Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 76 (2011) 115–125
Theriogenology
www.theriojournal.com

The effects of antioxidants on semen traits and *in vitro* fertilizing ability of sperm from the flat-headed cat (*Prionailurus planiceps*)

P. Thuwanut[a,b], K. Chatdarong[b,*], A.-S. Bergqvist[a], L. Söderquist[a], K. Thiangtum[c], D. Tongthainan[d], E. Axnér[a]

[a] *Division of Reproduction, Department of Clinical Sciences, P.O. Box 7054, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden*
[b] *Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Department of Companion Animals Clinical Sciences, Faculty of Veterinary Medicine, Kamphaengsaen Campus, Kasetsart University, Nakorn Pathom 73140, Thailand*
[d] *Khao Kheow Open Zoo, Chonburi 20110, Thailand*

Received 8 September 2010; received in revised form 23 December 2010; accepted 25 January 2011

Abstract

Since antioxidants can overcome the negative effects of reactive oxygen species (ROS) during sperm cryopreservation, post-thaw sperm quality in flat-headed cats (*Prionailurus planiceps*), an endangered species, might benefit from the addition of antioxidants to semen extender. The objectives of this study were to: 1) investigate semen traits; and 2) evaluate effects of the vitamin E analogue Trolox (vitamin E) and glutathione peroxidase (GPx) on the quality of frozen sperm from captive flat-headed cats in Thailand. Eight ejaculates were collected by electroejaculation from four flat-headed cats. Each semen sample was divided into three aliquots and re-suspended in a semen extender as follows: 1) without antioxidant supplementation (control); 2) supplemented with 5 mM vitamin E; or 3) supplemented with 10 U/mL GPx. All samples were cryopreserved and thawed. Subjective sperm motility, progressive motility, and the integrity of the sperm membrane, acrosome and DNA were evaluated at semen collection, after 1 h cold storage, and at 0 and 6 h after thawing. Mitochondrial membrane potential, early apoptotic cells, and embryo development by heterologous *in vitro* fertilization were evaluated after thawing. Captive flat-headed cats were affected by teratozoospermia. After 1 h cold storage, sperm membrane integrity in samples supplemented with GPx was higher than the control group (54.5 ± 13.7 vs 51.3 ± 13.9; $P < 0.05$; mean ± SD). Sperm frozen in extender with GPx had higher motility at 6 h and greater mitochondrial membrane potential at 0 and 6 h post-thaw incubation than the other groups ($P < 0.05$). In conclusion, GPx improved the quality of frozen-thawed sperm in flat-headed cats.

Keywords: Wild felids; Flat-headed cat; Sperm cryopreservation; Semen; Oxidative stress

1. Introduction

The flat-headed cat (*Prionailurus planiceps*) is a small wild cat with a long, sloping snout and flattened skull roof [1]. Like most species in the family Felidae, the flat-headed cat is included in Appendix I of the Convention on International Trade in Endangered Species. 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 [1,2]. The population size is predicted to decline continuously by at least 20% over the next 12 y, due to destruction of the forest for human settlement or

* Corresponding author. Tel.: + 662 218 9644; fax: +662 255 3910.
E-mail address: kaywalee.c@chula.ac.th (K. Chatdarong).

0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2011.01.024

draining for agriculture [1,2]. Although the exact population size cannot be estimated, it is suspected to be < 2,500 individuals along its habitat area [1]. In Thailand, only a few individuals are kept in captivity. Hunting of this species is now completely prohibited in Indonesia and Malaysia, as well as in Thailand, by national legislation [2]. Preservation of gametes, such as sperm, of this endangered species is therefore urgently needed to sustain and increase the population. Even though breeding management programs including gamete preservation, AI, IVF, and embryo transfer have been continuously developed in both domestic and non-domestic felids for several decades [3], the achievement in wild felids is limited. The problem may be partly due to a high proportion of morphologically abnormal sperm in at least 28 felid species [4], which may be associated with the poor *in vitro* fertility in wild felids i.e., cheetah [4].

In breeding programs, long-term preservation of sperm is considered the primary biotechnological technique to facilitate genetic variation, both *in situ* and *ex situ* [5,6]. However, several crucial factors have been reported to compromise cryopreserved sperm quality [6], including cold shock [7], osmotic stress [7], and oxidative stress [8]. In felid studies, both cold shock and osmotic stress factors have been evaluated [6], whereas little is known about oxidative stress. The term oxidative stress refers to the imbalance between reactive oxygen species (ROS) and antioxidants [8]. Antioxidants are generally abundant in seminal plasma [9,10], which is discarded during conventional sperm cryopreservation. Reactive oxygen species, free radicals (i.e., hydroxyl and hydroperoxyl radicals), are generated by metabolism of oxygen in all living mammalian cells [8], including sperm. Morphologically normal sperm produce ROS via the mitochondria [8,11,12], but abnormal sperm are considered to be the main source of ROS [13]. The ROS level was increased during sperm freezing [14]. Excessive ROS causes sperm membrane disruption, DNA damage, or failure of IVF, and can also induce a harmful reaction called lipid peroxidation [8]. It was noteworthy that this reaction, which is also one of the main causes of mammalian sperm damage, occurred during post-thaw incubation of epididymal cat sperm [15]. It may be one of the reasons that epididymal cat sperm had tail abnormalities which increased during epididymal transit [16].The effects of excessive levels of ROS and lipid peroxidation can be overcome by adding antioxidants [8,11]. Supplementation of non-enzymatic (cysteine, vitamin C, vitamin E) and enzymatic antioxidants (catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD)) to semen extenders had positive effects on some aspects of sperm quality, such as motility and integrity of membranes and DNA in cat [15,17], dog [18], boar [19], and human sperm [20]. As previously described, ejaculated sperm from wild felids generally have higher percentages of abnormal sperm than domestic cats [4], which might generate high levels of ROS. Furthermore, several types of antioxidants present in seminal plasma are completely removed during conventional cryopreservation. Thus, addition of exogenous antioxidants to semen extenders might improve post-thaw sperm quality in wild felids.

Due to the limited knowledge of semen traits in the flat-headed cat, the primary aims of this study were to evaluate: 1) the basic seminal characteristics of flat-headed cats (individually kept in two different zoos in Thailand); and 2) the effects of various supplements on sperm quality and their ability to fertilize heterologous oocytes from domestic cats *in vitro*. The supplements consisted of a non-enzymatic antioxidant (vitamin E analogue Trolox) and an enzymatic antioxidant (GPx) added to a conventional extender after cold storage of sperm.

2. Materials and methods

2.1. Experimental design

Four captive adult flat-headed tom cats, aged between 1.5 and 3 y, were used. One individual was kept at Songkhla zoo (Songkhla, Thailand) and the others in Khao Kheow Open Zoo (Chonburi, Thailand). All were born in captivity and two were proven as breeders prior to semen collection. Fresh pork and fish were provided as the primary diet for all cats. Semen collection was performed by electroejaculation between July and December, 2009, once from the cat maintained in Songkhla Zoo, and three times each from the others, with a 1–1.5 mo interval between collections. Only ejaculates with $>3.9 \times 10^6$ sperm and >30% motility were selected for use in this study; eight samples fulfilled these criteria. After semen collection, the sample was evaluated for sperm concentration, total number of sperm, morphology, pH, total and progressive motility, and membrane-, acrosome- and DNA integrity. Each sample was then divided into three equal aliquots, centrifuged to remove seminal plasma, and supplemented with: (1) egg yolk Tris buffer (EE-I; control); (2) egg yolk Tris buffer with 5 mM vitamin E analogue Trolox (Sigma Chemical Co., St Louis, MO, USA; EE-VitE); and (3) egg yolk Tris buffer with 10 U/mL GPx (Sigma; EE-GPx), respectively. Each sperm sample was slowly