Effect of human chorionic gonadotrophin supplementation during different culture periods on in vitro maturation of canine oocytes

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Abstract

The IVM of canine oocytes is characterized by low rates of metaphase II. The objective of this study was to evaluate the effects of hCG on meiotic development of canine oocytes for culture periods up to 96 h. Oocytes were collected after ovariohysterectomy. Only oocytes >110 μm in diameter, with a homogeneous dark cytoplasm and three or more layers of compact cumulus cells were used. For IVM, the COCs were cultured in TCM-199 + 10% fetal calf serum, without (medium A control) or supplement with 10 IU/mL of hCG (medium B), or with a combination of both media (treatment B/A). The COCs were randomly allocated into three groups. The first and second groups were cultured in either medium A or B, respectively for 24, 48, 72, and 96 h. Oocytes of the third group (treatment B/A) were incubated in medium with hCG (medium B) the first 48 h and then transferred to medium without hCG (medium A) for an additional 24 or 48 h. The proportion of COCs with cumulus cell expansion was also evaluated before fixation. Oocytes were stained with propidium iodide prior to nuclear assessment (with epifluorescence microscopy). COCs with cumulus expansion were evident after 48 h of culture. The proportion of COCs with cumulus expansion was higher ($P < 0.05$) for media containing hCG (B or B/A) than for meda lacking hCG (A); this difference was maintained for

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72 and 96 h in culture. In media A, B and B/A, 23.3, 31.7 and 29.5%, respectively, of oocytes were at metaphase II after 72 h, with 20.7, 33.1 and 43.4% at this stage after 96 h. The advancement of meiosis was directly proportional to the time of incubation; the highest percentage ($P < 0.05$) of oocytes at metaphase II was observed after 96 h of culture when 10 IU/mL hCG was present for only the first 48 h of culture.

Keywords: Canine; In vitro maturation; hCG; Oocytes

1. Introduction

The implementation of in vitro reproductive technologies in the domestic bitch would be useful for this species, as well as a model for reproductive studies of endangered canine species. Successful maturation of oocytes is a prerequisite for fertilization and further embryo development. Although in vitro protocols for nuclear maturation in canine oocytes have been conducted with various maturation media [1–6], IVM and IVF in canines have not been as successful as in other mammalian species (e.g. livestock and laboratory animals). This low efficiency may be due to differences in oocyte maturation; canine oocytes are spontaneously ovulated at the germinal vesicle stage as primary oocytes, whereas oocyte maturation occurs within the ovarian follicle in the majority of mammals [7]. In the bitch, completion of meiotic maturation to second metaphase occurs in the oviduct and requires an extended period (2–5 days) [8,9]. Acquisition of nuclear and cytoplasmic competence is controlled by intercellular communication between cumulus cell and the oocyte [10]. Another problem in obtaining in vitro maturation of canine oocytes may be related to the failure of cumulus cells to stimulate meiotic resumption [11].

Most of the protocols for IVM of canine oocytes have been adapted from those used in other mammals, without considering canine-specific requirements. Some culture conditions, such as protein concentration, co-culture with supplementary oviductal epithelial cells [2] and addition of glycosaminoglycan [12], are important factors for the successful in vitro maturation of canine oocytes. Hormonal supplementation of culture media has been previously attempted to improve oocyte development [13,14]. In other species, the addition of gonadotrophins to the IVM media enhanced nuclear maturation of oocytes [15–21]. In canines, however, there was no improvement in maturation rate when hormones were present during the entire incubation period [13,22]. In order to optimize IVM of canine oocytes, it is important to consider the dynamics of the preovulatory follicle and the physiological environment of the oviduct in the dog. The time of ovulation in canines and its relationship with the subsequent gamete development in vivo has been described [23]. Ovulation occurs 24–72 h after the surge of luteinizing hormone (LH) at the beginning of estrus [8,24] and the LH surge lasts 48–96 h [25]. Both pre- and post-ovulatory canine oocytes are exposed to varying concentrations of gonadotrophins; therefore, oocyte maturation may be influenced by decreasing levels of LH, which exerts its action through granulosa cells [26]. Yamada et al. [27] found that canine oocytes that had been exposed to gonadotrophins in vivo matured faster than controls. For IVM in canines, oocytes are removed from a gonadotrophin-rich environment; therefore, meiotic maturation of oocytes may be affected by the degree and duration of hormonal exposure.

The objective of the present study was to evaluate the effect of exposure of canine oocytes to human chorionic gonadotrophin (hCG; with LH-like activity) during different culture periods on IVM.

2. Materials and methods

2.1. Oocyte preparation

Ovaries were obtained from 70 healthy adult domestic bitches of different breeds by ovariohysterectomy. Ovaries were transported to the laboratory (within 2 h after removal) in pre-warmed saline solution at 37 °C (0.9% NaCl with 100 IU/mL penicillin). In the laboratory, cumulus-oocytes complexes (COCs) were released by slicing the ovarian cortex with a scalpel blade. After two washes in PBS (supplemented with 11.2 mg/mL pyruvic acid, 10% fetal calf serum and 50 μg/mL streptomycin (all from Sigma, St. Louis, MO, USA), the COCs were selected according to oocyte diameter (>110 μm), measured with a micrometer disc (Bausch and Lomb, USA), three or more compact cumulus cell layers, and darkly granulated cytoplasm (Fig. 1). The selected COCs were placed in 35 mm petri dishes (Falcon # 3001; Becton Dickinson, Lincoln Park, NY, USA), containing 0.5 mL of TCM-199 (Earle's salt, buffered with 25 mM Hepes; Invitrogen, Grand Island NY, USA), supplemented with 10% FCS and 11.2 mg/mL pyruvic acid.

2.2. In vitro maturation of oocytes

In each replicate, COCs were randomly allocated into three groups of 40–50 oocytes for culture under three different conditions. The first group of COCs were cultured in a

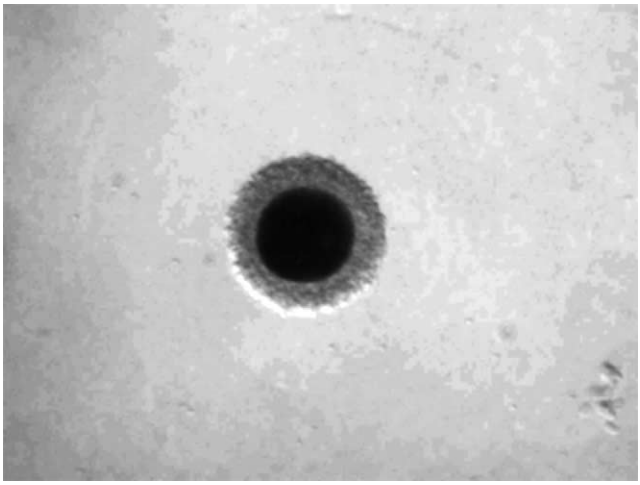

Fig. 1. Stereomicroscopic photograph of a selected bitch oocyte for in vitro maturation.

medium (Medium A) which was prepared with TCM-199 (Earle's salt, buffered with 25 mM Hepes; Invitrogen), supplemented with 10% FCS, 11.2 mg/mL pyruvic acid and 5 μL/mL antibiotic solution (12.2 mg/mL penicillin and 20 mg/mL streptomycin; Sigma), pH 7.4. The second group of COCs were cultured in the same medium, but with the addition of 10 IU/mL of hCG (CG-5, Sigma), designated as Medium B. Group 1 or Group 2 were cultured in media A or B, respectively for 24, 48, 72, and 96 h. The third group of COCs were cultured in Medium B (with 10 IU/mL of hCG) for the first 48 h, then subsequently transferred to Medium A (without hCG), for an additional 24 or 48 h (B/A). In each experiment, COCs of each treatment were placed in 100 μL culture drops, each of which contained not more than 8–10 COCs, covered with mineral oil (Sigma). All cultures were performed at 38.5 °C in a humidified atmosphere of 5% CO_2.

2.3. Assessment of oocyte maturation

The effects of hormonal culture conditions on cumulus expansion during each period were assessed by stereomicroscope examination, subjectively considering mucification when the oocyte was surrounded by completely dispersed follicle cells. For nuclear development evaluation in each treatment group, the COCs were removed from the drops and denuded by agitating in a test tube containing sodium citrate (5 mg/mL in PBS). Denuded oocytes were mounted and fixed in acetic acid/methanol/chloroform (3:6:2 v/v) [28,29] for 3 min and then in acetic acid/methanol (1:3 v/v) fixative for 3 d. The oocytes were stained with 0.1% propidium iodide (PI; Molecular Probes, Eugene, Oregon, USA) for nuclear assessment using an epifluorescence microscope Nikon Optiphot 2 (Nikon, Kawasaki, Japan).

Nuclear stages were classified as: (a) immature or germinal vesicle (GV), when the nuclear envelope was clearly visible; (b) resumption of meiosis or germinal vesicle break down (GVBD), when the nuclear envelope was no longer visible and the chromatin was dispersed; (c) metaphase I to telophase I (MI to Tel I), when chromosomes were condensed and present in equatorial view or when the chromosomes were migrating to the poles; and (d) mature (metaphase II), when chromosomes were in second metaphase with the extrusion of the first polar body. Oocytes with unidentifiable chromatin were discarded.

2.4. Statistical analysis

Oocytes were allocated into groups for media used and duration of culture. More than 1200 COCs were evaluated for cumulus cell expansion in 10 experimental replicates. Almost all those oocytes evaluated for cumulus expansion and additional oocytes in two more experimental replicates (1500 oocytes) were evaluated for meiotic development (12 experimental replicates). The proportion of oocytes with expanded cumulus cell (mucification) and the proportion of those reaching each stage of nuclear maturation in each treatment group and during each culture period were arc-sine transformed, and evaluated by ANOVA (Statistical Analysis System, SAS Institute, Cary, NC, USA) [30]. The model included the main effects of culture media, duration of culture, and their interaction for each stage of nuclear development ($P \leq 0.05$ was considered significant). Differences among means were located with Tukey tests.

Table 1
Mean ± S.E.M. (total) number of canine oocytes with cumulus cell expansion following in vitro culture (different media and duration of culture)

Medium	Duration of culture (h)			
	24	48	72	96
A	3.8 ± 1.7 (109)	21.7[a] ± 2.9 (120)	42.8[a] ± 3.3 (137)	42.2[a] ± 2.7 (115)
B	6.3 ± 1.9 (103)	36.5[a] ± 3.3 (170)	62.8[a] ± 3.2 (153)	64.1[a] ± 2.1 (122)
B/A	–	–	64.8[a] ± 2.0 (182)	70.1[a] ± 3.8 (153)

(A) Medium: TCM-199; (B) Medium TCM-199 with hCG (10 IU/mL); B/A: B medium first 48 h and A medium the last 24 or 48 h. There were effects of media ($P = 0.0001$) and duration of culture ($P = 0.0001$).
[a] Within a column, means with different superscripts are different ($P < 0.05$).

3. Results

The presence of hCG in the culture medium enhanced ($P < 0.05$) cumulus cell expansion after 48 h of incubation. Maximum percentages ($P < 0.05$) of oocytes with cumulus expansion were observed at 72 h after the start of incubation, with no further increase at 96 h (Table 1). The progression of meiosis (Table 2) was evaluated after staining with propidium iodide. The percentages of oocytes at the GV stage (Fig. 2a) were high at 24 h, irrespective of the presence of hCG in the culture medium. GVBD stages (Fig. 2b) were especially abundant at 48 h. Higher percentages ($P < 0.05$) of oocytes at MI-Tel I (Fig. 2c or d) and metaphase II (Fig. 2e) were observed after 72 and 96 h in culture (Table 3).

Meiotic maturation was evaluated in nearly all oocytes. After 72 h of culture, there were no differences ($P > 0.05$) in nuclear development when hCG was present for the whole period (Medium B) or only for the first 48 h (Media B/A). After 96 h, the proportion of oocytes arrested at the GV stage was low in all groups. At this time point, the percentage of oocytes at metaphase II was different ($P < 0.05$) among the three treatments; the percentage of oocytes that had reached metaphase II was highest in oocytes cultured with hCG for only the first 48 h, whereas the lowest percentage was in those cultured without hCG (Table 3).

The percentage of mature oocytes (metaphase II) increased ($P < 0.05$; Fig. 3) when culture time was extended up to 72 h in culture, irrespective of hCG supplementation. At 96 h, the percentage of metaphase II was significantly higher in medium containing hCG for the first 48 h, than that at 72 h in the same medium (B/A group) and than those in media A and B at 72 or 96 h.

Table 2
Number of canine oocytes examined for nuclear development in each media and for each culture time

Medium	Duration of culture (h)			
	24	48	72	96
A	109	159	116	125
B	103	238	150	142
B/A	–	–	116	315

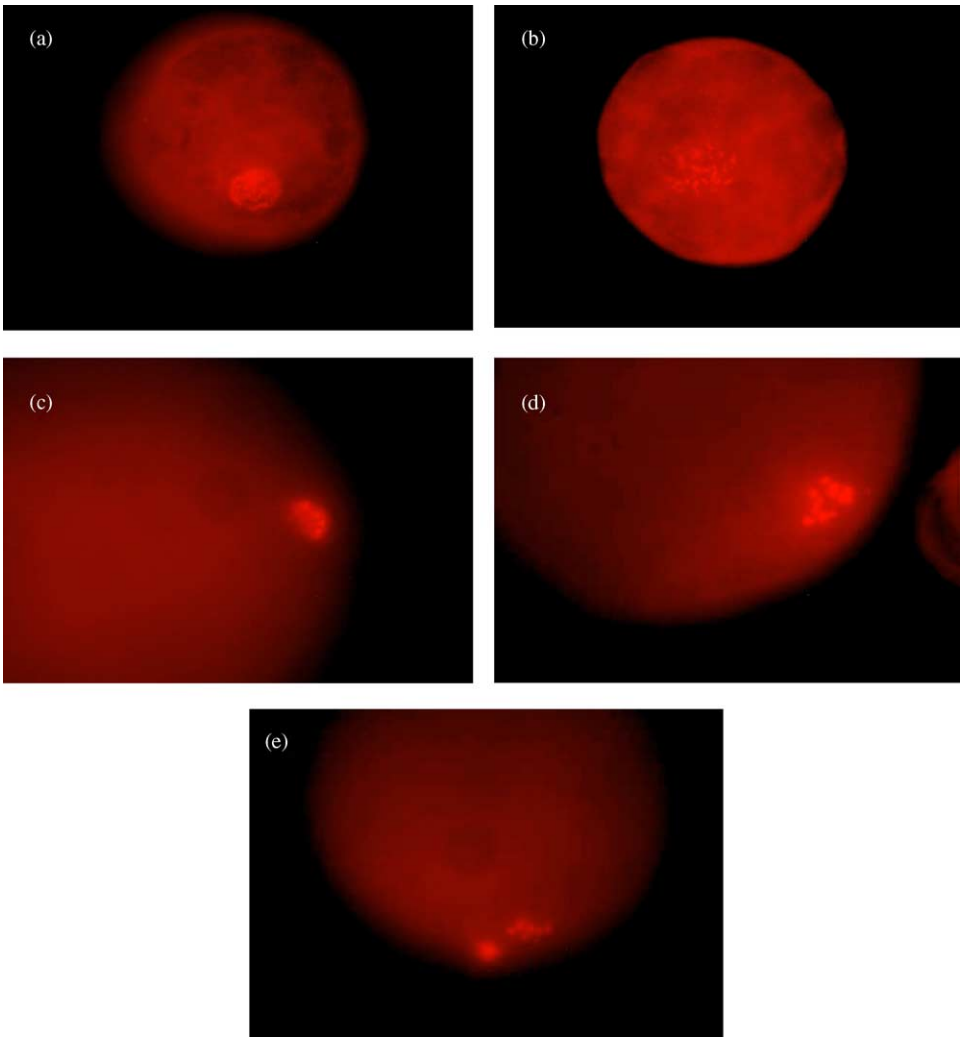

Fig. 2. Photomicrographs of canine oocytes stained with propidium iodide (PI) showing: (a) fluorescent germinal vesicle (GV) (×100); (b) fluorescent nuclear labeling with chromatin condensation and dissolution of the nuclear membrane. Germinal vesicle break down (GVBD) (×100); (c) fluorescent nuclear labeling with chromosomes in first metaphase (MI) (×200); (d) fluorescent nuclear labeling with chromosomes in first telophase (Tel I) (×400); (e) fluorescent nuclear labeling with chromosomes in second metaphase with extrusion of first polar body (×200).

4. Discussion

In this study, the time of exposure of canine oocytes to hCG affected in vitro maturation. The oocytes used were recovered from bitches at various reproductive stages; although the number of oocytes recovered during estrous may have been numerically higher than oocytes recovered during other stages, in most reports there was not association between

Table 3
Mean ± S.E.M. of canine oocytes with different nuclear status following in vitro culture (different media and duration of culture)

Nuclear status	Medium	Duration of culture (h)			
		24	48	72	96
GV	A	68.2 ± 3.4	11.1[a] ± 2.5	4.5 ± 1.7	6.2 ± 2.5
	B	61.7 ± 4.6	5.6[a] ± 1.2	3.0 ± 1.4	1.8 ± 1.3
	B/A	–	–	3.8 ± 1.7	3.2 ± 0.8
GVBD	A	28.5 ± 3.3	73.3[a] ± 3.1	59.2[a] ± 3.4	53.8[a] ± 2.6
	B	28.5 ± 3.8	62.9[a] ± 2.5	39.9[a] ± 2.7	33.4[a] ± 2.9
	B/A	–	–	45.1[a] ± 4.0	35.6[a] ± 2.4
MI-Tel I	A	0.9 ± 0.9	8.8 ± 1.5	13.0[a] ± 2.9	19.3[a] ± 2.6
	B	2.4 ± 1.2	8.1 ± 1.6	25.4[a] ± 2.3	31.7[a] ± 2.1
	B/A	–	–	21.7[a] ± 1.9	17.9[a] ± 1.8
MII	A	2.4 ± 1.3	6.8[a] ± 1.9	23.3[a] ± 3.2	20.7[a] ± 2.3
	B	7.0 ± 1.9	23.4[a] ± 2.6	31.7[a] ± 2.6	33.1[a] ± 2.3
	B/A	–	–	29.5[a] ± 2.8	43.4[a] ± 2.1

(A) Medium: TCM-199; (B) Medium TCM-199 with 10 II hCG; B/A: B medium first 48 h and A medium the last 24 or 48 h. GV: germinal vesicle; GVBD: germinal vesicle break down; MI-Tel I: metaphase I to telophase I; MII: metaphase II. There were effects of media ($P = 0.0001$) and duration of culture ($P = 0.0001$). Interaction media-duration of culture was significant in GVBD and MI-TI ($P < 0.05$).

[a] Within a column, means with different superscripts are different ($P < 0.05$).

meiotic competence and reproductive state [1,28,31,32]. Therefore, the parameters used in this study for selecting oocytes were those reported to favor meiotic competence, including oocytes >110 μm in diameter [1,5,29], and homogeneous dark cytoplasm with three or more layers of compact cumulus cells [33].

That canine oocytes are difficult to mature in vitro may be attributed to their in vivo maturation in the oviduct [34] and probably to the inability of cumulus cells to stimulate and control the resumption of meiosis [11]. In rat oocytes, there is a decrease in the net area of cumulus cell gap junctions after administration of hCG [35]; the loss of cumulus gap junctions has been associated with GVBD and cumulus expansion [36]. There are few reports on cumulus cell function during oocyte development in canids. Wen et al. [37] reported that hCG supplementation more effectively promoted cumulus cell development at 24 and 36 h of IVM of silver fox oocytes. In contrast, in the present study, few oocytes with cumulus cell expansion were observed at 24 h in each of the different culture systems. Nevertheless, after 48 h of culture, a greater proportion of oocytes with mucification were present, especially when cultured with hCG supplement. This is in accordance with in vivo studies in fox oocytes; gap junctions were present within the ovary and each junctional contact was disrupted 2–3 days after the LH surge [38]. Gap junction communications between the cumulus cells and the oocyte are involved in the regulation of oocyte meiotic maturation and development; this role of cumulus cells seems to be more essential in canids than in other mammals [10]. Even though the use of other gonadotrophins (e.g. FSH) has also been tried in canids [39] and other species [40–42], it caused a further arrest of meiotic progression in blue fox oocytes [13]. In this study, we excluded other hormones, so as not to confound the effects of hCG. Nevertheless, other gonadotrophins and steroids may have

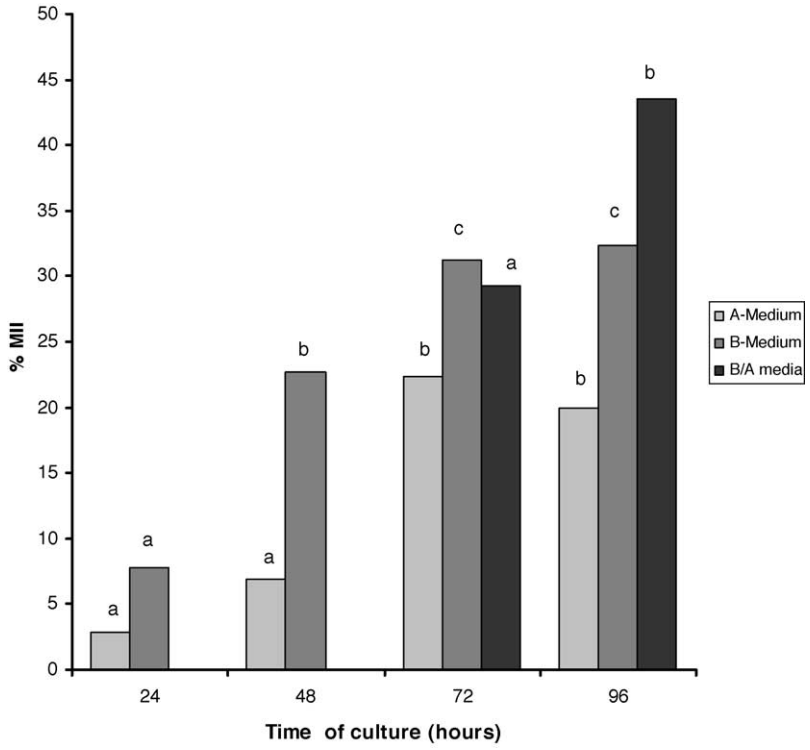

Fig. 3. Percentage of selected bitch COCs cultured in different maturation media reaching second metaphase, according to the time of culture.

been present in fetal serum products. Since all maturation conditions contain serum components, it becomes necessary to assess hormonal contributions in media with FCS or BSA in order to support normal development.

In the current work, both duration of culture and the addition of hCG had a highly significant effect upon the nuclear maturation of bitch oocytes. In contrast, Hewitt and England [22] reported that supplementation with LH and/or FSH did not affect the proportion of canine oocytes undergoing meiotic development. However, hormonal supplementation may vary among culture systems. Furthermore, the concentration of gonadotrophins is difficult to compare among studies due to potential differences in purity.

Duration of culture has been previously reported as influencing IVM of canine oocytes [1,9,27]. In blue fox females, oocytes in metaphase I have been found in oviducts 24 h after ovulation (i.e. 72 h post LH peak), and oocytes in the second metaphase have been observed after 48–72 h of ovulation [38]. Therefore, resumption of meiosis may be achieved at 24 h and is more evident at 48 h under physiological conditions, when the oocytes are still exposed to LH. This was consistent with our results; in the present study, almost 30% of oocytes resumed meiosis at 24 h of culture (GVBD stage), but it was not until 48 h of culture that a significant increase of meiotic development occurred. Prior to this time, the proportion of oocytes that remained at the germinal vesicle neared 70% and

no differences were observed between gametes cultured with or without hCG. Prolonged culture (up to 72 h) lead to a majority of oocytes that underwent GVBD and reached first and second metaphase in each culture media. Nevertheless, gametes which were under the influence of hCG resumed meiosis earlier and had a higher proportion with meiotic development versus those without hCG. One mechanism by which LH/hCG gonadotrophins may enhance IVM of oocytes is by modification of the nutritional environment to increase the energy available for oocyte development. In support of this theory, it is known that these gonadotrophins increase glycolysis and mitochondrial glucose oxidation in oocyte-cumulus complex [43] and enhanced glucose metabolism during IVM of bovine oocytes has been associated with developmental competence [44].

The change in gonadotrophin concentration during culture has not been previously described for canine oocytes; this may be an important factor, considering physiological events in vivo. In previous studies, the addition of hormones (including hCG) was restricted to the first culture period and improved the IVM and IVF rate in pig oocytes [45,46]. LH promotes follicular luteinization prior to ovulation in the bitch [24,47] and it has been suggested that the preovulatory development of canine oocytes (induced by exogenous gonadotrophin) may be important for subsequent maturation in vitro [9]. The removal of hCG supplement from maturation medium 48 h after the start of culture significantly improved the ability of bitch oocytes to mature to the second metaphase (at 96 h of culture), as compared to supplementation with hCG during the whole period or no hormonal supplementation. It is noteworthy that the acquisition of meiotic competence is a two-step process; the oocyte first achieves the ability to undergo germinal vesicle break down and later becomes progressively more capable of completing meiotic maturation to metaphase II [48]. Perhaps in the initial period of culture, the oocyte, via cumulus cells, may be more dependent on LH stimulation than in subsequent culture periods; therefore, hormonal supplementation (mainly LH) is not required throughout the entire culture period. Furthermore, canine oocytes that mature in vivo in oviducts presumably complete meiotic development to metaphase II in the absence of LH and FSH. Perhaps hormonal changes during maturation in vivo are of considerable importance for gamete development and may help explain the differences in developmental competence between oocytes matured in vivo and in vitro.

In conclusion, hCG supplementation was beneficial to meiotic resumption of canine oocytes; exposure to hCG during only the first 48 h improved the rate of metaphase II after 96 h culture. Further investigations regarding the dynamics of hCG (and potentially other hormones) on IVF are needed.

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References

[1] Hewitt DA, England GC. The effect of oocyte size and bitch age upon nuclear maturation in vitro. Theriogenology 1998;49:957–66.

[2] Hewitt DA, England GC. Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro. Anim Reprod Sci 1999;55:63–75.
[3] Otoi A, Fujii M, Tanaka M, Ooka A, Suzuki T. Effect of serum on the in vitro maturation of canine oocytes. Reprod Fertil Dev 1999;11:387–90.
[4] Saint-Dizier M, Renard JP, Chastant-Maillard S. Induction of final maturation by sperm penetration in canine oocytes. Reproduction 2001;121:97–105.
[5] Otoi T, Willingham L, Shin T, Kraemer DC, Westhusin M. Effects of oocyte culture density on meiotic competence of canine oocytes. Reproduction 2002;124:775–81.
[6] Rodrigues BA, Dos Santos LC, Rodrigues JL. Embryonic development of in vitro matured and in vitro fertilized dog oocytes. Mol Reprod Dev 2004;67:215–23.
[7] Wildt DE, Chakraborry PK, Panko WB, Seager SW. Relationship of reproductive behavior, serum luteinizing hormone and the time of ovulation in the bitch. Biol Reprod 1978;18:561–70.
[8] Renton JP, Boyd JS, Eckersall PD, Ferguson JM, Harvey MJ, Mullaney J, et al. Ovulation, fertilization and early embryonic development in the bitch (*Canis familiaris*). J Reprod Fertil 1991;93:221–31.
[9] Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Toyada Y. In vitro maturation and fertilization of preovulatory dog oocytes. J Reprod Fertil 1993;47(Suppl.):227–9.
[10] Luvoni GC. Current progress on assisted reproduction in dog and cats: in vitro embryo production. Reprod Nutr Dev 2000;40:505–12.
[11] Kalab P, Srsen V, Farstad W, Krogenaes A, Motlik J, Hafne AL. MAP kinase activation and RAF-1 synthesis in blue fox oocytes is controlled by cumulus granulose cells. Theriogenology 1997;47:400 (abstract).
[12] Rodrigues BA, Dos Santos BA, Rodrigues JL. The effect of hyalyronan concentrations in HST supplemented TCM 199 on in vitro nuclear maturation of bitch cumulus-oocyte complexes. In: Proceedings of 5th International symposium on Canine and Feline Reproduction; 2004. p. 256–7.
[13] Srsen V, Kalous J, Nagyova E, Sutovsky P, King WA, Motlik J. Effects of follicular stimulating hormone, bovine somatotrophin and okadaic acid on cumulus expansion and nuclear maturation of blue fox (*Alopex lagopus*) oocytes in vitro. Zygote 1998;6:299–309.
[14] Rodrigues BA, Rodrigues JL. Meiotic response of in vitro matured canine oocytes under different proteins and heterologous hormones supplementation. Reprod Dom Anim 2003;38:58–62.
[15] Hensleigh HC, Hunter AG. In vitro maturation of bovine cumulus enclosed primary oocytes and their subsequent in vitro fertilization and cleavage. J Dairy Sci 1985;68:1456–562.
[16] Younis AI, Brackett BG, Fayerer-Hosken RA. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res 1989;23:189–201.
[17] Mattioli M, Bacci ML, Galeati G, Seren E. Effect of LH and FSH on the maturation of pig oocytes in vitro. Theriogenology 1991;36:95–105.
[18] Eppig JJ, Schroeder AC, O'Brien MJ. Developmental capacity of mouse oocytes matured in vitro: effects of gonadotrophic stimulation, follicular origin and oocyte size. J Reprod Fertil 1992;95:119–27.
[19] Keefer CL, Stice SL, Dobrinsky J. Effect of follicle-stimulating hormone and luteinizing hormone during bovine in vitro maturation on development following in vitro fertilization and nuclear transfer. Mol Reprod Dev 1993;36:469–74.
[20] Kito S, Bavister BD. Male pronuclear formation and early embryonic development of hamster oocytes matured in vitro with gonadotrophins, amino acids and cysteamine. J Reprod Fertil 1997;110:35–46.
[21] Choi YH, Carnevale EM, Seider Jr EG, Squire EL. Effects of gonadotrophins on bovine oocytes maturation in TCM-199. Theriogenology 2001;56:661–70.
[22] Hewitt DA, England GC. Influence of gonadotrophin supplementation on the in vitro maturation of bitch oocytes. Vet Rec 1999;144:237–9.
[23] Tsutsui T. Gamete physiology and timing of ovulation and fertilization in dogs. J Reprod Fertil 1989;39(Suppl):269–75.
[24] Onclin K, Murphy B, Verstegen JP. Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. Theriogenology 2002;57:1957–72.
[25] Badiand F, Fontbonne A, Marel C, Siliart B. Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears. J Reprod Fertil 1993;47(Suppl.): 63–7.

[26] Eppig JJ. Oocyte control of ovarian follicular developmental and function in mammals. Reproduction 2001;122:829–38.
[27] Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Naito K, Toyada Y. Maturation, fertilization and development of oocytes in vitro. Biol Reprod 1992;46:853–8.
[28] Fujii M, Otoi T, Murakami M, Tanaka M, Une S, Suzuki T. The quality and maturation of bitch oocytes recovered from ovaries by the slicing method. J Vet Med Sci 2000;62:305–7.
[29] Otoi A, Fujii M, Tanaka M, Ooka A, Suzuki T. Canine oocyte diameter in relation to meiotic competence and sperm penetration. Theriogenology 2000;54:535–42.
[30] Statistical analysis system. Copyright© 1996, Cary, NC, USA: SAS Institute Inc.
[31] Otoi A, Ooka A, Muramaki M, Karma NW, Suzuki T. Size distribution and meiotic competence of oocytes obtained from bitch ovaries al various stages of the estrus cycle. Reprod Fertil Dev 2001;13:151–5.
[32] Rodrigues BA, Rodrigues JL. Influence of reproductive status on in vitro oocyte maturation in dogs. Theriogenology 2003;60:59–66.
[33] Nickson DA, Boyd JS, Eckershall PD, Ferguson JM, Harvey MJ, Renton JP. Molecular biological methods for monitoring oocyte maturation and in vitro fertilization in bitches. J Reprod Fertil 1993;44(Suppl.):231–40.
[34] Farstad W. Assisted reproductive technology in canid species. Theriogenology 2000;53:175–86.
[35] Larsen W, Wert S, Brunner G. A dramatic loss of cumulus cell gap junctions is correlated with germinal vesicle break down in rat oocytes. Dev Biol 1986;113:517–21.
[36] Wassarman PM. Oogenesis. In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, surgery and technology. Philadelphia, USA: Lippincott-Raven Publishers; 1996. p. 341–57.
[37] Wen XH, Feng HL, Sun QY. In vitro maturation of follicular oocytes of the silver fox (*Vulpes fulva* desm). Theriogenology 1994;41:333. abstr..
[38] Hyttel P, Farstad W, Mondain-Monval M, Bakke K, Smith AJ. Structural aspects of oocyte maturation in the blue fox (*Alopex lagopus*). Anat Embryol 1990;181:325–31.
[39] Krogenaes AK, Nagyova E, Farstad W, Hafne AL. In vitro maturation of blue fox oocytes and cAMP production in oocytes-cumulus cell complexes. Theriogenology 1993;39:250. abstract.
[40] Leifried-Rutledge L, Cristel ES, First NL. Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol Reprod 1986;35:850–7.
[41] Kameyama Y, Ishijima Y. Effect of cumulus cell on in vitro maturation of pig oocytes. Jpn J Fertil Steril 1994;39:66–9.
[42] Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon specific factors secreted by the oocyte. Dev Biol 1993;138:16–25.
[43] Zuelke KA, Brackett BG. Luteinizing hormone-enhanced in vitro maturation of bovine oocytes with or without protein supplementation. Biol Reprod 1990;43:784–7.
[44] Brackett BG, Zuelke KA. Analysis of factors involved in the in vitro production of bovine embryos. Theriogenology 1993;39:43–64.
[45] Funahashi H, Cantley T, Day BN. Different hormonal requirements of pig oocyte-cumulus complexes during maturation in vitro. J Reprod Fertil 1994;101:159–65.
[46] De los Reyes M, Lapierre L, Sáenz L, Crosby J, Barros C. Effect of the length of the hormonal supplementation on in vitro maturation of porcine oocytes. Theriogenology 2001;55:468. abstract.
[47] Concannon PW. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J Reprod Fertil 1989;39:3–25.
[48] Downs SM. Factors affecting the resumption of meiotic maturation in mammalian oocytes. Theriogenology 1993;39:65–79.