



Enhanced *in vitro* maturation of canine oocytes by oviduct epithelial cell co-culture



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ABSTRACT

Canine-assisted reproductive techniques have been successful for several years; however, the lack of an oocyte *in vitro* maturation system has limited their application. The aim of this study was to evaluate the effect of canine oviduct epithelial cells (cOECs) on canine oocyte maturation *in vitro*. Specifically, the method used for isolation of cOECs did not affect the expression of epithelial markers, E-cadherin and cytokeratin, on fresh, cultured and cryopreserved cells. Moreover, BrdU analysis showed that cOECs cultured in Medium 171 supplemented with mammary epithelial growth supplement were more proliferative than counterparts in advanced Dulbecco's modified Eagle medium or Medium 199. Maturation rate of canine oocytes collected from bitches at diestrus was significantly increased when oocytes were co-cultured with either fresh, cultured or frozen/thawed cOECs ($13.23 \pm 1.15\%$, $10.38 \pm 4.89\%$, or $10.54 \pm 2.96\%$, respectively) than that of control oocytes cultured without cOECs ($2.48 \pm 2.16\%$, $p < 0.05$). Additionally, the number of oocytes collected from bitches at estrus the reached metaphase II was increased ~4 fold in co-culture with fresh, cultured, or frozen/thawed cOECs ($47.2 \pm 3.82\%$, $45.4 \pm 7.34\%$, and $46.9 \pm 1.51\%$, respectively) as compared with oocytes cultured without cOECs ($11.9 \pm 3.18\%$, $p < 0.05$). Nuclear maturation was further confirmed by assessing the formation of normal metaphase-II spindles, whereas cytoplasmic maturation was confirmed by inducing parthenogenetic oocyte activation. Embryonic development to the 8-cell stage was similar between *in vivo* and *in vitro* matured oocytes. These results suggested that co-culturing immature canine oocytes with cOECs facilitated canine oocyte maturation and early stages of embryonic development.

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1. Introduction

Somatic cell nuclear transfer has been successfully applied to assisted reproductive techniques in numerous species, including sheep [1], goat, cattle [2], mice [3], pig [4], rabbit [5], camel [6], fox, and dog [7], albeit with limited efficiency. Several *in vitro*-matured oocytes are always necessary to improve the outcomes of animal cloning. For this reason, oocyte *in vitro* maturation (IVM) methods have been widely employed for animal cloning in several species; however, despite substantial research, none has been established for canines to date.

A primary hindrance to canine oocyte IVM is the lack of an

optimized culture medium. When oocytes are recovered from follicles and cultured in media supplemented with antioxidants [8], hormones [9], meiotic inhibitors [10], growth factors [11], or vitamins [12], only ~20% reach metaphase II stage after 24 h–96 h. On the other hand, some studies have tried to directly increase maturation promoting factor activity by gene knockdown [13] or phosphodiesterase inhibition [14], with only weak effects observed.

One reason for this low IVM rate might be attributed to the poor developmental ability of oocytes recovered at anestrus or small follicular-stage ovaries. However, even oocytes harvested from pre-ovulatory follicles show a depressed IVM rate of 32% [15]. The low IVM rate might also stem from the complex reproductive environment of canines. In most mammals, oocytes mature in the ovarian follicle until metaphase II and are then ovulated, whereas canine oocytes are released as germinal vesicles and continue to mature in the oviduct for 1–4 days [16]. A previous co-culture study

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using anestrous oviduct epithelial cells showed no effect on oocyte maturation [17]; therefore, we hypothesized that estrus canine oviduct epithelial cells (cOECs) might convey a positive effect on canine oocyte maturation *in vitro*.

Secretory and ciliated epithelial cells play important roles in oviduct function. Secretory cells produce oviductal fluid rich in amino acids and various factors that provide an optimal microenvironment for sperm capacitation, fertilization, embryonic survival, development, and oocyte maturation [18]. Analysis of trace components in the oviductal fluid is limited due to the poor sensitivity of current techniques and the difficulty of obtaining oviductal fluid from living animals. To overcome this issue, it is essential to culture oviduct epithelial cells on a large scale; however, there is no commercially available system for isolating or culturing these cells.

In general, oviduct epithelial cells have been isolated from the oviduct by mechanical, enzymatic, and mixed methods in rats [19], mice [20], chickens [21], pigs [22], bovines [23], and humans [24]. In these cases, oviducts are collected by surgery or from a slaughterhouse; however, new methods are necessary for canines due to limited organ sources and ethical considerations.

In this study, immature canine oocytes obtained from diestrus- or estrus-stage dogs co-cultured with fresh, cultured, or cryopreserved cOECs, resulted in increased maturation *in vitro*.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Advanced Dulbecco's modified Eagle medium (ADMEM), Medium 199 (M199), Medium 171 (M171), and mammary epithelial-growth supplement (MEGS; Cat. No. S-015-5) were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and specifically approved by the Institutional Animal Care and Use Committee of National Institute of Animal Science (NIAS), Korea (Approval No: NIAS-2015-145). Mongrel bitches from 1 to 5 years of age were bred in the laboratory animal unit, special canine research center of NIAS. After surgical collection of oocytes and cOECs, the donors were housed in a temperature-controlled room with proper darkness-light cycles, fed with a regular diet, and maintained by a veterinarian in the NIAS facility.

2.3. Canine oocyte collection and denuding

For the collection of diestrus oocytes, ovaries were removed from six diestrus dogs by surgery and transported to the laboratory in Dulbecco's phosphate-buffered saline (D-PBS) at 37 °C within 30 min. The ovarian cortex was immediately sliced with a razor blade in HEPES-buffered M199 medium supplemented with 1% fetal bovine serum (FBS). The cumulus oocyte complexes (COCs, $n = 317$) showing uniform cytoplasmic and compact cumulus were selected for experimentation.

Estrus oocyte collection was performed as previously described [25]. Briefly, serum progesterone levels ranging from 6 ng/mL to 15 ng/mL were defined as ovulation [26]. Oocytes were obtained by surgical procedure at 1 (immature, 9 dogs) or 3 days (mature, 6 dogs) after ovulation. Flushing medium (10 mL) was injected into oviducts from the uterus side using a venoclysis needle, and the flushing medium containing oocytes was recovered from the ovary bursa split open using negative pressure. Only oocytes with more

than three cumulus-cell layers were collected for culture and analysis. Total 132 immature oocytes and 71 mature oocytes were collected for experiment. To determine oocyte stage, cumulus cells were denuded before or after culture by repeated pipetting in HEPES-buffered M199 medium using a fine-flame, mouth-operated micropipette with an inner diameter slightly larger than that of the oocytes.

2.4. Mechanical methods and *vivi*-oviduct flushing for cOEC isolation, culture, and cryopreservation

Oviducts used for mechanical cOEC isolation were obtained from three healthy bitches that underwent tubal ligation at local veterinary clinics. Following isolation, oviducts were transferred to the lab within 20 min, and oviduct tissue was cut into small pieces and washed with D-PBS containing 0.1% polyvinyl alcohol (PVA-PBS) by centrifugation at 420g for 5 min.

Total 15 bitches were used for *vivi*-oviduct flushing methods. Each replicate consist of one single dog. The *Vivi*-oviduct flushing methods were modified according to a previously report [27]. Briefly, a gastric needle was inserted into the oviduct bursa from the bursa split, and 10 mL flushing medium (HEPES-buffered M199 containing 10% FBS and 1% penicillin/streptomycin) was injected into the oviduct from the uterus side of the oviduct using a venoclysis needle. The flushing medium containing cOECs was collected from the ovary bursa and separated using a 40- μ m cell strainer, followed by washing with PVA-PBS by centrifugation at 420g for 5 min to remove blood and small tissue fragments.

The cOEC fragments used for cell culture, cell passage, BrdU assay and cryopreservation experiment, were treated with treated with 5 mg/mL collagenase type IV for 5 min at 37 °C, washed with D-PBS and single cells were dissociated with TrypLE Express (Gibco, Grand Island, NY, USA) for 5 min at 37 °C. Single cells were washed with 0.5% bovine serum albumin (BSA-PBS) and cultured in different tissue culture medium according to experimental design.

For cryopreservation, singularized cells were mixed with Syntha-Freeze CTS (Gibco) at room temperature to final concentration of viable cells in the range between 10^6 and 10^7 cells/mL. The cells were then aliquoted into CryoTube™ (Nunc, Rochester, New York, USA) and stored in NALGENE™ Cryo Freezing container (Nunc) at -70 °C for 24 h. The cells were further stored in -196 °C in LN₂ tank.

For cell passage, cOECs fragments were cultured in 171 tissue culture media supplemented with 1% MEGS on 35 mm collagen coated dishes, until cells reached 100% confluence. Cells were then dissociated with TrypLE Express for 5 min at 37 °C, washed three times with 171 tissue culture media and centrifuged at 420 g for 5 min cell plates were re-suspended and plated on 4-well dishes with to a concentration of 5×10^6 cells/well. Cells were passaged four times.

For co-culture after flushing, 20 cOECs fragments were cultured in wells of 4-well dish with Medium 171/1% MEGS for 3 days until confluence. After culturing, cells were washed with IVM medium three times and made ready for use. All experiments were repeated three times.

2.5. Immunofluorescence and laser-scanning confocal microscopy

Oocytes or cOECs were washed three times in PVA-PBS, fixed in 4% formaldehyde for 20 min at room temperature, and permeabilized with PVA-PBS containing 0.5% Triton X-100 at 38.5 °C for 1 h in a humidified incubator. The fixed cells were then blocked in 3.0% BSA for at least 30 min. For immunostaining of epithelial-cell markers, cells were incubated with antibodies to E-cadherin (sc-1500; Santa Cruz Biotechnology, Dallas, TX, USA) or cytokeratin

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