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Studies on the IVM/IVF Rate of *In Vitro* Cultured Canine Oocytes

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개 난자의 체외성숙과 체외수정에 관한 연구

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SUMMARY

개 난자의 체외성숙과 체외수정을 통해 수정란을 획득하기 위하여 개 난소를 채취하여 난자의 형태, 난소의 채취시기 등이 체외성숙과 체외수정에 미치는 영향을 구명하기 위하여 수행하였다.

1. 난구세포 부착 및 미부착 난자를 48시간 배양했을 때 체외 성숙율은 42.0±3.4%, 24.4±4.1%였다. 난구 세포 부착 난자의 체외 성숙율이 미부착 난자의 체외 성숙율보다 유의하게 높게 나타났다(p>0.05).
2. 난소를 휴지기, 난포기 및 황체기로 구분하여 난자를 회수하여 배양했을 때 체외 성숙율은 각각 35.6%, 50.0%, 31.1%였고 체외 발생율은 각각 2.2%, 20.0% 및 8.9%로서 난구세포 부착 난자가 미부착 난자에 비해 유의한 체외 수정율을 나타냈다.
3. 난구세포 부착 및 미부착 난자를 이용하여 체외수정시켰을 때 체외 수정율과 체외 발생율은 각각 48.0%, 35.6% 및 22.5%, 13.3%로서 난구세포 부착 난자가 미부착 난자에 비해 유의하게 높게 나타났다.

(Key words : canine, oocyes, IVM/IVF, stages of reproductive cycle)

INTRODUCTION

Domestic pet animals have been raised for 2 million years and the current population exceeds 3 million. Raising of the pet has been turned into the and the concern of pet becomes greater (Freistedt *et al.*, 2001; Goodrowe and Hay, 1993). The reproduction of small pet dogs is mainly attained by natural copulation and the proliferation of bad genes has been in great anxiety.

The techniques of IVF and embryo transfer in small breed of canines needs to be developed in order to solve the problems of lower efficiency in fertilization and pregnancy. Among these techniques, the collection of ovum is a very difficult and important step. In domestic canine species, the techniques of *in vitro* maturation, *in vitro* fertilization and *in vitro* embryo culture as well as embryos transfer and cryopreservation have progressed during the last decade. The sperm penetration and *in vitro* fertilization of oocytes has been studied human (Liu and Baker, 1994), bovine (Fazeli *et al.*, 1993), porcine (Iva-

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nova and Mollova, 1993; Maertinez *et al.*, 1993), equine (Codde and Berger, 1995), feline (Goodrowe and Hay, 1993; Howard *et al.*, 1991, Otoi *et al.*, 2001), canine (Hay *et al.*, 1997a; Hay *et al.*, 1997b; Hewitt and England, 1997; Otoi *et al.*, 2000). The correlation betures sperm penetration rate *in vitro* fertilization rate was founded in human (Liu and Baker, 1994) and porcine (Ivanova and Mollova, 1993). Karja *et al.* (2002) reported for the feline oocytes recovered from ovaries that were collected at inactive, follicular and luteal stages and the GV stage was 98.1%, 81.8% and 94.2%, respectively. Hewitt and England (1999) reported that GVBD and M Ⅱ stage of caine oocytes cutured for 48 hours was 33.0% ~49.0% and 2.0%~6.0%, respectively. Hewitt and England (1999) and Bolamba *et al.* (1998) reported that *in vitro* maturaion rate of oocytes cutured in SOF medium supplemented with 3% BSA was a little higher than that of oocytes cultured other medium.

Therefore, study on these fields is urgently needed to obtain high IVM/IVF embryos cultured *in vitro*. This study was carried out to investigate effects of morphology and collection time oocytes on *in vitro* maturation and development rate of immature canine oocytes.

MATERIALS AND METHODS

1. IVM of Oocytes

Canine ovaries were kept in physiological saline and were maintained at 38℃ for 1~6 h before oocyte recovery. Oocytes were collected from fresh ovaries. The collected oocytes were cultured in TCM- 199 medium (Whittaker, U.S.A.) supplemented with 2 IU/ml hCG (Sigma, U.S.A.), 1 μg/ml β-estradiol (Sigma, U.S.A.) and 10% (v/v) FCS (Sigma, U.S.A.). Five oocytes were transferred to 50 μl drops of maturation medium covered mineral oil and cultured in a CO_2 incubator (5% CO_2, 95% air, 38.5℃) for 48 hrs. Cumulus-oocyte complexes and denuded oocytes were cultured for 48 h and investigated to GV, GVBD and M Ⅱ. The reproductive cycle stages of ovarian pairs were classified into inactive (ovaries with no follicles $\geq$ 2 mm in diameter), follicular (ovaries with follicled 22 mm in diameter) and luteal (one or more corpora luteal) on at least one ovary.

2. IVF of Oocytes

After IVM, five oocytes were transferred to 50 μL drops of maturation medium under mineral oil and cultured in CO_2 incubator for 24 h and transferred to each droplet of 50 μl fertilization medium. After swim-up treatment for 30 min, the supernatant was added with fertilization medium and centrifuged at 1,000 rpm for 10 min. The sperm pellet was diluted with 20 μl of heparin (Sigma, U.S.A.) solution and incubated for 15 min in CO_2 incubator. Two μl of capacitation-sperm suspension was added in to the fertilization medium containing matured oocytes.

3. Assay of IVF and IVD Rate

The *in vitro* matured oocytes were mounted on slide glass and fixed with ethanol : acetic acid (3 : 1) for 24 hrs and stained with 10 μg/ml bisbenzimide (Hoechst 33342). The stage of meiosis under fluorescence microscope. The IVF and *in vitro* development of oocytes was based on the cleavage for *in vitro* culture of 7 days in CO_2 incubator, respectively.

4. Statistical Analysis

For comparison of means, Duncan's multiple range test was performed using SAS package of General Linears Model (GLM) procedures (SAS Institute, 1996).

RESULTS AND DISCUSSION

1. *In Vitro* Maturation of Oocytes

The maturation of oocytes with or without cumulus cells cultured for 48 hrs as shown in Table 1.

The *in vitro* maturation rates of oocytes with or without cumulus cells were 42.0±3.4%, 24.4±4.1%, respectively. The IVM rate of oocytes with cumulus cells was higher than that of oocytes without cumulus cells.

This result was a little higher with Hewitt and England (1999) reported that the *in vitro* maturation rates of canine oocytes to GVBD and M Ⅱ stage was 33.0~49.0% and 2.0~6.0%, respectively. This results indicated that *in vitro* fertilization rate was higher when cultured fresh oocytes with excellent morphology and compact cumulus cell.

2. IVM and Development of Oocytes

In vitro maturation rate of oocytes recovered from ovaries that were collected at inactive, follicular and luteal stages is shown in Table 2.

IVM rate of oocytes recovered from ovaries inactive, follicular and luteal stage was 35.6%, 50.0%, 31.1%, respectively, and *in vitro* developmental rates to 16 cell stage were 2.2%, 20.0%, and 8.9% respectively.

This result was similar with Hewitt and England(1999), who reported that the into GVBD and M Ⅱ stage in canine oocytes was 45.0%, 6.0%, 36.0%, The result of *in vitro* develomental rate was also similar to or higher than those of Otoi *et al.* (2000), who reported that the *in vitro* maturation of canine oocytes. When canine oocytes in SOF medium supplemented with 3% BSA was cultured the IVM rate was a little higher than that of oocyte cultured in other medium(Hewitt and England, 1999; Bolamba *et al.*, 1998).

3. IVF and Development Rate of Oocytes

In vitro fertilization and developmental rates of oocytes with or without cumulus cells recovered from fresh ovaries for 48 hrs was shown in Table 3.

Table 1. IVM rates of oocytes with and without cumulus cells

Type of oocyte	No. of oocytes examined	No. of oocytes at			IVM rate (%)
		GV	GVBD	M Ⅱ	
Intact	50	5	24	21	42.0±3.4[a]
Denuded	45	13	21	11	24.4±4.1[b]

* Mean±S.D.

** [a,b] : Values with different letters between intact and denuded oocytes differ significantly ($p<0.05$).

Table 2. Nuclear status of fresh oocytes recovered from ovaries collected at different stages of the reproductive cycle

Ovarian stage	No. of oocytes examined	No. of oocytes at		Developmental rates (%)*
		GV	M Ⅱ	
Inactive	45	7 (15.6)	16 (35.6)	1 (2.2)[b]
Follicular	50	22 (44.0)	25 (50.0)	10 (20.0)[a]
Luteal	45	8 (17.8)	14 (31.1)	4 (8.9)[b]

* [a,b] : Values with different letters within same columns differ significantly ($p<0.05$).

** Oocytes developed to 16 cells.

Table 3. *In vitro* fertilization rate of oocytes with or without cumulus cells

Type of oocytes	No. of oocytes		
	Cultured	Fertilized (%)	Developed (%)*
Intact	50	24 (48.0)[a]	10 (22.5)[a]
Denuded	45	16 (35.6)[b]	6 (13.3)[b]

* [a,b] : Values with different letters within same columns differ significantly ($p<0.05$).
** Oocytes developed to 16 cells.

In vitro fertilization and developmental rates of oocytes with cumulus cells for 48 hrs was 48.0%, 35.6% and 22.5%, 13.3%, respectively. These rates of oocytes with intact cumulus cells were higher significantly than that of ocytes with denuded cumulus cells.

This result was similar or a little lower than Quan *et al.* (2004) who reported that the *in vitro* fertilization and cleavage rate of salt-stored feline ovaries was 22.3~57.1% and 23.3~33.0%. Hewitt and England (1999) and Bolamba *et al.* (1998) reported when cultured canine oocytes in SOF medium supplemented with 3% BSA was a little higher than that cultured other medium.

CONCLUSION

The studies were carried out to investigate the effects of morphology and collection time of oocytes on *in vitro* maturation and fertilization of canine oocytes.

The results were summarized as follows :

1. The *in vitro* maturation rates of oocytes with or without cumulus cells were 42.0±3.4%, 24.4±4.1%, respectively. The IVM rate of oocytes with cumulus cells was higher than that of oocytes without cumulus cells. The rate of oocytes with cumulus cells was higher than that of denuded oocytes.
2. IVM rate of oocytes recovered from ovaries inactive, follicular and luteal stage was 35.6 %, 50.0%, 31.1%, respectively. *In vitro* developmental rates to 16 cell stage were 2.2%, 20.0%, 8.9% respectively. *In vitro* fertilization rate of oocytes with cumulus cells were significantly lower than that of denuded oocytes.
3. *In vitro* fertilization and developmental rates of oocytes with cumulus cells for 48 hrs was 48.0%, 35.6% and 22.5%, 13.3%, respectively. These rates of oocytes with intact cumulus cells were higher significantly than that of ocytes with denuded cumulus cells.

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