Establishment of Cryopreservation of Leopard Cat Semen Collected by Electro-ejaculation Method

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ABSTRACT

The aim of this study was to evaluate the post-thawed characteristics of leopard cat semen. In this experiment, semen was collected from two leopard cats (A and B) at wild animal center in Seoul Grand Park in Korea. After collection, the sperms were washed with D-PBS and diluted by the freezing medium (Irvine science, USA) and stored in liquid nitrogen. The post-thawed concentration was 357×10^6sperms/ml for A and 97×10^6 sperms/ml for B. The viability of post-thawed sperm from A and B individual was 24.0% and 19.0%, respectively. Pre-freezing motility of A and B individual semen was 68.54% and 56.65. Leopard cat A had more normal sperm than that of B (69.5% vs. 54.5%). Acrosome integrity analysis detected live (14.5% vs. 9.0%), damage (39.0% vs. 44.0%) and dead (46.0% vs. 47.0%) in leopard cat A and B, respectively. The present results concluded that leopard cat semen can be collected successfully by electro-ejaculation method and cryopreserved successfullyfor future use in different assisted reproductive technologies. The cryopreservation protocol needs to be modified for increasing post-thawed viability of leopard cat spermatozoa.

(Key words : leopard cat, electro-ejaculation, semen, cryopreservation, felid)

INTRODUCTION

The leopard cat (*Prionailurus bengalensis*) is one of the most widespread species belong to the felidae family of Carnivora and widely distributed in the Asia. It occurs in forests from south Asia through east Asia to the Russian Far East and from southeast Asia to western Indonesia and the Philippines (Tamada *et al*., 2008). The leopard cat is little larger than a big domestic cat and has a base color that ranges from yellow/brown to grey/brown, found mostly in the north of its range. The under part, chest, and lower head are usually white as a large spot which is commonly found on the back of the otherwise black ears (Yin *et al*., 2006). Their average life span is about 21 years. The leopard cats deliver 2～4 kittens after a 60～70 days gestation period. The kittens reach in full maturation about 18 months of age. The male kittens acquire breeding ability at 7 months and that of female at 10 months. The population of leopard cats are rapidly declining and of under threat of immediate extinction (Lee *et al*., 2010). The Felidae's family does belong to 37 extant species, 36 of which are classified as threatened or endangered by extinction (CITES, 1973; Howard and Wildt, 1990). Leopard cat is one of 36 extant species. Historically, this species was successful reproduction of live kitten (Howard and Wildt, 1990). Moreover, recently leopard cat is described of individual number and a live individual is revealed serious inbreeding by fast destroy of natural-environment. Therefore, the present state is receiving protection supervision at zoo or wild animal center etc. in all parts of the world.

To overcome this situation, assisted reproductive techniques (ART) should be helped to conserve and restore these endangered species including of leopard cat. Several ART has been applied to conserve an endangered wild species including cryopreservation of germ cells (oocyte or sperm) or embryo, *in vitro* maturation, fertilization and culture, embryo transfer and somatic cell nuclear transfer (SCNT). Cryopreservation of semen plays pivotal roles in the preservation and transfusion of different valuable genes to the future generation and thus

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help to conserve and propagate endangered animals (Sontakke *et al*., 2004). Although semen characteristics and cryopreservation protocols for different farm animals and some wild species is well known (Sontakke*et al.,* 2004), there is scare of information on leopard cat species. The motility and ability to penetrate zona free hamster oocytes of post-thawed leopard semen declined about 50% compared to fresh semen (Shivaji *et al*., 2003).

Personality characteristic of leopard cat is different from domestic cat. They are rough and very sensitive compared with domestic cat. Therefore, application of natural artificial vagina method for semen collection is very difficult. In addition, some wild felids male reproductive functions are affected by season and age (Axner *et al*., 2007). The electric ejaculation method is commonly used for semen collection from wild species (Shivaji *et al*., 2003). This method also successfully used for leopard cat (Wildt *et al*., 1986).

Therefore, the aim of this study was to evaluate the semen collection by electro-ejaculation method and cryopreservation potential of individual leopard cat semen as the first step to establishing a species-specific reproductive database and developing an efficient strategy for genome resource banking and assisted reproduction.

MATERIALS AND METHODS

1. Animal

In this experiment 3 male leopard catswere selected (*Prionailurus bengalensis*), each weight 5.45 kg, 7.4 kg, 5.3 kg that was managed at wild animal center in Seoul Grand Park, Korea.

2. Semen Collection

Electro-ejaculation stimulation was used (304 PTEs 220 VACs, USA) for semen collection according to Howard *et al*. (1990). The leopard cat is laid on table after anesthetization using 10～15 mg/ml of Zoletil 50® (Virbac Co., French). The hair was removed from the surrounding areas of penis and testicles region and sterilized using 70% alcohol. The probe was lubricated and inserted 7.5 cm in to the rectum and the electrode positioned ventrally. A sterile 1.5 ml eppendolf tube was placed over the penis and gentle cranial pressure was applied to expose the penis. A total of 80 electrical stimuli were applied in three parts as described in Table 1. Part I and II consisted of 30 stimuli each, divided into three sets of 10 stimuli at 2, 3 and 4 volts (Part I) and at 3, 4 and 5 volts (Part II). The part III consisted of 20 stimuli (10 stimuli at 4 and 5 volts). The stimulus cycle constituted approximately 1 sec from 0 to the desired voltage, 2 to 3 seconds at the desired voltage, and an abrupt return to 0 volts for 3 second. The animal was rested for 5 min between two parts (Table 1, Fig. 1).

3. Semen Freezing and Thawing

After electro-ejaculation, the collected semen was diluted with Tris-Ext I and then transported to laboratory of Gyeongsang National University in 5℃ ice box for 6 hr. The semen was cooling in Tris-Ext I during transportation. When it arrived at the lab, semen was centrifuged and diluted with freezing medium (Irvine Science, USA). The semen was equilibrated in freezing medium at 5℃ refrigerator for 25 min and then loading in 0.25 ml straw. All of loaded straws were exposed stepwise on the LN_2 gas vapor that was 10 cm over LN_2 surface for 10 min by open the cap, same height for 10 min by cover the cap and then 5 cm for 10 min more by cover the cap. After these step, all of straws were immersed into LN_2 and then stored in LN_2 tank till thawing. The frozen semen was thawed in the 37℃ water for 30 sec and cleanup with dry

Table 1. Protocol of semen collection for electric ejaculation of leopard cat

Voltage (V) / stimulation (n)		
Part 1	Part 2	Part 3
2 volt / 10	3 volt / 10	4 volt / 10
3 volt / 10	4 volt / 10	5 volt / 10
4 volt / 10	5 volt / 10	

(Cited by Howard *et al*., 1990)

Fig. 1. Semen collection of leopard cat. (A) Spread gel on the electro-probe, (B) Semen collection with 1.5 ml eppendorf tube from leopard cat's penis.

paper tissue. Post-thawed semen was evaluated viability, motility, acrosome integrity and so on.

4. Evaluation of Concentration and Motility

Semen concentration was measured with Makler Counting Chamber (Sefi-Medical Instruments Ltd., USA) and calculated the forward movement, pendulum exercise, and turning movement with sperm morphology index (SMI): SMI = {(spermatozoa progressive motility × 20) + (% spermatozoa motility)} / 2.

5. Staining of Semen

Evaluation of live/dead sperm was evaluated by staining method modified previously described (Choi *et al*., 2010). To analyze sperm viability, the sperm smeared on to the glass slides were rapidly air dried after fixing with 5% eosin-B solution at 1:1 ratio of sperm and Eosin-B solution. Two hundred spermatozoa were counted randomly to evaluate percent live and dead sperm by optical microscope using more than 200 magnifications. Sperm were classified pink color to dead spermatozoa and no stained indicated live spermatozoa.

6. Morphology Evaluation of Sperm

Sperm morphology was evaluated by the Diff-Quik kit (SYSMEX Co., Kobe, Japan) method previously described (Mota and Ramalho-Santos, 2006). Briefly, about 10～20 μl sperm was smeared on to a glass slide and fixed for 5 sec in Diff-quik fixation solution and then the glass slide was air dried after washing with distilled water. Total 200 sperms were counted at more than 200 magnifications by an optical microscope.

7. Acrosome Integrity of Leopard Cat Semen

About 10～20 μl semen was smeared on to a glass slide and then air dried. Then slide was fixed with 95% ethanol. Dye solution was made by mixing of 95 μl FITC (L-7381, SIGMA) and 5 μl of PI (Propidium Iodine, INVITROGEN). Twenty μl of dye solution was infiltrated on to slide and then put in a cold refrigerator for 30 minutes. Finally, the slide was washed with distilled water and perfectly dried at cold refrigerator. DNA integrity was evaluated for fluorescent microscope.

RESULTS

1. Morphological Evaluation of Fresh Leopard Cat Spermatozoa

Even semen collection was tried in 3 leopard cats, two of them can be collected the semen by electro-ejaculation method in this experiment. Semen concentration was about four-times higher in leopard cat A than that in B (357×10^6 sperm/ml vs. 97×10^6 sperm/ml, Table 2). However, no differences were observed in sperm motility between A and B individual (68.54% and 56.65%). Post-thawed viability ofleopard cat A and B semen were 24% and 19% (Table 2, Fig. 2A).

2. Morphology of Post-thawed Leopard Cat Spermatozoa

Post-thawed spermatozoa analyzed the morphology by Diff-Quik method. The percent of normal spermatozoa in leopard cat A (69.5%) was higher than that in leopard cat B (54.5%). The percent of the mid-piece defection was more prominent than those of head and flagella defection in both leopard cat A and B (Table 3, Fig. 2B).

3. Acrosome Integrity of Post-thawed Leopard Cat Spermatozoa

The percent of acrosome integrity spermatozoa as live, damage and dead was 14.5%, 39.0% and 46.0% in leopard cat A and 9.0%, 44.0% and 47.0% in leopard cat B (Table 4, Fig. 3).

DISCUSSION

In this study leopard cat's semen can be collected by electro-ejaculation method and cryopreserved by conventional free-

Table 2. Morphological evaluation of pre-freezing and post-thawed semen of leopard cat

Leopard cats	Sperm conc. ($\times 10^6$/ml)	Motility at pre-freezing (%)	Post-thawed viability (%)
A	357	68.54	24.0
B	97	56.65	19.0

*Post-thawed viability was counted 100 sperm per individual leopard cat.

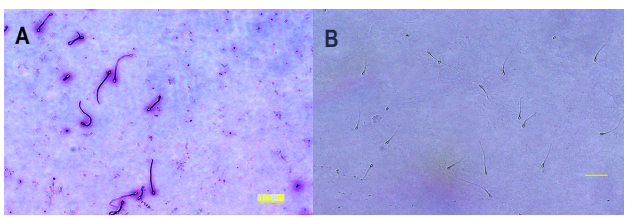

Fig. 2. Photograph of post-thawed spermatozoa. (A) eosin-B staining for live-dead sperm evaluation (200X). (B) Diff-Quik staining for normal and abnormal sperm evaluation (100X).

Table 3. Morphological characteristics of post-thawed leopard cat spermatozoa

Leopard cat	No. of morphological abnormal sperm (%)			No. of morphological normal sperm (%)
	Head	Mid-piece	Flagella	
A	1 (0.5)	59 (29.5)	1 (0.5)	139 (69.5)
B	3 (1.5)	87 (43.5)	1 (0.5)	110 (54.5)

Table 4. Acrosome integrity of thawed leopard cat spermatozoa

Individual leopard cats	No. of acrosome integrity of sperm (%)		
	Intact	Damage	Dead
A	29 (14.5)	79 (39.0)	92 (46.0)
B	18 (9.0)	88 (44.0)	94 (47.0)

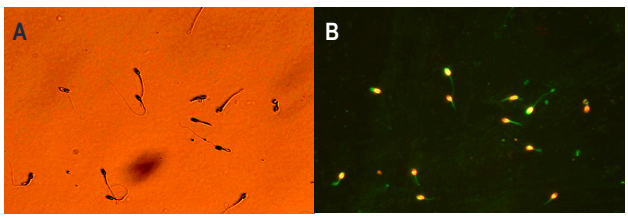

Fig. 3. Acrosome integrity of leopard cat sperm. (A) Sperm photograph via non-fluorescence, and (B) fluorescent microscope. All of sperm was divided 3 kinds of color of engrained head part (green: intact, red + green: damage, and red color: dead).

zing method. Several other scientists were reported semen collection by electro-ejaculation method in felid species (Scott *et al*., 1970; Platz *et al*., 1978; Howard *et al*., 1990; Ha *et al*., 2010) and established the cryopreservation of collected semen. This method can be applied in conservation and restoration of endangered wild animal species including of felid species and also artificial insemination, *in vitro* fertilization and intra-cytoplasm sperm injection.

In many parts, the leopard cat is very similar to a domestic cat including chromosome numbers (both have 38 pair chromosomes) as well as resembled face image. Semen characteristic of leopard cat was very similar with domestic cat, especially motility, post-thawed viability and morphology. High ratios of abnormal sperm is a common feature in felid's ejaculated semen (Wildt *et al*., 1988; Howard *et al*., 1993; Jayaprakash *et al*., 2001; Ha *et al*., 2010). The cat has very low concentration of testosterone resulting about 91% abnormal spermatozoa (Bader *et al*., 1988). A High percentage of abnormal sperm morphology was present in the cats with poor fertility, but some of the cats have to reproductive ability. Morphology of abnormal sperm was likely to have a negative impact on fertility (Axner *et al*., 2007).

In our study, one of three leopard cat showed dysfunction of testis and so could not collected semen. This should be causes obstacle of major physiological for propagation of wild animals and/or especially failure of electro-ejaculation. Semen collection and cryopreservation methods should be applied for reconstruction of endangered animals. For such reason, many investigators have contributed in the development of cryopreservation for felid's semen (Jayaprakash *et al*., 2001; Baudi *et al*., 2008; Silva *et al*., 2010). Baudi *et al*. (2008) demonstrated that some cells suffer from irreclaimable stress. It is related with temperature rate at cooling in other mammalian species. Phenotype of this was known as cold shock. Cold shock's changes motility and function of membrane integrity of spermatozoa including the change lipid phase of membrane. We analyzed various morphological parameters of post-thawed leopard cat semen using eosin-B, Diff-Quik, and FITC-PI staining. The semen collected by electro-ejaculationthat could bring the decline of quality and influence to regeneration of semen structurally ingredient with endocrine change for anesthesia (Carter *et al*., 1984; Zambelli *et al*., 2007). Excessive electro-ejaculation may influence seminal plasma composition of semen by stimulation accessory reproductive gland (Axnér *et al*., 2007).

In this study, we found different morphological abnormalities in post-thawed semenof leopard cat. The semen showed double head, misshapen head, elongated head, proximal droplet, distal droplet, coiled tail, and others abnormalities in its spermatozoa (Ha *et al*., 2010). The frequency of coiled tail was highest among others deformation in leopard cat semen. Several scientists were reported the similar abnormality of leopard and other cat semen (Carter *et al*., 1984; Axnér *et al*., 2007; Zambelli *et al*., 2007). Spermatozoa acrosome was damaged easily during freezing and thawing process. Acrosome plays an important role for sperm capacitation and fertilization. Therefore, semen process is very important for cryopreservation.

This study concluded that leopard cat's semen can be collected with electro-ejaculation method and cryopreserved successfully. However, the cryopreservation method needed to be improved for efficient and reliable use of leopard cat semen in the future, especially AI, IVF and ICSI.

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