Media Peternakan, April 2013, pp. 45-51
ISSN 0126-0472 EISSN 2087-4634
Accredited by DGHE No: 66b/DIKTI/Kep/2011

Online version:
http://medpet.journal.ipb.ac.id/
DOI: 10.5398/medpet.2013.36.1.45

The Quality of Stallion Semen in Skim Milk and Dimitropoulos Extenders Preserved at 5 °C and Ambient Temperature Supplemented with Different Sugar

R. I. Arifiantini[a,*], B. Purwantara[a], T. L. Yusuf[a], & D. Sajuthi[b]
[a]Division of Reproduction and Obstetric, Department of Veterinary Clinic, Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University
[b]Division of Internal Medicine, Department of Veterinary Clinic, Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University
Jln. Agatis, Kampus IPB Darmaga, Bogor 16680, Indonesia
(Received 31-10-2012; Reviewed 04-02-2013; Accepted 21-03-2013)

ABSTRACT

This study was conducted to evaluate the effects of sugars supplementation in skim milk based (SM) and dimitropoulos (DV) extenders on the sperm motility and viability in stallion semen storage at 5 °C and ambient temperature (24-29 °C). Semen samples were collected from 3 stallions; evaluate individually and each of them divided into 8 aliquots. Four out of eight aliquots were diluted 1:1 with SM, while the remaining four were diluted 1:1 with DV; all were then centrifuged at 1006 *g* (3000 RPM) for 15 min. The supernatants were discarded, and each pellet was re-diluted with SM (control), SM trehalose (SMT), SM-raffinose (SMR), SM-fructose (SMF), DV (control), DV-trehalose (DVT), DV-raffinose (DVR), and DV-fructose (DVF). The diluted semen were divided into 2 aliquots and stored at 5 °C or ambient temperature. The sperm motility and viability were evaluated every 3 h on chilled semen stored at ambient temperature, and every 12 h on those stored at 5 °C. Results of the experiments demonstrated that sperm motility and viability in DV extender significantly higher (P<0.05) in both temperature. The supplementation of fructose was the best on the motility and viability of the sperm at both temperatures compare to trehalose, raffinose, or the control group. The best extender and sugar combination was DVF, which the total motile sperm stored at 5 °C for 96 h was 45.1% followed by to DVT (40.2%) and DVR (39.2%). The sperm motility in DV and SMF were 35.3% and 35.6%, respectively; these were higher than those diluted with control (28.9%); SMT (30.3%), and SMR (29.6%). The study concluded that the supplementation of fructose in DV extender (DVF) was the best combination to preserve stallion sperm motility and viability stored at 5 °C or ambient temperature.

Key words: skim milk, dimitropoulos, sugars, chilled semen, stallion

ABSTRAK

Penelitian ini dilakukan untuk mengevaluasi pengaruh suplementasi gula dalam pengencer susu skim (SM) dan Dimitropoulos (DV) terhadap motilitas dan viabilitas sperma dalam semen cair kuda yang disimpan pada suhu 5 °C dan suhu ruangan (24-29 °C). Semen dikoleksi dari tiga ekor kuda, dievaluasi, dan diproses secara individu. Semen dari masing-masing kuda jantan dibagi menjadi 8 bagian sama banyak. Empat bagian diencerkan 1:1 dengan SM, sedangkan empat lainnya diencerkan 1:1 dengan DV, seluruh tabung yang telah diencerkan disentrifugasi 1006 g (3000 RPM) selama 15 menit. Supernatan dibuang, dan masing-masing pelet diencerkan kembali dengan SM (kontrol), SM trehalosa (SMT), SM-rafinosa (SMR) atau SM-fruktosa (SMF), DV (kontrol), DV-trehalosa (DVT), DV-rafinosa (DVR) atau DV-fruktosa (DVF). Setiap semen cair dibagi menjadi dua tabung dan disimpan masing-masing pada suhu 5 °C atau suhu ruangan. Motilitas dan viabilitas sperma dievaluasi setiap 3 jam pada suhu kamar dan setiap 12 jam pada suhu 5 °C. Hasil penelitian menunjukkan bahwa motilitas dan viabilitas sperma dalam pengencer DV lebih tinggi (P<0,05) dibandingkan dengan pengencer skim pada kedua suhu penyimpanan. Motilitas dan viabilitas sperma pada pengencer yang disuplementasi fruktosa lebih unggul dibandingkan dengan trehalosa, rafinosa atau kelompok kontrol. Pengencer terbaik adalah DVF, dengan total sperma motil dalam penyimpanan pada suhu 5 °C selama 96 jam adalah 45,1%; diikuti DVT (40,2%) dan DVR (39,2%). Motilitas sperma dalam pe-

*Corresponding author:
E-mail: Iis.arifiantinipurna@gmail.com

ngencer DV dan SMF masing-masing adalah 35,3% dan 35,6%, lebih tinggi daripada yang diencerkan dengan SM kontrol (28,9%), SMT (30,3%), dan SMR (29,6%). Penelitian ini menyimpulkan bahwa suplementasi fruktosa dalam extender DV (DVF) adalah kombinasi terbaik untuk preservasi semen cair kuda baik pada suhu 5 °C maupun suhu ruangan.

Kata kunci: susu skim, dimitropoulos, gula, semen cair, kuda

INTRODUCTION

Artificial insemination is an important tool in animal production. However, cooling and cryopreservation are known to induce non-regulated capacitation-like sperm modification in numerous domestic species (Bailey *et al.,* 2000). Storage of chilled semen at 5 °C induces a transition on sperm plasma membrane from the chilled crystalline to the gel phase. At body temperature sperm metabolism is maximal, while at ambient temperature (24-29 °C) it is lower. For each 10 °C temperature decrease, cellular metabolism is reduced by 50%; at 5 °C sperm metabolic activity is only 10% of what it would had been at body temperature (McKinnon, 1999). In the reduced metabolic state, sperm need energy to maintain their cellular activity.

Equine semen contains high sodium chloride concentration; this alters the osmotic pressure during cooling and freezing. To avoid this, the seminal plasma needs to be separated by centrifugation (Kopt *el al.,* 2005; Morrell *et al.,* 2009). Consequently, not only the sodium chloride, but other components such as fructose, sorbitol, enzyme and other energy sources are wasted. Besides the needs of energy (McKinnon, 1999), equine chilled semen stored in low temperature needs to be considered of sperm cold shock, which may alters the configuration of their plasma membrane (Ball, 2008; Neild *et al.,* 2005). The increased use of cooled semen in the equine industry has consequence to the need of suitable diluents availability.

The composition of extenders varies enormously, but they are normally based upon milk or egg yolk products plus antibiotics. Nutrients, buffer solution to neutralize metabolic waste of sperm and anti cold shock are the important factors to maintain the viability of sperm during storage at low temperature (McKinnon, 1999). Sugars serve as the nutrient for sperm. The most commonly used sugar to preserve and cryo-preserve equine semen is glucose (Bozkurt *et al.,* 2007; Pojprasath *et al.,* 2011). Fructose is easier than glucose to be metabolized by sperm, and is commonly included in chilled and frozen semen extenders in different species (Aboagla & Terada, 2004a; Silva *et al.,* 2005; Akhter *el al.,* 2010). Trehalose and raffinose are extracellular cryoprotectants which stabilize plasma membrane; both of these are commonly supplemented in frozen semen extenders (Hu *et al.,* 2010; Pojprasath *et al.,* 2011; Slanina *et al.,* 2012). Sperm plasma membrane damage not only occurs during freezing, but also during cooling (Aurich, 2005) refers chilling injury. Trehalose and raffinose in semen extenders are expected to reduce cold shock of sperm during storage at 5 °C**.** This study aims to study the effects of fructose, trehalose, and raffinose in each SM and Dimitropoulos (DV) extenders in maintaining the quality of equine chilled semen stored in ambient temperature and 5 °C.

MATERIALS AND METHODS

Animals

Three stallions (5–8 yr old) owned by Athena stable, Cinere-Depok, West Java, Indonesia used as semen source in the study. The stallions were a fourth generation (G4) of Thoroughbred, an American pinto, and a Swedish warm blood, each was in a good health and demonstrated the best quality on the daily sperm output.

Extenders

All reagents were obtained from Merck, KgaA (Darmstadt Germany) unless otherwise indicated. Extenders used in this study were skim milk (SM) based extender (Kenney *et al.,* 1975) and Dimitropoulos extender (Ijaz & Durcarme, 1999). SM was composed of 2.4 g SM and 4 g glucose, diluted in 100 mL water. Extenders were heated to reach 92-95 °C for 10 min, allowed to cool, filtered, and then added with 100 mg streptomycin and penicillin 100,000 units.

Dimitropoulos (DV)-based extender consisted of two parts, A and B solutions. A solution was composed of 2 g glucose and 2 g fructose diluted in 100 mL water, heated in a water heater for 15 min until it reached 95 °C, allowed to cool, and stored at 5 °C for a maximum of 1 wk. B solution was composed of 2 g sodium citrate, 0.94 g glycine and 0.35 g sulphanylamide diluted in 100 mL water. The mixed solution was heated to 100 °C, stored in ambient temperature for a maximum of 1 wk.

DV-based extender was a mixed of 30% A solution, 50% of B solution, and 20% of egg yolk; the mixed solution was centrifuged at 1200 x g, for 20 min. The supernatant was collected and added with 1000 unit penicillin and 1 mg streptomycin per mL solution. Extenders semen in this study were SM or DV-based extenders (control groups) and SM or DV-based extenders supplemented with either 50 mM trehalose (Sigma, Chemical Co, St. Louis, Mo, USA), 50 mM raffinose (BDH Chemical Ltd, Poole England), or 100 mM fructose.

Semen Collection and Processing

An ejaculate from each stallion was collected twice a week, using Nishikawa type artificial vagina which was

modified with semen collecting tube of Missouri type (Nasco, Fort Atkinson, WI). On the top of the semen collecting tube was covered with gauze to strain out the gel fraction of the ejaculates (Arifiantini *et al*., 2006)

Immediately after collection, semen sample was assessed for volume, color, consistency, pH, motile and viable sperm, sperm concentration, and sperm morphology. Sperm concentration was measured by counting chamber, using a dilution of 1:100 with 3% saline solution (Baracaldo *et al.,* 2007). Sperm morphology was examined by carbofuchsin eosin method (Arifiantini *et al.,* 2010; Arifiantini, 2012).

Only ejaculate with concentration greater than 150.10^6 sperm/mL having >65% progressively motile and less than 30% sperm abnormality selected for preservation. Chilled semen of each sample was prepared by dividing the semen equally into eight tubes; semen samples in the first four tubes were diluted 1:1 with SM based extender; semen in the remaining four tubes were diluted 1:1 with DV-based extender. All semen samples in tubes were centrifuged (Hettich, EBA 3S, Tuttlingen, Jerman) for 15 min at 1006 x g (3000 RPM), and then the supernatant was discarded. The pellet (sperm) in four tubes added with SM was each mixed with SM (control), trehalose (SMT), raffinose (SMR), or fructose (SMF). Similarly, the pellet in the remaining four tubes added with DV-based extender was each mixed with DV (control), trehalose (DVT), raffinose (DVR), or fructose (DVF). Final sperm concentration in each solution was 200 x 10^6 sperm mL^{-1}. All extended semen then divided into two tubes and stored at 5 °C and ambient temperature.

Assessment of Chilled Semen Quality

The assessment conducted every 3 h on each chilled semen stored at ambient temperature, and 12 h on those stored at 5 °C. Sperm motile was assessed subjectively under a light microscope (Olympus CH20, with 400x magnification) equipped with a heating stage adjusted to +37 °C. Motility estimations were performed from 5 different fields in each sample by the same person throughout the study.

The sperm viability was assessed using eosin-nigrosin solution (3.3 g eosin Y, 20 g nigrosin, 1.5 g sodium citrate in 300 mL of water, adjusted to pH 7.0) (Bart & Oko, 1989). Equal volumes of sperm suspension were mixed, 20 μL of this mixture were smeared into a clean microscope slide (pre warmed to 37 °C) and allowed to dry at heating stage (37 °C). Ten fields per slide (at least two hundred sperm) were directly counted using light microscope. Sperm colored pink were considered non-viable and unstained (clear) cells were counted as a live.

Statistical Analysis

All values were calculated as means ± standard deviation of the means (± SD), and p value < 0.05 was considered significant. Analysis used 2 x 4 factorial treatment (DV versus SM based extender; control versus fructose, trehalose and raffinose) on sperm motility and viability, with 3 stallions and 4 replication (n-12).

RESULTS AND DISCUSSION

Fresh Semen Quality

Fresh semen in the study was good quality; semen macroscopic evaluation demonstrated the mean volume without gel was 33.6±12.9 mL, pH was 7.0±0.2, thin consistency, and white-cloudy in semen color. The motile and viable sperm were 70.0%±5.4% and 83.3%±18.4%, respectively; sperm concentration was 213.0±21.9x10^6/mL; sperm with normal morphology was 75.2%±7.0%.

The Sperm Survival of Stallion Chilled Semen Stored at 5 °C

The proportion of motile cells, in all extender progressively decreased from the 2nd day of storage to reach a minimum value at day 4 (96 h). Since the second days of storage until day 4, the highest percentage of motile sperm was found in both extender supplemented with fructose ($P<0.05$). No significantly difference was found between trehalose and raffinose in DV extender, while in SM extender supplementation of fructose seem to be better extender compare to trehalose, raffinose, or control (Table 1).

During storage at 5 °C, the percentage of viable sperm in DV was decreased by 3.4% to 4.3% for every 12 h of observation; while it was decreased by 6.8% to 7.4% in semen samples diluted with SM. The viable sperm in DV control after 96 h of storage was 53.3%±2.4%; this was higher than SM which was only 23.3%±2.4%. The viable sperm in DVT was 59.6%±2.3%, significantly higher than DVR, DVF, DV control and SM supplemented with all sugar (Table 2).

Trehalose (disaccharide) and raffinose (trisaccharide) commonly reported to be useful in cryopreservation. In this study shown that trehalose maintained the sperm viability in DV better than other sugar. Viable sperm related with membrane integrity. In most studies revealed that trehalose supplementation in semen extender enhances the membrane integrity of sperm in farm animals (Hu *et al.,* 2009; Khalili *et al.,* 2009; 2010; Tonieto *et al.,* 2010; Jafaroghli *et al.,* 2011). The presence of trehalose in extenders is likely to modulate membrane fluidity by inserting itself into membrane phospholipids bilayer, thus it renders membrane more stable during cooling.

According to our study the sperm motility decreased by 22.2% for every 24 h of observation, regardless the types of sugar or extenders. This was high when compared with only 11%-17.5% in the same species (Masuda *et al.,* 2004). High individual variation of stallion semen was believed responsible for the results of the two studies; different equine breeds had different results. In ram chilled semen the percentage of motile sperm decrease only 12% for 24 h stored (Karahan *et al.,* 2006).

Table 1. Motile sperm of stallion chilled semen (%) in skim milk and dimitropoulos extenders stored at 5 °C supplemented with different sugar, values represent means (±SD)

Stored time (Hour)	Skim milk extender				Dimitropoulos extender			
	Control	Trehalose	Raffinose	Fructose	Control	Trehalose	Raffinose	Fructose
0	68.2±5.6	68.2±5.6	68.2±5.6	68.2±5.6	72.1±3.2	72.1±3.2	72.1±3.2	72.1±3.2
12	48.6±2.3^{a}	50.5±3.1^{a}	51.4±2.2^{a}	56.4±1.9^{b}	56.7±2.1^{b}	59.6±3.2^{bc}	58.3±1.8^{bc}	63.8±2.4^{c}
24	39.1±1.7^{a}	41.4±2.1^{ab}	41.4±3.3^{ab}	48.2±1.9^{b}	48.3±2.2^{b}	54.2±3.2^{bc}	52.5±1.8^{bc}	58.8±2.2^{c}
36	32.3±2.2^{a}	34.1±1.8^{a}	32.7±3.2^{a}	40.5±1.2^{b}	40.8±2.6^{b}	45.4±1.1^{c}	45.8±1.5^{c}	52.1±2.3^{d}
48	25.0±2.1^{a}	26.4±2.4^{a}	25.0±3.1^{a}	31.4±2.2^{b}	34.6±1.9^{b}	40.0±1.6^{c}	39.2±2.1^{bc}	46.3±2.3^{d}
60	19.1±3.2^{a}	19.6±1.7^{a}	19.6±2.3^{a}	25.0±1.8^{b}	27.9±2.4^{b}	34.2±2.1^{c}	32.5±2.3^{c}	39.6±1.5^{d}
72	13.0±2.7^{a}	14.5±1.6^{a}	13.5±3.2^{a}	20.0±1.5^{b}	19.6±2.3^{b}	26.3±2.2^{c}	23.6±1.9^{bc}	30.9±2.1^{d}
84	9.1±4.1^{a}	11.4±3.1^{a}	10.0±2.3^{a}	16.4±1.9^{b}	13.3±2.7^{ab}	18.3±2.2^{b}	16.7±1.9^{b}	25.0±1.5^{c}
96	5.9±3.2^{a}	6.4±2.1^{a}	5.0±3.3^{a}	11.8±2.1^{b}	6.7±1.3^{a}	12.2±2.3^{b}	11.7±2.1^{b}	17.1±1.8^{c}

Note: Means in the same row with different superscript differ significantly (P<0.05).

Table 2. Viable sperm of stallion chilled semen (%) in skim milk and dimitropoulos extenders stored at 5 °C, values represent means (±SD)

Stored time (Hour)	Skim milk extender				Dimitropoulos extender			
	Control	Trehalose	Raffinose	Fructose	Control	Trehalose	Raffinose	Fructose
0	78.8±3.0	78.8±3.0	78.8±3.0	78.8±3.0	79.9±4.0	79.9±4.0	79.9±4.0	79.9±4.0
12	59.9±1.7^{a}	64.8±0.9^{b}	59.5±3.1^{a}	66.5±2.7^{b}	72.6±3.0^{c}	73.8±1.7^{c}	74.4±2.4^{c}	73.5±3.0^{c}
24	51.7±2.3^{a}	54.6±1.5^{b}	51.6±1.8^{a}	54.9±2.6^{b}	70.1±2.4^{c}	69.0±1.9^{c}	69.3±2.9^{c}	70.7±1.7^{c}
36	44.8±2.8^{a}	47.9±3.8^{ab}	45.6±2.3^{a}	49.3±2.2^{b}	66.9±1.5^{c}	67.2±2.1^{c}	65.8±1.3^{c}	68.2±2.3^{c}
48	40.4±2.4^{a}	42.4±2.8^{a}	39.7±1.5^{a}	43.3±2.3^{ab}	63.5±3.0^{c}	64.8±2.1^{c}	63.8±2.0^{c}	64.3±1.6^{c}
60	36.7±2.2^{a}	37.5±1.2^{ab}	34.5±1.3^{a}	36.8±1.6^{ab}	61.4±0.4^{c}	62.4±3.4^{c}	61.3±9.2^{c}	62.2±1.9^{c}
72	33.7±3.3^{a}	31.3±4.3^{a}	31.0±2.6^{a}	33.0±2.8^{a}	57.5±3.1^{b}	60.8±2.2^{bc}	58.3±3.1^{b}	58.8±1.4^{b}
84	29.3±2.8^{b}	24.3±2.4^{a}	26.2±3.1^{ab}	27.4±2.6^{ab}	54.8±2.2^{d}	59.4±2.4^{d}	58.1±2.5^{d}	57.3±2.1^{d}
96	23.3±2.4^{b}	16.3±3.4^{a}	20.9±2.1^{ab}	20.8±3.1^{ab}	53.3±2.4^{c}	59.6±2.3^{d}	58.5±3.1^{d}	56.9±2.5^{cd}

Note: Means in the same row with different superscript differ significantly (P<0.05).

The Sperm Survival of Stallion Chilled Semen Stored at Ambient Temperature

At ambient temperature percentage of motile sperm decreased by 8.4% to 10.8% for every three hours stored. The highest decrease was 15.0% to 30.5%; this occurred during the first three hours. At the 18th h of observation, the percentage of motile sperm in DVF was 21.4%±2.1% higher than DVR (15.1%±3.2%), DVT (14.5%±2.2%) and DV (11.9%±2.3%), SMF (8.6%±2.1%), SMR (4.1%±1.8%) and SMT (5.5%±1.9%) (Table 3).

Viable sperm decreased by 7.0% to 9.1%, the decrease was smaller compared to motile sperm (Table 4). At the 18th h of stored, the viable sperm in DVF was 37.7%±1.5%; this was higher than DV (30.6%±1.8%), DVT (28.6%±2.7%) and DVR (28.6%±1.2%). No significant difference between DV, DVT and DVR. Supplementation of fructose and raffinose in SM were (29.5%±3.4%) and (27.0%±3.1%), both were significantly higher than trehalose (26.1%±2.1%), or SM (24.1%±1.2%).

In our laboratory the ambient temperature is between 24 °C until 29 °C. Sperm survival is very low; this is due to sperm reach nearly optimum cellular activity, consuming the energy substrates, leads to lactic acid accumulation as metabolic waste. According to Vishwanath & Shannon (1999), the main source of peroxidation occurring at ambient temperature in bovine sperm is oxidative deaminase of aromatic amino acid by aromatic amino acid aminase (AAAO), which is released from dead sperm plasma membrane. The enzyme is increased with increasing temperature and duration of storage. The amount of AAAO enzyme is increased along with the temperature increase and prolonged duration of storage, AAAO enzyme is inactive in viable sperm. This mechanism might be happen the same way in equine sperm. The explanation of low sperm survival at ambient temperature (only 18 h); this is related to the lack of energy substrates, lactic acid accumulation, and AAAO release by dead sperm plasma membrane.

Semen extender is a media for sperm during storage; the semen extender components directly influence the survival of sperm. Dilution with DV demonstrated better results than SM in semen samples; these were caused by the two types of sugar (fructose and glucose)

present in DV extender, whereas only glucose present in SM extender. DV extender also contains egg yolk, which has lecithin and phospholipids to protect sperm during cold shock (Aboagla & Terada, 2004b). Extenders containing egg yolk superior compared to extenders without egg yolk on the sperm movement after 48 h of storage (Katila *et al.,* 2004). Other than sugars and egg yolk, DV extender contains citrate buffer to neutralize sperm metabolism waste (Katila *et al.,* 2004).

The advantages of DV extender in this study were agreement with the ones reported by Yudi *et al.* (2008) and Parlevliet *et al.* (1992); this group of researchers demonstrated that DV extender showed progressive sperm percentage in Dutch warm blood stallion semen stored at 5 °C and 20 °C better when compared to SM extender.

Milk contains natural buffer (McKinnon, 1999), which may had less effects than citrate buffer. Sperm break down glucose, fructose, or mannose to lactic acid under anaerobic (absence of oxygen) condition. Glycolysis (fructolysis) allows sperm to survive during storage prior to insemination; this makes fructose (a simple sugar) in seminal plasma is the most important component to be metabolized by sperm. In this study, fructose had better effects than glucose (control) and raffinose or trehalose, in all extender stored at 5 °C or ambient temperature. These were believed that under semi anaerobic condition during semen storage at 5 °C or ambient temperature, fructolysis occurred and needed fructose. Fructose delivers energy faster because fructose might be directly converted to fructose-6-phosphate (6P); while glucose and mannose have to be converted to glucose 6P prior to conversion to fructose 6P, which then is converted to fructose-bisphosphate to produce ATP and lactic acid (Hammestad, 1993).

The osmotic pressure of DV-based extender and SM extender was 293 mosmol/kg and 349 mosmol/kg, respectively. Fresh semen osmotic pressure of each stallion in this study was 291 mosmol/kg, 302 mosmol/kg, and 312 mosmol/kg, with the mean of 301.6 mosmol/kg. In this study, SM extenders had higher osmotic pressure (more hypertonic) than DV. Osmotic pressure of the extenders affects the ion pump function of sperm plasma membrane. Generally, cells are more sensitive to hypotonic solutions, which may decrease the sperm viability by 40%-50% and may injure the sperm (Chow *et al.,*1986).

Each species of animal contains a different membrane composition. This causes different effects from cooling and subsequently different cryo sensitivity of sperm across various species. The characteristics of membranes that affect their sensitivity include cholesterol/phospholipids ratio, degree of hydrocarbon chain saturation and protein/phospholipids ratio (Medeiro *et*

Table 3. Motile sperm of stallion chilled semen (%) in skim milk and dimitropoulos extenders supplemented with sugars stored at ambient temperature, values represent means (±SD)

Stored time (Hour)	Skim milk extender				Dimitropoulos extender			
	Control	Trehalose	Raffinose	Fructose	Control	Trehalose	Raffinose	Fructose
0	68.2±2.2	68.2±2.2	68.2±2.2	68.2±2.2	71.7±2.0	71.7±2.0	71.7±2.0	71.7±2.0
3	40.0±1.9^{a}	41.4±2.3^{a}	37.7±3.1^{a}	45.9±1.7^{b}	50.4±2.1^{c}	51.3±2.1^{c}	52.5±1.4^{c}	56.7±2.3^{d}
6	29.6±3.1^{a}	32.3±1.7^{ab}	28.6±2.1^{a}	34.1±2.2^{b}	38.8±1.9^{c}	39.4±2.1^{c}	39.1±2.2^{c}	46.0±3.2^{d}
9	22.3±2.3^{ab}	25.0±3.0^{b}	20.5±2.4^{a}	26.4±2.2^{b}	30.8±1.8^{c}	32.8±2.3^{cd}	34.1±1.8^{d}	40.3±3.1^{e}
12	14.6±1.9^{a}	16.4±2.3^{ab}	14.1±2.1^{a}	18.2±1.9^{b}	25.1±3.1^{c}	27.5±2.1^{c}	27.6±1.4^{c}	32.8±2.2^{d}
15	9.1±3.1^{a}	11.4±2.9^{a}	9.1±3.1^{a}	13.6±2.2^{ab}	18.3±3.2^{b}	21.2±2.0^{b}	20.8±1.8^{b}	27.0±1.9^{c}
18	3.6±2.5^{a}	5.5±1.9^{ab}	4.1±1.8^{a}	8.6±2.1^{b}	11.9±2.3^{c}	14.5±2.2^{d}	15.1±3.2^{d}	21.4±2.1^{e}

Note: Means in the same row with different superscript differ significantly (P<0.05).

Table 4. Viable sperm of stallion chilled semen (%) in skim milk and dimitropoulos extenders storage at ambient temperature supplemented with different sugar, values represent means (±SD)

Stored time (Hour)	Skim milk extender				Dimitropoulos extender			
	Control	Trehalose	Raffinose	Fructose	Control	Trehalose	Raffinose	Fructose
0	78.8±5.7	78.8±5.7	78.8±5.7	78.8±5.7	79.9±4.0	79.9±4.0	79.9±4.0	79.9±4.0
3	65.2±3.2^{a}	66.5±1.7^{a}	65.3±3.7^{a}	70.2±3.5^{b}	70.8±2.0^{b}	69.9±1.4^{b}	67.8±1.1^{ab}	73.3±2.1^{c}
6	56.3±3.2^{a}	59.5±1.6^{a}	58.4±2.6^{a}	63.7±2.3^{a}	61.9±1.7^{ab}	61.4±2.2^{ab}	59.8±1.2^{a}	65.1±1.9^{b}
9	44.9±2.0^{a}	50.0±2.0^{b}	47.1±1.3^{ab}	54.4±2.7^{c}	53.7±1.8^{c}	53.9±1.9^{c}	51.9±2.3^{c}	60.0±1.7^{d}
12	38.0±2.5^{a}	40.6±2.4^{a}	40.5±2.6^{a}	45.6±8.1^{b}	46.2±2.4^{b}	44.8±1.7^{b}	42.2±1.6^{ab}	55.3±1.8^{c}
15	30.5±1.4^{a}	32.9±2.2^{a}	34.0±3.1^{ab}	37.9±3.1^{bc}	38.8±3.3^{bc}	37.3±1.0^{bc}	36.8±1.8^{b}	45.4±2.2^{d}
18	24.1±1.2^{a}	26.1±2.1^{ab}	27.0±3.1^{b}	29.5±3.4^{b}	30.6±1.8^{b}	28.6±2.7^{b}	28.4±1.2^{b}	37.7±1.5^{c}

Note: Means in the same row with different superscript differ significantly (P<0.05).

al., 2002). Stallion sperm are more fragile to cold shock than sperm of other species; this is caused by high stallion plasma membrane concentration of arachidonic fatty acid (Chow *et al.,* 1986). Bull, ram, and stallion sperm plasma membrane arachidonic fatty acid concentrations are 3.5%, 4.5%-5% (White, 1993), and 18.2% (Chow *et al.,* 1986). Arachidonic acid is an unsaturated fatty acid with a 20-carbon chain and four double bonds. High fatty acid concentration in stallion sperm causes the instability of plasma membrane making it is easily damaged by reactive oxygen species (ROS) leading to lipid peroxidation (Neild *et al.,* 2005; Ball, 2008). Lipid peroxidation increases membrane plasma rigidity, decreases enzyme binding affinity (includes disturbing ion pump), disturbs membrane receptor activity and permeability (Best, 2006).

Centrifugation to separate seminal plasma in the early processing is believed to be one of the factors contributing to the low stallion sperm survival (Kopt *el al.,* 2005; Morrell *et al.,* 2009). Three enzymes present in stallion seminal plasma are superoxide dismutase (SOD), catalase, and glutation (Baumber *et al.,* 2003); these enzymes may reduce lipid peroxidation induced by ROS, and preserve sperm metabolism. For instance, SOD combined with peroxidase convert superoxide anion to oxygen, water, and hydrogen (Best, 2006).

Both ROS and cold shock contributed to the sperm damage during storage at 5 ^{o}C. Compared to the storage at ambient temperature, a 4-5 ^{o}C decrease in temperature interrupts the sperm metabolism, prolonging survival of sperm. Cold shock alters normal configuration of sperm membrane to hexagonal configuration (Ball, 2008) this damages sperm plasma membrane. When the damage occurs in midpiece, aspartate aminotransferase (AspAT), which is the main enzyme in ATP production by mitochondria, is released to the seminal plasma. Loss of AspAT interrupts ATP production and disturbs sperm motility (Colenbrander *et al.,* 1992).

CONCLUSION

The dimitropoulos (DV) extender better preserved the quality of stallion chilled semen at both ambient temperature and 5 ^{o}C. Fructose supplementation improved the sperm motility of stallion chilled semen stored at both ambient temperature and 5 ^{o}C and trehalose protect viable sperm only in DV at 5 ^{o}C.

ACKNOWLEDGEMENT

Thanks to the Athena Stable owners and keeper for supplying the equine semen and Bondan Achmadi for technical help.

REFERENCES

Aboagla, E. M. & T. Terada. 2004[a]. Effects of supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa. Theriogenology 62: 809-818. http://dx.doi.org/10.1016/j.theriogenology.2003.12.003

Aboagla, E. M. & T. Terada. 2004[b]. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 62: 1160-1172. http://dx.doi.org/10.1016/j.theriogenology.2004.01.013

Ahmad, E. & M. Aksoy. 2012. Trehalose as a cryoprotective agent for the sperm cells: A Mini Review. Animal Health Prod. Hyg. 1: 123-129.

Akhter, S., M. S. Ansari, B. A. Rakha, S. M. H. Andrabi, M. Anwar, & N. Ullah. 2010. Effect of fructose addition in skim milk extender on the quality of liquid nili-ravi buffalo (*Bubalus bubalis*) Semen. Pakistan J. Zool. 42:227-231.

Arifiantini, R. I., T. L. Yusuf, & B. Purwantara. 2006. Daya tahan spermatozoa kuda hasil sentrifugasi dengan kadar plasma semen yang berbeda menggunakan pengencer Skim. Anim. Prod. 8:160-167.

Arifiantini, R. I., B. Purwantara, & M. Riyadhi. 2010. Occurrence of sperm abnormality of beef cattle at several artificial insemination centers in Indonesia. Anim. Prod. 12: 44-49.

Arifiantini, R. I. 2012. Teknik Koleksi dan Evaluasi Semen pada Hewan. IPB Press, Bogor.

Aurich, C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim. Reprod. Scie. 89:65-75. http://dx.doi.org/10.1016/j.anireprosci.2005.06.025

Bailey, J. L., J. F. Bilodeau, & N. Cormier. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl 21:1–7.

Ball, B. A. 2008. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. Anim. Reprod. Scie. 107: 257–267. http://dx.doi.org/10.1016/j.anireprosci.2008.04.014

Baracaldo, M. I., A. D. Barth, & W. Bertrand. 2007. Steps for freezing bovine semen: From semen collection to the liquid nitrogen tank. IVIS Reviews in Veterinary Medicine. International Veterinary Information Service, Ithaca NY.. www.ivis.org [5 December 2007]

Baumber, J., B. A. Ball, J. J. Linfor, & S. A. Meyers. 2003. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24: 621-628.

Best, B. 2006. Aging mechanism. http://www.benbest.com/cryonics/aging mechanism. html [7 December 2006].

Bozkurt, T., G. Türk, & S. Gür. 2007. The time-dependent motility and longevity of stallion spermatozoa diluted in different spermatozoal oncentrations and extenders during cool-storage. Revue Méd. Vét. 158: 67-72.

Chow, P. Y. W., I .G. White, & B. W. Pickett. 1986. Stallion sperm and seminal plasma phospholipids and glycerylphosphorylcholine. Anim. Reprod. Sci. 11: 207-213. http://dx.doi.org/10.1016/0378-4320(86)90122-3

Colenbrander, B., A. R. Fazeli, A. Van Buiten, J. Parlevliet, & B. M. Gadella. 1992. Assesment of sperm cell membran integrity in the horse. Acta Vet. Scand. Suppl. 88: 49-58.

Hammestad, R. H. 1993. Maintenance of bio energetic balance in sperm and preservation of lipid peroxidation: a review of the effect on design of storage preservation system. Reprod. Fertil. Dev. 5:675-90. http://dx.doi.org/10.1071/RD9930675

Hu, J. H., Q. W. Li, G. Li, Z. L. Jiang, S. H. Bu, H. Yang, & L. Q. Wang. 2009. The cryoprotective effect of trehalose supplementation on boar spermatozoa quality. Anim. Reprod. Scie. 112:107-118. http://dx.doi.org/10.1016/j.anireprosci.2008.04.009

Hu, J. H., L. S. Zan, X. L. Zhao, Q. W. Li, Z. L. Jiang, Y. K. Li, & X. Li. 2010. Effects of trehalose supplementation on semen quality and oxidative stress variables in frozen-thawed bovine semen J. Anim. Sci. 88:1657-1662. http://dx.doi.org/10.2527/jas.2009-2335

Ijaz, A. & R. Durcarme. 1999. Effect of various extender and taurine on survival of stallion sperm cooled at 5 ^{o}C. The-

riogenology 44:1039-1050. http://dx.doi.org/10.1016/0093-691X(95)00290-O

Jafaroghli, M., B. Khalili, A. Farshad, & M. J. Zamiri. 2011. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Rum. Res. 96: 58-63. http://dx.doi.org/10.1016/j.smallrumres.2010.11.010

Katila, T., T. Reilas, K. Güvenc, K. Alm, & M. Andersson. 2004. The effect of seminal plasma on motility characteristics and viability of spermatozoa after cooled storage. In: J. Müller, Z. Müller, & J. F. Wade (Eds.). Proc. 3rd Meeting EEGG. Havemeyer Foundation Monograph Series No. 13: 3-5.

Karahan, U., T. R. K. Gaffari, & G. R. Seyfettin. 2006. *In vitro* effects of prostaglandin F2a and metamizol on the motility of diluted Bull Semen. Turk J. Vet. Anim. Sci. 30: 271-278.

Kenney, R. M., R. V. Bergman, W. L. Cooper, & G. W. Morse. 1975. Minimal contamination techniques for breeding mares: Technique and preliminary findings. Proc. Am. Assoc. Equine Practr. 327-336.

Khalili, B., A. Farshad, M. J. Zamiri, A. Rashidi, & P. Fazeli. 2009. Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. Asian-Australasian J. Anim. Sci. 22: 1614-1619.

Knop, K., N. Hoffmann, D. Rath, & H. Sieme. 2005. Effects of cushioned centrifugation technique on sperm recovery and sperm quality in stallions with good and poor semen freezability. Anim. Reprod. Scie. 89:294-297.

Masuda, H., S. Nanasaki, & Y. Chiba. 2004. A new extender for preservation of equine spermatozoa at 5 °C. J. Equine Sci. 15: 1-5. http://dx.doi.org/10.1294/jes.15.1

McKinnon, A. O. 1999. Breeding and its technology - now and the future. www.harness.org.au/99wldcon/CONFEREN.HTM [22 October 2008]

Medeiro, C. M. O., F. Forell, A. T. D. Oliveira, & J. L. Rodrigues. 2002. Current status of sperm cryopreservation: Why isn't it better. Theriogenology. 57: 327-344. http://dx.doi.org/10.1016/S0093-691X(01)00674-4

Morrell, J. M., A. Johannisson, H. Strutz, A. M. Dalin, & H. Rodriguez-Martinez. 2009. Colloidal centrifugation of stallion semen: changes in sperm motility, velocity, and chromatin integrity during storage. J. Equine Vet. Sci. 29: 24-32. http://dx.doi.org/10.1016/j.jevs.2008.11.006

Neild, D. M., J. F. H. Brouwers, B. Colenbrander, A. Ero, & B. M. Gadella. 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. Mol. Reprod. Dev.72:230-238. http://dx.doi.org/10.1002/mrd.20322

Parlevliet, J., L. Malmgren, M. Boyle, A. Wockener, H. Bader, & B. Colenbrander. 1992. Influence of conservation method on the motility and morphology of stallion semen (An International Project). Acta Vet. Scand. Suppl. 88: 153-162.

Pojprasath, T., C. Lohachit, M. Techakumphu, T. Stout, & T. Tharasanit. 2011. Improved cryopreservability of stallion sperm using a sorbitol-based freezing extender. Theriogenology. 75:1742-1449. http://dx.doi.org/10.1016/j.theriogenology.2011.01.014

Silva, A. R., R. C. S. Cardoso, & L. D. M. Silva. 2005. Comparison between different dilution rates on canine semen freezing using tris-buffer with the addition of egg-yolk and glycerol. Arq. Bras. Med. Vet. Zootec. 57: 764-771. http://dx.doi.org/10.1590/S0102-09352005000600010

Slanina, T., M. Miškeje, L. Knížat, J. Mirda, & P. Massányi. 2012. The effect of different concentration of trehalose on turkey spermatozoa motility *in vitro*. J. Micro. Biotech. Food Sciences. 1: 573-582.

Tonieto, R. A., K. L. Goularte, G. D. A. Gastal, R. S. Schiavon, J. C. Deschamps, & T. Lucia Jr. 2010. Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen. Small Rum. Res. 93: 206-209. http://dx.doi.org/10.1016/j.smallrumres.2010.05.003

Vishwanath, R. & P. Shannon. 2000. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62: 23-53. http://dx.doi.org/10.1016/S0378-4320(00)00153-6

White, I. G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a Review. Reprod. Fertil. Dev. 5: 639-658. http://dx.doi.org/10.1071/RD9930639

Yudi, I. Arifiantini, B. Purwantara, & T. L. Yusuf. 2008 Daya tahan semen segar dan kualitas semen cair kuda dengan konsentrasi spermatozoa berbeda dalam pengencer Dimitropoulos yang dimodifikasi. JITV 13: 35-42.