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Studies on the Effects of Collection Time, Supplementation of EGF and Hormones on IVM Rates of Canine Oocytes

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개 난자의 채취시기, EGF 및 호르몬 첨가가 체외성숙율에 미치는 영향에 관한 연구

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SUMMARY

본 연구는 개 체외성숙 난자를 안정적으로 생산하기 위하여 채취시기, 난구세포 부착 여부 및 배양액에 EGF와 호르몬을 첨가 후 배양했을 때 체외성숙율에 미치는 영향을 조사하였다.

1. 미성숙 난포란을 TCM-199 배양액에서 24, 48시간 배양했을 때 체외성숙율은 각각 7.93%, 8.94%로서 48시간 배양했을 때 가장 높은 체외성숙율을 나타냈다.
2. 휴지기, 난포기, 황체기에 채취한 난소로부터 회수한 난자를 20 ng/ml의 EGF가 첨가된 TCM-199 배양액에서 배양했을 때 체외성숙율은 14.3%로서 0, 10 ng/ml의 EGF 첨가군(3.1%, 7.5%)에 비해 높은 체외성숙율을 나타냈다.
3. 난구세포 부착 및 미부착 난자를 48시간 배양했을 때 체외성숙율은 각각 18.8% 및 7.5%로서 난구세포 부착 난자가 미부착 난자보다 높은 체외성숙율을 나타냈다.
4. 난자의 체외성숙 배양 시 0.5 mg/ml FSH, 5 mg/ml LH, 1 mg/ml E_2와 FSH+LH, FSH+LH+E_2를 첨가한 TCM-199 배양액에서 배양했을 때 체외성숙율은 각각 1.2%, 10.0%, 2.0%와 10.0%, 31.2%로서 호르몬의 병용처리군이 높은 체외성숙율을 나타냈다.
5. 난자의 체외성숙 배양 시 EGF와 FSH, LH, E_2 및 EGF와 FSH+LH, FSH+LH+E_2를 첨가한 TCM-199 배양액에서 배양했을 때 체외성숙율은 32.3%, 27.0%, 3.0%와 36.2%, 69.4%로서 EGF와 호르몬 병용 처리군이 높은 체외성숙율을 나타냈다.

(Key words : EGF, hormones, incubation time, IVM, reproductive cycle)

INTRODUCTION

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 (Gunzel, 1986; Kim, 2001).

Generally, IVM and IVF rate of canine oocytes were lower than those of other animals (Farstad,

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2000). The maturation rate of canine oocytes was only about 50~60%, and 60~70% of oocytes were *in vitro* fertilized (Wood *et al.*, 1995). Farstad (2000) and Luvoni *et al.* (2001) reported that maturation rate to GV stage of canine oocytes were 0~58%, maturation to MII phase of oocytes was 20% (Holst and Phemister, 1971). Hewitt and England (1999) reported that the developmental rates to GVBD and MII stage of canine oocytes cultured 48 hrs were 33.0~49.3% and 2.0~6.0%, respectively. Freistedt *et al.* (2001), Spindler and Wildt (1999) reported that the *in vitro* development rate of canine oocytes was 20~30%. It was reported that season (Freistedt *et al.*, 2001; Goodrowe and Hay, 1991), culture condition (Johnston *et al.*, 1991), reproductive cycle (Freistedt *et al,*, 2001) and morphology of oocytes (Wood *et al.*, 1995; Goodrowe and Hay, 1991; Pope *et al.*, 1997) can affect oocytes *in vitro* development rate. Hewitt and England (1999) reported that developmental rates to GVBD and MII stage of canine oocytes cultured with SOF medium supplemented with 3% BSA and SOF medium supplemented with 4% BSA for 48 hrs. were 20/44 (45.0%), 2/33 (6.0%) and 15/ 44 (36.0%), 3/42 (7.0%), respectively.

EGF is important for cytoplasmic maturation : the addition of EGF to maturation medium stimulated meiotic maturation (Ding and Foxcroft, 1993; Abeydeera *et al.*, 1998; Chance *et al.*, 1979; Kim *et al.*, 2004; Gomez *et al.*, 1993; Lorenzo *et al.*, 1994). Bolamba *et al.* (2005) reported that maturation rate of canine oocytes cultured with TCM-199 medium supplemented with FSH, LH and FSH+LH, FSH+LH+17β-estradiol were 7.0~8.0% and 28.0~40.0%, respectively.

For the small-size dogs of sterility treatment, which were difficulty in sterility, it was very essential of transplantation of embryos. But unlike other animals, there were many difficulties when transplanting embryos. Specially, it is difficult to secure the ovaries temporarily, and also the *in vitro* fertilization technique is difficult, compares to those of other animal's.

The objective of this study was to produce *in vitro* fertilized embryos and solute canine sterile. This study was carried out to investigate the effects of collection time, supplementation of EGF and hormones on IVM rates of canine oocytes.

MATERIALS AND METHODS

1. Oocyte Collection and *in vitro* Maturation (IVM)

Canine ovaries were transported to the laboratory in 0.9% (w/v) NaCl containing 75 ul/ml penicillin G at 25℃. Oocytes were sliced with surgical blade and suspended with m-PBS and then collected. Collected oocytes were cultured with TCM-199 medium supplement with 0, 10, 20 ng/ml EGF or 0.5 mg/ml FSH, 5 mg/ml LH, 1 mg/ml 17β-estradiol (E_2) and 10% (v/v) FCS (Sigma, U.S.A.). Ten oocytes were transferred to 50μl drops of maturation medium covered mineral oil and cultured in CO_2 incubator (5% CO_2, 95% air, 38℃) for 0~72 hrs. 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 luteal present on one ovary) and follicular stage (one or more mature follicles were present on one ovary).

2. Assessment of *in vitro* Maturation Rate

After maturation 44~48 hrs, oocytes were freed from cumulus cells by gentle pipetting in the same IVM medium containing 0.1% hyaluronidase (Sigma, U.S.A.) solution for 1 min. and washed three times in TL-Hepes medium containing 1% BSA. Some of the denuded oocytes were fixed for 5 min in acetic acid : ethanol = 1:3) at room temperature. They were then stained with 10 ug/ml Hoechest 33258 (Sigma, U.S.A.) in mounting medium containing PBS and glycerol (1:1). Oocytes were mounted on slide and evaluated under fluorescent microscope (400~1,000×) to determine the stage of meiosis.

3. Statistical Analysis

The results were expressed by treatment as mean ±SD. For comparison of means, Duncan's multiple verification was performed using SAS package of General Linears Model (GLM) procedures (SAS Institute, 1996).

RESULTS AND DISCUSSION

1. Effects of Culture Time on the IVM Rate

This experiment was conducted to investigate the effects of culture time on the IVM rate of oocytes cultured in TCM-199 medium for 0~48 hrs, the *in vitro* maturation rate were shown in Fig. 1. The IVM rate of oocytes cultured in TCM-199 medium for 24, 48 hrs were 7.93%, 8.94%, respectively. When the oocytes were cultured in TCM-199 medium for 48 hrs, the IVM rate were higher than cultured 24 hrs. This result was higher than Hewitt and England (1999) reported that the maturation rate to MII stage of canine oocytes cultured for 48 hrs were 2.0~6.0%.

2. Effects of Oocytes Collected at Different Reproductive Stage on the IVM Rate

IVM rate oocytes recovered form ovaries that collected a inactive, follicular and luteal stages of the reproductive cycle cultured with TCM-199 medium supplement with 0, 10, 20 ng/ml EGF were shown in Fig. 2. Developmental rate to MII stage

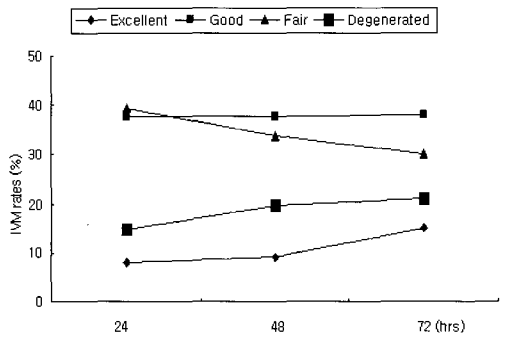

Fig. 1. IVM rates of canine oocytes incubated for 0~72 hrs.

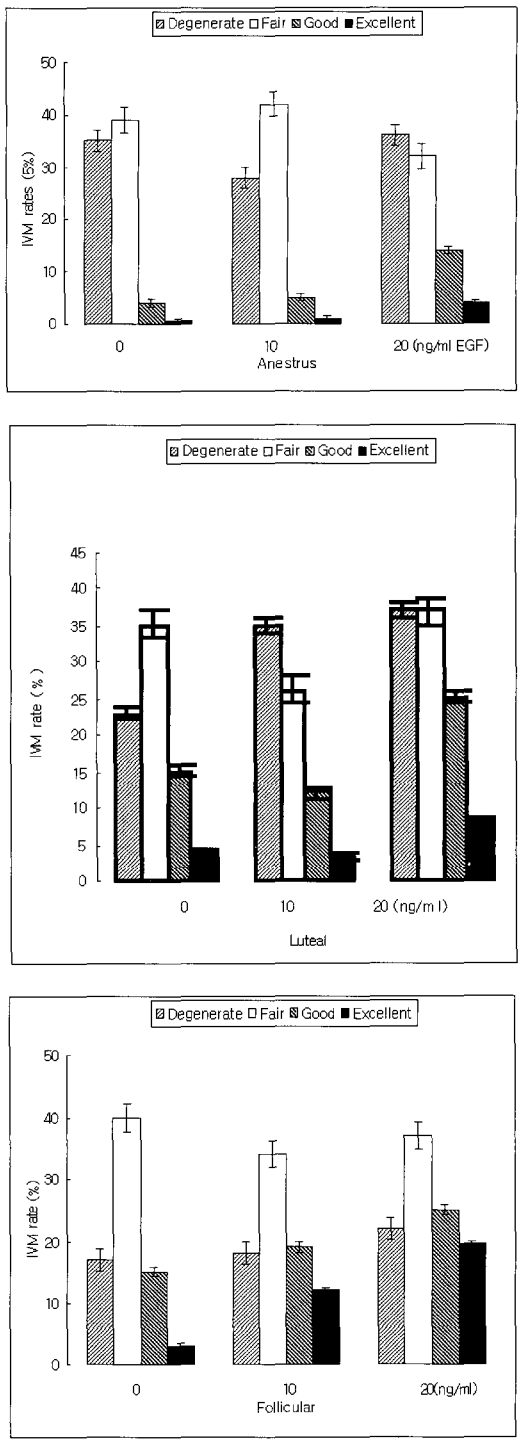

Fig. 2. IVM rates of *in vitro* cultured oocytes from ovaries collected at different stages of the reproductive cycle.

of cumulus-attached oocytes recovered from ovaries that collected at inactive, follicular and luteal stages of the reproductive cycle cultured with TCM-199 medium supplement with 0, 10, 20 ng/ml EGF were 3.1%, 7.5%, 14.3%, respectively. This results was higher than Otoi *et al.* (2000) reported that IVM rate to MII stage of canine oocytes cultures with SOF medium supplemented with 10 ng/ml EGF were 4.0~5.2%. Karja *et al.* (2002) reported that IVM rate to GV stage of cats oocytes recovered form ovaries that collected a inactive, follicular and luteal stages of the reproductive cycle cultured with SOF medium were 98.1%, 81.8%, 94.2%, respectively.

3. Effects of Oocytes with or without Cumulus Cells on the IVM Rate

The IVM rate of oocytes with or without cumulus cells cultures with TCM-199 medium for 0~48 hrs were shown in Fig. 3. The IVM rate of oocytes with or without cumulus cells cultured with TCM-199 medium for 0~48 hrs were 18.8% and 7.5%, respectively. The IVM rate of oocytes with cumulus cells was higher than that of oocytes without cumulus cells. This result was a little higher than with Hewitt and England (1999) reported that the IVM 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 IVM rate was higher when cultured for 43~46 hrs fresh oocytes with excellent morphology and compact cumulus cells. And this results similar with Lee and Kim (2003) reported that the IVM rate of oocytes with or without cumulus cells were 34.3% and 20.0%.

4. Effects of Hormones on the IVM Rate

This experiment was conducted to investigate the effects of hormones on the *in vitro* maturation of oocytes cultured in medium supplement with hormones. When oocytes were cultured 48 hrs in TCM-199 medium supplement with hormones, the IVM rate of oocytes were shown in Fig. 4. The IVM rate of oocytes cultured in medium supplement with 0.5 mg/ml FSH, 5 mg/ml LH, 1 mg/ml E_2 were 1.2%, 10.0%, 2.0%, respectively. The IVM rate of oocytes cultured in medium supplement with FSH+LH, FSH+LH+E_2 were 10.0%, 31.2%, respectively ($p<0.05$). This results was lower than with Bolamba *et al.* (2005) reported that the IVM rate of oocytes cultured in medium supplement with FSH, LH were 28.0~40.0%. The IVM rates of oocytes cultured in medium supplement with FSH+LH, FSH+LH+E_2 were significantly higher than cultures in medium supplement with FSH, LH (Bolamba *et al.*, 2005).

5. Effects of EGF + Hormones on the IVM Rate

When oocytes were cultured 48 hrs in TCM-199 medium supplement with EGF+hormones, the IVM

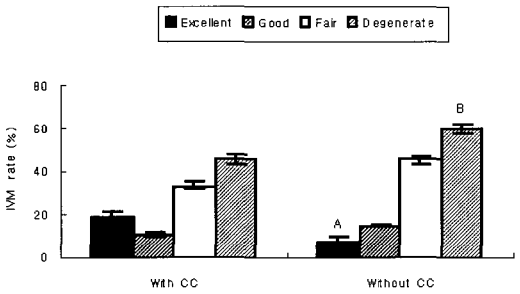

Fig. 3. IVM rate of oocytes with or without cumulus cell cultured for 48 hrs.

* Values with different superscripts in same columns were denoted significantly different ($p<0.05$).

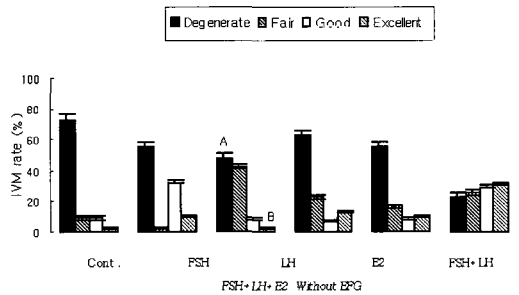

Fig. 4. IVM rate of oocytes recovered the follicular stage matured in TCM-199 supplement with FSH, LH and E_2.

* Values with different superscripts in same columns were denoted significantly different ($p<0.05$).

rate of oocytes were shown in Fig. 5.

The IVM rate to MII stage of oocytes cultured in medium supplement with FSH, LH, E_2+EGF were 32.3%, 27.0%, 3.0%, respectively. And the IVM rate to MII stage of oocytes cultured in medium supplement with FSH+LH, FSH+LH+E_2 were 36.2 %, 69.4% (p<0.05). The IVM rate to MII stage of oocytes cultured in medium supplement with hormone+EGF (36.2%, 69.4%) higher than that of supplement with hormones (3.0~32.3%). This results was similar with Bolamba *et al.* (2005) reported that the IVM rate of oocytes cultured in medium supplement with EGF+FSH, LH (28.8~40.0 %) higher than that of supplement with EGF+FSH, LH (7.0~8.0%).

CONCLUSION

The study was carried out to investigate the effects of collection time, morphology, supplementation of EGF, FSH, LH, E_2 and FSH+LH, FSH+LH+E_2 on IVM rate of canine oocytes. The IVM rates of oocytes with cumulus cell cultured for 24, 48 hrs in CO_2 incubator with 5% CO_2 in air at 38.5℃.

The results were summarized as follows :

1. The IVM rates of oocytes cultured in TCM-199 medium for 24 and 48 hrs were 7.93%, 8.94%, respectively. The rate of oocytes cultured for 48 hrs was higher than that oocytes cultured for 24 hrs.
2. The IVM rates oocytes recovered from ovaries collected at different stages of the inactive, follicular and luteal stage cultured in TCM-199 medium supplement with 20 ng/ml of EGF were 14.3%. The IVM rate of oocytes was higher than to those group with supplement of 0, 10 ng/ml of EGF (3.1%, 7.5%)
3. The IVM rates of oocytes with or without cumulus cell cultured in TCM-199 medium for 48 hrs were 18.8% and 7.5%, respectively. The IVM rate of oocytes with cumulus cell cultured for 48 hrs was higher than that of denuded oocytes.
4. When the oocytes were cultured in a medium supplement with FSH, LH, E_2, FSH+LH, FSH +LH+E_2, the IVM rates were significantly increased to 1.2%, 10.0%, 2.0%, 10.0%, 31.2 %, respectively.
5. When the oocytes were cultured in a medium supplement with EGF+FSH, LH, E_2, FSH+ LH, FSH+LH+E_2, the IVM rates were 32.3%, 27.0%, 3.0%, 6.2%, 69.4%, respectively. The rates showing that the IVM rates were significantly increased when both the EGF and hormone treatment were applied.

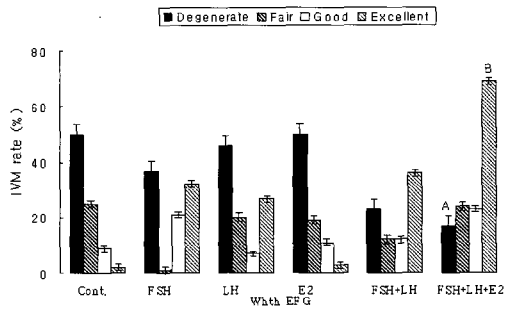

Fig. 5. IVM rate of oocytes recovered the follicular stage matured in TCM-199 supplement with 0~20 ng/ml EGF+hormones.

* Values with different superscripts in same columns were denoted significantly different (p<0.05).

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