The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa

Paweena Thuwanut

Department of Clinical Sciences
Faculty of Veterinary Medicine and Animal Science

Master of Science Programme in Veterinary Medicine for International Students
Swedish University of Agricultural Sciences

Uppsala 2007

Report- Master of Science Programme in Veterinary Medicine for International Students
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences
Report no.67
ISSN 1403-2201

The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa

Paweena Thuwanut

Department of Clinical Sciences
Faculty of Veterinary Medicine and Animal Science

Master of Science Programme in Veterinary Medicine for International Students
Swedish University of Agricultural Sciences
Uppsala 2007

The present thesis is a partial fulfillment of the requirements for the Master of Science (MSc) Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Animal Reproduction.

Paweena Thuwanut
Division of Reproduction
Department of Clinical Sciences, P.O.Box 7054
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences (SLU),
Center for Reproductive Biology in Uppsala (CRU),
SE-750 07 Uppsala, Sweden

To my beloved parents, family and teachers…..

We make a living by what we get, but we make a life by what we give…….
(Winston Churchill)

Abstract

Thuwanut, P.2007. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa. Master Thesis. ISSN 1403-2201 Report no. 67

Living cells stored under aerobic conditions require oxygen (O_2) to support their metabolism. Their metabolite products are reactive oxygen species (ROS). In general, the ROS need to be continuously inactivated to keep a minimal amount necessary to maintain normal cell function but an excessive accumulation can cause cell damage. In addition, ROS can be produced and accumulated during the freezing and thawing process of mammalian spermatozoa. However, an adverse effect of ROS can be reduced by antioxidants. The aim of this study was to investigate effects of antioxidant (cysteine or water soluble vitamin E analogue Trolox) supplementation of a tris egg yolk extender on post-thaw epididymal cat spermatozoa. Epididymal spermatozoa were collected from eight male cats. The sperm sample from each cat was divided into three aliquots, resuspended with (1) tris egg yolk extender I (EE-I), (2) tris egg yolk extender I with cysteine (EE-C) or (3) tris egg yolk extender I with vitamin E (EE-Ve) and extended with tris egg yolk extender with Equex STM paste (EE-II) for freezing. Sperm motility, progressive motility, membrane integrity stained with SYBR-14/EthD-1 and acrosome status stained with FITC-PNA/PI were evaluated after collection, prior to freezing at 4 ºC and 0, 2, 4, and 6 h post-thaw. Acridine orange was used to evaluate DNA integrity at 0 and 6 h post-thaw. Vitamin E supplementation had positive effects on post-thaw motility, progressive motility and membrane integrity ($P<0.05$), while cysteine supplementation improved post-thaw motility and DNA integrity ($P<0.05$). However, antioxidant supplementation had no significant positive effect on post-thaw acrosome integrity ($P>0.05$). These results demonstrated that cysteine and vitamin E supplemented to the egg yolk tris extender can improve post-thaw epididymal cat spermatozoa qualities such as motility, progressive motility, membrane integrity and DNA integrity but not acrosome integrity. In conclusion, when the tris egg yolk extender containing Equex STM paste is used, the addition of cysteine or vitamin E is recommended in order to protect post-thaw epididymal cat spermatozoa from ROS-induced sperm damage.

Keywords: Feline; Frozen-thawed sperm; Reactive oxygen species; Antioxidative enzyme; Non-enzymatic antioxidant

Author's address: Paweena Thuwanut, Division of Reproduction, Department of Clinical Sciences, P.O.Box 7054, Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences (SLU), Center for Reproductive Biology in Uppsala (CRU), SE-750 07 Uppsala, Sweden

Contents

Background, 9
Semen collection and utility of sperm preservation, 9
Causes of sperm damage, 11
Physiology and effects of oxidative stress on sperm functions, 12
Physiology and effect of antioxidants on oxidative stress and sperm functions, 13

Introduction to research report, 16

Aims of the study, 18

References, 19

Research Report, 23
Paweena Thuwanut, Kaywalee Chatdarong, Eva Axnér
The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa

Conclusions, 37

Additional comments and future prospects, 38

Acknowledgements, 39

Background

Of the 36 species of non-domestic cats in the world nine live in Thailand. These include Leopard cat (*Prionailurus bengalensis*), Fishing cat (*Prionailurus viverrinus*), Flat headed cat (*Felis planiceps*), Asiatic golden cat (*Profelis 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 which might impair fertility (Pukazhenthi et al., 2006). Because of the difficulties in captive breeding of wild cats and an increasing interest in breeding of pedigree cat, protocols for cat sperm preservation and gene banking for conservation are established and developed. The domestic cat (*Felis silvestris catus*) has proven to be a suitable model for development of assisted reproductive techniques (ARTs) (Donoghue et al., 1992) such as sperm preservation or artificial insemination. Reproductive biotechnologies might, however, also have an application in the domestic cat. There is an increasing interest in requiring pedigree domestic cats as pets. Pedigree cats are bred in isolated populations. Most pedigree male cats kept as pets are early castrated to decrease behavioral problems such as spraying. This contributes further to a tendency for decreasing genetic variation.

Sperm cryopreservation together with artificial insemination could contribute to a genetic exchange over long distances and could also be used together with other ARTs such as *in vitro* fertilization or embryo transfer. Cryopreservation of spermatozoa can be used to preserve both ejaculated and epididymal cat spermatozoa. Although the protocols for both ejaculated and epididymal domestic cat sperm cryopreservation have been continuously improved, several factors can impair sperm survivability and cause sperm damage after cryopreservation such as cold shock, osmotic (Watson, 2000) or oxidative stress (Alvarez & Storey, 1992; Aitken & Krausz, 2001; Sikka, 2001, Agarwal et al., 2003). Cryopreservation induced sperm damages are related to diminished fertilizing ability of the spermatozoa.

Semen collection and utility of sperm preservation

The main obstacles in the development of ARTs in the domestic cat are related to difficulties associated with sperm collection and handling. The volume of ejaculated semen and the total number of spermatozoa are low as shown in Table 1. Therefore experimental design is associated with limitations compared with development of ARTs is other species in which higher sperm numbers can be collected, such as dog, boar or stallion.

Feline spermatozoa can be collected by an artificial vagina, by electroejaculation of anesthetized male cats and from the cauda epididymides (Axnér & Linde-Forsberg, 2002). Sperm samples collected by electroejaculation or from the

epididymides have lower sperm concentration and lower total number of spermatozoa than samples collected by an artificial vagina as shown in Table 1. However, sperm collection by the electroejaculation and from the epididymides is often more practical than collection by an artificial vagina because the male cats do not need to be trained. Furthermore, collection of epididymal spermatozoa makes it possible to save valuable genetic material from felids that die unexpectedly or that are castrated. Epididymal cat spermatozoa showed lower initial motility and viability than ejaculated spermatozoa but there were no significant differences between ejaculated and epididymal spermatozoa in their sensitivity to chilling or freezing-thawing process (Tebet et al., 2006; Hermansson & Axnér 2007). In addition, cat spermatozoa can be maintained within epididymides stored at 4ºC for 24 h without any significant decrease of sperm quality compared to epididymal spermatozoa that were immediately harvested into storage media (Chatdarong et al., 2006). Therefore shipment of cooled testes can be a valuable alternative to conserve the genetic material from wild felids especially when the testis of wild felids can not be immediately transported to a laboratory for the cryopreservation process.

Table. 1. Semen parameters in male domestic cats

Parameter	**Semen collection method**		
	Artificial vagina	**Electroejaculation**	**Cauda epididymides**
Volume (mL)			
• Average	0.034 to 0.04	0.076 to 0.22	*
• Range	0.01 to 0.12	0.019 to 0.74	
Sperm concentration (10^6/mL)			
• Average	1730	168 to 361	37.8 to 80.3 (a)
• Range	96 to 5101	-	5.5 to 72
Total number of spermatozoa (10^6)			
• Average	57 to 61	12 to 30	18.9 to 122 (b)
• Range	3 to 117	9 to 153	2.25 to 160
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 50.6 (c)
Motility (%)	56 to 84		50 to 80 (d) Mean;51.9 to 80.8
pH	6.6 to 8.8		**
Osmolarity (mOsm/kg)			
• Average	320 to 339		**
• Range	274 to 390		

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

* Epididymal spermatozoa are collected into a collection media why the volume of the epididymal fluids is not measured
** There is no information available about pH and osmolarity in the feline cauda epididymidis (Axnér, 2006).

(a) Goodrowe & Hay, 1993; Axnér et al., 2004; Tebet et al., 2006; from research report part of this thesis
(b) Tsutsui et al., 2003; from research report part of this thesis
(c) Goodrowe & Hay, 1993; Axnér et al., 2004
(d) Goodrowe & Hay, 1993; Axnér et al., 2004; Kashiwazaki et al., 2005; Hermansson & Axnér, 2007

Furthermore, it was shown that, the addition of Equex STM paste to an egg yolk tris extender improved post-thaw acrosome integrity of epididymal cat spermatozoa (Axnér et al., 2004). The potential usefulness of frozen-thawed cat epididymal spermatozoa has been demonstrated by their ability to bind to homologous zona pellucida (Kashiwazaki et al., 2005) *in vitro* and for *in vivo* fertilization after artificial intrauterine insemination resulting in birth offspring (Tsutsui et al., 2003).

Causes of sperm damage

In felid species, a main barrier in the success of artificial insemination or other ARTs is poor sperm quality and low sperm survivability after cryopreservation (Pukazhenthi et al., 1999). Sperm cryopreservation involves several steps such as centrifugation, sperm 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 classified as (1) cold-shock (Watson, 2000), (2) osmotic stress (Watson, 2000) and (3) oxidative stress (Aitken & Krausz, 2001; Sikka, 2001, Agarwal et al., 2003). Cooling before the freezing process causes stress to the spermatozoa due to the temperature changes that result in sperm damage known as cold shock (Watson, 1981; Luvoni, 2006). In one study, a rapid or ultra rapid cooling rate (4°C/min or 14°C/min) resulted in acrosomal damage while a slow cooling rate significantly reduced the acrosomal damage (0.5°C/min) (Pukazhenthi et al., 1999).

The addition of cryoprotectants such as glycerol or dimethyl sulfoxide (DMSO) is used to minimize the formation of ice crystals. However, addition of cryoprotectants results in sperm shrinkage because water efflux from cells due to hyperosmotic environment and then spermatozoa swells due to water and cryoprotectant influx to cells for maintaining isotonic solution (Gao et al., 1997). Extensive sperm membrane damages occurred when cat spermatozoa were returned from hyperosmotic conditions to the isotonic solution (Phukazhenthi et al., 2000). Pukazhenthi et al. (2000) also demonstrated that cat sperm motility is sensitive to changes in osmolarity similar to human (Curry & Watson, 1994), ram (Curry & Watson, 1994) or stallion spermatozoa (Ball & Vo, 2001).

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

Physiology and effects of oxidative stress on sperm functions

The term of oxidative stress refers to an imbalance between 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 sperm cooling, freezing and thawing, require oxygen (O_2) to support their normal metabolism. Excessive metabolites of oxygen such as ROS can, however, cause cell damage or apoptosis (de Lamirande & Gagnon, 1995). The ROS are free radicals and thus have an unpaired electron. The unpaired electron finds other electrons to become paired. Thus, the free radicals always attack other molecules to complete their unpaired electron (Nogushi & Niki, 1999). Examples of ROS are superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) or hydrogen peroxide (H_2O_2) (Halliwell, 1997; Nogushi & Niki, 1999; Silva, 2006).

The ROS have been reported as a main cause in human diseases such as cardiovascular, cancer or neurological disease (Papas, 1999). Furthermore, an adverse effect of ROS on mammalian spermatozoa and reproduction was firstly reported by McLeod (1943). Since then several studies have demonstrated that ROS have been 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 and immotile or abnormal spermatozoa (Silva, 2006, Engel et al., 1999).

Generally, ROS must be continuously inactivated to keep a minimal amount of them necessary to maintain normal cell functions (Agarwal et al., 2003). Low levels of ROS are for example beneficial for the ability of spermatozoa to undergo the acrosome reaction and capacitation (de Lamirande & Gagnon, 1993; de Lamirande & O'flaherty, 2007; Roy & Atreja, 2007). An excessive accumulation of ROS such as superoxide radicals ($O_2^{\cdot-}$), or hydrogen peroxide (H_2O_2) can cause stallion (Baumber et al., 2000), bull (Chatterjee & Gagnon, 2001), and human sperm plasma membrane damage and DNA damage in both mitochondrial and nuclear genomes (Aitken & Krausz, 2001). Hydrogen peroxide (H_2O_2) has the most harmful oxidant property (Alvarez & Storey, 1989; de Lamirande & Gagnon, 1992). It can freely cross cell membranes and cause DNA damage (Halliwell, 1997; Baumber et al., 2003). Moreover, Baumber et al. (2003) showed that exogenous ROS generation by the xanthine-xanthine oxidase (X-XO) system can significantly increase the percentages of DNA fragmentation in equine spermatozoa which is similar to the study of Lopes et al. (1998) on human

spermatozoa. This indicates that hydrogen peroxide (H_2O_2), the product of X-XO system, is responsible for sperm DNA damage. In addition, hydrogen peroxide (H_2O_2) can detrimentally produce hydroxyl radicals ($OH^{\cdot}$) via the Fenton reaction (Silva, 2006). Hydroxyl radicals ($OH^{\cdot}$) can directly react with DNA components (Baumber et al., 2003) leading to DNA damage.

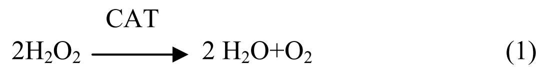
Furthermore, the mammalian sperm plasma membrane, rich in polyunsaturated fatty acids (PUFA) (Alvarez & Storey, 1995), can be easily damaged by the reaction between ROS such as hydroxyl radicals ($OH^{\cdot}$) and the polyunsaturated fatty acids. This mechanism is widely known as the lipid peroxidation 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 radicals from the initiation step attack other unsaturated fatty acid molecules on the cell membrane or steal electrons from oxygen (O_2) to become hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) can continue to attack other polyunsaturated fatty acids (PUFA) on the cell membrane. This step can continue so the lipid peroxidation reaction is also known as the chain reaction (Herrera & Barbas, 2001). In the termination step, which is the end stage of the lipid peroxidation reaction, there is combination of free radicals 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, 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 et al., 1989) and is also related to DNA strand breaks (Baumber et al., 2003).

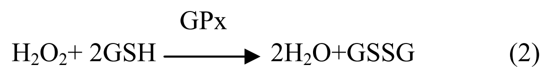
It has been demonstrated that free oxygen radicals such as superoxide radicals ($O_2^{\cdot-}$) are produced during the freezing-thawing process in human (Mazzilli et al., 1995), bovine (Chatterjee & Gagnon, 2001) and dog spermatozoa (Tselkas et al., 2000; Michael et al., 2007). In addition, the cycle of freezing and thawing has been reported to be responsible for a decrease in the level of antioxidants such as glutathione (GSH) or superoxide dimutase (SOD) in bovine (Bilodeau et al., 2000) and human (Alvarez & Storey, 1992) spermatozoa. This indicates that the ROS can cause sperm damage during the process of sperm freezing.

Physiology and effect of antioxidants on oxidative stress and sperm functions

Antioxidants act as protective agents against aerobic organism cell damage which is induced by ROS or other free radicals (Halliwell, 1997). The antioxidants are classified into two categories due to their function, 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 decomposition of the free radicals (Fig. 1) (Papas, 1999). The enzymatic antioxidants include catalase (CAT), superoxide dimutase (SOD) and glutathione peroxidase (GPx).

Fig. 1. The defense system of enzymatic antioxidants (catalase and glutathione peroxidase)





The first reaction (1) demonstrates that catalase (CAT) is a hydrogen peroxide (H_2O_2) detoxifier.
The second reaction (2) demonstrates that gluthathione peroxidase (GPx) decomposes hydrogen peroxide (H_2O_2) to be water and glutathione disulphide which is an important cellular antioxidant.

(These reactions are modified from Nogushi & Niki (1999) and Silva (2006))

The function of non-enzymatic antioxidants is to inhibit chain initiation and break chain propagation stages in the lipid peroxidation reaction (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-tocopheral) and uric acid (Nogushi & Niki, 1999; Silva, 2006).

The antioxidants can be found in mammal serum and cells and can also be found as a composition in food. A low level of antioxidants in the body system or an imbalance between antioxidants and reactive oxygen species level can lead to cell dysfunction and diseases. Therefore antioxidants can be used as medication in treatment of human disease (Papas, 1999). In human, it has been shown that infertile clients had lower levels or absence of some antioxidants in seminal plasma such as glutathione compared to fertile men (Lewis et al., 1995; Ochsendorf et al., 1998). Addition of antioxidants to semen extenders or oral feed medication with some antioxidants such as glutathione, cysteine, vitamin E or vitamin C have been reported to improve semen quality in human (Askasi et al., 1994; Sulieman 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).

Cysteine is included in Thiols (-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 glutathione (GSH) which functions as a cofactor of glutathione peroxidase to destroy hydrogen peroxide (H_2O_2) (Meister, 1994). In addition, it has been shown that glutathione (GSH) can denote hydrogen atoms to repair damaged DNA why glutathione (GSH) and the other Thiols (-SH) 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 chilled liquid 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 the seminiferous tubules of human (Erkkilä et al., 1998).

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 to prevent membrane peroxidation by scavenging the free radicals or lipid peroxyl radicals involved in the lipid peroxidation chain reaction (Herrera & Babas, 2000). Vitamin E reacting with lipid peroxyl radicals is formed to be vitamin E radicals but this free radical form is, however, stable because the free electron is delocalized to be an aromatic ring structure (Cadenas, 1995; Herrera & Babas, 2000). Thus, the radical reaction chain of lipid peroxidation is stopped. Moreover, it has been reported that in the biological system, one molecule of vitamin E can protect 1,000 molecules of unsaturated fatty acids (Kontush et al., 1996). Vitamin E supplemented to the semen extender has been reported to be more effective than vitamin C in improving post-thaw sperm motility in human (Askasi et al., 1994). The addition of vitamin E has also been shown to protect membrane integrity in chilled boar spermatozoa (Cerolini et al., 2000) while water soluble vitamin E analogue Trolox improved boar sperm motility and mitochondrial membrane integrity during post- thaw incubation (Peña et al., 2003).

Introduction to the research report

Semen preservation in domestic cats has been developed over the past years with the main aim to conserve the genetic variation in threatened wild felids. Cat spermatozoa can be collected by an artificial vagina, electroejaculation and from the epididymis (Axnér & Linde-Forsberg, 2002). Although epididymal cat spermatozoa had a lower concentration and motility than spermatozoa collected by electroejaculation, epididymal cat spermatozoa can be used to rescue and conserve genetic material from castrated or unexpectedly died male felids. Sperm collection and preservation techniques developed in the domestic cat can also be applied in endangered wild felid species (Donoghue et al., 1992). The protocols of epididymal cat sperm preservation have been continuously developed toward the ultimate goal to achieve high sperm quality. Although both chilled and frozen-thawed epididymal cat spermatozoa can be used to produce embryos after IVF (Kashiwazaki et al., 2005) or new offspring after artificial insemination (Tsutsui et al., 2003), sperm damage caused by the sperm preparation process such as cold shock (Watson, 2000), osmotic stress (Watson, 2000) and oxidative stress results in an impaired sperm function and reduced fertility (Alvarez & Storey, 1992; Aitken & Krausz, 2001; Sikka, 2001, Agarwal et al., 2003).

During the sperm preservation process, spermatozoa are generally stored under aerobic conditions. Spermatozoa require oxygen to support and maintain their normal cell functions and metabolism. The excessive oxygen metabolite products such as reactive oxygen species (ROS) are, however, considered as a cause of sperm damage such as loss of membrane fluidity, decrease in motility and increase in DNA breakage (Aitken, 1999; Baumber et al., 2000; Agarwal et al., 2003; Baumber et al., 2003). Excessive metabolite oxygen products can be eliminated or restricted by the function or the activity of antioxidants which can be generally found in the seminal plasma. An imbalance between the metabolite oxygen products and antioxidants level is known as oxidative stress (Sikka, 2001; Agarwal et al., 2003). The oxygen metabolites such as ROS can be eliminated by antioxidants. During the sperm preservation process, the seminal plasma is, however, removed and thus the antioxidants are absent. Chatterjee & Gagnon (2001) demonstrated that the ROS can also be found during the cycle of freezing and thawing bull spermatozoa which is similar in human (Mazzilli et al., 1995) and dog (Tselkas et al., 2000; Michael et al., 2007). Furthermore, when the spermatozoa were challenged with exogenous ROS such as hydrogen peroxide (H_2O_2), the sperm motility decreased and sperm DNA damage increased (Lopes et al., 1998; Baumber et al., 2003). This indicates that when spermatozoa are stored under aerobic conditions and exposed to ROS and when there is a lack of

antioxidants in the sperm preservation media, oxidative stress can cause sperm damage leading to poor sperm quality.

In order to improve the post-thaw epididymal cat sperm quality this study was conducted with the main purpose to gain knowledge about the effect of antioxidants on post-thaw epididymal cat spermatozoa quality.

Aims of the study

The overall aim of the present work has been to study the effects of antioxidants on the sperm quality in post-thaw epididymal cat spermatozoa. This could result in an improvement in the cat sperm preservation process leading to an increase in the post-thaw epididymal cat sperm quality. The specific aims of the study were

- To investigate the effects of two different type of antioxidants; cysteine and water soluble vitamin E analogue Trolox, on post-thaw epididymal cat sperm quality such as motility, progressive motility, viability, acrosome integrity and DNA integrity
- To compare the potency of cysteine and water soluble vitamin E analogue Trolox, on post-thaw epididymal cat sperm quality

References

Agarwal, A. Ramadan, A. Mohamed, A.B. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility*.79(4):829-843.

Aitken, R.J. 1989. The role of free oxygen radicals and sperm function. *Internal Journal of Andrology*. Apr; 12(2):95-107.

Aitken, R.J. Clarkson, J.S. Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of Reproduction*. Jul;41(1):183-197.

Aitken, R.J. 1999. The Amoroso Lecture. The human spermatozoon--a cell in crisis? *Journal of Reproduction and Fertility*. Jan;115(1):1-7. Review.

Aitken, R.J & Krausz, C. 2001. Oxidative stress, DNA damage and the Y chromosome. *Reproduction.* Oct;122(4):497-506.

Almeida, J. & Ball, B.A. 2005. Effect of alpha-tocopherol and tocopherol succinate on lipid peroxidation in equine spermatozoa. *Animal Reproduction Science.* Jul;87(3-4):321-337.

Alvarez, J.G. & Storey B.T. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Research*. May;23(1):77-90.

Alvarez, J.G. & Storey, B.T. 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservatio *Journal of Andrology.* May-Jun;13(3):232-241

Alvarez, J.G. & Storey, B.T. 1995. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Molecular Reproduction and Development.* Nov;42(3):334-346.

Askasi, H.A. Check, J.H, Peymer. N, Bollendorf. A. 1994. Effect of natural antioxidants tocopheral and ascorbic acids in maintenance of sperm activity during freeze-thaw process. *Archives of Andrology*; 33:11-15.

Axnér, E. & Linde-Forsberg, C. 2002. Semen collection and assessment, and artificial insemination in the cat. *Recent Advances in Small Animal Reproduction*. International Veterinary Information Service.

Axnér, E. Hermansson, U. Linde-Forsberg, C. 2004. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. *Animal Reproduction Science.*84(1-2):179-191.

Axnér, E. 2006. Sperm maturation in the domestic cat. *Theriogenology*; 66(1):14-24.

Ball, B.A. & Vo, A. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. *Journal of Andrology.* Nov-Dec;22(6):1061-1069

Baumber, J. Ball, B.A. Gravance CG., Medina ,V. Davies-Morel, M.C. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology*. 21(6):895-902.

Baumber, J. Ball, B.A. Linfor, J.J. Meyers, S.A. 2003. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *Journal of Andrology*. Jul-Aug;24(4):621-628.

Bilodeau, J.F. Chatterjee, S. Sirard, M.A. Gagnon, C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development*. 55(3):282-288.

Bilodeau, J.F. Blanchette, S. Gagnon, C. Sirard, M.A. 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*. 56(2):275-286.

Bilodeau, J.F. Blanchette, S. Cormier, N. Sirard, M.A. 2002. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*. Feb;57(3):1105-1122.

Cadenas, E. 1995. Mechanism of oxygen activation and reactive oxygen species detoxification. In. *Oxidative stress and antioxidant defenses in biology*. (edited by Ahmed, S.). Chapman and Hall. United state of America. pp. 26.

Chatdarong, K. Boonyen, T. Rungsuk, C. Chowtrakul, C. and Lohachit, C. 2006. Freezing of cat epididymal spermatozoa after cool storage. *Proceedings of the 2nd symposium of the Asian zoo and wildlife medicine and the 1st workshop on the Asian zoo and wildlife pathology*, October 26th-29th, 2006. Bangkok, Thailand. p. 20.

Chatterjee, S. & Gagnon, C. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Molecular Reproduction and Development*. 59(4):451-458.

Cerolini, S. Maldjian, A. Surai, P. Noble, R. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Animal Reproduction Science*. 58: 99-111.

Curry, M.R. & Watson, P.F. 1994. Osmotic effects on ram and human sperm membranes in relation to thawing injury. *Cryobiology*. Feb;31(1):39-46.

de Lamirande, E. & Gagnon, C. 1992. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *Journal of Andrology*. Sep-Oct;13(5):368-378.

de Lamirande, E. & Gagnon, C. 1993. Human sperm hyperactivation and capacitation as parts of an oxidative process.*Free Radical Biology & Medicine*. Feb;14(2):157-166.

de Lamirande, E. & Gagnon, C. 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Human Reproduction*. Oct;10 Suppl 1:15-21. Review.

de Lamirande, E. & O'flaherty, C. 2007. Sperm activation: Role of reactive oxygen species and kinases. *Biochimica et Biophysica Acta.* Sep 5

Donoghue, A.M. Howard, J.G. Byers, A.P. Goodrowe, K.L. Bush, M. Blumer, E. Lukas, J. Stover, J. Snodgrass, K. Wildt, D.E. 1992.Correlation of sperm viability with gamete interaction and fertilization in vitro in the cheetah (Acinonyx jubatus). *Biology of Reproduction*. Jun;46(6):1047-1056.

Engel, S. Schreiner, T. Petzoldt, R. 1999. Lipid peroxidation in human spermatozoa and maintenance of progressive sperm motility. *Andrologia*. Jan;31(1):17-22.

Erkkilä, K. Hirvonen, V. Wuokko, E. Parvinen, M. Dunkel, L. 1998. N-acetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. *Journal of Clinical Endocrinology and Metabolism*. 83(7):2523-2531.

Funahashi, H. & Sano, T. 2005. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. *Theriogenology*. 63(6):1605-1616.

Gao, DY. Mazur, P. Crister, J.K. 1997. Fundamental cryobiology of mammalian spermatozoa. In. *Reproductive tissue banking.* (A.M. Karow and J.K. Crister, Eds.), pp 263-328.

Goodrowe, KL. & Hay, M. 1993. Characteristics and zona binding ability of FRESH and cooled domestic cat epididymal spermatozoa. *Theriogenology*;40(5):967-75.

Halliwell, B. 1997. Antioxidants: The Basics-What they are and how to evaluate them. In. *Antioxidnats in disease mechanisms and therapy*. (edited by Sies, H.) Academic Press. Toronto. pp: 4.

Hermansson, U. & Axnér, E. 2007. Epididymal and ejaculated cat spermatozoa are resistant to cold shock but egg yolk promotes sperm longevity during cold storage at 4 degrees C. *Theriogenology*. Apr 15;67(7):1239-1248.

Herrera, E. & Barbas, C. 2001. Vitamin E: action, metabolism and perspectives. *Journal of Physiology and Biochemistry*. Mar;57(2):43-56. Review.

Kashiwazaki, N. Yamaguchi, R. Uesugi, R. Hishiyama, N. Kim, M. Nakatsukasa, E. Kojima, Y. Okuda, Y. Hisamatsu, S. Inomata, T. Shino, M. 2005. Sperm motility, plasma membrane integrity, and binding capacity to homologous zona pellucida of cryopreserved epididymal spermatozoa in the domestic cat. *Journal of Reproduction and Development*. 51(6):735-739.

Kontush, A. Finckh, B. Karten, B. Kohlschütter, A. Beisiegel, U. 1996. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. *Journal of Lipid Research*. Jul;37(7):1436-1448.

Lewis, S.E. Boyle, P.M. McKinney, K.A. Young, I.S. Thompson, W. 1995. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertility and Sterility*. Oct;64(4):868-870.

Lopes, S. Jurisicova, A. Sun, J.G. Casper, R.F. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Human Reproduction*. 13(4):896-900.

Luvoni, G.C. 2006. Gamete cryopreservation in the domestic cat. *Theriogenology*. Jul 1;66(1):101-111.

Mazzilli, F. Rossi, T. Sabatini, L. Pulcinelli, FM. Rapone, S. Dondero, F. Gazzaniga, P.P. 1995. Human sperm cryopreservation and reactive oxygen species (ROS) production. *Acta Eur Fertility*. Jul-Aug;26(4):145-158.

McLeod. 1943. The role of oxygen in the metabolism and motility of human spermatozoa. *J. American Journal of Physiology* -- Legacy Content, Vol. 138, Issue 3, 512-518.

Meister, A. 1994. Glutathione, ascorbate, and cellular protection. *Cancer Research*. 54:1969s-1975s.

Michael, A. Alexopoulos, C. Pontiki, E. Hadjipavlou-Litina, D. Saratsis, P. Boscos, C. 2007. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology.* Jul 15;68(2):204-212.

Nair, S.J. Brar, A.S. Ahuja, C.S. Sangha, S.P. Chaudhary, K.C. 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Animal Reproduction Science.* Nov;96(1-2):21-29.

Nogushi, N. & Niki, E. 1999. Chemistry of active ixygn species and antioxidants. In. *Antioxidant status, Diet, Nutrition, and Health* (edited by Papas, AM.) CRC Press. Washington, D.C.pp : 3-19.

Ochsendorf, FR. Buhl, R. Bästlein, A. Beschmann, H. 1998. Glutathione in spermatozoa and seminal plasma of infertile men. *Human Reproduction*. Feb;13(2):353-359.

Papas, A.M. 1999. Determinants of antioxidant status in humans. In. *Antioxidant status, Diet, Nutrition, and Health* (edited by Papas, AM.) CRC Press. Washington, D.C.pp : 24-30.

Peña, F.J. Johannisson, A. Wallgren, M. Rodiguez Martinez, H. 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science*. 15;78(1-2):85-98

Peris, S.I. Bilodeau, J.F. Dufour, M. Bailey, J.L. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. *Molecular Reproduction and Development*. Jul;74(7):878-892.

Pukazhenthi, B. Pelican, K. Wildt, D. Howard, J. 1999. Sensitivity of domestic cat (Felis catus) sperm from normospermic versus teratospermic donors to cold-induced acrosomal damage. *Biology of Reproduction*. Jul;61(1):135-141.

Pukazhenthi, B. Noiles, E. Pelican, K. Donoghue, A. Wildt, D.E. Howard, J.G. 2000. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. *Cryobiology*. Mar;40(2):139-150.

Pukazhenthi, B.S. Neubauer, K. Jewgenow, K. Howard, J.G. Wildt, D.E. 2006. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. *Theriogenology.* Jul 1;66(1):112-121.

Roy, S.C. & Atreja, S.K. 2007. Effect of reactive oxygen species on capacitation and associated protein tyrosine phosphorylation in buffalo (Bubalus bubalis) spermatozoa. *Animal Reproduction Science*. Jun 23.

Satorre, M.M. Breininger, E. Beconi, M.T. Beorlegui, N.B. 2007. α-Tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm *Theriogenology*. In Press, Corrected Proof, Available online 4 September 2007.

Sikka, S.C. 2001. Relative impact of oxidative stress on male reproductive function. *Current Medical Chemistry*. Jun;8(7):851-862. Review.

Silva, P.F.N. 2006. *Physiology of peroxidation process in mammalian sperm*. PhD Thesis. Utrech University, Ridderprint, Ridderkerk, pp. 5-36.

Storey, B.T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa.*Molecular Human Reproduction*. 3(3):203-213.
Suleiman, S.A. Ali, M.E. Zaki, Z.M. el-Malik, E.M. Nasr, M.A. 1996. Lipid peroxidation and human sperm motility: protective role of vitamin E. *Journal of Andrology*. Sep-Oct;17(5):530-537.
Tebet, J.M. Martins, M.I. Chirinea, V.H. Souza, F.F. Campagnol, D. Lopes, M.D. 2006. Cryopreservation effects on domestic cat epididymal versus electroejaculated spermatozoa. *Theriogenology*.Oct;66(6-7):1629-1632.
Tselkas, K. Saratsis, P. Karagianidis, A. Samouilidis, S. 2000. Extracellular presence of reactive oxygen species (ROS) in fresh and frozen-thawed canine semen and their effects on some semen parameters. *Dtsch Tierarztl Wochenschr*. Feb;107(2):69-82.
Tsutsui, T. Wada, M. Anzai, M. Hori, T. 2003. Artificial insemination with frozen epididymal sperm in cats. *The Journal of Veterinary Medicine Science*. Mar;65(3):397-9.
Van Zandwijk, N. 1995. N-acetylcysteine (NAC) and glutathione (GSH): antioxidant and chemopreventive properties, with special reference to lung cancer. *Journal of Cellular Biochemistry. Supplement*. 22:24-32.
Watson, P.F. 1981. The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. *Journal of Reproduction and Fertility*. Jul;62(2):483-492.
Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*. Jul 2;60-61:481-492. Review.

Research Report

The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa

Paweena Thuwanut[1,2], Kaywalee Chatdarong[2], Eva Axnér[1]

[1]Department of Clinical Sciences, P.O.Box 7054, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden
[2]Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

Abstract

Reactive oxygen species (ROS) are produced and accumulated during cryopreservation of spermatozoa. Their adverse effects can be reduced by antioxidants. The aim of this study was to investigate the effect of antioxidant (cysteine or water soluble vitamin E analogue Trolox) supplementation of a tris egg yolk extender on post-thaw epididymal cat spermatozoa. Epididymal spermatozoa were collected from eight male cats. The sperm sample from each cat was divided into three aliquots, resuspended with (1) tris egg yolk extender I (EE-I), (2) egg yolk tris extender I with cysteine (EE-C), or (3) tris egg yolk extender I with vitamin E (EE-Ve), and extended with tris egg yolk extender with Equex STM paste (EE-II) for freezing. Sperm motility, progressive motility, membrane integrity (SYBR-14/EthD-1), and acrosome status (FITC-PNA/PI) were evaluated after collection, prior to freezing, and 0, 2, 4, 6 h post-thaw. DNA integrity (Acridine orange) was evaluated at 0 and 6 h post-thaw. Vitamin E supplementation had positive effects on post-thaw motility, progressive motility, and membrane integrity ($P<0.05$), while cysteine supplementation improved post-thaw motility and DNA integrity ($P<0.05$). However, antioxidant supplementation had no significant positive effect on post-thaw acrosome integrity ($P>0.05$). These results demonstrate that cysteine and vitamin E supplementation of tris egg yolk extender can improve post-thaw epididymal cat spermatozoa qualities such as motility, progressive motility, membrane integrity, and DNA integrity, but not acrosome integrity. In conclusion, when egg yolk tris extender containing Equex STM paste is used, the addition of cysteine or vitamin E is recommended in order to protect post-thaw epididymal cat spermatozoa from ROS-induced damage.

***Keywords*:** Feline; Frozen-thawed sperm; Reactive oxygen species; Antioxidative enzyme; Non-enzymatic antioxidant

1. Introduction

Cryopreservation of epididymal cat spermatozoa has a potential as a tool for rescuing genetic materials from felids that die unexpectedly or that are castrated [1, 2]. However, sperm damage can occur during the freezing and thawing process [3], due to factors such as cold shock [4], and osmotic [5] or oxidative stress.

Living cells stored under aerobic conditions require oxygen (O_2) to support their metabolism; however, an excess of metabolites such as reactive oxygen species (ROS) can cause cell damage [6]. The ROS include superoxide radicals ($O_2^{\cdot -}$), hydroperoxyl radicals ($HO_2^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$) [7]. Excessive accumulation of ROS products such as hydrogen peroxide (H_2O_2) has been shown to be toxic to human [5] stallion [8, 9] and bull spermatozoa [10]. Hydrogen peroxide (H_2O_2), the end product of superoxide radicals ($O_2^{\cdot -}$), is considered to be the most harmful oxidant [11] and the main cause of sperm DNA damage in both human [12, 13] and stallion [14]. These ROS need to be continuously inactivated, retaining only the minimal amount necessary to maintain normal cell function [6]. ROS can be detected during the cycle of freezing and thawing of spermatozoa in bull [10] and stallion [15], resulting in a decrease in sperm function following cryopreservation [10].

The sperm cell membrane has a high content of polyunsaturated fatty acids, and so it is easily damaged by ROS, particularly the hydroxyl radical ($OH^{\cdot}$). This biomedical reaction is known as lipid peroxidation reaction [7]; it results in changes of sperm membrane fluidity, loss of membrane integrity and decrease of sperm motility [16].

ROS can be neutralized by the combined action of antioxidative enzymes and non-enzymatic antioxidants, resulting in a cellular oxidation/reduction reaction (redox) homeostasis [7]. Addition of cysteine has been shown to prevent loss of sperm motility by inhibition of lipid peroxidation caused by ROS (hydrogen peroxide; H_2O_2) in frozen-thawed bull semen [17]. Funahashi and Sano (2005) [18] have demonstrated that cysteine can enhance viability of boar spermatozoa in liquid preservation. Furthermore, a form of cysteine known as N-acetyl-L-cysteine has been shown to be advantageous for inhibition of programmed sperm cell death (apoptosis) in the seminiferous tubules of humans [19]. Therefore, cysteine is likely a useful effective potential antioxidant which can reduce the toxic effect of the ROS during sperm metabolism.

Vitamin A (retinol), vitamin C (ascorbic acid) and vitamin E (tocopherol) are non-enzymatic antioxidants [7]. Vitamin E can inhibit the propagation stage of lipid peroxidation in the membranes by scavenging peroxyl ($RO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and other lipid derived radicals [7]. Vitamin E has been reported to be more effective than vitamin C in improving post-thaw sperm motility in humans [6, 20]. Addition of vitamin E (α-tocopherol) has been shown to protect membrane integrity in chilled boar spermatozoa [21] while the water-soluble vitamin E analogue Trolox has been shown to improve boar sperm motility and mitochondrial membrane integrity during post-thaw incubation [22].

The protocol for freezing cat spermatozoa has been continuously developed towards the ultimate goal of achieving high post-thaw quality. Addition of Equex STM paste to an egg yolk tris extender has been reported to improve post-thaw acrosome integrity but decrease post-thaw sperm longevity [1]. The addition of antioxidative enzyme or non-enzymatic antioxidant such as cysteine and vitamin E to semen preservation media has been reported to improve chilled and post-thaw sperm longevity and quality in several animal species [17-18, 20-22] but not yet in cats. Thus, the aim of the present study was to investigate whether two selected antioxidants, cysteine and the water soluble vitamin E analogue Trolox, would improve the quality of cat epididymal spermatozoa during the process of cryopreservation and post-thaw incubation.

2. Materials and methods

2.1. Animals

The study included eight privately owned domestic male cats of mixed breeds, aged between 8 and 16 mo, subjected to routine castration at the Small Animal Hospital of the Swedish University of Agricultural Sciences. After castration, the testes were immediately transported to the laboratory at room temperature for processing.

2.2. Sperm recovered and cryopreservation media

The semen extenders were composed of two components, I and II (Table 1). Component I, prepared according to the protocol described by Axnér et al. [1,23], was used alone as a control (EE-I). To create the experimental extenders, component I was supplemented with 5 mM DL-cysteine (Sigma Chemical Co., St Louis, MO, USA) (EE-C) or 5 mM Trolox (Aldrich Chem. Co, WI, USA) (EE-Ve). Component II had the same composition as component I except that it contained 7% glycerol (v/v) and 1% Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA) (pH 6.5, 1352 mOsm) (EE-II). The tris buffered solution used as a medium for sperm recovery and thawing had the same composition as EE-I but it did not contain egg yolk or glycerol. The composition of components I and II, and the thawing medium are presented in Table 1. All media were prepared in the same batch at room temperature, and kept at -20˚C until used.

2.3. Collection of epididymal spermatozoa

The caudae epididymidum were dissected free of the visible blood vessels and connective tissues, placed in a 700 μL warmed tris buffer, and transversely cut into four pieces. After 10 min incubation at 38˚C, the tissue was removed and an aliquot of 20 μL of the fresh sample was evaluated for sperm motility, progressive motility, membrane integrity, acrosome integrity, and sperm concentration. The sample was then divided into three equal aliquots of approximately 200 μL, placed in 0.5 mL small Eppendorf tubes, and centrifuged at 600 X g for 6 min.

2.4. Freezing and thawing of spermatozoa

After removal of the supernatant, a sperm pellet was extended at room temperature (approximately 20˚C) with 85 μL of EE-I, EE-C, or EE-Ve. The

sperm sample was placed in a bench cooler and slowly cooled to 4 ˚C for 1 h. An aliquot of 20 µL of each sperm sample was taken for evaluation of sperm motility and progressive motility (5 µL), sperm membrane integrity (5 µL), and acrosome integrity (10 µL). A volume of 65 µL of EE-II was added to the sample, giving a final concentration of 5% glycerol and 0.5% Equex STM paste. For each cat, a volume of approximately 0.06 mL was loaded into each of two straws. Before loading of the extended sample, 10 µL of a 1:1 (v/v) mixture of the egg yolk tris extender (EE-I, EE-C, or EE-Ve) and EE-II was loaded into the straw to fill the cotton plug of the straw in order to prevent loss of the spermatozoa in the plug.

The straws were frozen as described by Rota et al. [23]. In brief, a maximum of six straws were put into a goblet and a cane. The goblet was put into a canister which was lowered into an Apollo SX-18 liquid nitrogen (LN_2) tank with a level of 16-18 cm of LN_2 (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 the liquid nitrogen.

For thawing, the straws were thawed in a water bath at 70˚C for 6 sec and emptied into a small Eppendorf tube containing 65 µL of warmed thawing medium (1:1 v/v). One straw was thawed for evaluation of motility, progressive motility, membrane, and acrosome integrity, while the other was used to assess DNA integrity. The sperm samples were kept dark at 38˚C for 5 min before evaluation. Motility, progressive motility, membrane integrity, and acrosome integrity were evaluated at 0, 2, 4, and 6 h post-thaw, and DNA integrity at 0 and 6 h post-thawing.

2.5. Sperm evaluation

2.5.1. Sperm concentration, motility and progressive motility

Sperm concentration was determined using a hemocytometer chamber (Boeco, Humburg, Germany). To evaluate sperm motility and progressive motility, a 5 µL aliquot of the sperm sample was placed on a pre-warmed glass slide, covered with a warmed cover slip, and subjectively assessed under a phase contrast microscope at magnification: X200. The motility was reported with the percentage of motile spermatozoa. The quality of progressive motility was scored on a scale from 0 to 5; with 0 being immotile spermatozoa and 5 spermatozoa that could move fast forward and straight.

2.5.2. Sperm membrane integrity

Sperm membrane integrity was determined according to the protocol described by Axnér et al. [1]. In brief, a 5 µL aliquot of the sperm sample was mixed thoroughly with 1 µL of 14 µM EthD-1 (Molecular probes Inc., OR, USA) in PBS and 1 µL of 0.38 µM SYBR-14 (Molecular probes Inc., OR, USA) in DMSO, and then incubated in the dark at 37˚C for 30 min. The sperm sample was placed on a glass slide and covered with a cover slip. Sperm evaluation was performed under epifluorescent microscope (Laborlux-11 Leitz, Jena, Germany) with objective lens set (ParaLens®) (Becton Dickinson; Lieden, The Netherlands). Two hundred spermatozoa were evaluated and classified into three categories — live, moribund, and dead spermatozoa — which stained green from SYBR-14, stained both green and red, and stained red from EthD-1, respectively.

2.5.3. Acrosome integrity

A 10 µL aliquot of the sperm sample was gently smeared on the glass slide, air-dried, and membrane permeabilised with 95% ethanol for 30 sec. FITC-PNA staining was used for evaluation of acrosome integrity as described by Axnér et al. [1]. Briefly, a 90 µL aliquot of FITC-PNA (100 µg/mL in PBS) was mixed with 5 µL of propidium iodide (PI, 340 µM in PBS, final concentration in 18 µM), and 20 µL of this solution was spread over the smeared slide. The slide was incubated in the dark in a humidity chamber at 4˚C for 30 min. After incubation, the slide was rinsed with 4˚C distilled water and air-dried in the dark at 4˚C. Two hundred spermatozoa were evaluated under the epifluorescent microscope and classified into three categories: intact acrosome (stained with bright green from FITC-PNA at the acrosomal cap), damaged acrosome (stained with green and red), and missing acrosome (stained with red from PI) [1, 24].

2.5.4. DNA integrity

The method for evaluation for DNA integrity of spermatozoa using an Acridine orange (AO) was modified from the method used for evaluation of human spermatozoa [25, 26]. Briefly, a 10 µL aliquot of sperm sample was gently smeared on a glass slide, and air-dried. The smeared slide was fixed overnight or for at least 3 h in daily prepared methanol-glacial acetic acid (Carnoy's solution; 3:1 v/v) at room temperature. The slide was removed from the fixative solution, air-dried, and then stained with 1% (100 mg/mL) AO [26] (Sigma) in distilled water for 5 min. After staining, the slide was gently rinsed by a stream of distilled water and covered with the cover slip. Two hundred spermatozoa were evaluated under an epifluorescent microscope. The heads of the sperm cells with normal DNA integrity (double-stranded) had 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. The AO staining solution was prepared by adding 10 mL of 1% AO in distilled water to 40 mL of 0.1 M citric acid (Merck, Darmstadt, Germany) and 2.5 mL of 0.3 M $Na_2HPO_4.7H_2O$ (Merck, Darmstadt, Germany) pH 2.5. The AO staining solution was prepared daily and stored in the dark at room temperature until use.

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Analysis Systems software package (Version 9, SAS Institute Inc., 2002, Cary, NC). Normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. The dependent variables (percentages of sperm motility, progressive motility, 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 a paired-t test when the data were distributed normally and the Wilcoxon test when the data were not distributed normally. The statistical models for all parameters

included the fixed effect of time and treatment. The interaction between time and the treatments in each parameter was analyzed using the General Linear Model (GLM). Values are presented as mean ± SD. The level of significance was set at $P \leq 0.05$.

3. Results

The range of sperm concentration from the eight male cats was 5.5-72.0 x 10^6/mL, while the mean sperm concentration was 37.8±23.9 x 10^6/mL. The effect of time was significant, with all sperm evaluation parameters decreasing with time. There was, however, no interaction between time and treatment in any of the sperm evaluation parameters.

3.1. Motility

The sperm motility in fresh samples ranged from 75% to 90% (80±4.6%). The motility of cooled samples did not differ from that of fresh samples ($P > 0.05$), and sperm motility in the cooled samples did not differ between treatments ($P > 0.05$) (Table 2). The post-thaw sperm motility after incubation for 0, 2, 4, and 6 h was higher in EE-Ve than in the control samples ($P < 0.05$). At 2 h incubation, the post-thaw sperm motility in both EE-Ve and EE-C was higher than in the control samples ($P < 0.05$).

3.2. Progressive motility

The progressive motility in fresh samples ranged from 4 to 5 (4.4±0.2). Progressive motility did not differ between the cooled samples and the fresh samples ($P > 0.05$), and progressive motility in the cooled samples did not differ between treatments ($P > 0.05$) (Table 2). After post-thaw incubation for 6 h, progressive motility was highest in the EE-Ve samples (Table 2).

3.3. Sperm membrane integrity

The percentage of spermatozoa with an intact membrane in the fresh samples ranged from 67.5% to 93% (78.7±7.9%). Sperm membrane integrity did not differ between the fresh and cooled samples ($P > 0.05$), and sperm membrane integrity in the cooled samples did not differ between treatments ($P > 0.05$). Immediately post-thawing, the spermatozoa in the EE-Ve sample had a higher proportion of intact membranes than those in the EE-C and control samples ($P < 0.05$). At 6 h post-thaw incubation, the EE-Ve sample had a higher percentage of sperm with intact membrane than the control sample ($P < 0.05$) (Table 3).

3.4. Acrosome integrity

The percentage of intact acrosomes in fresh samples ranged from 41% to 56.5% (47.9±6.1%). There were no significant differences in the percentage of sperm with intact acrosome before and after cooling, nor between control and treatment samples at any time after post-thaw incubation ($P > 0.05$) (Table 3).

3.5. DNA integrity

Immediately after thawing, the percentage of spermatozoa with intact DNA did not differ between treatments ($P > 0.05$) (Table 4). However, at 6 h after post-thaw incubation, the EE-C sample had a higher percentage of spermatozoa with intact DNA than the control sample ($P < 0.05$) (Table 4).

4. Discussion

Our results show that supplementation of the egg yolk tris extender with one of two antioxidants, cysteine or the water soluble vitamin E analogue Trolox, significantly improved post-thaw epididymal cat sperm quality after the freeze-thaw process. However, antioxidant supplementation did not improve sperm quality after cooling. Addition of vitamin E to the egg yolk tris extender resulted in improved post-thaw sperm motility and progressive motility immediately after thawing and at 2, 4, and 6 h post-thaw (Table 2). Sperm membrane integrity was significantly better at 0 and 6 h after thawing in vitamin E supplemented samples than in control samples (Table 3). The addition of cysteine to the egg yolk tris extender significantly improved progressive motility and DNA integrity at 6 h after thawing compared to control samples (Tables 2 and 4). However, acrosome integrity did not differ significantly between control and treatment samples (Table 3).

Addition of either antioxidant (cysteine or vitamin E) to the egg yolk tris extender did not improve sperm quality immediately after cooling (Tables 2, 3, and 4). Chatterjee and Gagnon [10] showed that levels of ROS products such as superoxide radicals ($O_2^{\cdot-}$) increased when bovine spermatozoa were cooled from 22˚C to 4˚C over a period of 2 h, but the antioxidative enzyme activity levels in stallion semen stored at 5˚C were maintained and had a positive effect on membrane integrity over and after a period of 24 h [27]. Moreover, no increase in the lipid peroxidation products was seen during this period [28]. It is interesting to note that a steady rise of ROS (superoxide anion; $O_2^{\cdot-}$) in frozen-thawed bull spermatozoa was observed immediately after thawing [10], while a significant reduction of antioxidative enzymes could also be noticed [29]. It may be that the ROS products can be accumulated during the freeze-thaw process, in a latent period of time and in inverse proportion to the antioxidative enzyme level which is reduced by the cryopreservation process and time.

In the present study, vitamin E supplementation improved post-thaw sperm motility and membrane integrity compared to control samples. The lipid peroxidation reaction has been reported as the main cause of sperm dysfunction, especially in terms of the loss of membrane fluidity or sperm membrane damage which can lead to decrease of sperm motility and viability [16]. Vitamin E is postulated as the most potent antioxidant, and it can be recycled to function again even when its concentration is low [6]. Addition of vitamin E to the freezing extender has been reported to enhance post-thaw motility for boar spermatozoa [22], and to improve post-thaw motility [20] and membrane integrity [30] for human spermatozoa.

During the freeze-thaw process, ROS products can induce premature acrosome capacitation, reaction, or damage [31, 32]. The presence of Equex STM paste in

the egg yolk tris extender has been shown to protect the acrosome of epididymal cat spermatozoa from the freeze-thaw process [1]. However, our results show that antioxidant supplementation in EE-I does not have an additional beneficial effect on post-thaw acrosome integrity (Table 3). This is in accordance with a study by Luvoni et al. [32], who showed that the addition of taurine did not improve the post-thaw acrosome integrity of cat sperm. Luvoni et al. [32] concluded that the process of cryopreservation was the main cause for the acrosome damage. Other researchers have concluded that the cause of live acrosome integrity improvement in stallion spermatozoa was not catalase supplementation, but the removal of seminal plasma before antioxidant supplementation [33]. Furthermore, Baumber et al. [8] showed that the acrosome integrity of stallion spermatozoa was not affected by exogenous ROS (xanthine(X)-xanthine oxidase (XO) system). However, vitamin E (α-tocopherol) supplementation of the freezing extender decreased post-thaw acrosome capacitation-like changes in boar spermatozoa [34]. This contradictory effect may be related to the type of antioxidant, or to the difference of oxidative stress susceptibility among species [8, 34].

In the present study, cysteine supplementation of the egg yolk tris extender had a protective effect on DNA integrity of epididymal cat spermatozoa after freezing and thawing (Table 4). Hydrogen peroxide (H_2O_2), a ROS product, has been reported to induce sperm DNA damage in horse [14] and human [6, 35]. In the present study, a higher percentage of intact DNA was observed when cysteine was added to the freezing extender. It should be noted that DNA integrity was evaluated by means of AO [25]. Our preliminary trials made use of the Sperm Chromatin Structure Assay (SCSA) by flow cytometry, but this technique did not produce reliable results, due to contamination by debris or red blood cells during the process of epididymal spermatozoa collection. We therefore chose to evaluate the samples with the epifluorescent microscopy. We tested AO concentrations of 0.001, 10, 50, and 100 mg/mL [26, 36, 37] (results not shown), and based on these tests and the study of Liu and Baker [26], we finally chose 1% (100 mg/mL) AO in distilled water. The most reliable concentration of AO for DNA integrity staining remains to be elucidated. This is the first description of DNA integrity stained with AO in the frozen-thawed epididymal cat spermatozoa. In a study by Hingst et al. [38], 86.5% of fresh epididymal cat spermatozoa had intact DNA when stained with 0.02% (2 mg/mL) AO, a similar proportion to our result immediately after thawing (Table 3). Comparison with, for example, the SCSA, might be used in further studies on cat sperm DNA integrity.

Further studies are needed to evaluate the antioxidative enzyme activity in seminal plasma, and the ROS levels generated during cooling and the freeze-thaw process. The optimal concentration of antioxidants and the effect of antioxidants on the *in vitro* fertility test under the restricted aerobic condition such as in *vitro* fertilization in the domestic cat would also merit further study.

In conclusion, antioxidant supplementation of the egg yolk tris extender containing Equex STM paste, prior to freezing, using 5 mM of either cysteine or the water soluble vitamin E analogue Trolox, improved the motility, progressive motility, membrane integrity, and DNA integrity — but not the acrosome integrity — of epididymal cat spermatozoa during post-thaw incubation.

5. Acknowledgements

Part of this study was founded by the Zoological Park Organization under H.R.H. the King, Thailand. The authors are grateful to Associate Prof. Dr. Padet Tummaruk and Assistant Prof. Jatesada Jiwakanon for their assistance with the statistical analyses, and to the veterinary surgeons and students at the Small Animal Hospital of the Swedish University of Agricultural Sciences for providing the reproductive tracts.

Table 1. *The composition of semen freezing and thawing extenders*

Extender	EE-I	EE-C	EE-Ve	EE-II	Tris
Tris [a] (g)	2.4	2.4	2.4	2.4	2.4
Citric acid [b] (g)	1.4	1.4	1.4	1.4	1.4
Glucose [c] (g)	0.8	0.8	0.8	0.8	0.8
Glycerol [d] (mL)	3	3	3	7	-
Egg yolk (mL)	20	20	20	20	-
Na benzyl pencillin [e] (g)	0.06	0.06	0.06	0.06	0.06
Streptomycin sulphate [f] (g)	0.1	0.1	0.1	0.1	0.1
Equex STM Paste [g] (mL)	-	-	-	1	-
DL-cysteine [h] (mM)	-	5	-	-	-
Water soluble Vitamin E analogue Trolox [i] (mM)	-	-	5	-	-
Distilled water (mL)	To 100 mL	To 100 mL	To 100 mL	To 100 mL	To 100 mL
pH	6.4	6.48	6.44	6.5	6.5
Osmolarity (mOsm)	877	939	919	1374	256

[a] Merk Eurolab AB, Stockholm, Sweden
[b] Sigma Chemical Co., St Louis, MO, USA
[c] Kebo-Lab., Stockholm, Sweden
[d] Kebo-Lab., Stockholm, Sweden
[e] Boehringer Ingelheim Vetmedia, Copenhagen, Denmark
[f] Sigma Chemical Co., St Louis, MO, USA
[g] Nova Chemical Sales, Scituate, Inc., MA, USA
[h] Sigma Chemical Co., St Louis, MO, USA
[i] Aldrich Chem. Co, WI, USA

Table 2. *Motility (%) and progressive motility of fresh, cooled and post-thaw epididymal cat spermatozoa in three different extenders; egg yolk tris extender I (EE-I), egg yolk tris extender I with cysteine (EE-C), egg yolk tris extender I with vitamin E (EE-Ve). Mean±SD. n=8 cats.*

Time (h)	Motility				Progressive motility			
	Tris	EE-I	EE-C	EE-Ve	Tris	EE-I	EE-C	EE-Ve
Fresh	80±4.6a				4.4±0.2a			
Cooled		76.3±8.8a	76.3±8.8a	77.5±6.5a		3.8±0.7a	4.1±0.4a	4.1±0.5a
0 h		59.4±11.5a	61.9±8.4a	69.4±5.6b		3.6±0.6a,b	3.7±0.4a	3.9±0.3b
2 h		45.6±15.9a	53.8±12.2b	58.1±9.9b		3 ± 0.7a	3.4 ±0.4a,b	3.6±0.3b
4 h		32.5±12.8a	33.8±14.1a,b	35.6±12.9b		2.2±0.9a	2.5± 0.6a,b	2.6± 0.6b
6 h		7±3.5a	11.9±5.9a,b	15±8.0b		0.9±0.5a	1.3±0.7b	1.5±0.7c

The motility was reported with the percentage of motile spermatozoa.
The progressive motility was scored with 0 to 5.
Means within row with different letters differ significantly (p<0.05)

Table 3. *Intact sperm membrane and intact acrosome of fresh, cooled and post-thaw epididymal cat spermatozoa in three different extenders; egg yolk tris extender I (EE-I), egg yolk tris extender I with cysteine (EE-C), egg yolk tris extender I with vitamin E (EE-Ve). Mean±SD. n=8 cats*

Time (h)	Intact sperm membrane				Intact acrosome			
	Tris	EE-1	EE-C	EE-Ve	Tris	EE-1	EE-C	EE-Ve
Fresh	78.7±7.9 a				47.9±6.1a			
Cooled		72.4±11.3a	73.6±9.2a	73.3±7.1a		51.6±8.9a	49.5±7.8a	50.3±8.2a
0		59.7±10.6a	61.4±7.6a	65.1±8.1b		47.2±7.6a	41.6±6.4a	44.9±5.1a
2		53.2±10.3a	56.9±5.5a	58.3±7.3a		33.2±6.1a	28.3±4.1a	32±7a
4		47.5±12.7a	53.8±5.9a	53.6±8.5a		20.6±5.9a	23.3±5.7a	24.1±9.1a
6		43.3±9.6a	49.1±3.7a,b	50.1±7.8b		16.7±6.8a	13.9±6.9a	15±7.1a

Means within row with different letters differ significantly (*p*<0.05)

Table 4. *DNA integrity of frozen-thawed epididymal cat spermatozoa stained with 1.0% Acridine orange (AO) in distilled water in three different extenders; egg yolk tris extender I (EE-I), egg yolk tris extender I with cysteine (EE-C), egg yolk tris extender I with vitamin E (EE-Ve). Mean±SD. n=8 cats*

Time after thawing (h)	EE-I	EE-C	EE-Ve
0	86.7±5.2	89.1±3.4	88.1±4.3
6	77.4±5.8a	84.1±4.4b	79.7±6.3a,b

Means within row with different letters differ significantly (*p*<0.05)

6. References

[1]Axnér E, Hermansson U, Linde-Forsberg C. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci. 2004;84(1-2):179-191.

[2]Kashiwazaki N, Yamaguchi R, Uesugi R, Hishiyama N, Kim M, Nakatsukasa E, Kojima Y, Okuda Y, Hisamatsu S, Inomata T, Shino M. Sperm motility, plasma membrane integrity, and binding capacity to homologous zona pellucida of cryopreserved epididymal spermatozoa in the domestic cat.J Reprod Dev. 2005;51(6):735-739.

[3]Aitken RJ., Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reproduction. 2001;122(4):497-506.

[4]Mazur P. Freezing of living cells: mechanisms and implications. Am J Physiol. 1984;247:125-142.

[5]Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci. 2000;60-61:481-492.

[6]Agarwal A, Ramadan A, Mohamed AB. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility. 2003; 79(4):829-843.

[7]Silva PFN. Physiology of peroxidation process in mammalian sperm. PhD Thesis. Utrech University Ridderprint, Ridderkerk, 2006 , pp.5-36.

[8]Baumber J, Ball BA., Gravance CG., Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl. 2000;21(6):895-902.

[9]Ball BA., Vo AT. Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C1l-BODIPY581/591. J Androl. 2002;23(2):259-269.

[10]Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev. 2001;59(4):451-458.

[11]Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 1989; 23(1): 77-90.

[12]Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa.Hum Reprod. 1998;13(4):896-900.

[13]Aitken RJ. The Amoroso Lecture. The human spermatozoon--a cell in crisis? J Reprod Fertil. 1999 ;115(1):1-7.

[14]Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl. 2003;24(4):621-628.

[15]Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine spermatozoa. Am J Vet Res. 2001;62(4):508-515.

[16]Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa.Mol Hum Reprod. 1997;3(3):203-213.

[17]Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology. 2001;56(2):275-286.

[18]Funahashi H, Sano T. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. Theriogenology. 2005;63(6):1605-1616.

[19]Erkkilä K, Hirvonen V, Wuokko E, Parvinen M, Dunkel L. N-acetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. J Clin Endocrinol Metab. 1998;83(7):2523-2531.

[20]Askasi HA, Check JH, Peymer N, Bollendorf A. Effect of natural antioxidants tocopheral and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Archives of Andrology 1994;33:11-15.

[21]Cerolini S, Maldjian A, Surai P, Noble R. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim Reprod Sci. 2000;58: 99-111.

[22]Peña FJ, Johannisson A, Wallgren M, Rodiguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane

potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci. 2003 ;78(1-2):85-98.
[23]Rota A, Strom B, Linde-Forsberg C, Rodiguez Martinez H. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38 degrees C. Theriogenology. 1997;47(5):1093-1101.
[24]Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J Androl. 1996 ;17(6):674-682.
[25]Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 1984 ;42(1):87-91.
[26]Lui DY, Baker HW. Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. Fertil Steril. 1992 ;58(6):1178-1184.
[27]Aurich JE, Schonherr U, Hoppe H, Aurich C. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. Theriogenology. 1997 ;48(2):185-192.
[28]Kankofer M, Kolm G, Aurich J, Aurich C. Activity of glutathione peroxidase, super oxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. Theriogenology 2005;63:1354-1365.
[29]Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev. 2000;55(3):282-288.
[30]Verma A, Kanwar KC. Effect of vitamin E on human sperm motility and lipid peroxidation in vitro. Asian J Androl. 1999;1(3):151-154.
[31]de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. Rev Reprod. 1997;2(1):48-54.
[32]Luvoni GC, Ruggiero C, Marinoni G, Kalchschmidt E. Effect of taurine containing diluent for cryopreservation of domestic cat epididymal spermatozoa. Theriogenology. 2002; 57(1) : 466.
[33]Ball BA, Medina V, Gravance CG, Baumbe J. Effect of antioxidants on preservation of motility,viability and acrosomal integrity of equine spermatozoa during storage at 5 degrees C. Theriogenology. 2001 Sep 1;56(4):577-589.
[34]Satorre MM, Breininger E, Beconi MT, Beorlegui NB. α-Tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm Theriogenology. In Press, Corrected Proof, Available online 4 September 2007.
[35]Donnelly ET, McClure N, Lewis SE. Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species.Mutagenesis. 2000;15(1):61-68.
[36]Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl. 2001;22(1):45-53.
[37]Virant-Klun I, Tomazevic T, Meden-Vrtovec H. Sperm single-stranded DNA, detected by acridine orange staining, reduces fertilization and quality of ICSI-derived embryos.J Assist Reprod Genet. 2002 ;19(7):319-328.
[38]Hingst O, Blottner S, Franz C. Chromatin condensation in cat spermatozoa during epididymal transit as studied by aniline blue and acridine orange staining. Andrologia. 1995 ;27(5):275-279.

Conclusions

The results of the present study can be summarized as follows:

- Supplementation of the egg yolk tris extender including Equex STM paste with antioxidants (cysteine or water soluble vitamin E analogue Trolox) resulted in significant positive effects on post-thaw epididymal cat sperm qualities such as motility, progressive motility, sperm plasma membrane integrity and DNA integrity. Supplementation with the antioxidants did not improve the epididymal cat sperm quality after a short period of cooling (1 h before freezing process).

- Supplementation with the water soluble vitamin E analogue Trolox improved motility, progressive motility, sperm plasma membrane integrity while cysteine supplementation improved motility and DNA integrity of post-thaw epididymal cat spermatozoa.

Additional comments and future prospects

The present study investigated the effects of antioxidants on post-thaw epididymal cat sperm quality based on the hypothesis that oxidative stress is a cause of sperm damage and that it can be eliminated or restricted by the positive effects of antioxidants. Although the oxidative stress has been studied and demonstrated as a cause of sperm damage in many mammalian species, this has not been studied in cat spermatozoa. Thus, the oxidative status in fresh semen or during the process of semen collection and preservation in the domestic cat should be considered.
Parameters that would be of interest to evaluate are;

- The level of reactive oxygen species (ROS) measured by using Luminal-dependent chemiluminescence assay. This should be evaluated in ejaculated, epididymal cat spermatozoa after cold storage and the freeze-thaw process.
- Detection of the lipid peroxidation reaction in chilled and frozen-thawed ejaculated or epididymal cat spermatozoa by using lipophilic fluorescent dye (C11-BODIPY$^{581/591}$).

These parameters could further confirm the results of this study. Furthermore, evaluation of other antioxidants and their optimal concentration could lead to further improvements in cat sperm preservation cryoprotocols.

Acknowledgements

The study was carried out at the Division of Reproduction, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden and Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The project was funded by SLU. A part of living expense was supported by the Zoological Park Organization on Royal Patronage, Thailand.

I would like to express my deepest gratitude to;

Prof. Björn Ekesten and Dr. Rauni Niskanen; the present and the former head of Department of Clinical Sciences for warm welcome and accepting me as the MSc student. I am thankful for putting the facilities of the department at my disposal.

Associate Prof. Dr. Lennart Söderquist, Prof. Heriberto Rodríguez-Martínez and Associate Prof. Dr. Karin Östensson; The present, the former head of Division of Reproduction and the Director of the International Master of Science Programme, for the warm welcome and giving me the good opportunity to continue my MSc study.

Dr. Eva Axnér; my scientific supervisor; I am sincerely grateful for all you have taught and advised me in both of laboratory and clinical work. I would like to take a moment to pass along a very warm thank you to your friendship, kindness, positive attitude, and especially for your helping hands when I was in time of needs. I am also deeply thankful for the constructive criticism on my manuscript. I enjoyed all our discussion both in scientific and non-scientific issues. Working under your supervision is very impressive.

Associate Prof. Dr. Kaywalee Chatdarong; my co-supervisor who guided me to 'the world of research'. I am eternally and deeply grateful for all of your everlasting helps not only at my profession but also my personal life. All you have done is very memorable. I am also truely thankful for all you have taught, your guidance, positive attitude, endless support and continuous encouragement over the years. Special thanks for the beautiful and fascinating friendship, leading me pass through all the troubles. These made you become one of the special persons in my life. The thousand words of thank you are not enough. **P Lee,** I owe you so much. Without your endless support, I cannot be there.

Prof. Catharina Linde-Forsberg; for all of your guides and teaching me especially on how to be a good scientist. I am sincerely thankful for the very nice chats and for always being patient with my mistakes on boiling and burning of the catheters. Finally, I also would like to convey my deepest gratitude for all your kind helps and supports in many ways that I have never expected before. Your kindness is appreciated so much.

Marie Sundberg and Birgitta Berner; for all your valuable helping hands in all the secretarial assistance.

Associate Prof. Dr. Padet Tummaruk and Assistant Prof. Jatesada Jiwakanon; for your excellent statistical analysis.

Karin Selin-Wretling, Annika Rikberg, Åsa Jansson, Hans Ekwall, Associate Prof. Dr. Anders Johannisson and Junpen Suwimonteerabutr; for the excellent in the laboratory support. **Dr. Jane Morell, Fernanda Saravia and Dr. Margareta Wallgren;** for the excellent in CASA teaching. **Kjell-Åke Ahlin;** for the excellent in computer support. Without all of your kindness and helping hands, my work could not be complete.

Staff and students at small animal at Swedish University of Agricultural Sciences and at Chulalongkorn University: especially for Prapai Intapihong; for being friendly, excellent surgeons and providing me the reproductive tracts.

All of my teachers who have taught and educated me with the knowledge since I was young. **Special thanks for Kru Day (Orawan Tharawan);** I am thankful for always being a true teacher and filling me with the valuable knowledge. You are not only my teacher but also the important part of my success.

Dalin family; for the warm and impressive traditional Swedish Christmas dinner night. I am also truly thankful for your kindness and friendship especially for giving me the best experiences with skiing and ice skating during the coldest winter time in Sweden. Your kindness is appreciated so much.

Tummaruk family; I would like to say 'Thank you' for your friendships and for everything that I cannot mention all here. Your generosity has made me speechless. **Special for my little niece 'Fah',** thanks for your bright smiles that made me feel happy all the time!

Kampa family; I am thankful for your kindness. Because of all of you, I could enjoy my life in Sweden.

My colleagues and friends in Thailand: Dr. Kongkiet and Dr. Sayamon Srisuwatanasagul, Dr. Sukanya Manee-in, Dr. Theerawat Tharasanit, Panisara Kunkitti, Kritnarong Wongbandoo, Nuttee Am-in, Yut, Oil, Give, Boui and Dang; I am sincerely thankful for your lovely friendships and big thanks for always with me in 'MSN' and updating all 'news' in Thailand. All you had done was the thing that made me could pass through the lonely home sick time. You all are like my family.

My Thai friends and Thai families in Sweden: especially for Na Salil, Na Tassanee, Na Amara, P Orn and UU students (P Jeab, P Joy, P Pum, P Jum, P Juk); Thank you for sharing me the good time and all of your friendships.

My MSc friends: especially Serbian women and Bosnian guy; Bojana, Aleksandra, Laki; Special thanks for your friendships, great parties and nice chatting time in the classes especially for all the non-scientific stories! All of you always made me smile and laugh.

My room mates: Assistant Prof. Jatesada Jiwakanon and Associate Prof. Dr. Renée Båge; Big thanks for creating the best atmosphere to stay and work with. I am also deeply thankful for your friendships and nice chatting especially for all joking stories. Special thanks for **P Jate** who always solved and listened to all my nonsensical problems. You are my big brother!

Yaohong Zhu, Fernando Saravia, Dr. Ann-Sofi Bergqvist, Dr. Seri Koonjaenak, all OG staffs and PhD students; for sharing me the nice time, nice parties and nice coffee breaks during I stayed there. I am thankful for all of your friendships.

Finally for my lovely family: my beloved mom and dad (Pranom and Ruengwit Thuwanut); I am extremely deeply truly grateful for your warm hugs and true loves. Truly thank you for always supporting and inspiring me ‘to be good and to do good’. These are wealthier than all precious things. Both of you are the most important persons in my life. I love you so much. **Deeply thank you for my aunts and uncles (especially for Raywadee-Jongsak Panichakul, Wiyada Chusai), my grandfather and grandmother (Raywat and Duangdee Thuwanut), my brother (Panwit Thuwanut) and all sisters,** I am very proud to be one part of this big family. Thank you for always sharing the good things, giving me the best opportunity and endless support. Without all your love, I cannot fly as far as this.