Effects of Follicle Stimulating Hormone and Human Chorionic Gonadotrophin on the *In Vitro* Maturation of Canine Oocytes

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ABSTRACT

The present study investigated the effects of follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) on the nuclear maturation of canine oocytes. Oocytes were recovered from mongrel female ovaries in various reproductive states; follicular, luteal or anestrous stage. Oocytes were cultured in serum-free tissue culture medium (TCM)-199 supplemented with various concentrations of FSH (Exp. 1: 0, 0.5, 1.0 or 10 IU) or hCG (Exp. 2: 0, 0.5, 1.0 or 10 IU) or both (Exp. 3: 1 IU FSH + 1 IU hCG) for 72 hr to determine the effective concentration of these hormones, and to examine their combined effect. After maturation culture, oocytes were denuded in PBS containing 0.1% (w/v) hyaluronidase by gentle pipetting. The denuded oocytes were stained with 1.9 μM. Hoechst 33342 in glycerol and the nuclear state of oocytes was evaluated under UV light. More ($p<0.05$) oocytes matured to MII stage when follicular stage oocytes were supplemented with 1 IU FSH (6.2%) compared with the control, 0.1 or 10.0 IU FSH (0 to 1.2%). Significantly higher ($p<0.05$) maturation rate to MII stage was observed in follicular stage oocytes supplemented with 1.0 IU hCG (7.2%) compared with the control or other hCG supplemented groups (0 to 1.5%). However, the combination of FSH and hCG did not improve the nuclear maturation rate of canine oocyte (2.4 %) compared with FSH (6.2%) and hCG alone (7.2%). In conclusion, FSH or hCG alone significantly increased the maturation of canine oocytes to MII stage.

(Key words : Canine oocytes, FSH, hCG, *In vitro* maturation)

INTRODUCTION

At the onset of puberty some primordial follicles in the ovary begin to grow. The primordial follicle remains arrested at prophase of the first meiotic division. In most mammals, *in vivo*, following the LH surge, the oocyte resumes meiosis. *In vitro*, spontaneous maturation occurs when oocytes and their associated cumulus cells are removed from the follicular environment (Pincus and Enzmann, 1934; Edwards, 1965). Growth, maturation and ovulation of the Graafian follicle depend on appropriate patterns of secretion, sufficient concentrations and adequate ratios of various reproductive hormones, especially follicle stimulating hormone (FSH) and luteinizing hormone (LH) hormones.

FSH is primarily responsible for follicle recruitment. FSH receptors are located exclusively to the granulosa cells of maturing follicles (Camp *et al.*, 1991; O'Shaughnessy *et al.*, 1996) which in turn are directly connected to the oocyte via gap junctions (Albertini and Anderson, 1974; Anderson and Albertini, 1976). FSH, both *in vivo* and *in vitro*, affects the number of oocyte centrosomes, suggesting that the hormonal environment can modulate the dynamics of centrosome-based microtubule assembly, including spindle formation and thus the nuclear maturation of oocytes (Albertini, 1992). Therefore, supplementation of culture medium with FSH may be beneficial for oocyte maturation.

It has been suggested that the preovulatory development of canine oocytes (induced by exogenous gonadotrophin) is important for subsequent maturation *in vitro* (Yamada *et al.*, 1993). Wen *et al.* (1994) reported that hCG more effectively promoted cumulus cell development at 24 and 36 hr of *in vitro* maturation of silver fox oocytes. In contrast, Hewitt *et al.* (1999) and Songsasen *et al.* (2002) reported that supplementation with LH and/or FSH did not affect the proportion of canine oocytes undergoing meiosis.

The estrous cycle of the domestic bitch is long com-

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pared with other domestic mammals (Jeffcoat, 1998). Each cycle is at least 5 months in duration with a relatively long anestrus phase. In the anestrus phase, ovaries are mostly inactive whereas in the follicular phase ovaries are under the stimulatory effect of FSH and the luteal phase is dominated by progesterone (Concannon, 1993). Thus, oocytes retrieved from different estrous cycle stages may not mature equally in *in vitro* culture medium.

In most mammalian species, a mature oocyte usually completes its first meiotic division and is arrested at MII stage at the time of ovulation; it then awaits further signals (i.e. fertilization or activation) necessary to complete the second meiotic division. However, canine oocytes are ovulated at prophase of the first meiotic division and undergo maturation in the distal part of the oviduct for at least 48 to 72 hr (Concannon *et al.*, 1989). Because of these pronounced differences, applying methods for IVM of other domestic species to canine IVM has been unsuccessful.

Accordingly, the present study was performed to investigate the effects of FSH and hCG (an LH analog) on *in vitro* nuclear maturation of canine oocytes. In addition, in order to investigate the effect of stage of the estrous cycle on the meiotic competence of canine oocytes matured *in vitro*, oocytes were collected from various reproductive states and matured *in vitro* in the presence of the gonadotrophins.

MATERIALS AND METHODS

Collection of Canine Oocytes

Reproductive tracts from bitches greater than 10 months of age were collected after ovariohysterectomy and transported to the laboratory within 1 hr in a physiological saline solution (0.9% sodium chloride) at 37℃. Estrous cycle stage was evaluated for each bitch by ovarian morphology, and bitches were categorized according to the stage of the estrous cycle (anestrus, follicular or luteal) prior to oocyte collection. Ovaries were separated from the tracts and washed several times in fresh phosphate buffered saline (PBS) then minced repeatedly with a #10 blade at room temperature in a handling medium [TCM-199 (Life Technologies, USA) supplemented with 25 mM HEPES (Life Technologies), 1% fetal calf serum (FBS; Hyclone, USA) and 1% penicillin-streptomycin solution (Life Technologies)]. Minced ovaries were washed in the handling medium and cumulus-oocyte complexes (COCs) were collected and washed three times in the handling medium prior to transfer to maturation medium.

In Vitro Maturation

Immature COCs were categorized into three groups according to the stage of the estrous cycle (anestrous, follicular or diestrous stage) prior to transfer into IVM culture medium. Three groups of COCs were incubated for 72 hr in a 500 μl drop of serum-free TCM-199 in the absence of steroid hormones at 38.5℃ in a humidified atmosphere of 5% CO_2 in air. In experiment 1, in order to investigate the effects of FSH (Tenka, Japan), three groups of COCs were cultured for 72 hr in IVM medium containing various concentrations of FSH (0, 0.5, 1.0 or 10 IU). In experiment 2, three groups of COCs were cultured for 72 hr in IVM medium with various concentrations of hCG (0, 0.5, 1.0 or 10 IU; Intervet, Netherlands). In experiment 3, in order to investigate the combined effects of FSH and hCG on nuclear maturation of canine oocytes, three groups of COCs were cultured in IVM medium supplemented with 1 IU/ml FSH and hCG.

Assessment of Meiotic Stage

At the end of the maturation culture, COCs were transferred to PBS containing 0.1% (w/v) hyaluronidase (Sigma-Aldrich, USA) for 1 min and the cumulus cells were subsequently removed by gentle pipetting. The denuded oocytes were fixed in a 4% formaldehyde-Triton X-100 solution (Sigma-Aldrich, USA) for 15 min and were mounted on a slide and stained with 1.9 μM. Hoechst 33342 (Sigma-Aldrich, USA) in glycerol (Sigma-Aldrich). Chromatin state and position as well as spindle formation of oocytes were evaluated under UV

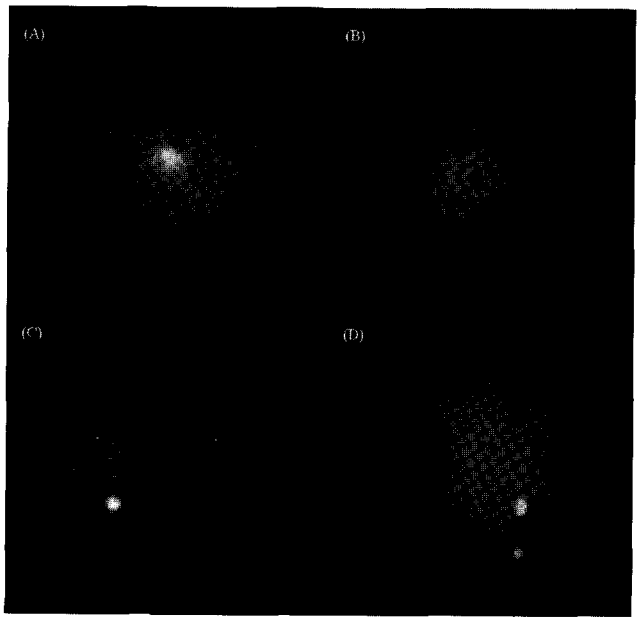

Fig. 1. Canine oocytes stained with Hoechst 33342 and visualized under UV light. GV (A); germinal vesicle (nuclear envelope) still intact, GVBD (B); organization and condensation of chromatin into chromosomes, MI (C); alignment of chromosomes on meiotic spindle and MII (D); meiotic division in chromosome number reduction and expulsion of first polar body. (×200).

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, and metaphase II (MII) stage (Fig. 1).

Statistical Analysis

Data were analyzed using the statistical analysis system (SAS, USA) program. Oocytes were randomly distributed into each experimental group and experiments were repeated four times. Experiments were first analyzed for interaction among experimental parameters. Because no interactions were found, the data were further analyzed by one-way ANOVA after arcsine transformation, followed by multiple pair-wise comparisons using a Duncan *t*-test. Differences of $p<0.05$ were considered significant.

RESULTS

Exp. 1: Effect of FSH Supplementation on Nuclear Maturation

As shown in Table 1, more oocytes ($p<0.05$) from the follicular stage of the cycle matured to the MII stage with 1 IU/ml FSH (6.2%) compared with the other FSH supplemented groups (0.0 to 2.3 %) or to the control (1.8%). The rates of maturation to the MI stage were significantly higher ($p<0.05$) in oocytes from the follicular stage with 0.5 (24.5%) or 1.0 IU/ml FSH (20.9%) compared with oocytes recovered from the anestrus or luteal stage groups.

Exp. 2: Effect of hCG Supplementation on Nuclear Maturation

As shown in Table 2, after 72 hr IVM culture, follicular stage oocytes matured in TCM-199 with 1.0 IU/ml hCG showed a higher maturation rate (7.2%) to MII ($p<0.05$) compared with the other hCG supplemented (0 to 1.5%) or the control (1.5%) groups. The GV rates were lower in oocytes from the follicular stage (1.5%) supplemented with 10 IU/ml hCG compared with the other groups.

Exp. 3: Effect of Combined Supplementation of FSH and hCG on Nuclear Maturation

As shown in Table 3, follicular stage oocytes matured in TCM-199 showed a higher maturation rate to MI (16.7 %) compared with oocytes recovered from the anestrus or luteal stages (9.5 and 12.1%, respectively). More oocytes from the follicular stage were matured to the MI stage in the presence of 1.0 IU FSH + 1.0 IU hCG ($p<0.05$) compared with luteal stage oocytes (14.3%

Table 1. Meiotic status of canine oocytes retrieved from various reproductive stages and cultured in TCM 199 supplemented with various concentrations of FSH

Reproductive stages	FSH concentration (IU)	No. of oocytes examined	% nuclear status of oocytes (mean±SD)			
			GV	GVBD^{NS}	MI	MII
Anestrous	Control	88	14.7±2.0^{c}	71.6±4.9	13.6±2.8^{ab}	0.0±1.3^{a}
	0.5	92	11.9±2.1^{bc}	78.3±4.8	9.7±2.9^{a}	0.0±1.3^{a}
	1.0	88	7.9±2.1^{b}	82.9±4.8	9.1±2.9^{a}	0.0±1.4^{a}
	10.0	86	0.0±2.0^{a}	90.7±4.7	9.3±2.9^{a}	0.0±1.3^{a}
Follicular	Control	106	3.7±2.2^{ab}	75.5±4.1	18.8±3.8^{bc}	1.8±1.9^{a}
	0.5	106	1.8±2.1^{a}	73.6±4.0	24.5±3.8^{c}	0.0±1.8^{a}
	1.0	94	0.0±2.2^{a}	77.7±4.0	16.0±3.7^{bc}	6.2±1.9^{b}
	10.0	86	0.0±2.2^{a}	76.7±4.1	20.9±3.8^{c}	2.3±1.8^{a}
Luteal	Control	91	2.1±2.5^{a}	89.1±4.2	12.1±3.7^{ab}	0.0±1.4^{a}
	0.5	92	4.3±2.5^{ab}	89.4±4.2	6.5±3.7^{a}	0.2±1.5^{a}
	1.0	85	3.5±2.4^{ab}	85.8±4.3	5.9±3.7^{a}	1.2±1.4^{a}
	10.0	78	2.5±2.4^{a}	85.9±4.2	10.3±3.6^{ab}	1.3±1.5^{a}

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.
NS, statistically not significant.
$^{a-c}$ Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 2. Meiotic status of canine oocytes retrieved from various reproductive stages and cultured in TCM 199 supplemented with various concentrations of hCG

Reproductive stages	hCG concentration (IU)	No. of oocytes examined	% nuclear status of oocytes (mean±SD)			
			GV	GVBD[NS]	MI	MII
Anestrous	Control	95	2.1±2.1[a]	86.3±4.8	11.6±4.2[a]	0.0±2.0[a]
	0.5	84	5.9±2.0[ab]	85.7±4.7	8.3±4.1[a]	0.0±1.9[a]
	1.0	82	2.4±2.0[a]	87.8±4.7	9.7±4.0[a]	0.0±1.9[a]
	10.0	84	0.4±2.1[a]	83.3±4.8	16.7±4.2[ab]	0.0±1.9[a]
Follicular	Control	65	9.2±2.6[b]	76.9±4.5	12.3±4.5[a]	1.5±2.2[a]
	0.5	73	9.5±2.5[b]	79.5±4.6	10.9±4.5[a]	0.0±2.0[a]
	1.0	71	2.8±2.5[a]	72.2±4.6	18.7±4.5[ab]	7.2±2.2[b]
	10.0	69	1.5±2.5[a]	73.9±4.6	23.2±4.6[b]	1.5±2.2[a]
Luteal	Control	63	6.3±2.4[ab]	82.5±4.9	11.1±4.2[a]	0.0±2.0[a]
	0.5	68	8.8±2.3[b]	82.4±5.0	8.8±4.3[a]	0.0±2.0[a]
	1.0	66	9.1±2.3[b]	78.8±4.9	10.6±4.2[a]	1.5±2.2[a]
	10.0	63	4.7±2.4[ab]	84.1±5.0	11.1±4.2[a]	0.0±2.0[a]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; NS, statistically not significant.
[a,b] Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 3. Meiotic status of canine oocytes retrieved from various reproductive stages and cultured in TCM 199 and supplemented with FSH and hCG

Supplement	Reproductive stages	No. of oocytes examined	% nuclear status of oocytes (mean±SD)			
			GV	GVBD	MI	MII
TCM-199	Anestrus	84	2.3±2.2[a]	88.1±4.2[ab]	9.5±3.2[ab]	0.0±1.7
	Follicular	60	15.0±1.9[c]	66.6±4.7[a]	16.7±3.6[b]	1.7±1.6
	Luteal	91	2.2±2.1[a]	85.7±4.5[ab]	12.1±3.3[ab]	1.1±1.7
1 IU FSH + 1 IU hCG	Anestrus	70	5.7±2.3[ab]	85.7±4.4 [ab]	8.6±3.2[ab]	0.0±1.7
	Follicular	84	7.1±2.0[b]	76.2±4.7[ab]	14.3±3.9[b]	2.4±2.1
	Luteal	84	1.2±2.1[a]	90.5±4.3[b]	6.0±3.3[a]	1.1±1.7

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.
[a~c] Values with different superscripts in the same column are significantly different ($p<0.05$).

vs. 6.0%, respectively). However, no significant increase in maturation to MII was observed among all groups of oocytes cultured with 1.0 IU FSH + 1.0 IU hCG.

DISCUSSION

The present study investigated the effect of FSH or hCG on IVM of canine oocytes collected from the various stages of the estrous cycle, and demonstrated that 1 IU FSH or 1 IU hCG significantly improved nuclear maturation of canine oocytes that were collected from the follicular stage of the cycle.

A number of studies have demonstrated an effect of stage of the estrous cycle on the meiotic competence of canine oocytes matured *in vitro*. Luvoni *et al.* (2001)

reported that canine oocytes obtained during late proestrus are capable of resuming meiosis and reaching MI or MII stage at higher rates than oocytes collected during anestrus. In the present study, higher proportion of oocytes collected from follicular stage ovaries reached MI and MII in the absence or presence of FSH and/or hCG (discussed below) compared to those collected from ovaries of anestrous and luteal stages, supporting the importance of oocytes recovery phase to select potentially meiotically competent canine oocytes for use of canine oocytes for *in vitro* experiment.

FSH plays a central role in the regulation of follicle growth and survival *in vivo* (Hillier, 2001), acting through FSH receptors expressed exclusively in granulosa cells (Camp *et al.*, 1991). Its importance for *in vivo* follicle development is clearly demonstrated in the absence of circulating FSH or competent FSH receptors, where follicle maturation is impaired and ovulation fails (Dierich *et al.*, 1998; Themmen and Huhtaniemi, 2000). There is increasing evidence that FSH also plays a role in the resumption of meiosis (Dekel and Beers, 1978; Downs *et al.*, 1988; Singh *et al.*, 1997). Thus, FSH is routinely included in IVM culture medium in several species. In mouse, addition of FSH to culture media promotes follicle survival and antrum formation in early preantral follicles (Cortvrindt *et al.*, 1997), and in follicles cultured from preantral to preovulatory stages (Nayudu and Osborn, 1992; Spears *et al.*, 1994). In the rat, FSH increases the growth of preantral follicles *in vitro* and increases viable follicle cell numbers when in combination with cyclic guanosine 3',5'-monophosphate (cGMP) or serum (McGee *et al.*, 1997). In human, FSH promotes antrum formation and estrogen production *in vitro* (Abir *et al.*, 1997). Furthermore, FSH appears to play an important role in follicular atresia. It is known that FSH prevents apoptosis in preantral and antral follicles of the mouse (Baker and Spears, 1997). In rat, FSH appears to be the most important survival factor for early antral follicles (Chun *et al.*, 1996), and suppresses apoptosis in serum-free culture in preantral (McGee *et al.*, 1997), antral (Tilly and Tilly, 1995) and preovulatory (Chun *et al.*, 1994) follicles. In isolated human preantral follicles, FSH was also seen to reduce atresia (Roy and Treacy, 1993).

In agreement with previous reports, the results of our study showed that 1 IU FSH improved nuclear maturation of canine oocytes collected during the follicular stage of the cycle. Although FSH did not improve nuclear maturation of canine oocytes collected from the anestrus or luteal stage to the MII stage, FSH (at concentrations of 1.0 or 10 IU) decreased GV rates in oocytes recovered from the anestrus or follicular stages. Taken together, our results suggest that FSH is involved in nuclear maturation of canine oocytes and that its effect depends both on the reproductive stage of oocytes and its concentration.

One mechanism by which LH/hCG may enhance IVM of oocytes is by the modification of nutritional environment to increase the energy available for oocyte development. In support of this idea, it is known that these gonadotrophins increase glycolysis and mitochondrial glucose oxidation in oocyte-cumulus complexes (Zuelke *et al.*, 1990), and enhanced glucose metabolism during IVM of bovine oocytes has been associated with developmental competence (Zuelk and Brackett, 1993). Mattioli *et al.* (1991) reported that LH and FSH accelerated and facilitated meiotic progression, and LH selectively improved cytoplasmic maturation which is required to promote the formation of a male pronucleus in porcine oocytes. In the present study, 1.0 IU LH increased the maturation of canine oocytes collected from the follicular phase to MII.

It is interesting that a gonadotrophin hormone was required to induce the maturation of canine oocytes at the follicular phase of the estrous cycle, when the follicle *in vivo* at this stage are already exposed the considerable other steroidal estrus hormone (eg. estrogen or progesterone) before ovulation.

As with FSH, the effect of LH also depends on its concentration and the reproductive stage of oocytes. Cumulus expansion of canine oocytes was maximal when 1 IU FSH and 1 IU hCG were added to IVM medium, but cumulus expansion again was not related to meiotic development. No synergistic effect of FSH and hCG on maturation of canine oocytes to MII was observed.

In conclusion, the present study demonstrated that supplementation of the culture medium with FSH or hCG alone significantly increased the maturation of canine oocytes from GV to MII. However, the combination of FSH and hCG had no effect on meiotic resumption in canine oocyte during *in vitro* maturation.

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