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In vitro immunomodulatory effects of microemulsions with levamisole delivery systems on blood phagocytes interacting with *Giardia lamblia*

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ABSTRACT

Background: Giardiasis is one of the main parasites that infect the gastrointestinal tract of humans, affecting hundreds of millions of people worldwide, particularly in developing countries. Antiparasitics administered to treat giardiasis are inefficient in 20% of the cases, usually because of parasite resistance and side effects. In this scenario, microemulsions are a promising pharmaceutical alternative as carriers of molecules with therapeutic action that stimulate the immune system.
Methods: The study evaluated the effects of a microemulsion delivery system with levamisole hydrochloride on the functional activity of MN phagocytes incubated with *G. lamblia*.
Results: The microemulsion formulated was incorporated with levamisole hydrochloride using distilled water, caprylic/capric triglyceride-Polymol 812®, Sorbitan Oleate-Span 80®, Polysorbate 80 - Tween 80® and 1-butanol. The activity of the microemulsion was analyzed by phagocytosis rate, microbicidal activity, apoptosis rate and intracellular calcium concentration. Phagocytosis rate, microbicidal activity and apoptosis index increased in the microemulsion treatment. The results suggest that the microemulsion improves the therapeutic efficacy of levamisole, increasing the functional activity of phagocytes.
Conclusions: The microemulsion with a levamisole delivery system is therefore an efficient alternative for treating giardiasis, acting as an immunomodulator that probably causes fewer side effects than conventional drugs.

1. Introduction

Although giardiasis is a public health problem worldwide, it is commonly neglected [1–3]. Global estimates indicate a prevalence of nearly 200 million cases of giardiasis per year, mostly in children [3]. The clinical manifestations of giardiasis can vary from asymptomatic to the occurrence of diarrhea and long-term malabsorption [2].

Giardiasis severity depends on the virulence factors of the parasite as well as the nutritional and immunological conditions of the host [4–6]. Other studies suggest that the body eradicates *G. lamblia* using defense mechanisms such as phagocytosis by macrophages and secretion of nitric oxide and mucins by epithelial cells [7]. Experimental studies indicate that macrophage recruitment is a form of parasite control during infection [8,9]. The mechanism of action of human mononuclear [MN] phagocytes during giardia infection is not fully understood, but they are known to phagocytize and eliminate opsonized *G. lamblia* [10–11].

The search for new drugs for the specific treatment of intestinal protozoa remains a challenge [12]. In the treatment of giardiasis, failure or cross-resistance between drugs occur in 20% of the cases [13–15]. In recent years, the demand for novel treatments has motivated the development of modified drug delivery systems that release biologically active molecules in specific sites, achieving significant therapeutic action with minimal side effects [16]. Previous studies report microemulsion systems capable of stimulating phagocytosis and the microbicidal activity of phagocytes against bacteria [17–18] and protozoa [19]. Microemulsion delivery systems with levamisole [MELe] are a promising therapy to combat parasites and act as an immunomodulating agent, but their efficacy against *Giardia* has yet to be tested.

Levamisole is rapidly absorbed from the gastrointestinal tract and metabolized in the liver. Maximum plasma concentrations are attained within 1.5 to 2 h and half-life is 3 to 4 h. About 70% of drug is excreted mainly in the urine and a small proportion in the faeces as metabolites and 5% as unchanged levamisole [20,21].

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Levamisole has immunostimulatory potential and can control different infections, including those caused by parasites [22–23]. Therefore, MELe likely affects phagocytes and activates intracellular mechanisms that modulate their interaction with *G. lamblia*. To investigate this issue, the present study evaluated the *in vitro* effects of MELe, assessing its action as a modulator of the functional activity of phagocytes in human blood incubated with *G. lamblia*.

2. Material and methods

2.1. Microemulsion systems preparation and drug incorporation

The microemulsions were formulated using distilled water, caprylic/capric triglyceride - Polymol 812®, HLB = 10.8 [Emfal®, Betim, Brazil], Sorbitan Oletado - Span 80® [SP], HLB = 4.3, [Emfal®, Betim, Brazil], Polysorbate 80 - Tween 80® [TW] - HLB = 15.0, [Vetec®, Rio de Janeiro, Brazil] and 1-butanol [BT], [Vetec®, Rio de Janeiro, Brazil], and the system was abbreviated as SP/TW/BT according to Ribeiro et al. [19].

Based on the average density apparent points of the system, Levamisole hydrochloride was dissolved, and the equivalent weight of the microemulsion aqueous phase was formulated under agitation followed by surfactant and oily phase addition. Levamisole was solubilized to provide 0.84 $mg \cdot mL^{-1}$ of the proportion of 95 mL microemulsion. This formulation was called the microemulsion levamisole [MLe] [19].

2.2. Blood sampling and blood cell separation

Blood Samples [10 mL] were collected from 55 healthy volunteers with negative serology for hepatitis, HIV and syphilis and who did not present chronic degenerative diseases such as diabetes and hypertension, male, aged 18 to 35 in tubes containing anticoagulant. This study was approved by the institutional Research Ethics Committee, and all of the subjects gave written informed consent before entering the experimental protocol. The samples were centrifuged at 160 $\times g$ for 15 min to separate plasma from the cells. Cells were separated over a Ficoll-Paque gradient [Pharmacia, Upsala, Sweden] to produce preparations with 95% pure mononuclear cells as analyzed by light microscopy. Purified MN phagocytes were resuspended independently in serum-free 199 medium at a final concentration of 2×10^6 $cells \cdot mL^{-1}$. The cells were used immediately for cellular viability phagocytosis, microbicidal activity, apoptosis rates and intracellular calcium assays.

2.3. Parasites

G. lamblia trophozoites, Portland 1 [P1, ATCC 30.888] were used in all of the experiments. The parasites were cultivated axenically in TYI-S-33-modified medium [24]. The parasites were cultivated from 2 to 5 days. Before the experiments, the culture tubes were centrifuged at 250 *g* for 5 min at 4 °C. The trophozoites were washed out and resuspended in medium 199.

2.3.1. Viability of the parasites in medium 199

In agreement with Hill and Pearson [8] the capacity of the parasites for survival in medium 199 was determined by the incubation of $1/10^6$ trophozoites/mL with 10 mL of medium 199 and also with TYI-S-33 medium, at 37 °C. After 2 h of incubation, the trophozoite movement and flagellum mobility were measured to determine the viability of the parasite. These experiments were carried out in triplicate.

2.4. Blood phagocytes treated with levamisole microemulsion [MELe]

The effects of MELe on MN cells were assessed by determining phagocytic and microbicidal activity, apoptosis and intracellular Ca^{2+} release. To that end, MN cells [2×10^6 $cells \cdot mL^{-1}$] were incubated with 25 μL of MELe (0.84 $mg \cdot mL^{-1}$ of the proportion of 95 mL microemulsion) for 60 min at 37 °C. The cells were then washed once with 199 medium at 4 °C and immediately used in the assays. A control treatment containing only 199 medium was also tested. To evaluate microemulsion use as carrier, cell suspensions were also incubated with 25 μL of levamisole solution [0.84 $mg \cdot mL^{-1}$] and 25 μL of microemulsion alone [ME] for 60 min at 37 °C. The phagocytes were then washed once with 199 medium at 4 °C and immediately used in the assays.

After treatment, the MN cells were used in the assays to test phagocytosis, microbicidal activity, apoptosis and intracellular calcium release.

2.5. Cellular viability and anti-parasitic assay

Cellular viability, phagocytosis and anti-parasitic activity were evaluated using acridine orange [Sigma, ST Louis, USA] method [25]. Equal volumes of parasites [4×10^4 $parasites \cdot mL^{-1}$] and cell suspensions [2×10^6 $cells \cdot mL^{-1}$ mononuclear phagocytes [pre-treated or not as described in item 2.4] were incubated at 37 °C for 120 min under continuous shaking. Phagocytosis was stopped by incubation on ice. The suspensions were centrifuged twice [160 $\times g$, 10 min, 4 °C]. Cells were resuspended in serum-free 199 medium and centrifuged. The supernatant was discarded, and the sediment was dyed with 200 μL acridine orange [Sigma, St. Louis, USA; 14.4 g/L] for 1 min. The sediment was resuspended in cold 199 medium and washed twice. Cellular viability, and phagocytosis as well as *G. lamblia* and leukocyte death were determined by fluorescence microscopy at 400× and 1000× magnification. In total, 100 parasites were counted per slide.

The viability index was calculated by counting the number of orange-stained [dead] and green-stained [alive] cells out of 100. Phagocytosis was considered to be positive when the cells contained internalized parasite. The anti-parasitic index was calculated as the ratio between orange-stained [dead] and green-stained [alive] *G. lamblia* × 100 [25]. All of the experiments were performed in duplicate.

2.6. Apoptosis assay

Annexin V staining was used to assess apoptosis. Untreated cells were used as negative control and cells treated with staurosporin [Sigma ST Louis, USA - [26]] were used to induce apoptosis, as positive control. Controls and MN cells [pre-treated or not as described in item 2.4] were incubated with *G. lamblia* were resuspended in 500 μL of binding buffer containing 5 μL of annexin V-FITC [Annexin V-FITC Apoptosis Detection Kit, Alexis TM, San Diego, USA] and then incubated for 10 min. at room temperature. Fluorescence of the cells was analyzed by flow cytometry [FACS Calibur system - BD, San Jose, USA].

2.7. Intracellular Ca^{2+} release determination

We performed fluorescence staining on the FACS Calibur [BD San Jose, USA] to assess intracellular Ca2 + release in MN cells. Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-Acetoxymethyl [Fluo3-AM– Sigma ST Louis, USA]. Cell suspensions [pre-treated or not as described in item 2.4] were mixed and incubated at 37 °C for 2 h under continuous stirring. Suspensions were centrifuged twice [160 $\times g$, 10 min, 4 °C] and resuspended in PBS containing BSA [5 mg/mL]. This suspension was incubated with 5 μL of Fluo-3 [1 μg/mL] for 30 min at 37 °C. After incubation, MN cells were washed twice in PBS containing BSA [5 mg/mL; 160 $\times g$, 10 min, 4 °C] and then analyzed by flow cytometry [FACS Calibur system - BD, San Jose, USA]. Fluo-3 was detected at 530/30 nm filter for intracellular Ca^{2+}. The rate of intracellular Ca^{2+} release was expressed as the geometric mean fluorescence intensity of Fluo-3.