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Cryopreservation of cartilage cell and tissue for biobanking ☆

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ABSTRACT

Preservation of cell and tissue samples from endangered species is a part of biodiversity conservation strategy. Therefore, setting up proper cell and tissue cryopreservation methods is very important as these tissue samples and cells could be used to reintroduce the lost genes into the breeding pool by nuclear transfer. In this study, we investigated the effect of vitrification and slow freezing on cartilage cell and tissue viability for biobanking. Firstly, primary adult cartilage cells (ACCs) and fetal cartilage cells (FCC) were cryopreserved by vitrification and slow freezing. Cells were vitrified after a two-step equilibration in a solution composed of ethylene glycol (EG), Ficoll and sucrose. For slow freezing three different cooling rates (0.5, 1 and 2 °C/min) were tested in straws. Secondly, the tissues taken from articular cartilage were cryopreserved by vitrification and slow freezing (1 °C/min). The results revealed no significant difference between the viability ratios, proliferative activity and GAG synthesis of cartilage cells which were cryopreserved by using vitrification or slow freezing methods. Despite the significant decrease in the viability ratio of freeze–thawed cartilage tissues, cryopreservation did not prevent the establishment of primary cell cultures from cartilage tissues. The results revealed that the vitrification method could be recommended to cryopreserve cartilage tissue and cells from bovine to be used as alternative cell donor sources in nuclear transfer studies for biobanking as a part of biodiversity conservation strategy. Moreover, cartilage cell suspensions were successfully cryopreserved in straws by using a controlled-rate freezing machine in the present study.

Introduction

Tissue and cell sample cryopreservation of endangered species is a part of biodiversity conservation strategy. The establishment and maintenance of biological resource centers requires careful attention to the implementation of reliable cryopreservation technologies to ensure that recovered cultures perform in the same way as the originally isolated culture [27].

The advances in low temperature biology have produced high viability preservation methods for many different cell suspensions. Nevertheless, methods that work for many different cells in suspension may not work for certain cell types and tissues. Moreover, there has not been any study reported on the freezing tolerances of different kinds of somatic cells. Well-established cryopreservation techniques with a high viability for different kinds of mammalian somatic cells would allow continued storage and availability for biobanking [14]. In the last two decades, conserving domestic animal diversity has become a global issue, as is biobanking. The first Global Plan of Action for Animal Genetic Resources was declared in Interlaken (3–7 September 2007). This action plan was adopted by 109 countries [8] including Turkey. In addition, the Turkish Ministry of Agriculture and Rural Affairs had initiated *in situ* and *ex situ* conservation programs for some selected breeds a few years before the Interlaken Declaration and has been expanding the program since then. Within this context, the Turkish Ministry of Agriculture and Rural Affairs has supported the national TURKHAYGEN-1 project [32] to establish a cryobank containing different type of somatic cells from native livestock breeds.

Ear tissue punctures taken from adult animals is the most appropriate and easily handled donor cell source for somatic cell nuclear transfer (SCNT). Although fibroblasts from ear tissue are accepted as the suitable donor cell type for nuclear transfer in conservation biology, we should consider the fact that the ear tissue consists of not only from fibroblasts but also chondrocytes in large proportions. Auricular cartilage isolated from ear tissue samples gives rise to cartilage cells in the culture [2,4,9]. Besides fibroblast cells, the cartilage cells derived from the ear tissue samples [2] as well as the articular cartilage [3] can be used for SCNT. That is why auricular and articular cartilage cell culture and cryopreservation could be very important in biodiversity conservation for biobanking.

Cartilage tissues from ovine, porcine and rabbit species have previously been cryopreserved to improve the tissue survival for transplantation [5,11,20,25]. Moreover, the cryosensitivity of

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cartilage cells has been studied in detail [20–22]. However, we could not find any report about cartilage cell and tissue cryopreservation for nuclear transfer studies and biobanking. Although cell culture from vitrified ear skin samples has been discussed in previous studies [26–28], the *in vitro* culture of cells from vitrified cartilage tissues has not yet been studied in the context of biobanking for endangered animal conservation.

Although numerous studies have investigated the cryopreservation of cartilage cells [23,30,34], vitrification was evaluated mostly for the cryopreservation of embryos, zygotes and oocytes [33]. The basic principle of vitrification is to vitrify the cell and prevent ice crystal formation by using high cryoprotectant concentration at high osmolarity. Despite its broad usage in gamete and tissue cryopreservation in assisted reproduction, vitrification has a limited usage in somatic cell cryopreservation. Recently, there have been a few studies reporting the usage of this technique for some special somatic cell types [7,10,19,24]. To the best of our knowledge, there is no detailed study published on the vitrification of chondrocytes isolated from tissue. It should be noted that vitrification has some drawbacks as it might cause toxic damage due to high cryoprotectant concentration in the cells. Likewise, slow freezing, another cryopreservation technique, might result in extreme cellular dehydration due to the exposure of the cells to a highly concentrated electrolyte solution. Thus, both vitrification and slow freezing can disrupt the metabolic activity of the cells.

The present study was part of a biobanking project and aimed at comparing the success and influence of different cryopreservation protocols on the viability of cartilage tissue and cells. For this reason, primary cell cultures and small tissue samples from articular cartilage were vitrified by a protocol evaluated for embryo and stem cell cryopreservation [6,19]. The efficiency of the vitrification was compared with the results of the conventional slow freezing technique to optimize the cryopreservation of primary bovine cartilage cells and tissues for biobanking for biodiversity conservation. For this reason, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay, the most commonly used assay for the detection of the metabolic activity and viability of cultured cells and tissues [16], was employed to examine the vitality of thawed tissues and the proliferative activity of thawed cells. Moreover, glycosaminoglycan amount, growth curve of cells, attachment rate and primary cell culture establishment ratio of tissues was employed to observe the success of the cryopreservation protocols for biobanking.

Materials and methods

Establishment of adult and fetal cartilage cell lines

Adult cartilage cells (ACCs) were isolated from the articular cartilage of the knee joints of a two-year old bovine obtained from the local slaughterhouse. Fetal cartilage cells (FCCs) were isolated from the articular cartilage of a three-month old bovine fetus. The cartilage tissues were washed in sterile phosphate buffered saline (PBS, pH 7.4, Cat No. D-5773, Sigma) supplemented with 1% penicillin/streptomycin (10.000 U/mL penicillin G, 10.000 mg/mL streptomycin, Sigma) (v/v) and cut into small pieces by surgical blades in a 100 mm culture dish. The small tissue pieces (1 mm^3) were seeded onto the tissue culture petri dishes and cultured with Dulbecco's Modified Eagle's Medium with high glucose (DMEM, Invitrogen 12800-082) supplemented with 20% (v/v) fetal bovine serum (FBS, Gibco 10270-106) and 1% (v:v) penicillin/streptomycin (A5955, Sigma) at 37 °C, 5% CO_2 and 100% humidity. The cells were harvested when they reached 80–90% confluence and were divided into petri dishes [15]. The muscle cells (MCs) were derived from bovine muscle tissue by explant culture as a control cell group.

Characterization of cells

Cartilage cells and muscle cells were labeled with antibodies raised against vimentin. Briefly, fixed cells were treated with a blocking solution composed of 5% goat serum and Triton-X in PBS for 45 min at room temperature. After being washed three times with PBS, the cells were incubated with primary antibodies (Vimentin, Sigma, 1:200). The next day, the cells were incubated with anti-mouse IgM secondary antibody dilution (1:128 Sigma) for 45 min at room temperature. Hematoxylin-eosin staining was employed to observe morphological differences.

Vitrification of cell and tissue samples

The cells and tissues were cryopreserved by vitrification process in two steps, a modification of the protocol used in blastocyst and mesenchymal stem cell preservation [19]. Firstly, the pellet of 2×10^5 cell/mL was first suspended in 50 μl equilibration solution for 5 min. Secondly, the suspension was mixed with 200 μl vitrification solution for 1 min. Then the cells were immediately loaded into 0.25 mL plastic straws and plunged directly into liquid nitrogen (−196 °C). The equilibration solution used was 20% ethylene glycol and the vitrification solution was composed of 40% ethylene glycol (EG, E9129, Sigma) 18% Ficoll 400 (F2637, Sigma) and 0.3 M sucrose (S1888, Sigma). All the solutions were based on dPBS containing 20% (v/v) FBS. After 3 weeks, the cells were thawed by rapidly immersing the straws in a water bath at 37 °C and were suspended serially in 0.5, 0.25 and 0 M sucrose in dPBS containing 20% (v/v) FBS. The tissue samples were vitrified as described using 0.5 mL plastic straws.

Slow freezing of cell and tissue samples

The ACCs and FCCs were cryopreserved by slow cooling in a controlled rate freezing machine (CL8800 CryoGenesis Standard Freezing Control) from room temperature to −60 °C at three different linear cooling rates: 0.5, 1 and 2 °C/min. The cells were trypsinized, stained with trypan blue and counted using a hemacytometer. After counting, 2×10^5 cells were equilibrated in DMEM/F12 media supplemented with 10% (v/v) dimethyl sulfoxide (Me_2SO; Sigma) and 50% (v/v) FBS immediately and then loaded into 0.25 mL plastic straws. The cells in the straws were stored in the vapor phase above the liquid nitrogen and then stored in liquid nitrogen until thawed. The tissue samples (1–2 mg) were loaded into 0.5 mL plastic straws in 10%, Me_2SO and 55% (v/v) FBS and then cooled to −80 °C at a rate of at 1 °C/min using a controlled rate freezing machine and then stored in liquid nitrogen. The straws were thawed in a water-bath at 37 °C and removed as the last ice melted.

Primary cell culture derived from cryopreserved tissue samples

The straws were stored in liquid nitrogen for a minimum of 3 weeks. Frozen-thawed tissue samples were seeded and cultured as described above. Briefly, the bovine articular cartilage were sliced using a scalpel in PBS supplemented with 2% antibiotics (Sigma). Small tissue samples (1–2 mg) were seeded into 24 well dishes. The tissue samples were cultured in 20% (v/v) FBS with DMEM high glucose for 30 days of maximum. The daily assessment of the cell culture from the onset until the subconfluence stage was performed under inverted microscope and the results were recorded.