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Short Communication

Study on Cryo-preservation of *Theileria annulata* Schizont Infected Cell Line Vaccine Strain S15

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ABSTRACT

The purpose of this study was to examine the potential application of Sorbitol, Sucrose and Gelatin in combination with glycerol in order to *Theileria annulata* infected cell line cryo-preservation. The efficiency of the freezing methods was compared by assessing the viability and plating efficiency of cells after resuscitation, dilution and equilibration in cell culture medium. As a result, the conventional cryo-preservative medium containing 5% PEG treated Bovine Serum and 10% glycerol successfully cryo-preserved cell as efficiently as the standard medium containing 80% FBS and 10% glycerol and was superior to all thirteen different experimental cryo-preservative media. A crucial result was obtained among the thirteen different serum-free media which showed that the 0.2M sorbitol+10% glycerol method yielded cells with viability of 54% and good plating efficiency results. It was concluded that the conventional method would be the best and ideal for the cryo-preservation and storage of *Theileria annulata*-infected cells for vaccine purposes to date.

Keywords: Cryo-preservation, *Theileria annulata*, Sorbitol, Sucrose, Gelatin, Glycerol

INTRODUCTION

The phenomenon of cell preservation is an extremely important aspect of cell culture. Freezing is the only effective mean of animal cells preservation, which can be accomplished with either liquid nitrogen or by employing deep freezers. The process of freezing (cryo-preservation) involves slowly reducing the temperature of prepared cells to -30 to -60 °C followed by a transfer to temperatures less than -130 °C. At these low temperatures (less than -130 °C), any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped (OPS Diagnostics 2007).

In 1963 Peter Mazur, showed that lethal intracellular freezing could be avoided if cooling was slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid (Mazur 1970). Phenomena which can cause damage to cells during cryo-preservation mainly occur during the freezing stage, and include: solution effects, extracellular ice formation, dehydration and intracellular ice formation. Many of these effects can be reduced by cryoprotectants.

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Cryo-preservatives are necessary additives to cell concentrates, since they inhibit the formation of intra and extracellular crystals and hence cell death. Cryoprotectants such as DMSO and glycerol are valuable to prevent cell dehydration during the freezing process (Stéphenne *et al* 2010).

Novel serum free cryo-preservative solutions were developed including different cryoprotectants: glucose, fructose (monosaccharides), maltose, trehalose, sucrose, lactose (disaccharides), maltitol, mannitol, xylitol, sorbitol, inositol (sugar alcohols), methyl cellulose, dextran, xantham gum (polysaccharides), proline, glutamine (amino acids), and sericin (Cell technology 2005).

The bovine Theileriosis vaccine is viably cryo-preserved at -196 °C in large liquid nitrogen tanks or -70 °C freezers at production facility and transported to farms in smaller liquid nitrogen containers (OIE 2008).
The aim of this study was to examine the potential application of Sorbitol, Sucrose and Gelatin in combination with Glycerol in order to *Theileria annulata* infected cell line cryo-preservation in comparison with standard method of animal cell freezing and conventional protocol of cell cryo-preserving used in process of bovine theileriosis vaccine production.

MATERIALS AND METHODS

To evaluate stability of the cryo-preserved cells during storage in liquid nitrogen, the viability and viable cell recovery of cryo-preserved *Theileria annulata* infected cell line have been studied throughout the twelve months storage.

***Theileria annulata* infected cell line.** *T. annulata* schizont infected cell line "S15" Iran vaccine strain was used in this study. S15 strain has been used for live attenuated vaccine production in Iran more than three decades (Hashemi-Fesharki 1998). This vaccine cell line is a local strain and has been attenuated after 260 passages (Hashemi-Fesharki 1988).

Cell culture. *T. annulata* infected cell line was grown as previously described (Hashemi-Fesharki 1988). Briefly, the working seed was cultured in complete Stoker medium (added 10% bovine serum, Penicillin 100 IU/ml and Streptomycin 100 µg per ml) and cultivated in 25-Cm^2 culture flasks at 37 °C, until they reached confluence. Preparing cell culture passage or subcultures could be made by simply transferring an adequate number of cells to establish a culture, into a new tissue culture flask and fresh medium was supplied after 4 to 5 days.

Experimental cryo-preservation media. Fifteen cryoprotectant combinations, listed in table 1, were compared in this study: 0.2M Sorbitol (Merck, Germany), 0.4M Sorbitol, 0.6M Sorbitol , 0.1M Sucrose (Fluka, Switzerland), 0.25M Sucrose, 0.5M Sucrose, 3% Gelatin (Sigma, USA), 4% Gelatin, 5% Gelatin, combination of 0.4M Sorbitol+ 0.1M Sucrose, combination of 4% Gelatin+0.4M Sorbitol, combination of 0.1M Sucrose+ 4% Gelatin, combination of 0.4M Sorbitol+4% Gelatin+0.1 M Sucrose, and 80% fetal calf serum (Biosera, UK) as standard control, and finally 5% PEG treated bovine serum (Razi Inst., IR Iran) as a conventional method of cell freezing in process of bovine theileriosis vaccine production. All cryoprotectant solutions were prepared in modified medium Stoker containing 10% glycerol (Merck, Germany).

Formulation and Cell Freezing. After counting the cells with a hemocytometer, cells were centrifuged at 400 g for 10 minutes and resuspended in different freezing medium combinations (table 1) at a concentration of 2.5×10^6 cells/ml. Then, the cells were aliquoted in cryotubes and cryovials, 1.5 ml/tube and 12 ml/vials respectively. The tubes and vials were placed upright in a cryobox in a -70 °C freezer. After 24 hours, the tubes and vials were transferred to liquid nitrogen (-196 °C) container.

Thawing. For the period of 12 months, the vials and tubes were taken out of the liquid nitrogen storage tank and were thawed rapidly by swirling in 37 °C water

Table 1. Thirteen cryo-preservative media were used for freezing the *Theileria annulata* schizont infected cell line vaccine strain S15, in comparison with standard as well as conventional vaccine cell cryo-preservation methods. The results of assay were obtained by cell viability test and cell culture.

	Cryo-protectant (CPA) Combination	Viability of cells for the period of 12 months freezing	
		Viability % (by dye exclusion)	The results of Culture §
1	Sorbitol 0.2M +10% Glycerol	54.13	2.38
2	Sorbitol 0.4M +10% Glycerol	38	1.63
3	Sorbitol 0.6M +10% Glycerol	32.56	0.75
4	Sucrose 0.10M +10% Glycerol	39.06	1.31
5	Sucrose 0.25M +10% Glycerol	43.69	1.06
6	Sucrose 0.50M +10% Glycerol	33.88	0.63
7	Gelatin 3% +10% Glycerol	63.69*	0
8	Gelatin 4% +10% Glycerol	67.31*	0
9	Gelatin 5% +10% Glycerol	68.06*	0
10	Sorbitol 0.4M, Sucrose 0.1M +10% Glycerol	44.25	0.81
11	Gelatin 4%, Sorbitol 0.4M +10% Glycerol	75.63*	0
12	Sucrose 0.1M, Gelatin 4% +10% Glycerol	69.69*	0
13	Sorbitol 0.4M, Gelatin 4%, Sucrose 0.1M +10% Glycerol	67.13*	0
14	80% FCS +10% Glycerol (Standard method)	86.56	4
15	5% BS +10% Glycerol (Vaccine method)	81.75	4

* The marked values are false because the gelatin has inhibited the dye infusion into the dead cells.
§ Cell culture was scored after five days by; 0: cells were dead, 1: cells were alive without growing, 2: cells were alive with a low growing (no pH shift), 3: cells were grown well (pH shift), and 4: cells were grown very well (severe pH shift).

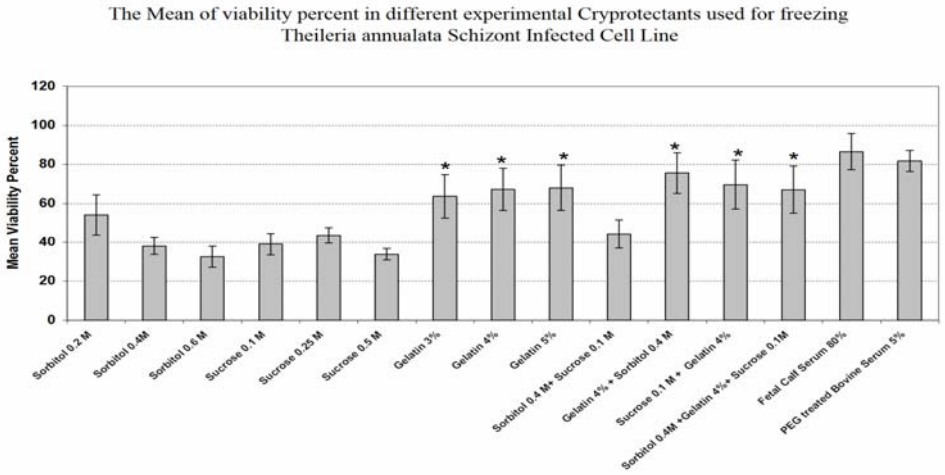

Figure 1. The mean viability percent of cryo-preserved cells were estimated by trypan blue dye exclusion method during 12 months freezing. The asterisk bars show samples including gelatin in media with false-positive results, because of no efficiency was obtained in viable cell recovery. Values are mean +/- 2SE.

bath. Then, the cells were subjected for viability assay and plating efficiency tests (Arikura *et al* 2002).

Viability Test. Viability test was performed as follows: suspension cell cultures were diluted into a trypan blue solution (0.04%), counted using a hemocytometer and scored for trypan positive and negative cells. These totals were compared to the total number of cells frozen down, and a percent viability calculated.

Plating Efficiency. This test was aimed to evaluate the level of cell growing, immediately after thawing. The assay was performed on the thawed cells by plating them into fresh target complete medium. Each aliquot of frozen cells was rapidly thawed at 37°C and added 1 ml of cell suspension to a tissue culture flask containing 4 ml complete Stoker medium (Razi Inst. Iran). TC flasks were incubated at 37°C incubator for 5 days and then have been observed by an inverted microscope (Olympus, NY) at 50× power.

The Statistical Analysis. The statistical analysis was performed with Analysis of Variance, paired t test, independent t test, and Dunnett t Test for multiple comparisons by using the statistical package SPSS 13.0 and s-plus 8.0 and p-value less than 0.05 considered as a significant level.

RESULTS AND DISCUSSION

The cell concentration of $2.5x10^6$ *Theileria annulata* infected cells/ml was used for all groups as well as two controls. The cryo-preservative was glycerol at concentration of 10%, moreover, Sorbitol, Sucrose and Gelatin were selected as cryoprotectants in different concentrations and combinations (Table 1). *Theileria annulata* infected bovine cells were frozen to -70 °C freezer and then transferred to -196 °C in liquid nitrogen. The efficiency of the freezing methods was compared by assessing the viability and plating efficiency of cells after thawing.

Viability test. The total cell count was determined using a hemocytometer. The viability percent of the frozen cells were estimated by trypan blue dye exclusion method. The mean viability of cryo-preserved cells was 86% for standard procedure of cell freezing, and 81% for the conventional protocol for freezing of bovine theileriosis vaccine (Table 1). The combination of Sorbitol 0.2M and glycerol 10% showed the highest level of viability percent (54%) between all proposed experimental cryoprotectants (Figure 1). To determine the effect of cryo-preserved cells vessel storage conditions on viability and viable cell recovery, paired samples of frozen cells were stored at −196 °C during 12 months in cryovials and cryotubes. The comparisons between these two methods of storage revealed no significant differences in cryo-preserved cells obtained from all 15 experiments, with respect to viability and viable cell recovery (correlation was 95%, the assay was carried out by paired t test, P value = 0.0).

Plating Efficiency. Plating efficiencies of the 13 cryoprotectants and two control groups were studied for the period of 12 months deep freezing. The mean values of plating efficiency tests of thawed cells were shown in table 1. The obtained results of two control groups of standard and conventional protocol of vaccine production were significantly higher than all experimental combinations (Figure 1). The statistical analysis (independent t test) has shown that there was no significant difference between two methods of standard procedure for cell freezing and conventional technique for Bovine Theileriosis vaccine production (P value >0.05). Analysis of variance for seven cryoprotectants (excluding 6 CPAs combinations having gelatin from all 13 experimental CPAs) showed there was significant difference (p value = 0.0). Although the combination of 0.2M Sorbitol+10% glycerol, and 0.25M Sucrose+10% glycerol exhibited high efficiency results between experimental cryoprotectants., but the combination of Gelatin in different percentages (3-5) with Sorbitol and or Sucrose had the lowest rate in plating efficiency post freezing in experimental groups (Table 1). Dunnett t test was used for multiple comparisons of the seven CPAs; first to sixth and tenth cryoprotectants. By this hypothesis that

the first CPA (Sorbitol 0.2M) has shown the highest percentage for viability test, as the criteria for this comparison; there was significant differences between the first and other cryoprotectants; 2, 3, 4, and 6 (P value = 0.01), but not for 5 and 10 (P value = 0.08 and P value >0.1 respectively).

Cryogenic preservation (storage below -70 °C) of the cell cultures is widely used to maintain reserves of cell cultures and remains the method of choice for long-term preservation of bovine theileriosis vaccine. The present study was planned to compare the effect of different cryoprotectants on cryo-preservation of *Theileria annulata* infected bovine cells. This investigation, conducted for 12 months, analyzed the efficiency of the freezing methods for bovine *Theileria annulata* infected cell line S15 vaccine strain. Thirteen experimental cryoprotectant combinations were analyzed for the period of twelve months to assess the stability of cryo-preserved cells. The efficiency of the freezing methods was compared by assessing the viability and plating efficiency of cells after resuscitation, dilution and equilibration in cell culture medium. In this study, *T. annulata* infected bovine cells were better preserved in 10% glycerol +5% bovine serum than in gelatin, sucrose and sorbitol combinations. But, unexpected the most terrible results were obtained when gelatin was used as cryoprotectant. The combinations 7-9 and 11-13 included gelatin had shown false-positive results for viability percent because of no efficiency was obtained in viable cell recovery (Table 1). The cryo-preservation of *T. annulata* infected bovine cells in 5% bovine serum resulted in percentage of standard protocol (80% FCS). Strict comparisons between our results and those of others were not possible, due to differences in cell types, species and cryo-preservation protocols. High FBS (Fetal Bovine Serum) concentrations may also help cells survive freezing. FBS (80% v/v) supplemented with 10% (v/v) Glycerol / DMSO is extensively used as a freezing medium for mammalian cells using conventional methods. However, FBS should ideally be avoided, owing to serious concerns regarding bovine spongiform encephalopathy and other infections such as viruses. Therefore, it is necessary to reduce the level of serum in cryoprotectant combinations. Thus the obtained results clearly showed the vaccine protocol for cell cryo-preservation is the best method among the experimental formulations even standard control used for *Theileria annulata*-infected cell cryo-preservation.

Hashemi-Fesharki and Shad-Del have reported long-standing preservation of *Theileria annulata* infected cell lines with 10% glycerol as cryoprotectant, but for *T.annulata* infected liver, spleen and blood tissue 13.5% glycerol was used and were frozen at -70°C for up to three years and finally no changes were occurred in the virulence of the cryo-preserved strains (Hashemi-Fesharki and Shad-Del 1973). The amazing results by Wathanga et al. who reported Bovine lymphoblastoid cell cultures infected with *Theileria annulata* have been frozen by different concentration of DMSO as a cryo-preservative using a programmable cooling apparatus. The results showed that the yielded cells with viability of 95 to 97% and a plating efficiency equal to that of unfrozen cells (Wathanga *et al* 1986). Kimbita et al. have used five different cryoprotectants 7.5% for glycerol, 5% for dimethyl sulphoxide (DMSO), poly vinylpyrrolidone (PVP) and poly ethylene glycol (PEG), and 2.5% for hydroxyethyl starch (HES)). They have shown the glycerol was significantly better than the others for preserving *T. parva* sporozoites (Kimbita *et al* 2001). Moreover, sucrose and glycerol were used for cryo-preservating of stabilates of *Theileria parva* sporozoites in liquid nitrogen (Mbao *et al* 2007). These two reports are in parallel of our results for using glycerol and Sorbitol for freezing the Theileria. One of the most interesting finding for cryo-preservation of trypanosomes was the report for Ndao et al. who revealed the glycerol yielded 90% viable parasites, whereas using dimethylsulfoxide, a more commonly used cryo-protectant, rendered the viability percent to only 35% (Ndao *et al* 2004). *Babesia microti* has been frozen at -196 °C using 19% glycerol as cryoprotectant by Gary and Phillips. They have reported that parasite

survival was increased by using a solution of sorbitol in saline as the diluent for the glycerol and for washing the recovered blood (Gray & Phillips 1981). Palmer et al. have cryo-preserved *B. bovis* by glycerol in -196 °C for 60 days with no appreciable reduction in plating efficiency (Palmer *et al* 1982). Since the cryoprotective additives (CPAs) used in the frozen storage of various microorganisms (viruses, bacteria, fungi, algae, and protozoa) include a variety of simple and more complex chemical compounds. The best CPA, or combination of CPAs, and the optimum concentration for a particular cryo-sensitive microorganism including protozoa and animal cell has to be examined and determined experimentally (Hubálek 2003). It was concluded that the conventional protocol of freezing for *T. annulata* schizont infected cell line by using 5% PEG treated bovine serum and 10% glycerol would be ideal for the cryo-preservation and storage of stocks of *T. annulata* schizont infected cells for vaccine purposes.

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