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Effect of cell-conditioned media on biomass production of *Leishmania* parasites

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Abstract: A large amount of parasite biomass production necessitates a higher volume of culture media, and this further increases the overall cost. For this reason, novel and alternative culture supplementary materials have been continuously investigated. The aim of this study was therefore to evaluate the effect of cell-conditioned media, collected as waste material during routine cell culture, on the growth and development of *Leishmania* parasites in vitro. In this study, for the first time, we show that cell-conditioned medium obtained from MCF-7 culture increased the growth of *Leishmania* parasites in vitro. These results show that the metabolites that are present in cell-conditioned media may play an important role in in vitro parasite culture. Cell-conditioned media may be used as an economic tool for isolation, cultivation, and obtaining biomass of *Leishmania* parasites in laboratory conditions.

Key words: Culture, medium, *Leishmania*, large-scale, biomass

Introduction

Leishmaniasis is a group of parasitic diseases transmitted by blood-sucking sand flies infected with *Leishmania* parasites (1). *Leishmania*, a protozoan parasite, is one of 21 known species responsible for leishmaniasis. The World Health Organization has declared leishmaniasis among 1 of the 6 major tropical diseases, and it is a major human and animal disease in the tropic and subtropic areas of world (1-4). *Leishmania* has 2 main life cycle morphologies: the intracellular amastigote in the mammalian host and the motile promastigote in the vector (2).

In vitro cultivation of protozoan parasites is very important for diagnosis, antibody production, assessment of parasite immune modulating capabilities, drug screening tests, improvements in chemotherapy, differentiation of parasites, molecular determination of strains, obtaining of purified antigen for vaccine production, development of attenuated strains, and investigation of host-parasite interactions. However, the in vitro culture of *Leishmania* parasites involves highly complex procedures because these parasites have very complex life cycles and, depending on the life cycle stage, may require different culture parameters (5). Amastigote cultures are carried out by using macrophage cells in liquid media, and promastigote cultures are carried out in liquid, biphasic, and semisolid culture media. *Leishmania* promastigotes were first grown on diphasic blood agar, and these nondefined diphasic media are still used today for adaptation and cultivation of *Leishmania* parasites directly isolated from both vertebrate and invertebrate hosts. Further progress has been made with the use of liquid monophasic media (6). However, these diphasic and monophasic media for parasite culture should be enriched with large amounts of certain amino acids, vitamins, hormones, and peptides (7-13).

Fetal bovine serum (FBS) is a common supplement to in vitro and ex vivo cell, tissue, organ, and *Leishmania* parasite cultures with concentrations varying between 10% and 30% (6). Depending on the number as well as the volume of the cultured material, the cost of FBS in a routine laboratory setting can in fact be very high. Especially for large-scale cultures, novel and alternative supplementary materials to FBS as well as serum-free methods have been continuously investigated (14,15).

Some studies showed that when protozoan culture was carried out together with mammalian cells, parasite growth was increased (16). However, the effect of mammalian cell metabolites on the growth of *Leishmania* parasites has not yet been investigated directly. In a routine cell culture laboratory, one of the most common cell lines used is human breast cancer cells (MCF-7). DMEM/F12 is generally used as a base medium in MCF-7 culture with FBS supplementation (17). During each passage, this medium, which also contains numerous crucial factors for cellular growth, is aspirated and discarded as culture waste. The investigation of the effect of this waste medium on parasite culture can therefore be very important, not only for the evaluation of the components in the waste medium, but also for examining its beneficial effects on the growth of *Leishmania* parasites in vitro. In this study, for the first time, the effect of a cell-conditioned medium that contains MCF-7 cell metabolites on *Leishmania* parasite development and culture was assessed.

Materials and methods

Leishmania promastigote culture

Leishmania tropica promastigotes (MHOM/TR/EP39) were kindly provided by Prof Dr Seray Özensoy Töz (Parasitology Department, Ege University), and 300,000 *L. tropica* promastigotes/mL were cultured in DMEM/F12 medium (10% FBS, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin) at 27 °C. Cultures were passaged after 4 days of incubation. Morphology and motility of parasites were observed by using an inverted microscope (Olympus CK40).

MCF-7 cell culture

In DMEM/F12 medium (2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin) supplemented with or without FBS at 37 °C and 5 % CO_2, 100,000 MCF-7 cells/mL were cultured. Cells were passaged twice a week by trypsin/EDTA.

MCF-7 cell-conditioned media collection

Cell-conditioned media with and without FBS were aspirated before each passage, generally on the third day of cell culture. Each collected conditioned medium was filtered (disposable sterile filters, 0.22 µm, Millipore Corporation), and volume and pH values were recorded before storing at 4 °C.

The effect of MCF-7 cell metabolites on the growth of *Leishmania* promastigotes

In the first phase of the study, *Leishmania* promastigote cultures were carried out with cell-conditioned media obtained from MCF-7 cell culture in DMEM/F12 without FBS, with varying ratios (25%, 50%, and 100% diluted with DMEM/F12 without FBS). An aliquot of 3×10^5 parasites/mL was inoculated in 2 mL of medium supplemented with varying ratios of metabolites.

In the second group of experiments, the effects of metabolites obtained from MCF-7 cells grown in medium with and without FBS were investigated. DMEM/F12 with 10% FBS and DMEM/F12 without FBS were used as control. Parasites were counted at 48 and 96 h.

Parasite counting

Parasites were diluted with formaldehyde (2%). The cell suspension was introduced into one of the V-shaped wells using a Pasteur pipette. The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. One entire grid on a standard hemocytometer was counted at 40× objective and the subsequent cell concentration per milliliter was determined by the following equation: cells per milliliter = average count per square × dilution factor × 10^4 (count 10 squares).

Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± standard deviation (SD). A parametric test (paired t-test) was used to evaluate the significance of the results. All data were analyzed using SPSS 16.0 for Windows, and values of $P < 0.05$ were considered statistically significant.

Results

In this study, the quantitative and qualitative effects of cell-conditioned media on *Leishmania* growth were investigated. We observed that parasites were more active, motile, potent, and healthy in medium containing MCF-7 cell-conditioned media compared to the control. Moreover, in the cultures with conditioned media supplementation, cluster formation, which is one of the common parameters that indicate physiological activity, was more intensive (Figure 1).

Figure 2 shows the effect of varying concentrations of metabolites on parasite growth. While the number of parasites was decreased in the control group, the number of parasites in the media containing metabolites was relatively increased with the increasing metabolite concentration. The number of parasites among groups at 96 h was significantly different ($P < 0.05$).

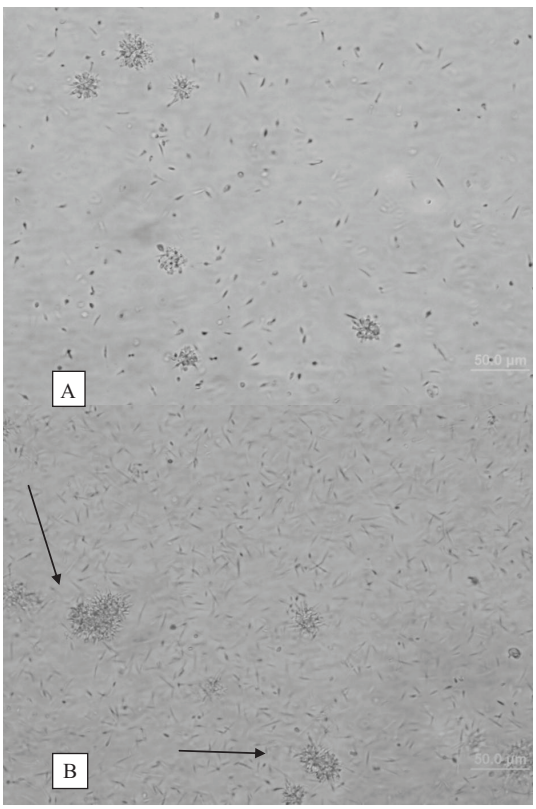

Figure 1. Microscopic morphology of the parasites that were cultured with different culture media compositions (20×): A) control, DMEM/F12 + 10% FBS; B) cell-conditioned media obtained from MCF-7 that was cultured with DMEM/F12 + 10% FBS.

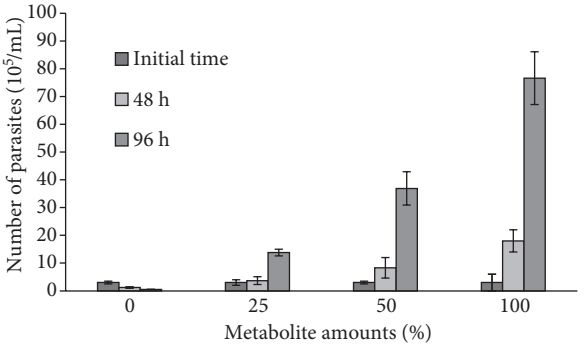

Figure 2. The effect of metabolites with different concentrations on the proliferation of *Leishmania* promastigotes (error bars ± SD).

The effect of cell-conditioned media with and without FBS on parasite growth is shown in Figure 3. In terms of parasite growth, the statistically nonsignificant but improved effect of cell culture metabolites was observed between the control (DMEM/F12 with 10% FBS) and the cell-conditioned medium (group 4). However, there was a difference between the control (DMEM/F12 without FBS) and metabolites obtained from MCF-7 cells that were cultured in medium without FBS (group 3).

Discussion

In the literature, together with FBS, numerous base media with varying formulas (199H, RPMI 1640, DMEM/F12, Schneider's *Drosophila* medium, and

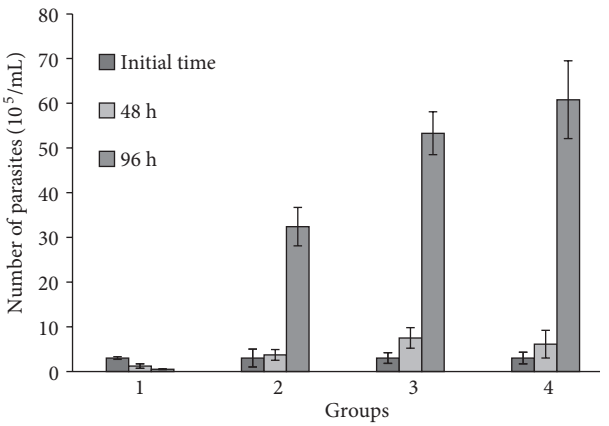

Figure 3. The effect of different culture media on the proliferation of *Leishmania* promastigotes: group 1, control, DMEM/F12; group 2, metabolite of MCF-7 grown in DMEM/F12 without FBS; group 3, control, DMEM/F12 + 10% FBS; group 4, metabolite of MCF-7 grown in DMEM/F12 with 10% FBS. Error bars ± SD.

Grace's insect tissue culture medium) have been reported for the cultivation of *Leishmania* parasites (18-22). FBS contains essential components such as hormones, vitamins, transport proteins, and attachment, spreading, and growth factors (14). About 500,000 L of serum are produced on an annual basis (23), and for this purpose, more than 1,000,000 bovine fetuses have to be harvested annually (24).

Large-scale culture of parasites for isolation and purification of the *Leishmania* immunogenic component is very important because an effective vaccine against the disease has not been developed yet (25). However, FBS is very expensive for large-scale culture for parasite biomass production (26). For this reason, decreasing the cost of culture media for *Leishmania* parasites is an important issue.

In our study, positive and growth stimulatory effects of cell-conditioned media may indicate that cancer cells may produce and secrete some factors into the cell culture media and that these factors provide positive and stimulatory effects on parasite proliferation in vitro.

The reason for this stimulation may be the fact that components released from mammalian cells induce metabolism of *Leishmania* parasites similar to other protozoa (16). Some studies indicated that metabolites of hepatocytes provide a stimulation of protozoa parasite *Plasmodium* spp. in culture (27). However, hepatocyte metabolites should only be obtained in primary culture; hence, continuous production of cell-conditioned media is not possible for such cells.

We have primarily chosen to use MCF-7 cell metabolites for the following reasons: long-term cultivation of these cells is possible; both MCF-7 cells and *Leishmania* parasites have been reported to grow in DMEM/F12 efficiently (18,19,26); and metabolites of MCF-7 cells can be obtained very easily because this cell line is used in a wide range of laboratory studies.

Development of a novel medium for parasite stimulation and growth is very important, not only for large-scale culture, but also for diagnosis of parasites by using parasite culture methods. Even if liquid media have a high ratio of FBS, sometimes this may not be enough to increase the sensitivity of the diagnosis for parasite isolation. Therefore, our results may indicate that MCF-7 cell-conditioned media can be used to increase the sensitivity of the diagnosis, the efficiency of the parasite isolation, and parasite multiplication in the culture.

As a result, for the first time, this study showed that waste medium with MCF-7 cell metabolites stimulated *Leishmania* parasites proliferation. Therefore, the medium may be useful for obtaining large amounts of parasite biomass. These findings may also imply that cell-conditioned media may be used for isolation, cultivation, and biomass production of other protozoa parasites. There are many culture laboratories in the world. If metabolites are used routinely in parasitology laboratories for parasite growth, cell metabolites may be easily obtained from cell culture laboratories that study different cancer cell lines.

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