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Research Brief

Optimization of *Entamoeba histolytica* culturing *in vitro*

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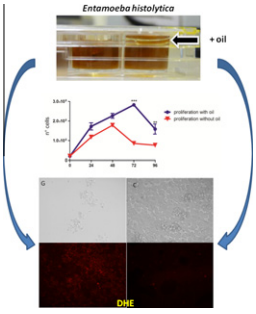
HIGHLIGHTS

- Mineral oil can significantly enhanced *Entamoeba histolytica* proliferation in microplates.
- Mineral oil covered medium-containing wells produced higher trophozoite numbers kept adherent and motile.
- Mineral oil addition reduces oxidative stress, down modulating reactive oxidative species production within trophozoites.

GRAPHICAL ABSTRACT



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ABSTRACT

Entamoeba histolytica is among the most deadly parasites accounting for the second highest mortality rate among parasitic diseases. Nevertheless, contrary to trypanosomatids, this protozoan in hardly studied by parasitology groups. This astonishing discrepancy is largely due to the remarkable intricate conditions required for parasite proliferation *in vitro*, particularly whenever large cell numbers are required. The present study was undertaken in order to optimize *E. histolytica* culturing harvest, using mineral oil layers preventing culture medium-air contact to maintain anaerobic conditions in culture plate wells. 2×10^4 trophozoites were plated on each well in 2.0 mL YI-S-33 medium, supplemented with bovine serum and 700 μL mineral oil. Parasites were daily quantified by light microscopy counting for up to 96 h and trophozoite motility was also assessed. We notice that *E. histolytica* cultures in 24-well plates reached several-fold higher cell densities, particularly whenever the mineral oil layer was placed on top of the medium surface, blocking the air interface.

At least 99% of the parasites were vigorously motile for 72 h in oil-containing wells, whereas only less than 5% displayed significant motility in oil-devoid wells.

In order to determine whether such different growth responses were due at least in part to the oxidative stress, we used the reactive oxidant species fluorescent probe dihydroethidium (DHE).

The remarkably higher DHE parasite labeling in oil-devoid cultures indicate that oxidative stress reduction can play a significant role in elevated growth rates observed in oil supplemented cultures. Propidium iodide and Trypan blue dye-exclusion assays indicate that parasite necrosis resulted from the stressing conditions.

The present study indicates that *E. histolytica* culturing in oil-sealed wells may comprise a valuable tool for bioactivity of antiparasitic compounds.

1. Introduction

Entamoeba histolytica is the protozoan parasite responsible for human amoebiasis which comprises a potentially fatal disease of cosmopolitan distribution, reaching up to 10% of world population,

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causing *circa* 100.000 deaths every year (Garcia-Zepeda et al., 2007; Upcroft and Upcroft, 2001; WHO, 2012).

The disease is established with the ingestion of food or water contaminated with parasite cysts and progresses with the proliferation of trophozoites in the large intestine lumen (Pritt and Clack, 2008; Upcroft and Upcroft, 2001). This luminal parasite mostly survive commensally feeding on bacteria, but can eventually injure the large intestine epithelial layer, ulcerate the mucosal tissues and reach other organs such as liver, lungs and even brain (Espinosa-Cantellano and Martínez-Palomo, 2000). There are innumerous unanswered questions on the amoebiasis pathophysiology. Many of them wait to be elucidated because of deficient experimental models. Despite the medical importance of this disease of high lethality and the relevant scientific questions regarding the control of virulence in a protozoan which may shift between an asymptomatic infestation to a deadly infection, *E. histolytica* is seldom approached experimentally. The tricky and burdensome isolation and cultivating procedures have usually hampered attempts to study this parasite.

E. histolytica dwell in low oxygen tension conditions, but immune responses and even the protozoan *per se* produce oxygen and nitrogen reactive species (Wang et al., 1994; Tekmani and Mehlotra, 1999) which may be toxic for the parasite, nevertheless little is known about the mechanisms employed to endure oxidative stress during tissue invasion or experimental handling. Therefore, cultivation of this parasite requires microaerophilic conditions, mimicking the human gut environment.

The present study was undertaken in order to standardize the anaerobic/microaerobic *in vitro* conditions adapting the early methodology of oil blocking culture medium surface to optimize *E. histolytica* axenic proliferation in multi-well plates.

2. Material and methods

2.1. Parasite cell culture

E. histolytica HM1-SS strain was kindly provided by the Dr. Edward Felix Silva and Dr. Maria A. Gomes, Parasitology Department, Universidade Federal de Minas Gerais (UFMG) and maintained in YI-S-33 medium.

2.2. Proliferation assays

E. histolytica routine growth was performed in 15 mL test tubes, containing 12 mL YI-S-33 medium supplemented with 20% (v/v) bovine serum, using 2×10^4/mL inoculum. The tube ice-cold-detached cells were centrifuged at 380*g*, 4 °C, for 10 min, washed in cold PBS, pH 7.2, before counting. Parasite proliferation was assessed by daily light microscopy direct counting on Neubauer chambers for 96 h.

For assays performed in 24-well plates, each well was seeded with 2×10^4/mL *E. histolytica* trophozoites, in 2 mL YI-S-33 medium supplemented with 20% (v/v) bovine serum. Some wells also received a 700 μL mineral oil (Nujol®) layer on top of the culture medium surface (Fig. 1). The oil layer was carefully laid on the center of the culture medium surface and whenever necessary the tip was gently used to lay it down regularly. Different mineral oil trademarks were tested, producing the same results (not shown). Living trophozoites were incubated with either 4% trypan blue (TB) or DAPI before counting on an inverted microscope.

Dye exclusion assays were performed with coverslip-adherent parasites, so that detached parasites were removed.

Statistical analysis and data plotting were performed using Graphpad Prism 5.0 employing ANOVA and Tukey as a post-test.

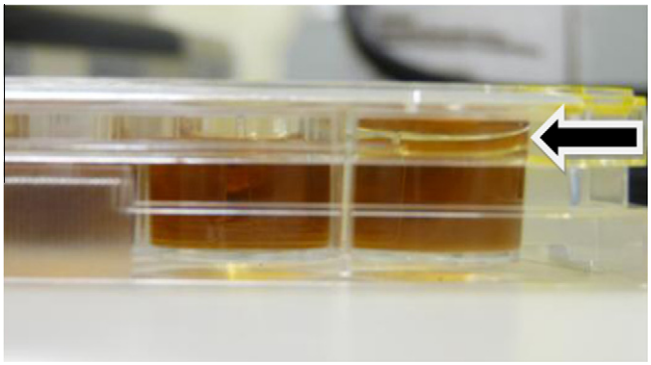

Fig. 1. Side view of a 24-well culture plate displaying wells with (arrow) and without added 700 μL mineral oil (Nujol®) layer on top of the 2 mL YI-S-33 culture medium, supplemented with 20% (v/v) bovine serum.

2.3. Detection of ROS

In order to detect reactive oxidant species (ROS) we used the probe DHE. Glass coverslip-containing wells were plated with 2×10^5 trophozoites/mL with and without mineral oil and cultured for 72 h. Living trophozoites were incubated with 5 μM/mL DHE for 10 min and directly observed under an Olympus BX51 fluorescence microscope and all micrographs were made in using the same capture parameters.

3. Results

E. histolytica proliferation in both tubes and 24-wells plates peaked within 72 h, but the growth in tubes was characterized by a long lag phase, whereas in plates the parasites entered an early log phase, therefore the cell densities achieved were threefold higher, although the medium volume used was 6 times lower.

We compared the *E. histolytica* proliferation in oil-covered medium within 24-well plates with cultures carried out in anaerobic jars. Interestingly no significant difference was observed but the handling was quicker and easier (particularly microscopical monitoring) in the former. The parasite growth in tissue culture plates displayed similar pattern in the first 48 h, but addition of a mineral oil layer on the top of the culture medium significantly ($p < 0.0001$) enhanced *E. histolytica* trophozoite numbers in 72 and 96 h timepoints (Fig. 2). This effect was not achieved by adding the same volume of medium. At 48 h the parasites grown in oil-covered medium displayed about 25% enhanced proliferation, higher motility and substrate adherence than the ones kept in oil-devoid wells. At 72 h, oil-containing *E. histolytica* cultures reached an almost complete confluency, covering most of the well bottom area and kept high motility with constant pseudopod emitting, whereas the oil-devoid wells presented mostly immotile, clustered and/or detached parasites (Fig. 3). At this time point mineral oil-containing cultures presented about 120% more adherent and fully motile trophozoites (Fig. 2).

Cell viability, was assessed by adherence, DAPI and TB dye exclusion. Proliferation in the oil-containing wells was maintained all over the assay and final cell counts were enhanced by *circa* 98% and less than 1% of cells were DAPI- (not shown) or TB-stained (Fig. 4). It is noteworthy that most of the cells cultured without oil were detached and therefore not counted.

We also approached the possible effects of oxidative stress on trophozoite viability. The fluorescent probe DHE was used for ROS detection. We observed that protozoans cultured without mineral oil were remarkably more labeled, indicating intense ROS production (Fig. 5).