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THESIS

EFFECT OF ANTIOXIDANT ON SEMEN CRYOPRESERVATION AND ARTIFICIAL INSEMINATION IN THE FISHING CAT (*Prionailurus viverrinus*)

KHONGSAK THIANGTUM

A Thesis Submitted in Partial Fulfillment of
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Khongsak Thiangtum 2012: Effect of Antioxidant on Semen Cryopreservation and Artificial Insemination in the Fishing Cat (*Prionailurus viverrinus*). Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Anuchai Pinyopummin, D.Vet.Med.Sc. 70 pages.

The objectives of this study were to 1) study the effect of antioxidant on motility, viability and acrosomal integrity of frozen-thawed domestic cat sperm 2) study the effect of antioxidant on frozen-thawed sperm quality in the fishing cat 3) preliminary study on laparoscopic artificial insemination in the fishing cat. In experimental 1, antioxidant catalase (CAT) and superoxide dismutase (SOD) were added in extender for domestic cat sperm cryopreservation. In experiment 2, CAT, glutathione peroxidase (GPx) and vitamin E were added in extender for fishing cat sperm cryopreservation. In experiment 3, female fishing cats had been induced for follicle stimulation and ovulation by exogenous hormone before conducted laparoscopic artificial insemination with fresh semen. Cryopreservation significantly impaired sperm motility, viability and acrosomal integrity ($p < 0.05$) in both domestic cat and fishing cat. However, motility, viability and acrosomal integrity of frozen-thawed sperm in extender with and without the antioxidants were not significantly different. Ovarian assessment and artificial insemination were performed successfully by laparoscopy. However, all recipients had no pregnant.

In conclusion, semen processing and cryopreservation had negative impact on frozen-thaw fishing cat spermatozoa. However, antioxidant did not improve motility and acrosomal integrity in cryopreserved fishing cat spermatozoa. Variable respond was found for follicle stimulation in fishing cat. Laparoscopic artificial insemination is helpful technique as one component of applied conservation effort in the fishing cat.

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Khongsak Thiangtum

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LIST OF ABBREVIATIONS

ROS	=	reactive oxygen spexies
OS	=	oxidative stress
CAT	=	catalase
SOD	=	superoxide dismutase
GPx	=	glutathione peroxidase
ART	=	assisted reproductive technique
IVF	=	*in vitro* fertilization
ET	=	embryo transfer
AI	=	artificial insemination
IU	=	international uint
µl	=	microliter
mg	=	milligram
kg	=	kilogram
mM	=	millimolar
h	=	hour
min	=	minute
^{o}C	=	degree Celsius
OH^{-}	=	hydroxyl radical
H_2O_2	=	hydrogen peroxide
O_2^{-}	=	superoxide anion
LPO	=	lipid peroxidation

EFFECT OF ANTIOXIDANT ON SEMEN CRYOPRESRVATION AND ARTIFICIAL INSEMINATION IN THE FISHING CAT (*Prionailurus viverrinus*)

INTRODUCTION

The fishing cat (*Prionailurus viverrinus*) is one of several felids species native to Southeast Asia that are threatened with extinction in the wild. This cat species is strongly adapted to an aquatic environment and its survival is depended on preservation of adequate habitat within wetland and riparian ecosystems (Nowell and Jackson, 1996). Because wetland are in precipitous decline throughout Southeast Asia due to conversion to aquaculture and rice cultivation, wild fishing cat populations presumably are exposed to high degree of risk for disappearance. Accordingly, the fishing cat is listed as endangered on the IUCN Red List (IUCN, 2012) and included on Appendix II of the Conservation on the Trade in Endangered Species (CITES, 2012).

Conservation of fishing cat and other ecologically-imperiled felid species could benefit from the application of genome resource banking, used in conjunction with assisted reproductive technology such as in vitro fertilization (IVF), embryo transfer (ET) and artificial insemination (AI) (Wildt and Roth, 1997; Wildt *et al*., 1997; Howard, 1999; Farstad, 2000; Holt *et al*., 2003). Maintenance of adequate genetic variation in ex situ (captive) and /or in situ (wild) populations could be facilitated by storage of frozen gametes and embryos, permitting easier transport for genetic exchange between distant locations and promoting long-term preservation to counter genetic drift and catastrophic loss (Wildt *et al*., 1997; Holt *et al*., 2003). Achieving the true potential of this technology, however, depends on establishing effective protocols for collection and cryopreservation of these fragile biological samples and then applying this genetic resource via assisted reproduction to generate viable offspring on routine and consistent basis.

However, using cryopreserved sperm is limited by its decrease in motility and fertilizing capacity related to sperm processing and cryopreservation techniques. There are many factors that have detrimental effects on fertilizing capacity of frozen-thawed sperm, one of which is oxidative damage to spermatozoa during sperm processing and storage. As spermatozoa membranes are rich in polyunsaturated fatty acids, they can easily undergo lipid peroxidation in the presence of reactive oxygen species (ROS). Peroxidation of sperm membrane phospholipids leads to change in membrane fluidity and results in loss of motility. Furthermore, the peroxidized sperm cells lack the membrane dynamics of fusogenicity and are deficient in fertilizing oocytes (Lenzi *et al*., 2002).

Physiologically, mammalian spermatozoa have an enzymatic antioxidant system to protect themselves against oxidative stress. This system consists of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), which physiologically control the balance between ROS production and neutralization. The amount of each enzyme in seminal plasma and spermatozoa is different among species (Lenzi *et al*., 2002).

Various antioxidants, such as vitamin E, vitamin C, taurine, acetylcysteine, CAT and SOD, have been tested in attempt to prevent oxidative stress in semen from a variety of species with controversial efficacy. In domestic cat, the antioxidants taurine, vitamin E and cysteine have been reported to have a positive effect on quality of frozen-thawed spermatozoa (Luvoni *et al*., 2002; Thuwanut *et al*., 2008). Moreover, GPx improved the quality of frozen-thawed sperm in flat-headed cats (Thuwanut *et al*., 2011). However, there are no reports about the efficacy of the enzymatic antioxidant CAT and SOD for domestic cat spermatozoa which usually been used as model for study in wild felid. Specifically in fishing cat, no antioxidants have been tested for sperm cryopreservation. Moreover, to achieve the maximum benefit from preserved semen, artificial insemination using frozen-thawed sperm should be applied in this species to produce heterogeneity offspring. However, there are no reports addressed on artificial insemination in fishing cat using ether fresh or frozen sperm. Therefore, the aims of this study were to: (1)

evaluate the effect CAT and SOD on motility, viability and acrosomal integrity of frozen-thawed cat spermatozoa; (2) evaluate the effect of addition of antioxidant CAT, GPx and Vitamin E into semen extender on quality of frozen-thawed fishing cat spermatozoa and (3) study the possibility of using laparoscopic artificial insemination in the fishing cat.

OBJECTIVES

1. To evaluate the effect of CAT and SOD on motility, viability and acrosomal integrity of frozen-thawed cat spermatozoa

2. To evaluate the effect of addition antioxidant CAT, GPx and Vitamin E in semen extender on quality of frozen-thawed fishing cat spermatozoa

3. To study the possibility of using laparoscopic artificial insemination in the fishing cat

LISTERATURE REVIEW

1. Fishing cat

The fishing cat is one of 37 wild cat species in the world. They are classified in

Order	Carnivore
Family	Felidae
Genus	Prionailurus
Species	Viverrina

Contradicting to the belief that cat don't like water, fishing cat are found in a number of water habitats, including marshy thickets, mangrove swamps, and densely vegetated area along rivers and stream. They are found in a range that spreads from northern India, Sri Lanka, across Burma, the Thai peninsular and down to Sumatra and Java, where record show the cat populations to be in critical decline. Powerful swimmers, they push themselves along with their webbed hind feet. They have been wading and swimming in shallow water, hunting for a variety of aquatic prey, including fish, frogs and toads, snails and crustaceans. They will also take small birds and mammals, snakes and domestic stock such as calves and young goats.

Figure 1 Fishing cats (*Prionailurus viverrinus*) maintained at zoo in Thailand.

Larger than a domestic cat, the fishing cat (Figure 1) is robustly built with a big, broad head, and a short tail. The short, coarse fur is a grizzled grey in color, and tinged with brown. There are elongate dark brown spots arranged in longitudinal rows extending over the entire body. There are six to eight dark lines running from the forehead to the neck, and the underparts are whitish and spotted. The head is relatively big and broad, the muzzle somewhat elongated. Their eyes have greenish irises, and the ears are rather short and rounded, with black backsides and prominent white spots in the middle. The legs are short with the forelimbs having two distinct elbow bars. Their claw sheaths are incomplete, which prevents the claws from being fully retracted. The tail is less than half the head and body length, is relatively thick, and has a series of incomplete rings with a black tip.

One remarkable feature is the layered structure of their fur, a crucial adaptation to life in the water. Next to the skin lines a layer of short hair so dense that water cannot penetrate it. Like snug-fitting thermal underwear, this coat helps keep the animal warm and dry even during chilly fishing expeditions. Sprouting up though the first coat is another layer of long guard hairs which give the cat its pattern and glossy sheen.

These cats are assumed to be polyestrus year round. They are said to have a characteristic mating call, but the call has not been described. Dens are constructed in dense shrubbery, reeds, hollow trees, in rocky crevices, or in other secluded locations. Kittens have been seen in the wild in April and June, and have been born at the Philadelphia Zoo in March and August. One to four, usually two, kittens are born after a 63-70 day gestation, and weigh around 170 grams at birth, their eyes are open by 16 days, meat is taken around 53 days, and kitten are weaned between four and six months. Adult size is attained at eight to nine months, and the young are independent between 12-18 months. It is thought that in the wild the adult male may help with the care and supervision of the young, but this is unverified. Captive individuals have lived to 12 years of age.

Wetland destruction is the primary threat facing this species, as over 50% of Asian wetlands are under threat and disappearing. Fishing cat are considered a food item in some areas of their range, and are also persecuted for taking domestic stock. Skins sometimes turn up in Asian markets, though far less frequently than other cats. They are protected over most of their range, with the exception of Bhutan, Malaysia and Vietnam. Although they are considered locally common around wetlands, their wild status overall is poorly known, and they have been placed on Appendix II of CITES.

2. Sperm cryopreservation in fishing cat

Sperm cryopreservation procedures, technique in assisted reproduction, are established on the basis of cellular physical characteristics in order to maintain viability and limit membrane damage that may occur during exposure to such non-physiological conditions as sub-zero temperatures, ice formation and high solute concentrations. A critical element of the cooling process is the passage of water across the cell membranes, the rate of which depends on permeability characteristics, cell surface-to-volume ratio, temperature and difference between extra and intracellular osmolality (Parks, 1997). The success of sperm cryopreservation using standardized protocols is known to vary among individuals and taxa (Holt, 2000), requiring careful extrapolation and assessment of freezing methods across species. For wildlife conservation, this technique has been promoted over the past 25 years as a potential means to conserve and manage threatened wildlife population (Swanson, 2006). However, in wild felids, gaining access to a sufficient number of animals for reproductive studies can be difficult owing to small population sizes and dispersal to distant geographic location. Sperm cryopreservation has been reported for fishing cat maintained in North America zoos (Howard, 1993) and in Thailand zoos (Thiangtum *et al*., 2006) which superior semen quality was found in Thai fishing cat. This may reflect the relatively inbred status of fishing cat in the North American population.

For cryopreservation, raw semen must be processed through a series of successive steps designed to removal seminal fluid, concentrate spermatozoa, resuspend the sample in a cryoprotectant solution and then cool and package the spermatozoa for freezing. Thermal and osmotic shock associated with sperm processing can damage acrosomal and plasma membranes and the actual freezing and thawing process can produce additional osmotic and thermal stress (Holt, 2000; Watson, 2000). In fishing cat, spermatozoa are fairly resilient to osmotic and temperature stress when samples are cooled slowly and glycerol is added in multiple steps. In addition, glycerol addition after slow cooling to 5^{o}C proved to be beneficial for improving the post-thaw longevity of sperm motility and the binding of spermatozoa to domestic cat oocytes (Thiangtum *et al*., 2006). Previous cryopreservation studies in other felid species have used either sperm pelleting on indentations in dry ice (Platz *et al*., 1978; Donoghue *et al*., 1992; Swanson *et al*., 1996) or straw freezing over liquid nitrogen vapor (Byers *et al*., 1989; Hey and Goodrowe, 1993; Swanson *et al*., 1996; Zambelli *et al*., 2002). Following stepwise protocol was reported for fishing cat sperm cryopreservation (Thiangtum *et al*., 2006).

Raw semen

↓

Diluted (1:1, v/v) with TEST-yolk buffer (TYB) without glycerol

↓

Centrifuged at 100g for 8 min

↓

Removed supernatant

↓

Resuspended in TYB

↓

Slowly cooled down from room temperature to 5°C (2.5-3 h time period)

↓

Diluted (1:1, v/v) with TYB containing 8% glycerol, divided into three aliquots and added at 15 min interval

↓

Sealed in straws

↓

Freeze using two step method (7.5 cm above the liquid nitrogen surface, for 1 min and lowered to 2.5 cm above the liquid nitrogen surface, for 1 min and then plunged into liquid nitrogen)

From above protocol, post-thaw fishing cat sperm motility was reported approximately range from 50-60% and sperm still had ability to fertilize domestic cat oocytes. Same report suggests that cryopreservation methods in the study are adequate for initial application with IVF procedures and the storage of frozen spermatozoa in genome resource bank as one component of applied conservation efforts. However, further improvements would be desirable, especially for the use of frozen-thawed spermatozoa with AI. Since many factors effect on post-thawed sperm quality, one of which is deteriorate effect from excessive reactive oxygen species (ROS) during sperm processing and cryopreservation. The increased generation of ROS and the consequent lipid peroxidation of membranes may also cause capacitation-like alterations and loss of

motility (Aitken *et al*., 1989). Among wild felid species, only one reported in flat-headed cat which adding glutathione peroxidase (GPx) in semen extender has positive effect on post-thawed sperm quality (Thuwanut *et al*., 2011).

3. Oxidative damage and sperm quality

3.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are highly reactive oxidizing agents belonging to the class of free radicals. A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electrons. The most common ROS that have potential implication in reproductive biology include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and the very reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radical nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in the reproduction and fertilization. The ultimate effects of (NO^-) depend upon its concentration and interactions with hydrogen peroxide. Peroxynitrite anion ($ONOO^-$) may be formed *in vivo* from superoxide and nitric oxide and actively reacts with glutathione, cysteine, deoxyribose and other thiols/thioethers. (Sikka, 1996).

Spermatozoa may generate ROS in two pathways: 1] the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane and 2] the NADH-dependent oxido-reductase (diphorase) at the level of mitochondria (Aitken *et al*., 1992; Gavella and Lipovac, 1992). Normally, these ROS must be continuously inactivated to keep only small amount necessary to maintain normal cell function. Once the normal physiological condition was impaired by over present of ROS, spermatozoa can undergo "oxidative stress" which has been attributed to affect the fertility status and physiology of spermatozoa. Oxidative stress (OS) is a condition associated with an increased rate of cellular damage induced by ROS. As described above, the production of ROS by sperm is a normal physiological process, but an imbalance between ROS generation and scavenging activity is detrimental to the

sperm and associated with male infertility. For example, when manipulated *in vitro* during assisted reproductive techniques, spermatozoa run the risk of generating and being exposed to supra-physiological level of ROS (Plessis *et al*., 2008). This phenomenon results in decreasing of sperm motility and fertilizing capacity. However, small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (Aitken *et al*., 1989). Low level of ROS can enhance the ability of human spermatozoa to bind with zona pellucid, stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion (de Lamirande and Gognon, 1995). The maintenance of suitable ROS level is, therefore, essential for adequate sperm functionality. Excessive ROS can cause adverse effects on the sperm plasma membrane, DNA, and physiological process, thereby, affecting the quality of spermatozoa. Generally, the term oxidative stress is applied when oxidants outnumber antioxidants, when peroxidation products develop and when these phenomena cause pathological effects.

3.2 Lipid peroxidation (LPO) of sperm plasma membrane

Lipid peroxidation (LPO) is broadly defined as "oxidative deterioration of polyunsaturated fatty acids (PUFA) which are fatty acids that contain more than two carbon-carbon double bonds. The LPO cascade occurs in two fundamental stages: initiation and propagation. The hydroxyl radical ($OH^{\cdot}$) is a powerful initiator of LPO. Most membrane PUFA have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene carbon-hydrogen bonds weaker, and therefore hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the rearrangement of the double bonds, which form a conjugated diene radical that can then be oxidized.

This means that lipids, which contain many methylene-interrupted double bonds, are particularly susceptible to peroxidation. Conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical ($ROO^{\cdot}$), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid hydroperoxides are stable

under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxides to generate alkoxyl and peroxyl radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell. Propagation of LPO depends on the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malondialdehyde (MDA) which has been used in biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa.

3.3 ROS and sperm motility reduction

The increased formation of ROS has been correlated with a reduction of sperm motility. The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (de Lamirande and Gagnon, 1992). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose--6phosphate dehydrogenase (G_6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of NADPH. This, in turn, is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken *et al*., 1997). Inhibition of G_6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione and reduced glutathione. This can reduce the antioxidant defenses of spermatozoa and peroxidation of membrane phospholipids (Griveau *et al*., 1995).

3.4 Oxidative stress-induced DNA damage

Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidant present in the seminal plasma. Studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free

site, deletions, frameshifts, DNA cross-link, and chromosomal rearrangement. Oxidative stress has also been correlated with high frequencies of single and double DNA strand break. Strong evidence suggests that high levels of ROS mediate the DNA fragmentation commonly observed in spermatozoa of infertile men (Agarwal and Said, 2003).

3.5 Oxidative stress and apoptosis of spermatozoa

Apoptosis, program cell dead, is a physiological phenomenon characterized by cellular morphological and biochemical alteration that cause a cell to commit suicide (Vaux and Flavell, 2000). Apoptosis appears to be strictly regulated by extrinsic and intrinsic factors and can be triggered by wide variety of stimuli. For examples, testicular irradiation is extrinsic stimuli. Previous studies have suggested that apoptosis is a key regulator of spermatogenesis in normal and pathological states. Spontaneous germ cell apoptosis has been identified in spermatogonia, spermatocytes, and spermatids in the testis of normal men and in patients with nonobstructive azoospermia (Jurisicova *et al*., 1999).

High levels of ROS disrupt the inner and outer mitochondrial membranes. This results in the release of cytochrome-C protein from the mitochondria that activates the caspases and induces apoptosis. On the other hand, Bcl-2, the inhibitor gene of programmed cell death, protects the cells from apoptosis, probably by mechanism that reduce ROS production (Gandini *et al*., 2000). Apoptosis in sperm may also be initiated by ROS-independent pathways involving the cell surface protein Fas (Lee *et al*., 1997). Fas is a type I membrane protein that belongs to the tumor necrosis factor-nerve growth factor receptor family and mediates apoptosis (Krammer *et al*., 1994). When Fas ligand or agonistic anti-Fas antibody binds to Fas, apoptosis occurs (Suda *et al*., 1993).

4. Antioxidant mechanisms and their benefit on spermatozoa

Antioxidants, in general, are free radical scavengers that suppress the formation of ROS and/or oppose their action (Sikka, 2004). In scope of reproduction, antioxidant

plays an important role as defense mechanism which protects spermatozoa from oxidative stress induced by ROS. Various antioxidants were found in seminal plasma and sperm itself. Starting from spermatogenesis in testes, testicular tissue itself has antioxidant enzymes compose of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) that protect spermatogenic and steroidogenic function of this organ from oxidative stress which impairing the ability to produce viable spermatozoa (Aitken and Roman, 2008). The fundamental biochemistry of these antioxidant enzymes is shown in (Figure 2).

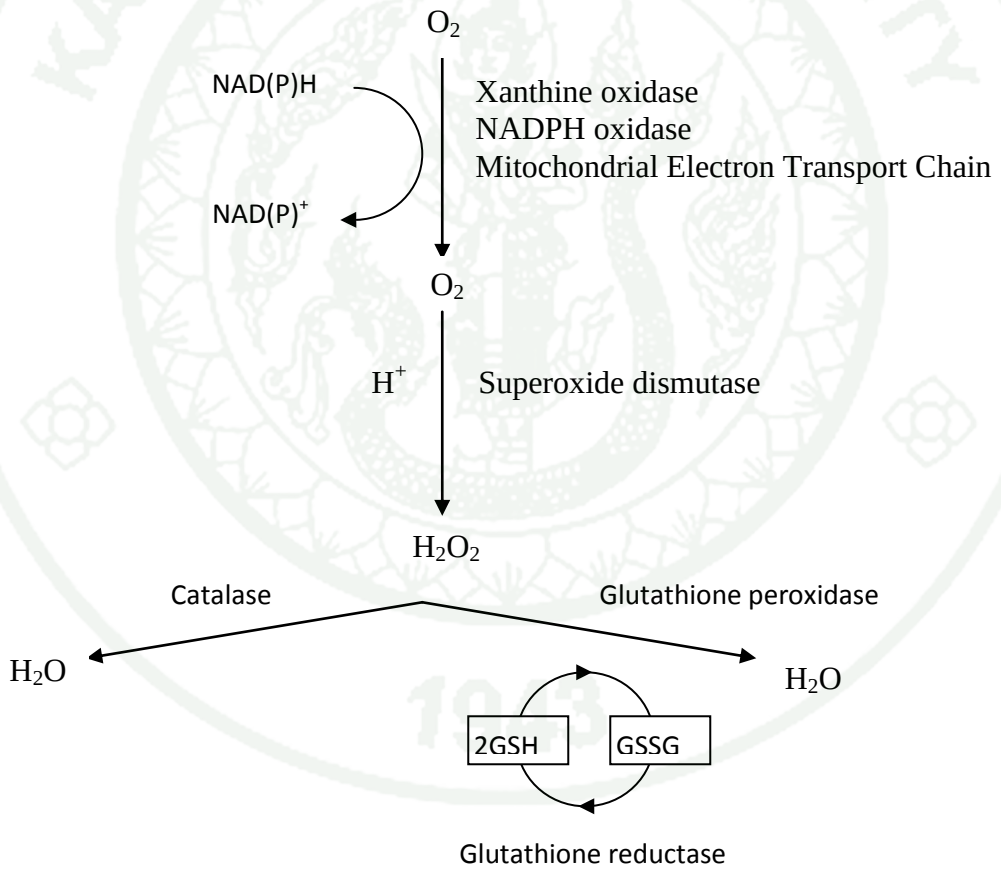

Figure 2 Major pathways of reactive oxygen species generation and metabolism (Aitke and Roman, 2008)

Throughout the post-testicular phase of sperm existence, extracellular antioxidants are secreted by the male reproductive tract. Such protection is apparent in

the epididymis (Vernet *et al*., 2004) but becomes particularly critical at the moment of ejaculation when spermatozoa inevitably face an increase in such stress as a consequence of the change in oxygen tension experienced by these cells as they move out of a hypoxic epididymal environment into the well vascularized lower female reproductive tract (Aitken, 2000). In order to counteract such stress, seminal plasma appears to be an important part which is well endowed with antioxidants. *In* 2000, Rhemrev *et al*. reported that the total antioxidant power of seminal plasma was estimated to be 10X higher than blood.

Antioxidants can be classified into two functional categories, enzymatic and non-enzymatic antioxidants. *Enzymatic antioxidants*, natural antioxidants, are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR). SOD, catalase, and GPx will convert superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to form O_2 and H_2O via the following equation:

$$2(O_2^-) + 2H^+ \xrightarrow{\text{SOD}} (H_2O_2) + O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + 1/2O_2$$

SOD protects against spontaneous O_2 toxicity and LPO (Fridovich, 1985). SOD and catalase also remove (O_2^-) generated by NADPH-oxidase in neutrophils and can play an important role in protecting spermatozoa during genitourinary inflammation (Baker *et al*., 1996). Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione as the electron donor, removes peroxyl radicals from various peroxides including H_2O_2 to improve sperm motility. Glutathione reductase regenerates reduced GSH from its oxidized form (GSSG).

$$2GSH + H_2O_2 \xrightarrow{\text{Peroxidase-(Se)}} GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{reductase}} 2GSH + NADP^+$$

Non-enzymatic antioxidants are also known as synthetic antioxidants or radical scavenging or dietary supplements. There are various non-enzymatic antioxidants such as vitamin E, vitamin C, zinc, taurine, hypotaurine and cysteine.

5. Laparoscopic artificial insemination in wild felids

The first successful AI in wild felids occurred in 1981 at the Zoological Society of London with puma cub produced by *in utero* deposition of sperm at laparotomy (Moore *et al*., 1981). Shortly thereafter, a Persian leopard (*Panthera pardus saxicolor*) was produced at the Cincinnati Zoo and Botanical Garden after a transcervical AI (Dresser *et al*., 1982). Procedural efficiency and effectiveness of AI in felids is related to having significant baseline information on: (1) site of insemination; (2) time of insemination; and (3) ovulation induction (Howard and Wildt, 2009). Base on previous studies in domestic cat since 1970 when the first kittens were born from females that were artificially inseminated vaginally with fresh sperm during a natural estrus (Sojka *et al*., 1970), AI technique was then applied in wild felids using both vaginal and intrauterine position sites of insemination (Wildt *et al*., 1986; Donoghue *et al*., 1996). However, compare between two sites of semen deposition, intrauterine insemination technique has superior results (Howard and Wildt, 2009). Major reason was discovered that sperm transport through the reproductive tract was poor in anesthetized felids, probably due to reduced uterine horn contractibility caused by anesthesia.

Laparoscopic intrauterine insemination technique was developed to ensure that sperm were deposited near to the site of fertilization (oviduct). This transabdominal approach involved inserting a laparoscope through a cannula device (3 cm skin/muscle opening) in the abdominal wall, followed by catheterizing the uterine lumen and depositing sperm in the cranial aspect of the uterine horn (Figure 3) (Howard *et al*., 1992). Laparoscopy also has been used extensively for characterizing felid reproductive tract anatomy and ovarian events, including documenting follicular development, presence of fresh (corpora hemorrhagica, CH) and mature corpora lutea (CL) and the effectiveness of various gonadotropin therapies (Howard, 1999). A major advantage of

intrauterine AI is the need for fewer spermatozoa than required during a vaginal insemination. This is important for wild felids that routinely produce comparatively low sperm densities (often with high accompanying pleiomorphisms). This is especially important in cases of using valuable thawed sperm that may well be further compromised as a result of cryopreservation/thawing (Pukazhenthi *et al*., 2006).

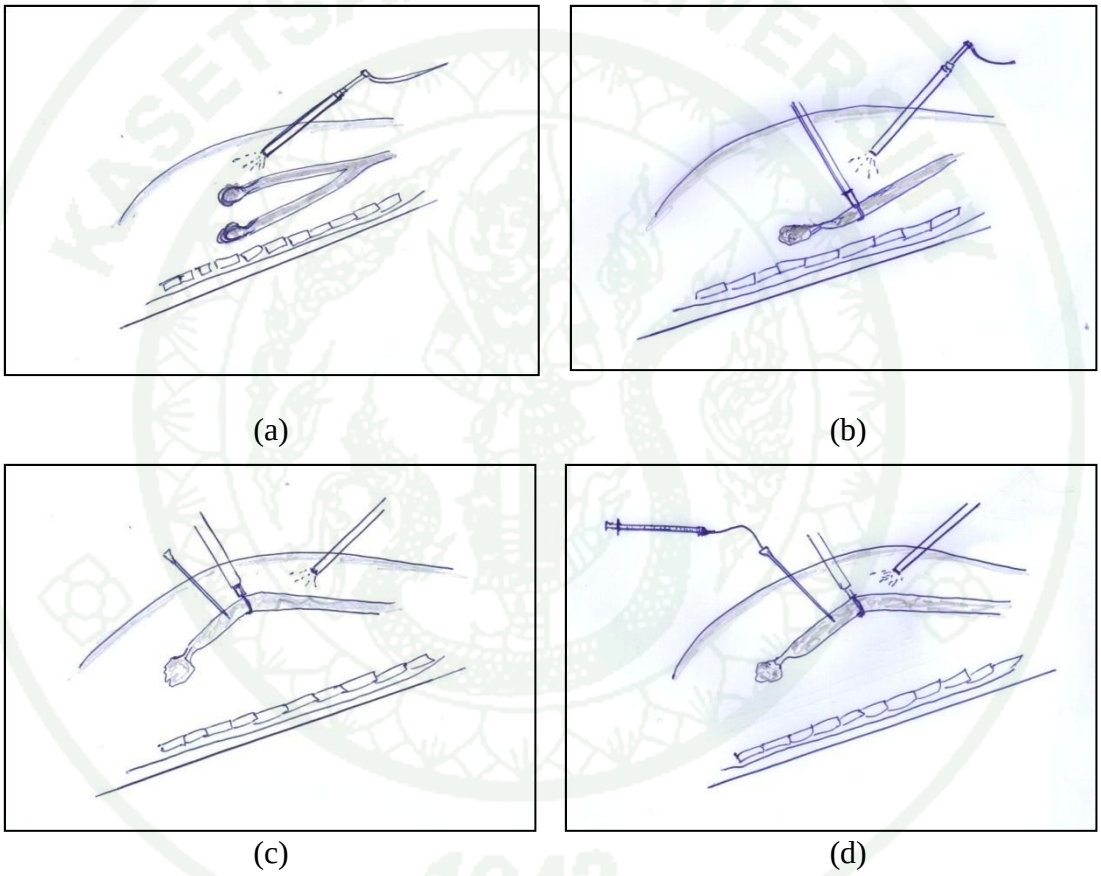

Figure 3 Laparoscopic artificial insemination in fishing cat. (a) The laparoscope is used to identify the reproductive tract; (b) accessory forceps are used to grasp the uterine horn; (c) the horn is elevated, and a catheter punctured through ventral abdominal wall is inserted into the uterine lumen; and (d) the stylette is withdrawn from the catheter and tubing containing spermatozoa is guided through the catheter into the uterine lumen for sperm deposition. (modify from Howard and Wildt, 2009).

6. Ovulation induction in wild felids

The most effective mean for scheduling and AI is to stimulate ovarian activity using exogenous gonadotropins (Howard and Wildt, 2009). Since many felids species are induced (rather than spontaneous) ovulation. Thus, ovulation can be controlled and will not normally occur in the absence of administrating an ovulation-inducing hormone, such as hCG. Data on ovulation induction has been reported in felids starting from domestic cat to wild felids (Platz *et al*., 1978; Phillip *et al*., 1982; Pelican *et al*., 2005). Table 1 show the success of ovulation induction and laparoscopic intrauterine artificial insemination (AI) conducted post-ovulation in various felids (Howard and Wildt, 2009).

Nonetheless, exogenous gonadrotropins can disturb endocrine profiles causing an aberrant follicular, oviductal or uterine environment and disruption in oocyte maturation, embryo development, or implantation (Ertzeid and Storeng, 2001). Using eCG and hCG, large foreign glycoprotein, can induce gonadotropin-neutralizing antibodies after subsequent eCG/hCG treatment. Too frequent administration results in refractoriness, which can be avoided by giving eCG or hCG treatments no more than once every 6-12 months (Swanson *et al*., 1996). At Smithsonian's zoological park, the most common mean of stimulating ovum release from ovarian follicle prior to AI is to give two I/M injection, the first eCG, followed by hCG 80-84 h later. There are rather remarkable differences among felids species in ovarian response to gonadotropin dosage. In the cheetah, high eCG/hCG dosages (400 IU/250 IU) result in abnormally small CL, whereas lower concentrations (200 IU/100 IU) produce larger, more structurally normal CL (Howard and Wildt, 2009). In tigrina, ovulation induction success when twice the dosage of eCG (200 IU) and hCG (150 IU) than the domestic cat and leopard cat (100 IU eCG/75 IU hCG) was applied. The ocelot, which is about one-fourth the body mass of a cheetah, requires 400-500 IU of eCG (twice the cheetah dosage) (Swanson *et al*., 1996). For fishing cat, species which has tendency of spontaneous ovulation, pre-treatment with the levonorgestrel implant before eCG/hCG has resulted in consistent CL numbers and quality (Pelican *et al*., 2008). However, the disadvantage of the levonorgestrel implant is

the need for two anesthetic procedures, to insert and to remove. Therefore, some later studies focus on oral progestin administration which gave good results in domestic cat.

Up to date, successful AI using fresh sperm after exogenous hormone administration was reported in domestic cat and eight wild felid species, including the leopard cat, tigrina, ocelot, clouded leopard, snow leopard, cheetah, puma, and tiger. More specifically, intrauterine insemination with frozen-thawed spermatozoa resulted in pregnancy in the leopard cat, ocelot and cheetah.

Table 1 Successful ovulation induction and laparoscopic intrauterine artificial insemination (AI) conducted post-ovulation in various felid (Howard and Wildt, 2009).

Species	Average body weight (kg)	Gonadotropin dosages		Time of ovulation post-hCG (h)	Type of sperm	No. pregnancies (%)
		eCG (IU)	hCG (IU)			
Domestic cat	2	100	75	25-30	Fresh	9/18 (50.0%)
Leopard cat	2	100	75	25-30	Fresh and frozen	2/2 (100.0%)
Tigrina	2	200	150	30-36	Fresh	1/4 (25.0%)
Ocelot	9	400	200	30-36	Fresh	1/4 (25.0%)
Ocelot	9	500	225	30-36	Frozen	1/4 (25.0%)
Clouded leopard	15	100	75	37-40	Fresh	1/20 (5.0%)
Snow leopard	30	600	300	~40	Fresh	1/15 (6.7%)
Cheetah	35	200	100	40-42	Fresh	6/13 (46.2%)
Cheetah	35	200	100	40-42	Frozen	3/11 (27.2%)
Puma	35	200	100	33-40	Fresh	1/8 (12.5%)
Tiger	250	1000	750	39-46	Fresh	1/10 (10.0%)

MATERIALS AND METHODS

Materials

1. Animals

Five adult male cats aged 3-6 years maintained at animal experimental house, Nippon Veterinary and Life Science University (Tokyo, Japan) were used in experimental 1. Each cat was individually housed in cage (size 50 x 50 x 50 cm) and was exposed to artificial light 14 h/day. Commercial cat food was provided twice a day and drinking water was given *ad libitum*. This study was conducted under the guideline of the Animal Care and Use Committee of Nippon Veterinary and Life Science University, Tokyo, Japan.

Six adult male fishing cats (estimated ages 2-5 years) maintained at Chiang Mai Night Safari (Chiang Mai, Thailand) were used in experimental 2 as semen donors for cryopreservation. Half the male were wild born and half were captive born. Ejaculates from six males were used for cryopreservation studies. Three from six male cats were also used as semen donor for artificial insemination in experimental 3. Four female fishing cats (estimated ages 2-5 years) at Chiang Mai Night Safari were used in experimental 3 for follicle stimulation and ovulation induction study and as recipient for artificial insemination. All fishing cats were provided with raw chicken meat (containing some bone) as their primary diet, supplemented with raw beef. Water was given *ad libitum*. Semen collection protocols in fishing cats were reviewed and approved by the International Animal Care and Use Committees at the National Zoological Park and the Cincinnati Zoo and Botanical Garden.

2. Equipments for Semen Collection

- Artificial vagina which modified from bottom part of microcentrifuge tube and dropper rubber (for collect semen in domestic cats)

- Electroejaculator (PT Electronics, Boring, OR, USA)
- Electroejaculator probe, 1.3 cm diameter, 24 length, three longitudinal electrodes (PT Electronics, Boring, OR, USA)
- Laboratory calipers for testicular measurement
- K-Y Gel
- Microcentrifuge tube
- Micropipett

3. Anesthetic Agents

- Zolazepam +Ttilatamine (Zoletil®, Virbac Co. Ltd.)
- Xylazine HCl (Ilium xylaxil®, Troy Laboratories)
- Isoflurane (Aerane®, Baxter Health Care Corp., Deerfield IL, USA)

4. Equipments for Semen Evaluation

- Micropipett
- Phase-contrast microscopy
- Fluorescent microscopy
- Slide warmer

- Glass slides and cover slips
- pH paper
- Counting chamber
- Hand count

5. Reagent for Evaluation of Sperm

- LIVE/DEAD spermatozoa viability kit (L-7011; Molecular Probes, Eugene, OR, USA)
- Modified Giemsa stain (Kwik Diff® kit, Thermo Fisher Scientific, Waltham, MA, USA)
- 0.3% Glutaraldehyde in phosphate-buffered saline
- 4% Paraformaldehyde
- Coomassie blue G-250 (Fisher Biotech, Springfield, NJ, USA)
- 0.1 M Ammonium acetate

6. Antioxidant Agents

- Bovine liver catalase (CAT) (Wako Pure Chemical Industries Ltd., Osaka, Japan)
- Superoxide dismutase from bovine erythrocytes (SOD) (EMD Biosciences, Inc., La Jolla, CA, USA)

- Glutathione peroxidase (GPx) (Sigma; Chemical Co., St Louis, MO, USA)
- Vitamin E analogue (Trolox) (Sigma; Chemical Co., St Louis, MO, USA)

7. Equipments for Semen Processing

- Microcentrifuge
- Plastic beaker
- Styroform ice chest
- Metal rack
- Liquid nitrogen tank
- 5^{o}C refrigerator
- Straw
- Straw powder

8. Equipment for Sperm Thawing

- 37^{o}C water bath
- Scissor
- Forceps
- Microcentrifuge tubes

9. Exogenous Hormone

- Pregnant Mare Serum Gonadotrophin (PMSG) (Follicon®, Intervet Schering-Plough Animal Health Ltd.)

- human Chorionic Gonadotrophin (hCG) (Chorulon®, MSD Animal Health)

10. Equipment for Laparoscopic Artificial Insemination

- Laparoscopic light source with monitor (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany)

- Rigid telescope, 7 mm diameter (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany)

- Trocar + trocar canular; 7 mm diameter (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany)

- Accessory forceps (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany)

- Cathetor

- Intradermic syring

Methods

Experimental 1: Effect of Catalase and Superoxide Dismutase on Motility, Viability and Acrosomal Integrity of Frozen-Thawed Cat Spermatozoa

1. Semen collection and assessment

Semen was collected by using an artificial vagina containing 50 µL of egg yolk Tris fructose citrate solution (EYT-FC, pH 6.5, osmotic pressure: 300 mOsm/l; Tsutsui *et al*., 2000). From each cat, semen was collected once weekly. After collection, fresh semen was evaluated for volume and then diluted with 100 µL of EYT-FC without glycerol. Diluted semen was evaluated for spermatozoa concentration, motility, viability, morphology and acrosomal integrity. The spermatozoa concentration was determined using a hematocytometer. Sperm motility was objectively determined by light microscope (VBS-FT-2; Nikon, Tokyo, Japan) at magnification 400x using the spermatozoa motility examination plate (NFA-71; Fujihira Industry, Tokyo, Japan) on a 37^{o}C pre-warmed stage (Type-III; Fujihira Industry). Progressive motility was scored as a percentage. Sperm viability was evaluated using the LIVE/DEAD spermatozoa viability kit (L-7011; Molecular Probes, Eugene, OR, USA). To determined dead or alive cells, at least 100 stained spermatozoa were examined on a glass slide using fluorescent microscopy.

For acrosomal evaluation, sperm samples were fixed in 0.5 ml of 4% paraformaldehyde solution at room temperature for 15 min then stored at 4^{o}C until processed for acrosomal staining. Fixed sperm then were centrifuged for 8 min at 3000g and the supernatant discarded. Pellets were washed twice with 0.5 ml of 0.1M ammonium acetate (pH 9.0) and the pellet resuspended in approximately 50 µL of the ammonium acetate solution. An aliquot of this suspension was smeared onto microscope slides and allowed to dry at room temperature. After drying, slides were flooded with Coomassie stain (0.22% Coomassie Blue G-250; Fisher Biotech, Springfield, NJ, USA; in 50% methanol, 10% glacial acetic acid and 40% deionised water) for 90 second

(Larson and Miller, 1999), rinsed with deionised water and then dried at room temperature. From each sample, 200 spermatozoa were assessed individually for acrosomal integrity using brightfield microscopy at 1000x. Acrosomal integrity was classified as: (1) intact (uniform dark staining overlying the acrosome region with light or no staining of the post-acrosomal region); (2) damaged (non-uniform or patchy staining overlying the acrosomal region); or (3) non-intact (total absence of acrosomal staining or staining only in the equatorial segment; post-acrosomal region is usually lightly staining blue).

2. Adding of CAT and SOD to extender and sperm cryopresetvation

The effect of CAT and SOD on frozen-thawed cat spermatozoa was studied in 10 ejaculates from five cats (two ejaculates/cat). After evaluation of diluted semen, sperm concentration was adjusted to 200 x 10^6 spermatozoa/mL and divided into three aliquots for the control and the two treatment groups. The diluted semen samples were transferred into microcentrifuge tubes, secured in a foam float and placed into a plastic beaker containing 300 mL room temperature (25^{o}C) water. The semen samples were cooled in 4^{o}C refrigerator for 1 h. After cooling, the semen samples were futher diluted (1:1 v/v) with chilled EYT-FC (containing 14% glycerol) adding 400 IU/ml bovine liver CAT (Wako Pure Chemical Industries Ltd., Osaka, Japan) for treatment 1 and 400 IU/ml SOD from bovine erythrocytes (EMD Biosciences, Inc., La Jolla, CA, USA) for treatment 2. In control group, no antioxidants were added. Diluted semen samples were loaded and sealed in 0.25-mL plastic straws before equilibration for 1 h in 4^{o}C refrigerator.

After equilibration, pre-freezing, spermatozoa motility was assessed. The straws were then cryopreserved using a one-step freezing method. Briefly, the straws were stored horizontally on a metal rack in a Styrofoam ice chest, 7 cm above the liquid nitrogen surface, for 10 min and then plunged into liquid nitrogen. Frozen straws were transferred into liquid nitrogen tank for storage until thawing.

3. Sperm thawing and assessment

For thawing, straws were immersed in a 37^{o}C water bath for 30 second. The straw content was then filled into a microcentrifuge tube. Immediately post-thaw, semen aliquots were evaluated for sperm motility, viability and acrosomal integrity. The methods used to assess the quality of frozen-thawed samples were the same as described for fresh semen evaluation.

4. Statistical analyses

Data were analyzed using repeated analysis of variance (ANOVA) with differences between control and treatments determined by student's *t*-test. A p-value < 0.05 was considered statistically significant.

Experimental 2: Effect of Adding Antioxidants into Extender on Quality of Frozen-Thaw fishing cat (*Prionailurus viverrinus*) spermatozoa

1. Semen collection and evaluation

Fishing cat were anesthetized with an intramuscular injection of xylazine-HCl (Ilium Xylaxil; Troy Laboratories; 0.25-0.5 mg kg^{-1}) and zolazepam + tilatamine (Zoletil®, Virbac Co. Ltd.; 5-7 mg kg^{-1}) maintained anesthetic condition with isoflurane inhalant anesthesia (Aerance; Baxter Health Care, Deerfield, IL, USA; 1-3%, v/v). Following anesthetic induction, the dimensions of each testis were measured (in cm) for length (L) and width (W) using laboratory calipers. The volume (V) of each testis was calculated as V=L x W^2 x 0.524 and total testes volume was determined by combining the volume of the left and right testes (Howard *et al*., 1990). The glans penis was exteriorized from the prepuce and assessed for morphological abnormalities and the presence or absence of penile spines. Semen was collected from each male using a standardized electroejaculation procedure (Howard *et al*., 1986), consisting of a total of 80 stimuli (2-6 V) divided into three series, delivered using a lubricated rectal probe (1.3

cm diameter, 24 length, three longitudinal electrodes; PT Electronics, Boring, OR, USA) and an electrostimulator (AC, 60-Hz sine wave; PT Electronics). Raw semen collected in each series was evaluated for ejaculate volume, pH, percentage of sperm motility (0-100%) and spermic samples from each series were combined for further processing. Sperm concentration was determined in semen samples using the haemocytometer method (Howard *et al*., 1986). Sperm morphology was assessed using light microscopy (1000x; 100-200 spermatozoa/sample) of stained spermatozoa with modified Giemsa, Kwik Diff®, staining. For acrosomal evaluation, sperm samples were stained with Coomassie blue stain and evaluated using same technique as described in experimental 1.

For cryopreservation processing, raw semen was initially diluted (1:1, v/v) with TEST-yolk buffer (TYB) without glycerol (Refrigeration Medium TYB; Irvine Scientific, Santa Ana, CA, USA) at 25^{o}C. Diluted semen was centrifuged (100g, 8min) to form a sperm pellet and allow removal of the supernatant containing seminal plasma and TYB. Sperm pellets were processed for cryopreservation.

2. Adding of antioxidants to extender and sperm cryopreservation

The effect of antioxidants on frozen-thawed fishing cat spermatozoa was studied in 11 ejaculates from six fishing cats (two ejaculates/fishing cat from 5 donors; one ejaculate/fishing cat from one donor). One month interval of semen collection was performed in all donors in this study. After raw semen was added with TYB (1:1 v/v) without glycerol, diluted semen was equally divided into four aliquots, transferred into microcentrifuge tubes and centrifuged at 100g for 8 min. Supernatant was discarded to allow the removal of seminal plasma. Sperm pellet was re-suspended to be concentration at 100 x 10^6 spermatozoa/ml with TYB containing different antioxidants as followed; 400 IU/ml of bovine liver catalase (CAT) (Wako Pure Chemical Industries Ltd., Osaka, Japan) for treatment 1, 10 IU/ml of glutathione peroxidase (GPx) (Sigma; Chemical Co., St Louis, MO, USA) for treatment 2, 5 mM of vitamin E analogue Trolox (Sigma; Chemical Co., St Louis, MO, USA) for treatment 3. In the control group, no antioxidants were added into TYB. Microcentrifuge tubes containing diluted sperm for control and all

treatment were secured in a foam float and placed into a plastic beaker containing 300 ml room temperature (25^{o}C) water. The sperm sample was cooled slowly (2.5-3-h time period) to 5^{o}C in a refrigerator. After cooling, the sperm sample was further diluted (1:1 v/v) with chilled (5^{o}C) TYB containing 8% glycerol and 400 IU/ml of bovine liver catalase (CAT) (Wako Pure Chemical Industries Ltd., Osaka, Japan) for treatment 1, 10 IU/ml of glutathione peroxidase (GPx) (Sigma; Chemical Co., St Louis, MO, USA) for treatment 2, 5 mM of vitamin E analogue Trolox (Sigma; Chemical Co., St Louis, MO, USA) for treatment 3. For control, no antioxidants were added into chilled TYB with 8% glycerol. In order to prevent sperm from high osmolarity change, chilled TYB with 8% glycerol containing deferent antioxidant in each treatment was divided into three aliquots (¼, ¼ and ½ of volume) and added at 15 min interval to provide a final glycerol concentration of 4%. After 15 min of final equilibration, the sperm sample was evaluated for sperm motility and acrosomal integrity before loading and sealing in 0.25-mL plastic straws within the deep confines of the refrigerator. The sperm straws then were cryopreserved using a two-step freezing method (Spindler *et al*., 2004). Briefly, sperm straws were suspended horizontally on a metal rack in a styrofoam ice chest, 7.5 cm above the liquid nitrogen surface, for 1 min and then quickly lowered to a second rack at 2.5 cm over liquid nitrogen for 1 min before plunging into liquid nitrogen. Frozen sperm straws were transferred into a liquid nitrogen tank for transport and storage.

3. Sperm thawing and assessment

For thawing, frozen straws were exposed to air (25^{o}C) for 10 second and immersed in a 37^{o}C water bath for 30 second before expelling the straw contents into microcentrifuge tube. Immediately post-thaw, sperm aliquots were evaluated for motility and fixed in 4% paraformaldehyde for acrosomal integrity assessment. The methods used to assess the quality of frozen-thawed samples were the same as described for fresh semen evaluation.

4. Statistical analyses

Basal seminal data for fishing cats were expressed as the mean ± S.E.M. Variation in sperm motility and acrosomal integrity for each sperm-processing step were assessed using a two-way ANOVA with differences between treatment determined using Tukey's means comparison (NCSS/PASS 2000; Dawson Edition, Kaysville, UT, USA).

Experiment 3: Preliminary Study on Laparoscopic Artificial Insemination in the Fishing Cat (*Prionailurus viverrinus*)

1. Follicle stimulation and ovulation induction

Four adult female fishing cats were used in this study to evaluate the efficacy of exogenous hormones, Pregnant Mare Serum Gonadotrophin (PMSG) and human Chorionic Gonadotrophin (hCG), for follicle stimulation and ovulation induction, respectively. To evaluate the efficacy of using high and low dosage of exogenous hormone, animal were divided into two groups (two fishing cats/group) which group I received 400 IU of PMSG (Folligon®, Intervet Schering-Plough Animal Health Ltd.) by intramuscular injection (I/M) followed with I/M 200 IU of hCG (Chorulon®, MSD Animal Health) 96-h post PMSG injection and group II received 200 IU of PMSG (Folligon®, Intervet Schering-Plough Animal Health Ltd.) by intramuscular injection (I/M) followed with I/M 100 IU of hCG (Chorulon®, MSD Animal Health) 96-h post PMSG injection

Timing of artificial insemination was also studied, two fishing cats which one received high dosage and another one with low dosage of exogenous hormone were anesthetized, ovaries were examined and intrauterine insemination was performed under laparoscopy at 41 h post-hCG. The remaining two fishing cats which one received high dosage and another one with low dosage of exogenous hormone were anesthetized, ovaries were examined and intrauterine insemination was performed under laparoscopy at 35 h post-hCG.

2. Ovarian examination and intrauterine insemination

Ovarian examination and intrauterine insemination were performed under laparoscopic vision. Female fishing cats were anesthetized with an intramuscular injection of xylazine-HCl (Ilium Xylaxil; Troy Laboratories; 0.25-0.5 mg kg^{-1}) and zolazepam + tilatamine (Zoletil®, Virbac Co. Ltd.; 5-7 mg kg^{-1}) maintained anesthetic condition with isoflurane inhalant anesthesia (Aerance; Baxter Health Care, Deerfield, IL, USA; 1-3%, v/v). Laparoscopic assessment of ovaries and intrauterine insemination were performed as described by Howard *et al*. (1992). Briefly, animal was laid back side on surgical table which head side tilted down about 30-45^{o} from horizontal plane. A pneumoperitoneum was created through a Verres needle (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany) attached to an automatic insufflators containing 100 CO_2. A 7 mm diameter trocar cannula (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany) was inserted through a 1 cm skin incision made 3-5 cm cranial to the umbilicus. The trocar was removed and replaced with a rigid, 7 mm diameter laparoscope (KARL STORZ GmbH & Co.KG, Tuttlingen, Germany) attached to a light source. All aspect of each ovary were examined for pre-ovulatory follicles and post-ovalatory corpora lutea. We use same criteria as in domestic cat to assess ovaries of fishing cat in this study. Pre-ovulatory vesicular follicles were clear in appearance, spherical in shape and generally flattened or only slightly raised above the ovarian surface. In contrast, the early developing post-ovulatory corpora lutea were opaque, dark red and prominently raised 2-3 mm above the ovarian surface. Cats demonstrating one or more pre-ovulatory follicles and no corpora lutea were classified as pre-ovulatory, whereas, females exhibiting at least one corpus luteum were classified as post-ovulatory.

For intrauterine insemination, an accessory forceps was inserted 3-5 cm lateral to the umbilicus and used for elevation of each uterine horn to the ventral body wall. The horn was cannulated with catheter inserted percutaneously into the proximal third of the lumen. The stylette was removed, polyethylene tubing (intradermic syringe) containing processed semen was inserted through the catheter into the uterine lumen and the semen expelled. After depositing semen into the contralateral horn, the catheter, tubing and

laparoscopic instruments were removed and the incision site sutured. In case that laparoscopic AI could not perform successfully, laparotomy was conducted to allow directly intrauterine deposit semen using catheter connected with intradermic syring. Blood samples were collected from all four recipients at time of laparoscopy for serum estradiol and progesterone level measurement.

3. Semen collection and processing for AI

Four male fishing cats were anesthetized using same protocol as for experimental 2. Each cat was designed to be donor for specific female recipient in order to increase genetic heterogeneity. Just before laparoscopy in female, semen collection was performed using same protocol as in experimental 2. Raw semen was added with 50 µl of TEST-egg yolk without glycerol to maintain sperm quality. Diluted semen was assessed for sperm motility and concentration. Processed semen was maintained at room temperature (25^{o}C) in darkness. Each uterine horn was inseminated with 100-200 µl of processed semen.

RESULTS AND DISCUSSION

Results

1. Results of experimental 1

Semen was collected successfully in all five cats using an artificial vagina and an estrus female cat for male cat mounting. Sperm motility could be evaluated objectively using light microscope. Sperm viability could be evaluated easily by LIVE/DEAD sperm viability kit and determined by fluorescent microscope (Figure 4). Coomassie staining proved to be an effective method for assessing acrosomal integrity in domestic cat. In fresh semen, the percentage of spermatozoa motility ranged from 70 to 95 (81 ± 2.6), and semen processing before cryopreservation had a negligible effect ($p > 0.05$) on motility. However, spermatozoa motility, viability and acrosomal integrity decreased significantly ($p < 0.05$) when semen diluted in EYT-FC with 7% glycerol was cryopreserved. However, there was no significant difference between the control and the two treatment groups ($p > 0.05$; Figure 5-7). The average percentage after thawing compared between control, treatment 1 and treatment 2 for motility were: 43.5 ± 3.2, 42 ± 4.1 and 38 ± 4.5; for viability: 44.8 ± 3.5, 50.6 ± 5.1 and 47.1 ± 4.1 and for acrosomal integrity: 45 ± 3.5, 44.9 ± 3.4 and 44.4 ± 3.3.

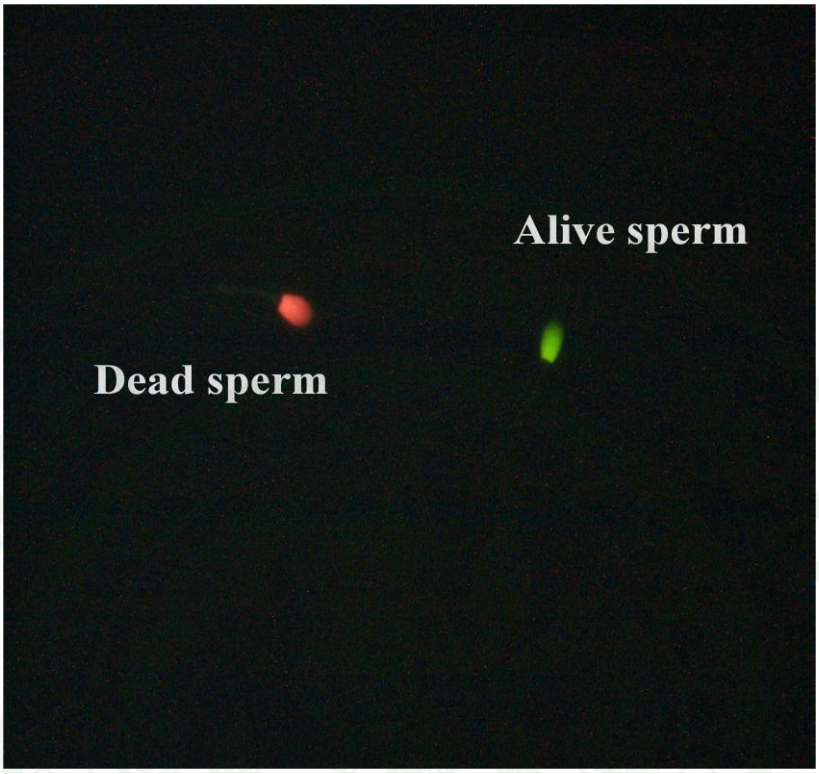

Figure 4 Viability of domestic cat spermatozoa using LIVE/DEAD® Sperm Viability Kit

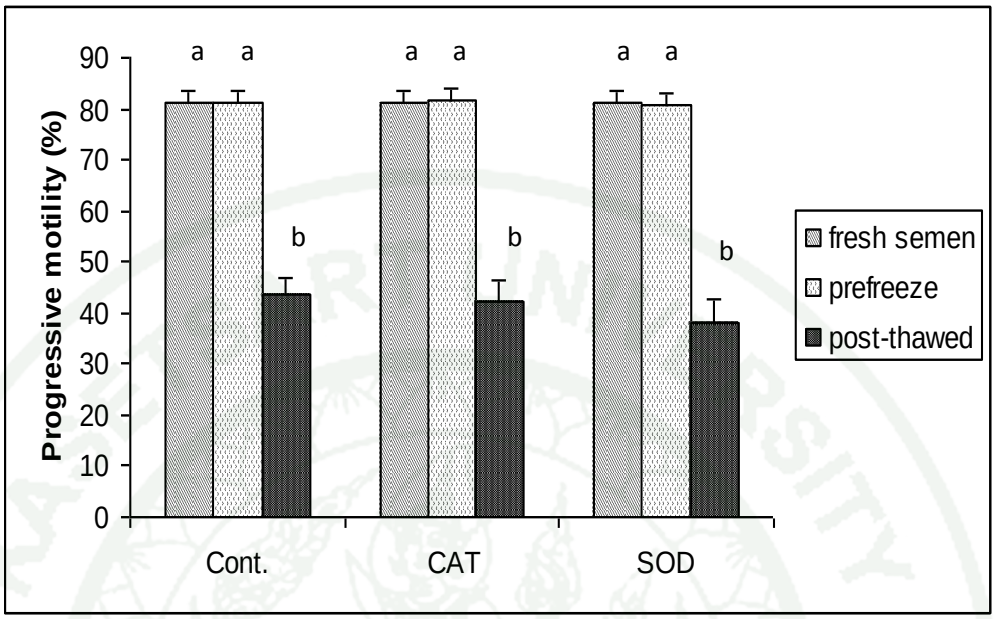

Figure 5 Progressive motility of domestic cat spermatozoa before freezing and after thawing. Cont. = control, CAT = treatment group 1 (freezing extender supplement with catalase), SOD = treatment group 2 (freezing extender supplemented with superoxide dismutase). [a,b]Different indices indicate significant differences within groups. There were no significant difference between group ($p > 0.05$).

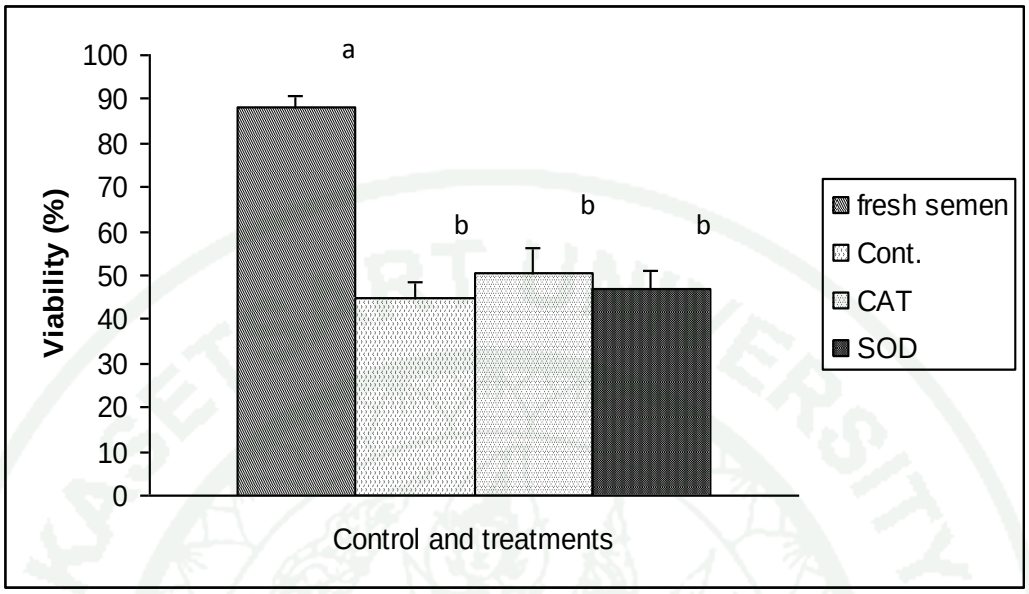

Figure 6 Viability of domestic cat spermatozoa before freezing and after thawing. Cont. = control, CAT = treatment group 1 (freezing extender supplement with catalase), SOD = treatment group 2 (freezing extender supplemented with superoxide dismutase). [a,b]Different indices indicate significant differences within groups. Thawing decreased viability significantly in all groups ($p < 0.05$); however, there were no significant difference between groups ($p > 0.05$).

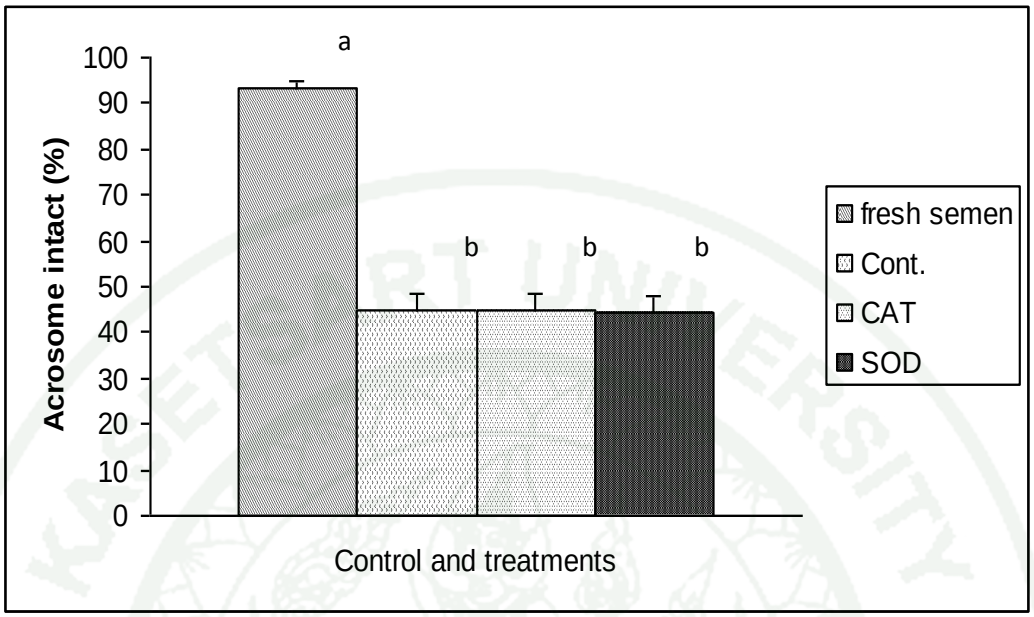

Figure 7 Acrosomal integrity of domestic cat spermatozoa before freezing and after thawing. CAT = treatment group 1 (freezing extender supplement with catalase), SOD = treatment group 2 (freezing extender supplemented with superoxide dismutase). [a,b]Different indices indicate significant differences within groups. Thawing decreased acrosomal integrity significantly in all groups ($p < 0.05$); however, there were no significant difference between groups ($p > 0.05$).

2. Results of experimental 2

All six male fishing cats in this study were each anesthetized safely for electroejaculation and recovered uneventfully (Figure 8). Each male possessed normal penile morphology with prominent keratinized spines present on the glans penis (Figure 9). Semen was collected successfully once a month for two months from five male fishing cats. Urine contamination occurred in one collection from one cat. However, the non-contaminated ejaculate (after one month interval) from same cat was used in this study. The injectible anesthetic regimen and maintained with low isoflurane inhalation provided a good anesthetic status enough for semen collection. Sperm ejaculates were recovered from all six males, sperm quantity and quality were variable among individuals. Mean (± S.E.M.) values for seminal trait are presented in Table 2. Males averaged 90.14 % morphologically normal spermatozoa, with primary and secondary abnormality at 4.5 ± 0.64 % and 5.36 ± 1.19 %, respectively. Sperm motility ranged from 80 to 95 (91 ± 1.5). Staining with Coomassie blue is convenient and proved to be an effective method for assessing sperm acrosomal status in the fishing cat (Figure 10). Dilution of raw semen with TEST-yolk buffer, centrifugation and slow cooling to $5^{o}C$ had a significant negative effect ($p < 0.05$) on sperm motility. Immediately after sperm thawing, sperm motility was reduced significantly ($p < 0.05$) from values observed in raw and pre-freez semen (Figure 11). The percentage of intact acrosome decreased ($p < 0.05$) immediately after thawing compared with raw and pre-freez semen (Figure 12). Comparing the adding 400 IU of CAT, 10 IU of GxP, 5 M of Trolox and no adding antioxidants in extender, sperm motility and acrosomal intact did not differ ($p > 0.05$) between control and all treatment either pre-freez and immediately post-thaw.

Figure 8 Fishing cat was anesthetized for semen collection.

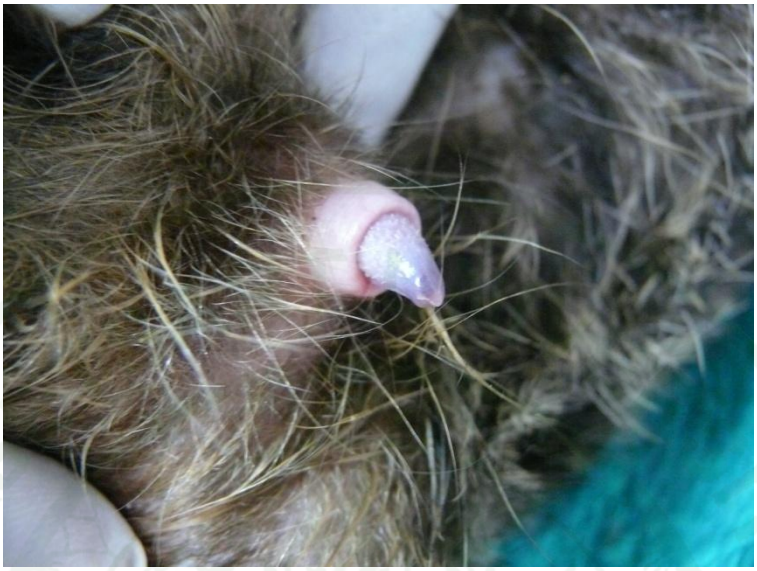

Figure 9 Presence of keratinized spines on the glans penis of a fishing cat.

Table 2 Seminal trait of 11 ejaculates collected from six male fishing cats (two ejaculates/cat + one ejaculate/cat) at Chiang Mai Night Safari, Thailand.

Parameter	**Mean (± S.E.M.)**	**Range**
Semen volume (ml)	0.30 ± 0.05	0.17 – 0.77
Semen pH	8.36 ± 0.07	8.00 – 8.50
Sperm concentration (x 10^6 ml^{-1})	419.00 ± 74.81	164 – 920
Total sperm (x 10^6)	118.31 ± 23.18	46.25 – 266.80
Sperm motility (%)	91.82 ± 1.55	80.00 – 95.00
Morphological normal sperm (%)	90.14 ± 1.40	80.00 – 95.00
Morphological abnormal sperm (%)	9.86 ± 1.40	5.00 – 20.00
• Primary abnormalities	4.50 ± 0.64	1.00 – 8.50
• Secondary abnormalities	5.36 ± 1.19	2.50 – 16.00
Intact acrosome (%)	93.18 ± 1.28	83.00 - 98.00
Total testicular volume (cm^3)	8.27 ± 0.48	6.45 – 10.71
Body weight (kg)	13.00 ± 0.58	10.00 – 15.55

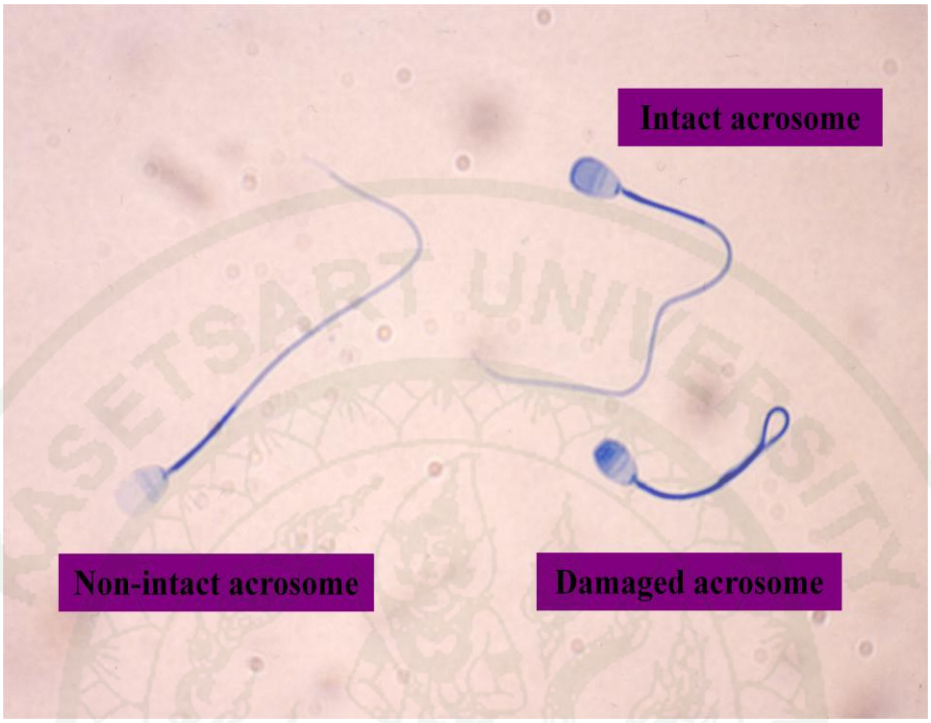

Figure 10 Acrosomal integrity of fishing cat spermatozoa using Coomassie blue stain.

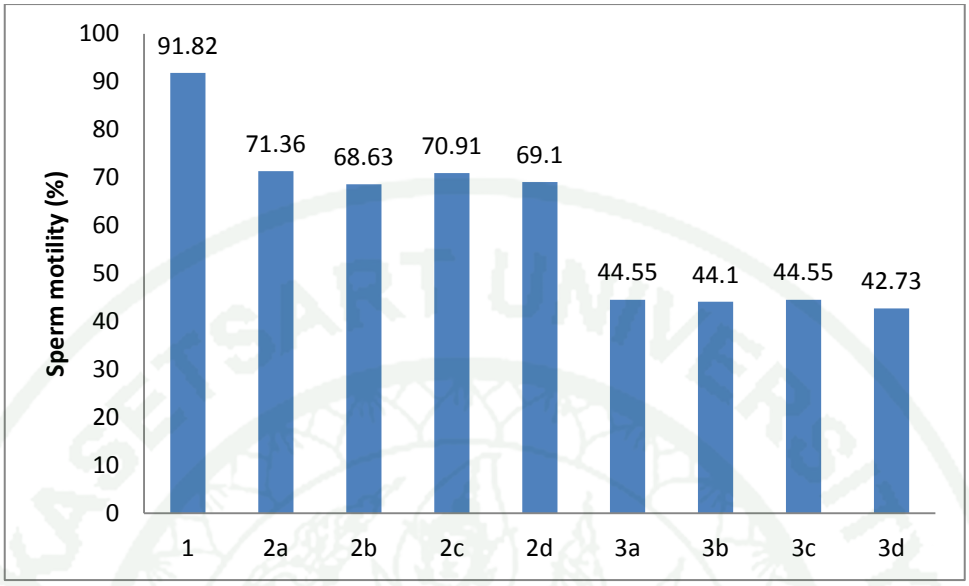

Figure 11 Sperm motility of fishing cat (n = 11 ejaculates) following procedural step in sperm processing and freezing: Step 1, raw semen (1); Step 2, fresh sperm in TEST-egg yolk buffer with no antioxidant (2a), CAT (2b), GPx (2c), vitamin E (2d) cooled to 5^{o}C (2a); Step 3, immediate post-thaw sperm in TEST-egg yolk buffer with no antioxidant (3a), CAT (3b), GPx (3c), vitamin E (3d).

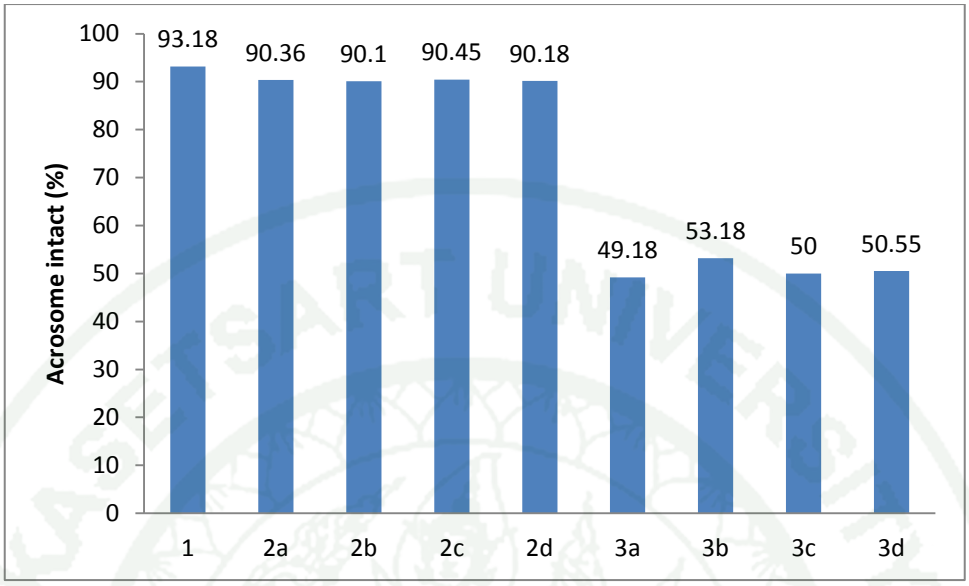

Figure 12 Sperm acrosomal integrity of fishing cat (*n* = 11 ejaculates) following procedural step in sperm processing and freezing: Step 1, raw semen (1); Step 2, fresh sperm in TEST-egg yolk buffer with no antioxidant (2a), CAT (2b), GPx (2c), vitamin E (2d) cooled to 5°C (2a); Step 3, immediate post-thaw sperm in TEST-egg yolk buffer with no antioxidant (3a), CAT (3b), GPx (3c), vitamin E (3d).

3. Result of experiment 3

After administration of high (400 IU of PMSG; 200 IU of hCG) and low dosage (200 IU of PMSG; 100 IU of hCG), No behavior change was observed in all four female fishing cat. However, the observation in this study was based on human observation and only during day time.

Laparoscopic assessment of ovaries was performed in all cats before intrauterine insemination of fresh sperm (Figure 13-14). Four to five pre-ovulatory follicles were found at ovaries of cats at 41 h post-hCG in both high and low dosage of exogenous hormone administered groups. At 35 h post-hCG, no follicles were found in both high and low dosage of exogenous hormone administered groups (Table 3).

Semen was collect successfully from three male donor cats. Unfortunately, urine contamination occurred in one from four donors and such semen could not use for AI. Approximately 50 to 100 x 10^6 total motile sperm, 0.2-0.4 ml in semen volume were inseminated in uterine horns of each recipient female cat.

Laparoscopic intrauterine insemination was successful performed in two cats (Figure 15). One cat could not inseminate semen via laparoscopy because of the difficulty to find the uterine horn under laparoscopic vision then laparotomy intrauterine insemination was conducted instead. One cat did not be inseminated due to the absence of follicle/corpora lutea on both side ovaries and the lack of enough semen from donor.

After AI/ovarian assessment, all cats recovered safely from anesthetic condition. Pregnancy diagnosis by ultrasound or x-ray did not perform due to the concern of stress and effect of anesthetic agents to, probably, pregnant cats. No inseminated cat was pregnant according to no pregnancy/parturition clinical sign was observed continuously 2 months post AI.

Table 3 Follicular stimulation, ovulation induction and laparoscopic/laparotomy intrauterine artificial insemination (AI) in fishing cat.

Fishing cat No.	Gonadocropin dosages		Time of AI post-hCG (h)	Type of sperm	No. follicle	No. CL	Estrogen level at AI (ng/ml)	Progesterone level at AI (ng/ml)	Pregnancy result
	PMSG (IU)	hCG (IU)							
2964	400	200	41.5	Fresh	5	0	0.10	<0.2	No
2087	400	200	35	Fresh	0	0	0.13	<0.2	No
3852	200	100	41.5	Fresh	4	0	0.07	<0.2	No
2271	200	100	34	-	0	0	0.08	7.3	-

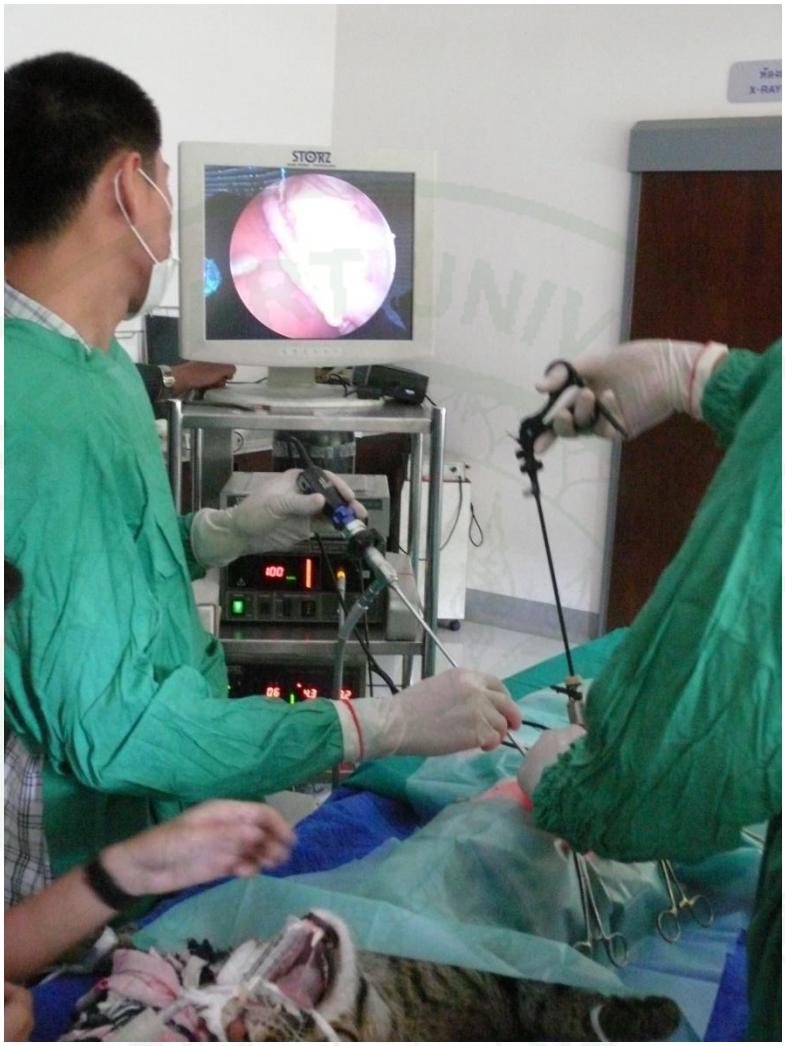

Figure 13 Ovaries assessment using laparoscopy in fishing cat.

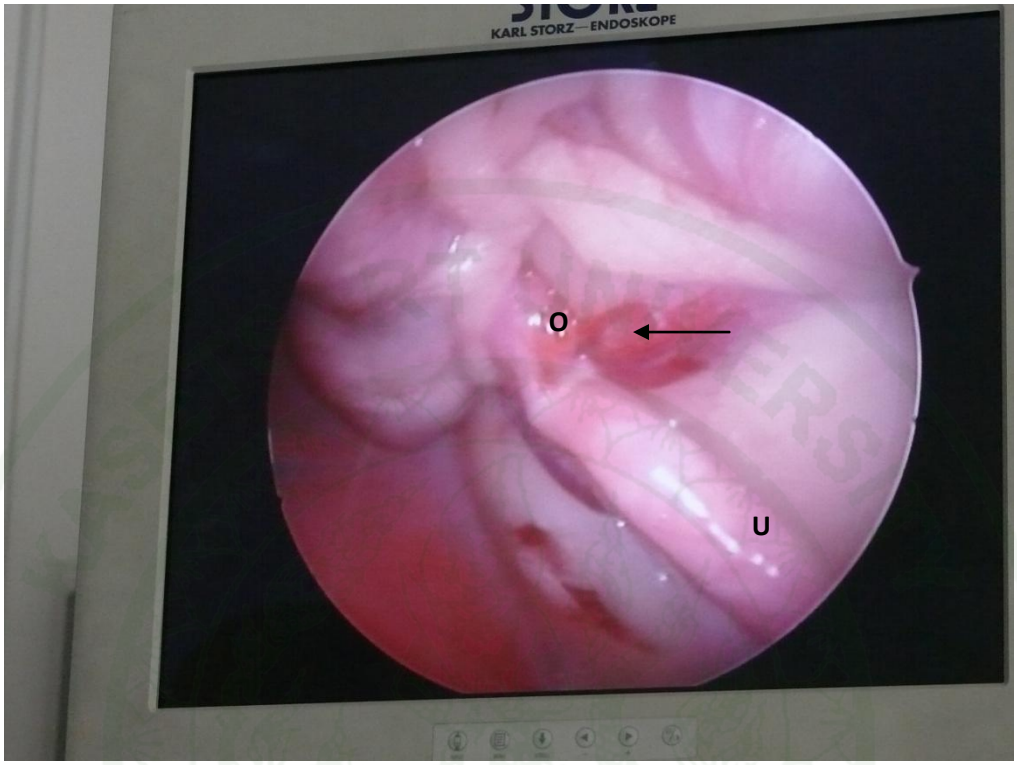

Figure 14 Fishing cat ovary (O) containing follicle (arrow) and uterine horn (U) after 400 IU PMSG/200 IU hCG administration.

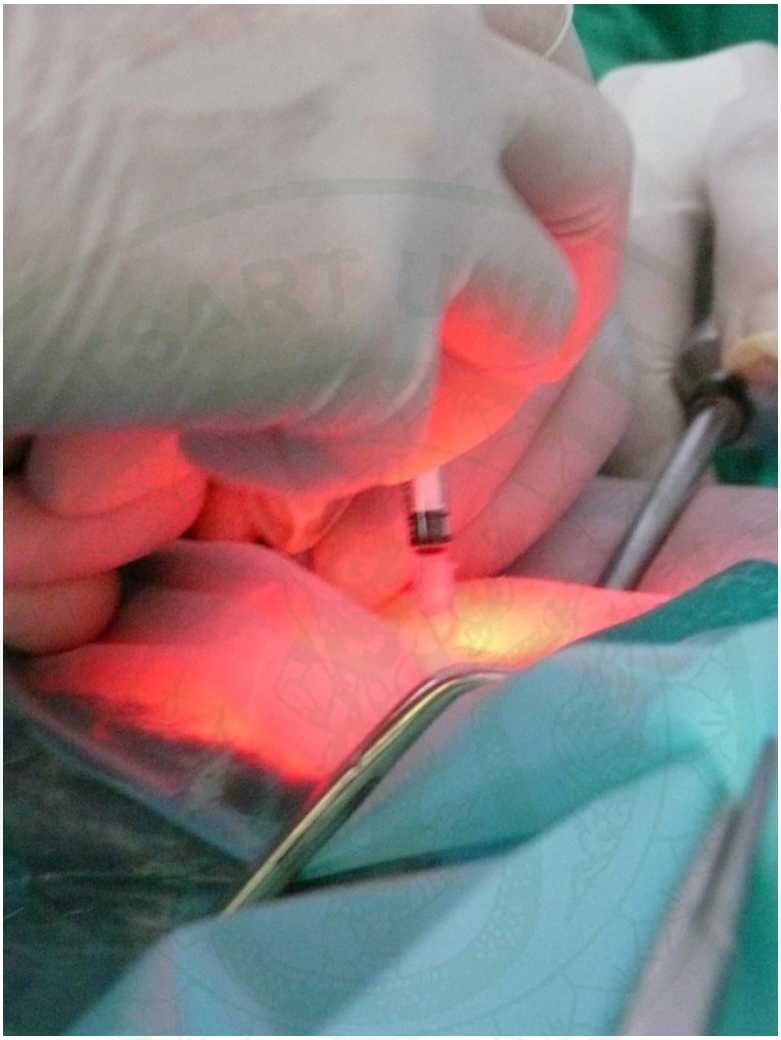

Figure 15 Semen contained in tuberculin syring was inseminated percutaneously into lumen of uterine horn.

Discussion

The domestic cat is a valuable model for conservation biology studies in wild felids because many species of wild felids are at risk of extinction and number in captivity is also limited. In this thesis study, effect of antioxidant CAT and SOD on frozen-thawed sperm quality was studied in *experimental 1* as a model for the study in fishing cat in *experimental 2*. In order to maximize the benefit of cryopreserved semen, these fragile gametes should introduce to female recipients by artificial insemination for the production of heterogeneity offspring. Therefore, the preliminary study of artificial insemination in fishing cat was performed in *experimental 3*.

Sperm cryopreservation is an important assisted reproductive technique (ART) for cats and wild felids, as it is a valuable tool for creating effective semen banking. However, it is well established that sperm motility, viability and acrosomal integrity are greatly affected by the freezing-thawing procedure. For cryopreservation, raw semen must be processed through a series of successive steps designed to removal seminal fluid, concentrate spermatozoa, resuspend the sample in a cryoprotectant solution and then cool and package the spermatozoa for freezing. Thermal and osmotic shock associated with sperm processing can damage acrosomal and plasma membranes and the actual freezing and thawing process can produce additional osmotic and thermal stress (Holt, 2000; Watson, 2000). In domestic cats, rapid cooling of spermatozoa to 5^{o}C produces significant acrosomal damage (Pukazhenthi *et al*., 1999). Similarly, osmotic stressors, such as rapid dilution in cryoprotectant or hypotonic medium, can result in reduced sperm motility (Glover and Watson, 1985; Pukazhenthi *et al*., 2002). The present findings indicate that fishing cat spermatozoa are fairly to poor resilient to osmotic and temperature stress when samples were cooled slowly and glycerol is added in multiple steps, as recommended for domestic cat spermatozoa. Different from our previous study which sperm processing has negligible effect on sperm motility and acrosomal integrity (Thiangtum *et al*., 2006), this study found the significant decreased of sperm motility after fishing cat sperm was processed, cooled and added cryoprotectant. The reason was

unclear but one possibility was the fluctuation of temperature in refrigerator used in present study. This may caused negative impact to cooled semen.

Generally, frozen-thawed sperm motility has been reported to decrease by 20-50% and acrosomal abnormalities increase by 15-30% (Luvoni, 2006). Similarly, frozen-thawed cat sperm motility and acrosomal integrity in this study decreased by 46% and 52%, respectively. For fishing cat, similar results were found in frozen-thawed sperm which motility decreased about 50% from raw semen. These results support previous studies stating that sperm processing and cryopreservation have a negative impact on frozen-thawed semen quality.

The CAT, SOD and GPx are enzymatic antioxidants which can be found in variety of aerobic cells (Fridovich, 1978). These antioxidants will convert superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to form O_2 and H_2O (Hugo, 1974). Including spermatozoa, these enzymes will control the balance between ROS production and neutralization (Lenzi *et al*., 2002). The amount of each enzyme which present in both seminal plasma and spermatozoa is different among species. Moreover, seasonal variation of theses antioxidant activity has been report in ram (Marti *et al*., 2007), boar (Koziorowska-Gilum *et al*., 2011) and bull (Asadpour *et al*., 2012). Besides of enzymatic antioxidants, the non-enzymatic antioxidant such as vitamin E also acted to inhibit the initiation of the chain reaction, break the chain propagationstage in the lipid peroxidation reaction and eliminate other ROS (Nogushi and Niki, 1999) In order to minimize the oxidative stress consequently from the increasing ROS level during sperm processing and cryopreserve, adding antioxidants into semen extender have tested. Various antioxidants, such as vitamin E, vitamin C, taurine, acetylcysteine, CAT and SOD, have been tested in attempt to prevent oxidative stress in semen from a variety of species with controversial efficacy. In domestic cat, the antioxidants taurine, vitamin E and cysteine have been reported to have a positive effect on quality of frozen-thawed spermatozoa (Luvoni *et al*., 2002; Thuwanut *et al*., 2008). Moreover, GPx improved the quality of frozen-thawed sperm in flat-headed cats (Thuwanut *et al*., 2011). The results from experimental 1 and experimental 2 in this study showed that adding CAT or SOD to an EYT-FC extender did

not improve the quality of frozen-thawed cat spermatozoa. Similarly, adding CAT, GPx or vitamin E analogue Trolox to TEST-egg yolk buffer extender did not improve the quality of frozen-thawed fishing cat spermatozoa.

Although the exact causes for this are unclear, proposed possible reasons are: *first*, endogenous antioxidant activities were high enough to neutralize exceeding ROS concentrations during semen processing and cryopreservation. Evidence to support this hypothesis is given by the finding of the antioxidant taurine in spermatozoa, seminal plasma and epididymal fluid of domestic cat (Buff *et al*., 2001). *Second*, the lipid peroxidation of spermatozoa membranes may not significantly increase during cat and fishing cat semen processing, cryopreservation and thawing which is similar to the finding in frozen-thawed epididymal cat spermatozoa (Thuwanut *et al*., 2010). *Third*, dosage of antioxidants used in this study might be improper concentration. In case of vitamin E, the protective effects against lipid peroxidation in boar spermatozoa depended on the concentration of the antioxidant supplement added to the semen extender (Breininger *et al*., 2005). *Lastly*, all possible reasons described above may concurrently occur during cryopreservation and thawing of domestic cat and fishing cat semen. Further studies are needed to confirm these hypotheses.

Successful, producing offspring, of laparoscopic intrauterine AI was report in 8 wild felids species including cheetah (Howard *et al*., 1992, 1997), clouded leopard (Howard *et al*., 1997), tiger (Donoghue *et al*., 1993), puma (Barone *et al*., 1994), leopard cat (Wildt *et al*., 1992), snow leopard (Roth *et al*., 1997), tigrina (Swanson and Brown, 2004) and ocelot.(Swanson *et al*., 1996) Pregnancy rate about 45% was reported only in cheetah whereas low rate (< 5%) remained in clouded leopard and tiger. Multiple attempts have failed in the fishing cat (Bauer *et al*., 2004). This present study is one more attempt to apply this technique to produce heterogeneity offspring in Thai fishing cat. Although, pregnancy was not found in this study but, at least, we can confirm the useful of standard laparoscopy technique for ovarian assessment and to deposit semen intrauterine horn in the fishing cat. The exact cause of pregnancy failure after AI has not been established, one contributor appears to be inconsistent ovarian response after

exogenous gonadotrophin are used to stimulate the ovary before insemination (Howard and Wildt, 2009). Results from this study showed the present of pre-ovulatory follicles in two fishing cat (50%) assessed by laparoscopy. However, due to very limited information in fishing cat about ovarian structure when it was stimulated by exogenous hormone or even during normal estrus cycle, we could not compare with others studies to classify the quality of pre-ovalatory follicles found in this study. The contrast between low level of estradiol and pre-ovulatory found in fishing cat no.2964 and no.3852 was unclear. The pre-ovulatory follicles found in this study might be an immature follicle resulted from less respond to exogenous hormone. This hypothesis will contrast with the results from fecal hormone study by Santymire *et al*. (2011) who suggested that fishing cat was hypersensitive to exogenous gonadotrophins. Further study needed to confirm in more fishing cat samples.

Study by analysis of fecal hormone indicated that fishing cat has spontaneous ovulation approximately 13% and no evidence of seasonal breeding (Santymire *et al*., 2011). Therefore, when follicular stimulation was conducted, it has possibility of given exogenous hormone to non quiescent ovary cat. From this reason, variable respond to exogenous hormone might be found. Similarly, our present study in four female fishing cats found, however, the less and variable respond to eCG/hCG administration. These cats might be not in quiescent period of ovary when eCG/hCG was introduced. To diminish the effect of unpredictable stage of ovarian cycle, pre-treatment with exogenous progestagen to temporarily suppress estrus cycle and attenuate ovulation was helpful method and given a better results compare with conventional procedures (Pelican *et al*., 2006). This protocol should be applied in fishing cat to stimulate follicle and induce ovulation for future study.

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this thesis study, the conclusion can be drawn as follow:

1. Approximately 50% of motility decreased when domestic cat and fishing cat sperm were processed and cryopreserved. This finding strength information that sperm processing and cryopreservation had deteriorated effect on frozen-thawed sperm quality.

2. The addition of CAT or SOD at dosage of 200 IU/ml to an EYT-FC extender did not have a positive effect on motility, viability and acrosomal integrity of frozen-thawed domestic cat spermatozoa.

3. Semen from fishing cats can collect successfully using standard electroejaculation procedure. The quality of semen was good compare with North America fishing cat. High percentage of morphological normal sperm (> 80%) was found in this study. This might reflect the high level of gene diversity among Thai fishing cat.

4. The addition of 400 IU/ml of CAT or 10 IU/ml of GPx or 5 mM of vitamin E in TEST-egg yolk extender did not have a positive effect on frozen-thawed fishing cat sperm motility and acrosomal integrity.

5. Exogenous gonadotrophin (PMSG) and humen chorionic gonadotrophin (hCG) hormone, can, however, stimulate follicle in fishing cat with variable results. The low dosage at 200 IU PMSG followed with 100 IU hCG and the high dosage at 400 IU PMSG followed with 200 IU hCG did not show different results for follicular stimulation in the fishing cat.

6. Ovarian assessment in hormone stimulated fishing cat could perform successfully by laparoscopy. Artificial insemination in fishing cat could benefit from using laparoscopy which semen was deposited intraluminal horn of uterus under vision of 7 mm telescope.

Recommendation

This study evaluated the effect of antioxidants both enzymatic (CAT, SOD, GPx) and non-enzymatic (vitamin E) on quality of frozen thawed fishing cat spermatozoa started with the study in domestic cat as a model. Base on the hypothesis that the process for semen cryopreservation lead to oxidative stress and antioxidant supplement may help to protect sperm from this harmful effect. Results from this study indicated that adding antioxidants to semen extender did not help to improve post thawed sperm quality. Apart from antioxidant, many factors involve in sperm quality after cryopreservation should be studied. Importantly, cryopreserved sperm should be used for artificial insemination to produce fishing cat offspring. Preliminary study of artificial insemination in this study marked an important requirement for this ART that is the understanding of normal reproductive hormone cycle and appropriate using of exogenous hormone to stimulate follicle and induce ovulation successfully in this species. In order to move forward the *ex situ* conservation of fishing cat, further research studies are recommended as follow;

1. Measurement the level of lipid peroxidation and oxidative stress during sperm were processed and cryopreserved in fishing cat. Natural activity of antioxidant in fishing cat semen should be addressed.

2. Sperm processing and cryopreservation procedure in fishing cat should be developed for more effective in field work.

3. Follicle stimulation and ovulation induction in this species needed an urgent study in order to provide an important information for artificial insemination success.

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