# Effects of Collection Time, Culture Time and Activation Treatment of Canine Oocytes on the IVM Rates

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## ABSTRACT

These study was carried out to investigate the effects of the collection time, culture time and activation of canine oocytes on *in vitro* maturation rates. The activated oocytes were cultured in 10% FCS+TCM-199 media containing hormonal supplements (10 IU/ml HCG, 10 IU/ml PMSG, 10 ug/ml gonadotropin) at 5% $CO_2$, 95% air, 38℃.

1. IVM rate of *in vitro* cultured cumulus-attached oocytes recovered from ovaries that collected at follicular and luteal stages of the reproductive cycles were 11.4% and 5.7%, respectively. IVM rate of oocytes recovered from ovaries that collected at follicular stages of the reproductive cycles was significantly higher than that of luteal stage ($p<0.05$).
2. When IVM was carried out at different periods of 40, 48, and 70 hrs, the IVM rates of oocytes matured *in vitro* were 2.9%, 8.6%, 5.7%, respectively. These results indicate that the IVM time between 48~70 hrs gives the highest maturation rate for the oocytes matured at the different stages.
3. IVM rate of oocytes matured *in vitro* for 10 hrs after single and combined activation treatment by ET, IP and CH and Ca+DMAP, CH+DMAP, ET+CH were 11.5±1.2%, 10.8±1.0%, 9.6±1.2% and 12.4±1.5%, 11.8± 1.5%, 11.2±1.4% respectively. This was higher than that in both single and combined stimulated groups compared to control group (6.2~7.2%).

(Key words : canine oocytes, collection time, culture time, activation, IVM rate)

## INTRODUCTION

Embryo development of canine oocytes matured and fertilized *in vitro* has recently been achieved. Although cleavage rates ranging between 8% and 37% have been reported (Hewitt and England, 1997; Otoi *et al.*, 2000; Songsasen *et al.*, 2002; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005), only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have developed in culture.

The low rates of canine embryo development testify to the inefficient developmental competence of oocytes matured *in vitro* and justify the main focus of research in the dog to be *in vitro* maturation (IVM). In the last decade, several attempts have been made to improve cultural conditions of maturation taking into account the main peculiar characteristic of the canine oocyte: the extra-follicular maturation that requires an extended period of time, 2~5 days (Holst and Phemister, 1971; Rsutsui, 1975). However, a recent report indicates that oocytes collected from ovaries at the follicular phase achieved 41% of maturation after 72 hrs of culture (Otoi *et al.*, 2004). These results are poor if compared with those obtained in other carnivores as the cat, in which up to 50% blastocyst rates can be obtained *in vitro* (Gomez *et al.*, 2003) or in ruminants as the bovine, in which blastocyst rates of 40~60% are routinely produced (Hansel, 2003). Yamada *et al.* (1992) reported that pre- ovulatory oocytes collected from canine ovaries treated with exogenous gonadotropins achieved the highest maturation rates after 72 h of culture (31.9%). After 12 h of coincubation with spermatozoa, 32% underwent monospermic fertilization and 37% of the total fertilized oocytes showed the male pronucleus. At 96 h after insemination, 13.3% of embryos reached beyond the 4-cell stage in culture. In situ pre-ovulatory priming might be helpful for canine oocytes to improve the acquisition of developmental competence and pre-ovulatory oocytes might be more competent than those collected any time during follicular phase of the oestrus cycle, which in the dog lasts for an extended length of time.

Ethanol, an agent that incites oocyte activation via a mono-

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tonic rise in calcium, was employed as a control (Bedford *et al.*, 2003; Loren and Lacham-Kaplan, 2006). To activate the enucleated oocytes, several calcium-elevating agents, such as ethanol (Minamihashi *et al.*, 1993) or Ca ionophore (Ware *et al.*, 1989; Shi and Jiang, 1993; Yang *et al.*, 1994) have been used.

The purpose of this study was to investigate the effects of the collection time, culture time and activation of canine oocytes on *in vitro* maturation rates.

## MATERIALS AND METHODS

### 1. Collection and Incubation of Oocytes

Canine ovaries were transported to the laboratory in sterile physiological saline containing 100 IUl/ml penicillin G, 100 ug/ml streptomycin at 25℃. Oocytes were sliced with surgical blade and suspended with mPBS and then collected. Collected oocytes were cultured with TCM-199 (Whittaker, U.S.A.) medium supplemented with 10% (v/v) FCS (Gibco. U.S.A.) and 1 mg/ml cysteine(Sigma, U.S.A.), 20 ng/ml $E_2$, 10 IU/ml HCG, 10 IU/ml PMSG and 10 ug/ml gonadotropin. Twenty oocytes were transferred to 50 ul drops of maturation medium covered mineral oil and cultured in a $CO_2$ incubator (5% $CO_2$, 95% air, 38℃).

### 2. Collection, Incubation and Activation of Oocytes

Depending on their morphology, the ovaries were divided into follicular stage (one or more mature follicles were present at least on one ovary) and luteal stage (one or more copora lutea present on one or both ovaries). Then each group of 50 oocytes was cultured in 50 $\mu$l of maturation medium. Oocytes were transferred into 50 $\mu$l drops of TCM-199 medium and cultured for 40, 48, 70 hrs. In order to activate oocytes at 24 hrs post onset of maturation, the oocytes were cultured with 10 ug/ml ethanol (ET), 10 $\mu$M/ml Ca or inophore (IP) for 5 min, 10 ug/ml CH for 6 hrs, 2.0 mM 6-dimethylaminopurine (DMAP) for 3 hrs alone or combination. After stimulations, the oocytes were removed from activation solution and cultured in maturation medium.

### 3. Assessment of Meiotic Stage

Oocytes were fixed in acetic acid : ethanol (1:3) solution for 24 h then stained using with 1% acetoorcein or 10 $\mu$g/ml *bisbenzimide (Hoechst 33342) and observed under an fluore*scence microscope. The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nuclear division, and *in vitro* development by investigating oocytes of development *in vitro*.

### 4. Statistical Analysis

The One-way ANOVA were used to determine the statistical significance of differences between values for the experimental and control groups. *P* values of 0.05 or less were considered as statistically significant.

## RESULTS AND DISCUSSION

### 1. Effect of Collection Time on IVM Rates

*IVM rates of in vitro cultured oocytes recovered from ca*nine ovaries that collected at follicular and luteal stages of the reproductive cycles were described in Table 1.

IVM rate to M Ⅱ stage of *in vitro* cultured cumulus-attached oocytes recovered from ovaries that collected at follicular and luteal stages of the reproductive cycles were 8.6% and 2.9%, respectively. IVM rate of oocytes recovered from ovaries that collected at follicular stages of the reproductive cycles was significantly higher than that at luteal stage ($p<0.05$). These result was lower than that of Otoi *et al.* (2004) report that the *oocytes collected from ovaries at the follicular phase* achieved 41% of maturation after 72 hrs of culture. However, only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have developed in culture.

### 2. Effect of Culture Time on IVM Rate

IVM rates of oocytes *in vitro* cultured at different incubation time were described in Table 2.

When IVM was carried out at different periods of 40, 48, and 70 hrs, the IVM rates of oocytes matured to the stage of M Ⅱ *in vitro* were 2.9%, 8.6%, 5.7%, respectively. These results indicate that the IVM time between 48~70 hrs gives the highest maturation rate for the oocytes matured at the different stages. These results was lower than that of Yamada *et al.*

Table 1. IVM rates of canine oocytes collected at different stages of reproductive cycle

| Collection time of oocytes | No. of oocytes examined | No. of oocytes developed to M Ⅱ stage |
|---|---|---|
| Follicular | 35 | 3 (8.6)[a] |
| Luteal | 35 | 1 (2.9)[b] |

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

Table 2. IVM rate of canine oocytes cultured at different incubation time

| Incubation time | No. of oocytes examined | No. of oocytes developed to MII stage |
|---|---|---|
| 40 | 35 | 1 (2.9) |
| 48 | 35 | 3 (8.6) |
| 70 | 35 | 2 (5.7) |

(1992) report, in which pre-ovulatory oocytes collected from ovaries of bitches treated with exogenous gonadotropins achieved the highest maturation rates after 72 h of culture (31.9%).

### 3. Effects of Oocytes Activation on IVM Rates

Effects of activation treatment of oocytes *in vitro* cultured on IVM rates were shown in Fig. 1.

IVM rate of oocytes matured *in vitro* for 48 hrs after single and combined activation treatment by ET, IP and CH and Ca+DMAP, CH+DMAP, ET+CH were 11.5±1.2%, 10.8±1.0%, 9.6±1.2% and 12.4±1.5%, 11.8±1.5%, 11.2±1.4%, respectively. This

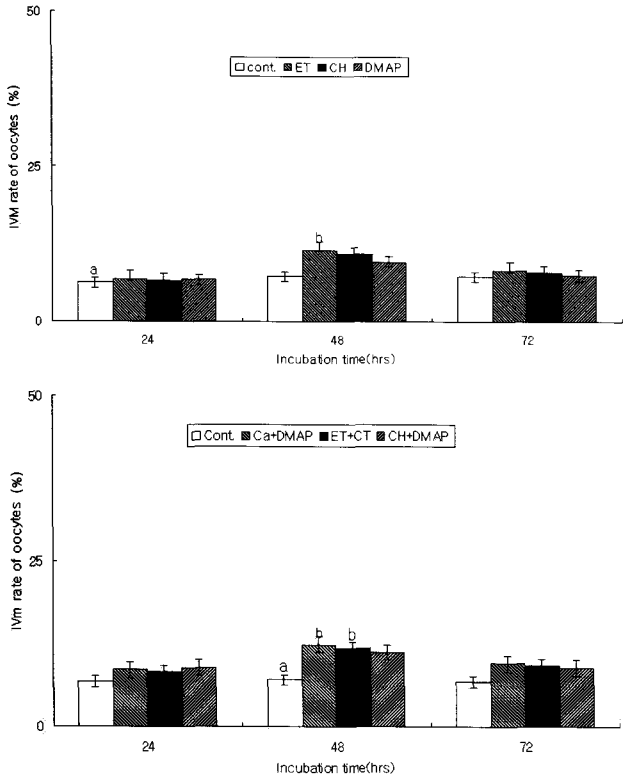


Fig. 1. Effects of single and combined activation treatment on IVM rate of oocytes. [a,b] Values within column with different superscript ($p<0.05$).

was higher than that in both single and combined stimulated groups compared to control group (6.2~7.2%). Although there was no similar report, this result was lower with Shi and Jiang (1993) reported that the survival rate of porcine oocytes activated with Ca-CH (22~48%).

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