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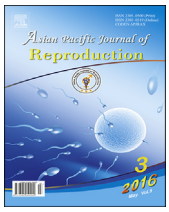

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Establishment, characterization and cryopreservation of Fars native goat fetal fibroblast cell lines

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ABSTRACT

Objective: To biologically develop and evaluate the caprine fetal fibroblast cell cultures before and after freezing.

Methods: Goat fetuses (ages 51, 53 and 55 d) were collected from slaughterhouse. Their skin was cut into small pieces (1 mm^3) and cultured in DMEM and FBS. When reaching 80%–90% confluence, cells were passaged. Cells of the 8th passage were cultured in 24-well plates (1.5×10^5 cells/well) for 9 d and three wells were counted every day. The average cell counts at each time point were plotted against day number and the population doubling time (PDT) was determined. Then, 42 vials of cells (2×10^6 cells/mL) were frozen. Samples were thawed and cultured after 1 month. Cell viability and PDT were evaluated after thawing.

Results: After eight passages, the goat fetal fibroblast cells had a latent phase of about 48 h and after an exponential phase, cells entered the plateau phase on day 5. Before freezing, PDT was about 22 h and after thawing it was about 28 h.

Conclusions: The goat fetal fibroblast cell culture can be established using the adherent culture method and can be cryopreserved, too. After thawing, growth and viability indices of these cells were acceptable.

1. Introduction

The variability of animal genetic resources is an important determinant of maintenance of biodiversity in farm livestock species. If these genetic resources are not protected from the extinction, not only they will be lost forever, but also research focused on the thorough explanation of biological mechanisms underlying proliferative activity, genetic stability, replicative senescence, physiological aging of cultured nuclear donor somatic cells and the subsequent epigenetic reprogramming of their cell nuclei both in the oocytes reconstructed by somatic cell nuclear transfer (SCNT) and in the resultant cloned embryos will have not been completed. Therefore, there is an urgent need to start protecting of endangered animals [1]. The present practical options for *ex situ* and *in vitro* conservation of endangered species are the protection of individual animals, semen cryopreservation, embryo, or oocyte freezing and vitrification, ovarian and testicular slices cryopreservation, whole ovary cryopreservation, somatic cells cryopreservation (as cell culture or as tissue slices and up to whole animal), stem cell cryopreservation and genomic libraries. These gametes, cells, and tissues freezing can only be performed for a limited number of species and needs customized techniques for each species [2]. Somatic cells cryopreservation is an alternative option for maintaining of genetic diversity in endangered animals *in vitro* [3]. In addition, cloning techniques have been developed for conservation of animal genetic materials using somatic cells as an attractive resource [4]. The development of

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somatic cell cloning technology in farm livestock species and the establishment of somatic cell banking for the purposes of recovery of endangered mammalian breeds and species threatened with extinction appear to be especially important. For each animal, tissue samples can be frozen and stored in liquid nitrogen as a method of choice for the rapid establishment of emergency cell banks.

The development of fibroblast cell banks, particularly for endangered species can provide an excellent resource for biological research and preserve valuable genetic materials [5]. Fibroblasts have been cultured from different species and tissues and have various applications including feeder layer of embryonic stem cells, nuclear transfer in cloning, tissue engineering, and wound healing researches. Isolation of ear marginal or fetal skin fibroblasts using adherent culture have been established for some species to develop fibroblast cell bank. Fibroblast cell banks establishment have been reported for some ruminant breeds such as Simmental cattle [6], Luxi cattle [7], Ujumqin sheep [8], Texel sheep [9], Mongolian sheep [10], Jining black grey goat [5,11], Taihang black goat [12], Liaoning cashmere goat [13], and Cashmere goat [14], as well as for laboratory animals such as guinea pig [15].

Goat is an important livestock species contributing to milk, meat and wool production [16]. Initial goat domestication is documented in the highlands of Zagros Mountains, Iran, at 10 000 calibrated calendar years ago [17]. Iran is one of the ten countries in goat keeping in the world with 25.7 million heads. About 30% of all goats in Iran are kept in Fars Province by migrating nomads and villagers [18]. Southern Zagros Mountains cover Fars Province. To preserve this valuable genetic resource, establishment of fibroblast banks have been proposed as a practical method. The purpose of this study was the establishment and *in vitro* evaluation of fibroblast cultures from skin of goat fetus.

2. Materials and methods

2.1. Fetus collection and skin preparation and culture

Six gravid uteruses of Fars native goat were collected from Shiraz Slaughterhouse, Iran and transported on ice to the laboratory. Seven fetuses (5 single and 1 twins) were dissected out using sterilized scissor and forceps. Sex of fetuses was visually determined. Linear measurement of the crown-rump length (straight distance between the occiput and the distal end of os coccygeus) to the nearest mm was recorded. Fetal age was estimated using the following equation [19]:

$$Y = \sqrt[2.49]{\frac{X}{0.002\,8}}$$

where X = crown-to-rump length (mm) and Y = age (d).

Fetuses were washed 4 to 5 times in sterile phosphate buffered saline (PBS; Gibco, cat. no. 18912-014, UK) containing 1% penicillin and streptomycin (Sigma cat. no. P-4687 and S-1277, St. Louis, USA). Slices of fetal skin were removed using sterilized forceps and were cut into small pieces (1 mm^2). Skin pieces were cultured in 88% Dulbecco's modified Eagles medium (DMEM; Gibco cat. no. 12800-116) containing 10% fetal bovine serum (FBS; Gibco, cat. no. 10270-106), 1% penicillin and streptomycin, and 1% L-glutamine (Sigma cat. no. G5840) and were cultured at 37 °C in an incubator with 5% CO_2 and saturated humidity. The medium was replaced after 48 h. When fibroblast cells reached 80%–90% confluence, the cells were harvested using 0.25% trypsin (Gibco cat. no. 15090-046). Fetal goat fibroblasts were passaged 8 times.

2.2. Cryopreservation and reseeding

In each passage, cells at the logarithmic growth phase were collected and counted with a hemocytometer, and then resuspended in freezing solution containing 10% dimethyl sulfoxide (DMSO; MP Bio cat. no. 196055) and 90% FBS, at a density of 2×10^6 cells/mL. The cell suspension was aliquoted into sterile plastic cryovials that were labeled with the fetus number, sex, freezing serial number, and the date. The vials were sealed and kept at −20 °C for 60 min to equilibrate the DMSO and then they were transferred to −70 °C for 24 h, and finally transferred to liquid nitrogen for long-term storage [20]. The cryovials were removed from the liquid nitrogen and quickly thawed in a 37 °C water bath. When the ice clump was almost thawed, 1 mL of cell culture medium (88% DMEM, 10% FBS, 1% penicillin and streptomycin, and 1% L-glutamine) was added, the vials were centrifuged at 240 ×*g* and the cells were transferred into flasks with gently blown into uniform single cell suspension, and cultured at 37 °C and 5% CO_2.

2.3. Cell viability

Before freezing and after thawing, viability was determined using the trypan blue exclusion test (0.4% trypan blue in PBS). The number of nonviable cells was determined by counting of 1 000 cells and then subtract the number of stained cells from the total and calculate unstained cells proportion (percent) from the total after 1 months of cryopreservation [21].

2.4. Growth curve analysis

Cells of the 8th passage before and after freezing were seeded in 24-well plates at a density of approximately 1.5×10^5 cells per well, cultured for 8 d, and counted every day (3 wells each time). The mean cell numbers at each time point were then plotted against time using GraphPad Prism version 5.01 for Windows (GraphPad software Inc., San Diego, CA, USA). Population doubling time (PDT) was determined based on this curve [22].

2.5. Karyotype analysis

The chromosomes were prepared, fixed, and stained following standard method [23]. Cells of the 8th passage were harvested when reaching 50%–70% confluence. After hypotonic treatment using 0.075 mol/L KCl (Merck, cat. no. 1.04936.1000, Darmstadt, Germany), fixation by acetic acid (Merck, cat. no. SAAR1021020LC) and methanol (Merck, cat. no. 1.02447.0500) (1:3), and Giemsa and Leishman staining (v:v, 1:3), chromosome number was counted for 50 metaphases under an oil immersion objective (×100) using a light microscope (Olympus IX51, Japan).