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Research Article

Effect of Slow and Rapid Freezing Method on the Viability of Cryopreserved Vero Cells

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Abstract: In this study, we examined the effects of slow and rapid freezing method on the viability of cryopreserved Vero cells. For this, Vero cells were cultured and maintained following the modified protocol of Ammerman *et al.,* (2008). After total cell counting, two frozen stocks were made simultaneously from the cell suspensions of same concentrations using DMSO and glycerol at a concentrations of 10% and preserved at liquid nitrogen temperature (-1960C) following slow and rapid freezing protocol. After 01 year of cryopreservation both frozen stocks were used providing same nutrients and environment for the revivability of the Vero cells. The Cell viability analysis was performed immediately after thawing by Trypan Blue Exclusion Test. Maximum cell viability rate was (90.4 % in 10% Glycerol & 70% in 10% DMSO) observed in case of slow freezing whereas, it was much less in rapid freezing irrespective to the nature of cryoprotectant. It is concluded that slow freezing method could be the best choice in cryopreservation of Vero cells.

Keywords: Vero cells; cryopreservation; dimethyl sulfoxide (DMSO); glycerol; slow & rapid freezing.

INTRODUCTION

Vero cells were originally isolated from the kidney of a normal adult African green monkey at the Chiba University in Chiba, Japan (Yasumura and Kawakita, 1962). Commercially available Vero cells are Vero, Vero 76 and Vero E6 were being used across the globe, primarily for virus isolation. Maintenance of frozen stocks allows researchers to discontinue regular subculturing, saving valuable time, culture medium, protection from infection/contamination and money and even provide a new source of cells during subsequent passages (Lieu *et al.,* 2007; Xiang *et al.,* 2007).The process of stabilizing biological materials at cryogenic temperatures is called cryopreservation, a practical application of cryobiology or the study of life at low temperatures (Rall, 1987). Advances in cryopreservation technology have led to methods that allow low-temperature maintenance of a variety of tissues, cell types and subcellular materials.

Miyata showed in a study on that glycerol is the best cryoprotectant at a temperature of -75°C (Miyata, 1975). On the other hand, the use of dimethyl sulfoxide (DMSO) as a cryoprotectant has increased because of higher penetration and a lower toxicity relative to other cryoprotectants (Miyata, 1973; Pozio and Rossi, 1988). The cryoprotective effect of glycerol was first discovered (Polge *et al.,* 1949) cryopreservation of spermatozoa has provided the most effective means of preserving genetic resources. However, the presence of glycerol can result in decreased sperm motility and fertility (Hammerstedt *et al.,* 1990).

Cryopreservation can be accomplished by slow freezing and vitrification methods. The major differences between the two are the concentrations of Cryoprotective agents (CPAs) and the cooling rates used. Theoretically, if cooling is sufficiently slow, cells could efflux intracellular water rapidly enough to eliminate super-cooling and thus prevent intracellular ice formation (Gao and Critser, 2000) 5. As a result of differences in the capacity of different cells to move water across the plasma membrane, optimal cooling rates will be dependent on cell types. Slow freezing first substitutes the water within the cytoplasm with CPAs which reduces cell damage and adjusts the cooling rate in accordance with the permeability of the cell membrane. Slow-cooling protocols involve a typical cooling rate of about 1◦C/min in the presence of less than 1.0M of CPA, with use of a high-cost controlled-rate freezer or a benchtop portable freezing container (Yong *et al.,* 2015; Mandawala *et al.,* 2016) .8, 9 The advantages of slow freezing are that it has a low risk of contamination during the procedures and does not demand high manipulation skills. However, slow freezing has a high risk of freeze injury due to the formation of extracellular ice. As an alternative to the slow-freezing technique, vitrification is a process by which cell suspensions are trans-formed directly from the aqueous phase to a glass state after direct exposure to liquid nitrogen (Ral and Fahy, 1985). 35The process requires cooling of the cells or tissues

to deep cryogenic temperatures (i.e., with liquid nitrogen) after their exposure to high concentrations of CPA (in the ratio of 40–60%, weight/volume), with subsequent rapid cooling to avoid ice nucleation. 18 Vitrification is largely dependent on three factors: (1) viscosity of the sample; (2) cooling and warming rates; and (3) sample volume (Yavin and Arav 2007). 18 Thus, a delicate balance must be maintained among all the relevant factors to ensure successful vitrification. There are two methods of vitrification: equilibrium and non-equilibrium. Equilibrium vitrification requires formulation of multimolar CPA mixtures and their injection into the cell suspensions. Non-equilibrium vitrification, which is further divided into carrier-based (including the former plastic straws, quartz microcapillaries, and cryoloops for obtaining a minimum drop volume18) and carrier-free systems, uses an extremely high freezing rate along with lower concentration of the CPA mixture. A major advantage of vitrification is the low risk of freeze injury, thereby ensuring a sufficiently high cell survival rate. However, the high potential of contamination with pathogenic agents is present, and the technique requires good manipulation skills.

Generally, the concentration, type and nature of the cryoprotectant and the cooling and thawing rates are known to be important factors that affect the viability of living materials after cryopreservation. Normally, the Vero cells are preserved in liquid nitrogen temperature for further used. During preservation to overcome cold shock glycerol or DMSO used as cryoprotectant. It is thought that, if the cells are placed directly in liquid nitrogen temperature the cells are supposed to get more cold shock, which negatively hampered the cells revivability. To reduce the problem Slow-cooling protocols involve a typical cooling rate of about 1◦C/min in the presence of less than 1.0M of CPA, with use of a high-cost controlled-rate freezer or a benchtop portable freezing container could be beneficiary. In absence of aforementioned controlled rate freezer gradual adaptation of cells at different temperature such as 2-4°C, -20°C and -80°C could be adopted before placing -196°C temperature. In this regard, no or least research had undertaken and no data is available. Therefore, in the present study, we used two cryoprotectant such as glycerol and DMSO at concentration of 10% but followed both slow and rapid freezing strategy in all cases to investigate in detail the effect of these two freezing strategy on Vero cells cryopreservation.

MATERIALS AND METHODS

Materials

Cell Line

Vero cells (CLS, Germany; order no. 605372) were brought from Germany and used in this study.

CELL CULTURE MEDIA AND REAGENTS

Cell culture medium-M-1999 (Gibco-Invitrogen, cat no. 11825), calf serum-FBS (Gibco-Invitrogen, cat no.10437), 0.25% Trypsin with EDTA (Gibco-Life technologies 20367,C13), DMSO (Gibco-Life technologies), Glycerol were used in this study.

CELL COUNTING CHAMBER

Vero cells were counted using hemocytometer with 0.4% trypan blue dye solution.

METHODS

Growth and Maintenance of Vero Cell Line

Continuous Vero cell culture and subculture were performed according to a modified protocol of Ammerman *et al.,* 2009. Briefly, when 80%–100% confluence was achieved in the cell cultures, medium in the cell culture flasks were carefully removed with a pipette and washed once with sterilized phosphate-buffered saline (PBS) (Hyclone). Then, 1mL of 0.25% Trypsin-EDTA solution (Gibco-Life technologies 20367, C13) was added to detach the cell monolayer. Then, 0.5mL of 50% FBS was added to terminate trypsinization. The cell suspensions were centrifuged at 3000rpm for 10min, and the supernatant removed. The harvested cells were resuspended with M-199 medium plus 10% FBS and 2% penicillin- streptomycin solution. In this study, seven successive subcultures were performed to obtain a relatively strong pure cell.

CRYOPRESERVATION OF VERO CELLS WITH DMSO & GLYCEROL

Vero cells in the logarithmic growth phase (80%–90% confluence) were harvested by rinsing the cell sheet three times with sterilized phosphate- buffered saline (PBS) (Hyclone), after which 2 mL of 0.25% trypsin- EDTA solution (Gibco-Life technologies 20367, C13) was added. Flasks were examined under a phase contrast inverted microscope (Olympus, CK2-TR, Japan) at 37^{0}C for 20–30 sec. Subsequently, the flasks were shaken gently to detach the cells, and 10% FBS was added to terminate trypsinization. Cell suspensions were centrifuged at 3000rpm for 10 min, and the supernatant was removed. The harvested cells were resuspended with freezing medium containing 10% FBS, 10% Glycerol or 10% DMSO and remaining percentage of M-199 and reached a final concentration of 1 - 2 X10^6 viable cells ml^{-1}. The resuspended cells were counted using a hemocytometer and dispensed in to 2.0 ml cryogenic vials (Corning) that were labeled with cell type, freezing medium name and date. Both DMSO & glycerol preserved cryogenic vials were kept in deep freezer at - 80^{0}C and liquid nitrogen (-196^{0}C) in Cryocane for a period of 01 year following slow and rapid freezing protocol.

SLOW FREEZING PROTOCOL

Properly labeled cryovial was first preserved at 2-4^{0}C for 03 hours, then -20^{0}C and -80^{0}C deep freezer for overnight subsequentially and then finally cryopresrved at liquid nitrogen (-196^{0}C) in Cryocane

RAPID FREEZING PROTOCOL

Properly labeled cryovials were preserved directly at liquid nitrogen (-196^{0}C) in Cryocane

CELL VIABILITY ANALYSIS BY TRYPAN BLUE EXCLUSION TEST

A cell viability analysis was performed with trypan blue dye exclusion staining method stated by Louis *et al.,* 2007. Vero cells cryopreserved with both DMSO and Glycerol, immediately after thawing were mixed with an equal volume of 0.4% trypan blue dye solution (LEA Gene Biotech, China). Then, approximately 20 µl of the cell mixture was transferred to both sides of the hemocytometer, covered with a cover glass, and observed under a light microscopy. The numbers of dead and viable cells were recorded based on the development of blue color. Survival rate of Vero cells was calculated using the following formula:

Cells Viability rate = number of viable cells (unstained cells) / total cell number (stained + unstained cells) ×100.

STATISTICAL ANALYSIS

The data of live cell count were analyzed statistically using t- test.

RESULTS AND DISCUSSION

A total of 60 vials containing preserved Vero cells (each 30 vials preserved with both DMSO and glycerol at a concentrations of 10% were used to determine post-thawing revivability. In case of glycerol revivability were 83.33 and 60 whereas in case of DMSO 73.33 and 53.33 percent at slow and rapid freezing respectively (Table-1).

Table-1: Post- thawing revivability of Vero cells

Cryopro-tectant Used	Preservation Method							
	Slow freezing				Rapid freezing			
	Total no. of vials containing Vero cells	No. of vials containing Vero cells (not revived)	No. of vials containing Vero cells (revived)	Post thawing revivability (%)	Total no. of vials containing Vero cells	No. of vials containing Vero cells (not revived)	No. of vials containing Vero cells (revived)	Post thawing revivability (%)
10% DMSO	30	08	22	73.33	30	14	16	53.33
10% Glycerol	30	05	25	83.33	30	12	18	60

The difference might be due to less toxic effect of glycerol than DMSO on Vero cells (Siddiqui *et al.,* 2015). The post thawing revivability is much more higher in case of both cryoprotectant at slow freezing than rapid freezing which indicates that some sorts of cold shock happened at rapid freezing.

The cell viability were 90.4 and 69 percent at both slow and rapid freezing in case of glycerol and in case of DMSO 76 and 57 percent at same freezing method and same cryoprotectant concentrations (Table-2).

Table-2: Percent of live and dead Vero cells counted after recovery from cryopreservation using DMSO and Glycerol

Cryoprotective agent	Cells viability	
	Slow freezing	Rapid freezing
10% Glycerol	90.4	69
10% DMSO	76	57

In this study, the cell viability is much more higher in slow freezing than rapid freezing with both cryoprotectants such as DMSO and glycerol which is a agreement of the findings of Masindi *et al.,* 2016 who found higher motility rate of Venda chicken sperm in slow freezing method and thawing at 5° compared to vitrification method.

The highest cell viability in case of 10% DMSO was 76% at slow freezing which found similar to the findings by Durrani *et al.,,,.* (2015) in a study using 10% DMSO for long-term storage of BHK-21 cells.

In this study, cells viability at 10% DMSO is much less than 10% Glycerol in both cases i.e in both slow and rapid freezing method. These findings are in accordance to the findings of our previous study (Siddiqui *et al.,* 2015). This might be due to DMSO above 4oC has a toxic effect on mammalian cells. Such effects of DMSO might play an important role in cell viability during cell counting. The toxicity of glycerol apparently is related to osmotic and non-osmotic effects. Glycerol exerted toxicity at concentrations of 3.5% or more: the maximal toxicity was observed at 5% in cryopreservation of stallion spermatozoa (Garcia *et al.,* 2012) but data in regard to Vero cells was not available. Although there may be some variation within a given lot, with constant storage conditions the number of recovered cells will generally be the same in all vials. Vial-to-vial variation may be an indication of problems occurring during storage and handling, which hamper cell viability, though this was not considered in this study.

CONCLUSION

It is concluded that slow freezing method could be the best choice in cryopreservation of Vero cells irrespective to the nature of cryoprotectant.

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