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Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and *in vitro* matured domestic cat oocytes

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ABSTRACT

Recent accomplishments in the field of somatic cell nuclear transfer (SCNT) hold tremendous promise to prevent rapid loss of animal genetic resources using *ex situ* conservation technology. Most of SCNT studies use viable cells for nuclear transfer into recipient oocytes. However, preparation of live cells in extreme circumstances, in which post-mortem material of endangered/rare animals is improperly retained frozen, is difficult, if not impossible. This study investigated the possibility of interspecies-SCNT (iSCNT) in Asiatic cheetah (*Acinonyx jubatus venaticus*), a critically endangered subspecies, using nuclei derived from frozen tissue in absence of cryo-protectant at −20 °C and *in vitro* matured domestic cat oocytes. No cells growth was detected in primary culture of skin and tendon pieces or following culture of singled cells prepared by enzymatic digestion. Furthermore, no live cells were detected following differential viable staining and almost all cells had ruptured membrane. Therefore, direct injection of donor nuclei into enucleated cat oocytes matured *in vitro* was carried out for SCNT experiments. Early signs of nuclear remodeling were observed as early as 2 h post-iSCNT and significantly increased at 4 h post-iSCNT. The percentages of iSCNT reconstructs that cleaved and developed to 4–16 cell and morula stages were 32.3 ± 7.3, 18.2 ± 9.8 and 5.9 ± 4.3%, respectively. However, none of the iSCNT reconstructs developed to the blastocyst stage. When domestic cat somatic and oocytes were used for control SCNT and parthenogenetic activation, the respective percentages of oocytes that cleaved (51.3 ± 13.9 and 77.3 ± 4.0%) and further developed to the blastocyst stage (11.3 ± 3.3 and 16.8 ± 3.8%) were comparable. In summary, this study demonstrated that enucleated cat oocytes can partially remodel and reactivate non-viable nuclei of Asiatic cheetah and support its reprogramming back to the embryonic stage. To our knowledge, this is the first report of iSCNT in cheetah using non-viable frozen cells.

1. Introduction

Present form of biodiversity on Earth is the legacy of about 3.5 billion years of evolution [1]. However, the last century has witnessed a rapid decline in biodiversity due to increased human activities [2]. Therefore, the current status of global diversity is facing crisis and threaten a considerable number of species to extinction [3].

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In situ conservation activities may play an indispensable key role in protection and maintenance of animal genetic bio-diversity [4]. In this regard, recent accomplishments in field of somatic cell nuclear transfer (SCNT) has lead to successful production of cloned offspring in more than 20 species. SCNT can be utilized to multiply species if they do not reproduce naturally and if other, more conventional, assisted reproductive techniques cannot be used successfully [5–7].

Successful cloning of a given animal species depends on the access to good sources of donor cells, recipient oocytes and surrogate mothers of the same species [8] or closely related species [2]. In this sense, the cloning of extinct species appears unachievable as live cells are not available in salvaged dead animals, frozen in permafrost for prolonged periods. Furthermore, the genetic integrity of cells is lost in this state [9]. Moreover, provision of recipient oocytes and surrogate mothers in the cases of endangered/extinct species is almost impossible [10].

Notwithstanding the aforesaid limitations, four recent studies reported successful production of cloned offspring [9,11,12] and blastocyst [13] using nuclei donor cells form frozen tissue kept at very low temperatures (−20 to −80 °C) for prolonged periods (1–16 years) without any cryoprotectant. Thereby, indicating that some cells within frozen tissue may maintain their complete genomic integrity for prolong period in absence of cryo-protecting agents. On the other hand, inter or sub-species-SCNT (iSCNT) have been successfully used for production of cloned offspring in wild cat [14], gray wolf [15], gaur [16], European mouflon [17] and Pyrenean ibex (the offspring died after caesarean section) [18] using oocytes and surrogate mothers from closely related species and sub-subspecies. With respect to these advancements in the field of *ex situ* conservation biology, scientists hope that iSCNT could be broadly used for cloning other endangered and even extinct animal species.

Cheetah (*Acinonyx jubatus*) is a large cat and also is the only present member of genus Acinonyx [19]. Although all six subspecies of cheetah are presently exist at incredibly low densities, the Asiatic cheetah (*Acinonyx jubatus venaticus*), also known as the "Iranian cheetah", is one of rarest feline and is now considered as a critically endangered subspecies by IUCN red-list owing to habitat, road accidents and persecution killings and poaching [20,21], with road accidents accounting for 40% of deaths [21]. The current total number of Asiatic cheetah has been estimated between 70 and 110 individuals [19]. At 3/6/2010, a female cheetah was found dead due to road accident in Yazd protected area, Iran (Fig. 1). The corpus was maintained in a −20 °C freezer for 10 days before sampling. This study investigated whether viable and culturable somatic cells could be retrieved from the corpus frozen without cryo-protectant. Subsequently, the feasibility of iSCNT using cheetah somatic cells as nuclei donor cells and *in vitro* matured cat oocytes as recipient cytoplasm was investigated.

Fig. 1. The Asiatic cheetah. The picture of the dead Asiatic cheetah which was found dead due to road accident. The skin sample was obtained ten days later when the corpus was kept at −20° Celsius.

2. Material and methods

Unless specified, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively. All animal care and surgical procedures were undertaken in strict accordance with the approval of the Institutional Review Board and Institutional Ethical Committee of Royan Institute.

2.1. Preparation of donor cells

2.1.1. Cheetah donor cells

At 3/6/2010, the corpus of a 2- to 3-y-old female Asiatic cheetah was found near a road by environment rangers of Yazd protected area, Iran (Fig. 1). The corpus was maintained in a −20 °C freezer for 10 days. A 1 × 1 cm^2 skin area of metatarsus was shaved and cleaned and a cubic-shaped incision was made on the skin of frozen corpus using a scalpel. The scalped skin was peeled off from subcutaneous loose connective tissue, placed in separate tubes containing Dulbecco's modified Eagle medium F-12 (DMEM/F-12) on ice and transferred to the laboratory within 10 h. The procedure of cell culture was carried out as described previously [22] with the exception that the initial procedures of tissue preparation and mincing were carried out on ice using cooled (4 °C) medium. Briefly, tissues were thoroughly washed in phosphate buffer saline (PBS) supplemented with 2 mM GlutamaxII (Invitrogen, USA), penicillin, streptomycin and fungizone. Tissues were minced to small pieces (about 1 mm^2) in supplemented PBS using a scalpel. The prepared pieces were used for cell culture using two methods, i) direct explant culture and ii) culture following enzymatic tissue digestion. The procedure of direct explant culture was carried out as described previously [22]. Briefly, skin pieces were cultured in 6-well dishes (Greiner CELLSTAR®, Austria) containing DMEM/F-12 with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin at 38 °C in a humidified atmosphere of 5% CO_2 in air. The cultures were assessed for contamination every day and for evidence of proliferating cells from day 7 onwards. The procedure used for enzymatic digestion was according to Wakayama et al. [11] with minor modifications. Briefly, skin pieces were transferred into 10 mL centrifuge tubes containing 5 mL of nuclear isolation medium (NIM) consisted of DMEM/F-12 with 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/mL collagenase type I. Then, tubes were incubated for 60 min at 37 °C in a shaker water bath. The enzymatic digestion was stopped by addition of 10% FCS. The cell suspension was filtrated through a 250 μm nylon mesh and the filtrate was centrifuged in NIM (250 × g for 5 min). The cell pellet was then used for cell culture (as above) or freezing as described by Jafarpour et al. [22]. In brief, cell pellet was diluted at 1 × 10^6 cells/mL in 2 mL cryotubes (Nunc, Switzerland) containing cooled (4 °C) DMEM/F-12 with 3 mM EDTA, 0.5 mM PMSF, 50% FCS, and 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen until a subsequent use.

2.1.2. Cat donor cells

Ear skin biopsies were obtained of a 2- to 3-y-old male cat that was brought to a veterinary clinic for routine orchiectomy by the environment office of municipality (Tehran, Iran). Domestic cat