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Effects of Ovary Status and *In Vitro* Maturation Condition on the Developmental Competence of Canine Oocytes

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ABSTRACT

In canine, oocytes are ovulated at the GV (germinal vesicle) stage and they have to fulfill maturation phase before reaching metaphase II stage. The efficiency of *in vitro* maturation is still very low. Therefore, the aim of this study was to investigate the effect of *in vitro* maturation on nuclear changes of immature canine oocytes recovered from different reproductive stages ovaries and different culture conditions. The oocytes were cultured in TCM-199 with supplement at 5% CO_2 and 38.5℃ for 72 h. The nuclear maturation of canine oocytes was evaluated with Hoechst 33342 stain under fluorescence microscope (Fig. 1). The results of this study detected differences in *in vitro* maturation rate between oocytes recovered from follicle status and non-follicle status ovaries. However, these differences were not significant as indicated in Table 1 and Fig. 2. In regard to the effect of culture condition with supplements, we did not found significant differences compared with control group (Table 2, Table 3). One of the reasons for this data could be the conditions that ovaries were exposed during slaughtering process or the long distant transportation of the ovaries. Although these data have not shown clearly significant differences results compared with control, furthermore the different reproductive status ovaries was beneficial for maturation of oocytes *in vitro* and can be a basic part of knowledge to improve *in vitro* maturation of canine oocytes.

(Key words : canine, oocytes, *in vitro* maturation, ovary, estrus cycle)

INTRODUCTION

The developmental competence and quality of embryos produced *in vitro* depends upon several factors in addition to the conditions of *in vitro* maturation, fertilization and culture. Especially, *In vitro* maturation (IVM) is one of the most important steps that determine the developmental competence of the oocytes (Eppig *et al*., 1994). IVM is a reproductive technology that enables oocytes to be matured *in vitro* from ovaries that have received either no or low levels of gonadotrophin stimulation (Edwards, 1965). Furthermore, IVM is an important assisted reproductive technologies (ART) as it has the potential to capture the vast supply of oocytes within an ovary (Gilchrist *et al*., 2008). In domestic animals except canine, IVM success rates are relatively high and therefore are more widely accepted. However, the efficiency of IVM is still very low in canine compared to other mammalian species. One of the reasons for low efficiency of IVM in this species may be due to its unique reproductive physiological characteristics (Kim *et al*., 2001). Unlike other species, 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 h (Lee *et al*., 2005; Songsasen and Wildt, 2007). Female dogs also have unusual characteristics including the follicular environment, extremely high lipid content representing a uniform dense, dark appearing cytoplasm, with highly compact, and unexpanded cumulus cells of ovulated oocytes (Hewitt and England, 1997).

Many researchers have examined the feasibility of IVM of canine immature oocytes (Yamada *et al*., 1992; Bogliolo *et al*., 2002; Bolamba *et al*., 2002; Songsasen *et al*., 2003; Hatoya *et al*., 2006; Saikhun *et al*., 2008; Apparicio *et al*., 2011; Lopes *et al*., 2011; Salavati *et al*., 2012; Songsasen *et al*., 2012).

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Nevertheless, the rates of maturation of canine oocytes to metaphase II (MII) remain low, especially when compared with those of many other mammalian species. Thus, there is no *in vitro* system has been developed to increase the maturation rates of canine oocytes comparable to *in vivo* one. A shortcoming that has been attributed to this low efficiency was the specific and highly complex requirements of *in vivo* canine oocytes maturation (B.A. Rodrigues JLR, 2010). Once research results improved, the reproductive techniques of *in vitro* maturation, *in vitro* fertilization, and embryo transfer promise to be useful tools for specific canine species conservation.

This study was conducted to evaluate the nuclear development of canine oocytes when collected from different reproductive stages ovaries or cultured with different supplements during *in vitro* maturation.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA), unless indicated.

1. Collection of Canine Ovaries and Cumulus-oocyte Complexes

Ovaries were obtained from canine females of various breeds and at different ages with unknown health status that underwent ovariohysterectomy at the veterinary hospital nearby Jinju city, Republic of Korea. The cumulus-oocyte complexes (COC) were released by repeated slicing of the ovarian cortex with the scalpel blades in TCM-199 medium with 25 mM HEPES supplemented with 0.1% bovine serum albumin (BSA) and 1% penicillin-streptomycin at 38.5℃.

2. *In Vitro* Maturation

The oocytes selected for this study had a perfectly spherical shape and an even, smooth, dark pigmented cytoplasm. The oocytes were cultured in TCM-199 supplemented with 1 μg/ml estradiol-17β, 10 μg/ml FSH, 0.6 mM cysteine, 0.2 mM Na-pyruvate, 10% FBS and 1% penicillin-streptomycin. The maturation was performed by culturing approximately 50~60 COCs in 500 μl of maturation medium in four-well dishes for 72 h at 5% CO_2 and 38.5℃.

3. Removal of Cumulus Cells and Assessment of Oocytes Nuclear Maturation

At the end of the maturation period, COCs were transferred to D-PBS buffer containing 0.1% hyaluronidase and the cumulus cells were removed by gentle pipetting with glass pipette. The denuded oocytes were washed several times in D-PBS with 0.1% polyvinyl alcohol (PVA) and transferred to 3.7% formaldehyde solution at room temperature before mounting on a slide with an overlay of 1.9 μM Hoechst 33342 in glycerol. The oocytes were evaluated under an inverted epifluorescence microscope with a UV light to determine the stage of meiosis, such as germinal vesicle (GV), germinal vesicle breakdown (GVBD), MI (metaphase I) stage, and MII (metaphase II) stage (Fig. 1).

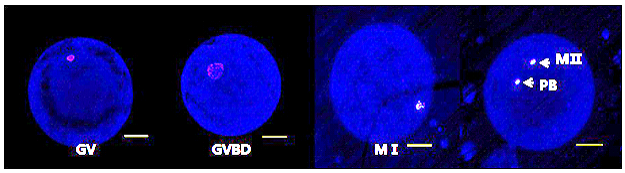

Fig. 1. Chromatin configuration of canine oocytes stained with Hoechst 33342. GV: germinal vesicle stage, GVBD: germinal vesicle breakdown stage, MI: metaphase I stage, MII: metaphase II stage, PB: first polar body. The bar is 50 μm.

4. Experimental Design

For the effect of reproductive stage ovaries, the cumulus-oocyte complexes (COCs) from non-follicle status (n=163) and follicles status (n=224) ovaries were released by repeated slicing of the ovarian cortex with the scalpel blades in TCM-199 medium with 25 mM HEPES supplemented with 0.1% bovine serum albumin (BSA) and 1% penicillin-streptomycin at 38.5℃. The oocytes were cultured in maturation medium at 38.5℃ of 5% CO_2 for 72 h.

For the different culture conditions, the oocytes from randomly stage ovaries were cultured in maturation medium with (n=54)/without (n=49) PMSG (0.5 IU/ml) & hCG (1 IU/ml) at 38.5℃ of 5% CO_2 for 72 h. The other group was cultured in maturation medium with (n=50)/without (n=20) MG132 (1 μM) at 38.5℃ of 5% CO_2 for 72 h.

5. Statistical Analysis

Data were expressed as the mean ± SEM by *t*-test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

1. The Effect of Ovary Status on Maturation Rate of Canine Oocyte

In domestic animals, the oocytes are recovered from slaugh-

tered animals that could be at different stages of estrus cycle and from different sizes of follicles. Even so, effects of ovary status on nuclear kinetics of canine oocytes were varied depending on *in vitro* maturation. The nuclear configuration of immature canine oocytes collected from different reproductive stage ovaries were evaluated after IVM as shown in Fig. 2. Overall, this study found that there were differences but not significant in maturation rate between oocytes recovered from follicle status and non-follicle status ovaries collected from different reproductive stages (Table 1. 24.6 ± 18.5%; Fig. 2-A vs. 21.4 ± 18.1%; Fig. 2-B, C). Oocytes recovered from follicle status ovaries reached metaphase stage in higher rate than that of recovered from non-follicle ovaries (Table 1). One of the reasons behind this result could be that there was no available information in regard to the health, and age of animals. Another possibility is that canine ovaries usually exposed to higher temperature in slaughterhouse, which could reduce the quality of the oocytes.

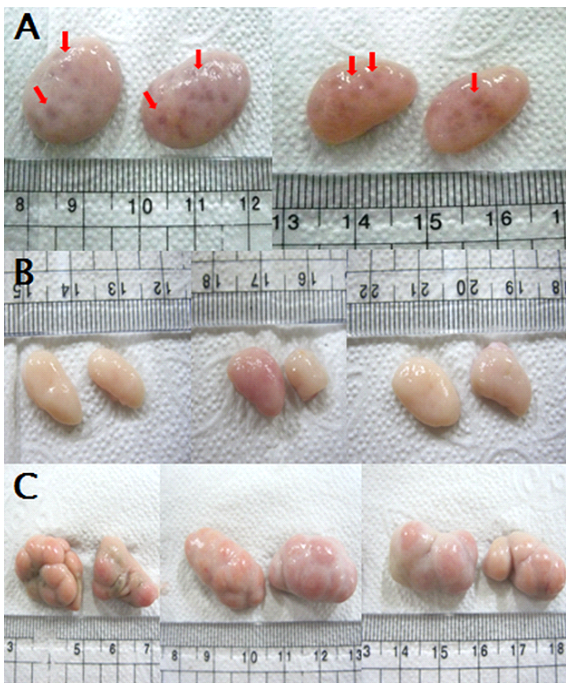

Fig. 2. Morphology evaluations of the canine ovaries. (A) follicular ovaries (red arrow), (B, C) non-follicular ovaries from different reproductive stages; (B) anestrus and (C) corpus luteum stage.

Oh *et al*. (2005) have reported that immature canine oocytes within follicles are exposed to high levels of E_2 and P_4 due to preovulatory luteinization of follicles which could influence their *in vitro* maturation. Following ovulation, canine oocytes are also exposed to high levels of P_4 and E_2 in the bursa and oviduct (Kim *et al*., 2005). In addition, size of the follicle is one of major factors influencing IVM success of the canine oocyte. The large follicles provide oocytes with higher meiotic competency compared to small ones. Songsasen and Wildt (2005) have reported also that nearly 80% of oocytes recovered from follicles > 2 mm have the capacity to achieve MII compared to that recovered from 1 to 2 mm (38%), from > 0.5 to 1 mm (26%) and from < 0.5 mm (17%) follicles. Therefore, oocytes collected from follicle status ovaries have a beneficial effect on the nuclear maturation *in vitro*.

2. The Effect of Culture Condition on Maturation RAte of Canine Oocyte

Oocytes aspirated from the follicle and exposed to an artificial environment such as a maturation medium will experience changes associated with the action of compounds to which they were subjected. Moreover, spontaneous apoptosis occurs in cumulus oocyte complexes (COCs) during suboptimal culture condition (Ikeda *et al*., 2003; Yuan *et al*., 2005). Specific commercial culture media can generate reactive oxygen species (ROS) depending on its composition, and media additives also are playing a crucial role in ROS induction (Agarwal *et al*.,

Table 1. Meiotic progression of canine oocytes from follicle and non-follicle status ovaries

Ovary status	No. of oocytes developed to (%)					
	GV	GVBD	MI	MII	Non	MI - II
Follicle*	47 (21 ± 20.1)	115 (51.3 ± 18.5)	28 (12.5 ± 5.6)	27 (12.1 ± 12.9)	7 (3.1 ± 4.1)	55 (24.6 ± 18.5)
Non-follicle**	9 (5.5 ± 1.4)	116 (71.2 ± 18.2)	25 (15.3 ± 11.2)	10 (6.1 ± 6.9)	3 (1.8 ± 2.0)	35 (21.4 ± 18.1)

* Follicle showed Fig. 2-A status, ** Non-follicle showed Fig. 2-B, Fig. 2-C status.
GV: germinal vesicle stage, GVBD: germinal vesicle breakdown stage, MI: metaphase I stage, MII:: metaphase II stage, PB: first polar body, Non: Not detected nuclear.

2006). Therefore, media additives may interfere with the extent of either cell death or survival of the oocytes. Nevertheless, nobody has been yet established efficient *in vitro* maturation system of canine oocyte as the well-established one of other domestic animal species.

We also have demonstrated that the effect of culture conditions on the *in vitro* maturation competence of immature canine oocytes when supplements were added. In this study, the various supplements specifically PMSG and hCG as hormone (Table 2) and in addition to MG132 as a proteosome inhibitor (Table 3) were added during *in vitro* maturation. The rates of maturation for the control, and hormone supplemented group (0.5 IU/ml PMSG and 1 IU/ml hCG) were 6.1% and 9.3%, respectively. The percentage of maturation to MⅡ stage was higher in the hormone treatment group that the control. But, there was no significant difference between control and hormone treatment groups. As the other study, the MG132 is a specific inhibitor of proteasome activity and prevents MPF inactivation by blocking cyclin B degeneration (Josefsberg *et al*., 2000; You *et al*., 2012). The rates of maturation for the control (non-treatment) and 1 μM MG132 treatment groups were 35% and 48%, respectively. The percentage of maturation to MⅡ stage was also higher in the MG132 treatment group than the control. But, there was no significant difference between control and MG132 treatment groups. Overall, no significant differences were observed on the *in vitro* maturation rate among the different supplements. Furthermore, we need investigate effect of MG132 treatment during oocyte maturation *in vitro* on the MPF activity in canine oocytes.

Chemical supplement using glucose in the maturation medium, seems to enhance the rates of meiosis resumption and metaphase stage of *in vitro* matured canine oocytes (Silva *et al*., 2009). Glucose is the predominant energy substrate used by canine oocytes (Wesselowsky, 2008). It has also been suggested that retinoic acid may promote embryonic development by preventing oxidative stress (Deb *et al*., 2011), and may also regulates the expression of several growth factors controlling genes during maturation of bovine oocytes (Gomez *et al*., 2004). Liang *et al*. (2012) reported that the influence of different concentration of 9-cis RA on nuclear maturation of canine oocytes during IVM. In the previous stuy, five nM 9-cis RA in the IVM medium was beneficial to nuclear and cytoplasmic maturation of canine oocytes (5 nM 9-cis RA vs. control: 18.3 ± 2.5 vs. 8.7 ± 1.5%) (Liang *et al*., 2012).

To date, several studies in canine oocytes have been performed to improve the rate of IVM by supplementation IVM medium with fetal bovine serum (Yamada *et al*., 1993), estrus bitch serum (Bogliolo *et al*., 2002), bovine serum albumin (Hewitt *et al*., 1998; Lee *et al*., 2003; Rodrigues and Rodrigues, 2003; Willingham-Rocky *et al*., 2003) or medium without serum (Songsasen *et al*., 2002) as well as collecting oocytes at various estrus phases (Yamada *et al*., 1993; Luvoni *et al*., 2001;

Table 2. Effect of hormonal supplementation on *in vitro* maturation rate of canine oocytes

Treatments	No. of oocytes developed to (%)					
	GV	GVBD	MI	MII	Non	MI - II
Control	9 (18.4)	16 (32.7)	2 (4.1)	1 (2.0)	21 (42.9)	3 (6.1)
PMSG & hCG	3 (5.6)	20 (37.0)	1 (1.9)	4 (7.4)	26 (48.1)	5 (9.3)

GV: germinal vesicle stage, GVBD: germinal vesicle breakdown stage, MI: metaphase I stage, MII: metaphase II stage, PB: first polar body, Non: Not detected nuclear.

Table 3. Effect of MG132 treatment on *in vitro* maturation rate of canine oocytes

Treatments	No. of oocytes developed to (%)					
	GV	GVBD	MI	MII	Non	MI - II
Control	9 (45.0)	3 (15.0)	6 (30.0)	1 (5.0)	1 (5.0)	7 (35.0)
MG132	15 (30.0)	6 (12.0)	21 (42.0)	3 (6.0)	5 (10.0)	24 (48.0)

GV: germinal vesicle stage, GVBD: germinal vesicle breakdown stage, MI: metaphase I stage, MII: metaphase II stage, PB: first polar body, Non: Not detected nuclear.

Willingham-Rocky *et al*., 2003). However the maturation rate has not been satisfactorily increased. In addition, the majority of *in vitro* matured canine oocytes have remained at the GV stage regardless of the culture conditions.

Likewise, many researchers have supplied the *in vitro* maturation media of canine oocyte with the several supplementations. However, there is still progress is urgently needed to establish more efficient *in vitro* maturation system that could help to advance reproductive technologies based on the mass production of canine embryos. Furthermore, efforts in this field are also required to increase the rates of metaphase stages of canine oocytes to compare with other domestic animals. This goal could be achieved through further studies that mainly focus on developing ideal *in vitro* culture condition suitable for canine oocytes.

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