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Deer Frozen Semen Quality in Tris Sucrose and Tris Glucose Extender with Different Glycerol Concentrations

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ABSTRAK

Penelitian tentang pengaruh penambahan karbohidrat dan gliserol pada semen rusa dilakukan dalam upaya meningkatkan kualitas semen beku rusa timor (*Cervus timorensis*). Semen dikoleksi dari lima rusa yang telah dewasa kelamin menggunakan elektroejakulator. Semen dievaluasi dan dibagi ke dalam enam tabung dan diencerkan menggunakan pengencer Tris sukrosa gliserol 10% (TSG10); Tris sukrosa gliserol 12% (TSG12); Tris sukrosa gliserol 14% (TSG14); Tris glukosa gliserol 10% (TGG10); Tris glukosa gliserol 12% (TGG12); Tris glukosa gliserol 14% (TGG14), kemudian dikemas dalam minitub 0,3 ml, diequilibrasi selama 4 jam pada suhu 5 °C, dan dibekukan dalam nitrogen cair selama 10 menit. Motilitas spermatozoa yang progresif, viabilitas, membran plasma, dan akrosom utuh dinilai pada semen segar, setelah equilibrasi dan *thawing*. Hasil penelitian menunjukkan bahwa persentase motilitas spermatozoa dalam TSG10, lebih tinggi (P<0,05) dibandingkan dengan yang diencerkan dengan bahan pengencer lainnya. Viabilitas spermatozoa tertinggi ditunjukkan oleh TSG10 (63,93±7,23%). Spermatozoa yang diencerkan dalam TSG10 dan TSG14 unggul dalam akrosom dan membran plasma utuh. Tris sukrosa dengan gliserol 10% melindungi sperma rusa Timor lebih baik dibandingkan dengan kombinasi lainnya.

Kata kunci: sukrosa, glukosa, gliserol, rusa timor, kualitas semen

ABSTRACT

In order to improve Timor deer (*Cervus timorensis*) frozen semen quality, the influence of sugar and glycerol concentration on semen characteristics of sperm was investigated. The semen was collected from five sexually mature Timor deer using an electroejaculator. The semen was evaluated and divided into six equal tubes and diluted with Tris sucrose glycerol 10% (TSG10); Tris sucrose glycerol 12% (TSG12); Tris sucrose glycerol 14% (TSG14); Tris glucose glycerol 10% (TGG10); Tris glucose glycerol 12% (TGG12); and Tris glucose glycerol 14% (TGG14). The diluted semen was packed in 0.3 ml minitub straw, equilibrated at 5 °C for 4 hours and frozen on liquid nitrogen vapor for 10 minutes. The total of forward motility, viability, acrosome integrity and membrane integrity were assessed in fresh, after equilibration and after thawing. Results demonstrated that the percentage of sperm motility in TSG10 was higher (P<0.05) than those diluted in other extenders. The highest viability of sperm was demonstrated in TSG10 (63.93±7.23%). The sperm in TSG10 and TSG14 extender were superior in acrosome as well as in membrane integrity. It was concluded that Tris Sucrose with 10% glycerol protected Timor deer sperm better than other combinations.

Key words: sucrose, glucose, glycerol, Timor Deer, semen quality

INTRODUCTION

The success of semen cryopreservation in a variety of livestock has been reported, but the information of deer semen is still limited. The successful cryopreserva-

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tion of deer semen has been reported with varying results between red deer (Soler *et al.,* 2003; Zomborszky *et al.,* 2005; Fernandez-Santos *et al.,* 2006; Martínez-Pastor *et al.,* 2009) and fallow deer (Soler *et al.* 2003; Fernandez-Santos *et al.* 2006). The natural mating process of deer is limited by antral cycle (Handarini 2006), therefore the availability of frozen semen is very important for the sustainability of deer reproduction through artificial insemination.

The principle of semen preservation is to suppress sperm metabolism by lowering the temperature as low as possible. When the temperature of sperms is reduced, for each 10 °C reduction, sperm metabolism is reduced by 50%; therefore, if spermatozoa are stored at 5 °C their metabolic needs are only about 10% of what they would have been at 37 °C (body temperature). Consequently, spermatozoa stored at 5 °C do not produce many waste products; lipid peroxidation occurs more slowly and the cells do not wear out as quickly (Mc.Kinnon, 1999). Even at a temperature of -196 °C, the activity of spermatozoa does not occur thereby extending the sperm viability.

Cooling, however, stresses spermatozoa and often causes cellular damage. Cellular injury can be caused directly by affecting cellular structure (such as rupturing membranes) or indirectly by altering cellular functions (such as slowing down metabolic processes). This damage can be minimized by including additives to the extenders and also by careful slow cooling through the temperature zone where the transition from the fluid phase to the solid phase occurs.

Simple carbohydrates such as glucose and fructose pay a role as an energy source, whereas large molecules carbohydrates can function as an extracellular cryoprotectant. The use of disaccharide (trehalose), trisaccharides (raffinose), and other oligosaccharides in semen diluents is able to protect the sperm in the freezing process (Yildiz *et al.,* 2000).

Glycerol is a preferable cryoprotectant for sperm freezing in most mammals, and it serves as a solute within the water and also penetrates into the sperm; the benefit of glycerol is because it functions as a solvent with a freezing point much lower than water. High concentrations of glycerol would be toxic; in contrast, at low concentrations the protective capability will be reduced (Silva *et al.* 2002). The concentration of glycerol used varies, depending on the semen and diluents, ranging from 4%-7% or 4%-4.5% in buck (Ritar *et al.,* 1990); 4%-8% in dog semen (Rota *et al.,* 2005; Prinosilova *et al.,* 2006; Silva *et al.,* 2005); 6.4% in bull semen (Arifiantini & Yusuf, 2006); 5% in stallion semen (Azizah & Arifiantini, 2009); 10% in deer semen (Nalley, 2006) and 14% in buck semen with skim milk as diluents (Ritar *et al.,* 1990). The possibility of increasing glycerol concentrations will improve the quality of frozen semen, and this encourages this research to determine the best glycerol concentration in protecting cells against damage of deer sperm during freezing in Tris sucrose and Tris glucose diluents.

MATERIALS AND METHODS

Five Timor stags aged 3-5 year olds, weight 64-102 kg on the hard antral stage were used as the source of semen. All stags have a proportional body condition, a pair of symmetrical and normal testicle, each of which was fed with 6-8 kg fresh forage and 250 g concentrates and water provided ad libitum.

Extender Preparation

The chemicals used in the study were purchased from Merck, KGaA, Darmstadt Germany. Semen diluents composition was modified from Asher *et al.* (2000) with the composition of buffer A (tris sucrose) and buffer B (tris glucose) (Table 1). The freezing diluents consisted of buffer, 20% eggyolk and 10%, 12%, or 14% glycerol (Table 2).

Semen Collection and Evaluation

Stags were anaesthetized before collection using combination of 1 mg xylazine and 2 mg ketamine kg^{-1} body weight (Fletcher, 2001). The Semen was collected

Table 1. Buffer tris composition

Composition	Buffer A	Buffer B
	Tris glucose	Tris sucrose
Tris (hydroxymethyl aminomethane) (g)	3.36	3.36
Citric acid (g)	1.99	1.99
Glucose (g)	0.50	-
Sucrose (g)	-	0.50
Aquadest ad (ml)	100	100

Table 2. Freezing extender composition

Composition	Tris glucose			Tris sucrose		
	G10	G12	G14	G10	G12	G14
Buffer (ml)	70	68	66	70	68	66
Egg yolk (ml)	20	20	20	20	20	20
Glycerol	10	12	14	10	12	14
Penicillin (IU/ml)	1,000	1,000	1,000	1,000	1,000	1,000
Streptomicin (1 mg/ml)	1	1	1	1	1	1

using an electroejaculator (CGS Intrument Pty, Ltd Australia) every 3 weeks. Electric stimulation was increased gradually from 2 volts to 18 volts with a maximum interval of 5 seconds (on-off) to obtain ejaculate.

After collection, semen was evaluated macroscopically for its volume (ml), color, consistency and pH (special pH indicator paper; Merck). Microscopic evaluation was conducted on the percentage of motile sperm by mixing 1 drop of semen with 4 drops of physiological saline and homogenized. One drop of the mixture was transferred to a clean warm glass object and covered with cover glass (18x18 mm). The sperm motile was assessed subjectively from 5 fields, 0 (all not moving) to 100% (all progressive motile). Sperm viability was assessed by mixing 1 drop of semen and stained with 4 drops of eosin nigrosin (Bart & Oko, 1989), smeared and placed in heating stage for 10 seconds fixation. Viable sperm was counted from 10 fields with a total of 200 cells count. The cells which absorbs the color were classified as dead sperm and unstained as a viable sperm. The sperm concentration was calculated using a Neubauer chamber (Kirkman-Brown & Björndahl, 2009) with 200x dilution in formolsaline. Sperm abnormality was assesed with eosin nigrosin staining (Barth & Oko, 1989). Membrane integrity was assesed with hyposmotic swelling test (Fonseca *et al.,* 2005), and the percentage of acrosome integrity was determined by evaluating the sperm smears stained with eosin nigrosin under phase contrast microscope at 1000× magnification under oil immersion objective and bright field (Yildiz *et al.,* 2000). A total of 200 spermatozoa were counted in at least three different fields for each treatment. Semen which has volume more than 2 ml, motility > 60%, and concentration > $500x10^6$ ml^{-1} were used in this study.

Semen Processing

Semen was individually processed by dividing it into six equal tubes. Three of them were diluted with Tris glucose with 10% glycerol (TGG10); 12% glycerol (TGG12); and 14% (TGG14). Three other tubes were diluted with Tris sucrose with 10% (TSG10), 12% glycerol (TSG12), and 14% glycerol (TSG14) at a dose of $200x10^6$ ml^{-1}.

After dilution, semen was packed into a 0.3 ml straw (Minitub, Germany) containing ± $50x10^6$ $straw^{-1}$, refrigerated to 5 °C, and held for equilibration at that temperature for 4 hours. At this point, sub-samples were taken for evaluation of motility viability, membrane and acrosome integrity, using the methods described previously. After the equilibration, straws were frozen for 10 min in nitrogen vapors 4 cm above the level of liquid nitrogen and immersed into liquid nitrogen (-196 °C) for further storage evaluation. The straws were kept for a minimum period of 24 hours in liquid nitrogen before thawing. For thawing, straws were placed in a 37 °C water bath for 30 seconds, and the contents were poured into a glass tube and assessed for sperm motility, viability, membrane and acrosome integrities as described above.

Statistical Analysis

The data were analyzed using a one-way analysis of variance, followed by the Duncan's multiple range tests to determine significant differences in all the parameters among groups. The differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

The fresh semen was 2.84±0.92 ml with a pH of 7.31±0.33, milky to cream in color and medium to thick in consistency. Microscopically, the mass movement ranged from 2+ and 3+, while motile and viable sperm 76.50±4.74% and 84.32±4.49% respectively. The sperm concentration was 900.50±139.7 10^6 ml^{-1} with 7.40±0.49% of total sperm abnormality. The membrane and acrosome integrity was 77.23±6.12% and 79.77±4.35%, respectively.

The Quality of Stags Semen after Equilibration

There was no significant difference ($P>0.05$) on the quality of the sperm after equilibration in all extenders; motile sperm, viable, membrane, and acrosome integrity ranged from 66%-71%; 75%-78%; 70%-73% and 71%-74% respectively (Table 3).

Compared to fresh semen, all variables decreased between 5.50% and 10.50% with the average of 7.83% in sperm motility, between 5.81% and 8.79% in viable sperm; while membrane and acrosome integrity decreased between 4.96% and 8.01% and 3.94% and 7.1% respectively (data not showed).

The Quality of Stags Semen after Thawing

The sperm motility in TSG10 was higher ($P<0.05$) than TGG10, TGG14 or TGG12 (52.50±5.89%). In all glycerol concentrations, TGG showed the lowest quality compared to the TSG. Sperm in TSG10 and TSG14 showed its superiority to all other parameters. Viable sperm in TSG10 and TSG14 extenders demonstrated significant increased compared to TSG12, TGG10, TGG12, and TGG14. Similar value was found in membrane integrity; TSG14 and TSG10 indicated higher value ($P<0.05$) compared to TGG12, but there were no difference in membrane inte-grity among other extender.

Plasma membrane will protect the organelles that exist inside the sperm; therefore, the integrity of the acrosome cap will greatly depend on the membrane integrity of sperm. In this study the value of sperm with acrosome integrity showed almost similar with the membrane intact. TSG10 and TSG14 showed the same value which was higher ($P<0.05$) compared to TGG12. Finally, no significant differences indicated between acrosome integrity among TGG10 and TGG14 and TSG12 (Table 4).

By the time of semen freezing and thawing, the sperm will be exposed to extreme temperature changes so that it will damage the viability of sperm. In general, semen quality will decline approximately 10%-40% (Parrish's, 2003) to 50% (Sorensen, 1987) during freezing

time. In this study the decrease was demonstrated at all parameters; 21% and 22.86% in motile and viable sperm; 21.42% and 17.54% in membrane and acrosome integrity, respectively.

Post thawed motility in this study ranged from 42.00±6.32% (TGG12) to 52.50±5.89% (TSG10). This results could be compared to those Soler *et al.* (2003) which reported post thawed motility in red deer, Pe`re David's deer and fallow deer sperm was 42.8±1.6% to 46.2±1.6%. Fernandez-Santos *et al.* (2006) reported that post thawed motility in Tris glucose was 61.7 ±3.5l %, but the semen was obtain from epididymis.

This research was conducted in the field, where the temperature could reach 30 °C. At this temperature the spermatozoa would move nearly optimally, so even the evaluation and semen processing were done in a short time, sperm quality in this study actually decreased when semen mixed with diluents. During semen processing, sperm exposed to diluents solution. In this study, diluents contained 10%, 12%, or 14% glycerol; consequently the diluents would have a high osmotic pressure. High osmotic pressure would affect the plasma membrane, known as osmotic stress (Anger *et al.* 2003). At equilibration time, the semen temperature was 5 °C. At this temperature sperm would be exposed to cold shock. Eggyolk in the extender consisting of phospholipids and phosphatidylcholin, would protect sperm from cold shock (Amirat *et al.* 2004), but some sperm still suffered from cold shock. Sperm quality declined during equilibration. This can be caused by high ambient temperatures during semen processing, changes in osmotic pressure after dilution and changes from ambient temperature upto the 5 °C.

The quality decrease of semen from dilution to equilibration was relatively small ranging only from 5.76% to 7.83% on all parameters tested. In stallion semen, the decrease was higher ranging from 6.7% to 10% (Arifiantini *et al.,* 2009), but in bull semen using Tris egg yolk diluents and Andromed, the reduction occurred less than 2% (Arifiantini & Yusuf, 2010). Based on these findings, there seem many factors that will affect the quality after equilibration, including the source of semen.

During freezing and thawing, sperm will encounter changes in extreme temperatures (Rubinsky, 2003). This will cause the decline in quality after thawing in all parameter. Cryopreservation-induced stress may result in membrane injury with the loss of sperm motility and viability. The temperature of liquid N_2 is-196 °C; at this temperature the spermatozoa will suppress the metabolism, but the temperature will return to 37° C when the thawing process accured. The repeated change of extreme temperature will cause the high decline of semen quality.

Tris sucrose with 10% glycerol demonstrated higher sperm motility after thawing than other glycerol concentration in Tris sucrose as well as in Tris glucose. This is probably due to the ability of sucrose as a large molecule carbohydrate that can function as a cryoprotectant extracellular (Naing *et al.,* 2010), so that the combination of 10% glycerol with sucrose which serves as intra and extracellular cryoprotectan could work together to protect deer sperm during the freezing process. The effect of sugar supplementation to semen freezing varies among species. This is attributed to differences in physical and biochemical composition of the sperm

Table 3. The quality of stags semen diluted with different glycerol concentration in tris sucrose and tris gructose after equilibration

	Diluents					
	TGG10	TGG12	TGG14	TSG10	TSG12	TSG14
% MS	66.00±7.38	69.00±5.16	69.00± 6.58	68.50±7.09	68.50±5.80	71.00±5.16
% VS	75.53±7.28	78.46±5.14	75.45±10.79	77.55±5.74	76.63±4.25	78.51±3.82
% MI	70.13±7.06	70.82±7.04	73.29± 4.96	70.57±7.25	71.86±4.40	72.13±5.39
% AI	71.76±7.20	71.86±7.06	74.81± 5.11	73.90±6.35	72.71±3.95	73.72±4.78

Note: TGG10= Tris glucose glycerol 10%; TGG12= Tris glucose glycerol 12%; TGG14= Tris glucose glycerol 14%; TSG10=Tris sucrose glycerol 10%; TSG12= sucrose glycerol 12%; TSG14= sucrose glycerol 14%. MS= motility semen; VS= viability semen; MI= membrane integrity; AI= acrosome integrity.

Table 4. The quality of stags semen diluted with different glycerol concentration in tris sucrose and tris gructose after thawing

	Diluents					
	TGG_{10}	TGG_{12}	TGG_{14}	TSG_{10}	TSG_{12}	TSG_{14}
% MS	$45.00±6.67^{cd}$	$42.00±6.32^{d}$	$46.00±6.58^{bcd}$	$52.50±5.89^{a}$	$49.00±6.58^{abc}$	$51.50±4.12^{ab}$
% VS	$60.08±7.90^{ab}$	$53.76±5.68^{b}$	$59.72±6.71^{ab}$	$63.93±7.23^{a}$	$58.32±6.78^{ab}$	$61.10±6.65^{a}$
% MI	$47.07±7.34^{ab}$	$42.72±9.58^{b}$	$48.25±12.66^{ab}$	$54.30±6.37^{a}$	$45.22±12.02^{ab}$	$54.08±8.42^{a}$
% AI	$50.14±6.97^{ab}$	$44.14±11.38^{b}$	$50.46±12.76^{ab}$	$55.53±6.69^{a}$	$53.25±10.49^{ab}$	$56.70±8.73^{a}$

Note: Means in the same rows with different superscript differ significantly (P<0.05). TGG10= Tris glucose glycerol 10%; TGG12= Tris glucose glycerol 12%; TGG14= Tris glucose glycerol 14%; TSG10=Tris sucrose glycerol 10%; TSG12= sucrose glycerol 12%; TSG14= sucrose glycerol 14%. MS= motility semen; VS= viability semen; MI= membrane integrity; AI= acrosome integrity.

membrane (Purdy, 2006), individual variation (Aisen *et al.*, 2002; Garde *et al.*, 2008), types of buffer (Arifiantini & Purwantara, 2010), or the composition of the diluents (Gutiérrez-Pérez *et al.*, 2009; Kozdrowski, 2009). In goat frozen semen, glucose showed the best sugars compared to others (Purdy, 2006), but in the study for Timor deer semen, sucrose proved to be better sugar than glucose.

CONCLUSION

Tris sucrose eggyolk with 10% glycerol concentration was the best diluents combination for freezing deer semen.

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