Effect of Various Caffeine Concentrations and Fertilization Time in *In Vitro* Fertilization of Canine Oocytes

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ABSTRACT

The techniques of IVM, IVF and IVC of canine oocytes may provide useful information for gamete salvage programs and the conservation of endangered canidae. This investigation has been made to determine the efficiency of *in vitro* maturation (IVM) as a basic experiment to study the development of canine oocytes after *in vitro* fertilization (IVF). The rate of oocytes developing to the MII stage was higher in the hormone treated group (10 IU/ml hCG+eCG, 14.7%, $p<0.05$) than in the control group (0 IU/ml hCG+eCG, 10.0%). The monospermy and pronuclear rates of canine oocytes were investigated after caffeine treatment on IVF. Canine oocytes were fertilized in the Fert-TALP medium supplemented with 0, 10, 20 or 30 mM caffeine (Fert I, Fert II, Fert III or Fert IV, respectively). The highest pronuclear formation rate was obtained in the Fert I for 24 h IVF (6.7%, 6/89). Therefore, it is believed that unlike in other mammals, caffeine in canine IVF does not increase the efficiency of fertilization rate, and is not an important factor.

(Key words : Canine, Oocyte, *In vitro* maturation, *In vitro* fertilization, Sperm capacitation)

INTRODUCTION

Research on reproductive technologies such as *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) has been conducted in a domestic bitch to improve knowledge regarding their reproductive biology, as well as to develop applications for conservation of endangered canine species. Therefore, an increased number of studies on IVM and IVF of canine oocytes have been reported. Nevertheless, the efficiency of IVM and IVF techniques are lower in canine species than in other species (Hewitt and England, 1999; Nickson *et al.*, 2000; Saint-Dizier *et al.*, 2001). And as well several investigators have reported that embryos processed from IVF in canines were cleaved and developed, although the rate was low (Otoi *et al.*, 2000; England *et al.*, 2001; Rodrigues *et al.*, 2004). The oocyte quality during IVM is the most important factor for determining successful fertilization and development *in vitro*. However, unlike other mammals whose oocytes complete their first meiotic division in the preovulatory follicle, canine oocytes ovulate at the GV stage of meiotic prophase I, due to the immaturity of oocytes (Concannon *et al.*, 1989; Tsutsui, 1989).

Capacitation involves the destabilization of the sperm head membrane rendering it more fusigenic. This change is facilitated by the removal of sterols (e.g. cholesterol) and non-covalently bound epididymal/seminal glycoproteins. It makes a more fluid membrane with an increased permeability to Ca^{2+}. An influx of Ca^{2+} produces increased intracellular cAMP levels and thus, an increase in motility. Hyper-activation is also part of capacitation and is the result of the increased Ca^{2+} levels.

Both heparin and caffeine have been in IVF as major capacitation factors. The efficiency of IVF in several studies has been found to be increased when heparin is added to the medium for a bull (Parrish *et al.*, 1988; Park *et al.*, 1989; Kurosaka *et al.*, 1990) and goat (Cox *et al.*, 1995) and dog (Lee *et al.*, 2007) *in vitro* fertilization. We treated heparin during IVF in the previous study, but heparin could not increase the rate of IVF in the canine (Lee *et al.*, 2007). Caffeine is a known phosphodiesterase inhibitor and has been used due to its effect on motility. Recently, several researchers have used caffeine as a stimulator to increase the efficiency of IVF in mammals, such as bovine, goat, and porcine (Park *et al.*, 1989; Izquierdo *et al.*, 1998; Funahashi *et al.*, 2001).

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In this study, the focus was on whether canine oocytes with sperm in a fertilization medium containing caffeine, which has been used as an insemination factor, progress to fertilization, cleavage, and subsequent development.

MATERIALS AND METHODS

Collection of Canine Oocytes

Reproductive tracts obtained after butchering normal and various bitches, aged more than 6 months, at a private abattoir, were kept in a physiological saline solution (PSS) composed of 0.9% NaCl, 0.06 mg/ml potassium penicillin G, and 0.1 mg/ml streptomycin sulfate maintained at 38.5℃, and were transported to the laboratory within 3 h. Ovaries removed from the tracts were washed free of blood 3 times in fresh PSS maintained at 38.5℃. Cumulus-oocyte-complexes (COCs) were aspirated from the ovaries with a 10 ml syringe containing a washing medium, consisting of TCM199 (Invitrogen, CA, U.S.A.) supplemented with 25 mM HEPES, 0.1% polyvinyl alcohol (PVA), 0.1 mM glutamine, 2.5 mM sodium pyruvate, 0.06 mg/ml streptomycin sulfate, and 0.06 mg/ml potassium penicillin G adjusted to 38.5℃. The COCs were washed twice in the washing medium and examined under a stereoscopic microscope (SZ51, Olympus, Tokyo, Japan). COCs were allocated to three groups according to the standard reported by Hewitt *et al.* (1998). Grade 1 COCs were darkly pigmented and completely surrounded by one or more layers of cumulus cells. Grade 2 COCs were lightly pigmented with incomplete layers of cumulus cells. Among these COCs, only grade 1 oocytes were washed 3 times in the washing medium prior to utilization in experiments. All chemical reagents were obtained from Sigma-Aldrich Corporation, unless otherwise indicated.

In Vitro Maturation

After the washing step for the oocytes, COCs were randomly allocated into four groups, according to four different conditions. The first group was cultured in a maturation medium, composed of TCM199 supplemented with 2.5 mM sodium pyruvate, 0.1 mM glutamine, 0.06 mg/ml streptomycin sulfate, 0.06 mg/ml potassium penicillin G and 0.1% PVA, pH 7.4. The second and third groups were cultured in a maturation medium supplemented with 10 IU/ml of hCG (CG-5) and eCG. All COCs used in this experiment were placed in 100 μl media drops, which contained not more than 20 COCs per drop, and were covered with mineral oil. All cultures were performed at 38.5℃ for 48 h in a humidified atmosphere of 5 % CO_2 in the air.

Sperm Preparation

Reproductive tracts containing testes and epididymis were removed from adult male dogs butchered at a private abattoir. The individual reproductive tracts were stored in sterile steel containers with tight sealing lids and maintained at 38.5℃. Epididymal spermatozoa were collected with a modified method of epididymodeferentectomy used by Cary *et al.* (2004). The cauda epididymis and vas deferens were isolated from each testicle, washed with a PBS solution, and sterilized with 70% ethanol. After the catheterization of the lumen of the vas deferens with an 18-gauge syringe, retrograde flushing of the vas deferens and cauda epididymis with 5 ml of the washing medium maintained at 38.5℃ was performed. The flushed suspension was collected in a warmed sterile 50 ml conical tube (Nunc, NY, U.S.A) at 38.5℃. The collected sperm of each dog was centrifuged at 120 g for 5 min. The supernatant was discarded, and the sperm pellet was resuspended with 10 ml of the washing medium. Suspended sperms were incubated to swim-up for 30 min at 38.5℃ and 5% CO_2 with maximum humidity. The supernatant (swim-up layer) was carefully collected and centrifuged at 120 g for 5 min. The supernatant was discarded, and the pellet was resuspended with 500 μl of the washing medium. Sperm collected from dogs were evaluated for motility and viability using a microscope. The percentage of total motile sperm (the number of sperm with progressive motility divided by the total number of sperm observed) was assessed in 5 μl drops of sperm suspension in a Makler counting chamber (Self-Medical Instrument, Haifa, Israel), using an inverted biological microscope (CKX31, Olympus, Tokyo, Japan). Only sperm with motility of more than 90% were used for IVF. After assessment of motility, the sperm concentration was adjusted to 1×10^7 sperm/ml with Fert-TALP.

Effect of Various Caffeine Concentration and Time in Insemination

The oocytes for IVM were processed for 48 h, randomly allocated to four groups, and cultured under different culture conditions. Group A, Group B, Group C, and Group D were fertilized in fertilization medium I (Fert I), fertilization medium II (Fert II), fertilization medium III (Fert III), and fertilization medium IV (Fert IV), respectively. Fert II consisted of Fert I supplemented with 10 mM caffeine. Fert III or Fert IV, consisted of Fert I supplemented with 20 or 30 mM caffeine. All groups were inseminated for 6, 12 and 24 h. First, *in vitro* matured oocytes were added to each droplet of 50 μl of each fertilization medium. These oocytes were added to each droplet as the rate of 20 oocytes and a prepared sperm (50 μl) was added to each droplet to maintain 5×10^6 ml as a final

concentration. Oocytes and sperm were co-incubated at 38.5℃ and 5% CO_2 with maximum humidity for 6, 12, and 24 h.

Assessment of Nuclear Stage of Maturation and Sperm Penetration

After 48 h of insemination, all oocytes in both groups were denuded of cumulus cells with TCM199 supplemented with 0.1% hyaluronidase for 5 min. These oocytes were washed with a PBS consisting of 8 mg /ml NaCl, 0.2 mg/ml KCl, 1.44 mg/ml Na_2PO_4, and 0.24 mg/ml KH_2PO_4. After washing, denuded oocytes were fixed in a fixation solution consisting of 2% formalin, 0.1% PVP, and 0.25% glutaraldehyde for 20 min. After fixing, oocytes were stained with 1.9 μM Hoechst 33342 in glycerol, and observed using a fluorescence microscope (BX50, Olympus, Tokyo, Japan) fitted with a digital camera (Coolpix4500, Nikon, Tokyo, Japan). Nuclear and sperm morphology in the oocytes was classified as pronuclear, monosperm and polysperm.

Statistical Analysis

Data were analyzed by chi-square analysis to determine the differences between groups. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of Gonadotropin on *In Vitro* Maturation

COCs cultured in either IVM medium control or hormone group were fixed and stained. As shown in Table 1, the percentages of oocytes that underwent maturation to the MII stage between the two culture media were very low. The rates of oocytes developing to the MII stage was higher in hormone treated group (14.7%, $p<0.05$) than in the control group (10.0%). However, the rates of meiotic resumption in oocytes were not significantly different in the hormone treated group (50.3%) than in the control group (49.9%). Especially, the rate of unidentified or degenerated oocytes were significantly higher in the control group (26.6%, $p<0.05$) than in the hormone treated group (21.3%). Oocytes observed after IVM are shown in Fig. 1.

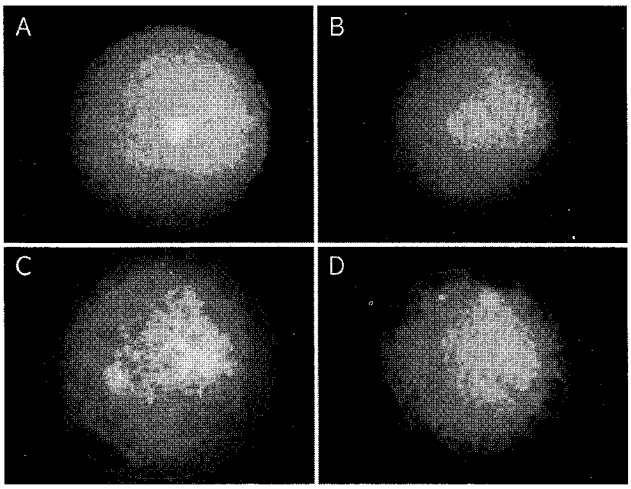

Fig. 1. Nuclear morphology in canine oocytes. Germinal vesicle (A), germinal vesicle breakdown (B), metaphase I (C), metaphase II (D).

Effect of Various Caffeine Concentration and Time in Insemination

The rate of sperm penetration after IVF in all groups is summarized in Table 2. The sperm penetration rate after IVF was detected at 24 h. The pronuclear formation rates under all culture conditions were very low. The overall pronuclear formation rates of oocytes cultured in 0 mM caffeine was the highest of all of the groups. However, the caffeine treated group was not significantly different. Especially, the rate of pronuclear formation of canine oocytes cultured in 0 mM caffeine for 24 h of insemination time (6.7%, $p<0.05$) had the highest of all of the culture conditions. Additionally, the rate of monospermy penetrated oocytes was significantly higher in 0 mM caffeine (31.5%) than other groups (3.3~26.7%). Canine oocytes inseminated in 0 mM caffeine for 24 h were observed as various morphologies for each nuclear stage of zygotes (Fig. 2). As summarized in Table 2, the rate of monospermic in canine oocytes inseminated in 0 mM caffeine for 24 h were high compared with any other culture conditions.

Table 1. Effect of gonadotropin for meiotic rates of canine oocytes

Condition	No. of oocytes	Culture period(h)	Nuclear stage (%)				
			GV	GVBD	MI	MII	DEG
Control	109	48	13(9.1)[a]	12(11.0)[a]	44(40.3)[a]	11(10.0)[a]	29(26.6)[a]
Hormone	122	48	15(12.2)[a]	20(18.3)[b]	43(35.2)[b]	18(14.7)[b]	26(21.3)[b]

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

Table 2. Effect of caffeine on insemination of canine oocytes

Caffeine	Insemination time	No. of oocytes	Insemination status (%)					
			MI	MII	Mono	Poly	PN	ND
0 mM	6	87	3(3.4)^{a}	1(1.1)^{a}	16(18.4)^{a}	24(27.6)^{a}	3(3.4)^{a}	40(46.1)^{a}
	12	89	2(2.2)^{a}	3(3.4)^{a}	21(23.6)^{b}	35(39.3)^{b}	3(3.4)^{a}	25(28.1)^{b}
	24	89	4(4.5)^{ab}	2(2.2)^{a}	28(31.5)^{c}	41(46.1)^{c}	6(6.7)^{b}	8(9.0)^{c}
10 mM	6	82	4(4.8)^{ab}	2(2.4)^{a}	13(15.8)^{a}	34(41.4)^{b}	0^{c}	29(35.3)^{ab}
	12	89	2(2.2)^{a}	1(1.2)^{a}	15(16.8)^{a}	42(47.1)^{d}	0^{c}	29(32.5)^{ab}
	24	86	5(5.8)^{ab}	3(3.4)^{a}	14(16.2)^{a}	50(58.1)^{e}	0^{c}	14(16.2)^{bc}
20 mM	6	83	3(3.6)^{a}	0^{b}	18(21.6)^{b}	29(34.9)^{b}	0^{c}	33(39.7)^{ab}
	12	86	2(2.3)^{a}	0^{b}	23(26.7)^{b}	20(20.9)^{a}	1(1.2)^{a}	40(46.5)^{a}
	24	88	1(1.1)^{a}	1(1.1)^{a}	12(13.7)^{a}	35(39.8)^{b}	0^{c}	39(44.3)^{a}
30 mM	6	90	2(2.2)^{a}	0^{b}	6(6.7)^{c}	42(46.7)^{d}	0^{c}	40(44.4)^{a}
	12	92	1(1.1)^{a}	1(1.1)^{a}	6(6.5)^{c}	45(48.9)^{d}	0^{c}	39(42.4)^{a}
	24	91	2(2.2)^{a}	2(2.2)^{a}	3(3.3)^{c}	51(56.0)^{e}	0^{c}	33(36.3)^{ab}

Mono; mono penetrate, Poly; poly penetrate, PN; pronuclear formation, ND; Non detected.
$^{a-e}$ Values with different superscripts in the same column differ significantly ($p<0.05$).

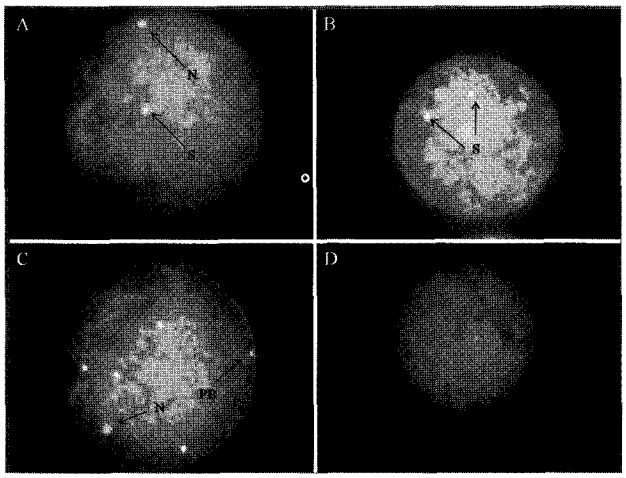

Fig. 2. Canine zygotes resulting from *in vitro* fertilization of oocytes matured *in vitro*. Monospermic (A), polyspermic (B, C), pronuclear formation (D). N; nuclear, S; sperm head, PB; polabody.

DISCUSSION

Previous studies investigated the efficiency of IVM of canine oocytes by various conditions, such as culture time from 48~72 h or gonadotropin supplementation (Hewitt and England, 1999; Lee *et al.*, 2007). However, the rate of IVM of canine oocytes was still very low.

In the present experiment, the control medium consisting of TCM199 supplemented with 10 IU/ml hCG and eCG did significantly influence the nuclear maturation of canine oocytes. This result is similar to previous reports that gonadotropin has been shown to positively affect both nuclear and cytoplasmic maturation (Songsasen *et al.*, 2003). Therefore, we insisted that gonadotropin does need to be contained in IVM of canine oocytes. However, the role of gonadotropin for canine IVM remains unclear because of the differences in research design by many investigators. It is hypothesized that the low MII efficiency and degeneration percentages in canine oocytes were caused by inadequate culture conditions or culture medium, among other variables. Until now, there have been few investigations of canine embryos generated by IVF and IVC after IVM. This may be due to the low rate of IVM of canine oocytes (Otoi *et al.*, 2000; Otoi *et al.*, 2004; De los Reyes *et al.*, 2006). England *et al.* (2001) obtained a single pregnancy after transplanting 2 embryos (2-cell stage) acquired from IVF following IVM of oocytes from 24~72 h. Until now, the only blastocyst after IVF following IVM was achieved by Otoi *et al.* (2004), who accomplished IVF for canine oocytes after IVM were cultured on a feeder layer of bovine cumulus cells. This experiment first showed that canine oocytes could develop to the blastocyst stage following IVM, IVF, and IVC.

As previously mentioned, although many researchers have investigated the effect of caffeine in IVF in va-

rious mammals, the role of caffeine in IVF of canines is not fully understood. Therefore, we investigated whether caffeine plays a role as a stimulator of sperm penetration in this study. A higher monospermy rate was observed in the caffeine-free condition for 24 h of insemination. In addition, differences among groups along with various concentrations were not revealed. From this result, we suggest that caffeine does not need to be present in IVF of canine oocytes. This result is similar to the assertion that heparin did not stimulate fertilization (Lee *et al.*, 2007).

The present study shows that canine viable oocytes obtained from bitches in various phases of the estrus cycles were cultured in a TCM199 medium with hormone for IVM. Although the rates of nuclear maturation in canine were very low, we tried to achieve embryonic development. Any embryo in the cleavage stage was not obtained following IVM, IVF and IVC. Therefore, it is concluded that the pronuclear formation from IVF after IVM in canine oocytes can be induced from IVF medium without caffeine.

These experiments, used abandoned oocytes obtained after a routine butchery of various bitches, unlike previous researches. Therefore, these results are applicable to IVM and IVF of wild dog. Especially, they may be an important tool to restore endangered and distinct canidae, such as the red wolf and bush dog.

Additionally, by investigating the function of caffeine in IVF of canine oocytes, it was found that the efficiency of embryonic development by caffeine is similar to the heparin-free condition. Although canine oocytes were cultured in various culture conditions for insemination *in vitro*, these oocytes failed to develop into cleavage and subsequent development. Therefore, it is hypothesized that capacitation and pronuclear formation in canine oocytes has unnecessary capacitation factors.

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