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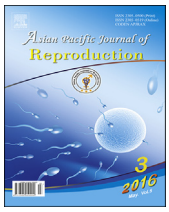

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Establishment, characterization and cryopreservation of Fars native goat fetal fibroblast cell lines

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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).

2.6. Statistical analysis

The mean and SE of counted cells in growth curve analysis before freezing and after thawing were subjected to the Kolmogorov–Smirnov test of normality and then were compared using independent sample *t*-test (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). Values with $P \leq 0.01$ were considered significantly different.

3. Results

3.1. Fetal age estimation

Three fetuses with ages of 51 (male), 53 (male), and 55 (female) d where selected based on crown-to-rump length (Table 1).

3.2. Morphological observation

At about 4–5 d after the tissue explants adhered to the flasks, fibroblast-like cells were observed sprouting from the margins of these tissue pieces (Figure 1a). The cells showed typical fusiform morphology with centrally located oval nuclei. The cells covered the bottom of the flasks within 3–4 d and formed a monolayer. The cells had fibroblastic characteristics with turgor vitalis cytoplasm, fibroblast-like radiating, and flame-like migrating patterns (Figure 1b–d).

Table 1
Estimated age of goat fetuses using crown-to-rump length measurement.

Fetus	Sex	CRL (mm)	Estimated age (d)
Fetus 1	Male	52	51
Fetus 2	Male	55	53
Fetus 3	Female	62	55

3.3. Growth curve analysis and cell viability

The growth curve of the Fars native goat fetal fibroblasts exhibited a typical "S" shape (Figure 2) and PDT was about 22 h before freezing and 28 h after thawing. In fresh and frozen-thawed samples, the latent phase was about 1 d, a result of trypsinization. This was followed by an exponential phase of 5 d before freezing and 3 d after thawing, which gave way to the stationary phase afterwards. There was no significant difference between cell concentrations in each day before and after freezing ($P > 0.01$). Viability of the culture was (89.78 ± 4.63)% before freezing and (88.32 ± 5.17)% after thawing.

3.4. Karyotype analysis

The chromosome number of the Fars native goat fetal fibroblasts was $n = 60$, comprising 58 autosomal and 2 sex chromosomes (Figure 3). For 50 metaphases of the 8th passage, the chromosome numbers per metaphases were

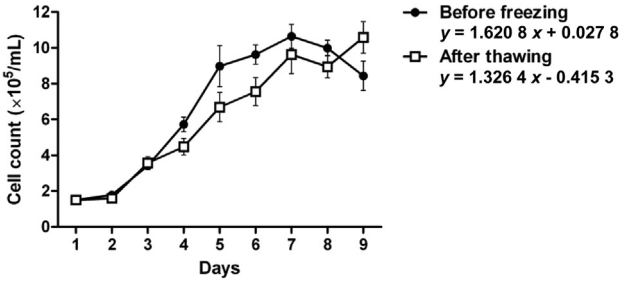

Figure 2. The growth curve and trend line equation of the 8th passages of the Fars native goat fetal fibroblasts before and after thawing ($n = 3$ fetus or 9 counted well/d; mean ± SE).

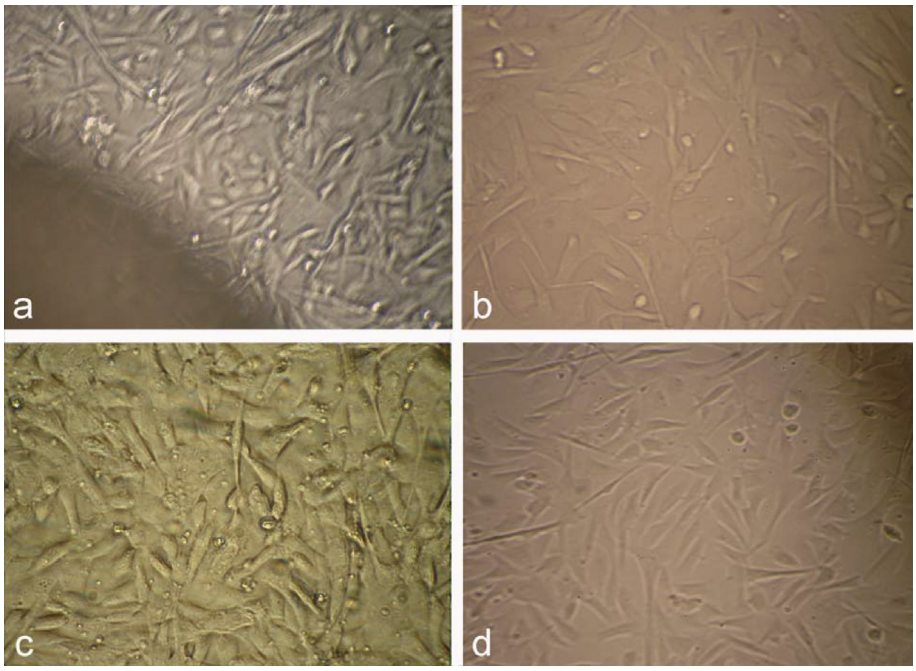

Figure 1. Morphology of the Fars native goat fibroblast cells *in vitro*.
a) Primary cells grew out on day 5 from fetal skin explants, b) 5 d after the first passage, c) near confluence, and d) cells at 24 h after recovery from cryostorage.

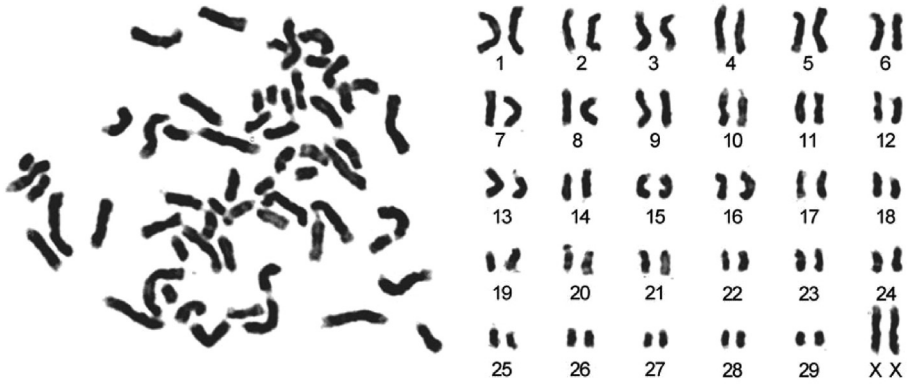

Figure 3. Chromosomes at metaphase (left) and karyotype (right) of the Fars native goat fetal fibroblasts. The karyotype of the Fars native goat fetal fibroblasts consisted of 30 pairs of chromosomes.

counted, and the results showed that 98% of the cells were diploid, supporting the conclusion that the cell culture was reproducibly diploid.

4. Discussion

The fetal fibroblast-like cell culture from the Fars native goat was established using the adherent culture method and these cells were frozen following eight passages. Somatic cells storage may be an option for *in vitro* conservation of species [3]. Somatic cell cryopreservation for every species is a cheap and fast way, and the method of choice for the rapid creation of cell banks. Fibroblasts may be trypsinized and adhered more easily and more readily than epithelial cells [24]. Because of these characteristic, a culture of pure fibroblast may be obtained after two to three passages [5,25,26]. Morphology, as the most important qualitative parameter of epidermal tissue reconstitution was evaluated by light microscopy. In our study, the cells had fibrous characteristics with turgor vitalis cytoplasm, and during growth, they showed typical fibroblast-like morphology as radiating, flame-like or whirlpool migrating shapes. Consistent with our findings in the Luxi cattle, fibroblasts, could be seen migrating from the tissue pieces five to 12 d after explanting [7].

Analysis showed that the population doubling time (PDT) for subculturing fibroblast-like cells with high rate of proliferation was approximately about 22 h before freezing and 28 h after thawing in accordance with the reports of Singh *et al.* who established three fibroblast cell lines from lower edge ear skin samples of healthy dairy goats with a population doubling time of 25 h without freezing [27].

Genetic stability of cell cultures is the most important aspect when preserving genetic resources. The cells must maintain the same diploid character as cells *in vivo*. *In vitro* cultured cells that keep their division capability but differentiation appears after successive cell divisions, so they cannot be used for breed conservation. A $n = 60$ frequency of 98% indicated that the Fars native goat fetal fibroblast cultures were stably diploid in accordance with the previous reports in goats [5,12–14,27–29]. Although hypodiploid and hyperdiploid cells, and some polyploid cells may emerge in the cultures with increasing passaging [30], the incidence of such cells was still very small in our study (below 2%). Hence, there was seldom a chromosome number variation in the Fars native goat fibroblasts.

It is not uncommon for cells to cease growth and show changes in biological characteristics or lose their diplont properties with time in cultures due to a variety of stimuli and factors. Effective measures are thus required to ensure diploid stability in cultures of cells that are used for preserving valuable genetic resources. The genetic characteristics of the cells may be changed by *in vitro* culture conditions after many passages, so a minimal number of passages are recommended to conserve them.

A fibroblast-like cell culture was established from explanted fetal skin tissue of the Fars native goat using standard tissue adherent culture and continuous passaging following trypsinization. We conclude that cell quality was similar among the cell cultures. We contend that our cell bank makes a valuable contribution to the preservation of the genetic resources of the Fars native goat and provides useful biomaterial for future studies in cell biology, medicine, genomics, postgenomics, and both genetic and embryonic engineering.

Conflict of interest statement

There is no conflict of interest.

Acknowledgments

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