Study on Development of Canine Oocytes Treated by *In Vitro* Fertilization and ICSI

Ji-Hoon Park, Young-Ho Chung[1] and Sang Keun Kim[†]

College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea

[1]Dept. of Companion Animal and Animal Resourses Science, Joongbu University, Chungnam 312-702, Korea

ABSTRACT

These study was to investigate the *in vitro* fertilization and viability of fresh and vitrified oocytes. Also, the developmental capacity of IVF and intracytoplasmic sperm injection (ICSI) oocytes were investigated. Then vitrification was performed with the use of 20% ethylene glycol + 20% DMSO + 0.5 M sucrose + 10% FCS + TCM-199 medium. Vitrification immature oocytes are cultured in vitrification solution for 10 min afterwards transferred to expose at room temperature for 5 min. and transferred to the ice water for 5 min. The oocytes were sealed in a 1.0 mm straw and placed in a LN_2 container. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in TCM-199 medium containing 0.5 M sucrose for 5 min each, respectively, at 38℃. After being washed for 2~3 times, using fresh medium the oocytes were cultured in TCM-199 medium supplemented with 5% FCS at 38℃ in 5% CO_2 and air. The normal morphology of fresh and vitrified-thawed oocytes were 87.1±2.1% and 54.8±2.5%, respectively. The viability rates of fresh and vitrified-thawed oocytes were 70.0±2.2% and 41.9±2.6%, respectively. Viability rates of vitrified-thawed oocytes were lower than that of fresh follicular oocytes ($p<0.05$). The *in vitro* maturation rates of fresh and vitrified oocytes were 45.1±3.6% and 28.9±4.4%, respectively. The IVF rates of fresh follicular and vitrified-thawed oocytes were 34.0±2.2% and 20.2±2.6%, respectively. The *in vitro* maturation and fertilization rates of vitrified-thawed oocytes were lower than those of the fresh follicular oocytes ($p<0.05$). A total of 350 oocytes were fixed and stained after co-incubation with spermatozoa, of which 88 had identifiable nuclear material. After IVF for 20 hrs, 25.1±3.4% of the oocytes found to have been penetrated by spermatozoas. Oocytes were fixed and stained after ICSI, and 105 oocytes contained identifiable nuclear material. After IVF and ICSI for 20 hrs, 34.3±3.4% and 59.0±2.0% of the oocytes were found to have been penetrated by spermatozoas. The developmental rates upon ICSI were significantly higher than those of the IVF method ($p<0.05$).

(Key words : Canine oocytes, Development, Vitrification, IVF, ICSI)

INTRODUCTION

It has been reported that *in vitro* maturation and IVF in canine oocytes are generally less efficient than when performed in other species (Hewitt and England, 1999; Otoi *et al.*, 2004). Embryonic development of canine oocytes that were matured and fertilized *in vitro* was recently achieved. The rates of cleavage are reported to be between 8% and 37% in culture (Bedford *et al.*, 2003, Songsasen *et al.*, 2002, 2005; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005). It is well known that the *in vitro* maturation rates and viability of fresh and vitrified canine oocytes are lower compared to other mammals. The *in vitro* maturation rate of canine oocytes is 5~30%, and few of them morula and blastocyst stage (Hewitt and England, 1999; Otoi *et al.*, 2004). Otoi *et al.* (2004) reported that oocytes collected from ovaries at the follicular phase have a maturation rate 41% after 72 hrs of culture. This result is higher than that found by Hewitt and England (1999), who reported that 45.0%, 6.0%, and 36.0% of canine oocytes are in GVBD (germinal vesicle breakdown) and MII stage. Hewitt and England (1999) reported that the rates of GVBD and MII stage canine oocytes after 48 hrs of culture are 33.0%~49.0% and 2.0~6.0%, respectively. Therefore, more research is urgently needed in these fields to obtain higher *in vitro* maturation and fertilization of canine oocytes *in vitro*. The cryopreservation of embryos (Sch-midt *et al.*, 1993; Leibo and Oda, 1993) or oocytes (Suzuki and Nishikata, 1992; Robinski *et al.*, 1991; van Blerkom, 1989) after frozen-thawing has been reported, but there was large differences among the various studies. Embryo vitrification was conducted by keeping the embryos cooled while preventing water hydration and ice crystal formation by the addition of high concentrations of cryoprotectants in vitrification solution (Rall and Fahy, 1985; Kasai *et al.*, 1990; Vajta *et al.*, 1998; Cuello *et al.*, 2004). Renard *et al.* (1984) reported that freezing with a short equilibrium time is capable of two-step freezing

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

by the addition of non-permeable sucrose. Further, freezing of early immature oocytes at different developmental stages is more appropriate than at the mature germinal vesicle stage. If oocytes with high fertilization and *in vitro* developmental rates after vitrification can be preserved, then they can be utilized for IVF and in other fields of biotechnology. However, the survival rates of canine immature oocytes or embryos are lower than those of experimental animals and thus must be improved.

The purpose of this study was to investigate the IVF and viability of fresh and vitrified oocytes as well as the *in vitro* development of oocytes following IVF and ICSI.

MATERIALS AND METHODS

Recovery and Culture of Oocytes

Ovaries were collected immediately after being slaughted and kept at 30℃ in saline solution containing 100 IU/ml of penicillin G and 100 μg/ml of streptomycin sulfate. Upon arrival at the laboratory, ovaries were washed three times with maturation medium. Follicular fluids was collected from 2~5 mm follicles using an 18 G syringe. Only cumulus-oocytes complexes (COCs) with more than two layers of intact cumulus cells, and with uniform cytoplasm were selected for use. The follicular oocytes were cultured in TCM-199 medium supplemented with 10% FCS, 1 μg/ml of FSH, 2 IU/ml of hCG, 1 μg/ml of β- estradiol, 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin sulfate at 38℃ in a 5% CO_2 incubator. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A.) except for those specifically described.

Vitrification and Thawing of Oocytes

Vitrification was performed with 20% ethylene glycol +20% DMSO+0.5 M sucrose +10% FCS +TCM-199 medium. The oocytes were sealed in a 1.0 mm straw. Immature oocytes cultured in vitrification solution for 10 min were exposed at room temperature for 5 min. and transferred to the in ice water for 5 min. The oocytes were then sealed in a 1.0 mm straw and placed in a LN_2 container. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in TCM-199 medium containing 0.5 M sucrose at 38℃ for 5 min each. After being washed 2~3 times, with fresh medium the oocytes were cultured in 10% FCS+TCM-199 medium, The COCs were then transferred to a 100 μl droplet of the maturation medium under mineral oil in a petri dish and cultured at 38℃ under 5% CO_2 in the air for 48 hrs.

IVF Procedure

Five mature Jindo dogs (4 years old) were used as the semen donors. The first fractions of ejaculate were collected by the massage method. To remove seminal plasma, whole semen was diluted with solution of physiological saline and Tris-buffer solution (1:3), centrifuged at 500 g for 6 min, and the supernatant was removed. Sperm pellets were diluted with fertile tyrode solution. TCM-199 medium containing 4 mg/ml of BSA (bovine serum albumin) was used for sperm washing, capacitation, and IVF. The semen was then centrifuged at 500 g for 5 min to remove the seminal plasma, after which the sperm pellet was washed twice under the same conditions. The washed spermatozoa were incubated at a concentration of $0.5\sim4.0\times10^8$ sperm/ml for 5 h at 38℃ in 5% CO_2, 95% air. The sperm suspension was then added to 0.4 ml of fertilization medium containing oocytes that had been cultured for 72 hrs. Over 80~85% were viable. The semen were thawed in a 25 ℃ water-bath for 2 min. The supernatant was then discarded, and motile and non-motile sperm were separated by the swim-method for 5 min. Motile sperm were diluted with mPBS medium to a concentration of a 1×10^6 sperm and then cultured at 38℃ for 4 hrs. Then a 10 ul droplet of the sperm was added to each 90 ul droplet of TCM-199 containing 10 oocytes that had been previously cultured by *in vitro* maturation for 48 hrs. The oocytes and sperm were then co-cultured in a CO_2 incubator for 20~24 hrs. To stain bound and penetrated sperm, oocytes were transferred to 1 ml of 100 μg/ml solution of bisbenzimide (Hoechst 33342) for 20 min. Examination of bound and penetrated sperm heads were carried out at 400× magnification with a fluorescent microscope (Nikon, Japan). The number of sperm remaining on or in the zona pellucida of each oocyte was recorded.

ICSI Procedure

Frozen semen was rapidly thawed in a water bath at 30℃. The spermatozoa were washed twice with 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 then resuspended in Hepes-buffered medium supplemented with 3 mg of BSA 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/ml of 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 of BSA. The sperm suspension was placed in a droplet of M_2 containing 7% polyvinylpyrrolidone. Each spermatozoon was injected into ooplasm using a micromanipulator (Narishige, Japan) immediately after immobilization.

Assessment of Survival and Developmental Rate

After thawing, the oocytes were denuded of cumulus cells in TCM-199 medium using a pipette by repeated aspiration and expulsion. The cumulus-free oocytes were stained with 20 ug/ml of propidium iodide (PI) in PBS containing 0.1% polyvinyl alcohol and incubated for 15 min. The oocytes were then examined under ultraviolet light using an epifluorescence microscope (Nikon, Japan), and plasma membrane integrity of oocytes was assessed. The oocytes with disrupted plasma membranes were dyed red with PI. Assessment of *in vitro* oocyte maturation and fertilization was carried out depending on the maturation criteria based on cellular and nuclear division and *in vitro* development by investigating embryo development.

Statistical Analysis

The results were expressed by treatment as mean±S.D. For comparison of means, Duncan's multiple verification was performed using SAS package of General Lineas's Model (GLM) procedures (SAS Institute, 1996).

RESULTS

Viability of Fresh and Vitrified-Thawed Oocytes

Viability of fresh and vitrified-thawed oocytes are shown in Table 1.

The normal morphology of fresh and vitrified-thawed oocytes were 87.1±2.1% and 54.8±2.5%, respectively. The viability rates of fresh and vitrified-thawed oocytes were 70.0±2.2% and 41.9±2.6%, respectively. Viability rates of vitrified-thawed oocytes were lower than that of fresh follicular oocytes ($p<0.05$).

IVM and IVF of Fresh and Vitrified Oocytes

The *in vitro* maturation and fertilization rates of fresh and vitrified-thawed canine oocytes are shown in Table 2.

The *in vitro* maturation rates of fresh and vitrified oocytes were 45.1±3.6% and 28.9±4.4%, respectively. The IVF rates of fresh follicular and vitrified-thawed oocytes were 34.0±2.2% and 20.2±2.6%, respectively. The *in vitro* maturation and fertilization rates of vitrified-thawed oocytes were lower than those of the fresh follicular oocytes ($p<0.05$).

Table 1. Viability of vitrified-thawed canine oocytes

Treatment	No. of oocytes examined	No. of normal oocytes (%)	
		Morphology	PI stain
Fresh	350	305(87.1±2.1)	245(70.0±2.2)[a]
Vitrified	356	195(54.8±2.5)	149(41.9±2.6)[b]

[ab] Values with different superscript are significantly different ($p<0.05$).

Table 2. Effects of kinds of oocytes on *in vitro* maturation

Treatment	No. of oocytes examined	No. of oocytes matured to (%)	
		MII	IVF
Fresh	350	158(45.1±3.6)[a]	119(34.0±2.2)[a]
Vitrified	356	103(28.9±4.4)[b]	72(20.2±2.6)[b]

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

Table 3. Developmental rates of canine oocytes fertilized at different fertilization methods

Fertilization methods	No. of oocytes examined	No. of oocytes IVF (%)
IVF	350	120(34.3±3.4)[a]
ICSI	105	62(59.0±2.0)[b]

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

In Vitro Development of Oocytes Following IVF and ICSI

The developmental rate of cultured oocytes with IVF and ICSI were shown in Table 3.

A total of 350 oocytes were fixed and stained after co-incubation with spermatozoa, of which 88 had identifiable nuclear material. After IVF for 20 hrs, 25.1±3.4% of the oocytes found to have been penetrated by spermatozoas. Oocytes were fixed and stained after ICSI, and 105 oocytes contained identifiable nuclear material. After IVF and ICSI for 20 hrs, 34.3±3.4% and 59.0±2.0% of the oocytes were found to have been penetrated by spermatozoas. The developmental rates upon ICSI were significantly higher than those of the IVF method ($p<0.05$).

DISCUSSION

It is well known that the *in vitro* maturation and viability rates of fresh and vitrified canine oocytes were lower than other mammals. The *in vitro* maturation rate of canine oocytes is 5~30%, and few of them morula and blastocyst stage (Hewitt and England, 1999; Otoi *et al.*, 2004). Freezing of early immature oocytes at different developmental stages is more appropriate than the mature germinal vesicle stage (Rall and Fay, 1985; Candy *et al.*, 1994; Toth *et al.*, 1994). If oocytes that have high fertilization rates and *in vitro* developmental rates after oocyte vitrification can be preserved,

it could be assumed that it will be highly utilized for IVF and other fields of biotechnology. However, an urgent subject need to be increasement of the survival rates of canine immature oocytes or embryos are lower than those of experimental animals.

The normal morphology of fresh and vitrified-thawed oocytes were 87.1±2.1% and 54.8±2.5%, respectively (Table 1). The viability rates of fresh and vitrified-thawed oocytes were 70.0±2.2% and 41.9±2.6%, respectively. Viability rates of vitrified-thawed oocytes were lower than that of fresh follicular oocytes ($p<0.05$). This result was higher than that found by Abe *et al.* (2008) who reported that the viability rate of vitrified-thawed canine oocytes treated with E30S vitrification is 17.6%. During vitrification, the factors that influence the survival of oocytes are the toxicity of cryoprotectants, the composition of the vitrification solution, and the freezing and thawing speed (Cuello *et al.*, 2004). Isachenko *et al.* (1998) reported that the maturation rate of vitrified-thawed oocytes is between 5.6~22.0%.

The *in vitro* maturation rates of fresh and vitrified oocytes were 45.1±3.6% and 28.9±4.4%, respectively (Table 2). The IVF rates of fresh follicular and vitrified-thawed oocytes were 34.0±2.2% and 20.2±2.6%, respectively. The *in vitro* maturation and fertilization rates of vitrified-thawed oocytes were lower than those of the fresh follicular oocytes ($p<0.05$). This result is similar to that of Fujihira *et al.* (2004), who reported that the nuclear maturation rate of vitrified-thawed oocytes treated with 7.5 μg CB for 30 min is 46.8%. However, This result is similar than that of Reyes *et al.* (2006) who reported that the IVF rate of *in vitro* matured canine oocytes using frozen-thawed dog semen is 26.4~34.2 %. These results indicate that an *in vitro* maturation and IVF time between 48~72 hrs provides the highest maturation and sperm penetration rates for oocytes matured at different stages.

A total of 350 oocytes were fixed and stained after co-incubation with spermatozoa, of which 88 had identifiable nuclear material. After IVF for 20 hrs, 25.1±3.4 % of the oocytes found to have been penetrated by spermatozoas (Table 3). Oocytes were fixed and stained after ICSI, and 105 oocytes contained identifiable nuclear material. After IVF and ICSI for 20 hrs, 34.3± 3.4% and 59.0±2.0% of the oocytes were found to have been penetrated by spermatozoas. The developmental rates upon ICSI were significantly higher than those of the IVF method ($p<0.05$). These results are sililar to or higher than those reported by Yamada *et al.* (1992) and Otoi *et al.* (2000), who demonstrated IVF of oocytes. Yamada *et al.* (1992) reported that oocytes are at the single cell stage after 24 hrs of insemination, whereas 6 of 41 (14.6%) oocytes. However, we could not find reports on ICSI of canine oocytes. The present results show that canine oocytes matured and fertilized *in vitro* in defined medium can develop to the blastocyst stages. We are now studying whether or not these oocytes can develop into fetuses.

REFERENCES

1. Abe Y, Lee DS, Kim SK, Suzuki H (2008): Vitrification of canine oocytes. J Mamm Ova Res 25:32-36.
2. Bedford SJ, Kurokawa M, Hinrichs K, Fissore RA (2003): Intracellular calcium oscillations and activation in horse oocytes injected with stallion sperm extracts or spermatozoa. Reproduction 126:489-499.
3. Candy CJ, Wood M, Whittingham DG, Merriman JA, Choudhury (1994): Cryopreservation of immature mouse oocytes. Hum Reprod 9:1738-1742.
4. Cuello C, Sntonia MG, Parrilla I, Tornel J, Vazquez JM, Roca J, Berthelot F, Martinat-Botte F, Martinez EA (2004): Vitrification of porcine embryos at various development stages using different ultra-rapid cooling procedures. Theriogenology 62:353-361.
5. Fujihira T, Kishida R, Fukui Y (2004): Developmental capacity of vitrified immature porcine oocytes following ICSI: Effects of cytochalasin B and cryoprotectants. Cryobiology 49:286-290.
6. Hewitt DA, England GCW (1999): Synthetic oviductal fluid and oviductal cell coculture for canine oocytes maturation *in vitro*. Anim Reprod Sci 55(1): 63-75.
7. Isachenko V, Soler C, Isachenko E, Perez-Sanchez F, Grishchenko V (1998): Vitrification of immature porcine oocytes: Effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. Cryobiology 36:250-253.
8. Kasai M, Komi JH, Takakamo A, Tssnoda H, Sakurai T, Machida T (1990): A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil 89:91-97.
9. Leibo SP, Oda K (1993): High survival of mouse zygote and embryos cooled rapidly or slowly in ethylene glycol plus polyvinyl pyrrolidone. Cryo-Letters 14:133-144.
10. Otoi T, Shin T, Kraemer D, Westhusin ME (2004): Influence of maturation culture period on the development of canine oocytes after *in vitro* maturation and fertilization. Reprod Nutr Dev 44:631-637.
11. Otoi T, Shimizu R, Naoi H, Wongsrikeao P, Agung B, Taniguchi M (2005): Meiotic competence of canine oocytes embedded in collagen gel. Reprod Domest Anim 41:17-21.
12. Rall WF, Fahy GM (1985): Ice-free cryopreservation of mouse embryos at −196℃ by vitrification. Nature 313:573-575.
13. Reyes M De, Carrion R, Barros C (2006): *In vitro* fertilization of *in vitro* matured canine oocytes us-

ing frozen-othawed dog semen. Theriogenology 66: 1682-1684.
14. Renard JP, Nguyen BX, Garnier V (1984): Two-step freezing of two-cell rabbit embryos after partial dehydration at room temperature. J Reprod Fert 71: 573-580.
15. Robinski B, Arav A, Devires AL (1991): Cryopreservation of oocytes using directional cooling and antifreeze glycoproteins. Cryo-Lettters 12:93-106.
16. Rodrigues BA, Carboneiro dos Santos L, Rodrigues JL (2004): Embryonic development of *in vitro* matured and *in vitro* fertilized dog oocytes. Mol Reprod Dev 67:215-223.
17. Schmidt M, Hyttle P, Grece T, Avery B (1993: Ultrastructure of frozen thawed bovine *in vitro* matured oocytes. Theriogenology 39:304.
18. Songsasen N, Yu I, Leibo SP (2002): Nuclear maturation of canine oocytes cultured in protein-free media. Mol Reprod Dev 62:407-415.
19. Songsasen N, Wildt DE (2005): Size of the donor follicle, but not stage of reproductive cycle or seasonality, influences meiotic competency of selected domestic dog oocytes. Mol Reprod Dev 72:113-119.
20. Suzuki T, Nishikata Y (1992): Fertilization and cleavage of frozen thawed bovine oocytes by one step dilution methos *in vitro*. Theriogenology 37:306.
21. Toth TL, Jones HW, Baka SC, Muasher S, Veeck LL, Lanzendorf SE (1994): Fertilization and *in vivo* development of cryopreserved human prophase I oocytes. Fertil Steril 61:891-894.
22. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen A, Greve T, Callesen H (1998): Open pulled straw vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 51:53-58.
23. van Blerkom J (1989): Maturation at high frequency of germinal vesicle-stage mouse oocyte after cryopreservation: Alterations in cytoplasmic, nuclear, nucleolar and chromosomal structure and organization associated with vitrification. Human Reprod 4:883-898.
24. Yamada S, Shimazu Y, Kawaji H, Nakazawa M, Naito K, Toyoda Y (1992): Maturation, fertilization, and development of dog oocytes *in vitro*. Biol Reprod 46: 853-858.

(Received: 7 May 2011 / Accepted: 30 May 2011)