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Effect of crude canine pituitary extract (cCPE) on the *in vitro* production of progesterone and nuclear maturation of canine oocytes

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We examined the effect of crude canine pituitary extract (cCPE) on the *in vitro* nuclear maturation of canine oocytes and production of progesterone by cumulus cells. cCPE was extracted from canine pituitaries and the concentrations of canine follicle stimulating hormone (FSH) and luteinizing hormone (LH) were determined. Cumulus oocyte complexes (COCs) were harvested from anestrus cycle ovaries and matured in NCSU-37 supplemented with 10% estrus bitch serum, 50 µg/ml gentamycin and 0, 40 or 400 µg/ml cCPE at 38°C in a humidified atmosphere of 5% CO₂ for 72 h. The nuclear maturation of the oocytes and the level of progesterone in the culture medium were evaluated. Development to metaphase I (MI) - metaphase II (MII) of canine oocytes in 400 µg/ml cCPE (15.4%) was significantly higher than in 0 and 40 µg/ml cCPE (4.3 and 8.7%), respectively. Treatment with 40 and 400 µg/ml cCPE also generated 0.33 and 0.65 ng/ml progesterone in the culture medium, respectively. Thus, the addition of cCPE to the culture medium promotes the nuclear maturation of canine oocytes and elevates the production of progesterone by cumulus cells.

Key words: *In vitro* maturation, pituitary extract, canine oocyte.

INTRODUCTION

To improve *in vitro* maturation rate of canine oocytes, various gonadotropin hormones have been added to the basic medium. These hormones included follicle stimulating hormone (FSH; Folltropin-V and FSH originating from porcine pituitary) (Hishinuma et al., 2004; Rodrigues and Rodrigues, 2003), human menopausal gonadotropin (hMG) (Otoi et al., 2006), luteinizing hormone (LH) (Hewitt and England, 1999), and human

chorionic gonadotropin (hCG) (Cui et al., 2006; Otoi et al., 2006). However, the rate with which canine oocytes progress to the metaphase II (MII) stage remains low despite these supplements (Songsasen and Wildt, 2007; Chastant-Maillard et al., 2011).

Here, we examined whether culture with crude canine pituitary extract (cCPE) improves the *in vitro* maturation (IVM) of canine oocytes. Supporting this possibility is that in horses, superovulation is routinely induced by using equine pituitary extract (EPE) as the homologous source of gonadotropin (Alvarenga et al., 2001; Scoggin et al., 2002; Squires et al., 2003). Moreover, when equine oocytes were matured in IVM medium supplemented with EPE, about 38% of oocytes develop to the MII phase (Brück et al., 1996). Furthermore, studies of *Xenopus* and fish oocytes have shown that the addition of pituitary extract to culture medium not only induces a resumption

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Abbreviations: cCPE, Crude canine pituitary extract; FSH, follicle stimulating hormone; LH, luteinizing hormone; COCs, cumulus oocyte complexes; MI, metaphase I; MII, metaphase II

of meiosis (Masui, 2001; Sen et al., 2002), but also induces maturation of oocytes.

FSH and LH were detected in the pituitary extract of horses (Guillou and Combarrous, 1983) and it has been shown that rat pre-ovulatory follicles express progesterone receptor mRNA when treated with either FSH or LH (Natraj and Richards, 1993; Park-Sarge and Mayo, 1994). Moreover, when porcine cumulus-oocyte complexes (COCs) were cultured with the progesterone synthesis inhibitor aminoglutethimide (AGT), the germinal vesicle (GV) breakdown rate was dropped (Shimada and Terada, 2002). These observations together suggest that the progesterone secreted by cumulus cells may induce the *in vitro* meiotic maturation of oocytes. In this study therefore, we prepared cCPE and examined its *in vitro* effect on cumulus cell expansion, progesterone production and nuclear maturation of canine oocytes.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. All procedures were conducted in accordance with the Ethics Committee of Yanbian University, Yanji, China.

Collection of canine ovaries and oocytes

Ovaries were harvested from crossbred bitches at the anestrus phase of the reproductive cycle by routine ovariohysterectomy at private clinics. The ovaries were kept in 0.9% saline containing 1% penicillin-streptomycin solution at 38°C and transported to the laboratory within 4 h (Lee et al., 2006). Oocytes were recovered by slicing the ovaries in D-PBS supplemented with 0.1% polyvinyl alcohol (PVA-PBS). Only oocytes with a diameter > 110 µm that exhibited homogeneous dark cytoplasm and had three or more layers of cumulus cells were selected for use (Otoi et al., 2002).

Extraction of cCPE

Canine pituitary glands were obtained from healthy female canines within 1 h of death after euthanasia at private clinics in Long Jing, China. In total, 20 g of pituitaries were collected and kept frozen at -80°C. To obtain cCPE, we modified the method used to purify equine FSH (Combarrous and Henge, 1981). Briefly, the canine pituitaries were homogenized in 40% ethanol and 6% ammonium acetate buffer (pH 6.0) and then shaken at 4°C for 3 h. The homogenized solution was then centrifuged at 10,000 × g at 4°C for 30 min and cold absolute ethanol was added to the supernatant to a final concentration of 80% ethanol to precipitate the cCPE. After centrifugation at 10,000 × g at 4°C for 30 min, the pellet was dissolved in 0.01 M phosphate buffer (pH 7.2) and dialyzed in 0.01 M phosphate buffer (pH 7.2) at 4°C over 24 h using Spectra/Por membrane tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a molecular weight cutoff of 12,000 to 14,000. The samples were then lyophilized and stored at -70°C until use. Hormone assays were performed by the NeoDIN Medical Institute (Seoul, S. Korea) using the Active(TM) LH IRMA, DSL-4600 and Active(TM) FSH IRMA, and DSL-4700 kits from Diagnostic Systems Laboratories, Inc. (USA). The concentrations of FSH and LH in cCPE measured 5.73 and 139.66 mIU/mg, respectively.

IVM of canine COCs

To evaluate the effect of cCPE, 30 COCs were cultured in 1 ml of basic medium NCSU-37 (Petters and Wells, 1993), supplemented with 10% estrus bitch serum and 50 µg/ml gentamycin, with 0, 40 and 400 µg/ml cCPE at 38°C in a humidified atmosphere with 5% CO₂ for 72 h.

Evaluation of the nuclear morphology of canine oocytes

After 72 h of IVM, the cumulus cells of the COCs were removed in PVA-PBS and 0.1% hyaluronidase by gentle pipetting using a small glass pipette. The denuded oocytes were then washed in PVA-PBS and fixed at room temperature for 30 min in PVA-PBS supplemented with 3.7% (w/v) formaldehyde and 10 µg/ml Hoechst 33342. The fixed oocytes were rinsed in PVA-PBS to remove the Hoechst 33342 and mounted onto slides to evaluate their nuclear morphology. The chromatin configuration was determined by fluorescent microscopy (Olympus, Japan). The nuclear morphology was categorized into three nuclear stages as shown in Figure 1. Briefly, in the GV stage, the nuclear membrane was visible; in the MI - MII stage, the chromosomes were condensed in pairs or formed an equatorial plate; in the case of MII, associated with the extrusion of the first polar body; in other stages, no chromosomes were visible (degeneration) or chromatin materials could not be identified (Downs, 1989).

Measurement of progesterone in medium

Thirty COCs were cultured in 1 ml basic medium supplemented with 40 or 400 µg/ml cCPE at 38°C in a humidified atmosphere containing 5% CO₂ for 72 h. After IVM, the medium was collected into 1.5 ml tubes, centrifuged at 3000 × g for 20 min, and the supernatants were stored at -70°C. The concentrations of progesterone (P4) were then analyzed at the NeoDIN Medical Institute (<http://www.neolab.co.kr/>) by using a DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., TX, USA) in combination with an Automatic Gamma Counter (Packard Cobra II Series, Packard Instrument Company, Downers Grove, IL, USA).

Statistical analysis

All data were analyzed by Chi-square analysis using SAS 8.0 software. Differences with a probability value less than 0.05 were considered significant.

RESULTS

To test whether cCPE improves the IVM of canine oocytes, the oocytes were cultured for 72 h with 0, 40, or 400 µg/ml cCPE (Table 1). The oocytes developed to MI-MII significantly more frequently when cultured with 400 µg/ml cCPE than with 0 or 40 µg/ml cCPE (15.4% vs. 4.3% and 8.7%, $p < 0.05$). Moreover, significantly fewer oocytes remained at the GV stage when cultured with 400 µg/ml cCPE compared to 0 or 40 µg/ml cCPE (23.1% vs. 43.5% and 34.8%, $p < 0.05$). The cumulus cells also showed greater expansion in 400 µg/ml cCPE than in 0 or 40 µg/ml cCPE (Figure 1a). Despite this, the corona radiata cells remained strongly attached to the zona

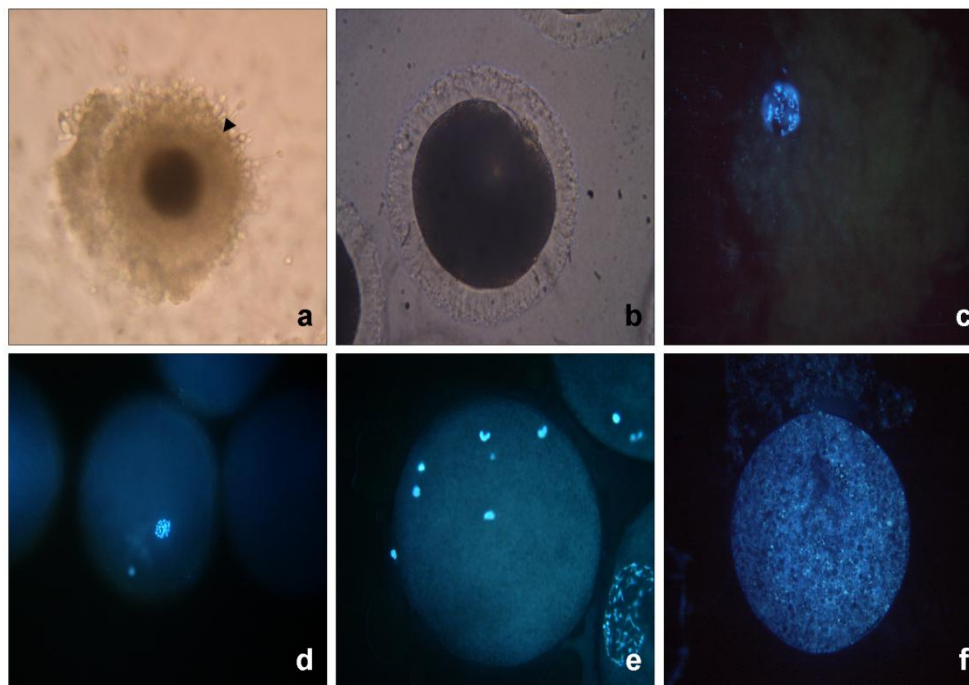


Figure 1. Morphology of (a) expanded cumulus cells (X 100), (b) canine oocyte at the MII stage in bright field(X 200), (c to e) different meiotic stages observed after Hoechst33342 staining at (c) GV stage (X 200), (d) metaphase II (MII) stage (X 200), (e) unidentified stage, and (f) a degenerated oocyte(X 200). The arrowhead indicates corona radiata cells.

Table 1. *In vitro* nuclear maturation of canine oocytes enclosed in cumulus-oocyte complexes (COCs) cultured with varying crude canine pituitary extract (cCPE) concentrations.

cCPE treatment ($\mu\text{g/ml}$)	Number of COCs examined for nuclear maturation	Cumulus cell expansion	Number (%) of oocyte at		
			GV	MI - MII	Other
Control	69	No	30 (43.5) ^a	3 (4.3) ^a	36 (52.2)
40	69	No	24 (34.8) ^{a,b}	6 (8.7) ^a	39 (56.5)
400	78	Yes	18 (23.1) ^b	12 (15.4) ^b	48 (61.5)

^{a,b}Values in the same column bearing different superscript differ significantly ($p < 0.05$). *All experiments were replicated at least three times.

pellucida and were not expanded at the end of the culture period, regardless of the amount of cCPE that was present (Figure 1a).

When the COCs were cultured with 40 or 400 $\mu\text{g/ml}$ cCPE for 72 h, the increase of the progesterone concentration was 0.33 or 0.65 ng/ml (Table 2). However, no progesterone release was observed for the control group.

DISCUSSION

The present study was conducted to determine whether CPE harvested from canine pituitaries can act as a homologous source of gonadotropin and promote canine oocyte IVM and secretion of progesterone. The pituitary

gland is a bean-shaped gland located below the brain at the base of the skull in an area called the pituitary fossa or sella turcica. It is a very important organ in the endocrinological system as it releases gonadotropins that are crucial for triggering oocyte growth and maturation (Thibault et al., 1993).

Pituitary extracts from zebrafish (Wang and Ge, 2003) and mares (Hofferer et al., 1993; Rosas et al., 1998) contain pituitary hormones such as gonadotropins, and early *in vivo* studies employed pituitary extracts to induce superovulation in mares (Scoggin et al., 2002), ewes (Boland and Gordon, 1982) and cattle (Staigmiller et al., 1989). Thus, pituitary extracts contain the gonadotropins needed to induce superovulation and oocyte maturation *in vivo* (Eppig, 1996). Studies have also shown that *Xenopus* and fish oocytes cultured with pituitary extracts

Table 2. Progesterone (P4) concentrations in the *in vitro* maturation (IVM) medium with canine cumulus-oocyte complexes cultured for 72 h with 40 or 400 µg/ml canine crude pituitary extract.

Type of media	Concentration of P4 (ng/ml)	
	Before IVM	After 72 h IVM (increased)
NCSU-37	0.01	0.01 ± 0.01 (0.0) ^a
NCSU-37 + 40 µg/ml cCPE	0.04	0.37 ± 0.05 (0.33) ^b
NCSU-37 + 400 µg/ml cCPE	0.09	0.74 ± 0.08 (0.65) ^c

^{a,b,c}Values in the same column bearing different superscripts differ significantly ($p < 0.05$).

resume meiosis (Sen et al., 2002; Masui, 1967), which indicates that pituitary extracts can also induce oocyte maturation *in vitro*.

Development to MI – MII of COCs in 400 µg/ml cCPE was significantly higher than in the untreated and 40 µg/ml-treated groups (15.4% vs. 4.3% and 8.7%, $p < 0.05$). However, over 400 µg/ml concentrations did not further improved the rate of oocyte maturation (unpublished data). Notably, previous studies have shown that 13.5% (Rodrigues and Rodrigues, 2003) and 16% (Hishinuma et al., 2004) of canine oocytes develop to the MI/AI-MII stages when cultured in IVM media supplemented with heterologous gonadotropin hormones such as FSH. Another study also showed that 15% of canine oocytes cultured in medium supplemented with 0.1 IU/ml FSH and 10 IU/ml LH reached the MI/AI-MII stages (Rota and Cabianca, 2004). Thus, cCPE is not superior to heterologous gonadotropins in inducing the IVM of canine oocytes.

The *in vivo* and *in vitro* maturation of mammalian oocytes is normally associated with cumulus cell expansion. The meiotic arrest of oocytes is induced by communications between oocytes and corona radiata cells, which occur via cytoplasmic extensions across the zona pellucida; communications from outer cumulus cells via gap junctions are also important for inducing oocyte meiotic arrest (Allworth and Albertini, 1993; Isobe and Terada, 2001). In the present study, we found that while 400 µg/ml of cCPE induced slight cumulus cell expansion, most of the corona radial cells remained strongly attached to the zona pellucida and appeared unexpanded 72 h after IVM; this was also observed when the IVM period was extended to five days (unpublished data). It has been shown that open communications between cumulus cells and canine oocytes are needed before the oocytes can complete meiosis (Luvoni et al., 2001). Our morphological analyses of the cCPE treated oocytes suggested that the gap junctions between corona radial cells and oocytes were not blocked. This may explain why the cCPE did not elevate the canine oocyte IVM rates to those observed with other domestic animals, even though cCPE promoted the nuclear maturation of oocytes to the MI-MII stages.

Progesterone is produced by cumulus cells and its production by cattle (Armstrong et al., 1996) and pig

(Shimada and Terada, 2002) cumulus cells is increased by stimulation with FSH or LH. The progesterone produced by cumulus cells plays an important role in the acquisition of developmental competence by rat oocytes (Zhang and Armstrong, 1989). When adding progesterone synthesis inhibitor AGT to gonadotropin-containing medium, low concentrations of progesterone were produced and remained a high frequency of the sheep and pig oocytes in the GV stage (Shimada and Terada, 2002; Osborn et al., 1986). This suggests that the ability of cumulus cells to produce progesterone is important for the meiotic maturation of COCs *in vitro*. Our data were consistent with these observations.

In conclusion, the progesterone that was secreted in response to cCPE treatment may have stimulated the nuclear development of canine oocytes and the expansion of cumulus cells. However, cCPE is not superior to heterologous gonadotropin sources in inducing canine oocyte IVM.

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