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CANINE OOCYTES' NUCLEAR COMPETENCY PROFILE BOTH WITH AND WITHOUT DIRECT CONTACT WITH BOVINE GRANULOSA CELL MONOLAYER

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ABSTRACT

The current study examined how the maturation of canine oocytes was impacted via direct interaction between oocytes and bovine granulosa cell monolayers (BGML). At 38.5°C in 5% CO_2, 5% O_2, and 90% N_2 for 72 hours, canine cumulus-oocyte complexes (COCs) were cultivated in DMEM (control), oocytes cultured in BGML fitted into 24-well plates (direct contact), and oocytes cultured in BGML using 24-well plates with multicell culture with inserts (indirect contact). Oocytes matured over direct contact with BGML had a greater total meiotic resumption rate (50.0%) than those matured over indirect contact with BGML (31.0%) and control (12.4%) ($P<0.05$). Similarly, oocytes that matured over direct contact with BGML had a larger proportion of oocytes that advanced past the germinal vesicle (GV) to metaphase I (MI) stages (22.8%) than those that matured over indirect contact with BGML (11.9%) and control (2.1%) ($P<0.05$). When oocytes were developed directly in BGML (12.9%), the proportion of them that came to the metaphase II (MII) stage was higher ($P<0.05$) than when they were matured indirectly with BGML (4.5%). It was concluded that cultivation of canine oocytes complex with direct contact to BGML leads to meiotic restart and enhancement of maturation rate than indirect contact to BGML.

Key words: Bovine granulosa cell monolayers, Canine, Oocyte, In vitro maturation

INTRODUCTION

Endangered species protection is linked to the possible advantages of assisted reproductive technologies (ARTs) in canine species. Biotechnologies used for reproduction in domestic and human animals have advanced significantly in recent years. However, the adaptation of biotechnological information acquired from other species have been made more difficult by the distinctive features of gamete physiology's innate physiological distinctiveness. Mammals typically ovulate mature oocytes. This species' distinct reproductive traits-mono-menstrual, poly-ovulatory, non-seasonal reproductive cycle, ovulating immature oocytes, and the oocytes in the oviduct going through a 48-to-72-hour postovulatory maturation phase-combine to make things more difficult and less prosperous (Concannon *et al.,* 1989; Tsutsui 1989; Songsasen *et al.,* 2003; and Bolamba *et al.,* 2006).

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Scientific study has focused on deepening our understanding of in vitro egg maturation and fertilization. Growth factors (Bolamba *et al.,* 2006), gonadotropins (Songsasen *et al.,* 2003), steroids (Kim *et al.,* 2005), and protein (Ali and Sirard, 2002) alterations in the maturation medium are some methods to enhance maturation. Rates of bitch oocytes in vitro maturation (IVM) have been reported to be between 20 and 25 percent, and such canine oocytes have low maturational and developmental competence. (Hewitt and England 1999; Ali and Sirard 2002; Willingham-Rocky *et al.*, 2003; Kim *et al.,* 2005; Vannucchi *et al.,* 2006). In canine in vitro fertilization (IVF) experiments, the lower rates have been linked to poor embryonic developmental competence (Rodrigues *et al.,* 2004).

To promote proper oocyte maturation and successful fertilization, how the oocyte and its companion granulosa cells interact, which is essential for the in vivo growth of each type of cell, is supposed to be advantageous. Numerous cell types have been co-cultured to encourage in vitro oocyte maturation (Rodrigues *et al.,* 2004; Hatoya *et al.,* 2006; and Vannucchi *et al.,* 2006). Because they can ensure that the oocyte's in vitro environment is similar to its in vivo conditions, the cells known as granulosa appear to be the most suitable among them. Granulosa cells have long been known to aid in the formation of cattle oocytes (Maeda *et al.,* 1996), sheep (Staigmiller *et al.,* 19984), goats (Teotia *et al.,* 2001), and camels (Khatir *et al.,* 2004). Abdel-Ghani *et al.* (2012) have demonstrated that adding bovine granulosa cell monolayers (BGML) to IVM medium enhanced canine oocyte maturation and positively affected general meiotic resumption. Furthermore, findings show a substantial relationship between in vitro oocyte maturation and cumulus cell abundance. Given that the connection in canine oocytes lasted in vivo until the morula stage, cumulus cells might have been more important in canids than in other species (Renton *et al.,* 1991).

Granulosa cell monolayers function during the meiotic resumption of canid oocytes. This must be clarified considering these observations. In this context, cell culture well inserts were used to analyze the nuclear competence profile of canine oocytes with and without direct contact between canine oocytes and bovine granulosa cells.

MATERIALS AND METHODS

Assembling and getting ready canine cumulus-oocyte complexes (COCs)

At nearby veterinary hospitals, ovaries were taken from healthy domestic bitches having standard ovariohysterectomy procedures. The soundness of bitches was evaluated. Bitches without systemic lesions or deformities were classified as suitable for the experiment, and those showing alterations or pathological lesions were excluded. The animals (n = 15) ranged in age from 5 months to 6 years and were of different breeds. Each bitch's two ovaries were brought to the lab in a thermos flask, bearded with physiological sterile saline at 37°C in less than an hour. The ovarian cortex was repeatedly sliced with sterile sharp scalpels at 37°C to release the COCs. Using a dissecting microscope (Nikon, Tokyo, Japan). These COCs were put in 35 mm Petri dishes (Falcon, NY, USA) that included PB1 medium (Whitingham 1974) augmented with 100 IU/mL of penicillin (Calbiochem, CA, U.S.A.), 100 µg/mL of streptomycin (MEIJI Co., Tokyo, Japan), and 3 mg/mL of bovine serum albumin (BSA; Sigma, St. Louis, USA). Following three rounds of washing in PB1 medium, COCs were selected based on pre-existing standards (De los Reyes *et al.,* 2005), while being observed using an inverted microscope (LEICA Co., Germany). More precisely, the characteristics were those that promote meiotic competency, including consistency of ooplasm, homogeneous dark

cytoplasm with more than three layers of compact cumulus cells, and oocytes >110 µm in diameter. The vitelline diameter of COCs was measured with a calibrated ocular micrometer. The donors' reproductive status was classified using the standards outlined by Otoi *et al.* (2002). Briefly, the anestrus stage when the ovaries have no follicles or pronounced luteal tissues. In a nutshell, the ovaries lack follicles and prominent luteal structures during the anestrus stage.

Preparation of BGML

Ovaries from cows were taken at a nearby slaughterhouse and brought to the lab in a thermoflask. Using an 18-gauge needle, an antral follicle measuring between 2 and 5 mm in diameter was aspirated to produce the granulosa cells. For five minutes, the granulosa cell-containing follicular fluid was centrifuged at 180 × g. The resultant granulosa cell pellet was centrifuged twice at 180 × g for 5 minutes using phosphate-buffered saline (PBS; WAKO, Tokyo, Japan) containing 100 IU/mL of penicillin and 100 µg/mL of streptomycin. The pellet was then immersed in a 0.1% hyaluronidase fluid at 38.5°C for 10 to 20 minutes. Following vigorous pipetting to encourage cell separation, the cells were centrifuged twice at 180 × g for five minutes using PBS.

Following the last washing, the cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) with 10% fetal calf serum (FCS, Sigma), one percent amphotericin (Sigma), 100 IU/mL penicillin, and 100 µg/mL streptomycin added. To reach the final cell concentration to 25 x 10^5 cells/mL, a culture medium was added. (measured using a hemocytometer). To create a confluent monolayer, the suspension was then put on a multicell plate coated with collagen type I (IWAKI, Tokyo, Japan) and cultivated for three to five days at 38.5°C in a humidified incubator with 5% CO_2 in the air.

Cryopreservation of BGML

Granulosa cells were cryopreserved and thawed, following Tirelli *et al.* (2005), after being cultivated for three to five days at 38.5°C in a humidified incubator with 5% CO_2 in the air to create a confluent monolayer. Centrifugation was used to pellet the granulosa cells, which were then resuspended in DMEM supplemented with 10% (v/v) FCS and 10% (v/v) DMSO (Sigma) in a cryovial (Nunc, Thermo Scientific, Denmark). The cells were then allowed to freeze overnight in a refrigerator set at -80°C before being submerged in liquid nitrogen (-196°C).

The cryovials' cryopreserved granulosa cells were thawed in a 37°C water bath. Following five minutes of centrifugation at 180 × g, the cells were pelleted down and resuspended in dimethylsulfoxide (DMEM; Sigma) with 10% (v/v) FCS added. To eliminate any remaining traces of the cryoprotectant (DMSO), this process was carried out twice. The addition of a culture medium brought the final cell concentration down to 25×10^5 cells/mL.

Canine COC maturation in vitro

After freezing, the intact COCs were cultivated in DMEM with or without BGML. 50 ng/mL of epidermal growth factor (EGF; Sigma), 10 µg/mL of estradiol-17β (E2758, Sigma), 0.1 IU/mL of human chorionic gonadotropin (hCG) (Sankyo, Tokyo, Japan), 0.1 IU/mL of follicular stimulating hormone (FSH; Sigma), 0.25 mM of pyruvic acid (WAKO), 100 µM of β-mercaptoethanol (WAKO), and antibiotics (100 IU/mL of penicillin and 100 µg/mL of streptomycin) were added to all media. Three treatment groups were randomly selected from among COCs; COCs in Group 1 (control group) were cultivated in DMEM devoid of BGML. COCs were cultivated in BGML that was placed onto 24-well plates (3526, IWAKI, Tokyo, Japan) for Group 2 (direct contact group). Using floating filters (0.4 µm

millicell culture well inserts; 353095, Falcon) placed onto 24-well dish plates, COCs were cultivated in BGML in Group 3 (indirect contact group). The COCs were incubated for 72 hours under mineral oil at 38.5°C in a humidified environment, with 5% CO2, 5% O2, and 90% N2 in every group of experimentation (groups 2 and 3 were not covered by mineral oil).

Evaluation of nuclear status

COCs were deprived by gently pipetting cumulus cells out after 10 minutes of exposure to 0.1% hyaluronidase (H3506, Sigma). For 15 minutes, the denuded COCs were permeabilized and fixed in PBS with 10% (v/v) formaldehyde (WAKO). Three washes in PB1 enriched with 3 mg/mL of bovine serum albumin (BSA) were performed on them. Then, the cumulus-free oocytes were stained with 10 µg/mL of propidium iodide (PI; Sigma) in PB1 containing 0.1% of polyvinyl alcohol (Sigma) and incubated for 15 min in darkness. They were then put on glass slides, given three PB1 washes, and covered with a coverslip. According to a study by De los Reyes et al. (2005), the chromatin state was assessed using a fluorescence microscope and UV light to identify the meiotic stage. The following were the stages: (a) immature (GV, Fig. 1-A), in which compacted chromatin encircled the nucleolus; (b) When the chromatin was distributed, the germinal vesicle breakdown (GVBD, Fig. 1-B) occurred; (c) MI (Fig. 1-C), in which chromosomes were moving to the poles and extremely compacted in a metaphasic plate; (d) mature or metaphase II (MII, Fig. 1-D), when the first polar body discharged and the chromosomes were in the second metaphase; (e) degenerated (deg, Fig. 1-E), when oocytes showed scattered chromosomes or a loss of membrane integrity; (f) Unclassified were those with unknown chromatin (Fig. 1-F).

Statistical analysis

Oocytes were distributed among the experimental groups at random. ANOVA was used to assess the percentage of oocytes in each treatment group that reached each stage of nuclear maturation. The LSD test was then used for post-hoc multiple comparisons. The mean ± SD was used to express the results. At a confidence level of $P<0.05$, all differences were deemed significant.

RESULTS

Table 1 indicates that oocytes matured over direct contact with BGML had a greater overall meiotic resumption rate ($P < 0.05$) than those matured with BGML indirectly (31.0%) and without BGML (12.4%). In the control group, the rate of oocytes stalled at the GV state (30.4%) was higher than the groups that matured in BGML (18.7%). Similarly, oocytes that matured through direct contact with BGML had a larger percentage of oocytes ($P < 0.05$) that advanced past GV to MI stages (22.8%) than those that matured through indirect contact with BGML (11.9%) and control (2.1%). Compared to oocytes that were incubated indirectly using BGML (4.5%), the proportion of oocytes attained the MII state was greater ($P<0.05$) when they were incubated directly using BGML (12.9%).

DISCUSSION

In the current study, oocytes that completed meiosis were 12.9% for oocytes co-cultured directly with BGML. This was substantially greater than the percentage of oocytes co-cultured indirectly with the control group and the BGML group.

Bovine granulosa cells produce a number of meiosis-promoting molecules, such as transforming growth factor beta, basic fibroblast growth factors, activin, and epidermal growth factor, which are likely to mediate the effect of BGML.

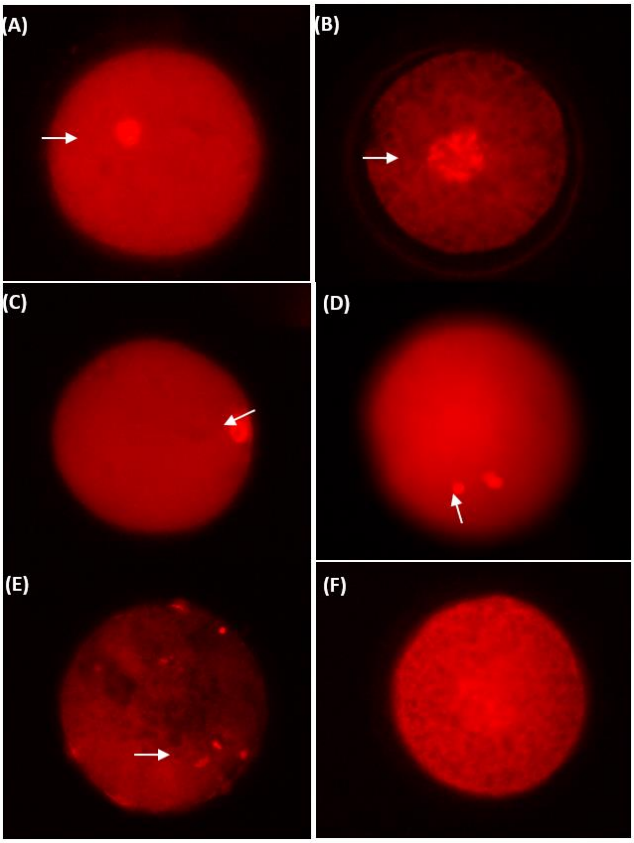

Figure 1: Fluorescence photomicrographs (X400) of canids oocytes stained with propidium iodide (PI stain) showing chromatin configuration. (A) Germinal vesicle: condensed chromatin (arrow); (B) Germinal vesicle break down: chromatin was dispersed (arrow); (C) Metaphase I: chromosomes were compact in a metaphasic plate and migrating to the poles (arrow); (D) Metaphase II: the extrusion of the first polar body (arrow); (E) Degenerated: dispersed chromosomes (arrow); (F) Unclassified: unidentifiable chromatin.

Table 1. Nuclear status of canine oocytes following in vitro culture with and without cell culture well insert for 72 h.

Stage of COCs donor	Treatment	No. oocytes examined	Meiotic stage (mean percentage ± SD)						
			GV	GVBD	MI	GVBD-MII	MII	Deg.	unclassified
Anesturs	Control *	73	30.4 ± 9.5[a]	6.5 ± 7.1[a]	2.1 ± 2.9[a]	2.2 ± 2.9	34.3 ± 13.6[a]	23.9 ± 17.9[a]	12.4 ± 8.5[a]
	With cell culture well insert **	68	27.7 ± 8.4[a]	14.9 ± 3.6[ab]	11.9 ± 2.6[b]	4.5 ± 4.3[a]	16.3 ± 9.4[b]	23.2 ± 8.4[a]	31.0 ± 7.9[b]
	Without cell culture well insert ***	58	18.7 ± 8.2[a]	16.6 ± 8.0[b]	22.8 ± 12.8[c]	12.9 ± 7.7[b]	11.2 ± 7.6[b]	18.1 ± 14.9[a]	50.1 ± 15.2[c]

[a–c] within a column, means without a common superscript differed ($P < 0.05$).

* DMEM without bovine granulosa monolayer (BGML).

** DMEM with BGML, no direct contact between canine oocytes and bovine granulosa cells.

*** DMEM with BGML, direct contact between canine oocytes and bovine granulosa cells.

**** GV, germinal vesicle; GVBD, germinal vesicle breaks down; MI, metaphase I; MII, metaphase II.

Cytokines and growth factors cooperate in an intricate system to ensure the oocyte's final differentiation, resulting in appropriate nuclear and cytoplasmic maturation. The idea that these growth factors promote *in vitro* maturation of oocytes in certain mammalian species has been presented by a large body of research, such as in rodents (Tsafriri *et al.,* 1989), bovine (Lonergan *et al.,* 1996), and humans (Goud *et al.,* 1998). The positive impact of BGML on oocyte meiotic maturation in the current investigation was probably caused by these BGML-derived factors.

Furthermore, when augmented with FBS or BSA, cells named granulosa in cows can produce large amounts of estrogen (E_2) and progesterone (P_4). Additionally, the profitable impacts of both hormones on oocyte maturation *in vitro* have been shown in domestic animals, including dogs (Mingoti *et al.,* 2005). Domestic dogs have similarly shown comparable outcomes (Kim *et al*., 2004). The continuation of meiosis and the development of the MII stage during IVM were enhanced when canine oocytes were cultured in a medium supplemented with either E_2 or a mixture of E_2 and P_4 (Kim *et al.,* 2004).

Additionally, the triggered impacts of E_2 and EGF on the meiotic resumption of oocytes were mediated by bovine granulosa cells. Furthermore, it is becoming clear that E_2 and EGF play a powerful role in the development of entire oocyte competency (Hatoya *et al.,* 2009). Likewise, cells of the granulosa are vital in raising intracellular glutathione concentration, while oocytes are maturing in the lab (Abeydeera *et al.,* 1998). According to Abeydeera *et al.* (1998), intracellular glutathione is linked to both nuclear and cytoplasmic maturation and plays a significant role in shielding cells from oxidative stress.

Oocytes co-cultured in BGML directly, however, matured to MII at a considerably higher proportion than those co-cultured using BGML with a cell culture well insert (Table 1). The influence of direct contact on the state of competence of gap junction communication (JGC) among oocytes and granulosa cells is most likely the rationale behind inequalities in meiotic competency of oocytes when using a cell culture insert. Additionally, communication between canine oocytes and cumulus cells decreases over time. In order to make up for the lost cells, BGML adheres to the canine oocyte. Therefore, when oocytes are cultivated in indirect contact with granulosa cells, there is no connection between cumulus cells and oocytes, and the presence of granulosa cell–oocyte communications durability is linked to the oocytes' capacity to resume meiosis. Accordingly, the GJC between BGML and oocytes is crucial for conduction of minor molecular substrates, such as ions, nucleotides, and amino acids, as well as meiosis-activating substances (Mori *et al.,* 2000). These findings thus support the significance of direct contact between bovine granulosa cells and canine oocytes for meiotic restart and are consistent with our earlier experiment employing bovine cumulus-oocyte complexes conditioned media (BCM) (Abdel-Ghani *et al.,* 2011).

Through the fluctuations in the cycle phase of canides, and inadequate harvesting frequently limit the recovery of this cell type, using freshly collected granulosa cells is challenging. For this reason, the study employed freezing to store granulosa cells. Granulosa cells must, however, be in ideal condition and maintain all of their unique characteristics, including proliferative activity, steroid synthesis, and FSH responsiveness, to promote oocyte maturation. Cryoinjury alters the structure of cells and metabolic pathways, especially the antioxidant system, in all cell types (Tirelli *et al.,* 2005). Consequently, cryopreservation may cause extreme stress to the cells, which could account for the low MII percentage observed at the moment, compared to earlier findings (Abdel-Ghani *et al.,* 2012). Furthermore, it has been

proposed that oocytes obtained from bitches that did not reach puberty have a lower capacity to initiate and complete meiosis (Haenisch *et al.,* 2003), and some oocytes were taken from dogs younger than six months. Prepubescent bitches' oocytes have high metabolic and transcriptional activity, but moderate protein synthesis, suggesting they have not yet fully developed the developmental competence (Haenisch *et al.,* 2003).

CONCLUSION

The current study's findings suggest that BGML's direct interaction with canine COCs promoted nuclear maturation. However, direct BGML-oocyte contact is essential for the continued development of canine oocytes during IVM, even if BGML co-culture with an insert can improve overall meiotic resumption. Since the prosperous progress and use of ARTs rely on fundamental biotechnologies for reproduction, especially the maturation of oocytes in vitro. Knowledge and advancements in the maturation of canids oocytes *in vitro* could provide a basis for further investigation into canine ARTs and reproductive physiology.

DECLARATION OF INTERESTS

We affirm that there are no conflicts of interest that would compromise the objectivity of the published research.

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تقييم كفاءة أنوية بويضات الكلاب سواء مع أو بدون اتصال مباشر مع مستزرع طبقة الخلايا الحبيبية البقرية

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تناولت الدراسة الحالية كيفية تأثر نضوج بويضات الكلاب من خلال التفاعل المباشر بين البويضات وطبقات الخلايا الحبيبية البقرية (BGML). زُرعت البويضات المحاطة بالطبقة الركامية الكلبية (COCs) في DMEM (مجموعة ضابطة) عند درجة حرارة ٣٨,٥ درجة مئوية في بيئة بتركيز ٥٪ من ثاني أكسيد الكربون، و٥٪ من الأكسجين، و٩٠٪ من النيتروجين لمدة ٧٢ ساعة، ووُضعت البويضات المزروعة في BGML في لوحات استزراع ذات ٢٤ تجويف (تلامس مباشر)، و داخل معزولات في BGML في لوحات استزراع ذات ٢٤ تجويف متعدد الخلايا (تلامس غير مباشر). أظهرت البويضات الناضجة من خلال التلامس المباشر مع BGML معدل استئناف انقسام منصف كلي أعلى (٥٠٪) من تلك الناضجة من خلال التلامس غير المباشر مع BGML (٣١,٠٪) والمجموعة الضابطة (١٢,٤٪) (P<٠,٠٥). وبالمثل، أظهرت البويضات التي نضجت عبر التلامس المباشر مع BGML نسبةً أكبر من البويضات التي تجاوزت مرحلة الحويصلة الجرثومية (GV) إلى الطور الاستوائي الأول (MI) (٢٢,٨٪) مقارنةً بتلك التي نضجت عبر التلامس غير المباشر مع BGML (١١,٩٪) والمجموعة الضابطة (٢,١٪) (P<٠,٠٥). عند نمو البويضات مباشرةً في BGML (١٢,٩٪)، كانت نسبة البويضات التي وصلت إلى مرحلة الطور الاستوائي الثاني (MII) أعلى (P <٠,٠٥) مقارنةً بنضجها غير المباشر مع BGML (٤,٥٪). وخلصت الدراسة إلى أن زراعة مجمعات البويضات الكلبية بالتلامس المباشر مع BGML تؤدي إلى إعادة بدء الانقسام الاختزالي وزيادة معدل النضج مقارنةً بالتلامس غير المباشر.