EVALUATION OF EFFECTIVE APPROACH FOR *IN VITRO* MATURATION OF CANINE OOCYTES WITH SEQUENTIAL ADDITION OF HORMONES

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ABSTRACT

About 134 canine ovaries were collected after routine ovariohysterectomy and 4,848 cumulus-oocyte complexes (COCs) were retrieved. Out of these, 1,080 good quality COCs were cultured in TCM-199 medium supplemented with 10% estrus bitch serum and incubated in 5% CO_2 at 37°C for 96 h. These COCs were matured either without (control) or with sequential addition of gonadotropic and steroidal hormones. After 96 h maturation period, the percent COCs expansion and oocyte maturation rate was better in groups in which hormones were added in maturation medium.

Keywords: Canine, COCs, *In vitro* maturation, Nuclear maturation, Oocytes

INTRODUCTION

In vitro maturation (IVM) of canine oocytes is very low compared to other mammalian species (Luvoni *et al.*, 2005). In fact, in canine species, the female gamete has unique characteristics. The oocyte is exposed to high concentration of progesterone in follicular environment, ovulates in dictyate state and resumes and completes meiosis in the oviduct. Therefore, the optimum conditions for IVM of canine oocytes differ from other mammalian models in which estrogen is the dominant hormone during dominant follicle stage and ovulates oocyte at the metaphase II stage of first meiotic division. A study has reported that sequential addition of hormones during IVM was beneficial (Evecen *et al.*, 2011). Therefore, the present study examined the impact of sequential addition of hormones on IVM of immature canine oocytes.

MATERIALS AND METHODS

Ovaries (n=134) from non-descript bitches were collected after routine ovariohysterectomy and were placed within 2h of surgery in polythene sachets containing PBS pre-equilibrated at 37°C. The slicing method was used to collect cumulus-oocyte complexes (COCs) from the ovaries and the COCs (n=4,848) were retrieved in PBS supplemented with gentamycin sulfate (50 µg/ml). Using 24G needle attached to a 2 ml plastic syringe, the COCs were transferred into a 35 mm petri dish containing media supplemented with or without hormones. The COCs with dark, round and uniformly homogenous cytoplasm, surrounded with several intact layers of cumulus cells and having cytoplasmic diameter >100 µm were considered of good quality (n=2,200). About 1,080 good quality COCs were randomly allotted to three groups based upon addition of hormones (Table 1). These were cultured in TCM-199 supplemented with 0.23 mM Sodium Pyruvate, 2 mM L-Glutamine and 50 µg/ml Gentamicin sulfate and 10% estrus bitch serum. The medium was pre-incubated in 5% CO_2 at 37°C for 1 h before its use. In each group, five or ten COCs were placed in 100 µl droplet of maturation medium in 35 mm petri dish and each time 20 or 40 COCs were respectively incubated. A total 6 incubations were conducted in each group. On each day before changing into new maturation medium, the COCs were washed twice in

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the control medium to remove the maturation medium of the previous day.

During and after incubation period (every 24 h), the COCs of all groups were examined for expansion through an inverted confocal microscope using magnification of 400X (Figure 1). Following expansion, the cumulus cells were denuded from the COCs by repeated pipetting through a fine bore glass pipette. If any cumulus cells were still adhering to the oocytes, the COCs were placed in 200 µl hyaluronidase solution for 1 minute and the cumulus cells were denuded off the oocytes by repeated pipetting through a fine bore glass pipette. The denuded oocytes were washed twice in filtered PBS to remove any hyaluronidase solution adhering to the oocytes. If the cumulus cells around the oocyte were spread over a large, short or little distance around the oocyte then the expansion was categorized as full, moderate or slight (Figure 1).

Thereafter, the evaluation of nuclear status of oocytes was carried out by placing in 200 µl propidium iodide solution for 15 min in dark place in normal atmosphere. Then, oocytes were washed twice in filtered PBS to remove excess of propidium iodide adhering to the oocytes. The stained oocytes were examined through an inverted confocal microscope at magnification of 400X with fluorescent illumination (green filter) equipped with an excitation filter 510-550 nm, emission filter 590 nm and dichromatic mirror 570 nm (excitation 530 nm and emission 615 nm for propidium iodide). The nuclear maturation of oocytes was evaluated by observing the Germinal Vesicle (GV, vesicle clearly visible or immature oocyte), Germinal Vesicle Breakdown (GVBD, chromatin was dispersed and initiating condensation or resumption of meiosis), Metaphase II (MII, presence of chromosomes in second metaphase phase with first polar body extruded named as mature oocyte), Degenerated Nucleus (DE, oocytes with irregular chromatin distribution) and Unclassified Nucleus (UC, abnormal chromatin) stages of the oocytes (Figure 2). The statistical analysis using SPSS software was done through one-way ANOVA followed by Descriptive and *Post Hoc* Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

After the end of 96 h maturation period, the better COCs expansion was observed in groups with sequential addition of hormones to maturation medium than the controls ($p<0.05$, Table 2). The proportion of partially and completely nuded oocytes increased as the culture period increased, while the proportions of intact oocytes decreased at 48 h after the start of culture (Table 2). In another study, at the end of culture period, the proportion of intact, partially nude and completely nude oocytes were 62.5, 22.9 and 11.9%, respectively (Otoi *et al.* 2002), However, in comparison, the expansion rate of cumulus cells is

Table 1: Addition of hormones to Cumulus-oocyte complex maturation medium

Group	Incubation period			
	0-24 h	24-48 h	48-72 h	72-96 h
I	-	-	-	-
II	2 µg/ml FSH, 20 µg/ml β-Estradiol	2 µg/ml FSH, 20 µg/ml β-Estradiol, 1 µg/ml LH	10 µg/ml FSH, 5 µg/ml β-Estradiol, 10 µg/ml LH, 4 µg/ml Progesterone	2 µg/ml FSH, 2 µg/ml Progesterone
III	Same as above			2 µg/ml FSH, 5 µg/ml LH, 4 µg/ml Progesterone

Table 2: *In vitro* expansion of cumulus cells around the canine oocytes with sequential addition of hormones to the maturation media

Groups	COCs (n)	COCs expansion		
		Full	Moderate	Slight
Culture drop of 5 COCs				
I	120	55 (45.8%)[a]	26 (21.7%)[b]	39 (32.5%)[b]
II	120	99 (82.5%)[b]	16 (13.3%)[a]	5 (4.2%)[a]
III	120	97 (80.8%)[b]	14 (11.7%)[ab]	9 (7.5%)[a]
Culture drop of 10 COCs				
I	240	118 (49.2%)[a]	54 (22.5%)[b]	68 (28.3%)[b]
II	240	202 (84.2%)[b]	30 (12.5%)[a]	8 (3.3%)[a]
III	240	195 (81.2%)[b]	34 (14.2%)[a]	11 (4.6%)[a]

Figures with different superscripts within a column differ significantly (p<0.05); COCs - Cumulus-oocyte complexes

Table 3: *In vitro* maturation rate (at the end of 96 h) of bitch cumulus-oocyte complexes (COCs) with sequential addition of hormones to the maturation media

Groups	Oocytes (n)	Stages of nuclear maturation				
		GV	GVBD	M II	DE	UC
Culture drop of 5 COCs						
I	111	17 (15.3%)[a]	22 (19.8%)[a]	1 (0.9%)[a]	4 (3.6%)[a]	67 (60.4%)[b]
II	107	15 (14.0%)[a]	35 (32.7%)[b]	4 (3.7%)[b]	3 (2.8%)[a]	50 (46.7%)[a]
III	113	15 (13.3%)[a]	37 (32.7%)[b]	3 (2.65%)[b]	4 (6.5%)[a]	54 (47.8%)[a]
Culture drop of 10 COCs						
I	219	35 (16.0%)[a]	44 (20.1%)[a]	3 (1.4%)[a]	6 (2.7%)[a]	131 (59.8%)[b]
II	222	30 (13.5%)[a]	75 (33.8%)[b]	9 (4.0%)[b]	8 (3.6%)[a]	10 (45.0%)[a]
III	229	33 (14.4%)[a]	78 (34.1%)[b]	7 (3.1%)[ab]	5 (2.2%)[a]	106 (46.3%)[a]

Figures with different superscripts within a column differ significantly (p<0.05); GV, Germinal Vesicle; GVBD, Germinal Vesicle Breakdown; M II, Metaphase II; DE, Degenerated Nucleus; UC, Unclassified Nucleus

more in the present study (Table 2). This could be a reason for low maturation rates of canine oocytes in this study.

After the end of 96 h maturation, the percentage of oocytes in GV stage between various groups was similar (p>0.05, Table 3). Others recorded COCs in GV stage in control, traditional and sequential hormonal supplementation groups as 14.9, 8.7 and 6.8%, respectively (Evecen *et al.,* 2011), which were lesser than the present study (Table 3). At GVBD and MII stage, the percentage of oocytes was lower in groups, both five and ten COCs matured per droplet, in which no hormones were added in maturation media compared to other groups (p<0.05, Table 3). Others reported 2.3 - 4.2 % oocyte maturation up to MII stage (Otoi *et al.*, 1999), which was lower than the present study (Table 3). The percentage of oocytes in DE stage were similar between groups (p>0.05), however, the oocytes in UC stage were higher in groups in which hormones were not added in maturation medium (p<0.05, Table 3). In brief, the sequential addition of hormones, mimicking *in vivo* hormonal milieu, in maturation medium lead to better *in vitro* maturation of oocytes.

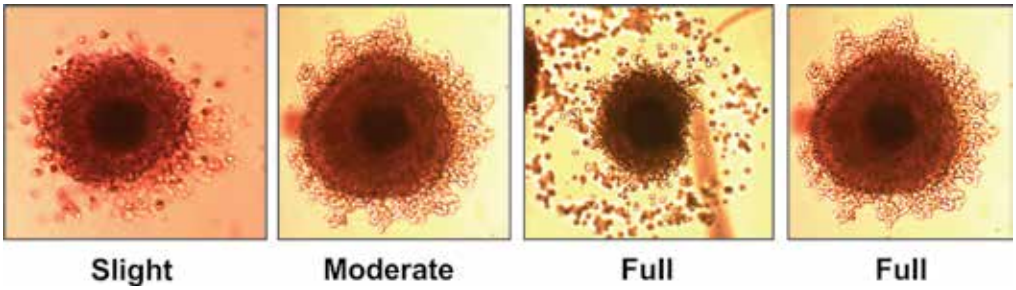

Figure 1: Cumulus-oocyte complex (COCs) expansion at the end of 96 h in vitro maturation

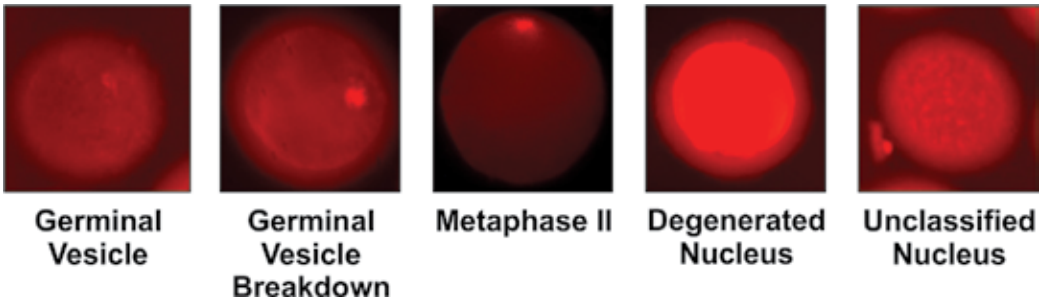

Figure 2: Canine oocytes showing different stages of nuclear maturation

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