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Abstract

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THE EFFECT OF CULTURE MEDIA AND CULTURE TIME ON THE *IN VITRO* MATURATION OF DOMESTIC CAT OOCYTES

Culture media and culture time play important roles in meiotic competence and *in vitro* development of domestic cats oocytes. This experiment was designed to investigate the effects of culture media (M199 versus DMEM) on the *in vitro* maturation of domestic cat oocytes, after culturing for 24, 36 and 48 h. Domestic cat ovaries were obtained from routine ovariohysterectomy at an animal hospital, cooled to 4°C and transferred immediately to the laboratory. The oocytes were collected by slicing ovaries in the collecting medium. Five to ten cumulus oocyte complexes (COCs) were cultured in small drops of 50 µl of M199 or DMEM for 24, 36 and 48 h, under light mineral oil, at 38.5°C and 5% CO_2, in a humidified atmosphere. The meiotic status of the cultured oocytes was assessed according to their chromosomal development and meiotic competence, after staining with basic fuschin and acetoglycerol and observation under a light microscope. The results showed that the maturation rate of domestic cat oocytes cultured in M199 was not significantly different (P>0.05) from those cultured in DMEM for 24, 36 and 48 h. However, the oocytes cultured in either M199 or DMEM for 36 h gave the best maturation rate (48.0% (49/102) and 48.1% (50/104) respectively), although not significantly different (P>0.05) when compared to other culture times. The results suggest that domestic cat oocytes can be matured *in vitro* in either M199 or DMEM and that 36 h is the appropiate time for culture rather than 24 and 48 h.

Keywords : *in vitro* maturation, domestic cat oocytes, culture media, culture time.

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บทคัดย่อ

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ผลของน้ำยาเลี้ยงตัวอ่อนและระยะเวลาการเลี้ยงต่ออัตราการเจริญพร้อมปฏิสนธิภายนอกร่างกายของโอโอไซต์แมว

น้ำยาเลี้ยงตัวอ่อนและระยะเวลาการเลี้ยงตัวอ่อนมีความสำคัญต่อความสามารถในการเกิดกระบวนการแบ่งตัวแบบไมโอซีสและการเจริญพร้อมปฏิสนธิภายนอกร่างกายของโอโอไซต์แมว จุดประสงค์ของการศึกษาเพื่อศึกษาผลของน้ำยาเลี้ยง ตัวอ่อนชนิด M199 และ DMEM และระยะเวลาเลี้ยง 24, 36 และ 48 ชั่วโมง ต่อการเจริญพร้อมปฏิสนธิภายนอกร่างกายของ โอโอไซต์แมว การศึกษานี้ใช้รังไข่จากแมวที่เข้ารับการผ่าตัดทำหมันด้วยวิธี ovariohysterectomy จากโรงพยาบาลสัตว์ เก็บรังไข่ไว้ที่อุณหภูมิ 4°ซ เก็บโอโอไซต์จากรังไข่ด้วยวิธีตัดย่อยรังไข่ในน้ำยาเก็บโอโอไซต์ เลี้ยงโอโอไซต์ที่มีเซลล์คิวมูลัส ล้อมรอบจำนวนมาก จำนวน 5-10 โอโอไซต์ในน้ำยาเลี้ยงปริมาณ 50 ไมโครลิตร ชนิด M199 หรือ DMEM เป็นเวลา 24, 36 หรือ 48 ชั่วโมง ในสภาวะ 38.5°ซ 5% คาร์บอนไดออกไซด์ ความชื้นสัมพัทธ์เต็มที่ประเมินสภาพพร้อมปฏิสนธิ โดยดูลักษณะของการแบ่งตัวแบบไมโอซีสของโครโมโซมในโอโอไซต์โดยย้อมด้วยสี basic fuschin และ acetoglycerol ภายใต้กล้องจุลทรรศน์ การศึกษาพบว่าอัตราการเจริญพร้อมปฏิสนธิภายนอกร่างกายของโอโอไซต์แมวที่ในน้ำยาเลี้ยงชนิด M199 ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติกับน้ำยาเลี้ยงชนิด DMEM เมื่อทำการเลี้ยงโอโอไซต์นาน 24, 36 หรือ 48 ชั่วโมง ($P>0.05$) โอโอไซต์ที่เลี้ยงใน M199 หรือ DMEM นาน 36 ชั่วโมงจะให้อัตราการเจริญพร้อมปฏิสนธิสูงสุด คือ 48.0% (49/102) และ 48.1% (50/104) ตามลำดับ แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) กับโอโอไซต์กลุ่มทดลองอื่นๆ จากการศึกษานี้สรุปว่าโอโอไซต์ของแมวสามารถเจริญพร้อมปฏิสนธิภายนอกร่างกายได้เมื่อเลี้ยงในน้ำยา M199 หรือ DMEM และระยะเวลาการเลี้ยงที่ 36 ชั่วโมง จะมีอัตราการเจริญพร้อมปฏิสนธิที่สูง แต่ไม่มีความแตกต่างเมื่อเปรียบ เทียบกับ 24 และ 48 ชั่วโมง

คำสำคัญ : การเจริญพร้อมปฏิสนธิภายนอกร่างกาย, โอโอไซต์แมว, น้ำยาเลี้ยงตัวอ่อน, ระยะเวลาการเลี้ยง

Introduction

Domestic felid species serve as a valuable model for reproductive studies on non domestic felid species (Wildt et al., 1986), allowing the rescue of genetic material from endangered cat species (Donoghue et al., 1990) and for human AIDS studies (Essex, 1995). The technology of *in vitro* maturation (IVM), *in vitro* fertilization (IVF), embryo culture, embryo transfer and cryopreservation has been widely developed in domestic felid species over the last decade (Farstad, 2000). In general, IVM efficiency using cat oocytes has been higher than that using dogs, but lower than that using livestock animals (Farstad, 2000). It has been demonstrated that factors such as oocyte quality (Farstad, 2000; Wood and Wildt, 1997), culture time (Johnston et al., 1989; Luvoni and Oliva, 1993; Pope et al., 1994), culture medium (Goodrowe et al., 1991) and season (Freistedt et al., 2001) influenced both meiotic competence and the *in vitro* development of domestic cat oocytes. Two culture media widely used for the culture of domestic cat oocytes *in vitro*, M199 (Gomez et al., 2003; Karja et al., 2002; Freistedt et al., 2001; Otoi, et al., 2001) and DMEM (Jewgenow, 1998). Both media containing essential substances supplying energy to the oocytes during the nuclear maturation process and induce domestic cat oocytes to reach the metaphase II stage (Freistedt et al., 2001). The percentage *in vitro* maturation rate for domestic cat oocytes using M199 is approximately 59.7% (Karja et al., 2002; Katska-

Ksiazkiewicz et al 2003; Otoi et al., 2001) and 57.4% using DMEM (Kitiyanant et al., 2003), taking into accout only grades 1 and 2 of the cumulus oocyte complex (COCs). However, it cannot be concluded that the *in vitro* maturation rate of domestic cat oocytes cultured in M199 is higher than that culture in DMEM because no reported experiments used M199 and DMEM under the same experimental conditions and the same laboratory conditions. Culture time is an influencing factor in the maturation of in vitro domestic cat oocytes. Although, domestic cat oocytes can reach the maturation stage as early as 24 h after the onset of oocyte culture and peak at 48 h (Goodrowe et al., 1989; Luvoni and Pellizzari, 2000), no reported experiments compared the optimum time for the culture domestic cat oocytes in M199 and DMEM, under the same laboratory conditions. The objective of this study was to investigate the effect of the culture media, M199 and DMEM, and the culturing times, 24, 36 or 48 h, on the *in vitro* maturation rate of domestic cat oocytes

Materials and Methods

Recovery of oocytes

Ovaries were collected from adult queens following routine ovariohysterectomy at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University. The ovaries were kept in phosphate buffer saline (PBS) (Gibco, USA) supplemented with penicillin 100 IU/ml and streptomycin 100 (g/ml (Gibco, USA.) and maintained at 4°C in a temperature controlled container, covered with an ice pack, before oocyte recovery. Within 1 h of collection, the ovaries were rinsed 3 times in Phosphate buffer saline (PBS) then placed in the collecting medium. The oocytes were recovered by slicing the ovaries in the collecting media: (i) Medium 199 (M199) (Sigma, USA.) or (ii) Dulbecco modified Eagle medium (DMEM) (Gibco, USA.) containing 10 mM HEPES, 0.026 g/ml pyruvate (Sigma, USA.), 0.292 g/ml L-glutamine (Sigma, USA.) and 0.4% bovine serum albumin (BSA; Fraction V). Only oocytes with more than 3 layers of cumulus masses and uniform, homogenous pigmented ooplasm were selected for culture.

Culture of oocytes

Five to ten cumulus oocyte complexes (COCs) were cultured either in 50 µl of (i) M199 or (ii) DMEM containing 0.026 g/ml pyruvate (Sigma, USA.), 0.292 g/ml L-glutamine (Sigma, USA.), 0.4% bovine serum albumin,100 IU/ml penicillin, 100 (g/ml, streptomycin, 1 (g/ml LH, 1 µg/ml FSH and 1 µg/ml oestradiol. The culture media were covered with a light mineral oil and the COCs were cultured at 38.5°C in a humidified atmosphere of 5% CO_2.The culture media were pre-equilibrated for 1-2 h at 38.5°C in a humidified atmosphere of 5% CO_2 before use.

Assessment of the meiotic status of oocytes

After culturing *in vitro*, the COCs were fixed in acetic alcohol (acetic acid: ethanol, 1:3) for 24-48 h and then stained by basic fuschin and acetoglycerol using a rapid staining method (Byun et al., 1991) to assess meiotic status and the development of oocytes, using the criteria described by Alberts et al. (1994) as:

Matured oocytes: oocytes which presented the first polar body in the perivitelline space and the metaphase plate.

Immature oocytes:

- *germinal vesicle*, oocytes with the nucleus surrounded by a nuclear envelope and without a divided nucleus;
- *germinal vesicle breakdown*, oocytes with the chromatin spread out, disappearing from the nuclear envelope;
- *metaphase I*, oocytes which show homologous chromosome arrangement;
- *telophase I*, oocytes which show the separation of homologous chromosomes and centromeres

Degenerated oocytes: oocytes with abnormalities in the cytoplasm and degradation of the oolemma

Parthenote oocytes: oocytes with spontaneous division and without fertilization or artificial activation

Experimental design

The effect of M199 or DMEM on the maturation rate after culture for 24, 36 or 48 h was oberved. The COCs were randomly devided into 6 groups according to the treatment; group 1: was cultured in M199 for 24 h, group 2: was cultured in M199 for 36 h, group 3: was cultured in M199 for 48 h, group 4: was cultured in DMEM for 24 h, group 5: was cultured in DMEM for 36 h and group 6: was cultured in DMEM for 48 h. Twenty replications were performed with approximatly 5-10 COCs per group in each replication. All groups were cultured at 38.5°C in a humidified atmosphere of 5% CO_2. After 24, 36 and 48 h of *in vitro* culture, the meiotic status of the oocytes from each group were assessed.

Statistical analysis

The maturation rate and chromosomal development seen in each group of oocytes were analyzed using Chi-square analysis. A p-values of less than 0.05 was considered significant.

Results

A total of 645 COCs were used to investigate the appropriate culture conditions for the in vitro maturation of domestic cat oocytes. The cultured oocytes were observed for their meiotic status as shown in Figure 1. The maturation and developmental rates are shown in Table 1. There was no significant differences in the maturation rates between M199 and DMEM after being cultured for different culture times ($p>0.05$). The highest maturation rate was found when the oocytes were cultured for 36 h in either M199 or DMEM (48.0% (49/102) and 48.1% (50/104) respectively), but without any significant difference compared to other culture times. The number of oocytes remaining in the germinal vesicle stage (GV) and the parthenogenic stage was not significant differrent ($p>0.05$) between M199 and DMEM. In contrast, the number of oocytes cultured in M199 for 24 h, reaching germinal vesicle breakdown (GVBD) to anaphase I (AI) stage, was significantly lower ($p<0.05$) compared to the other groups. The degeneration rate among all groups of oocytes varied from 1 to 16.8%.

Table 1: The developmental rate of domestic cat oocytes after culturing in M199 or DMEM for 24, 36 and 48 h

Culture medium	Time (h)	Total oocytes	Matured (%)	Immatured			
				GV (%)	GVBD-AI (%)	Degenerated (%)	Parthenote (%)
M199	24	107	49 (45.8)[a]	25 (23.4)[a]	12 (11.2)[a]	18 (16.8)[a]	3 (2.8)[a]
	36	102	49 (48.0)[a]	20 (19.6)[a]	32 (31.4)[b]	1 (1.0)[b]	0 (0.0)[a]
	48	120	45 (37.5)[a]	26 (21.7)[a]	32 (26.6)[b]	15 (12.5)[a]	2 (1.7)[a]
DMEM	24	106	44 (41.5)[a]	20 (18.9)[a]	27 (25.5)[b]	14 (13.2)[a]	1 (0.9)[a]
	36	104	50 (48.1)[a]	18 (17.3)[a]	33 (31.7)[b]	3 (2.9)[b]	0 (0.0)[a]
	48	106	45 (42.5)[a]	23 (21.7)[a]	29 (27.3)[b]	7 (6.6)[b]	2 (1.9)[a]

[a,b]Values within a column with different superscripts differ ($p<0.05$)

GV; germinal vesicle **GVBD;** germinal vesicle breakdown **AI;** anaphase I

Discussion

The study showed that the maturation rates of domestic cat oocytes were not affected by the culture medium (M199 or DMEM). M199 and DMEM are bicarbonate buffered, culture media that can maintain a pH of 7.2-7.4, in an atmosphere of air, enriched with 5% CO_2 (Nagy et al., 2002). Both M199 and DMEM contain L-glutamine and pyruvate that can be metabolized and used to supply energy to the oocytes during the nuclear maturation process (Freistedt et al., 2001). The maturation process of oocytes is especially dependent of pyruvate, rather than other substances (Spindler et al., 2000). Thus, the oocytes could be matured in vitro after culture in both M199 and DMEM. However, DMEM seems to provide slightly better oocytes maturation than M199, which may be because it contains more glucose, which is necessary for glycolysis and glucose oxidation and provides the energy for oocyte maturation (Spindler et al., 2000). Slightly similar experimental designs have been carried out by Katska-Ksiazkiewicz et al. (2003) who studied the effects of the culture media (TCM 199 versus SOF) and recording the maturation rate after culturing domestic cat oocytes for 24 h. No significant differences between TCM 199 and SOF were found.

Regarding the culture time, domestic cat oocytes begin to mature, *in vitro*, as early as 24 h after the onset of oocyte culture and reach a peak at 48 h (Goodrowe et al., 1989; Luvoni and Pellizzari, 2000). The results from this study showed that the oocytes reach the metaphase II stage without any significant differences ($p>0.05$) at 24, 36 and 48 h of culture time, when comparing them in either the same or different culture media (M199 or DMEM). This result may be explained by the two maturation waves of domestic oocytes. The first wave happens within the first 28 h and the second wave happens between 28-30 h of culture (Skrzyszowska et al., 2002). Katska-Ksiazkiewicz et al. (2003) suggested that oocytes cultured for a longer time show fragmentation of the first polar body debris in the perivitelline space and a clumping of chromosomes on the metaphase plate. Surprisingly, in our study, the oocytes cultured for 24 h in M199 or DMEM showed a significantly higher degeneration rate ($p<0.05$) compared to the 36 h cultures. This might be the individual response of oocytes to the degenerative processes, from the time the oocytes were collected from the ovaries, until they were cultured *in vitro*. Although the degeneration of domestic cat oocytes are not affected by temperature and the storage time, if the ovaries are kept at 4-24^{o}C for 24-72 h (Wolfe and Wildt, 1996) or 10^{o}C for 16-24 h (Katska-Ksiazkiewicz et al. 2003) before recovering the oocytes, and if temperatures below 4^{o}C are used, this may result in granulosa cell degeneration during cold storage which affects maturation, especially the diameter of the oocytes (Otoi et al., 2001). To avoid the effect of cold shock on the oocytes, ovaries were stored no longer than 1 h in this study.

Similarly, the percentage of parthenote oocytes in this study was not significant different ($p>0.05$) between each group of culture conditions and might not depend on the culture media and the culture times. The parthenogenetic process could spontaneously occur *in vivo* and *in vitro* in many species, due to the inability of oocytes to proceed into metaphase II arrest (Rougier and Werb 2001).

The oocytes in the germinal vesicle breakdown (GVBD) to the anaphase I (AI) stage, refers to the oocytes which developed from the resting stage, germinal vesicle, but failed to reach the maturation stage. Interestingly, if this GVBD to AI stage of oocytes could be induced to develop into the maturation stage, the maturation rate of oocytes would be higher. Accordingly, a method to increase the *in vitro* maturation rate of domestic cat oocytes should be further investigated. However, this study shows that domestic cat oocytes can be matured *in vitro* after culturing in either M199 or DMEM for 24, 36 or 48 h without incurring any statistical differences between them.

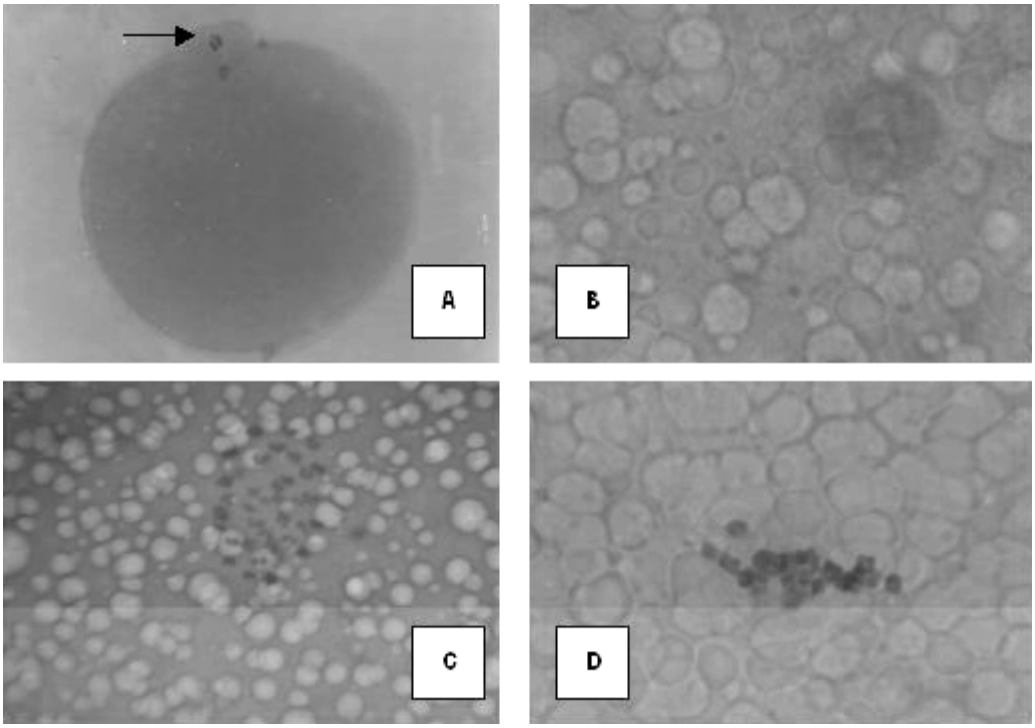

Figure 1 Matured oocyte (A) with first polar body (arrow), magnification X400. Immature oocyte (B-D), (B) germinal vesicle stage (C) metaphase I (D) anaphase I, magnification X1000.

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