

Development of Domestic Cat Embryo Produced by Preserved Sperms

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The ability to mature and fertilize oocytes of endangered species may allow us to sustain genetic and global biodiversity. Epididymis sperms may be the last chance to ensure preservation of genetic materials after injury or death of a valuable animal. Studies have been conducted to determine whether both epididymis sperms and oocytes can be used to produce viable embryos and offspring. The purpose of this study was to determine how long cats sperms contained in epididymis were remain motile and had intact membranes when preserved at 4 °C, and to determine whether such those preserved sperms are able to fertilize oocytes. Epididymis was preserved immediately in phosphate buffer saline at 4 °C for 1, 3, and 6 days. The observation of sperm quality and viability after preservation was performed by vital staining acrosom and Hoechst-Propidium Iodine. Biological functions of sperms were evaluated by *in vitro* culture technique for fertilization, micro fertilization and embryonic development rate in CR1aa medium. The results showed that average motility of sperms collected from ductus deferens, cauda and corpus epididymis decreased not significantly ($P > 0.05$) from 0, 1, 3, and 6 days of preservation times (from 83.0%, 80.2%, 79.0%; 80.9%, 75.0%, 75.5%; 52.0%, 63.2%, 55.0% to 34.6%, 34.6%, 33.3%, respectively). The general results showed that sperms from epididymis preserved for 1, 3, and 6 days can be used for IVF. The rate of embryonal cleavage produced by IVF technique using sperms collected from epididymis preserved for 1-, 3- and 6-days were 33.3, 26.7, and 20.0%, respectively and significantly different ($P < 0.05$) from that of control (50.0%). In conclusion, sperms contained in epididymis preserved at 4 °C in PBS (Phosphate Buffer Saline) for 1-6 days can be used to IVF and *in vitro* production of cat embryos.

Key words: gamet, preservation, *in vitro* fertilization

INTRODUCTION

The death of endangered animals in the marginal areas located far away from diagnostic laboratories would potentially result in the loss of genetic material sources in the form of sperms and oocytes. In this condition preservation becomes one of alternative ways could be used to save the genetic materials. It was assumed that preservation would minimize metabolism rate maintaining cell viability necessary for many biological functions.

One of animals considered as endangered species is Felidae family, both *big felids* (big cats from the group of *Panthera*) and *small felids* (small cats from the group of *Felis*). Almost all species of wild cats in Indonesia are now reduce in their population and some of them are in endanger condition including *Panthera tigris sumatrae* (Sumatran tigers), *Panthera pardus* (panthers), *Felis marmorata* (wild cats), *Felis temminckii* (gold cat), *Pardofelis nebulosa* (plant tiger), *Prionailurus planiceps* (dampak cat), *Felis bengalensis* (rock cat) (data from Leuser Management Unit). According to Pope (2000), more than 36 species of wild cat can be categorized as vulnerable or endanger animals from the hunting and the loss of natural habitats.

In order to support programs have been set up by the government to conserve protected endanger animals, some research have been conducted by using reproductive biotechnology for increasing population growth rate. These efforts need the use of animal models closely related both in family and physical characters. Wright and Walters (1980) divide species of cats into three genera namely *Panthera* (Great cats), *Acinonyx* (Cheetah), and *Felis* (Small cats). For this reason, domesticated local cat (*Felis catus*) can be used as an alternative representative animal model due to its close relationship. According to Pope (2000), domestic cat can be used not only as an animal model for developing *in vitro* technique, but also more importantly as recipient mother for embryos from *small wild cats*.

In this study epididymis preservation was carried out in phosphate buffer saline (PBS) solution in order to learn adaptation capability of epididymis in maintaining sperm viability. It is hoped that results of the study can be used as part of alternative solution for optimizing the use of genetic potential of wild animals, in particular those endangered species.

MATERIALS AND METHODS

The study was performed in the Laboratory of Embriology and the Laboratory of Anatomy, the Department of Anatomy,

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Physiology and Pharmacology of Veterinary Faculty of Bogor Agricultural University. Testis and ovarium used as samples were collected from 12 male and female cats aged 2-3 years old and 2-3.5 kg weight. Both testis and ovarium were collected by castration and ovariectomy surgery at the Surgery Laboratory, the Department of Clinic, Reproduction, and Pathology of Veterinary Faculty of Bogor Agricultural University.

Preservation and Sperm Collection of Ductus Deferens and Epididymis. Epididymis and ductus deferens were preserved in PBS medium and kept in a refrigerator of 4 °C. The left epididymis and ductus deferens were observed in the castration day (day 0), while the right epididymis and ductus deferens were preserved and then observed in the day one (day 1), three (day 3), and six (day 6) after preservation to evaluate the sperm concentration and motility. Observation of sperm viability was carried out until the 21st day. Sperm was aspirated from cauda and corpus epididymis 10X using 26 G of gauge connected with 1 ml of syringe containing of 0.2 ml of mPBS. The solution was then transferred into a vial and centrifuge at 500 g for 5 minutes. Ductus deferens was cut into 2 cm of fragments and flushed by using syringe containing of 0.2 ml of mPBS. *Flushing solution* was transferred into a vial and centrifuge again at 500 g for 5 minutes. The concentration, motility, and viability of sperms were then evaluated by using standard methods.

The percentage of motil sperms was counted by subtracting the number counted sperms with the number of dead sperms. Sperm viability was analyzed from the percentage of intact acrosomes observed microscopically by using eosin-B fast green vital staining (Aalseth & Saacke 1986).

Oocyte Collection. Oocytes were collected from a fresh ovarium obtained from ovariectomy surgery. After separating the ovary from connective tissues, oocytes was collected by slicing the cortex of ovary using a knife and put the slice in petridisk containing of mPBS. The number and quality of oocytes were observed by inverted microscope.

The oocyte quality was determined by visually evaluating the compactness and number of granulosa and homogeneity of cytoplasm condition as performed by Carolan *et al.* (1994). The grade A oocytes have homogenous cytoplasm surrounded by 5-10 layers of intact granulosa. The grade B oocytes have homogenous cytoplasm surrounded by 3-5 layers of intact granulosa. The grade C oocytes have homogenous cytoplasm surrounded by 1-2 layers of cumulus cells granulosa. The grade D oocytes have homogenous cytoplasm without cumulus cells.

Oocyte Maturation. Oocytes collected from the ovary were washed three times with CR1aa media (Rosenkrans and First

1994), added with *fetal bovine serum* (FBS) 10% (v/v), *bovine serum albumin* (BSA) 0.03%, *follicle stimulating hormone* (FSH) 10 mg/ml, *luteinizing hormone* (LH) 20 mg/ml, estradiol 1 mg/ml and antibiotic (10,000 IU of penicilline/ml and 10 mg of streptomycin/ml). Fifteen to ten grades A-B oocytes were placed in 50 ml of CR1aa dropping medium and covered with oil mineral. The oocytes were then cultured in an incubator containing of 5% CO₂ at 37 °C for 24 hours.

In Vitro Fertilization (IVF) and Embryo Production. Sperms collected from ductus deferens, cauda- and corpus epididymis were then processed for IVF. Each obtained sperm suspension was poured into a vial and centrifuged at 500 g for 5 minutes in CR1aa media enriched with 2.5 mM of caffeine, 36 ml of heparin and antibiotic. Sperm motility used for IVF was selected by *swim-up* technique (Younglai *et al.* 2001). Final concentration of sperms used for IVF was 2 x 10⁶/ml. Suspension containing of sperms was prepared in dropping medium of 100 ml and covered with mineral oil. The dropping medium was filled with 15 of oocytes and cultured in incubator containing of 5% CO₂ at 37 °C for 24 hours. After 18 hours incubated in fertilization media, oocytes were transferred into CR1aa culturing media. Observation of embryos development was performed after 96 hours of incubation. Parameters observed were the number of embryos developed into 2-8 of cells and morulla. Evaluation of data was carried out based on the percentage of developing embryos i.e. the number of developed embryos per the number of oocytes cultured.

Experiment Design and Data Analysis. This study has used complete random design with 6 and 3 replications for observation of sperm quality and development of embryos, respectively. Data of percentage of fertilization rate and embryo development obtained were analyzed by Analysis of Variance. Statistical analysis was continued by Tukey-Kramer HSD test if there was differences in any treatment groups (Steel & Torrie 1991).

RESULTS

Sperm Concentration and Motility. Concentration of sperms collected from ductus deferens, and cauda and corpus epididymis at the day 1, 3, and 6 after preservation was not significantly different ($P > 0.05$) from that control (Table 1). These results were different from those found by other researchers. According to Hafez and Hafez (2000), sperm concentration of cat was range from 1.5 x 10⁸/ml upto 28.0 x 10⁸/ml with the average of 13.0 x 10⁸/ml. Even though results obtained were different from that of Hafez, they were still representative enough to be used in the IVF since Risopatron *et al.* (1996) claimed that minimal concentration of sperms

Table 1. Concentration and motility of sperms collected from ductus deferens and epididymis of cat after preservation

Preservation of epididymis (day)	(n)	Sperm concentration (10 ⁶ /ml)			Sperm motility (%)		
		Ductus deferens	Cauda epididymis	Corpus epididymis	Ductus deferens	Cauda epididymis	Corpus epididymis
0 (control)	3	25.2 ± 0.5a	25.1 ± 0.4a	19.2 ± 0.2a	83.0 ± 0.5a	80.2 ± 0.2a	79.0 ± 0.5a
1	3	23.6 ± 0.6a	25.4 ± 0.6a	16.9 ± 0.2a	80.9 ± 0.5a	75.0 ± 0.5b	75.5 ± 0.3a
3	3	23.4 ± 0.2a	24.3 ± 0.3a	15.4 ± 0.4a	52.0 ± 0.3b	63.2 ± 0.2c	55.0 ± 0.2b
6	3	22.0 ± 0.2a	21.3 ± 0.4a	18.2 ± 0.3a	34.6 ± 0.2c	34.6 ± 0.4d	33.3 ± 0.3b

Number followed by the same superscript letters showed no significant different at 5% Turkey HSD probability.

needed for successful IVF was $1.0 \times 10^6/\text{ml}$, and that optimum concentration was $10.00 \times 10^6/\text{ml}$.

The motility of sperms collected from ductus deferens, cauda and corpus epididymis on the day 6 of preservation time were 34.6, 34.6, and 33.3%, respectively. These results were significantly different ($P < 0.05$) from that control (0H) that reaching of 80%, in the average. According to Hafez and Hafez (2000) frozen sperms should have at least 40% of motility to be used in artificial insemination program.

Sperm Viability. The success of fertilization was markedly influenced by sperm viability, especially the intact acrosom. In this study, observation of acrosom reaction was performed microscopically by using vital staining with eosin-B fast green (Figure 1). It showed that storage of epididymis and ductus deferens in a refrigerate temperature of 4 °C until day 6 could keep the quality and viability of sperms of having intact acrosom in minimal limit requiring for succesfull *in vitro* embryo production. The percentage of living sperm with intact acrosom from different sources as shown in Table 2.



Figure 1. Sperms appearance in vital staining, a. live sperms with intact acrosom, b. live sperms with lysed acrosom, c. dead sperms with intact acrosom, d. dead sperms with broken acrosom. Bar = 1 μm .

Although there was a decrease in sperm viability, storage of epididymis in low temperature (4 °C) could give better protection on chemical compound contained in epididymical fluid as well as good support for a longer viability of sperms in epididymis at low temperature, could protect chemicals contained in epididymical fluid and support sperm viability longer than that in higher temperature. It was appeared that low temperature (preservation) could minimize unacceptable change in comparison to preservation in room temperature. This, in turn, has a longer chance to protect sperms. According to Soler *et al.* (2003) quality of sperms collected from cauda epididymis kept in 5 °C for four days was significantly higher than that kept in room temperature.

Embryo Production of *In Vitro* Fertilization. Sperm capability of performing fertilization should be supported by fluidity and intact of its plasma membrane. These factors affected capacitation process, acrosom reaction and interaction of membrane of sperms with membrane of ovum (zona pellucida). Plasma membrane of sperms also played a role in maintaining their permeability, the capacity of membrane to transport compounds from inside and outside of sperms. In addition, plasma membrane was also needed in metabolic processes resulting energy for supporting sperm motility. Motility was very important for good *in vitro* and *in vivo* fertilization.

Oocytes fertilized by sperms collected from caudal epididymis preserved for day 1, 3, and 6 still have capability to reach cleavage stage (2-8 of cells) of 33.3, 26.7, and 20.0%, respectively (Table 3). Statistical analysis showed that capability of oocytes fertilized by sperms preserved for 1H to reach cleavage stage was not significantly different from that of control ($P > 0.05$). Capability of oocytes fertilized by sperms collected from that of control ($P < 0.05$). There was indication of reduction in cleavage rate of fertilized oocytes to reach cleavage stage of 2-8 cells. This phenomenon indicated that acrosom cap of sperms collected from ductus deferens

Table 2. Percentage of living sperms with intact acrosom or acrosom reaction after preservation from different sources

Source of sperm	(n)	Live sperm/intact acrosom (%)			
		Control	Day 1	Day 3	Day 6
DD	3	85.0 $\pm$ 5.16a	73.0 $\pm$ 6.24a	70.0 $\pm$ 6.01a	61.3 $\pm$ 2.71ab
CA	3	83.0 $\pm$ 6.06a	61.3 $\pm$ 7.09ab	73.0 $\pm$ 5.23a	55.0 $\pm$ 4.43b
CR	3	83.0 $\pm$ 3.08a	63.0 $\pm$ 1.00ab	61.3 $\pm$ 3.02ab	50.0 $\pm$ 6.21b
		Live sperm/acrosom reaction (%)			
		Control	Day 1	Day 3	Day 6
DD	3	12.0 $\pm$ 2.83a	20.0 $\pm$ 5.57ab	15.0 $\pm$ 2.34a	8.0 $\pm$ 6.07cd
CA	3	11.0 $\pm$ 5.02a	19.7 $\pm$ 7.09abc	20.0 $\pm$ 2.04a	10.7 $\pm$ 4.27d
CR	3	15.0 $\pm$ 3.45a	19.3 $\pm$ 5.21abcd	20.0 $\pm$ 3.35a	20.7 $\pm$ 3.05a

Number followed by the same letters showed no significant difference at 5% Tukey-Kramer HSD probability. DD: ductus deferens, CA: cauda epididymis, CR: corpus epididymis.

Table 3. Development of embryos produced by IVF method using preserved sperms

Preservation of ductus deferens (day)	(n)	Σ fertilized oocytes	Σ cleavage/2-8 of cells (%) oocytes	Σ morulla (%)
Control	3	50	25 (50.0) $\pm$ 0.57a	23 (46.0) $\pm$ 0.57a
1	3	30	10 (33.3) $\pm$ 0.57a	8 (26.7) $\pm$ 0.57a
3	3	30	8 (26.7) $\pm$ 0.57b	4 (13.3) $\pm$ 0.57a
6	3	30	6 (20.0) $\pm$ 0b	3 (10.0) $\pm$ 0a

Numbers followed by the same letters indicate no significant difference at 5% (Tukey-Kramer HSD).

preserved in 4 °C was still could be kept. However, longer preservation time would result in more reduction in quality and viability of sperms to perform fertilization. Intact acrosom caps was very important for the success of *in vitro* fertilization because vesicles contained in acrosom caps have some enzymes played significant roles fertilization process. If acrosoms were broken, these enzymes will be lost so that sperms capacity to pass barrier surrounding oocytes will be lost and there was no fertilizaitaion would be occurred.

DISCUSSION

Sperm Concentration, Motility, and Viability. It was assumed that the reduction in sperm motility occurred during preservation period resulted from accumulation of metabolic wastes. According to Nazlie (2004) during preservation sperms were still alive and performed their activities by using energy produced by metabolic processes. However, this energy gradually decreased, and by product of the metabolism such us lactic acids were in the high proportion. The lower pH due to from this lactic acid accumulation was toxic and had negative effect to some metabolic enzymes. As a consequence, all metabolic processes will be disrupted.

In addition to due to the metabolic wastes, the decrease in sperm motility, was due to reactive oxygen species (ROS) (Soeradi 2004). ROS could induce DNA damage (Fraser 2004). In this study, it was predicted that the low sperm motiliy was caused by the high proportion of DNA damage. According to Moustafa *et al.* (2004) there were three hypotesa could explain DNA damage: (i) DNA damage from the *packaging* and ligation processes during sperm maturation, (ii) oxydative stress resulted in DNA destruction and the increase of ROS formation such as 8-hydroxydeoxyguanosine in DNA of sperms, and (iii) DNA fragmentation caused by apoptosis. Factors causing DNA damage, however, were not deeply investigated in this study.

According to Fraser and Strzezek (2004) factors influencing DNA damage were species spesific and difficult to be explained. In pig, DNA destruction was caused by ROS formation during sperm ageing ultimately disturbing the integrity of other sperms. In this study, it was difficult to determine whether the long preservation time at low temperature (4 °C) can bring higher oxidative stress resulting in overproduction of ROS. This assumption will also contradict to the principle of oxidation-reduction. However, long preservation could create destructive effects. There was such indication that ROS was potentially formed during preservation because majority of Fellidae were reported having poor quality of sperms (Farstad 2000) and abnormality of ejaculated sperms > 60% (Penfold *et al.* 2003). Nazlie (2004) reported that abnormality of sperms collected from corpus epididymis of domesticated cats on day 0 (control) was as high as 25.75%. This will potentially trigger formation of ROS during preservation.

Moreover, it was suggested that chain reaction of lipid peroxidation and other free radical compounds could be

inhibited their formation by adding antioxidants such as glutathion, cystein, catalase, glutathion peroxidase, β -caroten, vitamin E (tocopherol), and vitamin C (ascorbic acid) in the medium. In this study, washing and capacitation media used were CR1aa containing of amino acid of MEM Non Essential Amino Acid (one of its component was cystein) and Minimum Essential Amino Acid (MEM-BME) that probably could play a role as antioxidant coumpounds as well as fixing plasm membrane of damaged sperms. As the results the reduction of sperm motility could be minimized during the washing of sperms.

Production of Embryos by IVF. By using IVF technique we obtained 26.67, 13.33, and 10% morulla for the preservation of day 1, 3, and 6, respectively. These results were not significantly different ($P > 0.05$) from control (46.00%). There is a suspicion that parthenogenesis has occurred in this study. From totally 302 oocytes, 15 (4.9%) oocytes cleavaged before fertilization. However, fertilization rates obtained in this study was higher than that of parthenogenesis, 33.3, 26.7, and 20.0% for day 1, 3, and 6 of preservation time respectively. The developed oocytes caused by *swim-up* sperms were used for *in vitro* fertilization. According to Younglai *et al.* (2001) DNA fragmentation in sperms prepared by *swim-up* method was lower than that of non *swim-up*. Howart *et al.* (1993) referred in Goodrowe *et al.* (2000) suggested that fertilization rate in domesticated cats by using freshly *swim-up* sperms was markedly higher (94.6%) than that of without *swim-up* (53.2%). Muratori *et al.* (2000) claimed that the high DNA fragmentation occurred in the unselected sperms and in *swim-up* sperms has positive correlation with abnormal morphology as well as with weak sperm tails. Barroso *et al.* (2000) performed sperm analysis to infertile men by using *terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (Tunnel) assay*, suggested that there was relationship between low sperm motility and the high DNA damage.

In this study, it was assumed that sperm preservation may caused DNA damage as reflected by the low sperm motility. This assumption was also supported by data of sperm observation by using *Hoechst-Propidium Iodine* staining. The data showed that *swim-up* became one of important treatments needed for fertilization rate even though in fact there were immotil sperms with intake DNAs.

In order to investigate the effect of preservation time on quality and viability of sperms collected from different sources, each sperm collected from the three sources was evaluated its fertility rate by measure the number of oocytes (in percentage) developed into 2-8 cells and the percentage of oocytes developed into morulla stage (Figure 2). This histogram showed that on the day 0 (control group) there was no significant difference ($P > 0.05$) in capability of oocytes fertilized by sperms collected from ductus deferens, caudal epididymis and corpus epididymis to developed into 2-8 cells i.e. 53.8% (ductus defferens), 52.4% (cauda epididymis), and 47.8% (corpus epididymis). Sperms collected from corpus epididymis have lower percentage of cleavage than that from ductus, deferens, and caudal epididymis. On the other hand,

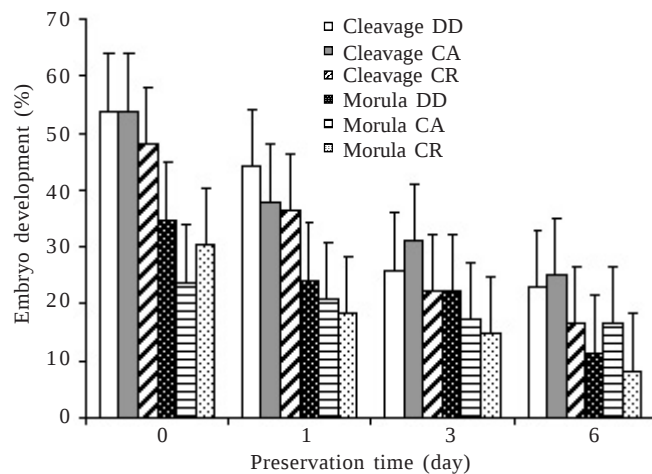


Figure 2. Histogram of embryo development after IVF with sperms from different preserved sources: ductus deferens (DD), cauda epididymis (CA), and corpus epididymis (R).

for preservation time of day 3 and 6, sperms collected from caudal epididymis have relatively higher percentage of development (2-8 cells) than that from ductus deferens and corpus epididymis. The occurrence of developing pattern above was probable caused by the higher capability of nutrition content of cauda epididymis to maintain better quality and viability of sperms. According to Tomes *et al.* (1979) fluid of caudal epididymis has lower sodium and chloride contents but higher potassium, phosphor, and protein than that of rete testis fluid. Epididymis also synthesizes carnitin, glycerilphosphorilcholin (GPC), fructosa, and glycoprotein (Johnson & Everitt 1995). Bearden and Fuquay (1997) said that the fluid of cauda epididymis has an optimum condition for sperms due to its low PH, high viscosity, high CO₂ concentration, high ratio of K⁺ and Na⁺, positive influence of testosterone on epididymis function, and combination of other factors resulting in low metabolism rate of this organ so that can prolong viability of sperms.

For the percentage of morulla stage development (Figure 3e,f), sperms collected from the three sources showed no differences. There was no embryo develop into blastocys since according to Skrzyszowska *et al.* (2002) in cat growth inhibition occurred in morulla stage. The proportion of cat embryos developed into blastocys produced by *in vitro* culture from the 2- to 4- cells was higher than that from the 1-cell (Figure 3a). This showed that embryonic growth stage started from *in vitro* culture has high impact to the increase in the development delay of cat embryo into morulla stage (Figure 3).

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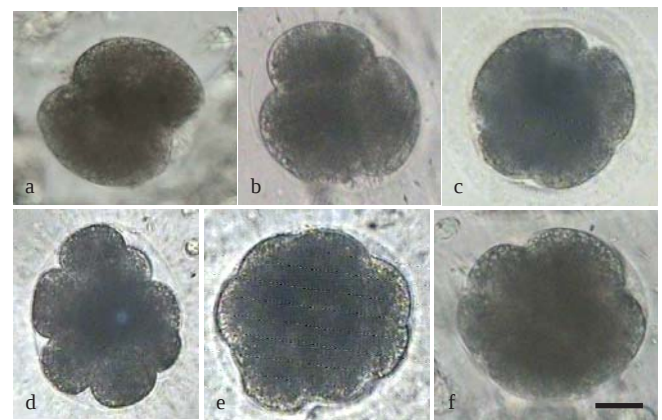


Figure 3. Development of embryo *in vitro* fertilized. a. phase 2-cells, b. phase 3-cells, c. phase 4-cells, d. phase 8-cells, e-f. phase compact morulla. Bar = 20 µm.

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