmol/lt; $MgCl_2(H_2O)_6$ 3.0 mmol/lt; $NaHCO_3$ 11.9 mmol/lt; NaH_2PO_4 8.0 mmol/lt; KH_2PO_4 20.0 mmol/lt; fructose 55 mmol/lt; sorbitol 1.0 gr/lt; BSA 2.0 gr/lt; Tris 133.7 mmol/lt; penicillin 1000 IUI; streptomycin 1 gr; and citrate acid mmol/lt. Measuring of extender osmolarity used electric osmolarity with osmolarity approximately 250-325 mOsm, and pH 6-7. Extender sterilized by using milipore membrane with size of 0.22 μm and supplemented with skim milk 5%, 10%, 15%, 20%, 25% and 30%.

Collection and Preparation of Semen

Fresh semen was collected from the AI Center in Singosari – Malang. The fresh semen was selected based on quality of spermatozoa that is fulfilling Indonesian National Standart (SNI) from fresh semen process to AI application. Several provisions from 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%. Fresh semen were diluted wit CEP-2 extender with and without skim milk. Spermatozoa were stored at refrigerator temperature in darkness with 25 x 10^6 concentration.

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 400x (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).

Sperm Viability

Method of observation for sperm viability uses eosin-negrosin staing. The advantages to this stain are that permanent slides can be made and the nigrosin provides a dark background for easier recognition of the non-stained, viable cells. Non-viable sperm have red or dark-pink heads and viable sperm have white or faintly-pink heads,as shown in the image on the right.

RESULT AND DISCUSSION

Sperm Motility

The observation result of sperm motility during storage in extender CEP-2 with and without supplementation of skim milk is shown in figure 1.

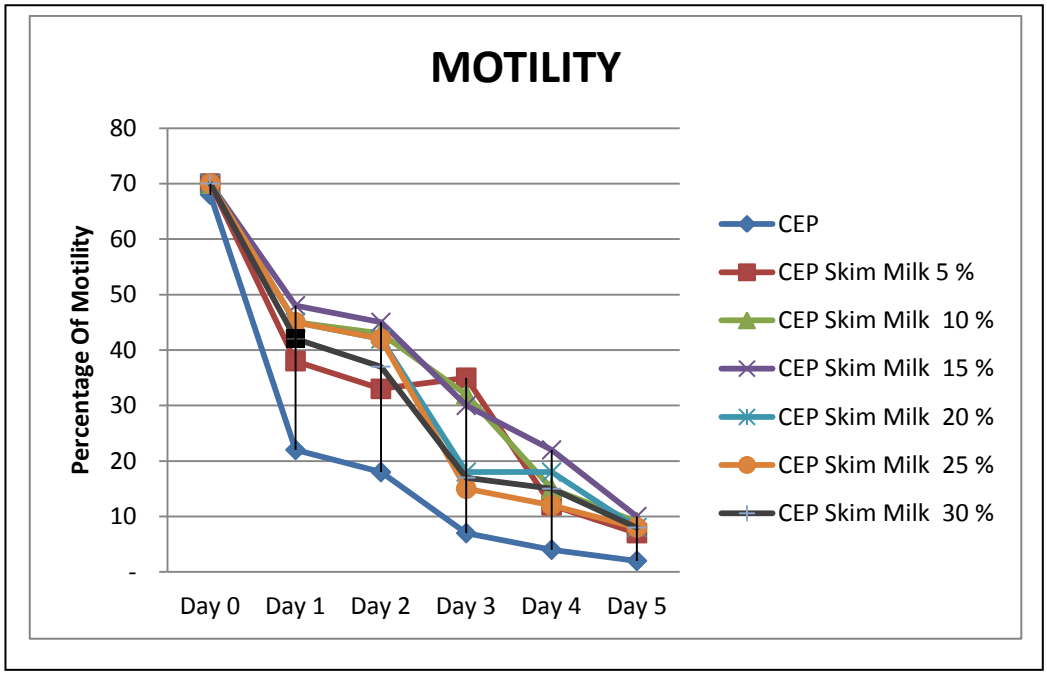

Figure 1. Graph of motility comparison Limousin bull sperm during storage in CEP-2 with and without skim milk at refrigerator temperature.

Analysis result of average and standard deviation shows motility difference between CEP-2 without supplementation skim milk compare CEP-2 with supplementation skim milk during 5 days . But, after 24 hour storage, sperm motility in CEP-2 without skim milk decreases faster than CEP-2 with skim milk.

Result of Duncan multiple comparison test 1) shows that there is no motility difference on the beginning of storage in extender CEP-2 with skim milk (70 ± 0.00%) and CEP-2 without skim milk (68 ± 0.67%). But on the day 1 after storage motility percentage is significant different in CEP-2 without skim (22 ± 8.19%) milk compares with CEP-2 skim milk 5% (38%), CEP2 skim milk 10% (45%), CEP-2 skim milk 15% (48%), CEP-2 skim milk 20% (45%), CEP-2 skim milk 25% (45%) and CEP-2 skim milk 30% (42%). Sperm motility on the day 2 shows significant different between CEP-2 without skim milk (and CEP-2 with skim milk 5% compares CEP-2 skim milk 10% (43%), CEP-2 skim milk 15% (45%), CEP-2 skim milk 20% (42%), CEP-2 skim milk 25% (42%) and CEP-2 skim milk 30% (37%). But, on day 3 storage, sperm motility in CEP-2 with skim milk supplementation 20%, 25% and 30% decreases faster than skim milk 5%, 10%, and 15%. Sperm motility shows biggest in CEP-2 with skim milk 15% until storage 5 days, whereas sperm motility decreases biggest in CEP-2 without skim milk. This shows that spermatozoa which stored in extender CEP-2 with supplementation of egg yolks still have a good quality during storage.

CEP-2 extender that developed by Verbackmoes et al. (2004, 2005) is imitated ion

composition, pH and osmolarity as in epididymis condition, and Ducha et al. (2012) have modivied method and composition. But, an semen extender requires extracellular protection of sperm during storage in low temperature as macromollecul that is egg yolk, skim milk. Ducha et al. (2012) have used egg yolk 20% to mix CEP-2 extender, and can maintenance sperm membrane until 8 days storage to fertilize oocyte. In this experiment uses skim milk for extracellular protection, and give protection of good motility until 3 days for skim milk 15%. This is different with result research Ducha et al. (2012) that can protect good of sperm quality until 8 days with use egg yolk.

Macromollecul component in skim milk or in egg yolk that can protect sperm are low density lipoprotein (LDL), phospholipid and cholesterol (Bergeron and Manjunath, 2006). But, in skim milk contain lipid macromollecul in big globul, so that can confine sperm motility.

Sperm Viability

The result of sperm viability can show in figure 2.

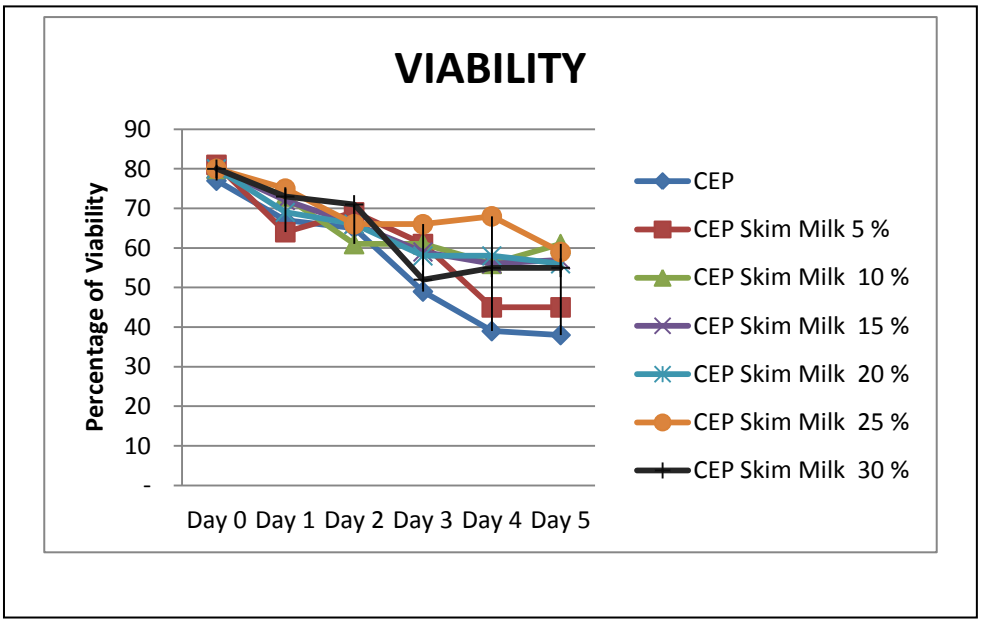

Figure 2. Comparison of sperm viability during storage in CEP-2 with and without skim milk at refrigerator temperatur

Average and standard deviation shows viability difference between CEP-2 without supplementation skim milk compare CEP-2 with supplementation skim milk during 5 days . Result of Duncan multiple comparison test shows that there is no motility difference on the beginning of storage in extender CEP-2 with skim milk (80 ± 0.00%) and CEP-2 without skim milk (78 ± 1.95%). Sperm viability is different from day 3, between sperm storage in CEP-2 with skim milk compare CEP-2 without skim milk, and the best good viability during storage is sperm that stored in CEP-2 with 10% skim milk.

Skim milk contains little lipid macromollekul, 0 - 0.5%. Lipid component is formed triasilglicerol, LDL, fosfolipid in lipid globul, and little cholesterol (Jhanwar, 2009). Limpid macomollecul can associate with sperm membrane and can give protection during storage (Evans and Setchell. 1978; Graham and Foote 1987).

CONCLUSION AND SUGGESTION

Suplementation of skim milk in CEP-2 extender can effect motility and viability of Limousin bull sperm during storage at refrigerator temperature. 15% skim milk can give best protection of sperm motility, eventhough 10% skim milk give best protection of sperm viability.

Result of this research can be background for follow research to study macromollecul component of skim milk can limited motility and viability of sperm.

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