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Birth of viable female dogs produced by somatic cell nuclear transfer

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Abstract

Since the only viable cloned offspring born in dogs was a male, the purpose of the present study was to produce female puppies by somatic cell nuclear transfer (SCNT). Adult ear fibroblasts from a 2-month-old female Afghan hound were isolated and used as donor cells. In vivo-matured canine oocytes surgically collected (approximately 72 h after ovulation) from the oviducts of 23 donors were used for SCNT. After removal of the cumulus cells, oocytes were enucleated, microinjected, fused with a donor cell, and activated. A total of 167 reconstructed SCNT embryos were surgically transferred (Day 0) into the oviducts of 12 recipient bitches (average 13.9 embryos/recipient, range 6–22) with spontaneous, synchronous estrous cycles. Three pregnancies were detected by ultrasonography on Day 23, maintained to term, and three healthy female puppies (520, 460, and 520 g), were delivered by Caesarean section on Day 60. These puppies were phenotypically and genotypically identical to the cell donor. In conclusion, we have provided the first demonstration that female dogs can be produced by nuclear transfer of ear fibroblasts into enucleated canine oocytes.
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Keywords: Female cloned dogs; SCNT; In vivo oocyte; Embryo transfer; Pregnancy

1. Introduction

Assisted reproduction technologies (ARTs) are less developed in the dog than in domestic livestock. Unique species-specific reproductive characteristics, i.e. monoestrual, polyovulatory and non-seasonal reproductive cycle, combine to increase the degree of difficulty and decrease the level of success. In contrast to most mammals that ovulate mature oocytes at the metaphase II stage, dogs ovulate immature oocytes at the germinal vesicle stage and the oocytes undergo a 48–72 h period of postovulatory maturation in the oviduct. Despite many studies focused on establishing a suitable system for in vitro maturation (IVM) of canine oocytes, efficiency is lower than that of other mammalian species [1–4].

To obtain mature oocytes for somatic cell nuclear transfer (SCNT), the most useful approach currently is the surgical collection of oocytes matured in vivo. However, collection of in vivo canine oocytes by a surgical approach, i.e. salpingectomy or flushing

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oviducts, is technically difficult because the oviduct wall is thin and the lumen is small. Similarly, laparoscopic oocyte retrieval by follicle aspiration was not feasible due to the presence of the ovarian bursa. Although in vitro maturation techniques can be used in some species to generate large numbers of mature oocytes, the number of in vivo matured canine oocytes that have been collected from oviductal flushing ranged from 7 to 10 oocytes per female [5].

The first cloned dog, a male named ''Snuppy'', was born from in vivo matured oocytes [6] since the necessary numbers of in vitro-matured oocytes could not be produced [7–10]. Thus far, ''Snuppy'' has not shown morphologically detectable abnormalities. The ultimate assessment of the normality of reproductive traits, such as libido, in cloned male dogs is mating with females produced either by natural mating or by cloning, to determine if pregnancies and live puppies can be produced. Therefore, the objective of the present study was to produce live cloned female dogs derived from SCNT using fibroblasts and enucleated in vivo-matured oocytes.

2. Materials and methods

2.1. Care and use of animals

In this study, a total of 35 mixed-breed female dogs (23 oocyte donors and 12 recipients) from 1 to 5 years of age were used as oocyte donors and embryo transfer recipients. Facilities for dog care and the procedures done met or exceeded the standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University. The study was conducted in accordance with recommendations described in ''The Guide for the Care and Use of Laboratory Animals'' published by Seoul National University.

2.2. Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.3. Recovery of in vivo matured oocytes

Beginning on the first day of spontaneous estrus, dogs were examined daily for vulvar swelling and serosanguinous discharge, and blood samples (3–5 mL) were collected and serum progesterone concentrations were determined with a DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., TX, USA). The day of ovulation was considered as the day that serum progesterone concentration reached 4.0–7.5 ng/mL [6]. Approximately 72 h after ovulation, oocytes were retrieved by laparotomy (using aseptic surgical procedures). Anesthesia was induced with 6 mg/kg ketamine HCl and 1 mg/kg xylazine, and general anesthesia was maintained with 2% isoflurane. While in dorsal recumbency, recipients were aseptically prepared for surgery and a mid-line ventral incision was made to expose the reproductive tract. 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; Fig. 1). The needle was held in place by a surgical ligature, which was tied using a quick-release device using 3 cm of plastic tube (diameter, 2 mm) and hemostatic forceps. To improve visualization of the oviductal lumen, digital pressure was applied to the lower portion of the isthmus of the oviduct (near the uterotubal junction), an intravenous catheter (24 gauge) was inserted, and 10 mL of Hepes-buffered TCM-199 (cat no. 11150-059, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM $NaHCO_3$ and 5 mg/mL BSA was introduced into the oviduct through the catheter and collected through the needle. In vivo-matured oocytes obtained from oviductal flushing were transported to the laboratory for SCNT within 10 min.

2.4. Donor cell preparation for somatic cell nuclear transfer

Adult fibroblasts were isolated from an ear skin biopsy of a female Afghan hound, 2-month-old. The small pieces of tissue were washed three times in D-PBS, minced with a surgical blade and dissociated in

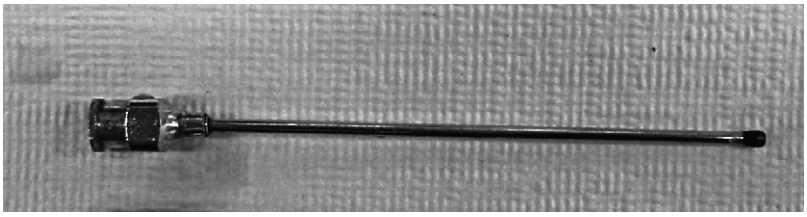

Fig. 1. Inverted flanged bulb steel needle (18 gauge, 7.5 cm) used for recovering canine oocytes from the oviduct.

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