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Influence of somatic cell donor breed on reproductive performance and comparison of prenatal growth in cloned canines

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ABSTRACT

Using *in vivo*–flushed oocytes from a homogenous dog population and subsequent embryo transfer after nuclear transfer, we studied the effects of donor cells collected from 10 different breeds on cloning efficiency and perinatal development of resulted cloned puppies. The breeds were categorized into four groups according to their body weight: small (≤9 kg), medium (>9–20 kg), large (>20–40 kg), and ultra large (>40 kg). A total of 1611 cloned embryos were transferred into 454 surrogate bitches for production of cloned puppies. No statistically significant differences were observed for initial pregnancy rates at Day 30 of embryo transfer for the donor cells originated from different breeds. However, full-term pregnancy rates were 16.5%, 11.0%, 10.0%, and 7.1% for the donor cells originated from ultra-large breed, large, medium, and small breeds, respectively, where pregnancy rate in the ultra-large group was significantly higher compared with the small breeds ($P < 0.01$). Perinatal mortality until weaning was significantly higher in small breeds (33.3%) compared with medium, large, or ultra-large breeds where no mortality was observed. The mean birth weight of cloned pups significantly increased proportional to breed size. The highest litter size was examined in ultra-large breeds. There was no correlation between the number of embryo transferred and litter size. Taken together, the efficiency of somatic cell cloning and fetal survival after embryo transfer may be affected significantly by selecting the appropriate genotype.

1. Introduction

For thousands of centuries, the domestic dog has been perceived as human's friend [1–3]. Domestic dogs evolved through a mutually beneficial relationship with humans, sharing living space and food resources. As a result, a number of diseases including cancers, blindness, heart disease, cataracts, epilepsy, hip dysplasia, and deafness are commonly seen in both canine and human populations, and clinical manifestations are often similar [2]. Kirkness, et al. [4] reported that more than 370 different genes in

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dogs share their mechanisms in diseases and dysfunctions with humans. This makes canines as an extremely important species for an animal research model for studying human diseases. However, many of these similarities with human disorders are restricted to a particular breed or a group of breeds by the underlying genetic variation of loci affecting phenotypic traits.

The domestic dog varies remarkably in size and conformation, resulting from intensive artificial selection throughout the history [5]. Their size varies from 1 kg for toy breeds to 90 kg for large breeds like the mastiff [6]. With these morphologic variations, differences in female reproductive parameters are also substantial in the dog family. A number of studies suggested that the mean litter size increased in accordance with the size of the breed [7–10]. A large-breed dog can give birth to a higher number of puppies. The relative size of the newborn compared with the small-breed bitch is larger than those of large-breed bitch regardless of biological factors such as limited space in the uterus.

Reproductive outcomes in artificial reproductive techniques are influenced by a complex process that consists of sequential maturation events of the oocyte, fertilization, embryo growth, and maternal recognition of pregnancy [11]. Among those factors, the type of donor nuclei is the one of the most important factors determining cloning efficiency in nuclear transfer. Fetal fibroblasts have been widely accepted to be the most selective donor nuclei for industrial animal cloning because of amenability to introduction of transgene, high efficiency of blastocyst development, and high embryo quality after nuclear transfer [12,13]. However, application of fetal cells has been limited to production of transgenic models. As donor nuclei for nuclear transfer, adult fibroblast with known identity and genetic background are valuable in preserving and conserving for future purpose.

Although the application of assisted reproductive techniques to dog species has been difficult to achieve because of peculiar reproductive characteristics, somatic cell nuclear transfer (SCNT) in dogs has been successful. An innovative technique for ovum flushed out from the oviduct allowed the production of cloned dogs [14]. A number of recent studies using *in vivo* oocyte collection protocols have been reported for successful generation of cloned small [15], medium [16], and large breed dogs [17], and production of one cloned dog from somatic cells that had been cryopreserved for a long period of time [18]. Recently, our previous studies have shown that dog ooplasm is a compatible host for somatic cell nuclei from very closely related species such as coyotes, and reconstructed coyote–dog interspecies embryos could undergo normal development to term after embryo transfer to surrogates dog [19]. Furthermore, we previously developed a transgenic dog model overexpressing Phosphoenolpyruvate carboxykinase (PEPCK) gene in the liver [20].

The relatively limited application of SCNT in canids is a consequence of the inappropriate culture condition for oocyte maturation and IVC of early preimplantation embryos. Moreover, reconstructed embryos using IVM oocytes were transferred to the oviducts of recipients immediately after nuclear transfer and activation. Therefore, prescreening developmental competence of cloned embryos during preimplantation period is deemed impossible. The aim of this study was to evaluate the influence of different breeds as somatic cell nuclear donors on reproductive performance after transferring reconstructed embryos into homogenous surrogates and comparing the perinatal growth of cloned puppies.

2. Materials and methods

2.1. Animals

From January 2009 to December 2011, 52 pregnancies in 454 bitches with transferred, cloned embryos were included in this study with adult somatic cells from Tibetan Mastiff (n = 2), Great Pyrenees (n = 1), Golden Retriever (n = 2), Labrador Retriever (n = 2), Greyhound (n = 1), German Shepherd (n = 2), Beagle (n = 2), Jindo Dog (n = 2), Boston Terrier (n = 1), and Pomeranian (n = 1). Donor breeds were categorized according to body weight (BW) at the time of biopsy as the following: small (≤9 kg), medium (>9–20 kg), large (>20–40 kg), and ultra large (>40 kg).

The female mixed breed dogs for surrogates, between the ages of 1 and 7 (BW 20–25 kg), were housed singly in an indoor kennel. To avoid biases from the maternal implication, all oocyte donors and surrogates were selected from the homogenous population, showing normal estrus cycle and providing approximately eight to 11 oocytes per each cycle [21]. They were fed standard commercial dog food once a day, and given water *ad libitum* in accordance with the animal study guidelines of Sooam Biotech Research Foundation's Accreditation for Laboratory Animal Care.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), unless otherwise stated.

2.3. Preparation of donor cell

Adult fibroblasts were obtained from biopsies under the owners' consent. The tissue samples measured approximately 1 × 3 cm, and were collected under light tranquilization and local anesthesia. Sections of the subcutaneous tissues were cut into small (approximately 1 mm^2) pieces and cultured in tissue culture medium at 37 °C in atmosphere of 5% CO_2 and air to obtain fibroblasts. Explants were maintained in culture until they approached 90% confluency. Cells were then trypsinized and reconstituted at concentrations of approximately 1 × 10^6 cells per mL and cryopreserved in cryovials containing Dulbecco's Modified Eagle Medium (DMEM) with 10% of dimethylsulfoxide (DMSO).

2.4. Laparotomy and collection of oocytes

The estrus of bitches was followed weekly by observing vulval bleeding to detect the onset of heat period, during which a blood sample (2 mL) was collected everyday at the same time by cephalic venipuncture, and serum progesterone levels were assayed by using Cobas E411 (Roche Diagnostics). The oocyte retrieval and cloned embryos transfer were performed under general surgical procedures