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Original Article

Development and Biological Characteristics of Brown Quail (*Coturnix ypsilophora*) Embryonic Fibroblast Primary Cells

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ABSTRACT

Using tissue explantation and cryopreservation biotechniques, a quail embryonic fibroblast primary cell line was successfully developed, which performed by using 20 quail embryo samples and a stock of 25 cryovials, each one containing 3.0×10^6 cells. Most of the cells were apparently fibroblasts in their morphology, and the population doubling time (PDT) was about 48 h. The cells were tested for microbial contamination and found free of infections from bacteria, fungi, viruses and mycoplasms. The total chromosome number of a diploid cell was 78 According to karyotyping and chromosome analysis. All the tests showed that the quality of the cell line conforms to the quality criteria of the ATCC (American type culture collection).This work succeeded not only in preserving the genetic resources of quail cells, but it also established a new protocol to preserve cell of avian breeds.

Keywords: Avian, Primary cells, Fibroblasts, Cryopreservation, Quality Control

INTRODUCTION

Cell culture is defined as growth of cells dissociated from the parent tissue by spontaneous migration or mechanical or enzymatic dispersal sense (Freshney, 1994). Primary cell culture refers to the cell culture initially derived from the parent tissue prior to any subsequent culture in vitro. Modern political and social situations also demand models alternative to live animals. Because of wide availability of chicken embryos and the ease of obtaining tissues from these embryos without resorting to sophisticated surgical procedures, chicken embryos are the ideal sources of a variety of primary cell cultures. Besides, the use of chicken embryos is not subjected to tight regulations as is the use of live animals and is not surrounded by the controversy as is the use of human fetus tissues in research. This circumstance allows the researcher to be devoted more to the research project itself.

Genetic diversity of poultry had been seriously reduced, and some high-quality species of poultry are on the brink of extinction, especially due to the exacerbation of environmental pollution and the development of animal husbandry (Thoraval et al., 1994). If these genetic resources are not preserved in any forms before their extinction, they will be irreparably lost; it is therefore crucial to undertaketake realistic measures to conserve endangered species (Love et al., 1994). Currently, semen, embryos, genomic and cDNA libraries are all conventional methods to preserve single animals. In addition, since the entire genome of a species is contained in the nucleus of somatic cells, modern cloning techniques have made fibroblasts an attractive resource for safeguarding animal genetic materials.2 In addition, the establishment of an animal somatic cell bank can not only preserve this genetic resource at the cell level, but also provide a precious experimental material for investigation in cell biology, genomics and post-genomics, and for embryonic engineering. Quail, is characterized by small body, high fecundity and tender flesh as one of the avian wiled breeds (Watanabe et al.1992).

In the present research, a fibroblast bank through primary explantation and programmed cryopreservation was successfully constructed and tested the quality of the cell lines obtained. The genetic resource of this valuable wiled breed has thus been preserved through somatic cells for long-term storage. In addition, this technical platform will provide a technical and theoretical support to conserve other animal genetic resources at the cell level.

Objective of this study:

To illustrate that primary cell culture can provide supplementary information to the larger models of live birds. These models can be used as a model for cellular responses in the future.

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MATERIALS AND METHODS

The 8-day old embryos of brown quail used in this research were provided by the chicken breeding farm of the Animal Production Department/Faculty of Agricultural Duhok University, Kurdistan-Iraq. The experiment was conducted since Oct. 2012 until Apr. 2013 at Animal biotechnology Lab., Scientific Research Center/ Faculty of Science, Duhok Univ. Except otherwise specifically mentioned; all reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation and culture of quail embryonic fibroblasts

Quail eggs incubated for 8 days were sterilized using alcohol swabs, and then the embryos were isolated and washed three times with phosphate buffered saline (PBS).Embryos were chopped into pieces of 1 mm^3 and placed onto the surface of a tissue culture flask, at 37°C in a humidified air atmosphere containing 5% of CO2, for 3-4 h. Modified Eagle's medium (MEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) was added into the inverted flask and cultured overnight. The medium was refreshed after 2-3 d. The cells were harvested at 80-90% confluence using 0.25% trypsin (m/v) solution and were separated into culture flasks at the ratio of 1:2 or 1:3 (Ren, 2002).

Cell viability test

Viabilities before freezing and after thawing were determined using the Trypan blue exclusion test. The number of non-viable cells was determined by counting 1000 cells (Qi et al. 2007).

Growth dynamics

Cells were plated onto 24-well microplates (Corning, Santa Clara, CA, USA) at the density of approximately 1.5×10^5 cells per well, cultured for 7 d and counted every day (3 wells each time) afterwards. The mean cell counts at each time point were then used to plot a growth curve, based on which the population doubling time (PDT) was calculated (Weingartl et al. 2002).

Microbial analysis

Detection of bacteria and fungi: The cells were cultured in complete MEM (Gibco, Carlsbad, CA, USA) media free of antibiotics and observed for the presence of bacteria and fungi at 3 d after subculture, according to the method described by (Doyle et al.1990).

Detection of mycoplasms: According to the protocol of the American type culture collection (ATCC), the cells were cultured in antibiotic-free medium for at least 1 week, and then tested for potential mycoplasma contamination by direct classical microbiological growth on selective media according to Upoff *et al.* (1992). Cells were cultured on PPLO Broth medium (Diffco®, UK) and incubated in capped tubes at 37°C (Thermo Forma incubator, USA) for three weeks. Culture medium with sample inoculums was monitored for 3 weeks for the development of color changed as indicated by pH indicator.

Virus detection: Hay's hemadsorption protocol was used to examine the samples for cytopathogenesis using phase-contrast microscopy (Olympus Corp., Tokyo, Japan) (Hay RI, 1992; Wu et. al. 2008).

Karyotyping and Chromosomal Analysis

Chromosome spreads were prepared, fixed and stained following standard methods by Suemori et al. (2006) with slight modification. The cells were harvested upon 80%–85% confluence and were treated with 0.04 mg/mL of colchine (Draje® , Turkey) for 4 h. Hypotonic treatment was conducted to obtain good metaphase spreading, the time of the hypotonic treatment was tightly controlled within 40 min. Cells were harvested when 80- 90% confluent and fixed. The cells were sampled for counting chromosome numbers of diploid cells. The chromosome numbers were counted from 50 spreads after Giemsa staining, using 20X an oil immersion objective.

Cells Cryopreservation

After two passages, the cultured cells were suspended in media containing 40% MEM, 50% fetal bovine serum and 10% dimethyl sulfoxide (DMSO). Cells in logarithmic growth phase were counted with a hemocytometer and adjusted to a density of 3×10^6 viable cells per mL(Hay RI, 1992). Aliquots of the cell suspension were transferred into sterile cryovials labeled with the breed name, gender, freezing serial number and date. The vials were sealed and kept at 4°C for 20-30 min to equilibrate DMSO, put into -30°C for one h, then into -80°C overnight, and finally transferred to liquid nitrogen for long-term storage.6 Vials taken from the liquid nitrogen were thawed in a 37°C water bath, then transferred to flasks with MEM containing 10% fetal bovine serum and cultured at 37°C with 5% CO2. The medium was renewed after 24 h.

RESULTS

Morphological Observation of Quail fibroblasts

Cells sprouted from small tissue pieces one day after being plated in the bottom of a tissue culture flask, and then continued to proliferate and were subcultured when 80-90% was confluent in about 3 days. The cells displayed typically fibrous and fusiform morphology with centrally located oval-shaped nuclei. Morphologically, epithelial cells were seldom present, too. The fibroblasts grew rapidly and replaced the epithelial cells gradually after 2-3 passages, and then a relatively pure fibroblast line was obtained (Fig. 1; A, B, C).

Cell Viability:

Depending on; initial seed density, the result of quail fibroblast cells showed high yield of viable cells (10^4 -10^5 cell/ml) with good ability of attachment and growth. The viabilities of quail fibroblasts before freezing and after recovery were 90 % and 85%, respectively, as estimated by the Trypan blue exclusion

test. The harvest and subsequent attachment of cells were graded using a scale as in table 1.

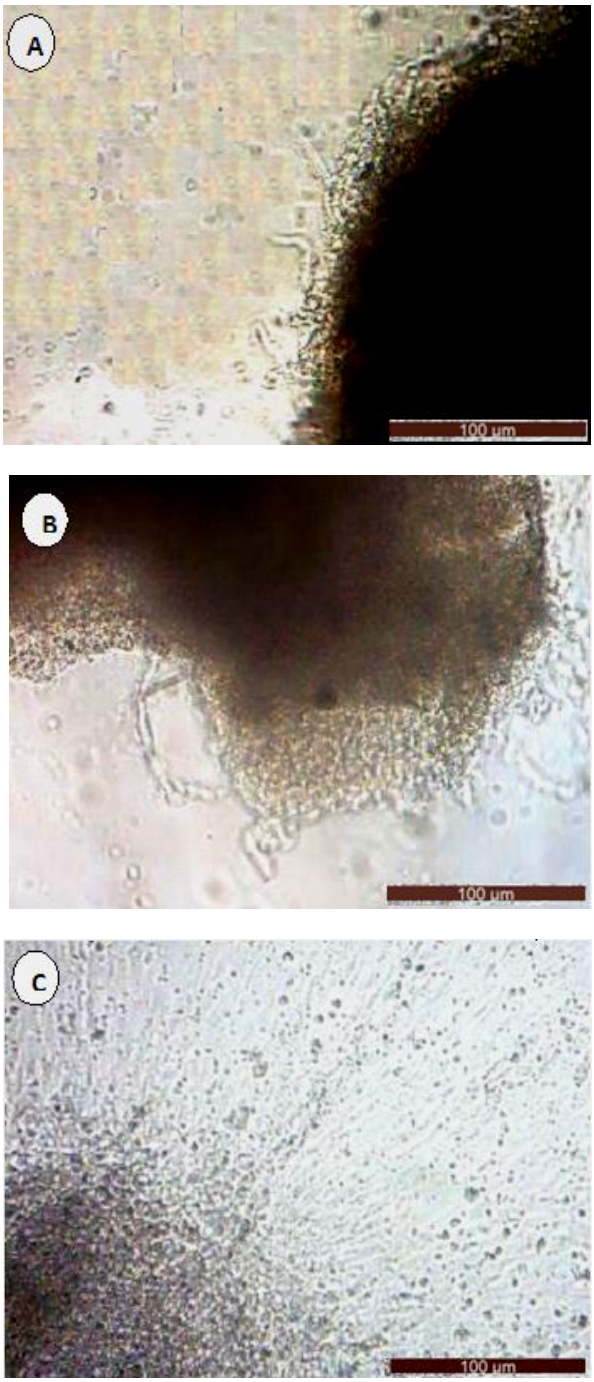

Fig. 1: Morphology of quail embryonic fibroblasts cultured *in vitro*. (A) 1 d after explanting; (B) 2 ds after explanting; (C) confluent monolayer in about 3-4 ds after explanting. Un stained cells. 100µm.

Table 1: Scoring system to record confluence and viable cell counting

Score	Score description	Percentage of substrate coverage (confluence)
-	No growth	0%
+/-	little	<1% (individual cells)
+	medium	5-30%
++	well	30%-50%
+++	excellent	<50%

Growth dynamics

The growth curve of quail fibroblasts appeared sigmoidal (Figure 2) and the population doubling time (PDT) was about 48 h. Latent phase lasted about 24 h, followed by an exponential phase 2 days after seeding, giving way to the stationary phase thereafter.

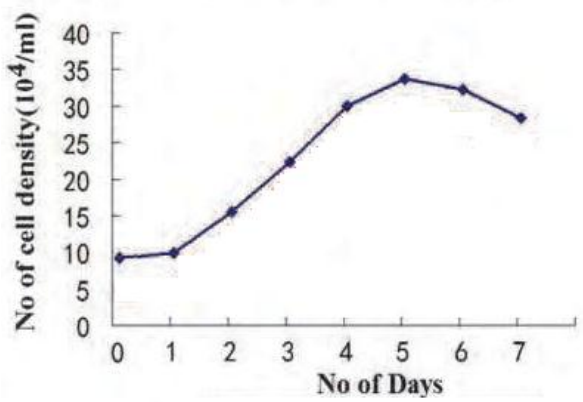

Fig.2: Growth curve of quail embryonic fibroblasts.

Microorganism detection

The medium remained clear for the whole period and no abnormalities could be observed under the microscope. The results indicated that the quail fibroblasts were free of bacterial contamination and the fibroblast cultures were apparently free of mycoplasms. The tests for virus contamination were both negative as well, as indicated by the cytopathogenic evidence.

Karyotyping

The results of chromosome counts of 50 metaphase primary quail fibroblast cells at P2 passage showed diploid number (2n) ranging from 60 to 87 with a model peak at 78 chromosomes (Fig. 3).

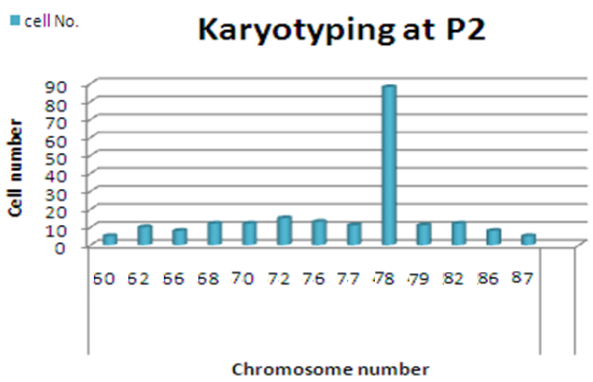

Fig. 3: Chromosome analysis of primary quail fibroblast cells showing the chromosome number distribution at passage P2 with model peak at 78

Cryopreservation

Quail Primary fibroblast cells recovered from storage in liquid nitrogen one month and after 6 months. Cells grew to confluence within 7 days post one month freezing while no confluence noticed post 6 months freezing (Table 2).

The growth curves before cryopreservation is generally consistent with that after resuscitation (Fig. 4).

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Table 2: Comparison of the viability and confluence of primary quail fibroblast cells before freezing and after freezing

Freezing time	Cell viability and confluence	No. of days to reach confluent
Before freezing	+++	5
1 month	+	7-10
6 months	+/-	-

(+++): Excellent growth, <10^6 viable cells, <80% confluence
(+): Medium growth, 10^3 viable cell, 20-40% confluence
(+/-): Little growth, 10-10^2 viable cells
(-): No growth, no confluence

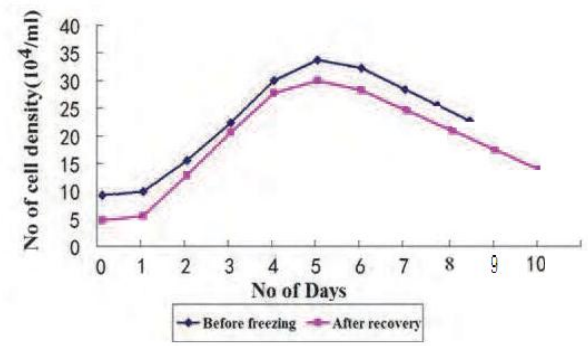

Fig. 4: Growth dynamics. Growth curves of primary quail fibroblast line, before cryopreservation and after resuscitation. Representative growth curve consist of latency phase, exponential phase, plateau phase and decline phase.

DISCUSSION

Quail embryonic fibroblasts were selected in this experiment as they offer many advantages, such as availability with high vitality and purity, easy access, low probability of bacterial contamination. Quail primary fibroblast line was successfully established from 20 embryo samples by direct explants in adherent culture. The biological and genetic characteristics may be altered by *in vitro* culture after many passages, so a minimal number of passages are recommended to protect the cell lines against degeneration. Morphological observation indicated that there were both epithelial cells and fibroblasts during the primary first passage of the explanted tissues. In cell cultures from primary explants, contamination may occur due to the undesired presence of cells from different species or tissues and due to their different tolerance to trypsinization, the fibroblasts detached from the flasks earlier when digested with trypsin and adhered again quickly after passage, whilst most epithelial cells were difficult to adhere, or only did so in an unstable manner and fell off when vibrated (Xue, 2001). For this reason, a purified fibroblast line could be obtained after 2-3 passages (MacLeod et. al. 1999). The genetic stability of cell line is critical to preserve the genetic resources, namely the fibroblasts must maintain the same diploidy as cells *in vivo* . A chromosome with its great potential of future achievements has become completely a new synthetic science due to advancement of technical aspects of the proper research on biochemistry, biophysics, cell physiology and genetics (Sharma, 1984). The shape and size of the chromosomes seem to be of great value in the cytotaxonomy and karyotypic evaluation. The chromosome number is an important datum for a species than any other characteristics seemed significantly stable to merit taxonomic significance (Garber, 1978). The chromosomal numbers in different species, length, shape index and centrometric types were studied because of the importance for taxonomy and evolution. In animals karyotype analysis using to identify heredity disease and analyzing the mechanism of pathological change (Huang *et al*., 1995).

In summary, a primary quail cell bank containing biologically normal and stable fibroblasts was successfully established, meeting the standards of cell line quality standards of the major international culture collection and centers.

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