Assessment of cryoprotectant toxicity on the viability of Cryptosporidium parvum

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Abstract
Cryptosporidium parvum is an obligate intracellular parasite that can cause life-threatening infections among immunocompromised individuals. In spite of multiple studies, successful cryopreservation of C. parvum has not been achieved which constitutes an obstacle to the establishment of standardized isolates or cloned stocks. Compounds commonly used as cryoprotectants may have detrimental effects on parasite viability, thus toxicity studies should be pursued before developing any cryopreservation method. The present study evaluated the toxic effects of different agents used in cryopreservation methods on the viability of C. parvum as determined by propidium iodide incorporation in oocysts, excystation of sporozoites, and infectivity of sporozoites in an in vitro infection model. Treatment of C. parvum oocysts with ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (DMSO), or sucrose for 30 minutes resulted in minimal incorporation of propidium iodide at concentrations ranging from 0.25 to 4 M. Moreover, excystation of sporozoites was not impaired by the treatment of oocysts with these compounds. Treatment of excysted sporozoites with 2 M concentrations of EG, PG, DMSO, or 0.25 M sucrose did not affect the infectivity of C. parvum as determined by RT-PCR, though excystation of sporozoites in infected HCT-8 cells was observed in all tested cryoprotectants. We have established a framework to monitor the toxic effects of cryoprotectants and other compounds on Cryptosporidium viability. Future experiments will examine the protective effects of these compounds at ultra low temperatures as we continue the pursuit of novel cryopreservation approaches for this parasite.

Introduction
Cryptosporidium are obligate, intracellular parasites that infect luminal epithelial cells of the digestive and respiratory tracts of humans and other vertebrates (1). Infections caused by Cryptosporidium parvum result in self-limiting diarrhea in immunocompetent individuals but life-threatening disease in immunocompromised patients (1,2). The life cycle of Cryptosporidium sp. features asexual and sexual stages with the production of thick-walled, environmentally resistant oocysts. Following ingestion by the host, oocyst release vesicles invade the epithelial lining of the intestines or lungs (3). Subsequent developmental stages of the parasite occur within a parasitophorous vacuole, between the cytoplasmic membrane and the apical membrane of the host cell (3).

Advances in in vivo culture systems for C. parvum have allowed the study of the various developmental stages of the parasite (1,4). However, methods that facilitate continuous maintenance of C. parvum in culture, generation of standardized isolates or cloned stocks of isolates are missing. Thus, researchers continue to rely on in vivo animal models for the replenishment of parasite stocks. Attempts to establish standardized isolates or cloned stocks have been particularly affected by the lack of cryopreservation techniques that would allow the storage of C. parvum under ultra-low temperatures.

Cryopreservation of tissues and cells is achieved by the use of compounds termed cryoprotectants. These include simple sugars and highly penetrating compounds such as dimethylsulfoxide (DMSO) which balance changes in osmotic pressure and prevent the crystallization of intracellular water, respectively (5). Importantly, misuse of cryoprotectants may cause cell damage thus must be taken in optimizing the concentration of the reagents. A number of studies have attempted cryopreservation of C. parvum with oocytes or sporozoites in vitro and excystation studies by freezing (6). The freezing process itself appears detrimental to the parasite, however, toxic effects of the cryoprotectants prior to the cooling process cannot be ruled out. The purpose of the present study was to examine the effects of common and non-conventional cryoprotectants on the viability of C. parvum as a preamble to the development of future cryopreservation methods for this parasite.

Materials and Methods
Isolates. Oocysts of Cryptosporidium parvum RNA were obtained from Smithsonian Parasitology Laboratory, University of Arizona. Oocysts were maintained at 4°C in an artificial ovum (4°C)Twenty, 100, and 100 μg/ml of Pergam, and 100 μg/ml of gentamicin were less than 8 weeks old when used in the experiments.

Treatment of oocysts. Oocysts (2 x 10⁷) were incubated with serial dilutions of ethylene glycol (EG), propylene glycol (PG), DMSO, glycerol, or sucrose in PBS for 30 minutes at room temperature. Concentrations ranged from 0.125 to 1 M for sucrose and 0.25 to 4 M for the remaining compounds. Following incubation, oocysts were centrifuged at 700 x g and washed 3 x in PBS. Oocysts were resuspended in 100 μl of a 1:2000 solution of propidium iodide (PI) in PBS, or 100 μl of excystation buffer (0.15 M potassium acid phosphate in PBS). Oocysts in PI solution were incubated for 30 minutes at room temperature in the dark. Oocysts in excystation buffer were incubated for 1 hour at 37°C. Samples were examined after 40 x magnification using an Axioskop® microscope (Zeiss, Oberkochen, Germany) coupled to a UV-light source.

Treatment of sporozoites. Oocysts (3 x 10⁷) were incubated in excystation buffer for 1 hour at 37°C. Excysted sporozoites were centrifuged at 300 x g, washed 3 x in PBS, and counted with a hemocytometer. Sporozoites (3 x 10⁷) were treated with EG, DMSO, or sucrose as described above in the oocyst experiments. Following centrifugation, sporozoites were resuspended in HCT-116 cell monolayers (900,000 cells) and incubated at 37°C in 5% CO₂ in DMEM supplemented with 1 μg/ml sodium pyruvate and 10% FBS. Infected cells were examined after 2 and 4 days for the presence of intracellular developmental forms of C. parvum by light and fluorescence microscopy, immunofluorescence assay was performed using a rabbit polyclonal antibody against the C. parvum glycophorin (glyco-specific) protein (85 kDa). In parallel experiments, infection of C. parvum in HCT cells was monitored by analysis of parasite gene expression using RT-PCR as described below.

Results

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum (cryoprotectant related-gemline system (TRAP))</td>
<td>TRAP-F (GTCAGTTTGGTCAGAATGGTC)</td>
<td>TRAP-R (CTCCACCAACTAAGAACGCC)</td>
<td>300</td>
</tr>
<tr>
<td>C. parvum (achieved)</td>
<td>CP18S-F (CTCCACCAACTAAGAACGCC)</td>
<td>CP18S-R (AGAGATTGGAGGTTGTTCCT)</td>
<td>300</td>
</tr>
<tr>
<td>C. parvum (achieved)</td>
<td>CP270-F (GTCAGTTTGGTCAGAATGGTC)</td>
<td>CP270-R (CTCCACCAACTAAGAACGCC)</td>
<td>300</td>
</tr>
<tr>
<td>C. parvum (achieved)</td>
<td>TRAP-F (GTCAGTTTGGTCAGAATGGTC)</td>
<td>TRAP-R (CTCCACCAACTAAGAACGCC)</td>
<td>300</td>
</tr>
</tbody>
</table>

Fig. 1. Propidium iodide incorporation of C. parvum oocysts following treatment with various cryoprotectants

Fig. 2. Excystation of C. parvum sporozoites following treatment of oocysts with various cryoprotectants

Fig. 3. Infectivity of C. parvum sporozoites following treatment with various cryoprotectants

Fig. 4. Expression of C. parvum genes in HCT-8 cells

Conclusions

- High concentrations of cryoprotectants have negligible effects on C. parvum oocyst viability as determined by PI incorporation and excystation assays.
- Infectivity of sporozoites using an in vitro cell model was also unaffected by cryoprotectant pre-treatment.
- Future studies need to examine the effects of cryoprotectant cocktails in C. parvum viability.
- Combinations of these assays will allow fine tuning the concentrations of cryoprotectants and cooling parameters as we work towards developing freezing methods for the long term storage of C. parvum under ultra-low temperatures.

References