Study on the Effects of the Recovery Time, Diameter of Canine Oocytes on *In Vitro* Fertilization and ICSI

Ji-Hoon Park[1], Man-Hee Rhee[2] and Sang-Keun Kim[1,†]

[1]*College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea*

[2]*College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea*

ABSTRACT

These study was carried out to investigate the effects of the recovery time, diameter of oocytes on *in vitro* fertilization or intracytoplasmic sperm injection (ICSI). The *in vitro* maturation rates to MII stage of oocytes recovered at the inactive, follicular and luteal stages matured for 72 h were 1.4±0.0%, 43.4±3.2% and 10.8±2.7%, respectively. The fertilization rates of *in vitro* cultured oocytes recovered from ovaries at the in active, follicular and luteal stages were 0.0±0.0%, 15.7±3.4% and 7.6±3.5%, respectively. The *in vitro* maturation rate of oocytes recovered from ovaries at the follicular stage of the reproductive cycle was significantly higher than those at the inactive and luteal stages ($p<0.05$). The penetration rate determined that the percentages of oocytes with diameters in the <100 μm, 100 to 100 μm and 110 to 120 μm ranges were 17.5±4.7%, 43.9±4.5%, 21.3±3.4%, respectively. The penetration rate of oocytes with diameters between 100 to 110 μm was significantly higher than that of oocytes whose diameters were 100< μm and 110~120 μm ($p<0.05$). The penetration rate of oocytes determined that the percentages of ovaries with diameters between 1 to 5 mm and 6 to 10 mm were 32.9±3.2% and 17.5±3.7%, respectively. Thus, the diameters of the ovaries were significantly higher at 1 to 5 mm ($p<0.05$). A total of 264 oocytes were fixed and stained after co-incubation with sperm, of which 72 had identifiable nuclear material. After *in vitro* fertilization for 20 hrs, 27.3% of oocytes were penetrated by spermatozoas. Oocytes were fixed and stained after ICSI, of which 38 oocytes contained identifiable nuclear material. After *in vitro* fertilization and ICSI for 20 hrs, to 27.3% and 67.9% of oocytes were penetrated by spermatozoas. The *in vitro* fertilization rates by ICSI was significantly higher than that *in vitro* fertilization method ($p<0.05$).

(Key words : Canine oocytes, Recovery time, Oocyte diameter, *In vitro* fertilization rate)

INTRODUCTION

Embryonic development of canine oocytes that were matured and fertilized *in vitro* was recently achieved. Although the rates of cleavage reported to be between 8% and 37% (Bedford *et al*, 2003, Songsasen *et al.*, 2002, 2005; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005), only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have been developed in culture. The low rates of canine embryonic development a testament to the inefficient developmental competence of oocytes matured *in vitro*. In the last decade, several attempts have been made to improve the cultural conditions of maturation by taking into account the fact that canine oocytes: undergo extra-follicular maturation that requires an extended period of time, 2~5 days (Holst and Phemister, 1971; Rsutsui, 1975). However, a recent report indicated that oocytes collected from ovaries at follicular phase achieve 41% maturation after 72 hrs of culture (Otoi *et al.*, 2004). These results are poor if compared to those obtained in other carnivores as in cat, in which blastocyst rates up to 50% can be obtained *in vitro* (Gomez *et al.*, 2003), or in bovine ruminants, in which blastocyst rates of 40~60% are routinely obtained (Hansel, 2003). Compared to the above report by Otoi *et al.* (2004) report that the oocytes collected from ovaries at the follicular phase achieved 41% of maturation after 72 hrs of culture, only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have been developed in culture. This result is higher than Hewitt and England (1999), who reported that 45.0%, 6.0% and 36.0 % of canine oocytes were in GVBD (germinal vesicle breakdown) and MII stage. Hewitt and England (1997) reported that there are no differences in maturation rate between oocytes collected from ovaries at the proestrous stages and estrous stages or at the metestrous and anestrous stages. However, our findings indicate that oocyte diameter is an important factor when assessing meiotic competence and sperm penetration of oocytes collected at different reproductive stages. Yamada et al. (1992) reported that oocytes were at the single cell sta-

† Corresponding author : Phone: +82-42-821-6754, E-mail: kskkim@cnu.ac.kr

ge at 24 h after insemination, whereas 6 oocytes out of 41 (14.6%) were at the 2 or 3 cell stage at 48 h after insemination, However, some oocytes were still at the germinal vesicle stage and had swollen sperm heads in the ooplasm.

The purpose of this study was to investigate the effects of collection time and diameter of oocytes on *in vitro* fertilization or ICSI.

MATERIALS AND METHODS

Rcovery and Incubation of Oocytes

Canine ovaries were transported to the laboratory in sterile physiological saline containing 100 IUl/ml penicillin G 100 ug/ml streptomycin at 25℃. Oocytes were sliced with surgical blade and suspended with mPBS and then collected. Recovered oocytes were cultured with TCM-199 (Whittaker, U.S.A.) medium supplemented with 10% (v/v) FCS (fetal calf serum) and 1 mg/ml cysteine, 20 ng/ml E_2, 10 IU/ml HCG, 10 IU/ml PMSG and 10 ug/ml gonadotropin. Twenty oocytes were transferred to 50 ul drops of maturation medium covered mineral oil and cultured in a CO_2 incubator (5% CO_2, 95% air, 38℃). Follicles were divided into groups according to their recorded dimeter size 1 to 5 and 5~10 mm. After incubation for 72 hrs, the oocytes were divided into groups according to their recorded diameter size 100 to 110 and 110 to 120 um. Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical (St Louis, USA).

In Vitro Maturation of Oocytes

Depending on their morphology, the ovaries were divided into follicular stage (one or more mature follicles were present at least on one ovary) and luteal stage (one or more copora lutea present on one or both ovaries). Then each group of 50 oocytes was cultured in 50 μl of maturation medium. Oocytes were transferred into 50 μl drops of TCM-199 medium and cultured for 40, 48, 70 hrs.

In Vitro Fertilization of Oocytes

Two mature Jindo dogs (4 years old) were used as semen donors. The first and second fractions of ejaculate were collected by massage methods. TCM-199 medium with 4 mg/ml BSA (bovine serum albumin) was used for sperm washing, capacitation, and *in vitro* fertilization. The semen was centrifuged at 500×g for 5 min to remove seminal plasma, then the sperm pellet was washed twice under the same conditions. The washed spermatozoa were incubated at a concentration of 0.5~4.0×10^8 sperm/ml for 5 h at 38℃ in 5% CO_2, 95% air. The sperm suspension was added to 0.4 ml fertilization medium containing oocytes that had been cultured for 72 hrs. Examination of bound and penetrated sperm heads were carried out at 400 x magnification with a fluorescent microscope. The number of sperm remaining on or in the zona pellucida of each oocytes was recorded.

ICSI of Oocytes

Fresh semen used in experiments. The spermatozoa were washed twice Hepes-buffered TCM-199 medium by centrifugation at 800 G for 10 min. The spermatozoa were then exposed to 0.2 uM inophore A23187 for 2 min and resuspended in Hepes-buffered TCM-199 medium supplemented with 3 mg BSA (bovine serum albumin) and 1 mM caffeine for 4~6 hrs at 38℃. Five to ten oocytes with a first polar body were loaded into 1.5 ml microcentrifuge tubes containing 500 ul of M_2 medium supplemented with 3 mg BSA and centrifuged at 12,000 g for 3 min to facilitate sperm injection. ICSI was carried out in 2 ul drops of M_2 containing 3 mg BSA. The sperm suspension was placed in droplet of M_2 containing 7% polyvinylpyrrolidone. Each spermatozoon was injected into ooplasm using a micromanipulator (Narishige, Japan) immediately after immobliization.

Assessment of Meiotic Stage and Sperm Penetration

Oocytes were fixed in acetic acid : ethanol (1:3) solution for 24 h then stained using with 1% acetoorcein or 10 μg/ml bisbenzimide (Hoechst 33342) and observed under an fluorescence microscope. The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nuclear division, and *in vitro* development by investigating oocytes of development *in vitro*. The oocytes were then fixed, stained, and examined for the meiotic stage of the oocytes and the state of the penetrating sperm head.

Statistical Analysis

The One-way ANOVA were used to determine the statistical significance of differences between values for the experimental and control groups. *P* values of 0.05 or less were considered as statistically significant.

RESULTS

In Vitro Fertilization of Oocytes at Different Reproductive Stages

In vitro fertilization rates of *in vitro* cultured oocytes recovered at the inactive, follicular and luteal stages of the reproductive cycles were shown in Table 1.

The *in vitro* maturation rates to MII stage of oocytes recovered at the inactive, follicular and luteal stages ma-

Table 1. *In vitro* maturation rates of oocytes collected at different reproductive stages

Collection time of oocytes	No. of oocytes examined	MII	IVF
Inactive	65	1.4±0.0	0.0±0.0[b]
Follicular	83	43.4±3.2	24.1±3.4[a]
Luteal	66	10.8±2.7	7.6±3.5[b]

[ab] Values within column with different superscript differ(p<0.05).

tured for 72 h were 1.4±0.0%, 43.4±3.2% and 10.8±2.7%, respectively. The fertilization rates of *in vitro* cultured oocytes recovered from ovaries at the in active, follicular and luteal stages were 0.0±0.0%, 15.7±3.4% and 7.6 ±3.5%, respectively. The *in vitro* maturation rate of oocytes recovered from ovaries at the follicular stage of the reproductive cycle was significantly higher than those at the inactive and luteal stages (p<0.05).

The Penetration Rate of Oocytes at Different Diameter

The penetration rate of oocytes classified diameter of oocytes and ovaries were shown in Table 2.

The penetration rate determined that the percentages of oocytes with diameters in the <100 μm, 100 to 100 μm and 110 to 120 μm ranges were 17.5±4.7%, 43.9± 4.5%, 21.3±3.4%, respectively. The penetration rate of oocytes with diameters between 100 to 110 μm was significantly higher than that of oocytes whose diameters were 100< μm and 110~120 μm (p<0.05). The penetration rate of oocytes determined that the percentages of ovaries with diameters between 1 to 5 mm and 6 to 10 mm were 32.9±3.2% and 17.5±3.7%, respectively. Thus, the diameters of the ovaries were significantly higher at 1 to 5 mm (p<0.05).

In Vitro Fertilization of Oocytes

Table 2. *In vitro* maturation rates of canine oocytes *in vitro* matured at different oocyte diameter

Diameter of ovaries & oocytes	No. of oocytes examined	No. of oocytes (%)	
		Penetrated	Unidentifiable
Oocytes(μm)			
100 to 110	82	43.9±4.5[a]	56.1±4.3
110 to 120	75	21.3±3.4[b]	78.7±4.5
Ovaries(mm)			
1 to 5	85	32.9±3.2[c]	67.1±4.4
6 to 10	80	17.5±3.7[d]	82.5±4.8

[a~d] Values within column with different superscript differ (p<0.05).

Table 3. *In vitro* fertilization rates of canine oocytes fertilized at different fertilization methods

Fertilization methods	No. of oocytes examined	No. of oocytes IVF (%)
IVF	264	72 (27.3)[a]
ICSI	56	38 (67.9)[b]

[ab] Values within column with different superscript differ(p<0.05).

The fertilization rate of cultured oocytes with *in vitro* fertilization and the ICSI were shown in Table 3.

A total of 264 oocytes were fixed and stained after co-incubation with sperm, of which 72 had identifiable nuclear material. After *in vitro* fertilization for 20 hrs, 27.3% of oocytes were penetrated by spermatozoas. Oocytes were fixed and stained after ICSI, of which 38 oocytes contained identifiable nuclear material. After *in vitro* fertilization and ICSI for 20 hrs, to 27.3% and 67.9 % of oocytes were penetrated by spermatozoas. The *in vitro* fertilization rates by ICSI was significantly higher than that *in vitro* fertilization method (p<0.05).

DISCUSSION

Development of canine oocytes that were matured and fertilized *in vitro* was recently achieved. Although the rates of cleavage were between 8% and 37% (Songsasen *et al.*, 2002; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005), only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have been developed in culture. The low rates of canine embryonic development are a testament to the inefficient developmental competence of oocytes matured *in vitro*.

The *in vitro* maturation rates to MII stage of oocytes recovered at the inactive, follicular and luteal stages matured for 72 h were 1.4±0.0%, 43.4±3.2% and 10.8± 2.7%, respectively. The fertilization rates of *in vitro* cultured oocytes recovered from ovaries at the in active, follicular and luteal stages were 0.0±0.0%, 15.7±3.4% and 7.6±3.5%, respectively (Table 1). The *in vitro* maturation rate of oocytes recovered from ovaries at the follicular stage of the reproductive cycle was significantly higher than those at the inactive and luteal stages (p<0.05). The *in vitro* fertilization rate of oocytes recovered from ovaries at the follicular stage of the reproductive cycle was significantly higher than that at the luteal stage (p<0.05). These results were lower than those reported by Lee and Kim (2006), who found that oocytes collected from ovaries at follicular phase achieved 50.0% maturation after 48 hrs of culture. However, these results were higher than those reported by Tsutsui (1975) and Otoi *et al.* (2004), who reported that

oocytes collected from ovaries at follicular phase achieved 41% maturation after 48 hrs. Further, the result of the *in vitro* developmental rate was similar to or higher than that reported by Otoi *et al.* (2000), who reported that 45.0%, 6.0% and 36.0% of canine oocytes were in GVBD and MII stage. The penetration rate determined that the percentages of oocytes with diameters in the <100 μm, 100 to 100 μm and 110 to 120 μm ranges were 17.5±4.7%, 43.9±4.5%, 21.3±3.4%, respectively (Table 2). The penetration rate of oocytes with diameters between 100 to 110 μm was significantly higher than that of oocytes whose diameters were 100< μm and 110~120 μm ($p<0.05$). The penetration rate of oocytes determined that the percentages of ovaries with diameters between 1 to 5 mm and 6 to 10 mm were 32.9± 3.2% and 17.5±3.7%, respectively. Thus, the diameters of the ovaries were significantly higher at 1 to 5 mm ($p<0.05$). These results suggest that canine oocytes acquire the ability to develop to MII stage and that sperm penetration occurs at an oocyte diameter of 100 to 110 μm. However, Hewitt and England (1997) reported that there were no differences in maturation rate between oocytes collected from ovaries at the proestrous stages and estrous stages, or at the metestrous and anestrous stages. However, our findings indicate that oocyte diameter was an important factor when assessing meiotic competence and sperm penetration of oocytes collected at different reproductive stages. These results indicate that *in vitro* maturation and *in vitro* fertilization times between 48~72 hrs give the highest maturation and sperm penetration rates for oocytes matured at different stages. These results were lower than those of Yamada *et al.* (1992), in which pre-ovulatory oocytes collected from ovaries of bitches treated with exogenous gonadotropins achieved the highest maturation rates after 72 h of culture (31.9%). Sperm penetration and *in vitro* fertilization of oocytes has been studied extensively in canines (Hay*et al.*, 1997; Hewitt and England, 1997; Reyes *et al.*, 2006). Hewitt and England (1999) reported that the percentages of canine oocytes in GVBD and MII stage for 48 hrs of culture were 33.0~49.0% and 2.0~6.0%, respectively. Bolamba *et al.* (1998) reported that the *in vitro* maturation rate of oocytes cultured in SOF medium supplemented with 3% BSA was a little higher than oocytes cultured in other media. A total of 264 oocytes were fixed and stained after co-incubation with sperm, of which 72 had identifiable nuclear material. After *in vitro* fertilization for 20 h, 27.3% of oocytes were penetrated by spermatozoas. Oocytes were fixed and stained after ICSI, of which 38 oocytes contained identifiable nuclear material (Table 3). The *in vitro* fertilization rates by ICSI was significantly higher than that *in vitro* fertilization method ($p<0.05$). The *in vitro* fertilization rates of oocytes were fixed and stained after ICSI, of which 38 oocytes had identifiable nuclear material. These results for the *in vitro* developmental rate were similar to or higher than those reported by Yamada *et al.* (1992) and Otoi *et al.* (2000), who found demonstrated the *in vitro* fertilization of oocytes. Yamada *et al.* (1992) reported that oocytes were at the single cell stage at 24 hrs after insemination, whereas 6 of 41 (14.6%) oocytes were at the 2 or 3 cell stage at 48 hrs after insemination; others were still at the germinal vesicle stage and contained swollen sperm heads in the ooplasm. However, we can not find reports on the ICSI of canine oocytes. The present results show that canine oocytes matured and fertilized *in vitro* in a defined medium can develop to the 8 and 16 cell stages. Weare now studying whether or not these oocytes can develop into blastocysts and fetuses.

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