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ABSTRACT

Cryoprotective agents (CPAs) protect sperm during cryopreservation. The objective of this study was to assess different CPAs on stallion semen cryopreservation. Skim milk (SM) and Dimitropoulos (DV) were the extenders used in this study; each was added by glycerol (Gly), combination of ethylene glycol-glycerol (EG+Gly) or dimethilformamide (DMF). Each semen sample was evaluated and divided equally into six tubes; semen in the three tubes was diluted 1:1 with (SM), while in the remaining tubes the semen was diluted 1:1 by DV. After being diluted, all tubes were centrifuged at 1006xg for 10 minutes. The supernatan discarded, the pellet was rediluted by SM trehalosa or DV trehalose, and added by G, EG+Gly, or DMF to reach the final sperm concentration of 200×10^6/ml. The extended semen was individually packed in 0.3 ml minitube, equilibrated at 4°C for 2 hours, frozen in liquid nitrogen vapor for 10 minutes, and then was stored in liquid nitrogen container at -196 °C. After 24 hours, the semen was thawed at 37 °C for 30 second. There were no significantly different ($p>0.05$) on the percentages of motile and viable sperm in SMT (21.7% and 43.4%, respectively) compared with those extended with DV T extender (26.9% and 50.8%, respectively). DMF demonstrated better results as CPA compared to the others; and DVTDMF combination had the best protection during cryopreservation in this study.
Keywords: cryopreservation, stallion sperm, sugar, cryoprotective agents

INTRODUCTION

Stallion semen has low freezing capability; only 24%-40% stallion sperm survived after freezing (Linfor *et al.*, 2002; Vidament *et al.*, 2002; Alvarenga *et al.*, 2004). Drastic changes in temperature during chilling, freezing, or thawing of semen, and changes in osmotic pressure during preservation with cryoprotectants cause significant damages on the sperm plasma membrane.

Cryoprotectant is a substance required in semen freezing. The ideal cryoprotectants have low molecular weight and low toxicity, and should be easily diluted in distilled water (Alvarenga *et al.*, 2005). Cryoprotectants can be classified based on the basic ingredient; alcohol group (ethylene glycol and alcohol) and amides (methylformamide and dimethylformamide). Glycerol penetrates bull sperm within 3-4 minutes (Berndtson and Foote, 1972); it is not surprising that glycerol is the most commonly used cryoprotectant to freeze bull, ram, buck, and stallion semens.

The cryoprotective capacity of a compound is dependent upon both the number of lone-pair electrons the compound contains, the spherical symmetry of the lone-pair electrons, and the solubility of the compound in water (Nash, 1996). The toxicity of compounds to cells depends upon both its chemical toxicity to cells (Nash, 1996), and its osmotic toxicity (Gao *et al.*, 1995), which is induced when the membrane permeability of a penetrating cryoprotectant is much slower than water (Gao *et al.*, 1995).

The first report on amides as cryoprotectants in the freezing of stallion semen was in the year of 2000; since then, studies on amides, such as methylformamide (MF) and dimethylformamide (DMF) have been popular, although the mechanism of their protection to stallion sperm during freezing was poorly understood.

Sugars such as glucose and fructose are the major energy source, while high molecular weight-sugars serve as extracellular cryoprotectants. Sugars are often used as non-

Tabel 1. Composition of Extender

Extender	amount	G	EG	DMF
Skim			(%)	
Skim milk powder (g)	2.4	5	3&3	5
Glucose (g)	4			
Mili-Q *water* (ml)	100			
Trehalosa (mM)	50			
Streptomycin (mg)	100			
Penicillin (IU)	100000			
Osmotic pressure (mOsm/kg)		1089	1317	1234
DV				
Lactose 11% (w/v) (ml)	50	5	3&3	5
Glucose – EDTA (ml)	25			
Trehalose (mM)	50			
Eggyolk (ml)	20			
Equex STM (ml)	0.5			
Streptomycin (mg)	100	100	100	100
Penicillin (IU)	100000	100000	100000	100000
Osmotic pressurev(mOsm/kg)		1380	1392	1158

Glukosa – EDTA consist of : Glucose 60 g; sodium citrate 3,70 g; Na2 –EDTA 3,70 g (Merck,KgaA, Darmstadt Germany) ; NaHCO3 1,20 g (Merck,KgaA, Darmstadt Germany) and milliQ water *ad* 1000 ml. G= glyserol: EG= etylen glykol and glyserol; DMF= dimethilformamid

penetrating cryoprotectants in combination with penetrating cryoprotectants. Differences in the cryoprotectant abilities of different sugars have been demonstrated for bulls and rams. Trehalose and sucrose, demonstrated a significant interaction between cooling rates and the presence of sugars (Woelders *et al.,* 1997). Trehalose is a high molecular weight-sugar, acts as an extracellular cryoprotectant (Rudolph and Crowe, 1985). Supplementation of trehalose and EDTA in ram semen demonstrated a better preservation to the percentage of motile sperm during cryopreservation than fructose (Aisen *et al.*, 2000).

However, equine spermatozoa cannot survive freezing without a cryoprotectant. Therefore, there is a need to evaluate other cryoprotectants that might be less toxic than glycerol to stallion spermatozoa. This study aimed to evaluate the quality of stallion frozen semen using different cryoprotective agents on skim milk and Dimitropoulus extender.

MATERIALS AND METHODS

Three stallions used in the study belong to Athena Stable, Cinere-Depok; a fourth generation (F4) Throughbred, an American pinto, and a warmblood Swedish. All stallions were 5-8 years old, healthy, and had demonstrated the best quality on the daily sperm evaluation.

An less state all chemical were obtained from Merck,KgaA, Darmstadt Germany. Two types of centrifugation extender used in this study were skim milk-glucose (Kenney *et al.,* 1975) and Dimitropoulos (DV) (Ijaz and Durchame, 1995). Skim milk-glucose extender was composed of 2.4 g skim milk (Tropicana slim, plain) and 4 g glucose, which were diluted in 100 ml Milli-Q water. The mixture was heated for 10 minutes at 92-95 °C, allowed to cool, and then it was filtered, added by 100 mg Streptomycin (Meiji, Japan) and 100,000 IU Penicillin (Meiji, Japan). DV extender consisted of two parts, solution A and solution B. Solution A was composed of 12 g glucose and 12 g fructose, which were diluted in 600 ml Milli-Q water; the mixture was heated for 15 minutes at 95°C, allowed to cool, and then it was stored in the refrigerator for a maximum of a week. Solution B was composed of sodium citrate, 9.4 g glycin and 3.5 g sulfanilamide, which were diluted in 1000 ml Milli-Q water; the mixture was heated until it reached 100°C, allowed to cool, and then it was stored at ambient temperature for a maximum of a week. DV centrifugation extender was made of 30% solution A, 50%

solution B, and 20% egg yolk; the mixture was centrifuged, and then 1000 IU Penicillin and 1 mg Streptomycin were added per milliliter supernatant.

Skim milk-based frozen semen extender was supplemented by 50mM trehalose. DV frozen semen extender was composed of 50 ml 11% (w/v) lactose, 25 ml glucose-EDTA solution (60 g glucose, 3.70 g sodium citrate, 1.20 g NaHCO3, diluted in 1000 ml Milli-Q water), 20 ml egg yolk, 0.5 ml equex *(orvus es paste*, Novo, USA), supplemented by 50mM trehalose. Each extender

Tabel 2. Mean (±S.E.M.) Percentage of Progressive Motile and Viable Sperm of Stallion Frozen Semen Diluted in Skim and DV with Different Cryoprotective Agent

Freezing step	Skim trehalosa			DV trehalosa		
	G	EG	DMF	G	EG	DMF
Raw semen						
Motile (%)	67.5±7.2 ^{a}	67.5±7.2 ^{a}	67.5±7.2 ^{a}	67.5±7.2 ^{a}	67.5±7.2 ^{a}	67.5±7.2 ^{a}
Viable (%)	78.4±7.7 ^{a}	78.4±7.7 ^{a}	78.4±7.7 ^{a}	78.4±7.7 ^{a}	78.4±7.7 ^{a}	78.4±7.7 ^{a}
After equilibration						
Motile (%)	56.0±9.1 ^{b}	52.0±9.2 ^{b}	57.5±8.9 ^{b}	55.5±9.9 ^{b}	52.0±11.1 ^{b}	57.0±9.8 ^{b}
Viable (%)	68.5 ±7.0 ^{b}	65.9±8.6 ^{b}	68.3±7.2 ^{b}	71.2±8.7 ^{ab}	72.2±7.1 ^{ab}	71.3±7.6 ^{ab}
After thawing						
Motile (%)	23.7±6.4 ^{d}	12.8±5.0 ^{e}	28.5±5.6 ^{d}	27.8±5.5 ^{d}	16.7±5.5 ^{e}	36.2±7.3 ^{c}
Viable (%)	45.2±8.5 ^{fg}	37.1±8.3 ^{h}	48.0±10.3 ^{ef}	53.4±10.5 ^{de}	39.6±9.0 ^{gh}	59.3±12.1 ^{dc}
RR (%)	35.1	19	42.2	41.2	24.7	53.6

Different letters in superscript at the same rowdeminstrate significant different ($P<0.01$); G (gliserol); EG (etilen glikol dan gliserol); DMF(dimethilformamide); SM (spermatozoa motil); SH (spermatozoa hidup) and RR (*recovery rate*)

Tabel 3. Effect of Extender, CPAs and its Combination on the Sperm Movement Evaluate with Sperm Vision

Parameter	Skim trehalosa			DV trehalosa		
	G	EG&Gly	DMF	G	EG&G	DMF
Total motil (%)	65.0±7.5b ^{c}	42.0±13.4 ^{cd}	71.6±11.6 ^{ab}	69.5±3.8 ^{b}	47.5±4.9 ^{c}	78.9±7.6 ^{a}
Progresif (%)	28.5±12.8 ^{b}	11.9±4.3 ^{d}	35.1±14.8 ^{ab}	28.5±3.7 ^{b}	19.5±4.1 ^{c}	41.5±5.5 ^{a}
DAP (μm)	16.2±4.5	14.8±2.9	16.1±1.5	16.5±4.5	19.1±2.6	17.6±0.8
DCL (μm)	19.9±11.5	23.5±5.5	28.2±3.3	27.2±10.0	29.6±5.3	32.3±2.6
DSL (μm)	11.7±2.1	11.2±2.1	12.0±0.8	11.3±1.5	12.6±1.5	12.5±0.8
VAP (μm/s)	40.4±9.4	37.9±8.3	40.1±3.9	41.8±11.1	47.9±7.6	43.0±2.4
VCL (μm/s)	59.9±14.3	61.0±15.8	68.9±9.3	69.3±19.8	74.1±14.2	76.9±7.8
VSL (μm/s)	29.8±4.4	29.13±6.0	30.3±2.1	28.7±3.6	31.9±4.4	30.4±2.4
STR (%)	72.0±0.1	79.0±0.1	75.0±0.1	69.0±0.1	71.0±0.1	71.0±0.0
LIN (%)	50.0±0.1	52.0±0.1	44.0±0.1	43.0±0.1	44.0±0.0	39.0±0.0
WOB (%)	66.0±0.1	66.0±0.1	58.0±0.0	61.0±0.1	66.0±0.0	54.0±0.0
ALH (μm)	3.9±1.0	4.0±0.8	4.7±0.3	4.3±0.3	4.3±0.4	5.0±0.4
BCF (freq)	17.6±3.4	17.7±6.4	17.4±1.3	17.5±5.8	19.3±4.6	17.7±1.1

Different letters in superscript in the same row demonstrate significant differences ($p<0.05$)

Gly: gliserol, EG: Etilen glykol, DMF : dimethilformamide

DAP: dance average path velocity, DCL: dance curvilinear velocity, DSL: dance straight line, VAP: average path velocity, VCL: curvilinear velocity, VSL: straight line velocity, STR: straightness, LIN: linearity, WO: wobble, ALH: Amplitude Lateral head displacement , BCF: Beat cross frequency was added by the cryoprotectant, which conwas 5% glycerol, ethylene glycol (3%)-glycerol (3%) combination or 5% dimethylformamide (DMF) (Table 1).

Semen was collected using a modified artificial vagina which was made of a Nishikawa type artificial vagina (Japan) with a Missouri type semen collecting tube (Nasco, Fort Atkinson, WI); the mouth opening of the collecting tube was covered by gauze to strain out the gel fraction of the ejaculates. Macroscopic evaluation on semen samples included volume (ml), color, consistency, and pH (pH-special indicator paper; Merck, interval 6-8, scale 0.2). Microscopic evaluation on semen samples included percentages of motile sperm and viable sperm, sperm concentration, and sperm morphology. Sperm concentration was measured using a Neubauer chamber; semen was diluted 1: 100 in 3% NaCl. Sperm morphology was evaluated on semen smears on glass slides stained with Williams. The good quality semen was equally divided into 6 tubes; skim milk-based centrifugation extender was added 1:1 into the first three tubes; DV centrifugation extender was added into the remaining three tubes. Extended semen in all tubes was then centrifugated for 15 minutes at 1006 x g; supernatant was discarded, and the pellet was added by the cryoprotectants, with the final sperm concentration was $200x10^6$/ ml. The pellet of three semen samples extended with skim milk was added by trehalose-glycerol (STGly), trehalose-ethylene glycol-gylcerol (STEG+Gly), or trehalose DMF (STDMF). Similarly, the pellet of three semen samples extended with DV was then added with trehalose-glycerol (DVTGly), trehalose-ethylene glycol-gylcerol (DVTEG+Gly), or trehalose DMF (DVTDMF). Semen samples were individually packed in 0.3 ml Minitub straws, which then arranged in cassettes, equilibrated for two hours at 4-5°C (Arifiantini *et al.,* 2007), and frozen in liquid nitrogen vapor (4 cm above the nitrogen level) for 10 minutes before being stored in liquid nitrogen container for 24 hours. Straws were thawed in a 37 °C water bath for 30 seconds. Motile sperm and viable sperm were evaluated subjectively (quantitatively) on raw semen, and after dilution, after equilibration, and after thawing. As comparison, the quality of thawed semen was evaluated with Spermvision (Minitüb, Tiefenbach, Germany) at Center of Artificial Insemination in Ungaran, Central Java, Indonesia.

Data were analysed as a 2x3 factorial analysis by random assignments of groups. Each of the two experiments had four replications. When significant differences among treatment were identified, comparisons between means were assessed using Duncan's Multiple Range Test (Walpole, 1982).

RESULTS and DISCUSSION

Effects of Extender on the Quality of Frozen Semen

There were no significant different (p>0.05) on the percentages of motile and viable sperm in skim milk trehalose (21.7% and 43.4%, respectively) compare with those extended with DV trehalose extender (26.9% and 50.8%, respectively)

Effects of the Cryoprotectants on the Quality of Frozen Semen

Subjective evaluation demonstrated that DMF demonstrated highest percentages of motile and viable sperm (40.5% and 67.8%

Tabel 4. Mean (±S.E.M.) After Thawing Quality Evaluate Subjective versus CASA

Extender	Cryoprotective agent	CASA		Subjective	
		Total motile (%)	Progressive Motile (%)	Progressive Motile (%)	Viable Sperm (%)
Skim Trehalose	Gly	65.0±7.5	28.5±12.8	27.4±3.4	48.4±5.1
	EG+Gly	42.0±13.4	11.9±4.3	15.5±3.1	39.7±6.7
	DMF	71.6±11.6	36.2±14.8	31.7±2.9	52.5±6.3
DV Trehalose	Gly	69.5±3.8	28.5±3.7	31.0±3.0	56.4±7.2
	EG+Gly	47.5±4.9	19.5±4.1	19.1±3.8	39.8±7.3
	DMF	78.9±7.6	41.5±5.5	40.2±4.0	64.8±7.0

ST : Skim rehalosa ; DVT : DV trehalosa; Gly: glycerol; EG :etilen glykol ; DMF : dimethilformamide; Data from 2 stallion respectively); followed by glycerol (31.5% and 56.8%, respectively), and combination of ethylene glycol-glycerol (19.3% and 40.1%, respectively). This result compromise with those conducted by CASA, DMF had the highest percentages of total motile sperm (74.3%) with progressive motility 37.8%), followed by glycerol (67.2% and 28.5%, respectively) and the lowest of total motile sperm and progressive motile was ethylene glycol-glycerol (44.1% and 14.8%, respectively).

Effects of Cryoprotectant and Extender on the Quality of Frozen Semen

Subjective evaluation demonstrated that semen extended with DVTDMF demonstrated post-thaw motility (36.2%), higher than those extended with STDMF (28.5%), DVTG (27.8%), STG (26.7%), DVTEG+Gly (16.7%) or STEG+Gly witch only 12.8% (Table 2)

Evaluation using CASA aggreement with the subjective evaluation; semen extended with DVTDMF had the highest percentage of progressive motility (41.5%), followed by those extended with STDMF (35.1%), STG (28.5%), or DVTG (28.5%) (Table 3). Semen extended with STEG+Gly had the lowest percentages of total motile sperm and sperm with progressive motility which were 42.0% and 11.9%, respectively.

Studies using Sperm Vision on the evaluation of stallion sperm were limited; some studies using CASA system reported that sperm with rapid average path were those having >30 µm/s curvilinear velocity (VCL). The mean of VCL in this study was 68.4>30 µm/s; the sperm had rapid velocity in all entender groups.

The mean percentage of sperm with progressive motility using CASA system was different by 1-2% than subjective evaluation; exception was in STEG or STDMF, whereas the difference was 4-5% (Table 4). This fact suggested that the evaluator's skill and experience were important on the subjective assessment of sperm motility.

Cryoprotectants can be classified by their role in cryopreservation into two main groups, namely penetrating agents, which maintain intracellular and extracellular solute concentration, and non-penetrating agents, which maintain only extracellular solute concentration (Woelders *et al.,* 1997). Based on the main component, cryoprotectants can be classified into alcohol groups (ethylene glycol, glycerol, etc.) and amides (dimethylformamide, acetamide, methylformamide, etc.) (Alvarenga *et al.,* 2005).

The mechanism of work, type, and concentration are the main three factors which influence the quality of cryoprotectants to protect sperm during cryopreservation. Cryoprotectants prevent ice-crystals forming; however, they are toxic to sperm during equilibration and post-thawing. Stallion sperm are known to be fragile; the right semen extender and cryoprotectant are certainly needed.

In this study, DMF in ST or DVT demonstrated a better protection on sperm during freezing than glycerol or ethylene glycol-glycerol. This result was different from what was reported by Squires *et al.* (2004), whereas 0.5 M glycerol had a higher percentage of motile sperm (61%) than those with methylformamide (40%) and dimethylformamide (38%). The percentage of motile sperm was increased to 48-54% when higher concentration of MF and DMF (0.6 M or 0.9 M) was used; this was similar to glycerol (52%). The results of this study suggest that DMF had the best protection on stallion sperm during cryopreservation; this was in agreement with some previous studies (Alvarenga *et al.,* (2004); Medeiros *et al.* (2002); Vidament *et al.,* 2002).

The choice of cryoprotectants is based on their ability to protect sperm during freezing, and their low molecular weight, which is important in reducing the high osmolarity-induced sperm toxicity by faster and easier cellular penetration. The molecular weights of ethylene glycol, DMF, and glycerol were 62.07; 73, and 92.10, respectively.

The osmotic pressures of STEG+Gly, STDMF, DVTG, DVTEG+Gly, and DVTDMF were 1089; 1317 ; 1234 ; 1380 ; 1392 and 1158 mosm/kg, respectively. According to Meyers *et al.* (2004), the volume of stallion sperm is 24.4 μm^3, with the tolerance on extender osmolarity varies from150 to 900 mosm/kg, based on these, semen extenders with osmotic pressure close to 900 mOsm/kg are STG and DVTDMF. In facts, semen extended with skim trehalose-glycerol had lower post-thawing motility than semen diluted with other extenders which have higher osmotic pressure. It is assumed that the extender's osmotic pressure, main component of extender, and cryoprotectant's toxicity influence the quality of frozen semen post-thawing.

DV extender combined with cryoprotectants appeared to protect the sperm better during freezing than skim milk extender, which was combined with the same cryoprotectants; this is due to the perfect components of DV extender, sodium buffer lechitin and lipoprotein from egg yolk. The lipid component of semen extender maintains the integrity of phospholipid bilayer of the cell membrane and protects sperm from cold shock (Parks and Graham, 1992). It is believed that Equex STM (*Orvus es paste*) is able to store more lipids from egg yolk in semen extender. In addition, DV extender contains EDTA which is a calcium chelating agent (Crabo, 2001).

Previous studies demonstrated that different types and concentrations of cryoprotectants and different breeds of the stallion had different results; the type and concentration of cryoprotectants are carefully selected for every semen samples prior to freezing. In this study, DVTDMF was the best semen extender-cryoprotectant combination, followed by DVT glycerol and SMTDMF. It was concluded that dimethylformamide was the best cryoprotectant of the stallion semen in this study.

Stallion sperm have a low tolerance to cold shock; this appears correlated to differences in the phospholipid composition on their plasma membrane. The arachidonic acid (unsaturated fatty acid) in stallion sperm is higher (18.2%) than in bull (3.5%) and ram (4.5-5%) sperm (Chow *et al.*, 1986; White, 1993). The inverse proportion of docosapentaenoic acid (DPA; 22:5) and docosahexaenoic acid (DHA; 22:6) on phosphatidylcholine and phosphatidylethanol-amine may responsible to the sensitivity of stallion spermatozoa to damage during cryopreservation (Gadella *et al.*, 2001). White (1993) reported that DPA in bull sperm is very low; while in stallion sperm it reached 17.2%. In contrast, Chow *et al.* (1986) reported that the DHA was as high as 61.3% and 61.4% in bull and ram sperm, respectively; while it was 7.6% in stallion sperm.

For most substances, melting and freezing points are approximately equal (Brown and Brown, 2000). The melting points of DHA, DPA and arachidonic acid are -44°C, -54°C and -49°C, respectively (VanderJagt *et al.*, 2003). The high DPA and arachidonic acid concentration on stallion sperm plasma membrane with their low melting points are believed contributing to the speed differences between extra- and intra-cellular freezings. The high freezing point of the fatty acid on stallion sperm plasma membrane causes lower tolerance to cellular damage than bull or rams sperm. Bull, ram, and stallion sperm have differences in osmotic water permeabilities, which are 10.5-10.8 $\mu m\ min^{-1} atm^{-1}$, 8.47 $\mu m\ min^{-1} atm^{-1}$, and 26,0 $\mu m\ min^{-1} atm^{-1}$, respectively (Noiles *et al.*, 1993). The lower melting point of plasma membrane along with the rapid movement of water from inside the cell during stallion sperm freezing, the faster extracellular freezing; this causes water to move out from inside sperm to the extracellular environment, and sperm become progressively dehydrated.

CONCLUSION

In this study, DMF demonstrated a better protection to the sperm during semen freezing than glycerol or glycerol-ethylene glycol combination and DV extender combined with DMF had the best results than other semen extender-cryoprotectant combination groups.

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