EFFECT OF MALTOSE CONCENTRATION IN TRIS DILUTION ON EPIDIDYMAL SPERMATOZOA QUALITY OF BALI BULL PRESERVED AT 5^0C

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ABSTRACT

The objective of research was to evaluate the effect of maltose concentration in Tris dilution on epididymal spermatozoa quality of Bali bull that preserved at 5^0C. Five testis of Bali bull collected from slaughter house were used in this study. Epididymal spermatozoa were collected through slicing and flushing methods, pressing cauda epididymal was conducted in NaCl physiology (NaCl 0.9%) emulsion. Spermatozoa which collected were divided into three reaction tube and each diluted by Tris dilution containing: Tris dilution + 20% of yolk (control); Tris dilution + 20% of yolk + 0.3 g of maltose/100ml (M0.3); and Tris dilution + 20% of yolk + 0.6 g maltose/100 ml (M0.6). Spermatozoa qualities observed were motile spermatozoa (MS), live-spermatozoa (LS) and intact-plasma membrane (IPM) that evaluated daily in refrigerator at temperature of 5^{o}C. Completely Randomized Design with three treatments and five replications was used in this study. Data was analyzed by analysis of variance. Examination on fresh spermatozoa showed that spermatozoa concentration was 11,222.5 million cell/ml, motile spermatozoa was 75.00%, live-sperm was 86.75%, abnormal spermatozoa was 10.50%, cytoplasmic droplet was 14.00% and IPM was 86.75%. At the seventh day of preservation, the percentages of MS, LS and IPM in M0.3 were 37.0 %, 49.2% and 50.4%, respectively, and M0.6 were 38.05%; 51.8 % and 52.0%, respectively that were significantly higher ($P<0.05$) than control (29.0%; 41.8% and 42.4%, respectively). It was concluded that maltose added into Tris dilution could lengthen epididymal spermatozoa quality of Bali bull which persevered at 5^0C.

Keywords: Maltose, Preservation, Epididymal Spermatozoa, Bali Bull

INTRODUCTION

Bali bull is one of superior's commodities which should become the development priority since it has several comparatives superior. Productivity improvements of Bali bull can be conducted by various ways and one of them is Artificial Insemination (AI) implementation. One advantage in implementing AI is increasing productivity and cattle-genetic quality. Spermatozoa from ejaculation that collected by artificial vagina was used in AI. Another alternative for AI is utilizing cauda epididymal spermatozoa. Cauda epididymal spermatozoa is mature spermatozoa , in which they experienced the maturation process at caput and epididymal corpus parts (Toelihere, 1981; Hafez and Hafez, 2000).

According to Rizal (2005), efforts of processing spermatozoa collected from the epididymal either in fresh or frozen semen for various reproduction technologies can be conducted at animal having superior genetic but its semen can not be collected, saving animal plasma-germ because of sudden death, and rare and wild animals. On some animal species, AI and in-vitro fertilization (IVF) by utilizing cauda epididymal spermatozoa was widely conducted with favorable result as: ram (Graham, 1994; Rizal, 2005), bull (Graham, 1994), boar (Kikuchi *et al*., 1998), stallion (Squires *et al*., 2000), red deer (Soler *et al*., 2003) and spotted buffalo (Rizal *et al*., 2007; Herdis *et al*., 2008).

White (1993) stated that semen preservation process in low temperature (3-5^{o}C and -196^{o}C) damaged the cell plasma membrane of spermatozoa. This was because of cold shock effect. To minimize the negative effect, it can be handled by adding certain substance (cryoprotectant) into semen dilution (Kayser *et al*., 1992), as *glycerol* (intracellular cryoprotectant) and some sugar variety

(extracellular cryoprotecant) (Supriatna and Pasaribu, 1992).

Monosaccharide, disaccharide and polysaccharide sugars are energy resources for spermatozoa and could be utilized as extracellular cryoprotecant to protect spermatozoa during frozen process (Rizal *et al*., 2003). Yildiz *et al*. (2000) stated that sugar role in dilution liquid was as the energy resource and cryoprotectant during semen frozen. Maltose as disaccharide sugar is expected to preserve spermatozoa quantity and quality that being diluted in yolk-Tris liquid semen dilution. Several kind of sugars that commonly used were : glucose from the ram frozen semen (Molinia *et al.*, 1993), and boar frozen semen (De Los Reyes *et al.*, 2005); Rafinose from Etawa buck frozen semen (Suwarso, 1999); trehalose from the Garut ram frozen semen (Herdis *et al*, 2005); Sucrose and Trehalose from the bull frozen semen (Woelders *et al.*, 1997); Lactose from the buck frozen semen (Singh *et al.*, 1995); Garut ram diluted semen (Rizal, 2006) and Maltose from the Garut ram frozen semen (Herdis, 2005).

This research was conducted to find out the effect of maltose added in the Tris dilution to preserve caudal epididymal spermatozoa of Bali Bull at 5°C. It is expected to minimize the damage of spermatozoa during preservation, so it may extend the vitality.

MATERIALS AND METHODS

Sperm Collection

Five Bali bull testis included the epididimal (used for replications) were used in this research. Testis was collected when the cow slaughtered at around 12.00 PM from the Cow Slaughterhouse in Ambon. It, then, was taken and carried to the Animal Production Laboratory, Animal Science Department, University of Pattimura. Spermatozoa collection was started at 7.00 AM, initially by the separating epididymal from the testis and washed by using NaCl physiological liquid (0.9% NaCl). Spermatozoa were collected by using combination of Slicing, Rinsing and Pressing Technique (rinse-press) in the NaCl physiological liquid to each tissue of cauda epididymal (Rizal, 2005). Before the "rinse-press" processed in the NaCl physiological liquid, spermatozoa concentration was counted using erythrocyte pipette.

Collected spermatozoa were divided equally into three reaction tube. Spermatozoa was diluted by different dilution materials according to the treatments: 80% Tris base dilution + 20% Yolk (control), 80% Tris base dilution + 20% Yolk + 0.3 g maltose/100 ml dilution (M0.3) and 80% Tris basic dilution + 20% yolk + 0.6 g maltose/100 ml dilution (M0.6). Tris basic dilution consisted of 3.87 g Tris (hidroxymethyl) aminomethane, 2.17 g citrate acid and 1.56 g fructose diluted by aquabidestillata until 100 ml and then added by penicillin and streptomycin each for 1000 IU/ml dilution. Spermatozoa were diluted until reaching 15 million spermatozoa motile/ml concentration. Then, test tubes were closed tightly and included into a goblet filled with clean water and preserved in refrigerator at 5 ^{o}C. Each treatment's quality was evaluated daily for six days.

Evaluation and Data Analysis

Spermatozoa's quality was evaluated after collection (fresh spermatozoa), that was: spermatozoa concentration, motile spermatozoa percentage, live spermatozoa percentage, abnormal spermatozoa percentage, cytoplasm droplet percentage and intact plasma membrane percentage. Meanwhile, evaluation on diluted (treated) spermatozoa and preserved was: motile spermatozoa percentage, live spermatozoa percentage and intact plasma membrane percentage.

Table 1. The Characteristics of Bali Bull Epididymal Spermatozoa

Variabels	Means ± SD
Sperm concentration (million/ml)	11.222,50 ± 455.21
Subjective Sperm Motility (%)	75.00 ± 0.00
Live Sperm (%)	86.75 ± 0.83
Abnormality Sperm (%)	10.50 ± 1.12
Cytoplasmic droplet (%)	14.00 ± 0.71
Intact Plasma Membrane (%)	86.75 ± 1.48

Motile spermatozoa percentage, percentage of spermatozoa with progressive motion (moving forward) were evaluated subjectively in eight different view fields with light microscope in 400 times magnification (Rasul *et al.*, 2001). Measurement criteria number ranges between 0 and 100 percent with 5 percent scale.

Live spermatozoa percentage was evaluated by 2 percent eosin B color (Toelihere, 1981). Living- and dead-spermatozoa was marked by transparent head, meanwhile dead spermatozoa was marked by red head. As minimum as 200 spermatozoa was evaluated by light microscope with 400 times magnification.

Intact Plasma Membrane (IPM) percentage was evaluated using *osmotic resistance test* (ORT) method (Revell and Mrode, 1994). Two hundred µl hypo-osmotic liquid was added by 20 µl semen and mixed until homogen and then incubated in 37°C for 45 minutes. Preparats made were wrapped in thin on object glass and then was evaluated by using light microscope with 400 times magnification towards minimum 200 spermatozoa. Spermatozoa with intact plasma membrane marked by encircling or swollen tail; meanwhile damaged plasma membrane was marked by straight tail. Hypo-osmotic liquid consisted of 0.9 g fructose + 0.49 g sodium citrate diluted by aquabidestillata to reach 100 ml volume.

Data was analyzed by variance analysis in the form of completely randomized design with three treatments and five replications. The difference between treatments was tested by least significant difference test (Steel and Torrie, 1993).

RESULTS AND DISCUSSION

Characteristics of Epididymal Spermatozoa

Bali bull epididymal fresh semen characteristics data (Table 1) showed that such semen had a good quality and had the prerequisites to be further processed as research sample. Research result showed that the average of motile spermatozoa was 75.00%, abnormal spermatozoa was 10.5% and intact plasma membrane was 86.75%. Fresh semen with good quality consisted of motile spermatozoa ≥ 70% (Evans and Maxwell, 1987), abnormal spermatozoa 6-10% (Delgadillo, 1992) and intact plasma membrane ≥ 60% (Revell and Mrodw, 1994).

The average of sperm concentration was 11.222,50 million cells /ml (range 10.590-11.780 million cells/ml). Such spermatozoa concentration was higher than other research in bull, that was 3.593-4.406 million cells/ml (Suhendra, 2002) and for Ongole crossbreed bull (PO) was 5.053,3 million cells/ml. Concentration of cauda epididymal spermatozoa concentration in mammals was reported about 10,000-50,000 million cells/ml (Senger, 1999) and approximately 10.445 million cells/ml in spotted buffalo (Yulnawati *et al*., 2008).

Bali bull cauda epididymal live sperm percentage in this research was approximately 86.75% (range 85.92-87.52%). Yulnawati *et al*. (2008) reported that live spermatozoa percentage

Table 2. Percentages of Motile Sperm, Live Sperm and Intact Plasma Membrane (IPM) of Bali Bull Epididymal Spermatozoa Preserved at 5 °C

Quality Variabel of Spermatozoa	Maltose (g/100 ml)	Preservation day						
		1	2	3	4	5	6	7
Motile Sperm (%)	0	75.00 ± 0.00^{a}	67.00 ± 2.74^{a}	53.00 ± 4.47^{a}	47.00 ± 2.74^{a}	42.00 ± 2.73^{a}	37.00 ± 2.74^{a}	29.00 ± 2.24^{a}
	0.3	75.00 ± 0.00^{a}	69.00 ± 2.24^{a}	59.00 ± 2.24^{b}	56.00 ± 2.24^{b}	50.00 ± 0.00^{b}	44.00 ± 2.24^{b}	37.00 ± 2.74^{b}
	0.6	75.00 ± 0.00^{a}	69.00 ± 2.24^{a}	59.00 ± 2.24^{b}	54.00 ± 2.24^{b}	50.00 ± 3.54^{b}	45.00 ± 3.54^{b}	38.00 ± 2.74^{b}
Live Sperm (%)	0	85.80 ± 1.10^{a}	78.20 ± 2.59^{a}	63.00 ± 3.54^{a}	58.00 ± 1.23^{a}	50.40 ± 3.05^{a}	47.00 ± 2.92^{a}	41.80 ± 1.30^{a}
	0.3	86.60 ± 1.10^{a}	80.00 ± 2.00^{b}	70.20 ± 1.79^{b}	66.60 ± 3.91^{b}	61.80 ± 2.38^{b}	54.80 ± 2.78^{b}	49.20 ± 1.64^{b}
	0.6	86.60 ± 1.10^{a}	82.80 ± 1.79^{b}	69.80 ± 2.05^{b}	66.00 ± 1.41^{b}	61.60 ± 2.30^{b}	55.00 ± 3.67^{b}	51.80 ± 4.15^{b}
IPM (%)	0	86.40 ± 0.89^{a}	77.80 ± 1.64^{a}	69.60 ± 0.89^{a}	61.20 ± 1.64^{a}	58.40 ± 3.51^{a}	49.60 ± 2.07^{a}	42.40 ± 2.07^{a}
	0.3	85.60 ± 0.89^{a}	80.00 ± 1.00^{b}	73.40 ± 1.14^{b}	68.00 ± 1.58^{b}	65.40 ± 1.14^{b}	56.40 ± 1.67^{b}	50.40 ± 1.14^{b}
	0.6	85.60 ± 0.89^{a}	80.40 ± 1.14^{b}	73.00 ± 0.71^{b}	68.60 ± 1.14^{b}	66.60 ± 2.30^{b}	55.40 ± 2.41^{b}	52.00 ± 2.24^{b}

^{a,b} Superscrips in a column indicating significant differences (P<0,05).

in spotted buffalo cauda epididymis was approximately 79.30%.

The abnormal spermatozoa percentage from this research was approximately 10.50% (range 9.38-11.62%) and cytoplasmic droplet percentage was approximately 14.00% (range 13.29-14.71%). Abnormal spermatozoa percentage from spotted buffalo was approximately 15.00% (Yulnawati *et al*., 2008). Abnormality in spermatozoa from Bali bull cauda epididymal in this research was in the normal range and those can be used for the next process, because according to Bearden and Fuquay (2000) that 8-10% abnormal spermatozoa has no significant effect on fertility.

Research result showed that the average percentage intact plasma membrane was 86.75% (Range 85.27-88.23%). Previously, researcher reported that lower intact plasma membrane percentage in spotted buffalo cauda epididymal spermatozoa was in the average of 80.80% (Yulnawati *et al*., 2008). Fresh semen with good quality consisted of intact plasma membrane ≥ 60% (Revell and Mrode, 1994).

Quality of Spermatozoa after Preservation at 5ºC

The Percentage of Motility Spermatozoa

The result showed that 0.3 g maltose addition and 0.6 g/100 ml Tris dilution produced higher real motile spermatozoa percentage than control treatment started from day 2 to the day 7 preservation (Table 2). This showed that maltose was able to give protection and at one time as the energy source substrate for spermatozoa during preservation. High motility in maltose treatment than control was caused by maltose as a disaccharide sugar that can be functioned as energy source. Spermatozoa motility is highly depends on the available energy source in dilute or semen plasma in the form of *adenosine triphosphate* (ATP) produced from metabolism. According to Gardner and Hafez (1997), the energy required for motility was apparently derived from extracellular stores of ATP. The use of ATP appears to be regulated by the endogenous level of *cyclic adenosine monophosphate* (cAMP). The cAMP not only regulates ATP breakdown but also has a direct effect on sperm motility. Brandt and Hoskins (1980), stated that cAMP was involved in the regulation of spermatozoa motility. Elevation of intracellular cAMP level appears to be related: to the acquisition of the potential for motility during epididymal maturation, to the actual initiation of motility upon ejaculation and to the surge of increased motility associated with capacitation. Woelders *et al*. (1997) demonstrated that an isotonic sugar medium in which Tris-citrate components were substituted by sucrose (disaccharide) and trehalose was significantly superior to a Tris-citrate egg yolk medium in preserving the motility and acrosome integrity of bovine spermatozoa.

Maltose as disaccharide sugar can be used as energy source through glycolysis process. Before glycolysis, disaccharide will be hydrolyzed by *disaccharidase* enzyme (Salisbury and Van demark, 1985). Maltase enzyme will break maltose into two glucose molecules. Spermatozoa will be easier to use glucose in its metabolism than other energy source use available in semen plasma that was fructose (Toelihere, 1993). According to Hammerstedt (1993), spermatozoa viability very depend on energy source which was found in semen plasma or dilution and could not be self syntheses energy that needed to metabolism and repair cell's damage. Protein in egg yolk takes part in support spermatozoa motility, because the protein was able to protect spermatozoa plasma membrane, in order to well functioned the cell organelle, included the metabolism process.

The Percentage of Live Spermatozoa

Salisbury and Van Demark (1985) suggested that viability spermatozoa was the spermatozoa's ability to active stationery after incubation in higher temperature from room temperature or after being kept in the lower temperature. The result of this research showed that the addition of maltose 0.3 g/ 100 ml and 0.6 g/100 ml Tris dilution provided presentation of live spermatozoa until 6 days preservation (during 5 days) were significant ($P<0.05$) than control. It indicated that the existence of maltose in Tris dilution provided energy source for spermatozoa so that their viability more than 5 days (Table 2), but according to Evans and Maxwell (1987) and Indonesia National Standard (SNI) about semen quality requirement used in AI programme must have minimum percentage of motile spermatozoa that is about 40%. In the control treatment, motile spermatozoa percentage in 40% can be defensible only until 5 days preservation (during 4 days), whereas by the adding of maltose (0.3 g and 0.6 g) can be defensible until 6 days preservation

(during 5 days). Spermatozoa that were kept in room temperature (37°C) have only several hour viability because of nutrition run out. Toelihere (1981) stated that the increasing of pH because of lactate acid accumulation and alteration which is occurred because spermatozoa's affected on spermatozoa's viability. Spermatozoa's viability *in vitro* was affected by essence contained in dilution and spermatozoa's quality. Good quality of spermatozoa had ability to outlive, because of having bigger ability to absorb nutrition (Gilbert, 1988; King, 1993). Spermatozoa with good quality completed plasmalemma and acrosoma, so they were able to absorb nutrition essence which were secreted by sertoli cell, whereas the high plasmalemma permeability needed to live on from pH alteration and osmosis pressured alteration (Lamming, 1990).

Membrane integrity was the vital importance for sperm viability. Pursel *et al*. (2009) reported that the results of five extenders used consisted of Tris (0.164 M) and citric acid (0.055 M) in combination with glucose, fructose, lactose, sucrose or raffinose (0.138 M) indicated that the sugar composition of the extender may effect the development of cold shock resistance during incubation. The sugar composition of semen extenders has previously been shown to be a significant factor during freezing of bull and ram spermatozoa (Salamon and Lightfoot, 1969).

The Percentage of Intact Plasma Membrane (IPM)

Maltose is extracellular cryoprotectant to protect spermatozoa's plasma membrane from damage caused by the cold shock effect during storage in 5°C. That was apparently from research result whereas intact plasma membrane percentage in real maltose treatment (0.3 g/100 ml and 0.6 g/100 ml) were significant ($P<0.05$) higher than control started from two days until 7 days of preservation (Table 2). Several researchers found that the incorporation of sugars in sperm diluents protects the spermatozoa of many species against freeze damage. Sugars probably play a key role in preventing deleterious alteration to the membrane during reduced-water states (Aimen *et al*., 2003). Pursel *et al*. (1972) stated that sugar and addition of egg yolk had a significant effect on cold shock resistance. According to White (1993), the influence of cold shock related to phospholipids alteration cell plasma membrane compiler, that the transformation was from liquid to gel which was occurred under 20° C temperature. The alteration of fat acid and protein in plasma membrane caused leakage or plasma membrane selectivity damaged that influence of ions entered cell openly. Supriatna and Pasaribu (1992) stated that carbohydrate was extracellular cryoprotectant which functioned to protect the cell plasma membrane from damage. Whole-cell plasma membrane passed to the positive effect of motility and viability spermatozoa. Spermatozoa's motility depends to energy supply of ATP from result metabolism. Metabolism will work out well if cell plasma membrane still complete. Because cell plasma membrane take part in arrange traffic substrate and electrolyte's entry and exit that needed in metabolism process in cell (Lehninger, 1994).

Cell plasma membrane contain carbohydrate which is alliance with lipid (glicolipid) or protein (glicoprotein) which is called cell cover or *glicokalics* (Lehninger, 1994). Carbohydrate is also known as extracellular cryoprotectant because estimated functioned in protection of glicokalics spermatozoa's cell plasma membrane from damage process during semen preservation. According to Viswanath and Shannon (2000) cryoprotectant composite carbohydrate category, like maltose have ability to change water molecule normally in hydrated polar class. The characteristics of maltose will help stabilize spermatozoa's cell plasma membrane during transition through the critic temperature zone, and change the character of dilution mechanic through viscosities. Aisen *et al*. (2002) reported that disaccharide carbohydrate categories act as water substitute on the surface of cell plasma membrane which direct related with dilution. Furthermore, they stated that maltose was able to interact with polar phospholipids center cluster during solidification and reduce bond interact Van der Walls between carbon chain.

Sugar contribute to stability of the membranes, as it has been found in a number of other cells dehydrated during freeze drying or low-rate freezing and thawing (Eiman *et al*., 2003). The presence of sugars in the diluents is likely to affect the pattern of crystallization and the shape and width of the channels of unfrozen solution (Nicollajsen and Hvidt, 1994), which could perhaps relieve or prevent fast-cooling damage to the spermatozoa. The presence of sugars leads to a lower salt concentration in the unfrozen water, consequently reducing the effects of the solution. It is possible that the sugars may

help prevent injurious eutectic freezing by trapping salts in an increasingly viscous or even glass-like phase. Giraud *et al*. (2000) reported that the recovery rate of viable or motile spermatozoa after freezing or thawing is superior for spermatozoa with high membrane fluidity before freezing.

CONCLUSION

The percentage of motile spermatozoa, live spermatozoa and intact plasma membrane in the 7th day of preservation were about 29.00%, 41.80% and 42.40% for treatment of 0.0 g maltose; 37.00%, 49.20% and 50.40% for treatment of 0.3 g maltose; 38.00%, 51.80% and 52.00% for treatment of 0.6 g maltose. Addition 0.3 g and 0.6 g/100 ml maltose in Tris dilution able to extending Bali bull epididymal spermatozoa quality which was preserved in 5°C temperature and qualified to benefit the AI program after the 5th day of preservation, whereas without added maltose can only preserve for 4 days.

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