Jour. Agri. Sci.
Chungnam Nat'l Univ., Korea
Vol.35(2): 167~176 (2008)

Influence of Reproductive Status, Serum Type and Estradiol-17β Supplementation on the *in vitro* Maturation of Canine Oocytes

Fibrianto Yuda Heru[1] · Minkyu Kim[2*]

ABSTRACT

Supplementation of serum and estrogen in *in vitro* maturation(IVM) medium was shown to improve embryo development and quality in several species. This study investigates the effect of ovarian estrus stage on canine oocyte quality and supplementation of medium with canine serum or estrogen on IVM of canine oocytes. As results, in experimental 1, IVM oocytes collected from follicular stage ovaries to MII stages(10.2±1.5%) was higher ($p<0.05$) with 10% canine estrus stage serum than control(1.3±1.6%), anoestrus stage serum(4.0±1.6%), luteal stage serum (2.7±1.7%) and 10% FBS(1.3±1.6). In experimental 2, 10% canine estrus stage serum supplementation has highest maturation rate to MII stages(10.0±1.8%) and there were significant differences($P<0.05$) with another treatment in follicular stages group. In order to investigate the synergic effect of estrous serum and estrogen supplementation, different estrous stage groups of oocytes were cultured with 2 ug/ml estrogen plus various concentrations of different reproductive stage serum and FBS(experimental 3). As results, the rate of maturation to metaphase II(MII) stage was significantly higher($p<0.05$) in oocytes from the follicular stage supplemented with estrogen and 10% canine estrus stage serum(11.5%) compared to the other groups(6.0 - 8.8%). The present study was demonstrated that canine serum and the estrus cycle of the bitch affect the meiotic competence of oocytes. Hormonal influences within the follicle may be one of the

Received Feb. 14, 2008; Accepted Jun. 5, 2008

[1] Faculty of veterinary physiology, Gadgah mada University, Karangmalang Yogyakarta 55281, Indonesia.
[2] Department of Animal Science and Biotechnology, College of Agriculture and Life Sciences, Chungngam National University, Daejeon 305-764, Korea
* Correspondence: Min-Kyu Kim(E-mail: kminkyu@cnu.ac.kr, Tel: +82-42-821-5773, Fax: +82-42-825-9754)

factors responsible for the greater proportion of maturation of oocyte to MII from bitches at the follicular phase.

Key words : Canine oocytes, *In vitro* maturation, Estrus serum, Estradiol 17-β

Ⅰ. INTRODUCTION

In canine, estrus cycle of bitch is unique that made further complicate the development of research methods to improve *in vitro* maturation(IVM) in this species. *In vitro* maturation of oocyte study has been limited to a small number of studies and the rate of maturation of oocytes to metaphase II still low(<40%)(Ottoi *et al.*, 2002). Successful maturation is a prerequisite for the techniques required for assisted reproduction of endangered canine species. The difficulty in obtaining high rates of *in vitro* matured bitch oocytes is probably due to the peculiar reproductive process of this speces including the hormonal environment and meiotic resumption and progression. In most mammals the oocytes is primarily exposed to high concentration of estrogen before ovulation and in the bitch, ovulated oocytes in the germinal vesicle stage and than completed meiotic maturation take place in oviduct and reach metaphase II by 3-5 days after LH peak (Tsuitsui, 1989).

A few studies have compared the development competence of oocytes from various stages of the estrus cycle (Yamada *et. al.*, 1993; Hewitt and England, 1997; 1999a; Ottoi *et al.*, 2001; Rodrigues and Rodrigues, 2003), but the result varies among them.

Serum or sources of protein addition in culture medium have been shown to improve the *in vitro* survival rate of mouse oocyte and to prevent hardening of the zona pelucida. In canine serum supplementation has been used by several researchers(Mahi and Yanagimachi, 1976; Yamada *et al.*, 1993; Hewitt and England, 1997; 1999a; Ottoi *et al.*, 1999; Bolamba *et al*; 2002). Hewitt *at al.*(1998) reported that supplementation of culture medium with 0.3% BSA had a positive effect on the IVM of canine oocytes, and Nickson *et al.*(1993) obtained the high rate of maturation of oocyte from bitches at various stage of the estrous cycle by supplementation of the culture medium with oestrous bitch serum and estradiol. Rodrigues *et al.*(2003) found that reproductive status of the female not influence *in vitro* nuclear maturation of dog oocyte.

Estrogen secretion is primarily by antral and preovulatory follicles, and reach peak concentrations as the follicles become competent to ovulate (Concanon, 1995), which indicate that these hormone may play a significant role in maturation of oocyte in most mammalian species.

The aim of this study was to evaluate more thoroughly the effect of stage estrus cycle, serum type and estrogen supplementation in culture medium on IVM of canine oocytes.

II. MATERIAL AND METHODS

1. Collection of Immature Oocyte and *In Vitro* Maturation

Reproductive tracts from normal bitches greater than 12 months of age were collected after routine ovariohysterectomy at private clinics, placed immediately into physiological saline solution at 37°C and transported to the laboratory within 1 hr. Ovaries removed from the tract were washed off blood in fresh PBS and minced with a shaving blade at room temperature in the bench medium consisting of M-199 (Life Technologies, Rockville, MD) with 25 mM HEPES (Life Technologies), 1% fetal calf serum (Hyclone, UT) and 1% Pennicillin-Streptomyocin solution (Life Technologies). Ovaries were classified into groups of anestrous, follicular and luteal stage based on present of follicle and luteal tissue. Only grade 1 oocytes (according to the criteria reported by Kim *et al.*, darkly pigmented and completely surrounded by one or more layers of cumulus cell) were selected and used for the experiments.

Immature oocytes were incubated for 72 hr in a 500ul drop of serum-free tissue culture medium (TCM)-199 (Life Technologies) in the presence or absence of steroid hormone as described in the experimental design at 39 °C in a humidified atmosphere of 5% CO_2 in air. At the end of the maturation culture, oocytes were completed denuded from cumulus cells by repeated pipetting in the same IVM medium containing 0.5 mg/mL hyaluronidase (Sigma-Aldrich) for 1 to 3 min.

2. Assessment of Meiotic Stage

Denude oocytes were fixed in a 4% formaldehyde-TritonX-100 solution (Sigma-Aldrich) for 15 min and washed in a solution of PBS. Fixed oocytes were mounted on a slide and stained with 1.9 mM Hoechst 33342 (Sigma-Aldrich) in glycerol (Sigma, St. Louis, MO). Chromatin state and position as well as spindle formation and migration of oocytes were evaluated under UV light to determine the stage of meiosis as follows (Kim *et al.*, 2004); germinal vesicle(GV) stage, germinal vesicle breakdown(GVBD), metaphase I (MI) stage, metaphase II (MII).

3. Experiment design

Collected immature oocytes were grouped according to the stage of the estrous cycle (anestrus, follicular or luteal stage), prior to transfer to maturation culture medium. In experiment 1, oocytes were randomly allocated into TCM-199 media as follows: (1) TCM-199; (2) TCM-199 supplemented 10% canine anestrus serum (CAS); (3) TCM-199 supplemented 10% CES; (4) TCM-199 supplemented 10%

canine diestrus serum (CDS); (5) TCM-199 supplemented 10% FBS. Experiment 2, (1) TCM-199; (2) TCM-199 supplemented 10% canine estrus serum (CES); (3) TCM-199 supplemented 20% CES; (4) TCM-199 supplemented 10% fetal bovine serum (FBS). In Experiment 3, oocytes were randomly allocated into TCM-199 as follows: (1) TCM-199; (2) TCM-199 supplemented 2μg estradiol-17ß (E2, Sigma-Aldrich); (3) TCM-199 supplemented 10% CAS and 2μg E2; (4) TCM- supplemented 10% CES and 2μg E2; (5) TCM-199 supplemented 10% CDS and 2μg E2; (6) and TCM-199 supplemented 10% FBS and 2μg E2.

4. Statistical analysis

Data were analyzed using the the SAS package (SAS Institute, Cary, NC). Random distribution of oocytes was made in each experimental group and experiments were repeated at least four times. Parametric analysis of the means between two or more populations was analyzed by an ANOVA followed by multiple pair-wise comparisons using a Duncan's multiple range test. Experiments were first analyzed for interaction among experimental parameters. As no interaction was found, the data were further analyzed using the ANOVA procedure followed by multiple pair-wise comparisons using a Duncan test. Differences with $P<0.05$ were considered significant.

III. RESULTS

In experiment 1, a total 1,400 oocytes were selected from bitches ovaries at different stages of estrus. After 72 hr cultured, in anoestrus group was only oocytes from canine estrus serum treatment have developed to MII stages(1.7±1.3%) but still no significant different($p>0.05$) in rate of meiotic resumption among treatment group. In follicular ovaries group, oocytes from 10% CES treatment has highest maturation rate to MII stages (10.2±1.5%) and there were significant differences($p<0.05$) with another treatment groups. From luteal ovaries group, there were no significant differences($p>0.05$) between serum treatment and rate of maturation to MII stages of oocytes were 2.3±1.5%; 3.2±1.4%; 3.1±1.4%; 1.0±1.4%; 2.0±1.4% for control, CAS, CES, CDS and FBS treatment, respectively(Table 1).

In experiment 2, a total 814 oocytes were collected from bitches ovaries at different stages of estrus. In follicular ovaries group, oocytes from 10% CES treatment has significantly higher($p<0.05$) maturation rate to MII stages(10.0±1.8%) than another treatment in follicular stages group(Table 2).

In experiment 3, a total 1,406 oocytes from different stages of estrous cycle were selected. In follicular stages of ovary were 7.8±1.3%;6.0 ± 1.3%; 11.5 ± 1.2%; 8.8 ± 1.1%; 2.8 ± 1.2% and 8.3 ± 1.5% for control, CAS, CES, CDS,

Table 1. Meiotic status of different reproductive stages recovered oocytes cultured in TCM 199 supplemented with canine serum and fetal bovine serum

Status stages	Treatment	No. of oocytes examined	% nuclear status of oocytes (mean ± SD)			
			GV^{NS}	GVBD	M I^{NS}	M II
Anestrus	Control	110	0.9 ± 0.8	68.1 ± 4.7^{a}	9.0 ± 3.0	0.0 ± 1.3^{b}
	10% CAS	118	1.6 ± 0.7	50.8 ± 4.5^{bcd}	8.4 ± 2.9	0.0 ± 1.2^{b}
	10% CES	112	1.7 ± 0.7	52.6 ± 4.6^{bc}	9.8 ± 3.0	1.7 ± 1.3^{b}
	10% CDS	111	0.0 ± 0.8	47.7 ± 4.6^{cd}	9.0 ± 3.0	0.0 ± 1.3^{b}
	10% FBS	113	0.8 ± 0.7	48.6 ± 4.6^{bd}	9.7 ± 3.0	0.0 ± 1.3^{b}
Follicular	Control	73	1.3 ± 0.9	61.6 ± 5.7^{ab}	17.8 ± 3.7	1.3 ± 1.6^{b}
	10% CAS	75	0.0 ± 0.9	37.3 ± 5.7^{d}	12.0 ± 3.7	4.0 ± 1.6^{b}
	10% CES	78	1.2 ± 0.9	41.0 ± 5.6^{cd}	12.8 ± 3.6	10.2 ± 1.5^{a}
	10% CDS	73	0.0 ± 0.9	45.2 ± 5.7^{cd}	12.3 ± 3.7	2.7 ± 1.6^{b}
	10% FBS	74	1.3 ± 0.9	48.6 ± 5.7^{bcd}	10.8 ± 3.7	1.3 ± 1.6^{b}
Leuteal	Control	85	0.0 ± 0.9	62.3 ± 5.3^{ab}	17.6 ± 3.4	2.3 ± 1.5^{b}
	10% CAS	91	0.0 ± 0.8	41.7 ± 5.1^{cd}	13.1 ± 3.3	3.2 ± 1.4^{b}
	10% CES	96	0.0 ± 0.8	40.6 ± 5.0^{cd}	15.6 ± 3.2	3.1 ± 1.4^{b}
	10% CDS	93	1.0 ± 0.8	45.1 ± 5.1^{cd}	11.8 ± 3.3	1.0 ± 1.4^{b}
	10% FBS	98	0.0 ± 0.8	39.7 ± 4.9^{cd}	9.1 ± 3.2	2.0 ± 1.4^{b}

$^{a-d)}$ Values with different superscripts are significantly different ($p<0.05$)
NS means that there is not significant difference between treatments
Control, TCM 199 alone, CAS, canine anestrus serum, CES, canine estrus serum and CDS, canine diestrus serum

Table 2. Meiotic status of different ovarian stages recovered oocytes cultured in TCM 199 supplemented with canine estrus serum(CES) and fetal bovine serum (FBS)

Status stages	Treatment	No. of oocytes examined	% nuclear status of oocytes(mean ± SD)			
			GV	GVBD	M I^{NS}	M II
Anestrus	TCM 199	87	16.0 ± 3.0^{b}	55.1 ± 5.2^{c}	12.6 ± 4.0	0.0 ± 1.5^{a}
	10% CES	82	10.9 ± 11.7^{b}	40.2 ± 5.3^{ab}	14.6 ± 4.1	2.4 ± 1.5^{ab}
	20% CES	85	11.7 ± 3.1^{b}	42.3 ± 5.2^{abc}	12.9 ± 4.0	1.1 ± 1.5^{ab}
	10% FBS	85	15.2 ± 3.1^{ab}	32.9 ± 5.2^{a}	12.9 ± 4.0	1.1 ± 1.5^{ab}
Follicular	TCM 199	59	8.4 ± 3.7^{ab}	52.5 ± 6.3^{bc}	15.2 ± 4.8	0.0 ± 1.8^{a}
	10% CES	60	5.0 ± 3.7^{a}	33.3 ± 6.2^{a}	30.0 ± 4.8	10.0 ± 1.8^{c}
	20% CES	57	15.7 ± 3.8^{b}	29.8 ± 6.4^{a}	26.3 ± 4.9	3.5 ± 1.8^{ab}
	10% FBS	60	8.3 ± 3.7^{ab}	46.6 ± 6.2^{abc}	18.3 ± 4.8	0.0 ± 1.8^{a}
Leuteal	TCM 199	63	4.7 ± 3.6^{a}	60.3 ± 6.1^{c}	17.4 ± 4.7	1.5 ± 1.7^{ab}
	10% CES	62	4.8 ± 3.6^{a}	32.2 ± 6.1^{a}	17.7 ± 4.7	4.8 ± 1.7^{b}
	20% CES	56	0.0 ± 3.8^{a}	39.2 ± 6.4^{ab}	14.2 ± 5.0	1.7 ± 1.8^{ab}
	10% FBS	58	3.4 ± 3.7^{a}	29.3 ± 6.3^{a}	18.9 ± 4.9	0.0 ± 1.8^{a}

$^{a-c)}$ Values with different superscripts are significantly different ($p<0.05$)
NS means that there is not significant difference between treatments
CAS, canine anestrus serum, CES, canine estrus serum and CDS, canine diestrus serum

FBS and E2 treatment respectively, and there were significant differences ($p<0.05$) between 10% CES and another treatment in this group. In luteal groups were significant differences ($p<0.05$) between 10% CAS with E2 and TCM-199 with E2 treatment group. Within estrus cycle, there were significant differences ($p<0.05$) between follicular stages and another stages (Table 3).

Within estrus cycle stage, there were significant differences ($p<0.05$) between follicular stages and another stages in this experiment.

Ⅳ. DISCUSSION

In this present study, addition of 10% CES in culture medium have a high development to MII stages than 20% CES and 10% FBS treatment. Previous reports conducted by Nickson *et al.* (1993) and Otoi *et al.*(1999) showed that *in vitro* cultured bitch oocytes supplemented with canine estrus serum achieve very high rates of maturation, but Rodrigues and Rodrigues(2003) have the opposite result of treatment with bitch estrus serum

Table 3. Meiotic status of different ovarian stages recovered oocytes cultured in TCM 199 supplemented with canine serum and fetal bovine serum with 2ug/ml estradiol-17β

Status stages	Treatment with 2 ug/ml estradiol -17β	No. of oocytes examined	% nuclear status of oocytes(mean ± SD)			
			GV	GVBD	M I	M II
Anestrus	TCM 199	110	11.4 ± 2.4^{b}	67.0 ± 5.2^{c}	6.8 ± 3.7^{ab}	0.0 ± 2.0^{a}
	10% CAS	118	18.3 ± 2.4^{c}	56.3 ± 5.3^{bc}	11.4 ± 3.7^{b}	1.1 ± 2.0^{a}
	10% CAS	118	18.3 ± 2.4^{c}	56.3 ± 5.3^{bc}	11.4 ± 3.7^{b}	1.1 ± 2.0^{a}
	10% CDS	111	25.5 ± 2.4^{d}	38.3 ± 5.3^{a}	11.6 ± 3.8^{b}	0.0 ± 2.0^{a}
	10% FBS	113	13.1 ±2.4^{bc}	59.3 ± 5.1^{bc}	0.0 ± 3.7^{a}	0.0 ± 1.9^{a}
Follicular	TCM 199	73	0.0 ± 2.8^{a}	43.7 ± 6.1^{a}	28.1± 4.4^{cd}	7.8 ± 1.3^{b}
	10% CAS	75	1.5 ± 2.8^{a}	53.0 ± 6.0^{abc}	31.8 ± 4.3^{d}	6.0 ± 1.3^{b}
	10% CAS	78	0.0 ± 2.7^{a}	55.0 ± 5.9^{bc}	21.7 ± 4.2^{cd}	11.5 ± 1.2^{c}
	10% CDS	73	0.0 ± 2.5^{a}	41.7 ± 5.5^{a}	29.1 ± 3.9^{cd}	8.8 ± 1.1^{b}
	10% FBS	74	1.4 ± 2.7^{a}	47.1 ± 5.9^{ab}	20.0 ± 4.2^{cd}	2.8 ± 1.2^{a}
Luteal	TCM 199	85	0.9 ± 2.2^{a}	61.7 ± 4.8^{c}	19.6 ± 3.5^{bc}	3.9 ± 1.8^{a}
	10% CAS	91	1.9 ± 2.2^{a}	44.5 ± 4.9^{a}	10.8 ± 3.5^{b}	6.9 ± 1.8^{b}
	10% CAS	96	0.9 ± 2.1^{a}	38.4 ± 4.8^{a}	15.3 ± 3.4^{b}	4.8 ± 1.8^{a}
	10% CDS	93	0.1 ± 2.2^{a}	48.1 ± 4.7^{ab}	13.8 ± 3.4^{b}	3.7 ± 1.8^{a}
	10% FBS	98	1.8 ± 2.3^{a}	44.8 ± 4.7^{a}	17.7 ± 3.4^{b}	1.8 ± 1.8^{a}

$^{a-d)}$ Values with different superscripts are significantly different ($p<0.05$)

CAS, canine anestrus serum, CES, canine estrus serum and CDS, canine diestrus serum

who found the low percentage of meiotic resumption to MII stages, Bolamba *et al.*(2002) also have found that serum supplement is not essential in his research with SOF medium. The reasons for this contradiction are not clear, as the percentage of the serum and media was similar. Ottoi *et al.*(1999) reported that supplementation of the culture medium with estrus serum is the optimal treatment for IVM, compared with anestrus, metestrus serum or BSA 0.3% and 10% serum concentration is higher in resumption of meiosis than in 5 or 20%. And also differences source of serum have influence in this study. FBS have no benefits for maturation rate in canine oocytes, this result similar with previous investigation(Bolamba *et al.*, 2002; Rodrigues and rodrigues, 2003) which shows that FCS serum from different species(cow) has no effect on rate of maturation. This result perhaps shows that specific species has important think in this species.

A few studies have compared the meiotic competence of canine oocyte from various known stages of the estrus cycle (Yamada *et al.*, 1993; Hewitt and England, 1997; Ottoi *et al.*, 2001; Rodrigues and rodrigues, 2003), the effect of the stage of the estrus cycle to IVM of canine oocyte remain ambiguous(Farstad, 2000). Hewitt and England(1997) have reported that there are no differences in the frequency of IVM between oocytes collected from ovaries of anestrus and diestrus bitches and also Rodrigues and Rodrigues(2003) found that *in vitro* maturation of dogs oocytes not influence by the in vivo reproductive status of the female. Yamada *et al.*(1993) showed that a higher proportion of oocyte from pre-ovulatory follicle of gonadotrophin-stimulated bitches reached MII compared with oocytes from follicle of non-stimulated and Ottoi *et al.*(2001) found a slightly higher proportion of oocytes from bitches at diestrus reached MII compared with oocytes from bitches at anestrus and also that the frequency of resumption of meiosis and maturation to MII of oocytes from bitches at the follicular phase was higher than for oocytes bitches at other stages of estrus cycle. In present study, there were found that the frequency of meiotic resumption of meiosis and maturation to MII of oocytes from follicular stage was higher than from luteal stage and anestrus stage($p<0.05$). Our results are supported by the result from the study by Yamada *et al.*,(1993) and Ottoi *et al.*,(2001). This result suggested that gonadothrophin and estrogen influences in maturation of canine oocyte *in vitro*. Advanced researches need for to know the effect of gonadothrophin on maturation of canine oocyte *in vitro*.

In the present study, estrogen supplementation on media has good effect on the rate of maturation of oocytes, its means high estradiol concentration supplement in culture medium have benefit effect on canine oocytes maturation. This result follows the previous study by Nickson et al. (1993) and Yamada et al. (1993) and Kim et al. (2005) which have

benefit effect of estradiol supplementation in culture medium, but on the other hand, Hewitt and England (1997) didn't found any benefits from supplementing estrogen or progesterone, alone or combination, to a culture medium containing BSA for canine oocyte maturation.

In the bitch, the proportion of estrogen and progesterone receptors varies depending on the presence of follicle or luteal tissue during the estrus cycle stage. The number of progesterone receptor in follicle relatively constant throughout the estrus cycle. Conversely, estrogen receptor concentrations vary during the estrus cycle phases. High levels of estrogen receptors were observed within primary and secondary follicles and in uterine cell during proestrus and anestrus (Rodrigues and Rodrigues, 2003).

The distribution and intensity of estrogen-receptor interaction in the uterus are more accentuated during the estrus phase of the cycle. The increase in ovarian capacity to produce estrogen and uterine sensitivity to estrogen even during late anestrus, a sexual quiescent stage, our partially explain that the rate of meiotic resumption in oocytes recovered during the follicular phase was different from that of oocytes recovered at the other reproductive states. It is suggesting that an *in vivo* environment with high estrogen level may contribute to resumption of meiosis in canine species, in agreement with previous reports (Luvoni *et al*., 2001).

In conclusion of this experiment was 10% canine estrus serum is the best type of serum for supplementation *in vitro* maturation media. The estrus cycle of the bitch affect the meiotic competence of oocytes especially in follicular stages. Hormonal influences within the follicle may be one of the factors that responsible for the great proportion of maturation of oocyte to MII from bitches at the follicular phase. The data presented here in confirm the findings reported by other authors and reinforce the need for research to establish efficient techniques to resolve the unknown features of the kinetics of *in vitro* meiotic progression in this species.

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