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The effect of different concentrations of Dimethyl sulfoxide (DMSO) and glycerol as cryoprotectant in preserving Vero cells

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Abstract

Different concentrations of dimethyl sulfoxide (DMSO) and glycerol were used for cryopreservation of Vero cells. After total cell count Vero cells were preserved in liquid nitrogen. Two frozen stocks were made simultaneously from the cell suspensions of same concentrations using DMSO or glycerol at concentrations of 2.5%, 5%, 10% and 15%. After six months of cryopreservation both frozen stocks were used providing same nutrients and environment for the viability of the Vero cells. The cell viability analysis was performed immediately after thawing by Trypan Blue Exclusion Test. Both cryoprotectants showed a protective effect on Vero cells. When Glycerol was used, a maximum cell viability rate of 89.4% and a lowest cell viability rate of 63% were achieved at concentrations of 10% and 2.5%, respectively. On the other hand, DMSO at a concentration of 10% had the highest effect on cryoprotectivity and showed highest cell viability (75%), while at 15% concentration it showed the lowest cell viability (53%). It is suggested that DMSO and glycerol are appropriate protective materials for the cryopreservation of Vero cells. The solutions at concentration of 10% of DMSO and glycerol could be the best choice of cryoprotectant for long-term (6 months) preservation of Vero cells. (*Bangl. vet*. 2016. Vol. 33, No. 1, 1 – 7)

Introduction

Vero cells were originally isolated from the kidney of a normal (non-diseased) adult African green monkey on March 27, 1962 by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. There are several lines of Vero cells commercially available (Vero, Vero 76, Vero E6), all ultimately derived from the same source. Vero cell lines have been used across the globe, primarily in virology laboratories. Maintenance of frozen stocks is extremely important when culturing cell lines. When actively growing cells are not required for an extended period, keeping frozen stocks allows researchers to discontinue regular sub-culturing, saving valuable time, culture medium, risk of infection/contamination and money (Lieu *et al.,* 2007; Xiang *et al.,* 2007). Also, very importantly, frozen stocks of cells provide a new source of cells during subsequent passages. In order to maintain an inventory of low-subculture Vero cells, frozen stocks should be prepared shortly after initiating cultures from

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frozen stocks. Freezing biological materials is called cryopreservation (Rall, 1987). Advances in cryopreservation allow maintenance of a variety of tissues, cell types and subcellular materials. Generally, the concentration and type of the cryoprotectant, and the cooling and thawing rates are important factors that affect the viability of living materials after cryopreservation. It was shown that glycerol is the best cryoprotectant at -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 than other cryoprotectants (Miyata, 1973; Pozio and Rossi, 1988). However, the effect of different concentrations of DMSO and glycerol on the survival rate of Vero cells is not known clearly. The cryoprotective effect of glycerol was first discovered (Polge *et al.,* 1949) in the cryopreservation of spermatozoa. However, glycerol can decrease sperm motility and fertility (Hammerstedt *et al.,* 1990). Until now, the effect of glycerol at different concentrations on non-human primate spermatozoa cryopreservation has only been demonstrated in cynomolgus monkeys (*Macaca fascicularis*) (Mahone and Dukelow, 1978; Sankai *et al.,* 1994) and 5–7% glycerol had shown to be suitable. Another penetrating cryoprotectant, DMSO, has been successfully used for cryopreservation of spermatozoa in the cynomolgus monkey (Feradis *et al.,* 2001): 6% DMSO level provided cryoprotection of sperm motility similar to that of glycerol. In the rhesus monkey, which is closely related to the cynomolgus monkey, 3% (Si *et al.,* 2000), 5% (Sanchez-Partida *et al.,* 2000; Si *et al.,* 2000), 7% (Leverage *et al.,* 1972), and 14% (Roussel and Austin, 1967) glycerol have been used for sperm cryopreservation. In only one study rhesus monkey offspring were produced using cryopreserved spermatozoa (Sanchez-Partida *et al.,* 2000). However, no study has compared the efficacy of different levels of glycerol or DMSO and no report has been published regarding Vero cell cryopreservation with glycerol. Therefore, in the present study, we used four different concentrations (2.5, 5, 10%, and 15%) of glycerol and DMSO to investigate the effect of these two cryoprotectants on Vero cells cryopreservation.

Materials and Methods

Cell line, culture media and reagents

Vero cells (CLS, Germany; order No. 605372) were purchased from Germany and 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.

Cell counting chamber

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

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 was carefully removed with a pipette and washed once with sterilized phosphate-buffered saline (PBS; Hyclone).

Then 1 mL of 0.25% Trypsin-EDTA solution (Gibco-Life technologies 20367, C13) was added to detach the cell monolayer. Then 0.5 mL of 50% FBS was added to terminate trypsinization. The cell suspensions were centrifuged at 3000 rpm for 10 min, and the supernatant removed. The harvested cells were re-suspended with M-199 medium plus 10% FBS and 2% penicillin-streptomycin solution. Seven successive subcultures were performed to obtain strong pure cells.

Cryopreservation of Vero cells with DMSO and 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 1 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^{o}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 3000 rpm for 10 min, and the supernatant was removed. The harvested cells were re-suspended with freezing medium containing 10% FBS, dimethyl sulfoxide (2.5, 5, 10% and 15%; DMSO; Sigma, USA) or Glycerol (2.5, 5, 10% and 15%) and made up to 100% with M-199 to reach a final concentration of 1 - 2 X10^{6} viable cells /mL. The re-suspended cells were counted using a haemocytometer and dispensed into 2.0 mL cryogenic vials (Corning). All vials were kept first at 2 - 8^{o}C for 3-4 hours, then at –80^{o}C overnight, and finally stored in liquid nitrogen (-196^{o}C) until use.

Post-thawing recovery of Vero cell lines

To recover and re-seed the cells, the cryogenic vials were removed from the liquid nitrogen after 6 months of cryopreservation and quickly thawed at 37^{o}C in a water bath. The thawed cells were transferred to 25 sq-cm cell culture flask (Corning), and 5 mL M-199 media was added slowly to equilibrate and exchange the DMSO or glycerol within 2 to 3 min. After centrifugation at 3000 rpm for 5 min, the pellet was re-suspended gently in M-199 medium containing 20% FBS and 2% penicillin-streptomycin solution and cultured at 37^{o}C at 5% CO_2 and 100% humidity.

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 DMSO or Glycerol at different concentrations (2.5, 5, 10% and 15%) were mixed immediately after thawing with an equal volume of 0.4% trypan blue dye solution (LEA Gene Biotech, China). Approximately 20 µl of the cell mixture was transferred to both sides of the haemocytometer, covered with a cover glass, and observed under a light microscope. The numbers of dead and viable cells were recorded based on the development of blue colour. Survival rate of Vero cells was calculated using the formula:

Cell 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 80 vial containing preserved Vero cells [20 vials, each preserved with DMSO or glycerol at different concentrations (2.5, 5, 10% and 15%)] were used to determine post-thawing viability. In case of glycerol, viability was 50, 65, 75% and 45% whereas in case of DMSO, viability was 40, 60, 65% and 30% at concentrations of 2.5, 5, 10% and 15%, respectively (Table 1). The difference might be due to less toxic effect of glycerol than DMSO on Vero cells.

Table 1. Post-thawing viability of Vero cells

Cryoprotectants	Concentrations (%)	Total No. of vials containing Vero cells	No. of vials containing Vero cells (not revived)	No. of vials containing Vero cells (revived)	Post-thawing viability (%)
Glycerol	2.5% glycerol	20	10	10	50
	5% glycerol	20	07	13	65
	10% glycerol	20	05	15	75
	15% glycerol	20	11	09	45
DMSO	2.5% DMSO	20	12	08	40
	5% DMSO	20	08	12	60
	10% DMSO	20	07	13	65
	15% DMSO	20	14	06	30

The cell viability was 63, 87.9, 89.4 and 79% in case of glycerol and in case of DMSO 61, 73, 75 and 53% at concentrations of 2.5, 5, 10 &15%, respectively ($P < 0.05$) (Table 2). The highest cell viability in case of DMSO was 75% at 10% concentration, similar to the findings by Durrani *et al.* (2015) using 10% DMSO for long-term storage of BHK-21 cells.

The highest cell viability in case of glycerol was 89.4% at 10% concentration. Very few similar studies have been conducted, but 10% glycerol was used by Visintin *et al.* (2000) for cryopreservation of rat morulae: embryos were cultured in Whitten's medium at 37°C, 5% CO_2 and 100% humidity; the *in vitro* development ranged from 56.6% to 100% after 72 hours of incubation. Effect of glycerol and DMSO on cryopreservation of rhesus monkey (*Macaca mulatta*) sperm was evaluated by Si *et al.* (2004) where 5% glycerol was better than 5% DMSO. It was found that spermatozoa cryopreserved with 15% DMSO showed the lowest post-thaw sperm motility, and spermatozoa cryopreserved with 15% glycerol and 15% DMSO showed the lowest plasma membrane integrity. These findings were in accordance with the findings of the present study.

Table 2. Percentage of live and dead Vero cells counted after recovery from cryopreservation using DMSO or Glycerol

Cryoprotective agents	Concentrations (%)	Cell viability (%)
Glycerol	2.5	63
	5	87.9
	10	89.4
	15	79
DMSO	2.5	61
	5	73
	10	75
	15	53

DMSO above 4°C has a toxic effect on mammalian cells. Such effects might play an important role in cell viability. The toxicity of glycerol apparently is related to 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.

In this study, we evaluated the protective properties of glycerol and DMSO at different concentrations for Vero cell cryopreservation. In comparison with lower (2.5%) and higher (15%) levels, both glycerol and DMSO at concentrations of 10% showed higher post-thaw viability. The results indicate that the best concentration of glycerol and DMSO for Vero cell cryopreservation is 10%, which confirms previous observations in cynomolgus monkeys' sperm cryopreservation (Mahone and Dukelow, 1978; Sankai *et al.,* 1994). We found that DMSO at concentrations of 2.5%, 5%, 10%, and 15% was inferior to 10% glycerol for preserving Vero cells, which indicated that glycerol could be a cryoprotectant of choice in long-term preservation of Vero cells. In our previous study Vero cell viability was 74 and 88.6% after one year of cryopreservation in 10% DMSO and glycerol, respectively (Siddiqui *et al.,* 2015), close to the findings of the present study. This indicated a difference in the efficacy of DMSO and glycerol in preserving Vero cells. This difference might be due to the differences in the mode of action.

Conclusions

DMSO and glycerol are the appropriate protective materials for the preservation of Vero cells: 10% DMSO or glycerol could be the best choice of cryoprotectant for long-term (6 months) protection of Vero cells.

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