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A Surgical Method for Collecting Canine Oocytes of *In Vivo* Matured from Uterine Tube

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수술적 방법을 이용한 개 난관내 성숙 난자의 회수에 관한 연구

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SUMMARY

산업동물의 복제와 더불어 개 또는 다른 애완동물의 복제는 많은 사람들의 관심의 대상이 되고 있다. 최근 애완동물로는 처음으로 고양이가 복제되었으며, 수백만 달러의 기금으로 개 복제의 연구가 수행되기도 하였다. 이러한 개의 체세포 복제(SCNT)를 비롯한 체외수정, 난자내 정자주입법(ICSI) 등의 생식보조술 성공률은 우수한 난질을 가진 난자가 절대적으로 필요하지만 낮은 핵 성숙률 때문에 체외성숙 난자를 이용하기에는 아직까지 요원한 게 현실이다. 따라서 자연 배란된 성숙 난자의 채취는 IVM으로 얻지 못하는 질 좋은 난자를 얻는데 매우 중요하며, 향후 IVM으로 수정 가능한 난자를 생산하여 번식에 이용할 때까지는 체내 성숙난자의 사용은 불가피하다. 하지만 개의 번식생리는 다른 동물들과 많은 차이를 보이고 있다. 특히 배란시의 난자의 형태는 다른 동물들과 달리 LH surge후 48시간 후에 난핵포 (germinal vesicle) 단계에서 배란되어 난관 내에서 48~72시간 동안 핵 성숙이 이루어지는 특징을 지니고 있다. 배란시기와 성숙난자의 채취시기는 질 상피세포 도말법과 혈중 LH 및 progesterone 농도의 변화상을 관찰하여 결정하는 것이 일반적이며, 채취방법은 정중 개복술에 의한 외과적 방법을 이용 난관을 절제(salpingectomy)하여 관류하거나 카데터를 난관채에 장착한 후 난관-자궁 접합부위에 주사침을 이용한 관류액의 주입으로 회수하는 방법이 사용되고 있다.

본 실험에서는 수술적 방법을 이용하여 난관 절제술을 실시한 성숙 난자의 회수율과 카데터를 이용한 난자 회수율 그리고 새롭게 고안된 개 난자 회수용 니들을 이용한 회수율을 비교하였으며, 각각의 회수 방법이 성숙난자의 외형에 미치는 영향을 조사한 결과는 12두에서 난관절제술로 채취한 성숙난자의 회수율은 89.7% 이었다. 수술적 회수방법에서는 본 연구실에서 개발한 난자 회수용 니들을 난관내에 삽입-결찰한 후 난관-자궁 접합부에서 난자 회수용 배지를 관류하는 방법으로 평균 83.0%의 회수율을 얻었다. 이 같은 결과는 Tom

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Cat 카테터를 이용한 회수율 (68.9%)과 난자 회수용 니들을 결찰하지 않고 관류한 방법 (73.5%) 보다 유의적으로 높은 회수율을 나타내었다 ($p<0.05$). 또한 난관 절제술과 각각의 수술적 방법으로 회수한 난자의 형태학적 차이는 관찰할 수 없었으나 난관 절제술과 난자 회수용 니들을 결찰하여 회수한 난자의 형태와 난질이 Tom Cat 카테터나 결찰하지 않은 니들을 이용하였을 때보다 영향을 덜 받는 경향을 나타내었다 (각각 72.0%, 73.8%와 62.8%, 69.6%).

(Key words : *in vivo* oocyte collection, bulbed needle and ligation method, canine ARTs)

INTRODUCTION

For many mammalian species, advancements of reproduction technologies such as *in vitro* fertilization(IVF), *in vitro* maturation(IVM), embryo culture and transfer(ET), and somatic cell nuclear transfer(SCNT) have already proven successful. Somewhat basic commercial technologies, such as semen cryopreservation, artificial insemination, and embryo transfer have been successfully reported in cats, dogs, and foxes(Kinney et al., 1979; Kraemer et al., 1982; Tsutsui et al., 1989; Jalkanen et al., 1998; England, 2001). However, the more advanced techniques of *in vitro* oocyte maturation, fertilization and embryo culture have been primary limited to a research setting(Kanda et al., 1995; Bolamba et al., 1998). For canids, limited research has been performed in the area of IVM. The reported rates of maturation beyond the GV stage range from 0~58% (Farstad, 2000a; Luvoni, 2000), while maturation rates to MII are around 20%, independent of the various treatments applied. These maturation rates are the result of oocytes cultured *in vitro*, which were harvested from canines of various estrous cycle stages, as well as different canids within the same family(i.e. different species of fox). Several factors influence the limited application of ARTs in companion animals. Some are related to basic problems involving the reproductive physiology of the dog. For example, dogs are monoestrus; generally exhibiting estrus only once every 6 month to 1 year. Methods for fertile estrus induction in bitches to date have proven unreliable or, at best, inconsistent and are generally most effective during late anestrus periods. Furthermore, surgical embryo collection and transfer is still the only proven method in dogs. In cats, surgical collection followed by non-surgical transfer has been developed but suffers from low pregnancy rates.

Cloned cat showed the possibility to cloned dog and more researcher have impetus on the investigating the companion animals. But, still now any live cloned cat and dog did show to public people. In canine, we have a limitation on the researching the ART due to hard approach to anatomical, physiological study. Now a day, in order to establish the *in vitro* maturation of canine oocyte, some researchers have tried so many times in various aspects. Then we thought that *in vivo* matured oocyte would be more useful in somatic cell nuclear transfer. But in that case, we have to consider the oocyte stage up to ovulation. While mammalian was ovulated at metaphase II stage, in canine oocyte was ovulated in GV stage, and matured MII stage to need the time for maturation in uterine tube.

The purpose of present study was based on the ovulation in bitch, we could obtain the *in vivo* matured oocyte from oviduct using the various flushing tools. With *in vivo* matured oocyte, this study will support the basic research about canine ARTs such as IVF, ET, ICSI and somatic cell nuclear transfer in dogs.

MATERIAL AND METHODS

1. Care and Use of Animals

In this study, Mixed breed female dogs ages 1.5 ~3 years with variable reproductive histories were used. The dogs were cared in facilities and using procedures that exceed the standards established by the Seoul National University for Accreditation of Laboratory Animal Care.

2. Vaginal Cytology and Progesterone Levels

All bitches were examined daily for vulval swelling and serosanguinous discharge from the start nature heat. Smears were obtained daily by beginning with the first sign of proestrus and until the surgery for oocyte flushing. Part the lips of the vulva and gently insert a swab at a relatively steep angle. Smear were collected, rolled on the slide-glass, stained, examined and classified as described in detail after by method of Concannon and DiGreorio(1986) using Diff-Quik® stain(International Chemical Co., Japan).

For progesterone level, blood samples(3~5 mL) were collected everyday by cephalic venupuncture into 5 mL syringe with 23 G needle, centrifuged at 3,000 rpm for 20 min and stored at -20℃ until assayed. The samples were analyzed via DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit(Diagnostic Systems Laboratories, Inc., TX, USA) with the assistance of Neodine VetLab, Seoul, Korea. The RIA Test Kit was calibrated before each use with controls.

3. Estimation of Day of Ovulation

Day of ovulation was estimated from peripheral blood progesterone concentrations as reported by Hase et al.(2000) and Jang(2004). The day on which the progesterone concentration initially reached 4.0~7.5 ng/mL was regarded as the day of ovulation. In vaginal cytology, the time of ovulation was superficial cells were usually greater than or equal to 90% of epithelial cells cornified index (C.I., Evans, 1970; C.I. = No. of cornified cells / Total No. of epithelial cells × 100) in the vaginal smear.

4. Anesthesia for Acquisition of Oocytes

48~72 hours after ovulation, both oviduct were excised and/or flushed from 131 dogs under general anesthesia. Atropine sulfate (0.05 mg/kg) and acepromazine maleate(0.025 mg/kg) were administered as a pretreatment, and ketamine(5 mg/kg) was administered for induction of anesthesia. The anesthesia was maintained by isoflurane.

5. Recovery of *In Vivo* Matured Oocytes

Bitches were placed under general anesthesia and the reproductive tract of each bitch was exposed by a mid-ventral laparotomy using aseptic surgical procedures. In excision method, both oviducts were excised from 12dogs. The excised oviducts were flushed downward to recover matured oocytes. A 24G indwelling needle connected to a 5 mL syringe was inserted into the tip of oviduct and 5 mL of flushing medium was infused downward (salpingectomy).

In surgical methods, the fimbriated end of the oviduct was manipulated by digital massage until extruded through the bursal slit. If necessary, the slit was expanded using fine forceps. The opening in the fimbriated end of the oviduct was located visually and open end Tom Cat™ catheter (3½Fr, Kendall Co., USA) was inserted 3~4 cm into the oviducts from 12dogs (Tom cat catheter method). The catheter was held in place by surgical ligature. The base of the oviduct, just above the uterotubal junction, was visualized using digital pressure to blanch the surrounding tissue and the oviductal lumen, and cannulated using a 24 gauge indwelling catheter attached to a syringe filled with embryo collection medium, tissue culture media 199(TCM 199) supplemented HEPES.

Fig. 1. Classification of collected oocytes by various tools. (a) Compact cumulus and homogenized cytoplasmic oocytes(Grade A, x200), (b) Denuded cumulus and crushed oocyte (Grade B, x200), (c) Free zona pellucida without cytoplasm (Grade C, x200).

We invented a new kind of flushing needle (we called "bulbed needle"). The opening in the fimbriated end of the oviduct was located visually and was cannulated using a bulbed needle from 20dogs. This method was did not ligature (bulbed needle method). The needle was held in place by a fine forceps. In bulbed needle and ligation method, the needle was inserted into the abdominal ostium of uterine tube and held in place by a surgical ligature (87 dogs), which was tied using a quick-release devise using 3 cm plastic tube and hemostatic forceps. The ligature suture material was passed and pulled the plastic tube and made clamp using hemostatic ferceps. The needle was retracted until stopped by the ligature. Approximately 7 mL of collection medium was injected through the rumen of the oviduct, through the bulbed needle and directed into a sterile plastic Petri dish. The needle was removed from the oviduct, the catheter lumen was flushed using the hypodermic needle into the collection dish. After flushing both oviducts, the abdominal incision was closed using two-layer closure followed by surgical adhesive on the skin incision.

6. Assessment of Retrieval Oocytes and Oocyte Classification

In vivo matured oocytes were recovered from the Petri dish with the aid of a stereomicroscope. Oocyte recovery rates were calculated as the ratio between the recovered oocytes and the number of ovulated corpus luteum.

The flushed ocytes were placed in TCM 199 with 2.5 mM Hepes (Gibco, USA) and classified, according to the degree of cumulus cell investment and the quality of the cytoplasm, into three groups: compact cumulus and homogenized cytoplasmic oocyte (Grade A, Fig. 1A), denuded cumulus and crushed oocyte (Grade B, Fig. 1B) and free zona pellucida without cytoplasm (Grade C, Fig. 1C).

7. Statistical Analysis

Data were analyzed using a statistical analysis system (SAS) program. The number of the oocytes compared with CL and the classification of the ocytes were expressed as percentage and were compared by analysis of variance (ANOVA). The percentage of CL and oocytes in each category were calculated for each of the observations (Tables 1 and 2).

RESULT

The result of recovery rate of different collection method for *in vivo* maturation of canine oocytes are shown in Table 1. The mean oocyte recovery rate 48~72hours after ovulation by excision method (salpingectomy) in 12 dogs was 89.7%. In surgical methods, the mean *in vivo* matured oocyte recovery rate was 83.0% by bulbed needle and

Table 1. Comparison of different collection methods for *in vivo* maturation of canine oocytes flushed from uterine tube

Collection methods		No. of dogs	Total no. of CL	Total no.(mean per dogs) of collected oocytes	Recovery rate(%)
Surgical methods	Tom cat cath.	12	74	51(4.3)	68.9[a]
	Bulbed needle	20	166	122(6.1)	73.5[a]
	Bulbed needle & ligation	87	801	665(7.6)	83.0[b]
Excision method	Salpingectomy	12	68	61(5.1)	89.7[b]

[a-b] Values with different superscripts are significantly different($p<0.05$).

Table 2. Morphological grading of *in vivo* maturation of canine oocytes flushed from uterine tube

Collection methods	Total no. of collected oocytes	Grade A oocytes(%)	Grade B oocytes(%)	Grade C oocytes(%)
Tom cat cath.	51	32(62.8)	15(29.4)	4(7.8)
Bulbed needle	122	85(69.6)	28(23.0)	9(7.4)
Bulbed needle & ligation	665	479(72.0)	163(24.5)	23(3.5)
Salpingectomy	61	45(73.8)	12(19.7)	4(6.5)

ligation methods. Oocytes collected by bulbed needle and ligation method was higher than that collected by Tom cat catheter method(68.9%) and bulbed needle method(73.5%) ($p<0.05$).

The matured oocytes yield was also significantly higher after collection by salpingectomy than ccollected by Tom cat catheter and bulbed needle method($p<0.05$).

The result of morphological different of collected oocytes showed that a non significantly between the grade of oocytes by excision method and that by surgical methods (Table 2). However, grade A oocytes by bulbed needle and ligation and salpingectomy method yielded better than others methods (72.0% or 73.8% vs 62.8% or 69.6%, respectively).

DISCUSSION

Oocytes matured *in vivo* can be collected surgically from bitches and used as recipient for IVF and nuclear transfer. However, the number of oocytes that can be obtained using this approach is very limited, until now typically a mean of approximately four good oocytes per collection (Westhusin et al., 2001). But the result of present study indicate that it is possible to obtained high percentage and quality *in vivo* oocytes from bitch for IVF and cloning dogs using our invented bulbed needle. Salpingectomy have spent more time for handling on operating and resulted in increasing bleed and more invasive than the surgical methods used with bulbed needle ligation. According to previous reports, Tsutsui et al. (2001) have showed that embryo was at 8-cell to blastocyst stage were collected by salpingectomy in ten bitches and recovery rate was 77.8%~100%. Our recovery rate was showed similarly(Table 1). In case of *in vivo* oocyte collection, Westhusin et al.(2001) collected mean 6.4 oocytes per dog using

a flanged intramedic catheter and all collected oocytes were used for producing the cloned puppy. At first time, to decrease the invasion for oviducts, we used the Tom Cat catheter but efficiency was low. To increase the recovery rate of *in vivo* oocytes, bulbed needle & ligation that was showed high efficiency was invented(Table 1).

In dogs, despite many resercher tried to attempt to apply for commercialization, still ARTs in canine was limited from involving the unique reproductive anatomy and physiology of the dog. Matured oocytes from *in vivo* or *in vitro* that played a crucial role for IVF, ICSI, SCNT were prerequisite. However, up to now, a lot of research team have tried to increase the *in vitro* maturation of caine oocytes but did not showed the good scores (Farstad, 2000b, Luvoni, 2000). To do breakthrough *in vitro* maturation limitation, as another methods, we collected *in vivo* matured oocyte with previous mentioned tools. In doing the collection *in vivo* oocytes, we found the fact that *in vivo* matured oocytes were very sensitive for flushing tools, flowing pressure of flushing medium in lumen of uterine tube. Although salpingectomy was thought to be good tools for obtaining the Grade A oocytes (73.8%, Table 2), as previous stated, it was more invasive and detrimental affect for bitches. Among the three tool for collecting the *in vivo* oocyte, we found the significant difference between tools methods in getting the Grade A oocyte. While we did not find the statistical difference in using the three tools, Tom cat catheter, bulbed needle, and bulbed needle ligation, we thought that bulbed needle ligation was better than others. Westhusin et al. (2001) reported that oocyte collections performed on 17 bitches resulted in 109 ova of which 63 (57.8%) were judged to be of good enough quality for use as recipients for nuclear transfer. One important things, using the bulbed needle ligation decreased the recovery of Grade C oocytes(3.5%, Table 2) compared to others methods.

In conclusion we suggested that these results showed high recovery rate of *in vivo* oocytes and reduced the detrimental effect to the oocytes with invented flushing needle. *In vivo* canine oocyte collected by that methods will be useful for canine ARTs.

REFERENCES

Bolamba D, Borden-Russ KD and Durrant BS. 1998. *In vitro* maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles. Theriogenology, 49:933-942.

England GCW, Verstegen JP and Hewitt DA. 2001. Pregnancy following *in vitro* fertilisation of canine oocytes. Vet. Rec., 148:20-22.

Evans JM and Savage TJ 1970. The collecting of vaginal smears from bitches. Vet. Rec., 7:598-599.

Farstad W. 2000a. Current state in biotechnology in canine and feline reproduction. Ani. Reprod. Sci., 60-61:375-387.

Farstad W. 2000b. Assisted reproductive thechnology in canids species. Theriogenology, 53:175-186.

Fulton RM, Keskintepe L, Durrant BS and Fayrer-Hosken RA. 1998. Intracytoplasmic sperm injection (ICSI) for the treatment of canine infertility. Theriogenology, 48:366.

Hose M, Hori T, Kawakami E and Tsutsui. 2000. Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by u;trasonographic diagnosis system in dogs. J. Vet. Med. Sci., 62:243-248.

Jalkanen L and Lindeberg H. 1998 Successful embryo transfer in the silver fox. Ani. Reprod. Sci., 54:139-147.

Jang G. 2004. Determination of ovulation timing and development potential of cloned embryo in dogs. A Thesis for the degree of master science,

Seoul National University, Seoul.

Kanda M, Oikawa H, Nakao H and Tsutusi T. 1995. Early embryonic development *in vitro* and embryo transfer in the cat. J. Vet. Med. Sci., 57:641-646.

Kinney GM, Pennycook JW, Schriver MD, Templeton JW and Kraemer DC. 1979. Surgical collection and transfer of canine embryos. Biol. Reprod., 20:170(abstract).

Kraemer DC, Kinney GM and Schriver MD. 1982. Embryo transfer in dogs and cats. 2nd International congress on Embryo Transfer and *In Vitro* Fertilisation, Annency, France p.223-234.

Luvoni GC. 2000. Current progress on assisted reproduction in dogs and cats: *in vitro* embryo production. Reprod. Nutr. Dev., 40:505-512.

Tsutsui T, Hori T, Okazaki H, Tanaka A, Shiono M, Yokosuka M and Kawakami E. 2001. Transfer of canine embryos at verious developmental stages recovered by hystrectomy or surgical uterine flushing. J. Vet. Med. Sci., 63:401-405.

Tsutsui T, Shimada K, Nishi M, Kubo N, Murao I, Shimizu T and Ogasa A. 1989. An experimental trial on embryo transfer in the dog. J. Vet. Sci., 51:797-800.

Westhusin ME, Burghardt RC, Rugila JN, Willingham LA, Liu L, Shin T, Howe LM and Kraemer DC. 2001. Potential for cloning dogs. J. Reprod. Fertil., 57:287-293.

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