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# THE EFFECT OF GLYCEROL CONCENTRATION IN TRIS GLUCOSE EGG YOLK EXTENDER ON THE QUALITY OF TIMOR DEER FROZEN SEMEN

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## ABSTRACT

The aims of study was to compare the glycerol concentration in Tris glucose egg yolk (TGEY) diluents on the quality of deer frozen semen. Semen was collected from 5 Timor deer using electroejaculator. Immediately after collection the semen was evaluated macroscopic and microscopically. After initial evaluation, the semen was divided into three tubes and extended with Tris egg yolk with three different glycerol concentrations, which were 10% ($TGEY_{10}$); 12% ($TGEY_{12}$) and 14% ($TGEY_{14}$). The sperm motility, viability, acrosome intact and membrane intact were evaluated in raw semen, after equilibration and after thawing. The results showed that there were no differences ($p>0.05$) on the sperm motility, viability as well as sperm acrosome intact. Sperm membrane intact in $TGEY_{10}$ (52.50±5.89%) and $TGEY_{14}$ (51.50±4.12 %) were higher ($p<0.05$) than in $TGEY_{12}$ (49.00±6.58). It was concluded that 10, 12 or 14% glycerol concentration can be used for Timor deer semen cryopreservation.

*Keywords: glucose, glycerol, Timor deer semen, Tris*

## INTRODUCTION

Cryopreservation of spermatozoa from endangered species is a valuable tool for genetic management. Semen preservation and artificial insemination could become powerful tools for the genetic management of deer semen programmers since these assisted reproductive techniques that would allow the storage of semen from genetically valuable animals (Watson and Holt, 2001).

Few studies have addressed on semen preservation in deer. Soler *et al*. (2003) reported the effect of thawing in red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and Pe`re David's deer (*Elaphurus davidianus*). Zomborszky *et al.* (2005) applied the cryopreservation of sperm obtain from cauda epididymis and vas defferentes of red deer (*Cervus elaphus hippelaphus*) and fallow deer (*Dama dama*). Martınez-Pastor *et al.* (2009) tested extenders and freezing protocols for Iberian red deer (*Cervus elaphus hispanicus*) semen.

Damage of sperm during cryopreservation has been attributed to cold shock, ice crystal formation, oxidative stress, membrane alteration, cryoprotectant toxicity, and osmotic changes (Watson, 1995). The survival of spermatozoa during freezing and thawing is affected by many factors, such as cyoprotectan, equilibration time, including the composition of the cryodiluent (Watson, 2000; Leibo and Songsasen, 2002). The amount of glycerol and the presence or absence of sugars in the cryodiluent, may also be important (Salamon and Maxwell, 2000; Cheng *et al.*, 2004). Glycerol is considered the most effective cryoprotectant for ruminants (Leibo and Songsasen, 2002). Nevertheless, an optimal concentration for Timor deer (*Cervus timorensis*) species has not been defined. The aim of this study was to evaluate the quality of Timor deer semen on Tris glucose egg yolk extender with different glycerol concentration.

## MATERIALS AND METHODS

### Reagents and Media

All chemicals were obtained from Merck, Germany. Tris buffer was prepared by mixing 3.36 g Tris (*hydroxymethyl aminomethane*), monohydrate citric acid 1.99 g and 0.5 g glucose dissolved in 100 mL aquadest (Asher *et al*. 2000). There were three extenders: Tris glucose egg yolk Glucose (TGEY) added by glycerol 10% ($TGEY_{10}$), glycerol 12% ($TGEY_{12}$) or glycerol 14% ($TGEY_{14}$) (Table 1).

### Semen sample

Samples were obtained every three weeks from 5 mature Timor deer (*Cervus timorensis*). A total of 50 ejaculates were individually frozen (Ten replications from each of five stags). Animals (4-5 years-old; 64-102 kg body weight) were housed in a semi-free ranging regime at private farm at Pondok Gede. In order to prevent variations due to multiple electroejaculations, the samples used in this study were always obtained from the first electroejaculation. Stags were restrained and anaesthetized by an intramuscular injection of 1 mg *xylazine* and 2 mg ketamin/kg body weight (Fletcher, 2001). The rectum was cleaned from feces and the prepuce area was shaved and washed with physiological saline. Electroejaculation was performed using a rectal probe which was connected to a battery power source that allowed to control the voltage and ampere. Ejaculation occurred in the average values of 2 to 18 voltages.

### Semen evaluation and processing

The semen samples were assessed for volume, color, consistency, mass activity, sperm concentration, sperm morphology, sperm viability and the percentage of motile sperm, sperm with membrane intact (MI) and sperm with acrosome intact (AI). Only ejaculates with a concentration more than $500 \times 10^6$ sperm/mL, having >65% progressively motile sperm and >80% of the sperm with normal morphology were selected for this study.

After evaluation, each of raw semen was equally divided into three tubes, and diluted in one of three extenders: $TGEY_{10}$, $TGEY_{12}$ or $TGEY_{14}$ to reach the total semen concentration of 100 x $10^6$/mL (25 x $10^6$ /straw). The extended semen was individually packaged in 0.25 ml straws, and equilibrated at 4°C for 4 hours. The straw was frozen in a styrofoam box at 5 cm above the liquid nitrogen surface for 10 minutes. The frozen semen was stored for 24 hours in liquid nitrogen for further evaluations. The frozen semen straws were placed in a 37°C water bath for 30 second and then the contents was poured into a glass tube.

### Assessment of the quality

The percentage of motile, viable, MI and AI sperm were evaluated after diluted, after equilibrated and after thawed. The motile sperm was evaluated by mixing the semen gently and placing a 10 µL drop of semen on a warm slide and covered with a glass cover slip (18 x18mm) from five selected representative fields. The mean of the five estimations was recorded as final motility score. Sperm viability was assessed using eosin-nigrosin stain (Bart and Oko, 1989), by diluting 10 µL drop semen on a slide glass and adding 40 µL drop of eosin-nigrosin, and then was smeared on a new slide glass and dried quickly in heating stage (37°C). Microscopes observation area were selected randomly from ten fields, with total of 200 cells. Individual sperm were recorded as being viable (unstained) or dead (stained). Samples were also taken to assess membrane integrity by means of the hypo-osmotic swelling (HOS) test. Briefly, 5 mL of diluted sperm suspension was mixed by 50 mL of hypo-osmotic sodium citrate solution (100 mOsmol/L) and was incubated the mixture at room temperature for 30 min and was evaluated

Table 1. Extender Composition (v/v)

| Component | $TGEY_{10}$ | $TGEY_{12}$ | $TGEY_{14}$ |
|---|---|---|---|
| Buffer Tris (%) | 70 | 68 | 66 |
| Egg yolk (%) | 20 | 20 | 20 |
| Glycerol (%) | 10 | 12 | 14 |

$TGEY_{10}$ (Tris glucose egg yolk glycerol 10%; Tris glucose egg yolk glycerol 12% ($TGEY_{12}$) and Tris glucose egg yolk glycerol 14% ($TGEY_{14}$)

under Olympus microscope CH 20 at 400 magnifications (Fonseca *et al.,* 2005). The sperm membrane was considered membrane intact if the sperm tail coiled at the end of the assay and the result was expressed as HOS positive (%). For acrosome integrity, stained smears were prepared according to Nagy *et al.* (2001).

**Statistical Analysis**

Data were statistically analyzed using a one-way analysis of variance, followed by the Duncan's Multiple Range Test to determine significant differences in all the parameters between groups using the SPSS software system (Version 17.0). Differences with values of $P < 0.05$ were considered to be statistically significant.

## RESULTS AND DISCUSSION

Raw semen demonstrated a variation in the quality between the stags. The average of semen volume was 2.84±0.92 mL, it was cloudy to creamy white in color, 7.31±0.33 of pH, and having thin to thick in consistency. The mean of mass activity was 2.7±0.46 with the percentage of sperm abnormality was 9.40±0.94% and 1200.50±139.57 million/mL in sperm concentration. The percentage of motile, viable sperm, membrane and acrosome intact were 76.5±4.74; 84.32±4.49%; 77.23±6.12% and 79.79±4.36%, respectively (Table 2)

After 4 hours equibration, the sperm motility, viability, membrane and acrosome intact of the sperm were found no differences ($p>0.05$), compared to those in raw semen. The sperm quality after equilibration decreased by 5.50 to 8.00% on the sperm motility and 5.60 to 7.68% on viable sperm. The membrane intact and acrosome intact decreased by 4.60 to 6.16% and 6.27 to 7.46%, respectively. There were no significant differences ($P>0.05$) on the quality of deer semen after thawing. The range of sperm motility and viability were 49 to 52.50% and 58.32 to 63.93% respectively. This finding was demonstrated by membrane and acrosome intact as well, with the range of 45.22 to 54.30% and 53.25 to 56.7%, respectively (Table 2).

Indirectly, analyzes of sperm motility are expected to provide cues on the potential fertility of spermatozoa. Decreasing the sperm motility during freezing were highly significant ($P<0.01$).

Table2. The Quality of Deer Semen after Thawing in Tris Glucose Egg yolk with Different Glycerol Concentration

| Parameter | Glycerol Concentration | | |
|---|---|---|---|
| | 10% | 12% | 14,00% |
| | Raw semen | | |
| Motile sperm (%) | 76.50±4.74[a] | 76.50±4.74[a] | 76.50±4.74[a] |
| Viable sperm (%) | 84.32±4.49[a] | 84.32±4.49[a] | 84.32±4.49[a] |
| Membrane intact (%) | 77.23±6.12[a] | 77.23±6.12[a] | 77.23±6.12[a] |
| Acrosome intact (%) | 79.79±4.36[a] | 79.79±4.36[a] | 79.79±4.36[a] |
| | After equilibration | | |
| Motile sperm (%) | 68.50±7.09[a] | 68.50±5.80[a] | 71.00±5.16[a] |
| Viable sperm (%) | 77.55±5.74[a] | 76.63±4.25[a] | 78.51±3.82[a] |
| Membrane intact (%) | 70.57±7.25[a] | 71.86±4.40[a] | 72.13±5.39[a] |
| Acrosome intact (%) | 73.90±6.35[a] | 72.71±3.95[a] | 73.72±4.78[a] |
| | After thawing | | |
| Motile sperm (%) | 52.50±5.89[a] | 49.00± 6.58[a] | 51.50±4.12[a] |
| Viable sperm (%) | 63.93±7.23[a] | 58.32± 6.78[a] | 61.10±6.65[a] |
| Membrane intact (%) | 54.30±6.37[a] | 45.22±12.02[b] | 54.08±8.42[a] |
| Acrosome intact (%) | 55.53±6.69[a] | 53.25±10.49[a] | 56.70±8.73[a] |

Superscript in the same row indicates significantly differences ($p<0.05$)

Table 3. The Sperm Motility on Raw Semen, After Equilibration and After Thawing of Timor Deer Extended in Tris Glucose Egg Yolk with Different Glycerol Concentration

| | Extender | | |
|---|---|---|---|
| | $TGEY_{10}$ | $TGEY_{12}$ | $TGEY_{14}$ |
| Raw semen | $76.50 \pm 4.50^a$ | $76.50 \pm 4.50^a$ | $76.50 \pm 4.50^a$ |
| After equilibration | $68.50 \pm 7.09^b$ | $68.50 \pm 5.80^b$ | $71.00 \pm 5.16^b$ |
| After thawing | $52.50 \pm 5.89^c$ | $49.00 \pm 6.58^c$ | $51.50 \pm 4.12^c$ |
| Recovery rate (%) | 68.63 | 64.05 | 67.32 |

Superscript in the same row indicates significantly differences ($p<0.05$)

As mentioned before, the motility decreased during equilibration, but the high level of decrease demostrated during freezing and thawing by 16 to 19.50%. The freezability of sperm was indicated in recovery rate (RR) value. Recovery rate was measured by dividing after thaw to raw semen motility and multiplied by 100% (Hafez, 1993). The RR of three different glycerol concentrations in Tris Egg yolk glucose was similar those were 68.63% ($TGEY_{10}$), 64.05% ($TGEY_{12}$) and 67.32% ($TGEY_{14}$), respectively (Table 3).

Post thawed motility of 49 to 52.50% obtained in this study was higher than those in research of Martınez-Pastor *et al.* (2009) on Iberian deer in Trilady extender which was only 42.20± 4.80%. After thawed motility was also influenced by source of sperm. Fernandez-Santos *et al.* (2007) reported that after thaw motility was 61.10±2.90%, if the sperm obtained from epididymal. The cryopreservation can be affected by various extender components such as buffer system, concentrations of egg yolk or glycerol, or the inclusion of different types of sugars (Salamon and Maxwell, 2000; Leibo and Songsasen, 2002).

The extender composition in this study consisted of Tris which was known as universal buffer and widely used for farm animals including buck and bull semen (Yusuf *et al.* 2006; Arifiantini and Purwantara, 2010). Egg-yolk is considered to be a beneficial component of freezing extenders, probably by stimulating adenylatecyclase and stimulating the sperm motility (Okamura *et al.* 1991). Lipoproteins from egg-yolk are proven to prevent spermatozoa from cold shock (Manjunath *et al.*, 2002). Glucose as monosaccharides preserved spermatozoa, whereas fructose improved acrosomal and mitochondrial status, mannose and glucose showed a membrane-stabilizing ability (Nicolajsen and Hvidt, 1994). Glucose addition to the extender conferred better improvement in forward motility percentage than the other disaccharides addition after cryopreservation in Boer goat (Naing *et al.*, 2010). Sugars performed several functions in the extender, such as adding osmotic pressure to the medium and act as cryoprotectants (Watson, 1979). The main effect of sugars and glycerol is their ability to replace the water molecule and stabilize the membrane during transition through the critical temperature zones (Crowe and Crowe, 1984; Crowe *et al.* 1985).

Glycerol is commonly used as a permeating cryoprotectant in sperm freezing; its toxicity can cause loss in sperm viability and fertility (Rudenko *et al.* 1984). According to Asher *et al.* (1992) cited by Semiadi (1998), the sperm motility after freezing decreased only 14% by adding 14% glycerol concentration to TEY extender. There were no differences on the semen quality of Iberian red deer (*Cervus elapsus hispanicus*) after freezing by adding 4 or 8% glycerol concentration in TEY extender (Garde 2000). The glycerol concentration in this study was higher compared to bull, ram or stallion frozen semen (Van Wagtendonk-Leeuw *et al.* 2000; Soylu *et al.* 2007; Azizah and Arifiantini, 2009). The result of this study indicated that 10, 12 or 14% of glycerol concentration can be used for freezing Timor deer spermatozoa with comparable post thawed quality.

## CONCLUSION

It was concluded that 10, 12 or 14 % of glycerol concentration in Tris egg yolk glucose was suitable for Timor deer cryopreservation.

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