

## **Comparative effectiveness of Dimethyl Sulphoxide (DMSO) and Glycerol as cryoprotective agent in preserving Vero cells**

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### **Abstract**

Vero cells have been derived from the kidney of African green monkeys (*Cercopithecus aethiops*) as mammalian continuous cell lines for biological research. Continuous culturing of Vero cells as well as maintenance of frozen stock is important for future use. Dimethyl sulphoxide (DMSO) and glycerol are two common cryoprotectants used widely to maintain frozen cell lines. DMSO, as a cryoprotectant in cryopreservation procedures, is detrimental to cells. A study was carried out to compare the effect of DMSO and glycerol as cryoprotectants on Vero cells. Vero cells were cultured and maintained using same nutrients and environment, following the modified protocol of Ammerman *et al.* (2008). After total cell counting the Vero cells were preserved in Cryocane at liquid nitrogen temperature (-196°C). Two frozen stocks were made simultaneously from the cell suspensions of same concentrations using DMSO and glycerol. After one year of preservation post-thawed cell viability with glycerol was higher than with DMSO. Solutions of 10% glycerol could be the best choice as cryoprotectant for long-term (1 year) preservation of Vero cells. (*Bangl. vet.* 2015. Vol. 32, No. 2, 35 – 41)

### **Introduction**

Vero cells were originally isolated from the kidney of healthy adult African green monkeys (*Cercopithecus aethiops*) in 1962 by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. By the end of the 1960s, Vero cell lines were being used across the globe, primarily in virology laboratories. Vero cells are one of the most common mammalian continuous cell lines used in biological research. This anchorage-dependent cell line has been used extensively in virology, and in the propagation and study of intracellular bacteria (*e.g.*, *Rickettsia* spp.; UNIT 3A.4) and parasites (*e.g.*, *Neospora*), and assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level. Vero cells have been licensed in the United States for production of live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines, and throughout the world Vero cells have been used for the production of other viruses, including Rabies virus, Reovirus and Japanese encephalitis virus. There are several lines of Vero cells commercially available (*e.g.*, Vero, Vero 76, Vero E6), all ultimately derived from the same source.

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Maintenance of frozen stocks is extremely important when culturing cell lines. When actively growing cells are not required for three weeks or more, keeping frozen stocks allows researchers to discontinue regular sub-culturing (Lieu *et al.*, 2007; Xiang *et al.*, 2007). In order to maintain an inventory of low-subculture Vero cells, new frozen stocks should be prepared shortly after initiating cultures from frozen stocks. Techniques are available for the cryopreservation of microorganisms, tissues, primary cells, established cell lines, small multicellular organisms, embryos, as well as nucleic acid and proteins (Mazur, 1984; Rall, 1987; Simione and Brown, 1991). Many compounds have been tried as cryoprotective agents, including sugars, solvents and even serum. DMSO and glycerol have been widely used and demonstrated to be the most effective agents for preserving living cells and organisms (Areman *et al.*, 1990; Lee *et al.*, 2005; Fleming and Hubel, 2006). Other cryoprotectants include polyethylene glycol, propylene glycol, polyvinylpyrrolidone, sorbitol, dextran and trehalose (Loken and Demetrick, 2005; Buchanan *et al.*, 2005).

DMSO is the most frequently used cryoprotectant in research and clinical purposes. However, there is no consensus on its optimal concentration. Defining the right concentration is important, because DMSO can cause severe adverse reactions. The recent trend is reducing DMSO concentration and/or replacing DMSO by other non-toxic cryoprotectants. About 10% of DMSO is widely used, but some centres have started to test lower DMSO concentrations.

Glycerol acts by retaining water within the cell, avoiding excessive dehydration due to the exposure to concentrated solutions. Higher concentrations of glycerol are most protective (Mazur and Kleinhan, 2008). On the other hand cryoprotectant toxicity has been claimed to be the limiting factor for the success of cryopreservation (Fahy, 2010). To freeze mammalian sperms glycerol is the most widely used cryoprotectant, however glycerol may have toxic effects on the spermatozoa in a dose-dependent manner (Meryman, 1971). Equine semen is frozen using glycerol at concentrations ranging from 2 to 5% (Loomis *et al.*, 1983; Vidament *et al.*, 2000; Loomis and Graham 2008), however few controlled studies have addressed the toxicity of different concentrations of glycerol (Gao, 1992; Ball and Vo, 2001). The nature of the cryoprotective agent and the freezing process are important factors affecting the recovery of cryopreserved mammalian cells (Baust, 2002). This study has been conducted to find a suitable cryoprotective agent for the long-term cryopreservation of Vero cells as well as enhancement of their recovery.

## Materials and Methods

### *Cell line*

Vero cell line (CLS, Germany) was bought from commercial source.

### *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.

### ***Cell counting***

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

### ***Growth and maintenance of Vero cells***

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) (Sigma Aldrich). 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 trypsinisation. 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 a relatively strong pure cell.

### ***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: Sigma Aldrich), after which 0.1 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°C for 20–30 sec. The flasks were shaken gently to detach the cells, and 10% FBS was added to terminate trypsinisation. 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, 10% DMSO (Sigma, USA) or 10% glycerol and 80% M-199 and reached a final concentration of  $1 - 2 \times 10^6$  viable cells/mL. The re-suspended cells were counted using a haemocytometer and dispensed into 2.0 mL cryogenic vials (Corning). DMSO-preserved cryogenic vials were transferred directly into liquid nitrogen and glycerol-preserved vials were kept first at 2 - 8°C for 3 - 4 hours, then at - 80°C overnight and finally stored in liquid nitrogen.

### ***Thawing of Vero cell lines***

To recover and reseed the cells, the cryogenic vials were removed from the liquid nitrogen after one year of cryopreservation and quickly thawed in a 37°C 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 media containing 20% FBS and 2% penicillin-streptomycin solution and cultured at 37°C in 5% CO<sub>2</sub> and 100% humidity.

### ***Cell viability analysis by Trypan Blue Exclusion Test***

A cell viability analysis was performed using the trypan blue dye exclusion staining method of Anjomshoa *et al.* (2009). Vero cells cryopreserved with DMSO or glycerol

were mixed with an equal volume of 0.4% trypan blue dye solution (LEA Gene Biotech, China) immediately after thawing. Approximately 20 ml of the cell mixture was transferred to both sides of the haemocytometer, covered with a cover glass, and observed under light microscopy. 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:

$$\text{Cells viability} = \frac{\text{Number of viable cells (unstained cells)}}{\text{Total cell number (stained + unstained cells)}} \times 100$$

### Statistical analysis

The data of live cells count were analysed using *t*-test.

## Results and Discussion

A total of 40 vials containing preserved Vero cells (20 preserved with 10% DMSO and 20 with 10% glycerol) were used to determine post-thawing viability. In case of 10% DMSO viability was 60% whereas in case of glycerol 70% (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

Cryoprotective agent	Total no. of vials containing Vero cells	No. of vials containing Vero cells (not revived)	No. of vials containing cells (revived)	Post-thawing viability (%)
10% glycerol	20	06	14	70
10% DMSO	20	08	12	60

The cell viability was 14.6% more in case of glycerol than DMSO (Table 2) ( $P < 0.05$ ) similar to the findings by Durrani *et al.* (2015) in a study using 10% DMSO for long-term storage of BHK-21 cells. The result is dissimilar to the findings of Merten *et al.* (1995) who developed a serum-free medium to which 10% DMSO and 0.1% methylcellulose or 3% polyvinyl pyrrolidone were added, for the cryopreservation of animal cells.

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

Cryoprotective agent	Cell viability
10% DMSO	74%
10% glycerol	88.6%

The cell viability in case of glycerol was 88.6%. Very few similar studies have been conducted, but 10% glycerol was extensively used in a study conducted by Visintin *et al.* (2000) for cryopreservation of rat morulae: embryos were cultured in Whitten's

medium at 37°C, 5% CO<sub>2</sub> and 100% humidity; the *in vitro* development ranged from 56.6% to 100% after 72 hours of incubation. Effect of glycerol and dimethyl sulfoxide 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. In contrast, Feradis *et al.* (2001) found that glycerol and DMSO at 6% concentration produced equivalent survival rates of cynomolgus epididymal sperm after cryopreservation. Lusianti *et al.* (2013) observed that storage of red blood cells (RBCs) in a refrigerated state allowed a shelf-life of a few weeks, whereas RBCs frozen in 40% glycerol had a shelf-life of 10 years.

DMSO above 4°C has a toxic effect on mammalian cells: that is why cryopreserved cells should be re-suspended as soon as possible after thawing at 37°C to reduce the adverse effect of DMSO, such adverse 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. Another factor that can affect the recovery of cryopreserved Vero cells was the freezing process. The slow freezing protocol in case of glycerol preservation might improve Vero cell viability.

Glycerol has been used widely as a cryoprotectant for mammalian sperm cells but has not been used frequently to preserve other mammalian cells like Vero or BHK. BHK or Vero cells are genetically mutated, and less sensitive than sperm cells. Glycerol is cheaper and more available than DMSO in Bangladesh. Therefore, glycerol could be as effective as DMSO for the long-term preservation of Vero cells in liquid nitrogen.

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