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Semen characteristics of genetically identical male cats cloned via somatic cell nucleus transfer

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Abstract

We investigated the sperm characteristics of four cloned male cats (*Felis catus*) to assess their reproductive potential. Fresh and frozen-thawed sperm were assessed for motility, viability, and morphology, and their functional competence was evaluated by in vitro fertilization (IVF) of domestic cat oocytes. All fresh semen characteristics varied among cats and collection times. Sperm concentration ($\times 10^6$/mL) of Cat A (512 ± 140, range 368 to 685) was significantly higher, whereas that of Cat C (335 ± 92, range 274 to 469) was significantly lower than that of Cloned B (459 ± 159, range 336 to 510) and control cats (680 ± 452, range 360 to 479). After thawing, motility and progressive motility of sperm from Cat B were significantly lower than that of the other cloned and control cats. The curvilinear, straight line, and average path velocities of sperm from Cat B were significantly higher, whereas the straightness was lower, than that of the other cloned and control cats. Frozen sperm from Cats A, B, and C successfully fertilized oocytes (cleavage = 74.4%, 71.4%, and 86.2%, respectively) and produced embryos that developed to the blastocyst stage after IVF/*In vitro* culture (IVC) (34.4%, 26.7%, and 48.0%) at frequencies similar to the cleavage rate (82.0%) and blastocyst rate (43.9%) obtained with sperm from the control male. In conclusion, seminal characteristics of cloned male cats did not differ markedly from those of our noncloned, control male cats.

Keywords: Cloned cat; Cloning; In vitro fertilization; Semen cryopreservation; Sperm characteristics

1. Introduction

The preservation of endangered wild animals is extremely important, as just one endangered species can disrupt an entire ecosystem [1]. Thirty-six feline species are currently listed as endangered, threatened, or vulnerable in all or part of their ranges [2]. Several reproductive biotechnologies, including artificial insemination (AI), embryo transfer, in vitro fertilization (IVF), gamete/semen cryopreservation, and somatic cell nucleus transfer (SCNT), are used for preservation of endangered and threatened wild species [1]. These techniques have also been successfully applied to nondomestic feline species [3–7].

We previously successfully produced cloned male cats using SCNT [8] and demonstrated their fertility [9]; however, their semen characteristics were not examined. Fertility and semen characteristics of other cloned species have been demonstrated to be

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normal [10,11]. Therefore, the purpose of this report was to investigate semen characteristics of cloned cats. Frozen-thawed sperm were assessed for motility, viability, and morphology, and their functional competence was evaluated by IVF of domestic cat oocytes.

2. Materials and methods

2.1. Source of reagents and institutional approval

All chemicals used were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated.

2.2. Animal housing and care

Three cloned male cats (Turkish Angora), A, B, and C, two controls (noncloned) male cats, and four female cats were used. The domestic female cats were used as ''teaser queens'' for semen collection. All male cats were housed individually in stainless steel cages measuring $1.8 \times 0.7 \times 0.65$ m and fed with dry food and water available ad libitum. All cats were maintained in a controlled room at 21 ± 2 °C, with a 14-h light:10-h dark photoperiod, and light onset at 0600. At the start of the project, the four cloned male cats were 59 and 61 wk of age, and their average weight was 4.9 ± 0.3 kg. Cats used were cared for in our facility, using procedures that exceeded the standards established by the Gyeongsang National University Association for the Accreditation of Laboratory Animal Care.

2.3. Semen collection

Semen was collected from cloned male cats and controls using an artificial vagina (AV), as described previously [12,13]. Briefly, we used a teaser queen in estrus induced by means of 100 IU equine chorionic gonadotropin (eCG; Daesung Microbiology Lab Inc., Seoul, Republic of Korea). Semen was collected from each male once each week for 1 mo. Ejaculated semen was immediately transferred to the laboratory for assessment. Semen samples that were contaminated with urine or blood were discarded.

2.4. Assessment of semen quality

We evaluated semen volume, pH, osmolality, concentration, viability, and morphology of fresh and frozen-thawed semen. The semen volume was measured by micropipette. Sperm concentration and total number of sperm were measured by hemocytometer, and osmolality was measured with a micro-osmometer (model 3300; Advanced Instruments, Norwood, MA, USA). Sperm samples were placed on a prewarmed microslide, and motility was evaluated using a phase-contrast microscope at $\times$ 400. To analyze membrane integrity (viable), samples were stained using a 5% eosin B solution (1 part sample to 1 part stain). One drop of stained sample was placed on a microslide, overlaid with a coverslip, and viewed with bright-field microscopy. Sperm were classified into two groups: membrane damaged (dead, head stained pink) and membrane intact (live, head not stained).

Diff-Quik (SYSMEX Co., Kobe, Japan) stain kits were used to assess morphologic abnormalities of fresh and frozen-thawed sperm, as previously described [14]. Stained slides were air-dried, and head, midpiece, and tail abnormalities were viewed and photographed with light microscopy at $\times$ 400, and abnormalities subsequently determined by computer. Total motility, progressive motility, and viability of frozen-thawed sperm were measured by the Hamilton-Thorne computer-associated sperm analysis (HTR Ceros 12.1; Hamilton Thorn Research Inc., Beverly, MA, USA), as described previously [15].

2.5. Semen cryopreservation

The collected semen was washed by Dulbecco's Phosphate Buffered Saline (D-PBS) and centrifuged at $700 \times g$ for 5 min to remove seminal plasma. Sperm pellets were diluted with Tris buffered extender containing 20% egg yolk (Extender 1) [16]. After dilution, samples were cooled to 4 °C over 2 h and then diluted with Extender 2 containing 8% glycerol for 1 h. After dilution, the sperm sample was immediately loaded into a 0.25-mL straw (1.0×10^7 sperm/straw) and laid 5 cm above liquid nitrogen for 15 min. The straw was then put into the liquid nitrogen for cryostorage until used for examination or IVF.

2.6. Assessment of acrosomal integrity

For analysis of acrosomal integrity, we used the double staining technique with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and propidium iodide (PI; Molecular Probes Inc., Eugene, OR, USA) as previously described [17,18]. Briefly, frozen-thawed sperm sample was smeared on microslide glass, air-dried, and slides were dipped in 95%