Effects of different concentrations of DMSO and glycerol on cryopreservation of *Trichomonas gallinae.*

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Abstract: Conventional methods for the propagation and preservation of parasites in vivo or in vitro have limitations, which include the need for labor, the initial isolation and loss of strains, bacterial and fungal contamination, and changes in the original biological and metabolic characteristics of the organism. All of these disadvantages are reduced considerably by cryopreservation. In this study, we examined the effects of different concentrations of dimethyl sulfoxide (DMSO) and glycerol on the survival rate of *Trichomonas gallinae*. Both of the two cryoprotectants examined at different concentrations showed a protective effect of *T. gallinae*. When DMSO was used as the cryoprotectant, a maximum survival rate (70%) was achieved at a concentration of 10% over a short-term duration of freezing (24 h). Glycerol at a concentration of 10% had the highest effect on cryoprotectivity after 70 days, while DMSO 20% showed the lowest cryopreservation for *T. gallinae.* In conclusion, DMSO and glycerol are appropriate protective materials for the cryopreservation of *T. gallinae*. The solutions of DMSO 10% and glycerol 10% could be the best choice of cryoprotectant for short-term (24 hours) and long-term (70 days) protection, respectively.

Keywords: Cryopreservation, *Trichomonas gallinae*, DMSO, glycerol

Introduction

The limitations of current conventional methods of propagation and preservation of parasites in vivo or in vitro include the need for technicians, the initial process of isolation, loss of strains, bacterial and fungal contamination and changes in the original biological and metabolic characteristics of the parasite (Gill and Redwin, 1995; Mutetwa and James, 1984; Uga and Matsumura, 1979). Since the first successful cryopreservation of *Plasmodium* spp. in 1939, some species of parasites have been successfully cryopreserved, including protozoa such as *Toxoplasma gondii (*Booth *et al.,* 1996*)*, *Entamoeba histolytica*(Farri *et al*., 1983), *Trypanosoma cruzi* (Filardi and Brener, 1975), *Giardia lamblia* (Layman and Marchin, 1984), and *Trichomonas vaginalis* (Uga and Matsumura, 1979), as well as helminths such as trichostrongylidae (Gill and Redwin, 1995), filarioidea (Lowire, 1983), *Trichinella* spp. (Pozio and Rossi, 1988), and *Toxacara canis* (Ramp *et al*., 1987). For example, Weathersby and McCall (1981) reported that if *Plasmodium gallinaceum* was frozen without a cryoprotectant, the organism remained as infective

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as organisms that had not been frozen. Lyman and Marchin (1984) reported that 90% of *G. lamblia* organisms survived after cryopreservation. On the other hand, some parasites show very low survival rates after freezing. In the case of *E. histolytica*, the survival rate was only 10% (Farri *et al*., 1983), and muscle-stage larvae of *Trichinella* spp. showed motility after freezing but were not infective (Pozio and Rossi, 1988). To obtain higher survival rates, further experiments to determine the optimum conditions for cryoprotectants, as well as cooling and thawing rates are needed (Dalgliesh, 1972).

Generally, the concentration and type of the cryoprotectant, and the cooling and thawing rates are known to be important factors that affect the viability of parasites after cryopreservation. Miyata showed in a study on *T. vaginalis* 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 (Pozio and Rossi, 1988; Miyata, 1973). However, the effect of different concentrations of DMSO and glycerol on the survival rate of *Trichomonas gallinae* is not known clearly at this stage.

In this study, we examined the effects of different concentrations of DMSO and glycerol on the survival rate of *T. gallinae*. We also evaluated the effect of freezing time after cryopreservation on the viability of *T. gallinae*.

Materials and Methods

Parasites: In our study, we used *T. gallinae* parasites that were isolated from a pigeon in 2002. Between $1– 5 \times 10^5$ trophozoites/ml were used. They were cultured in diamond media and passaged every two weeks. Ringer's solution with a pH of between 7.2 - 7.4 was used to preserve the parasites.

Cryoprotectants: Two cryoprotectants were used in this study: DMSO (Merck, Germany) and glycerol (Merck, Germany). To assess the effects of various concentrations of these cryoprotectants, three different concentrations (5%, 10% and 20%) of each cryoprotectant were prepared. Different concentrations of glycerol and DMSO were prepared by the dilution of the chemical with sterile Ringer's solution. These were then mixed with the suspension that contained the parasites. The solution was divided in 2 ml cyrotubes (NuNu, Denmark) and incubated for ten minutes at room temperature. The tubes were then kept at -80 °C until use.

Assessment of viability: After the samples were thawed in a temperature of 37 °C, the suspensions were observed using a light microscope (Olympus Optical, LTD with 400× magnification) to check the viability and morphology of the protozoa. The viability of *T. gallinae* was assessed by observation of the flagellated Trichomonas under microscope to determine that the organism is alive.

Statistical analysis: A one-way analysis of variance (ANOVA) was used to compare different concentrations (5%, 10% and 20%) of DMSO and glycerol. In order to detect significant changes over a time period, repeated measures analysis of variance were employed. A value for p of less than 0.05 was considered to be significant.

Results

The effects of the type and concentrations of cryoprotectant, as well as the effects of the time course after cryopreservation on the survival rate of *T. gallinae,* are shown in Table 1 and Figures 1 and 2. Of the two cryoprotectants that were examined in different concentrations, both DMSO and glycerol showed a protective activity for *T. gallinae*. When DMSO was used as the cryoprotectant, a maximum survival rate (70%) was achieved at a concentration of 10% over a short-term period (24 h). On the other hand, the maximal survival rate of *T. gallinae* for longer durations, including 32 and 70 days, was achieved when 10% of glycerol was used as the cryoprotectant (Figure 2). There were no significant difference in the survival rate of *T. gallinae* with different times after cryopreservation induced by 5% glycerol ($p<0.05$). The organism did not survive in DMSO at concentrations of 20% (Figure 1).

Table 1: Effects of type, concentration, and freezing time of cryoprotectants on the survival rate of *T. gallinae.*

Type of cryoprotectants	Concentrations of cryoprotectants (%)	Time after freezing (day)			
		1	15	32	70
DMSO	5	38	19	17	11
	10	70	61	33	30
	20	0	0	0	0
Glycerol	5	23	18	17	12
	10	65	62	54	50
	20	55	49	40	35

Discussion

The temperature of preservation is an important factor in viability of an organism. At -80°C, the levels of protein denaturation and metabolism are very low. Ice crystals that form in temperatures as low as -130°C can stop protein denaturation (Levine and Anderson 1966; Diamond, 1995). The usefulness of glycerol as a cryoprotectant is a matter of controversy. For example, when *T. vaginalis* and *Plasmodium chabaudi* were cryopreserved using 10% glycerol, no protective effect was observed for the former (Miyata, 1975), but a protective effect that was similar to that of DMSO was obtained for the latter (Mutetwa and James, 1984). The protective effect of both DMSO and glycerol on frozen cells requires intracellular permeation. The key difference between the mechanism of action of DMSO and glycerol is that glycerol permeates the cell significantly more slowly than DMSO.

In our study, DMSO and glycerol were examined at different concentrations. DMSO has been used as a cryoprotectant for the cryopreservation of many species of protozoa, and its optimum concentration was previously reported to be approximately 10%. For example, the maximum survival rate of *E. histolytica* (10%) was obtained when it was cryopreserved in the presence of 7.5% DMSO (Farri *et al*., 1983). When 6.5% DMSO was used, 90% of *G. lamblia* survived (Layman and Marchin, 1984). Furthermore, an optimum DMSO concentration of 12.5% was

Figure 1: The survival rates of *T. gallinae* in different concentrations of DMSO.

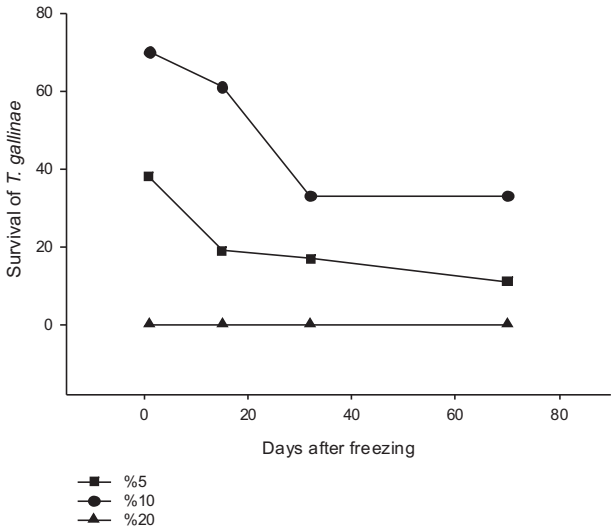
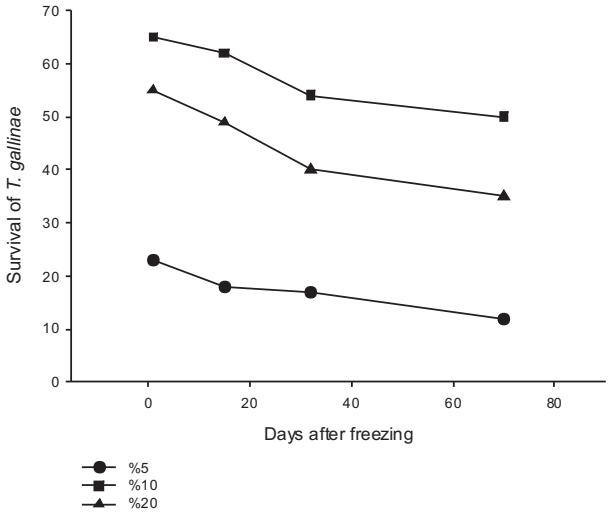


Figure 2: The survival rates of *T. gallinae* in different concentrations of glycerol.



obtained for the cryopreservation of *T. gondii* (Booth *et al.,* 1996). In the case of *T. vaginalis*, the optimum concentration of DMSO was 5–10%. These results suggest that, regardless of the species of parasite, DMSO shows an optimum protective effect at concentrations of between 5% and 12.5%.

In our study, the organism did not survive in DMSO at a concentration of 20%. This finding is in agreement with the previous study of Miyake *et al*. (2004). In conclusion, DMSO and glycerol are appropriate protective materials for the

cryopreservation of *T. gallinae*. The solutions of 10% DMSO and 10% glycerol could be the optimal choice for use as cryoprotectants over short-term (24 hrs) and long-term (70 d) periods of protection, respectively.

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