

Accomplishment of canine cloning through *in vitro* matured oocytes: a pioneering milestone

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Competing interests

No potential conflict of interest relevant to this article was reported.

Abstract

The *in vitro* maturation (IVM) rate of canine oocytes remains low compared to other mammals due to their unique reproductive characteristics. This study aimed to explore the effect of hormone supplementation during the IVM of canine immature oocytes on nuclear maturation and subsequently assess its potential application in canine somatic cell nuclear transfer (SCNT). Immature oocytes were collected and cultured in an IVM medium supplemented with hormones (follicle-stimulating hormone [FSH] and progesterone [P4]) or without hormones (control) for 24 hours. The maturation rates of oocytes in the hormone-treated group ($94.92 \pm 3.15\%$) were significantly higher than those in the control group ($61.01 \pm 4.23\%$). Both *in vitro* and *in vivo* matured oocytes underwent NT to evaluate their utility, and the fusion rates were higher in the *in vitro* matured group than those in the *in vivo* matured group, not significant between *in vivo* and *in vitro* matured group (73.28% and 82.35%, respectively). As a result, 14 fused embryos from the *in vitro* matured group were transferred into two surrogates, with one surrogate achieving a successful pregnancy and delivering four puppies. Whereas in the *in vivo* matured group, 85 fused embryos were transferred to 8 surrogate mothers, leading to three surrogates becoming pregnant and delivering one, four, and two puppies. The pregnancy rates were not significant between both groups (50% and 37.50%), but the number of offspring exhibited a significant difference (28.57% and 8.23%). In conclusion, we achieved a remarkable milestone by successfully producing cloned puppies using *in vitro* matured oocytes, underscoring the feasibility of canine cloning from *in vitro* recovered oocytes. It is important to note that this study focused only on immature oocytes after ovulation and only during the estrous stage. Further research targeting other stages of the estrous cycle could potentially enhance canine cloning efficiency.

Keywords: Canine cloning, *In vitro* maturation, Immature oocyte, Hormone treatment

INTRODUCTION

Since the birth of Dolly the Sheep, the first cloned mammal, in 1997 [1], successful cloning of dogs

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Ji K, Kim M.

Data curation: Ji K.

Methodology: Ji K, Park K, Kim M.

Validation: Kim E, Kil T.

Investigation: Ji K, Kim D.

Writing - original draft: Ji K.

Writing - review & editing: Ji K, Park K, Kim D, Kim E, Kil T, Kim M.

Ethics approval and consent to participate

The experimental procedures and methods used in this study were approved by the Animal Welfare and Ethics Office (CNU-01089) of Chungnam National University and performed according to the Guide for the Care and Use of Laboratory Animals published by the IACUC of Chungnam National University and ARRIVE guidelines.

was reported in 2005 [2]. Over the past 15 years, canine somatic cell nuclear transfer (SCNT) technology has found diverse applications in reproductive biotechnology, including: 1) propagating specific dogs with elite working abilities [3,4], 2) preserving endangered canine species [5,6] and companion dogs [7,8], and 3) generating disease model dogs for studying disease mechanisms and therapies [9–11]. Despite its utility, dog cloning remains a formidable challenge due to difficulties in obtaining satisfactory results in *in vitro* oocyte maturation, estrus induction, and superovulation [12,13]. The challenges stem from the unique reproductive physiology of canines, such as ovulation at the germinal vesicle stage, mono-estrus, the hormonal environment, and the lack of reliable *in vitro* maturation (IVM) systems [14,15].

To overcome these obstacles, canine SCNT studies have utilized oocytes matured *in vivo* [16,17]. Matured oocytes are collected surgically by flushing oviducts at 72 h post-ovulation, with the timing of natural ovulation predicted based on serum progesterone concentration in the oocyte donor (ovulation occurs at 4.0–9.9 ng/mL) [2]. Typically, six to 12 oocytes are collected from one female dog in natural estrus through oviduct flushing, yielding an average recovery rate of 93.8% [18]. However, not all oocytes recovered through this method undergo complete nuclear maturation, and at least 3% to a maximum of 33% of the oocytes remain immature, lacking the first polar body [19,20]. If immature oocytes can be matured *in vitro*, the availability of oocytes would be maximized, thereby enhancing SCNT efficiency.

Unlike other mammalian species, canine oocytes are ovulated in an immature state and are exposed to high levels of progesterone (P4) and estradiol (E2) in the bursa and oviduct. Nuclear maturation is completed in the oviduct over 48 h–72 h through exposure to P4 and E2 [14,15,21]. Various factors have been identified to influence the IVM of canine oocytes, with hormones playing an important role in the meiotic resumption in canine oocytes. The oocytes matured to the metaphase II (MII) stage significantly increased under supplementation with gonadotropins (such as follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) and/or steroid hormones (such as E2 and P4) [22–26]. Interestingly, oocytes reaching the MII stage during IVM were higher in the estrus stage than in the anestrus and diestrus stage [18,22,27]. Nevertheless, the production of *in vitro* matured canine oocytes remains limited in quantity and quality, rendering them unsuitable for successful canine SCNT.

In this study, we hypothesize that *in vivo*-immature oocytes from female dogs in the estrous stage possess the sufficient ability to resume meiosis when subjected to IVM under appropriate hormonal conditions. We selected P4 and FSH as the hormones for this study, and their concentrations were determined based on previous studies [22–26], and aimed to determine the combination effect of these hormones (P4 + FSH) on the IVM of immature oocytes recovered from oviduct flushing of naturally ovulated female dogs. Additionally, we sought to evaluate the suitability of these hormonally treated oocytes as donors for canine SCNT.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise specified.

Animal ethical statement

The experimental animals consisted of 1 to 6 years old mixed-breed female dogs in the estrus stage, which were used as oocyte donors and surrogate mothers. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Chungnam National

University (Approval Number: CNU-01089). All dogs were individually housed in controlled temperature and ventilation conditions, provided a commercial diet once a day, and had access to water *ad libitum*.

Preparation of donor cells

Preparation of donor cell was performed as previously described [28]. The donor cell was acquired from a 16-year-old male Pomeranian through a post-mortem skin biopsy for the establishment of fibroblasts. Muscle tissues were washed three times and minced in phosphate-buffered saline ([PBS] Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The tissue pieces were washed twice by centrifugation at 400×g for 5 minutes and cultured in a culture medium composed of Dulbecco's modified Eagle's medium ([DMEM] Gibco) supplemented with 20% (v/v) fetal bovine serum ([FBS] Gibco) and 1% penicillin-streptomycin. After being cut into small pieces, the cells were cultured at 39°C in a humidified atmosphere of 5% CO₂ and 95% air. When the cells reached confluence after 5–7 days, they were harvested after treated with 0.25% trypsin-EDTA (Gibco) for 2 minutes. The established cell line was frozen in 10% DMSO in FBS and stored in liquid nitrogen at -196°C until further use. Cells from passages 5–7 were trypsinized before SCNT.

Oocyte collection

The estrus stage in female dogs was determined based on serum progesterone (P4) concentration analysis using the Vet chroma™ progesterone kit (Anivet, Chuncheon, Korea). The day of ovulation was regarded when the P4 concentration exceeded 8 ng/mL. At 72 hours after assumed ovulation, all oocytes of the oocyte donors were recovered using the oviduct flushing method through a surgical procedure. Briefly, the female dogs were initially anesthetized with 6 mg/kg ketamine and xylazine and maintained under anesthesia with 2% isoflurane. The fimbria of the oviduct was accessed through the bursal slit and cannulated using an inverted flanged bulb steel needle (18 gauge, 7.5 cm). A 24-gauge intravenous (IV) catheter (Angiocath™ Plus, Becton Dickison Korea, Seoul, Korea) was inserted into the isthmus of the oviduct near the uterotubal junction. HEPES-buffered TCM-199 (Gibco) supplemented with 10% FBS, 2 mM NaHCO₃, 5 mg/ml Bovine serum albumin (BSA) and 1% penicillin-streptomycin was introduced into the oviduct through the IV catheter. Following the surgery, Cumulus-oocyte complexes (COCs) were promptly transported to the laboratory. A total of 282 oocytes were flushed from 25 oocyte donor dogs. Among these, 164 mature oocytes were used for SCNT, while the others were used for IVM.

Classification of oocytes

The cumulus cells were removed from the retrieved COCs by repetitive pipetting in TCM-199 containing 10% FBS and 0.1% hyaluronidase. After the removal of cumulus cells, the oocytes were analyzed under a stereoscopic microscope. Oocytes with the first polar body in the perivitelline space were classified as mature, while oocytes lacking the first polar body were classified as immature. A total of 282 oocytes were collected from 25 dogs. Among them, 164 oocytes were classified as mature and used for SCNT, while Among the oocytes recovered from tubal flushing, 98 oocytes without the first polar body were classified and subjected to IVM. Immature oocytes were cultured for 24 h in IVM medium in the presence or absence of hormones (P4 + FSH).

In vitro maturation of canine oocytes

For the IVM of *in vivo*-immatured oocytes, two types of culture medium were used as follows: 1) Non-treated (Control) group: TCM-199 containing sodium bicarbonate, 10% FBS, and 1% penicillin-streptomycin solution; 2) Hormone-treated group: TCM-199 containing 0.2 mM

sodium pyruvate, 10% FBS, 5 µg/mL FSH (folltropin-V®, Bioniche, Belleville, Canada), and 2 ng/mL P4. Approximately 15 immature oocytes were cultured in a 40 µL drop of each medium at 39°C under 5% CO₂ in 95% humidified air for 24 hours. The extrusion of the first polar body (MII) was evaluated under the stereomicroscope to assess IVM.

Somatic cell nuclear transfer and embryo transfer

SCNT was performed as previously described [2]. Briefly, enucleation of mature oocytes were performed through removing the first polar body and MII chromosomes by aspiration and assessed by staining with Hoechst 33342 (bisbenzimidazole). After enucleation, injection of donor cells was performed. A donor cell with a smooth cell surface membrane was selected and transferred into the perivitelline space of an enucleated oocyte. Following injection, the oocyte donor cell complexes were equilibrated in fusion medium containing 0.26 M mannitol, 0.1 mM HEPES, 0.5 mM MgSO₄, and 0.05% BSA, and then fused by two pulses of direct current (32 V for 15 µs) using an Electro-Cell Fusion apparatus (NEPA gene, Chiba, Japan). The success of fusion was confirmed after 30 minutes, and activation was performed using 10µM calcium ionophore (product No. C7522, Sigma-Aldrich) for 4 minutes, followed by 1.9 mM 6-dimethylaminopurine in PZM-3 for 3 hours and 30 minutes. The fused embryos were surgically transferred into the oviducts of estrus-synchronized surrogate mothers. Pregnancy was confirmed by ultrasonic diagnosis 30 days after embryo transfer.

Statistical analysis

Statistical analyses were performed using SPSS (version 26.0.0.0, IBM, USA), and significant differences in oocyte maturation were analyzed using the t-test, while data related to pregnancy rate and birth rate were analyzed using the chi-squared test. The significance level was set at $p < 0.05$.

RESULTS

In vitro maturation of *in vivo*-immature oocytes

We investigated the impact of the IVM medium in the presence or absence of the hormone combination (P4 + FSH) on the nuclear maturation of canine *in vivo*-immature oocytes. As shown in Fig. 1, nuclear maturation rates in the hormone-supplemented group (94.92 ± 3.15) were significantly ($p < 0.05$) higher than those observed in the control group (61.01 ± 4.23).

Fusion rate of *in vitro* matured oocytes in somatic cell nuclear transfer

The results of the comparison of fusion rates between SCNT utilizing *in vivo* and *in vitro* matured oocytes are shown in Table 1. Among the 116 reconstructed embryos, 85 (73.28%) successfully fused in the *in vivo* group, whereas 14 out of 17 reconstructed embryos (82.35%) successfully fused in the *in vitro* group. No significant difference in the fusion rate was observed between the two groups.

Pregnancy rate and delivery rate

The influence of different oocyte sources on the pregnancy rate with canine cloned embryos was also examined. Following embryo transfer, pregnancy was detected in three out of eight recipients that received cloned embryos in the *in vivo* group (37.5%) and in one out of two recipients that received cloned embryos in the *in vitro* group (50%) (Table 2). Notably, the number of offspring was significantly ($p < 0.05$) higher in the *in vitro* group (28.57%) than that in the *in vivo* group (8.23%). Among the three pregnant recipients in the *in vivo* group, six live puppies and one dead

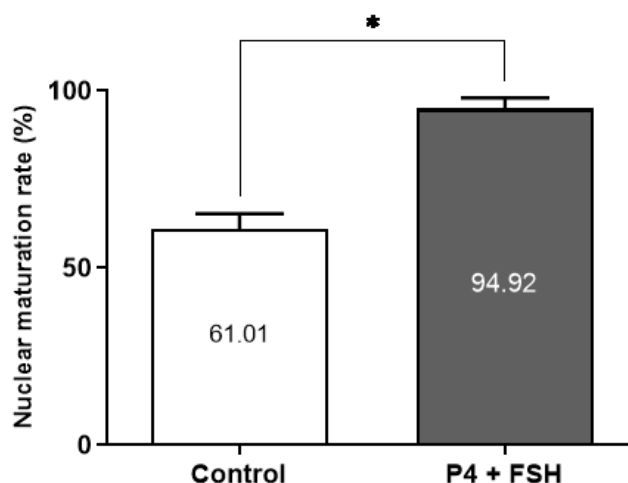


Fig. 1. Effect of hormones combination (P4 + FSH) on nuclear maturation of canine immature oocytes. In the control and hormone groups, 55 and 43 oocytes, respectively, were used for 5 independent replicates. *Significant differences between the control and hormones ($p < 0.05$). FSH, follicle-stimulating hormone.

Table 1. The efficiency of embryo fused by SCNT using *in vitro* or *in vivo* matured oocytes

Group ¹⁾	No. of oocyte donor dogs	No. of oocytes	No. of embryos		Fusion rate (%) ²⁾
			Injected	Fused	
<i>In vivo</i>	15	164	116	85	73.28
<i>In vitro</i>	2	20	17	14	82.35

¹⁾*In vivo*, nuclear matured oocytes with 1st polar body retrieved from oocyte donor dog; *In vitro*, *in vitro* mature oocytes obtained by culturing immature oocytes without 1st polar body retrieved from oocyte donor dogs in IVM medium supplemented with hormone combination for 24 h.

²⁾Percentage calculated from number of injected oocytes.

SCNT, somatic cell nuclear transfer.

Table 2. *In vivo* development of SCNT embryos derived from *in vivo* or *in vitro* matured oocytes

Group ¹⁾	No. of transferred embryo	No. of recipients	No. of pregnancies (%) ²⁾	No. of offspring (%) ³⁾
<i>In vivo</i>	85	8	3 (37.50)	7 (8.23) ^a
<i>In vitro</i>	14	2	1 (50.00)	4 (28.57) ^b

¹⁾*In vivo*, nuclear matured oocytes with 1st polar body retrieved from oocyte donor dog; *In vitro*, *in vitro* mature oocytes obtained by culturing immature oocytes without 1st polar body retrieved from oocyte donor dogs in IVM medium supplemented with hormone combination for 24 h.

²⁾Percentage calculated from number of recipients receiving embryos.

³⁾Percentage calculated from number of embryos transferred.

^{a,b}Values with different superscript in the same column differ from each other ($p < 0.05$).

SCNT, somatic cell nuclear transfer.

puppy were delivered, and the bodyweight of live pups were 112, 109, 121, 124, 103 and 121 g, respectively. Whereas the pregnant recipient in the *in vitro* group delivered three live puppies and one dead puppy, and the bodyweight of live pups were 124, 126 and 111 g, respectively. The necropsy of the dead puppies showed no specific pathological findings. All cloned dogs grew up healthy. Fig. 2 shows one -year-old cloned pups derived from the *in vivo* group (Fig. 2A) and the *in vitro* group (Fig. 2B).

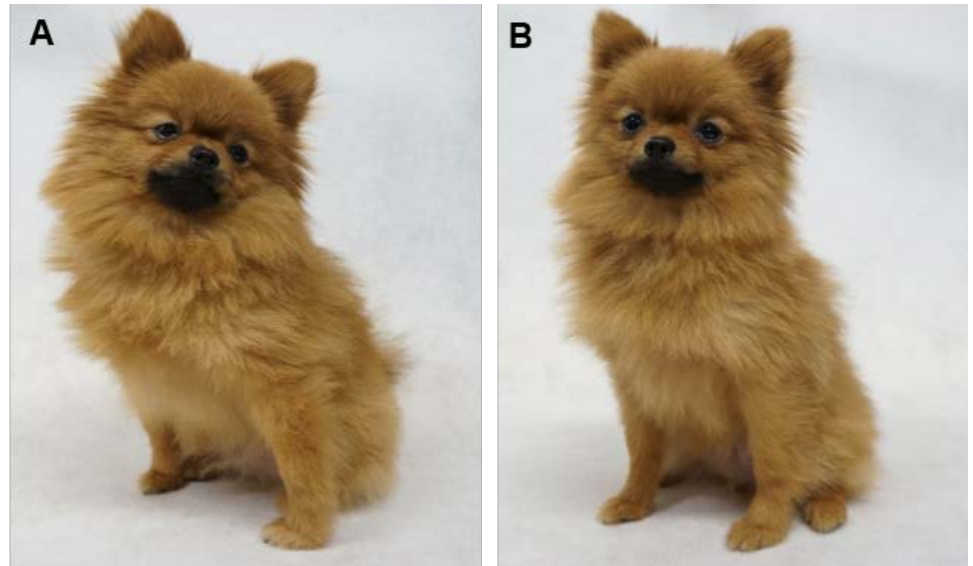


Fig. 2. One-year-old cloned dogs derived from SCNT. (A) Dog cloned using mature oocytes *in vivo*. (B) Dog cloned using mature oocytes *in vitro*. SCNT, somatic cell nuclear transfer.

DISCUSSION

This study aimed to investigate the effect of hormone supplementation (P4 and FSH) in the IVM medium on immature oocytes obtained via oviduct flushing of naturally ovulated female dogs and assess their suitability for SCNT as oocyte donors. Our findings indicate that the groups supplemented with hormones exhibited significantly higher maturation rates than those exhibited by the control groups.

P4 is an intra-follicular steroid hormone that plays critical roles in ovulation, implantation facilitation, and pregnancy maintenance [29]. In canines, P4 production is initiated within the follicle before ovulation, and its levels rise above 1–3 ng/mL during the onset of the preovulatory LH surge and further increase after ovulation [30,31]. Willingham-Rocky et al. reported that supplementation with 200 ng/mL of P4 showed an increased meiotic resumption or maturation rate between groups, though not significantly [26]. Kim et al. reported a positive effect on the percentage of oocytes progressing to the MII stage in oocytes obtained during the follicular stage supplemented with 0.5–2 µg/mL of P4 [22]. Similarly, Vannucchi et al. reported that supplementation with 20 µg/mL P4 in the maturation medium positively affected meiotic resumption /but was insufficient to complete it [25]. Hu et al. found that a combination of 1.0 IU/mL FSH, 5 IU/mL P4, and 5 IU/mL E2 in the supplementation group led to a higher maturation rate of oocytes than that in the control group [24]. In this study, we expected that the immature oocytes after ovulation would have already been sufficiently affected by P4. Therefore, P4 was supplemented to the IVM medium at the minimum concentration maintained after ovulation.

FSH is widely used in IVM protocols for various mammalian species. FSH supplementation induces oocyte maturation [32,33] and is involved in cumulus cell expansion [34]. Hewitt and England reported that the supplementation of gonadotropins such as FSH and LH in the maturation medium for canine IVM resulted in a high maturation rate, though not statistically significant, with 1 µg/mL of FSH supplementation [35]. Kim et al. found that supplementing the maturation medium with 1 IU of FSH improved the maturation rate of canine oocytes [36].

Additionally, Lee et al. reported that supplementing various concentrations of FSH (ranging from 0.5 to 50 $\mu\text{g}/\text{mL}$) in the IVM medium significantly enhanced the expansion of cumulus cells, with the 5 $\mu\text{g}/\text{mL}$ group showing the highest result for canine oocytes [23]. Hence, we adopted a supplement concentration of 5 $\mu\text{g}/\text{mL}$ for FSH.

The combination effect of P4 and FSH on immature oocytes recovered through oviduct flushing after ovulation demonstrated sufficient capacity for meiotic resumption, leading to a higher maturation rate to the MII stage than that reported in other reports. This difference may be ascribed to the method of oocyte recovery. Previous reports on canine IVM mainly employed ovariectomy to recover immature oocytes. However, in this study, we performed the recovery of immature oocytes through oviduct flushing 72 h after the assumed ovulation. Oocytes retrieved through ovariectomy were selected based on morphological classification for good quality [37], whereas oocytes retrieved from the oviduct had been exposed to *in vivo* conditions for a longer duration, at least 72 h, which may have conferred them with higher meiotic competence than that of those recovered by ovariectomy.

The stage of the estrous cycle may have an effect on the maturation rate of oocytes. In this study, all the recovered oocytes were focused on the follicular stage, specifically after ovulation. Interestingly, even at the same follicular stage, immature oocytes recovered from the oviduct after ovulation demonstrated higher meiotic competence than that demonstrated by immature oocytes retrieved directly from the follicles. Previous research has explored the effect of canine IVM across various estrus cycles [22,36,38,39]. Kim et al. reported that the stage of the estrous cycle for oocyte recovery is an important factor for successful meiotic competence of canine oocytes, with high glutathione (GSH) contents observed in *in vitro* matured oocytes collected from the follicular stage and *in vivo* matured oocytes [22,39]. Oh et al. reported similar results, showing a higher proportion of oocytes reaching MI and MII stages, regardless of the stage of the canine serum during the follicular stage [38]. In this study, we exclusively focused on oocytes recovered during the follicular stage, precisely after ovulation. The findings suggest that even at the same follicular stage, immature oocytes obtained from the oviduct after ovulation possess a higher meiotic competence than those retrieved directly from the follicles.

Subsequently, we conducted SCNT to investigate the viability of *in vitro* matured oocytes for cloning purposes. Interestingly, the difference between *in vivo* and *in vitro* oocyte maturation did not affect the fusion rate in SCNT. This result coincides with other reports in mammals [40–42]. Regarding the *in vivo* development of SCNT embryos between the *in vivo* and *in vitro* groups, the pregnancy rate did not show significant differences, but the number of offspring was significantly higher in the *in vitro* group than that in the *in vivo* group. These results suggest that *in vitro* groups have the same feasibility for the full-term development of cloned embryos as *in vivo* groups. Additionally, we successfully produced healthy cloned dogs in both groups. Notably, the cloned dogs in the *in vitro* group represent the first successful production of cloned puppies using *in vitro* matured oocytes.

In conclusion, this study marks the first report of an IVM system for canine immature oocytes recovered *in vivo*, leading to the successful production of cloned puppies using *in vitro* matured oocytes. To date, there have been no reports on the production of live puppies using *in vitro* matured oocytes. These results indicate that immature oocytes recovered *in vivo* hold promise as oocyte donors for SCNT and exhibit comparable full-term developmental potential to *in vivo* matured oocytes. However, it is important to note that the present study solely focused on immature oocytes after ovulation. Further research should consider investigating other estrous stages, which could potentially enhance canine cloning efficiency.

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