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Physiological effect of cryoprotectants in freezing of embryonic fibroblast cells

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Abstract
Fibroblast cells are the most common cells of connective tissue and form structural framework. In the present study embryonic fibroblast cells up to three subcultures were cryopreserved and their viability was studied using three freezing media. The viability at equilibrium of freezing media-3 (10% DMSO + 0.9 M Trehalose), 1(10% DMSO), 2 (0.9M Trehalose) was found to be 90.39±0.084, 89.75±0.047 and 87.69±.082 respectively by using trypan blue exclusion dye test. Trypan blue is an azo-based hydrophilic, tetra-sulfonated blue acid dye which is used to determine the number of viable cells present in a cell suspension. A viable cell will have clear cytoplasm and non-viable cell will have a blue cytoplasm. Media 3 reach confluency 60% after post thaw re-cultured in lower time of 4 days as compared to freezing media 1 and 2. The confluency timing was consequently found better from 2 days onwards in both freezing media -1 and freezing media - 2. Morphologically the isolated cells had fibroblast character like typical fusiform shape, turgor Vitalis cytoplasm, centrally located nuclei and flame like migration pattern.

Keywords: Confluency, cryopreservation, dimethyl sulfoxide, fibroblast cells, Trehalose, trypan blue

Introduction

Fibroblasts are large, flat, elongated (spindle-shaped) cells processes extending out from the ends of the cell body. The cell nucleus is flat and oval. Fibroblasts produce tropo collagen, which is the precursor of collagen and ground substance, an amorphous gel-like matrix that fills the spaces between cells and fibres in connective tissue. Fibroblasts plays an important role in wound healing, and its activity is thought to be regulated by cells known as fibrocytes residing in the tissue stroma. When tissue injury occurs, fibroblasts migrate to the site of tissue damage, where it deposits new collagen and facilitate the healing process. Virchow (1858) [13] were the first to describe fibroblast cell. Ever since the establishment of HeLa cells by George in the year 1951, scientists established several other fibroblast lines for different purposes, such as the fibroblasts NIH3T3 from Swiss mouse derived embryos and subsequently fibroblast cell are isolated mouse fibroblast cells (Takahashi and Yamanaka, 2006) [12]. The chicken embryo fibroblast (CEF) cultures were prepared by Mustaffa-Babjee (1970) [7]. Similarly, many cultures of turkey embryo fibroblasts (TEF) and goose embryo fibroblasts (GEF) were prepared. A fibroblast line from embryos of Xiaoshan chicken was established successfully by direct culture of explants and cryopreservation techniques (Guan *et al.* 2012) [4]. The fibroblast cell line from the Wenchang chicken was successfully established by the attachment culture method and the cryopreservation biotechniques (Pei *et al.* 2014) [9] .Cryopreservation allows virtually indefinite storage of biological materials without deterioration over a large time scale of at least several thousands of years, and probably much longer. Establishment of somatic cell banks using cryopreservation techniques was a novel and effective approach to conserve and maintain the diversity of domestic animals (Guan *et al.*, 2003) [3]. Establishing somatic cell banks of special livestock and poultry species could not only conserve and preserve the genetic resources at the cell level effectively and permanently, but can also provide valuable experimental material for research in the life sciences of these animals, such as cell biology, genomics, post-genomics and embryo engineering.

Materials and Methods
The proposed experiment will be conducted in the Department of Veterinary Physiology, College of Veterinary Science, AAU, Khanapara, Guwahati-781022. The duck embryonic somatic cells will be taken as experimental subject of evaluation. The duck eggs will be procured from the Experimental Farm of Department of Veterinary Physiology. The eggs will be wiped with sterile cotton soaked with antiseptic solution and will be allowed to dry. The eggs will be incubated at 37 °C and humidity of 75-80% for 12 days. On 12th day of incubation eggs will be washed with ethanol and kept on a small sterile beaker. A large circular hole will be made at the blunt end of the egg to remove the shell and air sac and thereby the embryo with sterile forceps. The embryo is then dissected and macerated to isolate cells for seeding in the culture flask containing culture media. The flasks will be incubated at 37 °C at 5% CO_2 till it gets adhered to the surface. Medium will be replaced at around 48-72 hours interval without disturbing the attached cells. The cells will be observed daily for occurrence of substantial outgrowth of cells from the tissue pieces; cells will be harvested at 80–90% confluence subculture. The cells will be maintained up to third subculture and then it will be cryopreserved in three different freezing media.

Freezing Media

Freezing Media 1	10% DMSO + 50% FBS + 40% MEM
Freezing Media 2	0.9M Trehalose in MEM
Freezing Media 3	10% DMSO and 0.9M Trehalose at 1:1

The cells will be eluted and washed at least three times in PBS containing 5% FBS. After final wash, the cell pellet will be suspended in freezing media adjusting the cell density to 4 $\times 10^6$/ml. The single cell suspension will be dispensed into 1ml aliquots in 2ml cryovials. The cryovials will be placed sequentially at 4 °C and then at -20 °C for equilibration subjected viability. Finally, will be transferred to liquid nitrogen for long-term preservation The cryopreserved cells will be thawed after 7, 14, 21 and 28 days of freezing using standard protocol as per Su *et al.* (2011) [11] for post freezing evaluation. The cryoprotectant will be removed by serial dilution and centrifugation methods. Finally, cells will be suspended in culture medium and will be seeded in culture flask for its growth and development. Immediately after thawing, cryoprotectant will be removed and re-suspended in culture media for post freezing culture.

Quality assessment of duck embryo fibroblast cells
Live and dead cell count will be made at equilibration and after thawing. The cell viability will be assessed by determining live and dead percentage staining with trypan blue dye exclusion test. (Yadav *et al.*, 2013) [14]. Multimorphological characteristic evaluation will be made by microscopic observations. After thawing, the cells will be re-cultured to study the revival of cells from freezing shock by observing cell attachment, and maintenance of fibroblast characteristic.

Statistical Analysis: The data generated in the present study were analyzed by using the Statistical Package (SPSS, version 20.0 Chicago, USA) by one way and two way ANOVA. The effects were considered to be significant at $p<0.01$.

Results and Discussion
Quality assessment of duck embryo fibroblast cells
Viability evaluation of duck embryo fibroblast cell
Trypan blue is an azo based dye that is cell membrane impermeable and therefore only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins thereby rendering the cells a blue colour. The trypan blue staining assay allows for a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population. The viability percentage of duck embryo fibroblast cells grown in three different freezing media (10% DMSO, 0.9 M Trehalose and 10% DMSO + 0.9M trehalose) were evaluated by staining with trypan blue stain in equilibration and at post thaw culture at 7, 14, 21, 28 days, and the findings are presented in Table 1. J.L. Hanslick *et al.* (2009) [5] studied that DMSO apoptosis in the developing central nervous system of C57BI/6 mice of varying postnasal ages, are known to have significant cytotoxic effects both in short term and long term. DMSO has been reported to cause translocation of apoptosis-inducing factors from mitochondria to nucleus and poly-(ADP-ribose)-polymerase (PARP) activation. Na *et al.* (2010) [8] also reported in Qingyuan partridge chicken fibroblast line that the mean viability before cryopreservation was 96.8% and it was 92.6% after recovery using 10% DMSO, 50% FBS, and 40% MEM to a final cell density of 8 × 10^6 viable cells/ml. Guan *et al.* (2012) [4] also reported in fibroblast line from Xiaoshan chicken that freezing medium containing 40% DMEM, 10% DMSO and 50% FBS to a final concentration of (3 to 4) × 10^6 viable cells/ml and the viabilities of Xiaoshan fibroblasts before freezing and after recovery evaluated through trypan blue exclusion tests were 98.36± 1.41 and 91.17± 0.92% respectively. Solocinski *et al.* (2017) [10] also reported that reducing the availability of water molecules to join a tetrahedral hydrogen network that plays a formative role in creating ice crystals during freezing, an overall smaller ice crystal size may be observed in presence of water, trehalose, and DMSO compared to the binary water DMSO system. High DMSO concentrations were found to be embedded between ice crystals while trehalose seems to be more ubiquitously distributed throughout both the ice and DMSO rich regions of the sample. A report has been found that addition of 0.2-0.6 M trehalose to a CPA containing 10% DMSO has been demonstrated to increase both post-thaw cell viability and plating efficiency in several mammalian cell types including primary human hepatocytes (Karterz *et al*., 2007) [6], human embryonic cells (Buchanan *et al*., 2004) [2] and pancreatic islets (Battie *et al*., 1997) [1].
Statistical analysis (ANOVA) revealed a significant ($p<0.05$) difference between the viability percentage between the group among the three-freezing media and found that freezing media 3 (10% DMSO + 0.9 M Trehalose) was found better than freezing media 1(10% DMSO) and freezing media 2 (0.9 M Trehalose).

Characteristic evaluation
The characteristic evaluation of the embryo fibroblast cells was done before cryopreservation and after post thaw culture was evaluated Fibroblast cells depicting the typical morphological characteristics were used for

cryopreservation. The fibroblast cell was observed migrating from the explant at 24 hours. Fig 1. The cell growth was observed at every 12-hour interval. The growth was observed for up to third passaging for 80-90% confluence. Fig 2. The cells were maintained up to three sub culture in which purified form is maintained. After post thawing, the growth of the cell was observed for 5 days at 55-60% confluency. The cells show fibroblast like characteristics with turgor vitalis, fusiform appearance, monolayer formation. Na *et al* (2010) [8] studied on a Qingyuan partridge chicken embryonic fibroblast line, the cells were cryopreserved after three passages. Fibroblasts were whirlpool migrating, frame-like, or radiating shapes with turgor vitalis cytoplasm were viable and grew well when thawed. Guan *et al*., (2012) [4] was reported that fibroblast line from Xiaoshan chicken sprouted out 1 day after being plated continued to proliferate and were sub-cultured at 80-90% confluence within 3 days. The cells display typical fibrous and fusiform morphology with centrally located oval-shaped nuclei. The fibroblast grew rapidly and replaced the epithelial cells gradually after 2 to 3 passages.

***In vitro* competence of cryopreserved embryonic fibroblast cells**

Time (hours) require attainment of confluence at different time interval of post thaw culture:

The time required (hours) for confluency of post thaw culture were presented in Table 2 Within different post thaw confluency timing 24 hours takes longer than 48 hours because initially the cells were in cryo-shock so the cells require time to revive from the cold shock, once they revive, they adequately grow among different percentage of confluence. Na *et al*. (2010) [8] on biological characteristics of Qingyuan partridge chicken fibroblast line the growth curve of the QPCE fibroblast line before cryopreservation and after recovery displayed a typical “S” shape. A lag phase of approximately 24 h was observed after the cells were plated which correspond to the recovery period against trypsin damage. Afterward, the cells proliferated rapidly and enter the exponential growth phase until the stationary phase after about 5 days. Solocinski*et al*. (2017) [17] found that after an initial lagging phase of about 3 days, cells numbers increased rapidly over the next 2-3 days, followed by reduced proliferation rates due to contact inhibition.

Analysis of variance between different confluence revealed that the time required among different confluence differed significantly ($p \leq 0.05$) within a group. The confluence at 24 hours and 48 hours was less than 72 hours, 84 hours and 96 hours and between the freezing media 1, freezing media 2 and freezing media 3 differed significantly (0.05%).And freezing media 3 (10% DMSO + 0.9 M Trehalose) and freezing media 2 (0.09 M Trehalose).

Table 1: The Cell Viability % (Mean± SE) Of Fibroblast Cells At Equilibrium And At Post Thaw Cryopreservation.

Timing	10% DMSO + 0.9 M Trehalose	10% DMSO	0.9 M Trehalose
Before cryopreservation	90.75±0.047	90.75±0.047	90.75±0.047
At equilibrium	90.39 ± 0.089^{a}	89.75 ± 0.047^{b}	$87.69\pm.082^{c}$
At 7 days	89.78 ± 0.068^{a}	89.61 ± 0.064^{b}	86.73 ± 0.014^{c}
At 14 days	89.40 ± 0.066^{a}	89.30 ± 0.035^{b}	$86.66\pm.040^{c}$
At 21 days	89.71 ± 0.13^{a}	89.06 ± 0.011^{b}	86.42 ± 0.04^{c}
At 28 days	$89.68\pm.021^{a}$	88.69 ± 0.14^{b}	86.00 ± 0.06^{c}

Means in a row bearing different superscripts (a, b) differ significantly.

Means in a row bearing similar superscripts do not differ significantly. ($p<0.05$).

Table 2: (Mean ± SE) Attainment of confluence at different time of post thaw culture

Time	10% DMSO + 0.9 M Trehalose	10% DMSO	0.9 M Trehalose
24 hrs.	$14.12\pm1.65_{E}^{y}$	$17.68\pm0.97_{E}^{x}$	$9.72\pm0.08_{E}^{z}$
48 hrs.	$26.44\pm1.93_{D}^{y}$	$32.32\pm0.99_{D}^{x}$	$14.84\pm1.14_{D}^{z}$
72 hrs.	$40.44\pm2.72_{C}^{X}$	$43.12\pm1.12_{C}^{x}$	$25.20\pm1.20_{C}^{y}$
84 hrs.	$50.64\pm2.89_{B}^{X}$	$49.56\pm0.18_{B}^{x}$	$44.56\pm0.30_{B}^{y}$
96 hrs.	$59.32\pm0.23_{A}^{x}$	$59.28\pm0.14_{A}^{x}$	$53.76\pm0.10_{A}^{y}$

Means in a rows bearing different superscripts (x,y,z) differ significantly. ($p \leq 0.05$)

Means in a column bearing different subscripts (A, B, C, D, E) differ significantly. ($p \leq 0.05$)

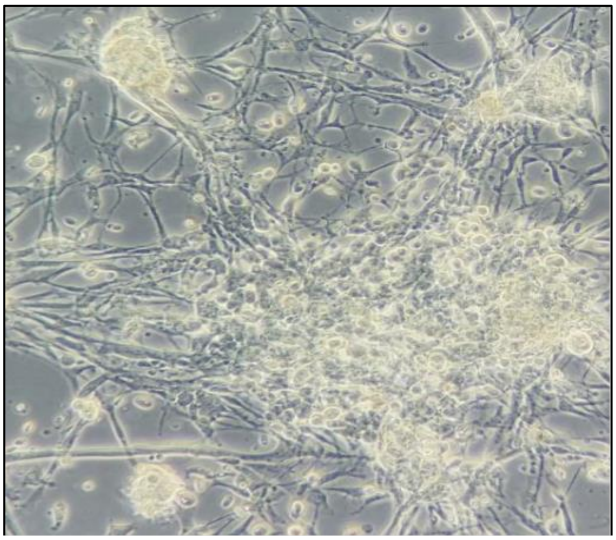

Fig 1: Explant at 24 hours

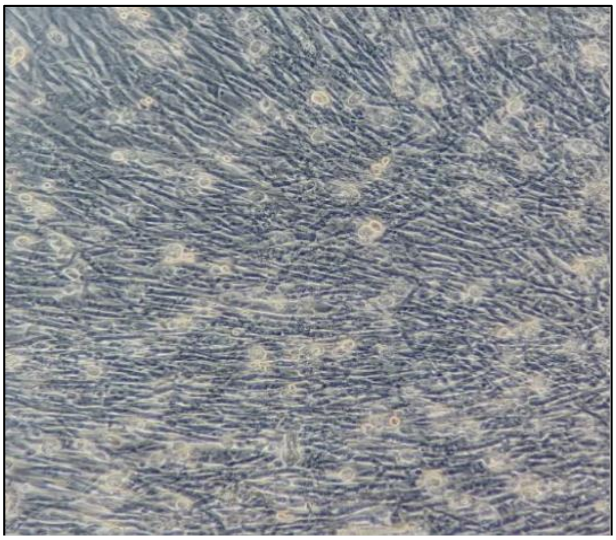

Fig 2: Confluency at third passaging

Conclusion

The present study was conducted to study the quality assessment of duck embryo fibroblast cells and in-vitro competence of cryopreserved embryonic fibroblast cells. Duck eggs were collected from experimental farm of department of Veterinary Physiology C.V.Sc, Khanapara. The embryo was processed and tissue explant cultures were done for isolation and development of fibroblast cells in culture containing Minimum Essential Media (MEM) supplemented with FBS (10%). The media was changed after every 48-72 hours interval. After growth up to third subculture, the cells were processed for cryopreservation. Out of the three freezing media, freezing media 3 (10% DMSO + 0.9 M Trehalose in 1:1) was found to be better in cryopreservation of duck embryonic fibroblast cells followed by freezing medium 2 (0.9 M Trehalose) and freezing medium 1 (10% DMSO). The cell viability before cryopreservation, at equilibrium 4 °C for 15 mins and -20 °C for 20 minutes and post thaw evaluation at 7,14,21,28 days of cryopreservation was found to be optimum resulting healthy growth and development at post thaw culture. Further study is needed to ascertain to the potential application of embryonic duck fibroblast cells for long term cryopreservation and made them available at the time of need.

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