Cryopreservation of *Blastocystis hominis* with Dimethylsulphoxide (DMSO)

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Abstract

Very few cryopreservation studies have been carried our on the protozoan parasite, *Blastocystis hominis*. The present study compares the cryoprotecrive ability of two cryoprotectants, mannitol in glycerol with and without dimethylsulphoxide (DMSO). The study also aims at selecting a good growth medium with appropriate supplement which can support cryopreserved parasites and to determine the minimum number of parasites needed for successful cryopreservarion. The study showed that the cryoprotectant using DMSO in mannitol and glycerol had better cryoprotective ability. The initial culture medium using Iscoves Minimum Dulbecco's medium (IMDM) should have 20% horse serum as nutrient supplement in order to resuscitate cryopreserved parasites. The critical concentration for cryopreservation of parasites should exceed 200 x 10^6 parasites per ml in order to obtain a good recovery of parasite in culture after crypreservarion .

Key Words: *Blastocystis hominis*, cryoprotection, dimethylsulphoxide

Introduction

Blastocystis hominis, (Brumpt, 1912), an intestinal protozoan parasite, is largely prevalent in humans and most animals. This strict anaerobic parasite has recently been described in clinical reports to be a cause of diarrhoea (Doyle *et al.*, 1990).

In vitro maintenance of the parasite was first successfully carried out in 1921 (Barret, 1921) and since then, the parasite have been shown to grow in Boeck and Drbohlav's (BD) medium (Boeck & Drbohlav, 1925), Dobell and Laidlaw's medium covered with Ringer's solution containing 20% human serum (Silard, 1979), Loeffler's medium (Silard *et al.*, 1983), Diamond's trypticase-panmedeserum (TP-S-1) medium (Moler *et al.*, 1981), Minimal essential medium (MEM) (Diamond, 1987) and lately the Iscoves Modified Dulbeccos Medium (IMDM) (Ho *et al.*,1993).

In our laboratory we maintain human and animal isolates of *Blastocystis* using the monophasic medium IMDM but continuous culture maintenance is tedious, time consuming, expensive and the parasites may be prone to genetic drift. There is therefore a need to cryopreserve these isolates for biochemical, molecular and other biological studies; but very little is known about the cryopreservation of this extremely fragile parasite. The only other study was by Zierdt who used Dimethylsulphoxide (DMSO) to cryopreserve *Blastocystis* (Zierdt, 1991) but extreme care was shown to be needed especially during the process of slow cooling and the subsequenr maintenance of parasites in liquid nittogen. Attempts to cryopreserve the parasites in DMSO containing IMDM and horse serum were unsuccessful (unpublished observation).

We have used mannitol in glycerol and 0.85% normal saline but only less than 10% of the cryopreserved vials containing the parasite were successfully recultured (unpublished observation). It is highly likely that the initial growth of cryopreserved parasites in culture medium may require a higher nutritious supplement. Furthermore the initial concentration of parasites intended for cryopreservation may also be critical for a good yield.

The present study compares cryoprotective ability of two cryoprotectants, mannitol in glycerol with and without DMSO . The study also aims at selecting a good growth medium with appropriate supplement which can support cryopreserved parasites and furthermore determine the minimum number of parasites needed for successful cryopreservarion.

Materials and Methods

In vitro maintenance of Blastocystis hominis

An axenic culture of *Blastocystis hominis* (isolate C) was obtained from the Department of Microbiology, National University of Singapore. The culrures were intially obtained fiom the stools of a *Blastocystis*-infected patient. The parasites are currently maintained in our laboratory and continuously cultured in Iscoves Modified Dulbeccos Medium (IMDM) with 10% horse serum. The cultures were maintained in anaerobic chambers at 37°C.

Cryoprotectants

Cryoprotectant "A", was prepared by adding 1.51 g of mannirol and 36 ml of 0.85% sodium chloride into 14 ml of Glycerol. The solution was stored in universal bottles at 4°C.

Cryoprotectant "B" consisted of cryoprotectant "A" with the addition of 10% dimethylsulfoxide (DMSO) and this was subsequently stored at 4°C.

Parasite Count

Parasites from anaerobic cultures of axenic isolate C were

centrifuged and the sediments containing the parasites were pooled together. Parasite concentration was made up to 50 x 10^6, 100 x 10^6 and 200 x 10^6 per ml in IMDM.

Several preparations of each concentration were added into cryovials (Nunc) (Table 1) containing the cryoprotectants and subsequently allowed to stand at room temperature for 20- 30 minutes. They were then placed in a polystyrene box and transferred to a -20°C freezer for two hours. The polystyrene box with these cryovials was subsequently stored at -70°C overnight before transferring the vials to liquid nitrogen for storage.

Table 1. Distribution of cryovials containing various parasite concentrations in the two cryoprotectants*

Cryoprotectant	Parasite concentration (x 10^6/ml)	No. of cryovials
A	50	10
	100	12
	200	25
B	50	30
	100	16
	200	29
Total		112

*A = mannitol and sodium chloride in glycerol; B = mannitol and sodium chloride in glycerol with DMSO

Thawing Procedures

Batches of cryovials containing parasites in both cryoprotectants were taken out at 2 and 8 weeks post-cryopreservation. Cryovials taken out from the liquid nitrogen tank were thawed in a polystyrene box at 37°C for 1.5 - 2 minutes for cryovials containing 50 x 10^6 and 100 x 10^6 parasites/ml. Cryovials containing 200x10^6 cells/ml took about three minutes to thaw completely. The contents of each of the cryovials were quickly transferred under sterile conditions to the respective culture tubes containing the pre-reduced IMDM. The culture tubes containing the cryopreserved parasites were centrifuged for two minutes at 800 rpm (MISO-minor centrifuge). The supernatant was discarded and the sediment containing the parasite was transferred to the respective culture tube containing the fresh pre-reduced IMDM with supplements as listed below:

a) IMDM plus 10% horse serum;
b) IMDM plus 20% horse serum;
c) IMDM plus 20% horse serum and 10% trypticase;
d) IMDM plus 20% horse serum and 20% trypticase;
e) IMDM plus 20% horse serum and 30% trypticase;
f) IMDM plus 20% horse serum with egg slant; IMDM with egg slant.

The above culture media and nutrient supplement were prepared in five culture tubes respectively. The culture tubes were then maintained at 37°C in anaerobic chamber. Growth of the parasite in the various culture media was then monitored and parasite count was made on day 3 of the culture.

Results

Preliminary studies using 50 x 10^6 parasites per ml in cryoprotectant A and B showed no growth in IMDM supplemented with horse serum after two weeks of cryopreservation. Parasites preserved in cryoprotectant containing DMSO showed a mean parasite number of 0.00031 x 10^6 on day 3 post-culture when supplemented with 20% horse serum. Parasites in the same cryoprotectant gave a yield of of 0.02 x 10^6 parasites on day 3 when cultured in egg slant medium with IMDM .

The presence of trypticase in culture medium did not seem to enhance the growth of cryopreserved parasites.

In vitro culture of 8 weeks-old cryopreserved parasites from both cryoprotecants showed no growth when cultured in IMDM medium with 10% horse serum (Tables 2 & 3). When horse serum supplement was increased to 20%, cryovials containing 100 and 200 x 10^6 parasites per ml in cryoprotectant A gave 0.044% and 18.75% yields respectively on Day 3 postculture. A higher yield (38%) was obtained when 200 x 10^6 parasites per ml were cryopreserved in cryoprotectant B in IMDM medium with 20% horse serum.

Doubling the parasite concentration from 100 to 200 x 10^6 in cryoprotectant A and B increased the parasite yield on day 3 from 1.2 % to 21% and 3.3 to 33.1 % respectively when cultured in IMDM, egg slant medium and 20% horse serum. In all instances the percentage yield after 3 days of culture was higher when the initial concentration of 200 x 10^6 was used when compared to 100 x 10^6 parasites per ml.

Parasites that survived in day 3 cultures after cryopreservation in both the cryoprotectant did not get distorted. Most of the forms seen were vacuolar, with distinct peripheral nuclei, and were mainly 4-8 µm in size.

Discussion

The present study was aimed at assessing the cryoprotective ability of DMSO and also the type of culture medium with nutrient supplement which could successfully resuscitate cryopreserved parasites and give a high yield of parasite in cultures.

Blastocystis hominis is an extremely fragile parasite. It is little wonder that parasites from only less than 10%

Table 2. Percentage yield of *Blastocystis hominis* in cryoprotectant A (without DMSO) after 8 weeks of cryopreservation

Parasite concentration/ml ($x10^6$)	IMDM culture medium with nutrient supplement	Mean no. of parasites ($x10^6$)/ml on Day 3	Percentage Yield
100	10% HS	0	0
100	20% HS	0.04 +0.02	0.04
100	20%HS + 10%T	0	0
100	20%HS + 20%T	0	0
100	20%HS + 30%T	0	0
100	20%HS + egg slant	1.20 ± 0.10	1.2
200	20%HS + egg slant	42.0 ± 12.50	21.0
200	10%HS	0	0
200	20%HS	37.5 ± 8.60	18.7
200	20%HS + 10%T	0	0
200	20%HS +20%T	0	0
200	20%HS + 30%T	0	0.00

Table 3. Percentage yield of *Blastocystis hominis* in cryoprotectant B (with DMSO) after 8 weeks of cryopreservation.

Parasite concentration/ml ($x10^6$)	IMDM culture medium with nutrient supplement	Mean no. of parasites ($x10^6$) /ml on Day 3	Percentage Yield
100	10% HS	0	0
100	20% HS	0.0087 ± 0.0001	0.0087
100	20% HS + 10%T	0.05 ± 0.0002	1
100	20% HS + 20%T	0.9 ± 0.004	0.9
100	20%HS + 30%T	1 ± 0.0006	1
100	20%HS + egg slant	3.3 ± 2.10	3.3
200	20%HS + egg slant	66.2 ± 16.6	33.1
200	10% HS	0.15 ± 0.05	0.075
200	20%HS	77.7 ± 30.74	38.85
200	20% HS + 10%T	1.00 ± 0.005	0.5
200	20%HS +20%T	2.00 ± 0.007	1
200	20%HS + 30%T	0.16 ± 0.006	0.08

of the cryovials successfully grew after cryopreservation using the conventional cryoprotectant containing mannitol in glycerol (unpublished observation). A similar cryoprotectant had been used previously for successfully cryopreserving malaria and *Acanthoemba* parasites (John *et al.*, 1994).

DMSO is the cryoprorectant most commonly used in freezing protozoa . Glucose has been used togerher with DMSO for freezing trypansomes (Polge & Soltys, 1960) and amoeba (Diamond, 1964). The present study shows that DMSO in mannitol and glycerol is a berter cryoprorectant for *Blastocystis* . The procedure for the cryopreservation in our study was very much simpler than that used by Zierdt (1991), in which he described a procedure where the sediment from 3-day old cultures of *Blastocystis* in egg slant medium was treated by first submerging a 1 ml piperte rip through rhe overlay to the sediment at the base of rhe egg slant and then releasing 0.1 ml of glycerol. This was followed by the addition of 0.1 ml of DMSO in the same manner. The overlay was then covered with 3 ml of sterile mineral oil and the culture was immediately frozen slowly to -70°C. In the present study DMSO was simply added into mannitol and glycerol and sediment from parasite culture was added inro cryovials containing this cryoprotectant.

50 x 10^6 cryopreserved parasites from both cryoprotectants did not grow in rhe IMDM culture medium supplemented with 10% horse serum suggesting that the parasite concentration for cryopreservation

should exceed 50 million parasites. However no growth was obtained even when the parasite concentration was increased to 200 x 10^6, in the monophasic IMDM culture medium which has been shown to maintain *B. hominis* in *in vitro* (Ho *et al.*, 1993). Increasing the nutrient supplement from 10 to 20% horse serum however increased the percentage yield of parasites maintained in cryoprotectant B containing DMSO by 0.044%. It appears that increasing the nutrient supplement aids in the resuscitation of frozen parasites if the concenttation of parasites exceeds a critical limit.

Trypticase has been used previously in an encystation medium to induce cyst-like stages *in vitro* (Suresh *et al.*, 1994). This nutrient supplement however did not exert any effect on the growth of the parasites. Perhaps other nutrient supplements should have been attempted. A combination of egg slant medium and IMDM with 10% horse serum showed a 1.2% yield. This increased to 21% when the parasite concentration for cryopreservation was doubled from 100 to 200 million. Almost similar results were seen with cultures in egg slant and IMDM with 20% horse serum. The results show that IMDM medium with egg slant and 20 % horse serum can be used as an initial culture medium to resuscitate cryopreserved parasites but obtaining a good yield of parasites without contamination of proteins from the egg slant medium for future biochemical and molecular studies may be hindered.

Ho *et al.* (1993) have shown in their studies that IMDM with 10% horse serum could provide berter parasite growth when compared to RPMI 1640, Minimum Essential Medium and Eagles Basal Medium but in our present study it was shown that 20% horse serum supplement in IMDM was needed to resuscitate parasites cryopreserved in DMSO.

The cooling process appeared to be vital in the present study. Other cryopreservation studies have shown thar rapid cooling yielded viable trophozoites of *Entamoeba invadens* and *Naegleria* sp. while slow cooling was used successfully for *Entamoeba histolytica* (James, 1988). In the present study, slow cooling appeared to result in successful growth for *Blastocystis*.

The thawing process was crucial to obtaining a high percentage of parasite recovery. Correct timing in each step readily conditions the cell to gradual change in temperature without damage to its cell wall structures. Delayed thawing using ordinary room temperature caused cell lysis which resulted in parasite death.

In the study by Zierdt *et al.* (1967), the tubes were first wrapped in a few layers of tissue paper and enclosed in cardboard mailing tube during the process of thawing. However in our study the cryovials taken out from the liquid nitrogen were transferred in a polysterene box and thawed immediately in a 37°C water bath.

The present study shows that the cryoprotectant using DMSO in mannitol and glycetol showed better cryoprotective ability. The initial culture medium using IMDM should have 20% horse serum as nutrient supplement and the critical concentration of parasites should exceed 200 x 10^6 parasites per ml in order to obtain a good recovery after cryopreservation. This may require pooling together a large number of culture tubes which is not practical when many isolates are to be cultured. There is still a need to search for a better cryoprotectant which needs a lesser parasite concentration to produce a higher yield. Furthermore studies must be done to assess if the present findings are consistent if the parasites are subjected to prolonged cryopreservation.

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