Study on the Factors Influencing Fertilization and Developmental Rate of *in vitro* Cultured Cat Oocytes Recovered from Ovaries Collected at Different Stages of the Reproductive Cycle

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번식주기의 단계별로 회수한 고양이 난자의 체외발생에 미치는 요인에 관한 연구

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ABSTRACT

The study was carried out to investigate the effects of morphology, reproductive cycle, incubation time and activation of oocytes on *in vitro* maturation of cat oocytes and development of IVM/IVF embryos.

The results were summarized as follows:

1. When recovered from ovaries collected at different stages of the reproductive cycle (inactive, follicular and luteal stage), the developmental rates of oocytes to GV and MI stage were 72.5% and 27.5%, 57.5% and 7.5%, 62.5% and 17.5%, respectively.

2. The developmental rates of oocytes with cumulus cells to GV and MI stage in different conditions of incubation (5% CO_2, 95% O_2 and 10% CO_2, 90% O_2) were 70.0% and 27.5%, 52.5% and 20.0%, 55.0% and 12.5%, respectively.

3. The developmental rates to GV and MI oocytes when cultured at different time of incubation (17~20, 21~24, 25~28 and 29~32 h) were 67.5% and 20.0%, 67.5% and 30.0%, 62.5% and 22.5%, 65.0% and 15.0%, respectively.

4. The fertilization and cleavage rates of freshly collected oocytes with and without cumulus cells were 72.5% and 25.0%, 37.5% and 7.5%, respectively. The rates were greater in oocytes with cumulus cells than those without cumulus cells.

5. The fertilization and cleavage rates of oocytes recovered from ovaries collected at different stages of the reproductive cycle (inactive, follicular and luteal stage) were 75.0% and 25.0%, 40.0% and 7.5%, 50.0% and 15.0%, respectively.

(Key words : Cat, Developmental rate, Reproductive cycle, Incubation time)

Ⅰ. INTRODUCTION

Domestic pet animals have been raised for 300 million years, and the current population exceeds 500 million. Raising of the pet has been turned into the generalization, and the concern of pet becomes greater (Freistedt et al., 2001; Goodrowe and Hay, 1993).

There are only a few reports published on cat ovum (Goodrowe and Hay, 1993; Howard et al., 1991; Otoi et al., 2001). It has been reported that efficiencies of IVM and IVF in cat oocytes are generally lower in comparison with those of other species (Farsted, 2000). Only about 50~60% of cat oocytes achieve maturation and, among them, 60~70% of oocytes are fertilized. About 20~30% of fertilized oocytes

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develop into blastocysts *in vitro* (Freistedt et al., 2001; Spindler and Wildt, 1999). To date it has been demonstrated that factors such as season (Freistedt et al., 2001; Goodrowe et al., 1991), culture conditions (Johnston et al., 1991), reproductive cycle (Freistedt et al., 1990) and morphological quality of oocytes influence the development of domestic cat oocytes *in vitro* (Wood et al., 1995; Goodrowe et al., 1991; Pope et al., 1997). For small cats, the techniques of IVF and embryo transfer needed to be developed in order to solve the problems of lower efficiency in pregnancy and fertilization. Among these techniques, the collection of ovum is a difficult and important step.

In this study, we investigated the factors that influence *in vitro* fertilization and *in vitro* development rate. In addition, *in vitro* ability of maturation and fertilization of ovum collected from different stages of reproductive cycle were tested.

Ⅱ. MATERIALS AND METHODS

1. Oocyte recovery and culture

Ovaries were sliced repeatedly with a scalpel blade. After suspended in m-PBS media, oocytes were collected, and groups of 5 oocytes were cultured in an oil-covered 50-ul drop of TCM-199 maturation media supplemented with 2 IU/ml hCG, 1 μg/ml β-estradiol and 10% FCS at 38.5℃ in CO_2 incubator. Oocytes with or without cumulus cells recovered from fresh cat ovaries were cultured for 24 h for *in vitro* maturation. Depending on their morphology, the ovaries were divided into inactive stage (the diameter of follicles are below 2 mm), luteal stage (one or more corpora lutea (CL) present on one or both ovaries) and follicular stage (one or more mature follicles were present at least on one ovary).

2. Pretreatment of sperm

The pretreatment of sperm was performed by the methods described previously (Kim, 2001). Briefly, epididymal tail was removed from cat testes and sliced. After suspended in m-PBS medium at 37℃, the medium containing sperm was centrifuged at 500×g for 5 min and supernatant was removed. The sperm motility was examined. Only sperm samples with motility over 50% were preserved by freezing method. Sperm suspension was diluted with extender R-1 and 2. After equilibrium for 5 min, the sperm suspension was added in 0.25 ml straw and froze using a cell freezer. Finally, the frozen sperm was preserved in LN_2 container. For thawing cryopreserved semen, the straw was thawed in water-bath and placed in Tris buffer for 2 min. Only samples with sperm motility above 50% were used for following IVF experiments.

3. *In vitro* fertilization

Frozen-thawed spermatozoa were diluted with 500 ul of BO medium and swam up for 15 min in CO_2 incubator. After centrifugation at 500 ×g for 5 min, the supernatant was removed and the sperm pellets were treated with 0.6% BSA and 20 μg of heparin (Sigma, USA). IVF was carried out by adding the sperm into culture medium containing mature oocytes and following co-incubation for 12 h.

4. Assessment of fertilization and developmental rate

The oocytes obtained from IVF was treated with 0.2% hyaluronidase (Sigma, U.S.A.) for 1~5 min. After cumulus cells were removed, denuded oocytes were fixed in acetic acid:ethanol(1:3) solution for 24 h and stained with 1% aceto-orcein or 10 μg/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.). The judgement of oocyte maturation *in vitro* was carried out depending on the criteria of fertilization and cleavage by investigating the stained oocytes or embryo development by *in vitro* culture in CO_2 incubator.

5. Statistical analysis

The SAS mixed linear model program was used to analyse the data. Treatment means were compared for differences through the use of Duncan's Modified Multiple Range Test (Duncan, 1955). Differences were considered statistically significant at $p<0.05$.

Ⅲ. RESULTS AND DISCUSSION

1. Developmental rates of ovum collected at different reproductive stages

Developmental rates of *in vitro* cultured oocytes recovered from ovaries that collected at inactive, follicular and luteal

stages of the reproductive cycle were described in Table 1.

Developmental rate to GV and MI stage of *in vitro* cultured cumulus-attached and denuded oocytes recovered from ovaries that collected at inactive, follicular and luteal stages of the reproductive cycles were 72.5% and 27.5%, 57.5% and 7.5%, 62.5% and 17.5%, respectively. The developmental rate to GV state obtained in our laboratory was lower in comparison with the previous report by others in which the developmental rate to GV by using oocytes recovered from ovaries collected at inactive, follicular and luteal stages of the reproductive cycles were 98.1%, 81.8%, 94.2%, respectively. However, the mature ability of oocytes collected from the different stages of reproductive cycles was similar.

2. Developmental rates at different culture conditions

The developmental rates at different culture conditions were described as Table 2.

When culture the oocytes in the different culture conditions as follows: 5% CO_2, 95% O_2; 10% CO_2, 90% O_2 and 10% CO_2, 90% O_2, *in vitro* maturation rate to the GV and MI stages were 70.0%, 27.5%, 52.5% and 20.0%, 55.0%, 12.5%, respectively. Among them, when oocytes were cultured at the conditions of 5% CO_2, 95% O_2, the best maturation rate was obtained.

3. IVF time and fertilization rate

In vitro fertilization rates of oocytes influenced by the IVF time were described as Table 3.

Table. 1. Developmental rate of *in vitro* cultured oocytes recovered from ovaries collected at different stages of the reproductive cycle

Type of oocytes	Cultured oocytes	Developmental stage of oocytes		
		GV	D	MI
Inactive	40	29(72.5)	0(0.0)	11(27.5)[a]
Follicular	40	23(57.5)	14(35.0)	3(7.5)[b]
Luteal	40	25(62.5)	8(20.0)	7(17.5)

[ab] Values within a column with different superscript differ ($p<0.05$).

Table 2. Developmental rates of *in vitro* cultured oocytes recovered from ovaries collected at follicular stage of the reproductive cycle

Type of oocytes	Cultured oocytes	Developmental stage of oocytes		
		GV	D	MI
5% CO_2 95% O_2	40	28(70.0)	1(2.5)	11(27.5)[a]
10% CO_2 90% O_2	40	21(52.5)	11(27.5)	8(20.0)
10% CO_2 90% O_2	40	22(55.0)	13(32.5)	5(12.5)[b]

[ab] Values within a column with different superscript differ($p<0.05$).

Table 3. Fertilization rates of oocytes *in vitro* cultured at different time of the incubation

Time of incubation (hrs)	Cultured oocytes	Developmental stage of oocyte (%)		
		GV	D	MI
17 ~ 20	40	27(67.5)	5(12.5)	8(20.0)
21 ~ 24	40	27(67.5)	1(2.5)	12(30.0)
25 ~ 28	40	25(62.5)	6(15.0)	9(22.5)
29 ~ 32	40	26(65.0)	8(20.0)	6(15.0)

Table 4. Developmental rate of fresh-stored oocytes with and without cumulus cells

Type of oocytes	Examined oocytes	Fertilized oocytes(%)	Cleaved oocytes(%)
Intact	40	29(72.5)[a]	10(25.0)[a]
Denuded	40	15(37.5)	3(7.5)[b]

[ab]: Values within a column with different superscript differ ($p<0.05$).

Table 5. Fertilization rates of *in vitro* cultured oocytes recovered from ovaries collected at different stages of the reproductive cycle

Stage of reproductive cycle	Cultured oocytes	Fertilized oocytes(%)	Cleaved oocytes(%)
Inactive	40	30(75.0)	10(25.0)[a]
Follicular	40	16(40.0)	3(7.5)[b]
Luteal	40	20(50.0)	6(15.0)

* Values within a column with different superscript differ ($p<0.05$).

When IVF was carried out at different periods of 17~20, 21~24, 25~28 and 29~32 hours, the fertilization rates of oocytes matured to the stages of GV and MI *in vitro* were 67.5%, 20.0%; 67.5%, 30.0%; 62.5%, 22.5% and 65.0%, 15.0%, respectively. These results indicate that the IVF time between 24~26 h gives the highest fertilization rate for the oocytes matured at different stages.

4. The relationship between morphology and developmental rate

Developmental rates to GV and MI stages by using freshly collected oocytes with and without cumulus cells were described in Table 4.

The development and cleavage rates of oocytes with and without cumulus cells were 72.5%, 25.0%, and 37.5%, 7.5%, respectively. The fresh oocytes with cumulus cells showed higher development and cleavage rate in comparison with denuded oocytes. There has been no report on development and division rates of cat oocytes. In dogs, when oocytes were cultured in the condition of salt at 4℃ for 48 h, the developmental rates to GVBD and MII were 33.0%~49.0% and 2.0%~6.0%, respectively, and the cleavage rate were 22.4 ~32.2% (Hewitt, 1997). The development and cleavage rate of cat oocytes obtained at our experimental conditions were similar to or greater than those of dog oocytes.

5. The relationship between fertilization rate and reproductive cycles

Fertilization rates of oocytes recovered from ovaries that collected at different stages of reproductive cycle were described in Table 5.

For the oocytes recovered from ovaries that were collected atinactive, follicular and luteal stages, the fertilization rate were 75.0%, 40.0%, and 50.0%, respectively, and the cleavage rate were 25.0%, 7.5%, 15.0%, respectively. In comparison with the report, in which the fertilization were 55.3~57.9%, and the developmental rate to the blastocyst stage was 20.6%~38.1% when the oocytes were cultured for 24 h (Karja et al., 2002), the fertilization and developmental rates obtained at our experimental conditions were slightly lower. However, fertilization and developmental rates of the oocytes with cumulus cells collected from the ovaries at inactive stage are similar to the report by Karja et al.(2002).

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