SHORT REPORT

Effect of different cryopreservation temperatures on recovery of goat skin derived fibroblast cells

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Cryopreservation is an important technology to preserve livestock germplasm for future use. How different freezing temperatures affect the post-freezing recovery of cells is not precisely known. In this study, we report the isolation of live fibroblast cells from goat skin after 14 days of animal death and the effect of 4 different freezing temperatures on the post-cryopreservation cell recovery. The primary outgrowing cells were cultured for three passages and cryopreserved at -20°C, -80°C, Liquid nitrogen (LN2) (-156°C), and -80°C overnight followed by storage in LN2 (-80°C/LN2) group. After 4 weeks of storage, cryovials in triplicate for each group were thawed, cultured, and analyzed. Mean and standard deviation of live cells recovered after 5 days of *in vitro* culture was 1.0 ± 0.71 x 10^5, 7.60 ± 2.88 x 10^5, 1.40 ± 0.55 x 10^5, and 14.80 ± 1.30 x 10^5 cells/mL for -20°C, -80°C, LN2, and -80°C /LN2 groups, respectively. Recovery of cells stored at -80°C/LN2 group was significantly higher than that in -80°C, -20°C, and LN2 groups ($P < 0.05$). Recovery was least upon freezing cells directly into LN2 or in -20°C. Although, storing cells in -80°C was significantly higher than that in -20°C and LN2 groups, it was significantly less than that in -80°C/LN2 group ($P < 0.05$). Based upon these results, we conclud that step cooling (-80°C overnight followed by LN2) was the most effective method for cryopreservation of goat fibroblast and perhaps other mammalian cells. This study highlighted the importance of storing biological tissues and cells at -80°C, especially at small two- and four-year teaching institutions or at limited resource farms, where the cost of maintaining liquid nitrogen supply is prohibitive.

Keywords: cryopreservation; primary fibroblasts; goat; liquid nitrogen; freezing temperatures.

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Introduction

It is well known that refrigerating temperatures slow metabolic rates of living organisms and thus reduce spoilage of biological samples [1]. In fact, this is the very reason food items are stored in refrigerating conditions to increase their shelf life. Freezing temperatures below 0°C further reduce cellular metabolism and other biochemical processes, however, these temperatures also destroy the cellular membranes and thus integrity of cells due to crystallization of water in the cells, which is a major component of the cells [2]. Scientists use various permeable and non-permeable chemicals, called cryoprotecting agents (CPAs) such as dimethyl sulfoxide (DMSO), sucrose, glycerol, trehalose, ethylene glycol, *etc*., to protect cellular integrity. These CPAs usually increase solute concentration in cells to reduce

crystallization of water and, thereby, preserve membrane integrity [3]. Cryopreservation is an essential technology and holds great promise in biological, medical, and agricultural sciences. Cryopreserved cells and tissues can be stored for long period of time without losing their viability and proliferative potential and thus are used for conservation of germplasm to preserve diversity and to bring back the lost genetics at a desired time in future [4-6]. Leon-Quinto *et al*. described cryobanking using skin biopsies in *Iberian lynx*, an endangered wild cat species of Europe, to preserve genetic diversity [4]. Hoshino *et al*. resurrected a bull by cloning from -80°C frozen organs [6]. However, the recovery of cells and tissues after cryopreservation, to ensure nuclear integrity, presents a challenge and need cell and tissue specific optimizations [2, 7]. It is known that the lower the temperature, the longer the cells and tissues can survive. However, the effect of different freezing temperatures on post-freezing recovery of cells is not adequately studied. Here we report the isolation of live fibroblast cells from goat skin after 2 weeks of animal death, and the effect of 4 different freezing temperatures on the post-cryopreservation recovery to enhance the understanding of cryopreservation as a method to conserve germplasm for future utilization.

Materials and Methods

Explant culture

Ear skin tissue used in this study was procured from a 4-day old female goat kid from university pastures (Fort Valley, GA, USA), which was accidentally killed by black vulture attack. Goat kid was born after 152 days of breeding of a mixed Kiko does and a pure Spanish buck. For cell culturing, small (2–3 mm^2) explants were excised aseptically from ear tissue that was stored for 14 days postmortem at 4-6°C in a lab refrigerator. Ten explants were adhered onto two 35-mm-diameter Falcon dishes (BD Biosciences, Oxnard, CA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Suwanee, GA, USA), 50 units/mL of penicillin, 50 µg/mL of streptomycin, and 2.5 µg/mL of fungizone at 37°C, 5% CO_2, and 95% humidity. Media was changed twice a week on Mondays and Thursdays. The dishes were observed for any microbial or fungal contamination, explant dislodging, and for the outgrowth of cells under an inverted microscope. Images were captured in Nikon TS100 Inverted Phase Contrast Microscope (Nikon Corporation, Kōnan, Tokyo, Japan).

Primary cell cultures and cryopreservation

Primary outgrowing cells were trypsinized at 70–80% confluence and cultures were established following our previous reported method [8]. Briefly, the cells were washed twice with 2.0 mL of phosphate buffered saline (PBS) without calcium and magnesium (Gibco, Carlsbad, CA, USA) and incubated with 1.0 mL of 0.25% trypsin for 5–10 min at 37°C. The trypsinized cells were neutralized with 5 volume of 10% serum containing DMEM media, counted to assess cell viability in hemocytometer [9], and pelleted at 200 × g for 7 min. The cells were resuspended in Synth-a-Freeze® (Life Technologies Corp., Carlsbad, CA, USA) media and aliquoted into twelve cryogenic storage vials (50,000 cells/vial) for each experiment. Triplicate vials were then stored at 4 different freezing temperatures including -20°C, -80°C, -156°C (Liquid nitrogen (LN2)), and -80°C overnight followed by storage in LN2 (-80°C/LN2).

Recovery of cells and cell viability assessment

After four weeks of storage, the frozen cryovials were thawed at 37°C. The cells were mixed slowly with 10 volume of the DMEM media and pelleted at 200 × g for 7 min. The pellet was dissolved in 1.0 mL of DMEM media containing 10% FBS. The cells were cultured in 12-well microtiter plates at 37°C, 5% CO_2, and 95% humidity. After 5 days of culture, the comparative images of cells were captured, and semi-quantitative estimation of confluence was performed and recorded by a single observer. Subsequently, the cells were trypsinized and the number of live and dead cells were counted by using Trypan Blue Dye Exclusion

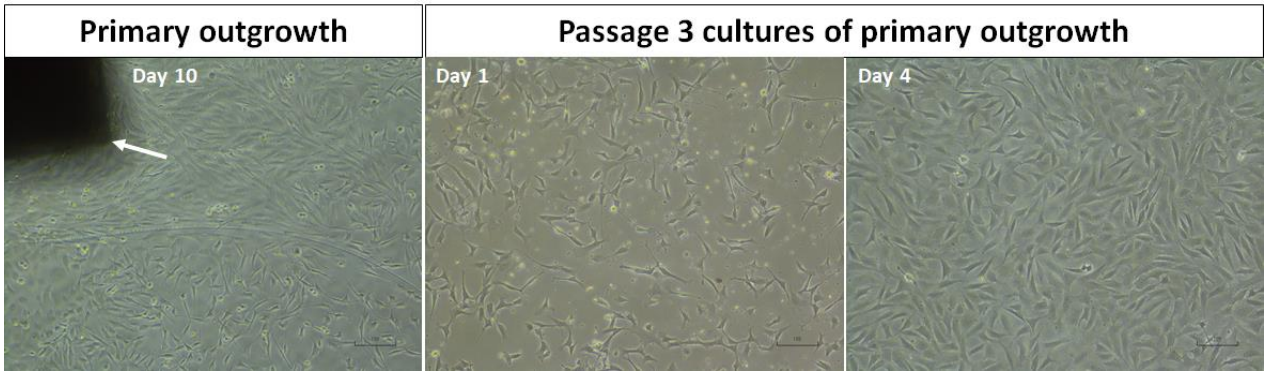

Figure 1. Explant culture from ear skin stored at 4-6°C for 14 days. Primary outgrowth panel: the outgrowing fibroblast-like cells (near arrow marked black skin explant) after 10 days of culture. Passage 3 cultures of primary outgrowth panels: day 1 and day 4 cultures, respectively, from passage three cells of primary outgrowth.

Assay [9]. Briefly, a cell suspension was mixed with Trypan Blue Dye (Millipore Sigma, St. Louis, Missouri, USA) and then visualized under microscope to determine whether cells taking up or excluding the dye. A viable cell should show a clear cytoplasm whereas a non-viable cell might demonstrate a blue cytoplasm due to uptake of dye.

Statistical analysis

The mean and standard deviation of viable cell counts for different temperature groups were calculated by using GraphPad Prism Version 10.0.2 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance between categories was calculated by using unpaired t-test. Data were considered statistically significant at $P < 0.05$.

Results and discussion

The primary outgrowth of cells around the skin explants was almost 100% confluent within 2 weeks (Figure 1). These cells were healthy and displayed elongated, fibrous, and spindle shape morphology, which is typical of fibroblasts. In an earlier study on similar cells by using immunofluorescence, the cells were stained positive with anti-vimentin antibodies confirming their fibroblastic nature [10]. These skin tissue derived fibroblast cells are easily accessible, comparatively easy to culture, and have enormous potential for reprogramming. They have been used to clone animals and thus are ideally suited for long term preservation of germplasm to conserve genetic diversity.

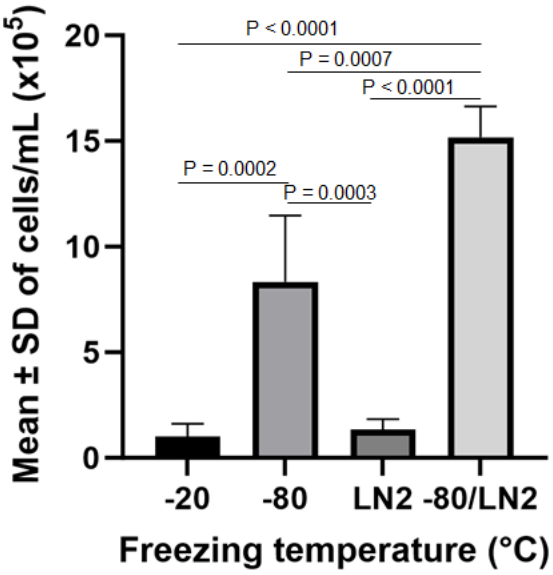

Figure 2. Post-freezing recovery of live cells from cultures prepared from cryovials stored at 4 different temperatures.

In an effort to test the effect of freezing temperatures on post-cryopreservation recovery of cells, we cryopreserved cells at 4 different temperatures and cultured them *in vitro* after 4 weeks. The means and standard deviations of live

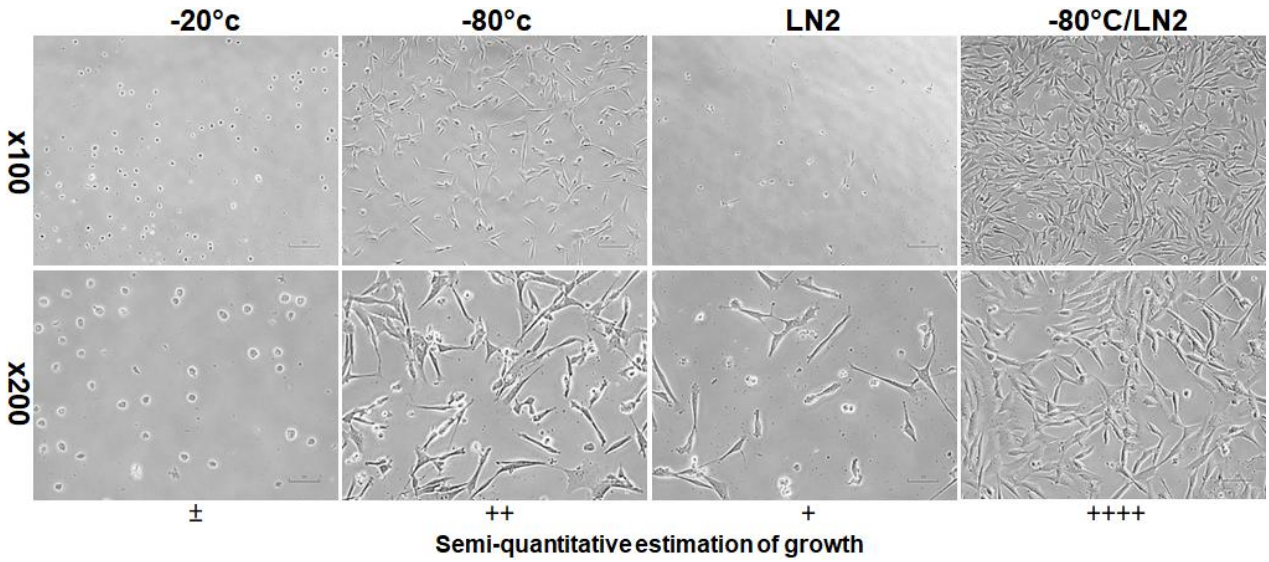

Figure 3. Comparative confluence of cells on day 5 of culture recovered from cryovials stored at 4 different cryopreservation temperatures. Scale bar was 100 µM.

cells recovered after 5 days of *in vitro* culture were 1.0 ± 0.71 x 10^5, 7.60 ± 2.88 x 10^5, 1.40 ± 0.55 x 10^5, and 14.80 ± 1.30 x 10^5 cells/mL for -20°C, -80°C, -156°C (LN2), and -80°C/LN2 groups, respectively (Figure 2). The results showed that the recovery of cells stored at -80°C/LN2 group was significantly higher than that in -80°C, -20°C, and LN2 group ($P < 0.05$). Recovery was the least upon freezing cells directly into LN2 and/or -20°C (Figure 2). These results were in agreement with the semi-quantitative estimation of cell growth by using the images of cell confluence as the unit of measurement captured prior to recovering cells from 12-well micro titer plates for quantitative analysis with no difference in the morphology of cells from different temperature groups (Figure 3). However, the number of visible fibroblast cells differed in different temperature groups. Although, storing cells in -80°C was significantly higher than that in -20°C and LN2 groups, it was significantly less than -80°C/LN2 group ($P < 0.05$). This study showed that plunging cells directly into liquid nitrogen was disadvantageous due to quick crystallization of water in cells damaging cellular membranes, and thus their integrity. Storing cells at -20°C was also not useful since cell survival was extremely low from this group which could be due to the long cryopreservation storage time of 4 weeks. Based upon these results, we conclud that step cooling (-80°C overnight followed by LN2) was the most effective method for cryopreservation of goat fibroblasts and perhaps other mammalian cells as well. This study highlighted the importance of storing biological tissues and cells at -80°C, especially at small two- and four-year teaching institutions or at limited resource farms, where the cost of maintaining liquid nitrogen supply is prohibitive.

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