Study on the *In Vitro* Maturation and Sperm Penetration Rates of Canine Oocytes

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ABSTRACT

The purpose of this study was to investigate the effects of the collection time, co-culture and sperm penetration of canine oocytes on *in vitro* maturation and fertilization. The oocytes were cultured in TCM-199 media containing hormonal supplements (10% FCS, 10 IU/ml HCG, 10 IU/ml PMSG) at 5% CO_2, 95% air, 38℃. The *in vitro* maturation rate to MII stage of *in vitro* oocytes recovered from ovaries that collected at follicular, luteal and inactive phases of the reproductive phase for 44~72 hrs were 19.2%, 12.2%, and 6.0%, respectively. Follicular phases oocytes had a significantly higher *in vitro* maturation rate than oocytes collected at luteal and anestrus stage ($p<0.05$). The *in vitro* maturation rates to the MII stage of canine oocytes after 48 hrs of culture with glutathione, pyruvate, or glutathione + pyruvate were 12.5%, 10.7%, and 17.5%, respectively. This was higher than that in both alone or the combination of the two compared to the control group (19.0%). The sperm penetration rates of *in vitro* matured oocytes by fresh and frozen semen were 29/80 (36.3%) and 18/80 (22.5%), respectively. Although there are limited reports about canine oocytes co-culture and *in vitro* fertilization, our results on *in vitro* maturation is comparable to the results from other researches.

(Key words : Collection time, Co-culture, *In vitro* maturation, Sperm penetration)

INTRODUCTION

It is well known that the *in vitro* maturation rate and vitrification of canine gametes is 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). In addition, the survival rate of canine semen after vitrification is very low, which is due to some semen enzymes such as, sperm cytoplasmic droplets and lysosomes. In order to overcome this, special vitrification method is needed to be developed (Kim, 2001).

Domestic pet animals have been raised for 1,500 thousand years, and the current population exceeds 3,000 thousand (Kim, 2001). Raising of the pet has been turned into the generalization, and the concern of pet becomes greater (Freistedt *et al.*, 2001). The small pet dog's reproduction is mainly completed by unconfirmed male dog of natural copulation and the proliferation of bad gene was worried. So the utility of artificial insemination was requested urgently. But canine semen has very big physiological difference with common domestic animal's seminal fluid, and when freezing the semen the viability and the fertility rate were quite low. So the artificial insemination utilized the frozen sperm is not used commonly and it was needed urgently to develope high viability of freezing method. The canine seminal plasma contains an enzyme harmful to the spermatozoon and the cytoplasmic droplets in seminal plasma contain lysosomal enzyme. Unless these are removed prior to preservation, it will be harmful to spermatozoa survival. There are only a few published reports on canine ovum. It has been reported that efficiencies of *in vitro* maturation and *in vitro* fertilization in canine oocytes are generally lower in comparison with those of other species (Hewitt and England, 1999; Otoi *et al.*, 2004). *In vitro* fertilization and embryo transfer techniques need to be adapted for use in small breed of canines in order to solve the problems of lower efficiency in fertilization and pregnancy (Lee and Kim, 2006; Otoi *et al.*, 2006). Otoi *et al.* (2004) reported that the oocytes collected from ovaries at the follicular phase achieved 41% of maturation after 72 hrs of culture. However, only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) developed in culture. The sperm penetration and *in vitro* fertilization of oocytes has been studied canines (Hay *et al.*, 1997; Hewitt and England, 1997; Reyes *et al.*, 2006). Hewitt and England (1999) reported that GVBD and MII stage of canine oocytes cultured for 48 hrs were 33.0~49.0% and 2.0~6.0%, respectively. Bolamba *et al.* (1998) reported

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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.

Therefore, more research is urgently needed in these fields to obtain higher *in vitro* maturation and fertilization of oocytes cultured *in vitro*. The purpose of this study was to investigate the effects of the collection time, co-culture and sperm penetration of canine oocytes on *in vitro* maturation and fertilization.

MATERIALS AND METHODS

Recovery of Oocytes

All chemicals and reagents were purchased from Sigma Chemical Company (St Louis, MO. USA), unless otherwise stated. Canine ovaries were transported to the laboratory in sterile physiological saline containing 100 IUl/ml penicillin G, 100 μg/ml streptomycin at 25℃. Oocytes were sliced with a surgical blade, suspended with mPBS, collected and cultured with TCM-199 medium supplemented with 10%(v/v) FCS. Oocytes were transferred to 50 μl drops of maturation medium under mineral oil and cultured in a CO_2 incubator (5% CO_2, 95% air, 38℃). Depending on their morphology, the ovaries were divided into follicular phase (one or more mature follicles were present at least on one ovary) and luteal phase (one or more copora lutea present on one or both ovaries), and inactive phase (follicle diameter are below 2 mm).

In Vitro Maturation of Oocytes

Collected oocytes were cultured with TCM-199 (Whittaker, USA) medium supplemented with 10% (v/v) FCS (Gibco. USA), 10 IU/ml HCG, 10 IU/ml PMSG and 10 μg/ml gonadotropin. Oocytes were transferred into 50 ul drops of maturation medium covered by mineral oil and cultured in a CO_2 incubator (5% CO_2, 95% air, 38℃). Oocytes were transferred into 50 μl drops of TCM-199 medium and cultured for 48~72 hrs. The oocytes were co-cultured with 2.0 mM pyruvate, 1.0 mM glutathione for 72 hrs alone or the combination of the two.

Sperm Penetration with Fresh and Frozen

Semen was collected from the tail of epididymis of 5 dogs. In order to remove seminal plasma, whole semen was diluted with a solution of physiological saline and Tris-buffer solution (1:3), centrifuged at 700 g for 6 min, and the supernatant was removed. Sperm pellets were diluted with fertile Tyrode solution. Prior to use in an experiment, sperm motility, the number of viable sperm and a morphologic examination was conducted by a sperm analyzer. Over 80~85% were viable, The semen, frozen by Kim (2001), were thawed in a 25℃ water-bath for 2 min. The supernatant was discarded, and motile and non-motile sperm separated by a swim-method for 5 min. Motile sperm were diluted with m-PBS medium to a concentration of a 1×10^7 sperm, and cultured at 38℃ for 4 hrs. 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 co-cultured in a CO_2 incubator for 20 ~24 hrs. To stain bound and penetrated sperm, oocytes were transferred to 1 ml of a 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. The number of sperm remaining on or in the zona pellucida of each oocytes was recorded.

Assessment of Meiotic Stage

Oocytes were fixed in a solution of acetic acid and ethanol (1:3) for 24 hrs stained using with 10 μg/ml bisbenzimide and observed under an fluorescence microscope. The judgment of *in vitro* oocytes maturation was carried out depending on the criteria of maturation by cell and nuclear division. Assesment of oocytes after *in vitro* fertilization, denuded oocytes were fixed and stained by the methods of Otoi *et al.* (2004). Briefly, denuded oocytes were fixed and permeabilized for 15 min at room temperature in TCM-199 supplemented with 3.7% (w/v) paraformaldehyde and 1% Triton-X 100. Oocytes were then transferred into TCM-199 supplemented with 90% (v/v) glycerol (Gibco. USA) and 10 μg/ml bis-benzimide, and observed under a fluorescence microscope.

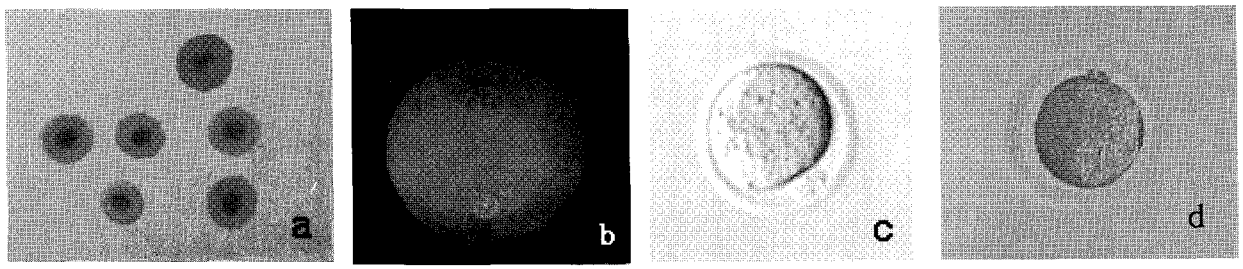

Fig. 1. (a) Non-mature oocytes, (b), (c) MII stage of oocytes and (d) Sperm penetration.

Statistical Analysis

All experiments were repeated at least five times. 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

Nuclear Developmental rate of Oocytes

IVM rates of *in vitro* cultured oocytes recovered from canine ovaries collected at the reproductive phase were shown in Table 1. The *in vitro* maturation rate to MII stage of *in vitro* oocytes recovered from ovaries that collected at follicular, luteal and inactive phases of the reproductive phase for 44~72 hrs were 19.2%, 12.2%, and 6.0%, respectively. Follicular phases oocytes had a significantly higher *in vitro* maturation rate than oocytes collected at luteal and anestrus stage ($p<0.05$).

IVM Rates of Oocytes Co-cultured with Glutathione and Pyruvate

IVM rates of canine oocytes collected from ovaries at the follicular phase and cultured with glutathione and pyruvate for 72 hrs are shown in Table 2. The *in vitro* maturation rates to the MII stage of canine oocytes after 48 hrs of culture with glutathione, pyruvate, or glutathione+pyruvate were 12.5%, 10.7%, and 17.5%, respectively. This was higher than that in both alone or the combination of the two compared to the control group (19.0%).

Table 3. Penetration rates of *in vitro* matured canine oocytes by fresh and frozen sperm

Penetration of oocytes	Semen of	
	Fresh	Frozen
Penetrated(%)	29/80(36.3)[a]	18/80(22.5)[b]

[a,b] Different letters within a column indicate significant differences ($p<0.05$).

Penetration Rate of Oocytes by Fresh and Frozen Sperm

The sperm penetration rates of *in vitro* matured oocytes inseminated the fresh and frozen sperm are shown in Table 3. A total of 160 oocytes were fixed and stained after insemination. The rate of oocyte penetration by sperm after insemination, the number of sperm remaining on or in the zona pellucida of each oocytes was recorded under fluorescent microscope. The sperm penetration rates of *in vitro* matured oocytes by fresh and frozen semen were 29/80(36.3%) and 18/80 (22.5%), respectively.

DISCUSSION

Recently, the maturation and fertilization of canine oo-

Table 1. *In vitro* maturation rates of canine oocytes

Collection phase	No. of oocytes examined	No. of oocytes developed to			
		GV	MI	Degen.	MII
Follicular	125	24(19.2)	52(41.6)	28(22.4)	24(19.2)[a]
Luteal	90	19(21.1)	18(20.0)	42(46.7)	11(12.2)[b]
Inactive	150	28(18.7)	16(10.7)	97(64.7)	9(6.0)[b]

[a,b] Different letters within a column indicate significant differences ($p<0.05$).

Table 2. IVM rates of canine oocytes after 72 hrs of culture with glutathione and pyruvate

Collection phase	No. of oocytes examined	No. of oocytes developed to			
		GV	MI	Degen.	MII
Control	100	26(26.0)	28(28.0)	25(25.0)	19(19.0)[a]
Glutathione	120	29(24.2)	28(23.3)	48(40.0)	15(12.5)
Pyruvate	122	27(22.1)	32(26.2)	50(41.0)	13(10.7)
Glu.+Pyr.	120	34(28.3)	24(20.0)	41(34.2)	21(17.5)[b]

[a,b] Different letters within a column indicate significant differences ($p<0.05$).

cytes have been achieved *in vitro*. Although cleavage rates ranging between 8% and 37% have been reported (Hewitt and England, 1997; Otoi *et al.*, 2000; Songsasen *et al.*, 2002; Rodrigues *et al.*, 2004;), only one morula (Otoi *et al.*, 2000) and one blastocyst (Otoi *et al.*, 2004) have been developed in culture. The low rates of canine embryo development testify to the inefficient developmental competence of oocytes matured *in vitro* and justify the focus *in vitro* maturation. Canine semen has a large physiological difference with common domestic animals, the viability and the fertility rate of frozen semen are quite low. Therefore, artificial insemination utilizing frozen sperm is not commonly used and a freezing method that increase viability is urgently needed. In the last decade, several attempts have been made to improve the maturation culture conditions taking into account the main peculiar characteristic of the canine oocyte: the extra-follicular maturation that requires an extended period of time, 2~5 days (Holst and Phemister, 1971; Tsutsui, 1975). However, a recent report indicates that oocytes collected from ovaries at the follicular phase achieved 41% of maturation after 72 hrs of culture (Otoi *et al.*, 2004). We found the IVM rate to MII stage of *in vitro* oocytes recovered from ovaries that collected at the follicular, luteal and inactive phases of the reproductive phase for 44~72 hrs to be 19.2%, 12.2%, and 6.0%, respectively. IVM rate of oocytes recovered from ovaries collected at the follicular phases was significantly higher than oocytes collected at luteal and inactive stages ($p<0.05$). These results were lower than reported by Otoi *et al.* (2004), that the oocytes collected from ovaries at the follicular phase achieved 41% of maturation after 72 hrs of culture. However, only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2000) have developed in culture. Our result was a little higher that Hewitt and England (1999) reported that the *in vitro* maturation rates of canine oocytes to GVBD and MII stage was 33.0~49.0% and 2.0~6.0%, respectively. These results indicated that *in vitro* maturation rate was higher, when cultured fresh oocytes with excellent morphology and compact cumulus cell. The rates of IVM to MII stage of canine oocytes after 48 hrs of culture with glutathione, pyruvate or glutathione + pyruvate were 12.5%, 10.7%, and 17.5%, respectively. Our results was higher than that in both alone or the combination of the two compared to the control group (19.0%, Table 2). Our results were lower than that of Songsasen *et al.* (2007), who reported that the nuclear maturation to MII phase of dog oocytes cultured for 48 hrs in TCM-199 medium containing 0.25~2.5 mM pyruvate or 1.0~4.0 mM glutamine were 17.3±5.4~18.3± 6.5% and 11.7±4.2~22.6±7.4%, respectively. Also, this reports suggestes that pyruvate played an important role earlier in the maturational process, and less glutamine was oxidized as the oocyte neared nuclear maturation. Our result was also similar or a little lower than Hewitt and England (1999) and Bolamba *et al.* (1998), who both reported when cultured canine oocytes in SOF medium supplemented with 3% BSA was a little higher than that those cultured in other media. The sperm penetration rates of *in vitro* matured oocytes by fresh semen were 29/80(36.3%) and the zona pellucida penetration by fresh sperm was higher than that the penetration of frozen sperm (18/80(22.5%, Table 3), respectively. Table 3). These results were lower than that of Reyes *et al.* (2006), who reported that the oocytes cultured with sperm frozen in extenders A (tris-buffer sol.), B (Saline sol.) and C (tris-buffer and Saline sol.) were 26.4%, 33.1%, and 34.2%, respectively. Also, Hatoya *et al.* (2006) reported that the canine oocytes for non-penetrated, monospermy and polyspermy cultured with mouse embryonic fibroblast cells or canine embryonic fibroblast cells were 7.1~7.4% and 7.2~8.7%, respectively. Also, Yamada *et al.* (1992) reported that pre-ovulatory oocytes collected from canine ovaries treated with exogenous gonadotropins achieved the highest maturation rates after 72 hrs of culture (31.9%). After 12 hrs of coincubation with spermatozoa, 32% underwent monospermic fertilization and 37% of the total fertilized oocytes showed the male pronucleus. At 96 hrs after insemination, 13.3% of embryos reached beyond the 4-cell stage in culture.

Although there are limited reports about canine oocytes co-culture and *in vitro* fertilization, our results on *in vitro* maturation is comparable to the results from Otoiet *et al.* (41%, 2004) and Hewitt and England (33.0 ~49.0%, 1999). This is due to the difficulty in obtaining canine oocytes and the difference in experimental techniques, which remains to be studied further.

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