

Role of Oxidative Stress and Antioxidants in Domestic and Non- Domestic Cat Spermatozoa

With special reference to cryopreservation

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Cover: “The cats” by my beloved persons

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Abstract

Living cells, including spermatozoa, stored under aerobic conditions require oxygen to support their normal metabolism. Excessive levels of metabolites (reactive oxygen species; ROS) can cause cell damage or lipid peroxidation. The ROS and lipid peroxidation can be detected during preparation and cryopreservation of mammalian spermatozoa. The negative effects of ROS can be partially ameliorated by the action of antioxidants. The aims of this thesis were to 1) study the effects of antioxidants on cryopreserved epididymal spermatozoa from the domestic cats and to apply the results from domestic cats to cryopreserved wild felid ejaculated spermatozoa (flat-headed cats; *Prionailurus planiceps*) in Thailand; 2) detect lipid peroxidation and its effects on post-thaw epididymal cat spermatozoa and 3) evaluate basic seminal characteristics of captive flat-headed cats. In the first study, the semen extender was supplemented with two types of non-enzymatic antioxidants (cysteine and a water soluble vitamin E analogue Trolox). Vitamin E had positive effects on post-thaw sperm motility, progressive motility and membrane integrity. In the second study, centrifuged post-thaw epididymal cat spermatozoa were resuspended in media with or without egg yolk and with or without a transition metal (Fe^{2+}) (which induces lipid peroxidation). Lipid peroxidation was measured by using a lipophilic dye probe (BODIPY^{581/591} C11). The results demonstrated that BODIPY^{581/591} C11 could be used to detect lipid peroxidation in cat spermatozoa. This reaction did not significantly increase during incubation unless induced by Fe^{2+} . In the third study, enzymatic antioxidants (catalase, glutathione peroxidase; GPx and superoxide dismutase) were added to the semen extender. Lipid peroxidation was induced after thawing. Supplementation of GPx resulted in positive effects on post-thaw motility, linear motility, mitochondrial activity, membrane and DNA integrity. Although antioxidants had positive effects on sperm quality, the concentration of antioxidants used in this study did not reduce lipid peroxidation. In the last study, the basic seminal traits of captive flat-headed cats were evaluated, as were the effects of adding the vitamin E and GPx to the semen extender for flat-headed cat spermatozoa. The results demonstrated that the captive flat-headed cats were affected by teratozoospermia. The GPx had positive effects on the post-thaw quality of flat-headed cat spermatozoa in a similar manner to domestic cat sperm quality but it did not significantly affect *in vitro* heterologous fertility tests. In conclusion, oxidative stress might be one of the factors causing damage during preservation of domestic and non-domestic cat spermatozoa. Post-thaw sperm quality was enhanced by the action of antioxidants.

Keywords: Feline, Sperm cryopreservation, Oxidative stress, Antioxidant

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Dedication

To my family and teachers

นัยของคำว่าขอบคุณ...

ขอบคุณความไม่มี ที่ทำให้รู้วิถีลูกชิ้นสู้
ขอบคุณความไม่รู้ ที่ทำให้รู้จักครูที่ชื่อประสบการณ์
ขอบคุณความคิดพลาด ที่ทำให้ฉลาดยิ่งกว่าเดิม
ขอบคุณความคิดหวัง ที่ทำให้ตั้งสติเพื่อลุกขึ้นใหม่
ขอบคุณความล้มเหลว ที่ทำให้เกิดความเชื่อขงชาญ
ขอบคุณศัตรูที่แกร่งกล้า ที่ทำให้รู้ว่าเรายังไม่ใช่มี้ออาชีพ
ขอบคุณคำวิพากษ์วิจารณ์ ที่ทำให้ผลิบานอย่างไร้ข้อตำหนิ

ว.จิริเมธี (W.Vajiramedhi)

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I. Thuwanut P, Chatdarong K, Techakumphu M and Axnér E. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. *Theriogenology* 70(2): 233-240, 2008.
- II. Thuwanut P, Axnér E, Johannisson A and Chatdarong K. Detection of lipid peroxidation reaction in frozen-thawed epididymal cat spermatozoa by using BODIPY^{581/591} C11. *Reprod Domest Anim Suppl* 2: 373-376, 2009.
- III. Thuwanut P, Chatdarong K, Johannisson A, Bergqvist AS, Söderquist L and Axnér E. Cryopreservation of epididymal cat spermatozoa: effects of *in vitro* antioxidative enzymes supplementation and lipid peroxidation induction. *Theriogenology* 73(8): 1076-1087, 2010.
- IV. Thuwanut P, Chatdarong K, Bergqvist AS, Söderquist L, Thiangtum K, Tongthainan D and Axnér E. The effects of antioxidants on semen traits and *in vitro* fertilizing ability of spermatozoa from the flat-headed cat (*Prionailurus planiceps*). (Manuscript submitted for publication).

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Abbreviations

AI	Artificial insemination
AO	Acridine orange
ALH	Lateral head displacement
ARTs	Assisted reproductive techniques
BODIPY ^{581/591} C11	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid
CASA	Computer-assisted sperm analysis
CAT	Catalase
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
ET	Embryo transfer
Fe ²⁺	Ferrous ion
GPx	Glutathione peroxidase
GPx4	Glutathione peroxidase protein type 4
GPx/GRD	Glutathione peroxidase/reductase pair
GSH	Glutathione
GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
IVC	<i>In vitro</i> culture
IVM	<i>In vitro</i> maturation
IVF	<i>In vitro</i> fertilization
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide
LIN	Linear motile
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
O ₂	Oxygen
O ₂ ⁻	Superoxide radicals
OH [·]	Hydroxyl radicals

PBS	Phosphate buffered saline
PI	Propidium iodide
PROH	1,2-propanediol
PUFA	Polyunsaturated fatty acid
RO·	Alkoxy radicals
ROO·	Lipid peroxy radicals
ROOH	Lipid hydroperoxides
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
VAP	Average path velocity
VSL	Straight line velocity
X-XO	Xanthine-xanthine oxidase

1 Introduction

Of the 40 species of non-domestic cats in the world, nine live in Thailand. These include the leopard cat (*Prionailurus bengalensis*), fishing cat (*Prionailurus viverrinus*), flat-headed cat (*Prionailurus planiceps*), asiatic golden cat (*Captopuma temminckii*), leopard (*Panthera pardus*), jungle cat (*Felis chaus*), marbled cat (*Pardofelis marmorata*), clouded leopard (*Neofelis nebulosa*), and indochinese tiger (*Panthera tigris*). Several felid species or subspecies are classified as threatened or endangered due to poaching or habitat loss. Non-domestic cats are often affected by teratozoospermia (> 60% of abnormal sperm morphology such as bent mid-piece or coiled tail) which might impair fertility (Luvoni, 2006; Pukazhenti *et al.*, 2006). Because of the difficulties in captive breeding of wild cats and an increasing interest in breeding of pedigree cats, protocols for cat sperm preservation and gene banking for conservation are being developed and established. The domestic cat (*Felis silvestris catus*) has proved to be a suitable model for development of assisted reproductive techniques (ARTs) (Donoghue *et al.*, 1992) such as semen preservation or artificial insemination (AI). Reproductive biotechnologies might also have an application in the domestic cat, since there is an increasing interest in acquiring pure-bred domestic cats as pets. Pure-bred cats are bred in isolated populations. Most pure-bred males kept as pets are castrated when young to decrease behavioral problems such as urine spraying. This contributes further to a tendency for decreasing genetic variation.

Conservation is urgently required for the flat-headed cat (*Prionailurus planiceps*), since it is one of the nine endangered felid species in Thailand included on Appendix I of the Convention on International Trade in Endangered Species. The flat-headed cat is a wild cat with a long, sloping snout and flattened skull roof (Hearn *et al.*, 2010). The population of this species is distributed around wetlands or swamp areas in the lowland forest of Indonesia, Malaysia and the extreme southern part of Thailand (Hearn *et al.*, 2010). The population size has been predicted to decline continuously by at least 20% over the next 12 years due to the destruction of the forest for

human settlement or draining for agriculture. Although the exact population size cannot be estimated, it is suspected to be fewer than 2,500 individuals throughout its habitat area (Hearn *et al.*, 2010). In Thailand, only a few individuals are kept in captivity. Hunting of this species is now prohibited by national legislation in Indonesia and Malaysia as well as in Thailand (Norwell & Jackson, 1996). Preservation of gametes, such as spermatozoa, of this endangered species is now urgently needed to aid reproduction and increase the population size.



Figure 1. Captive flat-headed cat (*Prionailurus planiceps*) in Khoa Kheaw Open Zoo, Chonburi, Thailand (Courtesy of Daraka Tongthainan, DVM)

Breeding management programmes using ARTs (including gamete preservation, AI, *in vitro* fertilization (IVF) and embryo transfer (ET)) have been continuously developed in both domestic and non-domestic felids for several decades. Of these techniques, cryopreservation of ejaculated semen and spermatozoa collected from the epididymides could facilitate a genetic exchange over long distances and has been reported to be successful in domestic cats. Success in wild felids is, however, still limited, possibly due to the high proportion of morphologically abnormal spermatozoa seen in at least 28 out of all felid species studied (Pukazhenthii *et al.*, 2006). This phenomenon is suggested to be correlated to the poor fertility results (Howard *et al.*, 1993). In addition, during cryopreservation of spermatozoa, several factors can impair sperm survival and cause sperm damage after cryopreservation, such as cold shock, osmotic stress (Watson, 2000) or oxidative stress (Alvarez & Storey, 1992; Aitken & Krausz, 2001; Sikka, 2001, Agarwal *et al.*, 2003). Cryopreservation-induced sperm damage is

related to reduced sperm fertilizing ability. The effects of cold shock and osmotic stress on spermatozoa have been studied in both domestic and wild felids for decades. Although the post-thaw sperm quality in wild felids has been improved after adjustments in the cryopreservation process, further improvements are still required. Oxidative stress has been identified as a crucial factor for induced mammalian sperm damage but its importance for cat sperm cryopreservation is not known. Studying the effects of oxidative stress in domestic cat sperm cryopreservation might contribute to improved sperm quality in this species as well as being applicable to wild felids species.

1.1 Semen collection and utility of sperm preservation

Although several studies related to the preservation of semen from the domestic cat have been performed, limited progress seems to have been made. One reason for this is probably the small semen volume and low total number of spermatozoa that the domestic cat produces (as shown in Table 1) compared to other species such as the dog, boar or stallion. This can cause difficulties in the experimental design of a research study.

In general, cat semen can be collected by an artificial vagina, by electroejaculation of an anesthetized tomcat and by slicing or squeezing the caudae epididymides post mortem or after castration (Axnér & Linde-Forsberg, 2002). In addition, semen collection by urethral catheterization after medetomidine administration was recently reported (Zambelli *et al.*, 2008). Semen samples collected by electroejaculation, from the epididymides, or urethral catheterization have lower sperm concentration and lower total number of spermatozoa than samples collected by an artificial vagina (as shown in Table 1). Semen collection by the former methods is often more practical than collection by artificial vagina, since male cats do not need to be trained and the presence of an oestrous female cat is not required (Zambelli *et al.*, 2008). Electroejaculation is also a practicable method for semen collection in wild felids. However, collection of epididymal spermatozoa makes it possible to save valuable genetic material from felids that are castrated or that die unexpectedly especially when the testis of wild felids cannot be immediately transported to a laboratory for the cryopreservation process. The fertilizing ability of spermatozoa obtained by the urethral catheterization technique, did not differ from spermatozoa collected by electroejaculation but the semen volume obtained with this technique was relatively low (Table 1), leading to difficulties in study design i.e. the number of treatments or experimental groups (Zambelli *et al.*, 2008). In addition, semen collection by urethral catheterization has not, to the author's knowledge, been reported in wild felid species. This thesis study focused on using epididymal cat spermatozoa

as a model to evaluate sperm damage and to develop a preservation protocol for wild felids including application of the electroejaculation technique in an endangered wild felid, the flat-headed cat.

Cat spermatozoa collected from the epididymides have been reported to have lower initial motility and viability than ejaculated spermatozoa, although there were no significant differences between the two types of spermatozoa in their sensitivity to cooling or post thawing (Tebet *et al.*, 2006; Hermansson & Axné, 2007). Furthermore, cat spermatozoa can be maintained within the epididymides and stored at 4°C for 24 h without any significant decrease in sperm quality compared to spermatozoa in samples extracted from the epididymides immediately and stored in an extender (Chatdarong *et al.*, 2006). Recently our group has shown that epididymal sperm quality was improved by the use of a sperm separation technique, single layer-centrifugation through a colloid (Chatdarong *et al.*, 2010). This technique resulted in a relatively low percentage of abnormal tail defects as well as a low degree of contamination of red blood cell originating from the procedure used at epididymal sperm collection compared to untreated sperm samples (Chatdarong *et al.*, 2010). The protocols for cryopreservation of epididymal sperm samples from the domestic cat differ among laboratories, but post-thaw acrosome integrity has consistently been reported to be low (Hay & Goodrowe, 1993; Lengwinat & Blottner, 1994). The percentage of intact acrosomes decreased about 30-50% after cryopreservation of epididymal sperm samples (Hay & Goodrowe, 1993). The supplementation of Equex STM paste to an egg-yolk Tris extender during the cryopreservation process has been reported to improve the post-thaw acrosome integrity of epididymal cat spermatozoa (Axné *et al.*, 2004). Frozen-thawed cat epididymal spermatozoa have the ability to bind to homologous zona pellucida (Kashiwazaki *et al.*, 2005) and embryo development to the blastocyst stage has been achieved (Thuwanut *et al.*, unpublished data) using spermatozoa cryopreserved by the protocol described by Axné *et al.* (2004). In addition, intrauterine insemination of cryopreserved epididymal cat spermatozoa has been reported to result in offspring (Tsutsui *et al.*, 2003).

Table. 1. Semen parameters in male domestic cats

PARAMETER	SEMEN COLLECTION METHOD			
	Artificial vagina	Electro- ejaculation	Cauda epididymides	Urethral catheterization
Volume (mL)				
• Average	0.04	0.22	*	0.0105 (a)
• Range	0.01 to 0.12	0.019 to 0.74		-
Sperm concentration (10 ⁶ /mL)				
• Average	1730	168 to 361	37.8 to 114.6 (b)	1868.4 (a)
• Range	96 to 5101	-	5.5 to 72	-
Total number of spermatozoa (10 ⁶)				
• Average	61	12 to 33.6 (c)	5.4 to 58.2 (d)	21(a)
• Range	3 to 117	9 to 153	-	-
Sperm morphology (% of normal spermatozoa)	38.2 to more than 90 normal spermatozoa. This average differs between studies probably due to different fixation and classification methods.		49 to 90.5 (e)	91.8 (a)
Motility (%)	56 to 84		50 to 80 (f) Mean;51.9 to 80.8	78.1 (a)
pH	6.6 to 8.8		**	7 (a)
Osmolarity (mOsm/kg)				
• Average	339		**	**
• Range	274 to 390			

This table is modified from Axnér & Linde-Forsberg, 2002

* Volume of cauda epididymal spermatozoa depends on the collected media.

** No data available

(a) Zambelli *et al.*, 2008

(b) Goodrowe & Hay, 1993; Axnér *et al.*, 2004; Thuwanut *et al.*, 2008

(c) Axnér & Linde-Forsberg, 2002; Chatdarong *et al.*, 2007; Zambelli *et al.*, 2008

(d) Cocchia *et al.*, 2009, not shown result from Thuwanut *et al.*, 2008 and Thuwanut *et al.*, 2010

(e) Goodrowe & Hay, 1993; Axnér *et al.*, 2004, Thuwanut *et al.*, 2010

(f) Goodrowe & Hay, 1993; Axnér *et al.*, 2004; Kashiwazaki *et al.*, 2005; Hermansson & Axnér, 2007

1.2 Cause of sperm damage

In felid species, one of the main barriers to the success of AI or other ARTs is poor sperm quality and low sperm survival after cryopreservation (Pukazhenti *et al.*, 1999). Semen cryopreservation involves several steps such as centrifugation, dilution, cooling, freezing and thawing (Luvoni, 2006). Each of these steps can cause sperm damage which impairs normal sperm function and fertilizing potential. The main causes of sperm damage during cryopreservation are (1) cold-shock (Watson, 2000), (2) osmotic stress (Watson, 2000) and (3) oxidative stress (Aitken & Krausz, 2001; Sikka, 2001; Agarwal *et al.*, 2003).

Cooling, as well as the freezing process, can cause stress to the spermatozoa due to temperature changes that result in sperm damage known as “cold shock” (Watson, 2000; Luvoni, 2006). A decrease in motility, progressive motility and frequency of intact acrosomes was observed after cooling or freezing (Glover & Watson, 1985; Pukazhenti *et al.*, 1999; Luvoni *et al.*, 2003). It was hypothesized that the temperature reduction (cold shock) during the cryopreservation process caused a change in cell membrane permeability (Watson, 1995; Luvoni, 2006). In one study, a rapid or ultra-rapid cooling rate (4°C/min and 14°C/min, respectively) resulted in acrosomal damage, whereas a slow cooling rate (0.5°C/min) significantly reduced acrosomal damage in cat spermatozoa (Pukazhenti *et al.*, 1999). However, in contrast, other studies demonstrated that cat sperm quality was not affected by a rapid or slow cooling rate (Zambelli *et al.*, 2002; Axnéer *et al.*, 2004; Hermansson, 2006). Thus, it appears that damage to cat spermatozoa mainly arises during the freezing process rather than during cooling (Hermansson, 2006). Therefore development of improved cryoprotective extenders is needed to improve sperm quality after cryopreservation.

Osmotic stress is yet another crucial factor that can cause sperm damage (Watson, 2000). During equilibrium, cooling of biological cells including mammalian spermatozoa to low subzero temperatures, extracellular ice crystal formation is induced (Picton *et al.*, 2000). The amount of ice crystals increases as the temperature decreases (Picton *et al.*, 2000), resulting in an osmotic gradient leading to shrinkage and dehydration of the cell (Picton *et al.*, 2000). Both the ice crystal formation and the osmotic gradients are liable to be the cause of cell damage. The addition of cryoprotective agents (CPA) such as glycerol, dimethyl sulfoxide (DMSO), 1,2-propanediol (PROH) or ethylene glycol are used to minimize the formation of ice crystals (Picton *et al.*, 2000). The CPA exert their protective function by penetrating cell membranes, thus preventing lethal ice formation in the cells (Picton *et al.*, 2000). However, a high concentration of CPA causes changes

in osmolarity and hence an osmotic effect (Luvoni, 2006). The addition of cryoprotectants causes first shrinkage of spermatozoa because of efflux of water from cells due to the hyperosmotic environment, and then swelling due to the influx of water and cryoprotectant to maintain the isotonic solution (Gao *et al.*, 1997). As an example of this effect, extensive sperm membrane damage occurred when cat spermatozoa were returned from hyperosmotic conditions to an isotonic solution (Phukazhenti *et al.*, 2000). Generally, cat spermatozoa were resistant to hypertonic exposure, but abnormal cat spermatozoa were less resistant (Phukazhenti *et al.*, 2002). Cat sperm motility was found to be affected by changes in osmolarity (Pukazhenti *et al.*, 2000) in a similar manner to human, ram and stallion spermatozoa (Curry & Watson, 1994; Ball & Vo, 2001). One reason for this is that mitochondrial function could be affected by the osmotic change, leading to decreased motility (Gao *et al.*, 1997).

Apart from cold shock and osmotic stress, oxidative stress is another important cause of sperm damages (Aitken & Krausz, 2001; Sikka, 2001; Agarwal *et al.*, 2003). Several studies have shown that oxidative stress induces damage to sperm membranes and DNA in human, stallion, ram and bull (Aitken, 1999; Agarwal *et al.*, 2003; Baumber *et al.*, 2000; Bilodeau *et al.*, 2001; Bilodeau *et al.*, 2002; Baumber *et al.*, 2003; Nair *et al.*, 2006; Peris *et al.*, 2007).

1.3 Physiology and effects of oxidative stress on sperm functions

The term “oxidative stress” refers to an imbalance between the excessive production or accumulation of reactive oxygen species (ROS) and an impaired antioxidant mechanism (Sikka, 2001; Agarwal *et al.*, 2003). Living cells stored under aerobic conditions, such as during cooling, freezing and thawing, require oxygen (O₂) to support their normal metabolism (Agarwal *et al.*, 2003). Excessive metabolites of oxygen such as ROS can, however, cause cell damage or apoptosis (de Lamirande & Gagnon, 1995). The ROS are free radicals, each having an unpaired electron, which is a highly unstable situation. The unpaired electron requires pairing with another electron to become stable. Thus, free radicals always attack other molecules, capturing electrons stabilize their unpaired electron (Nogushi & Niki, 1999). Examples of ROS are superoxide radicals (O₂⁻), hydroxyl radicals (OH[•]), and also hydrogen peroxide (H₂O₂), which is the end product of ROS reactivity and is considered to be the most toxic oxidants (Halliwell, 1997; Nogushi & Niki, 1999; Silva, 2006).

The ROS have been reported as a main cause of human diseases such as cardiovascular disease, cancer or neurological disease (Papas, 1999). The adverse effect of ROS on mammalian spermatozoa and reproduction was first reported by McLeod (1943). Since then several studies have demonstrated that ROS are involved in male infertility (Aitken & Krausz, 2001; Sikka, 2001; Agarwal *et al.*, 2003). A high level of ROS in human seminal plasma is related to poor sperm morphology, poor motility and a low sperm concentration (Aitken, 1989). Excessive ROS can be generated by (1) leukocytes and endothelial cells, (2) lack of antioxidants in seminal plasma and spermatozoa (3) normal spermatozoa, as well as immotile, abnormal and dead spermatozoa (Engel *et al.*, 1999; Bailey *et al.*, 2000; Silva, 2006.). Furthermore, ROS can be generated during the centrifugation of spermatozoa when removing seminal plasma or washing spermatozoa during preparation for ARTs (Sikka *et al.*, 1995).

1.3.1 ROS generation

Sperm themselves can produce ROS via two pathways: the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system in the sperm membrane and the NADPH-dependent oxido-reductase in the sperm mitochondria (Gavella & Lipovac, 1992; Agarwal *et al.*, 2003). The sperm mitochondria, located in the mitochondrial matrix rather than the intermembranous space, are the major source of ROS generation (Agarwal *et al.*, 2003; Koppers *et al.*, 2008). Generally, ROS must be continuously inactivated so that only a minimal amount of them are present at one time to maintain normal cell functions (Agarwal *et al.*, 2003). Low levels of ROS are, for example, necessary for sperm capacitation and the acrosome reaction (de Lamirande & Gagnon, 1993; de Lamirande & O'flaherty, 2008; Roy & Atreja, 2007). An excessive accumulation of ROS such as O_2^- or H_2O_2 can cause sperm plasma membrane damage in stallion (Baumber *et al.*, 2000), bull (Chatterjee & Gagnon, 2001), and human and sperm DNA damage in both the mitochondrial and nuclear genomes (Aitken & Krausz, 2001). The inner and outer mitochondrial membrane can be disrupted by a high level of ROS, leading to the activation of caspase, a series of cytosolic cysteine proteases which play a role in apoptosis induction (Agarwal *et al.*, 2003). This was confirmed by the study of Lozano *et al.* (2009) who demonstrated that H_2O_2 causes apoptosis in human spermatozoa by activation of caspase-3. The H_2O_2 has the most harmful oxidant properties (Alvarez & Storey, 1989; de Lamirande & Gagnon, 1992), being able to cross cell membranes freely and cause DNA damage (Halliwell, 1997; Baumber *et al.*, 2003). *In vitro* incubation with H_2O_2 caused a decrease in buffalo sperm motility (Garg *et al.*, 2009). Moreover, exogenous ROS generation by the xanthine-xanthine oxidase (X-XO) system can significantly increase the percentage of DNA fragmentation in equine spermatozoa (Baumber *et al.*, 2003) and human

spermatozoa (Lopes *et al.*, 1998). This observation indicates that H_2O_2 , the product of X-XO system, is responsible for sperm DNA damage. In addition, H_2O_2 can produce OH^\cdot via the Fenton reaction leading to lipid peroxidation induction (Silva, 2006). The OH^\cdot can also directly react with protein and DNA components leading to DNA damage (Baumber *et al.*, 2003). Furthermore, it has been demonstrated that free oxygen radicals such as $O_2^{\cdot-}$ are produced during the freezing-thawing process in human, bovine and canine spermatozoa (Mazzilli *et al.*, 1995; Tselkas *et al.*, 2000; Chatterjee & Gagnon, 2001; Michael *et al.*, 2007). Moreover, the cycle of freezing and thawing has been reported to be responsible for a decrease in the level of antioxidants such as glutathione (GSH) or superoxide dismutase (SOD) in human and bovine spermatozoa (Alvarez & Storey, 1992; Bilodeau *et al.*, 2000). This decrease in antioxidants would enable ROS to cause sperm damage during the freezing process.

1.3.2 Lipid peroxidation

The mammalian sperm plasma membrane, which is rich in polyunsaturated fatty acids (PUFA) (Alvarez & Storey, 1995), can be easily damaged by the reaction between ROS, such as OH^\cdot , and the polyunsaturated fatty acids. This mechanism is widely known as the lipid peroxidation reaction (Agarwal *et al.*, 2003). Lipid peroxidation is composed of three major steps; initiation, propagation and termination (Nogushi & Niki, 1999). The initiation step is the process of producing lipid radicals by ROS. In the propagation step the lipid radical from the initiation step attacks other unsaturated fatty acid molecules on the cell membrane or steals the electron from O_2 to form lipid peroxy radical (ROO^\cdot). The ROO^\cdot abstracts the hydrogen atom from other lipid molecules resulting in lipid hydroperoxides ($ROOH$) which are stable until attacked by a transition metal, generating alkoxy (RO^\cdot) and ROO^\cdot which can continue to attack other PUFA on the cell membrane. This step is self-perpetuating, which is why the lipid peroxidation reaction is also known as a chain reaction (Herrera & Barbas, 2001; Agarwal *et al.*, 2003). In the termination step, which is the end stage of the lipid peroxidation reaction, free radicals combine to form paired stable electrons. This step can be stopped earlier by antioxidants that can trap free radicals (Silva, 2006). The lipid peroxidation reaction results in changes in sperm membrane fluidity and loss of membrane integrity as well as irreversible loss of sperm motility (Storey, 1997). Lipid peroxidation has been reported to reduce the ability of human spermatozoa to penetrate zona-free hamster oocytes (Aitken, 1989) and is also related to DNA strand breaks (Baumber *et al.*, 2003). Furthermore, OH^\cdot can be generated by the Fenton reaction to H_2O_2 , which reacts with the transition metal i.e. ferrous ion (Fe^{2+}), promoting lipid peroxidation.

The lipid peroxidation reaction on the sperm cell membrane can be detected by two different methods: either using thiobarbituric acid reactive substances (TBARS) or using a lipophilic fluorescence probe (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY^{581/591} C11) (Baumber *et al.*, 2000; Aitken *et al.*, 2007). The TBARS method is used to measure the malondialdehyde (MDA) level, which is the end-point reaction product of lipid peroxidation (Sikka *et al.*, 1995; Baumber *et al.*, 2000). However, the TBARS technique has disadvantages. It cannot indicate the localization of lipid peroxidation and also has low sensitivity (Brouwers & Gadella, 2003). Therefore, BODIPY^{581/591} C11 has been developed (Pap *et al.*, 2000) to increase the accuracy, sensitivity and specificity of membrane oxidation detection. This probe is a fatty acid analogue, which fluoresces red when intercalated into the cell membrane and shifts towards green upon lipid peroxidation (Ball & Vo, 2002; Brouwers & Gadella, 2003; Silva, 2006). Therefore, BODIPY^{581/591} C11 directly detects sperm membrane lipid peroxidation and is now widely accepted as a highly sensitive indicator of the physiological relevant exposure of membrane fatty acid to ROS (Brouwers & Gadella, 2003). The BODIPY^{581/591} C11 can be evaluated by a fluorescence microscope, microplate reader, confocal microscopy or flow cytometry (Pap *et al.*, 2000; Ball & Vo, 2002). This technique has been applied successfully to detect lipid peroxidation in human (Aitken *et al.*, 2007) and red deer spermatozoa (Domínguez-Rebolledo *et al.*, 2010).

1.4 Physiology and effects of antioxidants on oxidative stress and sperm functions

Antioxidants act as protective agents against cell damage induced by ROS or other free radicals (Halliwell, 1997). They are classified into two functional categories, enzymatic and non-enzymatic antioxidants (Silva, 2006). The antioxidants can also be classified due to their water- or lipid solubility (Papas, 1999). The function of enzymatic antioxidants is to suppress the formation of free radicals by decomposing them (Papas, 1999). The enzymatic antioxidants include catalase (CAT), glutathione peroxidase/reductase pair (GPx/GRD) and superoxide dismutase (SOD) (Irvine, 1996). Antioxidants can be found in mammal serum and cells and can also be found as a component in food. A low level of antioxidants in the body system or an imbalance between antioxidants and ROS levels can lead to cell dysfunction and diseases. Therefore, antioxidants can be used as medication in treatment of human diseases (Papas, 1999).

In the scope of animal reproduction, seminal plasma is an abundant source of antioxidants i.e. α -tocopherol, vitamin C, uric acid, CAT and SOD

(Thérond *et al.*, 1996). Furthermore, GPx and SOD are present in the epididymis (Irvine, 1996; Thérond *et al.*, 1996), and the GPx/GRD pair and SOD have been reported to be present in the cytoplasm of the sperm mid-piece (Irvine, 1996). However, natural antioxidant levels in spermatozoa are not sufficient to protect completely against ROS-induced damage (Irvine, 1996). Besides, it has been shown that infertile clients had lower levels, or absence, of some antioxidants such as GSH in their seminal plasma compared to fertile men (Lewis *et al.*, 1995; Ochsendorf *et al.*, 1998). Furthermore, seminal plasma enriched in antioxidants is normally discarded during the cryopreservation process. Hence, several *in vitro* and *in vivo* clinical trials and studies have been conducted in human and animals using antioxidants to minimize the toxic effects of ROS and lipid peroxidation on spermatozoa.

Supplementation of semen extenders, oral medication or food with antioxidants such as GSH, cysteine, vitamin E or vitamin C have been reported to improve semen quality after cold storage or thawing in human (Askari *et al.*, 1994; Suleiman *et al.*, 1996), boar (Peña *et al.*, 2003; Funahashi & Sano, 2005; Sartorre *et al.*, 2007), bull (Bilodeau *et al.*, 2001) stallion (Almeida & Ball, 2005), and dog (Michael *et al.*, 2007). However, oral supplementation with vitamin E did not improve human sperm quality in fresh semen (Bolle *et al.*, 2002). Thus the effect of antioxidants on sperm quality is still contradictory and requires further investigation, especially in wild felid species which are affected by teratozoospermia. The abnormal spermatozoa might produce a high level of ROS, which in combination with an inadequate antioxidant system, could result in a burst of oxidative stress.

1.4.1 Enzymatic antioxidants

To complete the ROS scavenging system, aerobic organisms are enriched with enzymatic antioxidants. They are composed of CAT, GPx and SOD which is called the CAT/GPx/SOD catalytic triad (Drevet, 2006). These antioxidants work together to reduce the levels of free radicals (Drevet, 2006; Silva, 2006).

Catalase (CAT)

Catalase has been reported as a potential H₂O₂ detoxifier (Silva, 2006) but its presence in mammalian semen is still controversial.



Figure 2. The reaction demonstrates that catalase (CAT) is a hydrogen peroxide (H_2O_2) detoxifier by converting hydrogen peroxide (H_2O_2) to water (Silva, 2006).

Catalase is present in human (Khosrowbeygi & Zarghami, 2007) and rabbit (Foote & Hare, 2000) seminal plasma and in human spermatozoa (Jeulin *et al.*, 1989). The sertoli cells and the accessory sex glands are the source of CAT in the rabbit (Ihrig *et al.*, 1974; Foote & Hare, 2000). However, CAT is absent in mouse and bovine spermatozoa (Alvarez & Storey, 1984; Bilodeau *et al.*, 2000). Generally, CAT potentially functions together with another biological enzymatic antioxidant; SOD (Sikka *et al.*, 1995). Superoxide dismutase constitutes the first line defense mechanism by converting O_2^- to H_2O_2 whereas CAT is the H_2O_2 detoxifier. The addition of CAT to the semen extender had a positive effect on frozen-thawed dog spermatozoa (Michael *et al.*, 2007). The CAT supplementation together with SOD, was reported to improve sperm quality and fertilizing ability in the boar (Roca *et al.*, 2005). An incorrect concentration of CAT is, however, deleterious to ram spermatozoa (Maxwell & Stojanov, 1996).

Glutathione peroxidase (GPx)

The various GPx proteins are found either in spermatozoa or expressed throughout the epididymis of certain mammals such as horses, cattle and pigs (Drevet, 2006; Chabory *et al.*, 2010). Human infertility has been correlated to failure of GPx expression in spermatozoa (Drevet, 2006), which is also in accordance with the occurrence of subfertility or infertility in the GPx-knockout mouse (Liang *et al.*, 2009; Chabory *et al.*, 2010).

Glutathione peroxidase is a selenium-containing antioxidative enzyme with GSH as the electron donor (Sikka *et al.*, 1995). It plays a key role in removing $\text{ROO}\cdot$, which cause the lipid peroxidation chain reaction, as well as scavenging H_2O_2 (Sikka *et al.*, 1995). Although both CAT and GPx can potentially act as detoxifiers of H_2O_2 , CAT is weakly present or even absent in different animal species. Hence, GPx is the dominant enzymatic antioxidant protecting mammalian spermatozoa from oxidative stress compared to CAT (Drevet, 2006).

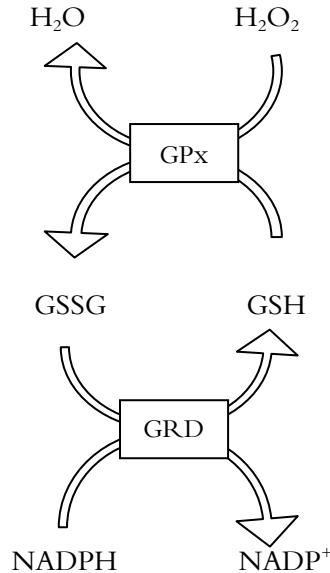


Figure 3. Glutathione peroxidase (GPx) decomposes hydrogen peroxide (H_2O_2) to water by using glutathione (GSH), an abundant non-protein thiol in mammalian cells (Irvine, 1996), as the electron donor (Chabory *et al.*, 2010). GSSG: oxidized glutathione; GRD: glutathione reductase; $NADP^+$: nicotinamide adenine dinucleotide phosphate and NADPH; reduced form of $NADP^+$. This figure is cited from Irvine (1996).

Supplementation of semen extenders with GPx resulted in positive effects on the acrosome integrity of ram spermatozoa (Maxwell & Stojanov, 1996) as well as an improvement in motility in both ram (Maxwell & Stojanov, 1996) and bull sperm samples (Bilodeau *et al.*, 2001). Furthermore, the glutathione peroxidase protein (GPx4) is essential for regulating mitochondrial apoptosis induced by oxidative stress (Liang *et al.*, 2009). Mice that lack this protein have structurally abnormal spermatozoa (Liang *et al.*, 2009), which might be the main source of ROS generation.

Superoxide dismutase (SOD)

Superoxide dismutase constitutes the first line defense mechanism against oxidative stress in living cells (Silva, 2006). The activity of this enzyme can be observed in donkey and stallion seminal plasma (Mennella & Jones, 1980) and in dog spermatozoa (Cassani *et al.*, 2005). The level of SOD is greater than GPx in bull spermatozoa (Bilodeau *et al.*, 2000). The major source of this enzyme is the male accessory glands such as the prostate gland and seminal vesicles (Nonogaki *et al.*, 1992; Chen *et al.*, 2003).

Superoxide dismutase plays an antioxidant enzyme role by catalyzing or removing O_2^- from the cytosol of living cells, thus generating H_2O_2 (Silva, 2006).



Figure 4. The antioxidative enzyme; superoxide dismutase (SOD) catalyses or removes superoxide radical (O_2^-), generating hydrogen peroxide (H_2O_2) and oxygen (O_2) (Sikka *et al.*, 1995).

Superoxide dismutase generally works together with CAT or GPx due to their H_2O_2 scavenging ability. Supplementation of SOD to semen extenders has been reported to improve sperm quality, i.e. sperm motility, mitochondrial activity, acrosome and DNA integrity in ram (Maxwell & Stojanov, 1996), human (Rossi *et al.*, 2001) and Iberian red deer (Maria *et al.*, 2007). Furthermore, SOD incorporated with CAT also improves the quality of cryopreserved human spermatozoa (Rossi *et al.*, 2001).

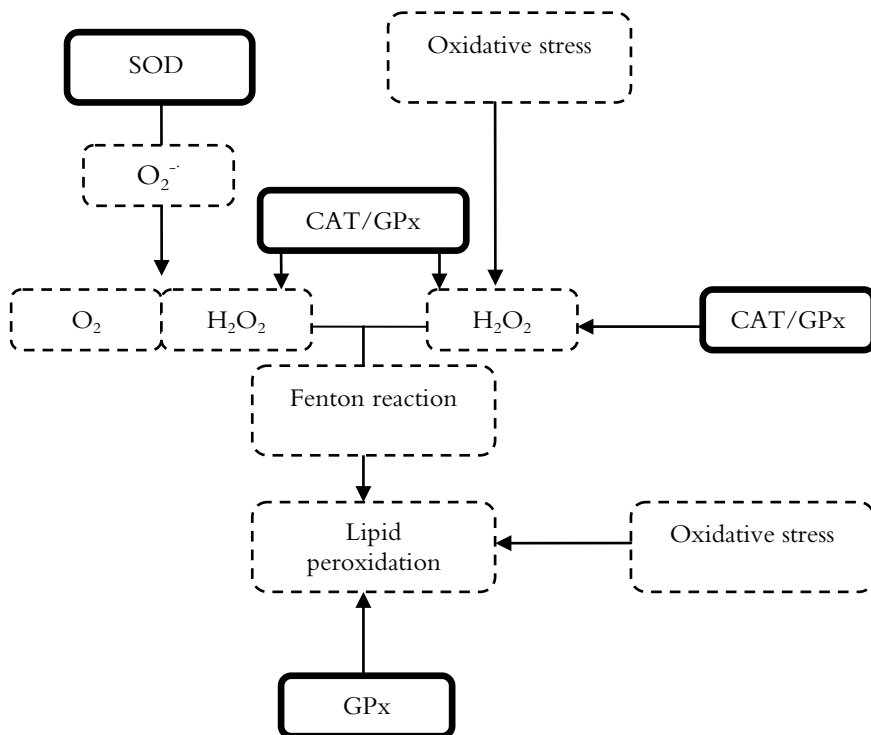


Figure 5. The function of three different types of enzymatic antioxidants against oxidative stress. Diagram is modified from Irvine (1996).

1.4.2 Non-enzymatic antioxidants

The function of the non-enzymatic antioxidants is to inhibit the initiation of the chain reaction, break the chain propagation stages in the lipid peroxidation reaction and eliminate other ROS (Nogushi & Niki, 1999). The non-enzymatic antioxidants are also called radical scavenging antioxidants and are classified into two sub-types; hydrophilic and lipophilic antioxidants (Nogushi & Niki, 1999). They include vitamin A (retinol), vitamin C (ascorbic acid), vitamin E (α -tocopherol) and uric acid (Nogushi & Niki, 1999; Silva, 2006). This thesis focuses on two types of non-enzymatic antioxidants; cysteine and vitamin E.

Cysteine

Cysteine is included in the thiol (-SH) group which is a large class of antioxidants (van Zandwijk, 1995). Cysteine is an α -amino acid and is a precursor in the production of intracellular GSH which functions as a cofactor of GPx to destroy H_2O_2 (Meister, 1994). In addition, it has been shown that GSH can donate hydrogen atoms to repair damaged DNA, which is the reason why GSH and the other thiol compounds such as cysteine or N-acetyl-L-cysteine may be important substances to protect cells from DNA damage. Addition of cysteine to the semen extender prevents loss of sperm motility by inhibition of lipid peroxidation caused by ROS in frozen-thawed bull semen (Bilodeau *et al.*, 2001). Funahashi & Sano (2005) showed that cysteine can enhance viability of boar spermatozoa in cold-stored preservation. Furthermore, N-acetyl-L-cysteine (one form of cysteine) has been shown to be advantageous for inhibition of programmed sperm cell death (apoptosis) in human seminiferous tubules (Erkkilä *et al.*, 1998).

Vitamin E

Vitamin E is classified as the most potent non-enzymatic antioxidant (Silva, 2006). The major component of vitamin E is α -tocopherol which is well-known as a potent substance for preventing membrane peroxidation by scavenging free radicals or $ROO\cdot$ involved in the lipid peroxidation chain reaction (Herrera & Barbas, 2001). Vitamin E (α -tocopherol) has been detected in human seminal plasma (Moilanen *et al.*, 1993).

Although vitamin E radicals are formed when vitamin E reacts with $ROO\cdot$, these are stable because the free electron is delocalized in an aromatic ring structure (Cadenas, 1995; Herrera & Barbas, 2001). Thus, the radical reaction chain of lipid peroxidation is stopped. In biological systems, one molecule of vitamin E can protect 1,000 molecules of unsaturated fatty acids (Kontush *et al.*, 1996). It was observed that vitamin E was more effective than vitamin C in improving post-thaw human sperm motility when used as a supplement to semen extenders (Askari *et al.*, 1994). The addition of vitamin E has also been shown to protect membrane integrity in cooled boar spermatozoa (Cerolini *et al.*, 2000), while the water soluble vitamin E analogue Trolox, improved both boar sperm motility and mitochondrial membrane integrity (Peña *et al.*, 2003).

2 Aims of the thesis

The overall aim of the present work was to investigate the effects of antioxidants (enzymatic and non-enzymatic antioxidants) and oxidative stress on sperm quality in cryopreserved semen samples collected from the epididymides of domestic tomcats and their application to semen samples collected by electroejaculation from a wild felid; the flat-headed cat (*Prionailurus planiceps*).

Specific aims were to:

- Investigate the effects and to compare the potency of two different types of non-enzymatic antioxidants, cysteine and the water soluble vitamin E analogue Trolox, on post-thaw sperm quality in semen samples collected from the epididymides of domestic cats
- Detect the lipid peroxidation reaction in cryopreserved epididymal spermatozoa from the domestic cats by using a lipophilic dye probe (BODIPY^{581/591} C11)
- Investigate the effects of three selected antioxidative enzymes (CAT, GPx, SOD) on sperm quality in semen samples collected from the epididymides of domestic cats, with or without lipid peroxidation induction (by a transition metal; Fe²⁺) during the cryopreservation process or during post-thaw incubation
- Measure the levels and effects of the lipid peroxidation reaction induced by the transition metal (Fe²⁺) in post-thaw sperm samples collected from the epididymides of domestic cats
- Evaluate basic seminal characteristics of flat-headed cats (*Prionailurus planiceps*), individually housed in two different zoos in Thailand

- Compare the effects of one non-enzymatic antioxidant (vitamin E analogue Trolox) and one enzymatic antioxidant (GPx) on cryopreserved flat-headed cat spermatozoa collected by electroejaculation and assess sperm fertilizing ability *in vitro* using heterologous oocytes from domestic cats

3 Methodological considerations

3.1 Animals

Semen samples were collected from the caudae epididymides of 55 male cats after routine castration (**Papers I, II and III**), and ejaculated spermatozoa were obtained from one domestic cat and four captive flat-headed cats (**Paper IV**). The healthy domestic cats were of mixed breeds aged between 8 and 16 months (**Papers I and IV**), or of unknown age in **Papers II and III**. The captive flat-headed cats aged between 1.5 and 3 years and two of them had sired offspring by natural mating (**Paper IV**).

Ovaries were collected from more than 65 domestic female cats of unknown age and breed after routine ovariohysterectomy (**Paper IV**).

3.2 Collection of spermatozoa

3.2.1 Epididymal spermatozoa

Testes with epididymides were collected after routine castration at the University Animal Hospital at the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden (**Paper I**) and the Veterinary Public Health Division of the Bangkok Metropolitan Administration in Bangkok, Thailand (**Papers II and III**). The caudae epididymides were dissected free from visible blood vessels and connective tissues, placed in warm (37°C) Tris buffer, and transversely cut into small pieces to release the spermatozoa. After 10 min incubation at 37°C to 38°C, the tissue segments were removed.

3.2.2 Ejaculated semen

Collection of semen using electroejaculation was performed in **Paper IV**. The domestic cats were injected with a combination of 10 mg/kg ketamine HCl (Calypsol, Gedeon Richter, Budapest, Hungary), xylazine 2 mg/kg (Rompun®, Bayer, Seoul, Korea) and 0.04 mg/kg atropine (Atropine, A.N.B. Laboratories Co.Ltd., Bangkok, Thailand). General anaesthesia was induced in all flat-headed cats by intramuscular injection of 7-8 mg/kg ketamine HCl (Gedeon Richter) and maintained with isoflurane inhalation. Electroejaculation was performed according to a standard protocol by stimulation of the prostate gland with three series of a total of 80 stimuli (2–5 V), delivered via a lubricated rectal probe (1.3-cm diameter, 12 cm long) connected to an electrostimulator.

3.3 Sperm recovery and cryopreservation media

Semen extenders, recovery and thawing media were prepared according to a protocol described by Axnér *et al.* (2004). Briefly, semen was diluted with egg-yolk Tris extender I and II in two steps (**Papers I, III, III and IV**). Extender I was added to the sperm samples at room temperature and was used to test various treatments with different types of antioxidants; non-enzymatic antioxidants (cysteine and vitamin E analogue Trolox) (**Paper I**), enzymatic antioxidants (CAT, GPx and SOD) (**Paper III**) and both enzymatic and non-enzymatic antioxidants (GPx and vitamin E analogue Trolox) (**Paper IV**). Extender II, which was added after cooling at 4°C, had the same composition as semen extender I except that it contained 1% Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA) and 7% glycerol. A Tris buffer, with the same composition as extender I, but without glycerol and egg yolk, was used as a medium for sperm recovery and thawing. The semen extenders and the Tris buffer were prepared as a single batch in each study and stored at -20°C until used.

3.4 Cryopreservation of spermatozoa

Extended samples were placed in a bench cooler (**Papers I, II and III**) or portable (**Paper IV**) cooler and slowly cooled to 4°C for 1 h. Semen extender II with an equal volume of semen extender I was added to the sample. Straws (0.25 mL) were filled manually with a mixture of extender I and II (1/1; v/v) to saturate the cotton plug of the straw (to prevent loss of

spermatozoa into the plug), and were subsequently filled with the diluted sample. The straws were frozen as described by Rota *et al.* (1997) (**Papers I, II and III**). In brief, the straws were placed in a goblet on a cane. The goblet was put into a canister which was lowered into an Apollo SX-18 liquid nitrogen tank with a level of 16-18 cm of liquid nitrogen (MVE Cryogenetics®, New Prague, MN, USA) in three steps, with the top of the goblet held 7, 13, and 20 cm below the opening of the tank for 2, 2, and 1 min, respectively. Finally, the canister was plunged into liquid nitrogen. In **Paper IV**, the straws with extended semen were frozen 4 cm above liquid nitrogen for 10 min and plunged into liquid nitrogen. The straws were stored in liquid nitrogen until evaluation. The straws were thawed in a water bath at 70°C for 6 sec (**Papers I and II**) or 37°C for 15 sec (**Papers III and IV**) and were subsequently emptied into Eppendorf tubes containing warm (37°C) thawing medium of an equal volume as the frozen-thawed samples.

3.5 Sperm incubation and post-thaw

After thawing, the semen sample was incubated at 37°C for 0, 2, 4 and 6 h (**Papers I and III**), 0 h, 30 min and 6 h (**Paper II**) and 0 and 6 h (**Paper IV**). The first group of semen samples was diluted with semen extenders with or without antioxidant supplementation (**Papers I, II, III and IV**). The second group was diluted with only Tris buffer (**Paper II**) and the third group was diluted with the semen extenders with or without antioxidant supplementation plus a transition metal (Fe^{2+} ; lipid peroxidation inducer) (**Papers II and III**).

3.6 Semen evaluation

3.6.1 Volume and pH

The volume of the ejaculated semen was measured using a micro pipette, whereas pH was determined using a pH paper (**Paper IV**).

3.6.2 Concentration and morphology

Sperm concentration was determined using a hemocytometer (Boeco, German) (**Papers I, II, III and IV**). Spermatozoa stained with Williams stain were assessed for head morphology while spermatozoa fixed in formol saline were evaluated for mid-piece and tail morphology (**Papers I, III and IV**).

3.6.3 Motility

To evaluate subjective sperm motility and progressive motility, an aliquot of each semen sample was placed on a pre-warmed glass slide, covered with a pre-warmed cover slip, and subjectively assessed using a phase contrast microscope at magnification X200. The motility was reported as the percentage of motile sperm while progressive motility was scored from 1 to 5, with 1 as the lowest and 5 the highest score (**Papers I, III and IV**).

To evaluate sperm motility patterns, a pre-warmed 10 μm -deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) was loaded with an aliquot of each sperm sample and sperm motility was assessed by computer-assisted sperm analysis (CASA) using a Strömberg-Mika Cell Motion Analyser (SM-CMA; MTM Medical Technologies, Montreux, Switzerland). Five randomly selected optical fields of each sample were measured for the motility patterns as follows: percentage of linearly motile sperm (LIN); straight line velocity (VSL; $\mu\text{m/s}$); average path velocity (VAP; $\mu\text{m/s}$); curve linear velocity (VCL; $\mu\text{m/s}$); and amplitude of lateral head displacement (ALH; μm). The parameter setting of the SM-CMA software was as follows: (1) spermatozoa presented in a minimum of 14 out of 32 frames were counted; (2) the velocity limit for immotile spermatozoa was less than 10 $\mu\text{m/s}$; (3) the velocity limit for local motile spermatozoa was less than 30 $\mu\text{m/s}$; (4) circular motility was considered at spermatozoa with radius lesser than 100 μm ; and (5) linearly motility was set at a sperm deviation less than 10% from a straight line. The settings had previously been chosen after evaluation of the trajectory patterns of the spermatozoa (**Paper III**).

3.6.4 Membrane integrity

Sperm membrane integrity was determined by aniline blue staining (**Paper IV**) or a double-fluorescent labelling technique, according to the protocol described by Axnér *et al.* (2004) (**Papers I, III and IV**). For aniline blue staining, a 5 μL sample was mixed with an equal drop of stain, smeared on the warmed glass slide and air dried. Two hundred spermatozoa were assessed under light microscope with x1000 magnification and classified as live (unstained) or dead (stained with red). For the fluorescent labelling technique, a 5 μL aliquot of the sample was thoroughly mixed with 1 μL of 14 μM EthD-1 (Molecular probes Inc., OR, USA) in phosphate buffered saline (PBS) and 1 μL of 0.38 μM SYBR -14 (Dead/Alive Kit, Molecular probes Inc.) in DMSO, and incubated in the dark at 37°C for 30 min. Sperm evaluation was performed using an epifluorescent microscope. Two

hundred sperm were counted from each sample and classified according to the following categories: live (stained only green from SYBR-14), moribund (stained both green and red) and dead sperm (stained only red from EthD-1).

3.6.5 Acrosome integrity

A 10 μL aliquot of the semen sample was smeared on a glass slide, air-dried, and treated with 95% ethanol for 30 sec to permeabilize the sperm membranes. FITC-PNA staining was used for evaluation of acrosome integrity as previously described by Axnér *et al.* (2004). Briefly, a 90 μL aliquot of FITC-PNA (100 $\mu\text{g}/\text{mL}$ in PBS) (Sigma Chemical Co., St Louis, MO, USA) was mixed with 5 μL of PI (340 μM in PBS, final concentration of 18 μM) and 20 μL of this solution was spread over the smeared slide. The slide was incubated in the dark in a humidified chamber at 4°C for 30 min. After incubation, the slide was rinsed with cold distilled water and air-dried in the dark at 4 °C. Two hundred spermatozoa were evaluated using an epifluorescent microscope and classified into three categories: intact acrosome (stained bright green from FITC-PNA at the acrosomal cap), damaged acrosome (stained bright green and red), and missing acrosome (stained red from PI) (**Papers I, III and IV**).

3.6.6 Mitochondrial activity and membrane potential

Sperm mitochondrial activity (**Paper III**) was determined by double staining using the fluorochromes Mitotracker Green FM (Molecular Probe Inc.) and PI (Sigma). The concentration and incubation time for Mitotracker Green FM and PI were modified from methods used for evaluation of boar (Bussulleu *et al.*, 2005) and cat spermatozoa (Axnér *et al.*, 2004), respectively. Briefly, a 5 μL aliquot of the sperm sample was thoroughly mixed with 1 μL of 200 nM Mitotracker Green FM in DMSO (Sigma), 1 μL of 18 μM PI in PBS, and incubated in the dark at 37°C for 30 min. The sample was placed on a glass slide and covered with a cover slip. Sperm evaluation was performed using an epifluorescent microscope. Two hundred spermatozoa were evaluated and classified into two categories: (1) heads with negative PI staining and mid-pieces with bright green positive staining of Mitotracker Green FM were recorded as live spermatozoa with intact mitochondria sheath; and (2) heads with positive or negative staining of PI and mid-pieces with weak green positive staining or negative staining of Mitotracker Green FM were recorded as live/dead spermatozoa with loss of mitochondrial function.

Sperm mitochondrial membrane potential (**Paper IV**) was determined using the fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probe Inc.). The JC-1 probe enables the mitochondrial membrane potential status (high and low) to be determined. Since the evaluation of mitochondrial membrane potential using JC-1 staining has not been reported in cat spermatozoa previously, three concentrations (2, 10, 15 μM) of JC-1 were evaluated to enable the correct concentration of staining to be identified. In short, a 10 μL aliquot of the sample was thoroughly mixed with 1 μL of 2, 10 or 15 μM final concentration of JC-1 in DMSO (Sigma) and then incubated in the dark at 37°C for 30 min. The sample was placed on a glass slide and covered with a cover slip. Sperm evaluation was performed using an epifluorescent microscope. Two hundred spermatozoa were evaluated and classified into two categories: high (stained with bright orange through the sperm mid-piece), and low mitochondrial membrane potential (stained with patchy pale orange or all green through the sperm mid-piece). The criteria used in selection of the JC-1 concentration were based on the correlation to sperm motility, or the absence of overstraining, such as presence of staining sediment in the background.

3.6.7 Early apoptosis

Annexin V-CY3 (Annexin V-CY3 Apoptosis Detection Reagent; Abcam plc, Cambridge, UK) together with application of 6-CFDA (Sigma), which stain live cells with absence of early apoptosis, were used to detect the transition of phospholipids of the sperm plasma membrane. Annexin V-CY3 staining was prepared following the manufacturer's protocol. Briefly, a total of 5×10^5 spermatozoa were centrifuged and resuspended in 0.5 mL cold 1X binding buffer. The 6-CFDA (2.5 μL with 0.5 mM final concentration) was added to the sample and incubated in the dark at room temperature for 5 min. After the incubation, 1 μL Annexin V-CY3 was added, the suspension was thoroughly mixed and incubated for 5 min in the dark at room temperature. A drop of the semen sample was placed on a glass slide and covered with a cover slip. Sperm evaluation was performed using the epifluorescent microscope. Two hundred spermatozoa were evaluated and classified into two categories: absence (stained only green from 6-CFDA), or presence (stained both green and red or orange from 6-CFDA and Annexin V-CY3) of early apoptosis (**Paper IV**).

3.6.8 DNA integrity

Sperm DNA integrity was evaluated using Acridine orange (AO) (Sigma) following the method described by Tejada *et al.* (1984) and Thuwanut *et al.* (2008). Briefly, an aliquot of 10 μL of the semen sample was gently smeared on a glass slide, and air-dried. The smeared slide was fixed in a freshly-prepared mixture of methanol and glacial acetic acid (Carnoy's solution; 3/1; v/v), for at least 3 h or overnight at room temperature. The slide was removed from the fixative solution, air-dried, and then stained with AO staining solution (1% AO diluted in distilled water) for 5 min. After staining, the slide was gently rinsed in a stream of distilled water and covered with a cover slip. Two hundred spermatozoa were evaluated using an epifluorescent microscope. The heads of the sperm cells with normal DNA integrity (double-stranded) emitted green fluorescence, while those with denatured or single stranded DNA showed orange, yellow, or red fluorescence. The stained slide was evaluated within 1 h after staining (**Papers I, III and IV**).

3.6.9 Lipid peroxidation reaction

The lipid peroxidation reaction was determined by using a lipophilic dye probe fluorescence assay (BODIPY^{581/591}C11; 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; Invitrogen Singapore Pte Ltd, Singapore). Before loading the BODIPY^{581/591} C11, all post-thaw samples were centrifuged at 600 x g for 6 min to remove any extender and each sample was then resuspended in 200 μL Tris buffer. BODIPY^{581/591} C11 was added to the samples at a final concentration of 10 μM and incubated for 30 min. Any unbound dye probe or excessive probe was removed by centrifugation at 600 x g for 6 min, after which 200 μL Tris buffer was added. The samples were evaluated for lipid peroxidation using a BD LSR flow cytometer (Beckon Dickinson, San José, CA, USA), using settings according to Aitken *et al.* (2007). Briefly, excitation was produced with an argon-ion laser (488 nm) HeCd laser (325 nm). Detection of green fluorescence was measured with an FL1 band pass filter (530/30 nm), and red fluorescence was measured using an FL3 long pass filter (>670 nm). A total of 50,000 sperm specific-events were evaluated and calculated as percentages (**Paper II**). To prevent false positive results from egg yolk particles (if some extender was still remaining in the samples), 17.8 μM Hoechst (Sigma) in DMSO was used to label sperm-DNA, detecting the fluorescence an FL5 long pass filter (> 380 nm) (**Paper III**).

3.6.10 Heterologous *in vitro* fertilization

Oocyte recovery, in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) media

All chemicals were purchased from Sigma except for fetal calf serum (Invitromex B.P. GmbH, Geilen Kirchen, Germany).

Oocyte recovery and IVM media were prepared according to a protocol described by Thongpakdee *et al.* (2010), whereas IVF and IVC media were prepared as described by Takahashi & First, (1992). Briefly, the oocyte recovery media was composed of 100 mL TCM 199 Hepes, 0.4 g bovine serum albumin, 0.0292 L-glutamine, 0.0026 g pyruvate and 1% penicillin (v/v). IVM media had the same composition as the oocyte recovery media, with the addition of 1 µg/mL estradiol, 10 µg/mL follicle stimulating hormone (FSH) and 10 ng/mL epidermal growth factor (EGF). The IVF media was composed of 0.6 g bovine serum albumin, 0.6294 g sodium chloride (NaCl), 0.0534 g potassium chloride (KCl), 0.0162 g potassium phosphate mono basic anhydrous (KH₂PO₄), 0.21 g sodium bicarbonate (NaHCO₃), 0.0033 sodium pyruvate, 0.0146 g L-glutamine, 0.0251 g calcium chloride dihydrate (CaCl₂·2H₂O), 0.01 g magnesium chloride dihydrate (MgCl₂·2H₂O), 2% essential amino acid (v/v), 2% non-essential amino acid (v/v), 1% penicillin streptomycin (v/v), 10% fetal calf serum (v/v) (Invitromex B.P.) in 100 mL double distilled water. The IVC media had the same composition as the IVF media with the addition of 0.4 g bovine serum albumin and 5% or 10% of fetal calf serum (v/v) (Invitromex B.P.) (**Paper IV**).

Oocyte collection and in vitro maturation (IVM)

A total of 384 oocytes was collected from the ovaries of domestic cats. Each ovary was kept in isotonic normal saline supplemented with 1% penicillin streptomycin (storage media) (Sigma) and transported to the laboratory within 4 h. The ovary was washed in clean storage media, placed in warmed oocyte recovery media and mechanically minced. Oocytes with more than two layers of compact cumulus cells and presence of dark homogeneous ooplasm (Grade I and II oocytes) were collected using a stereo microscope (X2). The selected oocytes were washed three times in IVM medium. Around 10–22 oocytes were transferred to a drop of 100 µL IVM medium and cultured at 38.5°C in 5% CO₂. After 24 h, cumulus cells were partially removed by repeated pipetting. The oocytes were washed three times and cultured in a drop of IVF medium at 38.5°C in 5% CO₂ (**Paper IV**).

Sperm preparation, in vitro fertilization (IVF) and in vitro culture (IVC)

Cryopreserved ejaculated domestic cat spermatozoa were used as a control for the IVF/IVC system. After thawing, flat-headed cat sperm samples from each group and samples from the domestic cat were washed twice by centrifugation and swim-up in IVF media. The sperm concentration was adjusted to $0.5 \times 10^6/\text{mL}$. After the IVM process the oocytes were inseminated with a washed sperm sample (final sperm concentration of $0.5 \times 10^6/\text{mL}$) and co-incubated at 38.5°C in 5% CO_2 for 18 h, after which all the cumulus cells were removed. The oocytes were washed three times and transferred to IVC media at 38.5°C in 5% CO_2 for 6 h. The cleavage rate was recorded after 24 and 48 h post-insemination, and uncleaved oocytes were removed. The developed embryos were transferred to a drop of IVC media supplemented with 5% and 10% fetal calf serum (v/v) 72 and 96 h after insemination, respectively. The embryo developmental stages (2, 4, 8, 8-16 cells, morula and blastocyst) were recorded consecutively until 144 h post-insemination (**Paper IV**).

3.7 Statistical analysis

Statistical analysis was performed using the Statistical Analysis Systems software package (Version 9, SAS Institute Inc., 2002, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. In **Paper I**, the dependent variables (percentage of sperm motility, level of progressive motility, percentage of membrane integrity, acrosome integrity, and DNA integrity) were evaluated using analysis of variance (ANOVA). The differences between fresh, cooled, and post-thaw sperm parameters and between control and treatment groups were compared using Tukey-Kramers test. The statistical models for all parameters included the fixed effect of time and treatment. The interaction between time and the treatment in each parameter was analyzed using the General Linear Model (GLM). In **Paper II**, the statistical models included the effects of treatment with lipid peroxidation promoter, post-thaw incubation (0 h, 30 min and 6 h) and resuspended samples (with and without the removal of semen extender). The dependent variable (the percentage of lipid peroxidation reaction) was evaluated using ANOVA, and a paired-t test was used to compare differences. In **Paper III**, the statistical models included the fixed effects of replication, time (fresh, cool and 0, 2, 4 and 6 h after thawing), types of extender and the interactions between time and extender. The dependent variables (percentage of lipid peroxidation reaction, percentage of subjective sperm motility, motility patterns observed by CASA, percentage

of mitochondrial activity, intact membrane, acrosome, and DNA) were evaluated using analysis of variance (ANOVA), and the differences between treatments were compared using the Tukey-Kramer test. In **Paper IV**, the statistical models included the fixed effects of individual flat-headed cats, time and extenders. The dependent variables (percentage of motility, levels of progressive motility, percentage of intact membrane, acrosome, DNA, mitochondrial membrane potential and early apoptotic cells) were analyzed by ANOVA (GLM procedure). Differences between treatments for each sperm quality parameter were compared using a Tukey-Kramer test. The embryo development was analyzed by a binomial score (1 or 0) based on the presence or absence of the morula and blastocyst stages. Values are presented as mean \pm SD. The level of significance was set at $P < 0.05$ (**Papers I, II, III and IV**).

4 Main results and discussion

It is widely accepted that various types of antioxidants can eliminate ROS-induced damage and prevent lipid peroxidation of living cells, including mammalian spermatozoa. Our current studies demonstrated that supplementation of semen extenders with enzymatic and non-enzymatic antioxidants can enhance the quality of epididymal cat and ejaculated wild felid (flat-headed cat) spermatozoa after the cryopreservation process. Moreover, this is also the first study on the presence and effect of lipid peroxidation in cryopreserved epididymal cat spermatozoa.

4.1 The effects of adding non-enzymatic and enzymatic antioxidants to cryopreserved epididymal cat and ejaculated flat-headed cat spermatozoa (Papers I, III and IV)

4.1.1 Vitamin E (Papers I and IV)

Addition of the vitamin E analogue Trolox, to the semen extender did not improve the quality of epididymal domestic cat spermatozoa after cooling but had positive effects on sperm quality after freezing and thawing. Motility (at 0, 2, 4 and 6 h after thawing), level of progressive motility (2, 4 and 6 h after thawing), and the percentage of intact sperm membrane (0 and 6 h after thawing) were significantly higher compared to control samples without the vitamin E analogue Trolox (**Paper I**). In contrast, adding vitamin E analogue Trolox to extenders for cryopreserving flat-headed cat spermatozoa enhanced the motility of cooled spermatozoa before cryopreservation compared to the control group, but had no effects after thawing (**Paper IV**). The lipid peroxidation reaction, induced by ROS, was suspected to be the main cause of sperm dysfunction, especially in loss

of membrane fluidity or sperm membrane damage, which can decrease sperm motility and viability (Storey, 1997). Generally, vitamin E functions as potent inhibitor of lipid peroxidation by neutralizing $\text{ROO}\cdot$ and $\text{RO}\cdot$ radicals (Agarwal *et al.*, 2003). These radicals are involved in the propagation stage of lipid peroxidation in the sperm membrane (Agarwal *et al.*, 2003). Breininger *et al.* (2005) demonstrated that the protective effects of vitamin E (α -tocopherol) against lipid peroxidation in boar spermatozoa depended on the concentration of the antioxidant supplement added to the semen extender. At an incorrect concentration, it failed to protect spermatozoa after the cryopreservation process (Breininger *et al.*, 2005). Similarly, in another study, it was confirmed that the effect of the vitamin E analogue Trolox on boar spermatozoa was dose-dependent (Peña *et al.*, 2003). Although the vitamin E analogue Trolox performed a protective effect for epididymal domestic cat spermatozoa, it did not improve the quality of post-thaw ejaculated flat-headed cat spermatozoa. The failure of vitamin E analogue Trolox to inhibit oxidative stress (**Paper IV**) might be due to an incorrect concentration of this antioxidant. In addition, the sperm quality i.e. normal sperm morphology of flat-headed cats investigated in this study was lower than previously report for domestic cats (Thuwanut *et al.*, 2010). The ROS levels and the induction of lipid peroxidation depend on the percentage of abnormal spermatozoa (Agarwal *et al.*, 2003). Therefore, it can also be hypothesized that the excessive level of ROS produced from morphologically abnormal spermatozoa in flat-headed cats could not be neutralized by the vitamin E analogue Trolox at the same concentration as for domestic cats and induced lipid peroxidation.

4.1.2 Cysteine (Paper I)

Supplementation with cysteine resulted in similar findings to supplementation with the vitamin E analogue Trolox, i.e. supplementation did not improve epididymal cat sperm quality after cold storage but improved sperm quality after post-thaw incubation for 6 h (level of progressive motility and DNA integrity) compared to the control group. Hydrogen peroxide, the ROS end-product, is considered to be the main cause of loss of motility and DNA damage in equine spermatozoa (Baumber *et al.*, 2003) and human spermatozoa (Lopes *et al.*, 1998; Agarwal *et al.*, 2003). Furthermore, DNA fragmentation was increased after long-term incubation with exogenous ROS, both in humans (Lopes *et al.*, 1998) and horses (Baumber *et al.*, 2003). Cysteine is a precursor in the production of intracellular GSH which can be found in the epididymis and functions as a cofactor of GPx to destroy H_2O_2 (Meister, 1994). Thus it is possible that although the level of H_2O_2 increased during post-thaw incubation of

epididymal cat spermatozoa, any negative effects on sperm motility and DNA were reduced by cysteine.

4.1.3 Catalase (Paper III)

Although CAT has been reported to be a potent H₂O₂ detoxifier and to have a positive effect on post-thaw sperm DNA integrity, it compromised the sperm motility after thawing. Supplementation of the semen extender with CAT had deleterious effects on linear motility (evaluated by CASA) and subjective epididymal cat sperm motility both immediately and 2 h after thawing, whereas sperm motility patterns (VAP and ALH) were higher than in the control group at 0 and 6 h after thawing. Moreover, CAT improved sperm DNA integrity after 4 h post-thaw incubation. Several studies have reported the effects of CAT on mammal spermatozoa in various concentrations. Concentrations of 400 and 800 U/mL of CAT were deleterious to ram sperm motility (Maxwell & Stojanov, 1996) while 200 U/mL (the same concentration as used in the present study) did not improve stallion sperm quality i.e. motility, mitochondrial activity, membrane and acrosome integrity (Baumber *et al.*, 2005). A study on cryopreserved ejaculated cat spermatozoa found that supplementation of 400 U/mL CAT did not enhance post-thaw motility, membrane and acrosome integrity (Thiangtum *et al.*, 2009). Hence, optimization of CAT concentration to reveal any potential benefits to cat spermatozoa may be required in further studies. Even though CAT decreased epididymal cat sperm motility, sperm mitochondrial activity was not influenced by CAT. It might be possible that the flagellar movement of cat spermatozoa mainly derives energy from the glycolysis pathway (Mortimer, 1997) rather than from sperm mitochondria. Furthermore, sperm motility patterns were higher in the CAT supplemented group compared to the control while the percentage of intact acrosomes was slightly decreased at the same time point. An increase in ALH is an indicator of sperm hyperactivated motility (Mortimer, 1997) which indicates sperm capacitation, a prerequisite for the acrosome reaction (Mortimer, 1997). Catalase supplementation might induce hyperactivation of post-thaw epididymal cat spermatozoa. However, since the value of ALH as an indicator of sperm hyperactivation and the implication of the increase in VAP have not been studied in cat spermatozoa, further studies of these parameters are needed.

4.1.4 Glutathione peroxidase (Papers III and IV)

Post-thaw epididymal cat sperm quality i.e. subjective motility (0 and 6 h), linear motility (6 h), mitochondrial activity (6 h), membrane (2 and 6 h) and

DNA integrity (4 h), was positively affected by the supplementation of GPx, compared to the control group (**Paper III**). Supplementation of semen extender with GPx at the same concentration as used in domestic cats, had positive effects on sperm motility (after cold storage for 1 h and 6 h after thawing) and mitochondrial activity (0 and 6 h after thawing) in flat-headed cat (**Paper IV**). Although the percentage of embryo development (blastocyst rate) from heterologous IVF derived from flat-headed cat spermatozoa supplemented with GPx did not significantly differ from the control and vitamin E analogue Trolox groups, it was four-times higher than in both groups (**Paper IV**).

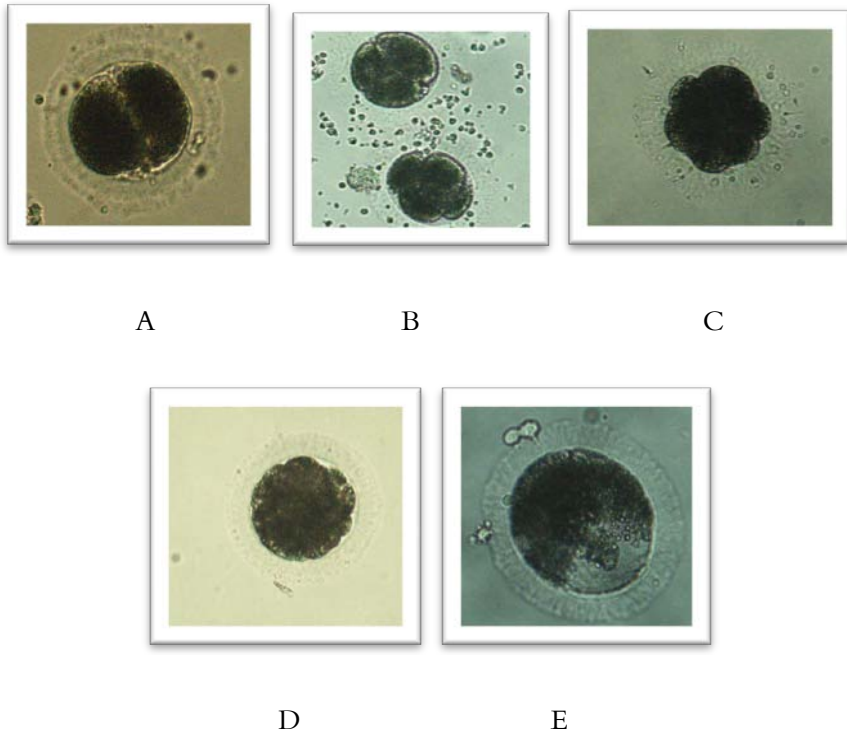


Figure 6. Embryo development derived from heterologous *in vitro* fertilization using domestic cat oocytes and cryopreserved flat-headed cat spermatozoa supplemented with glutathione peroxidase; A= 2-cell stage; B=4-cell stage; C=8-cell stage; D=morula stage, and E=blastocyst stage.

Glutathione peroxidase is an enzymatic antioxidant, normally present in human spermatozoa and in the epididymis of horses and cattle (Drevet, 2006). Lack of a subtype in the GPx protein family i.e. the GPx4 protein, is the main cause of sperm abnormality in mice (Liang *et al.*, 2009).

Glutathione peroxidase plays a key role as a defense mechanism against H_2O_2 (Drevet, 2006). In general, GPx uses GSH, a non-protein thiol found in epididymal spermatozoa, as an electron donor, converting GSH to GSSG at the same time as H_2O_2 is converted to water (Agrawal & Vanha-Perttula, 1988). Glutathione peroxidase also has a preventive effect against the decrease in sperm motility induced by spontaneous lipid peroxidation (Chabory *et al.*, 2010). Glutathione peroxidase can function even in small quantities of ROS substrates (Drevet, 2006). This might be the main reason why GPx had more potent antioxidative effects on spermatozoa than other antioxidants such as CAT and SOD (**Paper III**) and the vitamin E analogue Trolox (**Paper IV**). Supplementation of semen extenders with GPx had positive effects on sperm motility in rams (Maxwell & Stojanov, 1996) and bulls (Bilodeau *et al.*, 2001). In addition, supplementation of a sperm culture medium with GSH or exposure of sperm to GSH increased human sperm motility (Irvine, 1996), and had a positive effect on blastocyst development in pigs (Jeong & Yang, 2001). Glutathione peroxidase also had a protective effect on the mitochondrial membrane potential of cat spermatozoa (Thuwanut *et al.*, 2010). This might be because sperm mitochondria are the major site of intracellular ROS formation (Agarwal *et al.*, 2003) and can cause damage to the mid-piece (Koppers *et al.*, 2008). The presence of GPx4 protein in spermatozoa is essential for preventing mitochondrial apoptosis (Liang *et al.*, 2009); lack of this protein can cause sperm abnormalities (Liang *et al.*, 2009). Thus, it can be hypothesized that in the flat-headed cat a high incidence of abnormal spermatozoa might be due to insufficient or lack of GPx to protect sperm from ROS (**Paper IV**). When exogenous GPx was added, sperm mitochondria were protected (**Papers III and IV**). We also demonstrated that even though mitochondrial membrane potential was enhanced by the action of GPx, this parameter was not correlated to sperm motility (**Paper IV**). The mechanism by which ROS affects sperm motility is still being debated. It has been shown that the energy to support sperm flagella movement (adenosine triphosphate; ATP) is derived from two different mechanisms; oxidative phosphorylation in mitochondria and the glycolysis pathway in the principal piece of sperm flagellum (Mortimer, 1997). The results of our study (**Paper IV**) are similar to those of studies on mice (Miki *et al.*, 2004) and rhesus macaque spermatozoa (Hung *et al.*, 2008) which showed that the glycolysis pathway seems to be the major source of ATP to support sperm motility rather than oxidative phosphorylation from mitochondria.

4.1.5 Superoxide dismutase (Paper III)

Although supplementation of the semen extender with SOD had a positive effect on the membrane integrity of epididymal cat sperm at 2 h after thawing, it significantly reduced membrane integrity after cooling, linear motility at thawing and acrosome integrity at 2 h after thawing. The deleterious effects of SOD can be explained by H₂O₂ generation. Superoxide dismutase removes O₂⁻ from the cytosol of living cells, generating H₂O₂ (Silva, 2006). However, studies in ram (Maxwell & Stojanov, 1996), human (Rossi *et al.*, 2001) and Iberian red deer (Maria *et al.*, 2007) have shown that sperm motility, mitochondrial activity, acrosome and DNA integrity improved by supplementation with SOD. Contradictory effects may be related to different levels of susceptibility to H₂O₂ among species. An alternative method of enhancing the positive effect of SOD is by combination with other enzymatic antioxidants i.e. CAT or GPx, because they can detoxify H₂O₂ to water (Silva, 2006). The combination of CAT and SOD improved the percentage of viable Iberian red deer spermatozoa after cold storage (Maria *et al.*, 2007). Thus further studies combining either CAT or GPx with SOD to protect cat spermatozoa from oxidative stress could be informative.

4.2 Detection of lipid peroxidation by using a lipophilic dye probe BODIPY^{581/591} C11 and effects of lipid peroxidation in cryopreserved epididymal cat spermatozoa (Papers II and III)

Lipid peroxidation was detected in cryopreserved epididymal cat spermatozoa using the lipophilic dye probe; BODIPY^{581/591} C11 (**Papers II and III**). Its level was not significantly increased during post-thaw incubation of epididymal cat spermatozoa in Tris buffer (**Paper II**) or an egg yolk extender (**Paper III**), whereas a transition metal (Fe²⁺) induced an increase in lipid peroxidation after 6 h incubation (**Papers II and III**). Induction of lipid peroxidation by Fe²⁺ negatively affected cryopreserved epididymal cat sperm quality i.e. motility, mitochondrial activity, percentage of intact sperm membranes and DNA integrity (**Paper III**). However, the three selected types of enzymatic antioxidants could not significantly neutralize the level of lipid peroxidation induced by the transition metal (**Paper III**). The length of cold storage and post-thaw incubation is a crucial factor affecting the amount of lipid peroxidation. Cold storage of equine spermatozoa for 48 h resulted in a significant increase in the lipid peroxidation reaction (Ball & Vo, 2002) whereas 72 h cold storage was required to produce a significant increase in bovine spermatozoa (Nair *et al.*,

2006). Frozen-thawed human spermatozoa incubated for 15- and 60-min intervals at 37°C showed an increase in lipid peroxidation (Engel *et al.*, 1999). Although there was, on average, twice as much lipid peroxidation (**Paper II**) at 6 h incubation than immediately after thawing in our study (**Paper III**), this was not significantly different from the controls. The transition metal was shown to be capable of inducing lipid peroxidation in post-thaw epididymal spermatozoa via the Fenton reaction which was in accordance with a study in human spermatozoa (Aitken *et al.*, 2007). It can be hypothesized that H₂O₂ can be generated by epididymal cat spermatozoa and may provoke the oxidative stress system. Although antioxidative enzymes (CAT, GPx and SOD) could control the level of lipid peroxidation in semen during 24 h of cold storage (Kankofer *et al.*, 2005), the lipid peroxidation occurred naturally, which is in contrast to our study. The level of induced lipid peroxidation in our study might be too high to be neutralized by the selected concentrations of the enzymatic antioxidants.

4.3 Basic seminal characteristics of the captive flat-headed cat (Paper IV)

The present study is the first to report some basic seminal characteristics of captive flat-headed cats in Thailand, this species being an endangered small wild felid. The average semen volume and pH collected from the flat headed cats in this study were 121.2±72.1 µL and 8.0 respectively. The average total number of spermatozoa was 6.7±2.2 ×10⁶, which was relatively lower than in fishing cats (Thiangtum *et al.*, 2006) and ocelots (Stoops *et al.*, 2007), while being higher than in the Iberian lynx (Gañán *et al.*, 2009). Other sperm quality parameters studied, such as motility (56.3±19%), progressive motility (3.1±0.3) and acrosome integrity (30.5±7.2%), in fresh semen of flat-headed cats were also lower than reported for fishing cats (Thiangtum *et al.*, 2006), black footed cats (Herrick *et al.*, 2010) and sand cats (Herrick *et al.*, 2010). In addition, the percentages of viable spermatozoa and sperm DNA integrity were 48.7±15.4% and 81.5±7.5% in fresh spermatozoa.

Furthermore, the average percentage of morphologically normal spermatozoa obtained from these captive wild cats was lower than 40%, hence the reason that the flat headed cats in this study were regarded as suffering from teratozoospermia (> 60% morphologically abnormal spermatozoa) (Luvoni, 2006; Pukazhenthii *et al.*, 2006). Teratozoospermia in felids has been suggested to be caused by inbreeding. For the flat-headed cats included in the present study there was, however, no history of inbreeding (based on the stud book from each zoo). Two of them were also proven breeders.

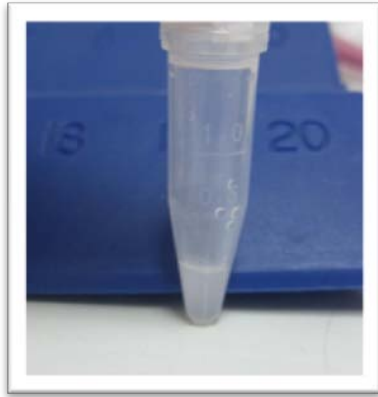


Figure 7. Fresh semen collected from flat-headed cat by electroejaculation

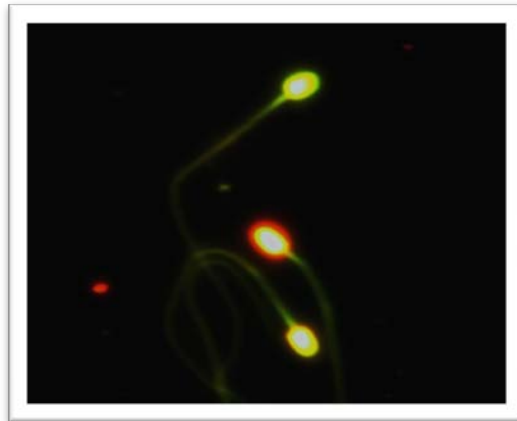


Figure 8. Flat-headed cat spermatozoa with intact DNA (green fluorescence) and damaged DNA (orange or red fluorescence)

5 Conclusions

- The addition of two different types of antioxidants; 5 mM cysteine and the water soluble vitamin E analogue Trolox, to a semen extender prior to freezing improved post-thaw epididymal cat sperm quality i.e. motility, progressive motility, membrane integrity and DNA integrity. The vitamin E analogue Trolox had a more potent antioxidative effect than the same concentration of cysteine.
- Lipid peroxidation in post-thaw epididymal cat spermatozoa could be detected with the lipophilic dye probe (BODIPY^{581/591} C11).
- The incorporation of 10 U/mL GPx to a semen extender resulted in improved quality of frozen-thawed epididymal cat spermatozoa, with regard to motility, mitochondrial activity, membrane integrity and DNA integrity. However, antioxidative enzymes at the concentrations used in the present study could not protect spermatozoa from the pronounced lipid peroxidation induced by Fe²⁺.
- The lipid peroxidation induced by Fe²⁺ had deleterious effects on post-thaw epididymal cat sperm quality. Furthermore, the level of lipid peroxidation (with or without induction by Fe²⁺) tended to increase during post-thaw incubation of epididymal cat spermatozoa.
- Semen from flat-headed cats (*Prionailurus planiceps*) could be collected using a conventional electroejaculation technique, and evaluated for basic seminal traits. However, the sperm quality was poor, indicating that this species may be affected by teratozoospermia.

- Glutathione peroxidase was a more potent antioxidant than the vitamin E analogue Trolox and improved sperm quality i.e. motility and mitochondrial membrane potential in cryopreserved flat-headed cat spermatozoa. Although these spermatozoa showed fertilizing ability *in vitro*, using heterologous oocytes from domestic cats, the percentage of embryo development did not differ among different antioxidant groups.

6 Future research

The present study investigated the role of oxidative stress and antioxidants on spermatozoa from domestic and non-domestic cats, based on the hypothesis that oxidative stress occurs during cryopreservation of spermatozoa, and is a cause of sperm damage which can be eliminated or restricted by antioxidants. Although the oxidative stress indicator, lipid peroxidation, was evaluated together with the effects of different types of antioxidants on cryopreserved domestic and non-domestic cat spermatozoa, several areas would be of interest for further research studies;

- The level of ROS during cryopreservation of domestic and non-domestic cat spermatozoa (normospermic and teratozoospermic cats) and its relation to the level of lipid peroxidation measured by using the Luminal-dependent chemiluminescence assay
- The presence of natural antioxidative enzymes (CAT, GPx/GRD or SOD) and their level in domestic and non-domestic cat spermatozoa (normospermic and teratozoospermic cats) and in seminal plasma
- The effects of the various concentrations and types of antioxidant supplements (i.e. cysteine, glutathione, vitamin A, vitamin C, vitamin E, CAT, GPx/GRD or SOD) added to semen extenders for both chilled and frozen-thawed domestic and non-domestic cat spermatozoa
- To optimize the composition of semen extenders for the preservation of domestic and non-domestic cat spermatozoa (for commercial purposes and conservation purposes respectively) for AI, IVF and ET

7 Popular scientific summary

Breeding programs for both pure-bred and wild cats have been investigated over the past decades. There is an increasing demand for pure-bred cats as pets as well as for conservation of wild cats, which show continuously declining population sizes caused by the destruction of the forest for human settlement, draining for agriculture and illegal hunting. The breeding programs are composed of several scientific techniques such as freezing of spermatozoa, artificial insemination or *in vitro* fertilization (popularly known as test tube babies) and embryo transfer. Sperm freezing confers several advantages: (1) it can help to increase genetic variation, for example, frozen spermatozoa can be used for artificial insemination when a female cat and a suitable male are in different locations, or to avoid inbreeding in small population of wild cats in fragmented habitats which means that freezing of spermatozoa can facilitate a genetic exchange over a long distance and increase a genetic variation, (2) it can be used to overcome physical problems during natural mating or behavioral incompatibility i.e. wild cats which sometimes can kill each other during attempted natural mating in zoos, (3) frozen semen from one male cat can be used several times in several matings with the same or different female cats, and (4) there is the possibility of producing offspring long after the male cat is dead. Moreover, scientific progress has made it possible to use frozen semen in *in vitro* fertilization when natural mating and artificial insemination cannot be achieved.

Sperm quality after freezing and thawing is one parameter that affects the chance of success in producing offspring after artificial insemination or *in vitro* fertilization. However, several factors involved in sperm freezing, can impair sperm quality such as cold shock, osmotic and oxidative stress. The negative effects of the “cold shock” factor can be avoided by using an optimal sperm cooling/freezing/thawing rate, whereas the “osmotic stress” factor can be avoided by adding optimal concentrations of cryoprotective agents to the semen extender. The “oxidative stress” factor has not been studied to a great extent in cats, in contrast to other species. Oxidative stress

is caused by an imbalance between oxidants (so called reactive oxygen species or ROS and lipid peroxidation) and antioxidants in a specific, defined environment. During sperm freezing, the sperm cells' artificial environment is the so-called semen extender. Semen extenders are added to spermatozoa to provide nutrients and to protect against sperm damage induced by the freezing process. However, semen extenders used for cat spermatozoa still need to be improved to achieve better sperm quality after freezing and thus to increase the probability of obtaining offspring. Therefore, the scope of this study was the development of semen extenders that could reduce the damage caused by oxidative stress during cryopreservation of cat spermatozoa. Freezing of epididymal domestic cat spermatozoa was used to develop the extender as a model for wild felid spermatozoa, because cat spermatozoa can be collected after routine castrations and are therefore more readily available than the spermatozoa of rare felid species. Domestic cats have been shown to be a relevant reproductive model for wild cats. The knowledge obtained from studies on domestic cat spermatozoa was applied to an endangered wild cat species, the flat-headed cat, in the last part of this thesis.

The hypothesis of this study was that cat spermatozoa can be damaged by oxidants produced during the cryopreservation process and that the negative effects of these oxidants on cat spermatozoa could be restricted or eliminated by the action of antioxidants. Antioxidants can be classified into two types; non-enzymatic (e.g. cysteine, vitamin E analogue Trolox) and enzymatic antioxidants (catalase, glutathione peroxidase, superoxide dismutase). In the present study, lipid peroxidation was observed in domestic cat spermatozoa after freezing and thawing, as well as after 30 minutes and 6 hours incubation post-thawing, indicating that oxidative stress might be one important cause of cat sperm damage. After supplementation of the semen extender with non-enzymatic (cysteine, or the vitamin E analogue Trolox) or enzymatic antioxidants (catalase, glutathione peroxidase, superoxide dismutase), we found that most of the post-thaw epididymal sperm quality parameters such as motility, viability and DNA integrity were improved compared to control groups without antioxidants. When the findings from domestic cat spermatozoa were applied to extenders for flat-headed cat spermatozoa, the addition of glutathione peroxidase had more positive effects on post-thaw sperm quality than supplementation with vitamin E. Also the rate of successful embryo development (*in vitro* fertilization) was higher for semen diluted in an extender supplemented with glutathione peroxidase than for supplementation with the vitamin E analogue Trolox, although this difference was not significant. In conclusion, of the antioxidants evaluated in this thesis, glutathione peroxidase improved semen quality most after freezing and thawing for both domestic and non-domestic cat spermatozoa. The findings from this study might be potentially

applicable for both domestic cat breeders and for conserving endangered wild cats worldwide.

8 Populärvetenskaplig sammanfattning

Forskning som syftar till att ta fram avelsprogram som omfattar spermafrysning, artificiell insemination (AI), *in vitro* fertilisering (populärt kallat provrörsbefruktning) och embryoöveföring för tamkatter och vilda kattdjur har pågått under årtionden. Många populationer av vilda kattdjur minskar ständigt framför allt på grund av skogsavverkning, torrläggning av mark för jordbruk och illegal jakt. Frysning av sperma är en basal teknik som medför flera fördelar. Till exempel kan frysta spermier användas för artificiell insemination när: 1) en hona och en hane är åtskilda av ett stort geografiskt avstånd; 2) det är svårt att få till stånd en parning, exempelvis kan vilda kattdjur ibland attackera och i värsta fall döda varandra när de sätts ihop för parning i en djurpark; 3) fryst sperma från en hane ska användas till flera inseminationer av samma eller olika honor; 4) man vill få avkommor efter att hanen är död. Vetenskapliga framsteg har gjort det möjligt att använda fryst sperma för *in vitro* fertilisering när naturlig parning eller artificiell insemination inte är ett alternativ.

Möjligheten att få avkommor efter artificiell insemination eller *in vitro* fertilisering påverkas av den kvalitet spermerna har efter frysning och upptining. Flera faktorer involverade i spermafrysningsprocessen kan försämra spermakvaliteten. Exempel på sådana faktorer är köldchock, osmotisk stress och oxidativ stress. Genom att optimera kyl-, frys- och upptiningshastigheten kan man reducera skadorna på spermerna orsakade av köldchock, medan osmotisk chock kan minskas genom att tillsätta kyl- och frysskyddande substanser (t ex glycerol) till den spädningvätska som man tillsätter innan spermerna fryses. Effekterna av köldchock och osmotisk chock har tidigare studerats ingående vid konservering av spermier från kattdjur. Oxidativ stress har studerats utförligt vid spermakonservering hos andra djurslag, men kunskapen är bristfällig när det gäller tamkatt och vilda kattdjur. Oxidativ stress uppstår genom en obalans mellan oxidanter (s.k. fria radikaler och lipidperoxidering) och antioxidanter i en specifik miljö. Under spermafrysningen utgör de spädningvätskor som tillsätts den specifika miljön för spermerna. Spädningvätskornas syfte är att ge spermerna näring och att

skydda dem från skador orsakade under frysningsprocessen. För att uppnå bästa möjliga spermiekvalitet efter frysning och upptining behöver spädningvätskorna, som används till spermier från tamkatter och vilda kattdjur, förbättras vilket skulle öka möjligheterna att få avkommor efter användning av AI. Syftet med denna studie var därför att utveckla spädningvätskor, som minskar skador på spermier orsakade av oxidativ stress under frysningen. Tamkatter har tidigare visat sig vara en bra modell för vilda kattdjur när det gäller basala reproduktionsstudier. Spermier från bitestikeln från tamkatter har därför använts som en modell för vilda kattdjur. Tamkatter kastreras rutinmässigt varför det är lätt att få tag på spermier från bitestiklarna efter kastration, medan det är svårare och mindre lämpligt att använda vilda kattdjur för basala studier av spermiefunktionen. Resultaten från dessa studier av spermier från tamkatten applicerades sedan i den sista artikeln på ett vilt kattdjur, platt huvudkatt (*Prionailurus planiceps*).

Hypotesen i avhandlingen är att spermier skadas av fria radikaler, som produceras under frysprocessen, och att de negativa effekterna av de fria radikalerna kan minskas genom att tillsätta antioxidanter till spädningvätskorna. Antioxidanterna kan delas in i två klasser; icke-enzymatiska antioxidanter (t ex cysteine och vitamin E analogen Trolox) och enzymatiska antioxidanter (t ex katalas, glutathione peroxidase och superoxid dismutas). Lipidperoxidation observerades i tamkatt spermier direkt efter upptining och efter 30 samt 60 minuters inkubation efter upptining av fryst sperma. Detta tyder på att oxidativ stress kan vara en orsak till spermieskador. Icke-enzymatiska (cysteine och Trolox) eller enzymatiska (katalas, glutathione peroxidase och superoxid dismutas) antioxidanter tillsattes spädningvätskan. Flera parametrar för spermiekvalitet såsom spermierörlighet, livsduglighet och DNA-integritet var bättre när Trolox eller glutathione peroxidase ingick i spädningvätskan än i kontrollprov utan tillsats av antioxidanter. När de bästa resultaten från studierna av tamkatt spermier applicerades på spermier från platt huvudkatt hade glutathione peroxidase mer positiva effekter än vitamin E. Även andelen embryon som utvecklades efter *in vitro* fertilisering var högre med tillsats av glutathione peroxidase än med vitamin E. Denna skillnad var dock inte statistiskt signifikant. Slutsatsen är att av de antioxidanter som utvärderades i denna studie förbättrade glutathione peroxidase spermiekvaliteten mest efter frysning och upptining, både för spermier från tamkatt som platt huvudkatt. Tillämpning av resultaten från denna studie kan vara till nytta såväl vid assisterad avel av tamkatter som för bevarande av hotade vilda kattdjur.

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