Quality of Frozen-Thawed Spermatozoa in an Asian Elephant (*Elephas maximus*) Collected after the Musth Period: Short Communication

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ABSTRACT

Elephant semen has a lower freeze-tolerance than semen from other species. This study investigated the effects of cooling rate and supplementation of Orvus Es Paste (OEP) on the post-thaw quality of spermatozoa collected from an Asian elephant (*Elephas maximus*) during before and after the musth period. Semen with ≥ 60% motility was cooled from room temperature to 5ºC by fast (1ºC/min) or slow (0.1ºC/min) cooling rate and then frozen with or without 0.74% OEP. The cooling rate and OEP supplementation did not

improve the post-thaw quality of spermatozoa. A reliable semen freezing protocol is necessary to improve the post-thaw quality of Asian elephant spermatozoa with a low quality after freezing.

Keywords: Asian elephants, cooling rate, cryopreservation, Orvus Es Paste, semen

INTRODUCTION

The population of Asian elephant (*Elephas maximus*) has been decreasing for the past 20 years. The transportation of bull elephants for natural breeding is expensive, increases the chance of injuries to the animal, and is often opposed by animal rights organizations, indicating the necessity of artificial insemination (AI) using chilled or frozen-thawed semen. However, most semen samples exhibit poor quality which, in combination with age and seasonal variations, constrains the implementation of effective preservation protocol (Thongtip *et al.*, 2008). Elephants are not considered seasonal breeders, but they routinely enter a temporary aggressive state called 'musth' that is characterized by elevated testosterone levels (Jainudeen *et al.*, 1972). The non-musth and full-musth of serum testosterone levels were significantly different (0.2-1.4 and 29-62 ng/ml). The motility of spermatozoa has been reported to be affected by the musth period (Thongtip *et al.*, 2008). The better semen quality was found in winter than summer and the winter was the musth period in Asian elephant in Thailand. An increased concentration of testosterone has been reported in the circulation of Asian elephant during the musth period (Niemuller and Liptrap, 1991). Controversy, in Iberian ibex, it has been suggested that testosterone levels are associated with sperm freezability (Coloma *et al.*, 2011). In winter, however, when the plasma testosterone concentration fell to baseline, the negative effects of cryopreservation on the percentage of motile spermatozoa and on the integrity of the plasma membrane of frozen-thawed sperm cells were significantly less intense. The cooling rate during cryopreservation affects spermatozoa physiology and induces biological changes. A cooling rate that is too rapid can cause cold shock, which reduces the metabolic rate of spermatozoa and their progressive motility and changes the membrane permeability (Lemma, 2011). Elephant semen cryopreservation revealed the acceptable post thaw quality by using the 1°C/min cooling rate (Thongtip *et al.,* 2004). However, the better quality might be obtained if the cooling rate is improved. Orvus Es Paste (OEP) as a freezing extender has been reported to improve the quality of post-thaw semen in various species.

The major ingredient of OEP is sodium lauryl sulfate (SLS), which contains a water-soluble anionic detergent that solubilizes active molecules. In the present study, we tried to improve the post-thaw quality of spermatozoa that were collected after the musth period, in which the spermatozoa had a low quality after freezing, using different cooling rates and OEP supplementation.

MATERIALS AND METHODS

Six ejaculate samples were collected by manual collection from an Asian elephant bull (EM1; 39 years of age) who was housed at Khao Kheow Open Zoo (Cholburi, Thailand) between October 2014 and August 2015. Each ejaculate was immediately evaluated for volume, sperm concentration, progressive motility, sperm viability and pH using pH paper strips. Sperm head was assessed by Diff-Quick staining (Domosławska *et al.,* 2019). Viability and acrosome abnormality were assessed using eosin-nigrosin staining. Membrane functional integrity was assessed using the hypo-osmotic swelling test (HOS Test) (Jeyendran *et al.,* 1992). Of which one sample was collected before the musth period (October 2014) and others were collected after the period. For fresh semen quality of before musth (N=1), the percentage of progressive motility, sperm concentration, pH, viability, normal acrosome, normal membrane functional integrity, normal head morphology were 70%, 795x10^6 sperm/ml, 8, 71%, 89.5%, 54% and 88%, respectively. For fresh semen quality of after musth (N=5), the mean values (± SEM) of progressive motility, viability, membrane functional integrity, and acrosome intact of semen samples were 61.7 ± 3.1%, 66.3 ± 8.2%, 47.2 ± 10.5%, and 85.6 ± 4.0%, respectively. Each semen sample collected after the musth period was frozen by TEST + glycerol, which has been used for the successful cryopreservation of Asian elephant, according to the methods described by Thongtip *et al.* (2004). All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise. The extenders were prepared for semen cryopreservation as follows: 1) TEST: TEST that consisted of 5.54% Tes (N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid), 1.15% Tris-(hydroxymethyl)-aminomethane, 0.4% glucose, and 20% egg yolk was supplemented with 5% glycerol. 2) TEST + OEP: TEST was supplemented with 6.5% glycerol, and 0.74% OEP (Miyazaki Kagaku, Tokyo, Japan). Each extender was divided into two parts. One extender did not contain glycerol and OEP (extender I). Another one contained either 10% glycerol or 13% glycerol + 1.48% OEP (extender IIa and IIb, respectively). Initially, extender I was slowly added to a semen sample (1:1) at room temperature (25°C). After mixing the extender

with the semen, the mixture was divided into two groups. For the fast-cooling rate (1°C/min) group, one of the semen mixtures was cooled from room temperature to 5°C using two chilled styrofoam boxes. The semen mixture tube was placed in a 600-mL glass beaker containing 400 mL of water at room temperature. The beaker was then transferred into the smaller styrofoam box, which contained water at 5°C. This styrofoam box was then placed into a larger styrofoam box. Ice cubes were placed between the two boxes to maintain the temperature. The total time for cooling from room temperature to 5°C was 30 min. For the slow cooling rate (0.1°C/min) group, the semen mixture was cooled from room temperature to 5°C using a 5°C-controlled refrigerator. The semen mixture tube was placed in a 600-mL glass beaker containing 400 mL of water at room temperature. The total time for cooling from room temperature to 5°C was 210 min. The semen mixture cooled by each cooling rate was divided into two parts and maintained at °5C. After cooling by the two cooling rates, extender IIa and extender IIb were added to semen mixtures (1:1) cooled by the fast and slow cooling rates. Subsequently, the semen mixture was equilibrated at 5°C for 1 h. The equilibrated mixtures were manually aspirated into a 0.5 mL plastic straw (Kruuse, Ltd., Leeds, UK) and sealed with the sealing powder (ARSTM, Chino, CA). They were vaporized for 10 min in a styrofoam box containing liquid nitrogen by the mixtures on a holding rack that was 2.5 cm above the liquid nitrogen level. Finally, they were kept in liquid nitrogen at least 7 days before thawing.

After thawing in a 37°C water bath for 30 sec, the samples were expelled into a 1.5 mL tube and maintained at 37°C through the evaluation process. Analyses of the motility, viability, plasma membrane integrity and acrosome integrity were conducted using computer-assisted sperm analysis (CASA), eosin B-nigrosin staining (Bjorndahl *et al*., 2003), hypo-osmotic swelling (HOS) test (Jeyendran *et al*., 1992; Perez-Llano *et al*., 2001) and Coomassie staining (Larson and Miller, 1999), respectively.

The mean values of sperm quality were analysed by analysis of variance (ANOVA) tests followed by Fisher's protected least significant difference (PLSD) tests. Differences with a probability value (P) of 0.05 or less were regarded as significant. Every time of semen collection, the body weight of elephant was measured using electric scale.

RESULTS

When the semen sample collected before the musth period (October 2014) was frozen by the TEST + OEP with fast cooling rate, the values of progressive motility, viability,

membrane function integrity and acrosome intact in the frozen-thawed spermatozoa were 23.0, 75.3%, 11.3%, and 84.5%, respectively. As shown in Table 1, however, the post-thaw parameters of spermatozoa collected after the musth period decreased and were significantly lower ($P < 0.05$) than those of fresh spermatozoa. Moreover, the cooling rate and OEP supplementation did not improve the post-thaw quality of spermatozoa. The bodyweight of elephant during each semen collection times were 3500 kg during before musth and 2700, 2700, 2700, 2700, 2960 kg during after musth.

DISCUSSION

In this study, we found that the post-thaw motility of spermatozoa collected after the musth period had very low values as compared with that of spermatozoa collected before the musth period, irrespective of cooling rate and OEP supplementation. It seems that the poor motility of frozen-thawed spermatozoa probably did not result from the inappropriate freeze-thawing protocol used in the study because the values of viability and acrosome integrity were similar to the results of a previous study

Table 1 The mean values of post-thaw parameters of semen frozen by fast and slow cooling rates with or without Orvus Es Paste (OEP). Data expressed as the mean ± SEM. Ejaculate samples were collected 5 times from an Asian elephant bull after the musth period.

Parameter	Fresh	Freezing group**			
		Fast	Slow	Fast + OEP	Slow + OEP
Motility (%)***	64.0 ± 2.4[a]	2.8 ± 1.0[b]	2.4 ± 1.1[b]	6.2 ± 4.5[b]	3.0 ± 1.5[b]
Viability (%)	61.0 ± 7.6[a]	39.1 ± 2.2[b]	30.4 ± 2.8[b]	45.5 ± 3.6[b]	40.4 ± 7.2[b]
Membrane function integrity (%)	43.8 ± 17.3[a]	11.6 ± 2.1[b]	8.1 ± 1.0[b]	12.8 ± 3.3[b]	9.6 ± 3.0[b]
Acrosome intact (%)	84.2 ± 5.3[a]	45.4 ± 9.7[b]	59.2 ± 11.5[a,b]	43.0 ± 7.4[b]	58.6 ± 7.2[b]

**Fast, fast cooling rate (1°C/min), Slow, slow cooling rate (0.1°C/min).

***The motility analysis of fresh semen was performed using a microscope by two investigators, because the laboratory was far from the zoo.

[a-b]The values with different superscript letters in the same column are significantly different ($P < 0.05$).

(Thongtip *et al*., 2004) that used the same freezing extender and cooling rate. Imrat *et al*. (2012) has suggested that the poor post-thaw motility relates to inter- and/or intra-individual differences in sperm cryosensitivity of the animals. Therefore, the poor motility of frozen-thawed spermatozoa might result from an individual characteristic of the elephant bulls. In the present study, the fresh samples collected after the musth period had good motility, but the most spermatozoa were immotile after freezing and thawing. The values of viability in post-thaw spermatozoa were greater than those of sperm motility. Therefore, these results imply that the membrane of the sperm head was still intact, but the sperm mid-piece related to the motility might be damaged. As another reason, the body weight of the elephant used in the present study was highest before the musth period (3500 kg). From the musth period until early of the rainy season, the elephant could not gain their weight to that highest level. The loss in body weight during musth might affect the frozen-thawed semen quality. However, it remains to be unclear why the post-thaw motility of spermatozoa collected after the musth period decreased as compared with spermatozoa collected before the musth period.

In conclusion, the cooling rate and OEP supplementation during freezing procedure could not improve the post-thaw quality of spermatozoa collected from an elephant bull with low quality after freezing and thawing. This result was obtained from one case, but the finding may be helpful for further strategy of elephant semen cryopreservation and artificial insemination.

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