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Effectiveness of hyperbaric oxygen for experimental treatment of schistosomiasis mansoni using praziquantel-free and encapsulated into liposomes: Assay in adult worms and oviposition

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ABSTRACT

The treatment of schistosomiasis depends on a single drug: praziquantel (PZQ). However, this treatment presents limitations such as low and/or erratic bioavailability that can contribute to cases of tolerance. Improvements to the available drug are urgently needed and studies with a controlled system of drug release, like liposomes, have been gaining prominence. The present study evaluated the activity and synergy between liposomal-praziquantel (lip.PZQ) and hyperbaric oxygen therapy (HBO). Mice received doses of 60 or 100 mg/kg PZQ or lip.PZQ, 50 days post-infection, and after the treatment, were exposed to HBO (3 atmosphere absolute – ATA) for 1 h. The viability of adult worms and oviposition were analyzed, by necropsy and Kato-Katz examination performed after 15 days of treatment. A concentration of 100 mg/kg of lip.PZQ + HBO was more effective (48.0% reduction of worms, 83.3% reduction of eggs/gram of feces) and 100% of the mice had altered of oograms (indicating interruption of oviposition) compared to other treatments and to the Control group (infected and untreated). It is known that PZQ requires participation of the host immune system to complete its antischistosomal activity and that HBO is able to stimulate the immune system. The drug became more available in the body when incorporated into liposomes and, used with HBO, the HBO worked as an adjuvant. This explains the decreases of oviposition and worms recovered form hepatic portal system.

1. Introduction

Schistosomiasis (a debilitating parasitic disease caused by intravascular digenetic trematodes of the genus *Schistosoma*) and several other helminthes, protozoa, viral and bacterial diseases remain neglected (Hotez et al., 2006, 2007; Utzinger et al., 2009, 2011). According to Utzinger et al. (2011), there is no global organization to combat schistosomiasis, unlike other diseases. The neglect of schistosomiasis and other diseases reflects the close association of these diseases with poverty, the geographic isolation of certain affected areas, and the lack of political will and global financing mechanisms in affected populations (Utzinger et al., 2011). According to Steinmann et al. (2006) and King (2010), schistosomiasis is the most important waterborne disease typically associated with poverty, reflecting the lack of access of endemic populations to clean water and sanitation. It is estimated that more than 207 million people are infected with this disease, and 800 million people are at risk of infection, worldwide (Steinmann et al., 2006).

In Brazil, where only the species *Schistosoma mansoni*, which causes schistosomiasis mansoni, has been identified, approximately 43 million people live in areas at risk of infection, and 7 million people are infected. Brazil is the country most affected by this type of schistosomiasis in the Americas (Lambertucci, 2010; WHO, 2010). As a treatment for this disease, chemotherapy

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might be restricted as a consequence of the limited availability of drugs. Currently, praziquantel (PZQ) is the only drug used for the chemotherapy of this disease (Parise-Filho and Silveira, 2001; Ferrari et al., 2003). PZQ is a broad-spectrum drug used in the treatment of all types of schistosomiasis. Although PZQ has a low potential for mutagenic, carcinogenic and toxic effects (Kramers et al., 1991; Montero et al., 1994; Montero and Ostrosky, 1997), this treatment has limitations, including low and/or erratic bioavailability, poor solubility in aqueous systems (Mourão et al., 2005) and reduced activity against younger forms of the parasite. Clinical cases of tolerance and experimental development of resistance in the treatment of schistosomiasis (Fallon and Doenhoff, 1994) have been reported. Thus, it is necessary to propose new therapeutic measures to develop new drugs or improve existing treatments.

A recent study reported the encapsulation of PZQ in soybean phosphatidylcholine liposomes (lip.PZQ), comprising amphiphilic two-carbon chain compounds that upon dispersal in water, tend to form one or more spherical and concentric bilayers separated by aqueous compartments, reaching diameters of 80 nm–100 μm (Weiner et al., 1989; Mourão et al., 2005). Improvements in the solubility of lip.PZQ *in vitro* have been demonstrated, and the results of pre-clinical experiments have shown this treatment to be more effective than free PZQ in reducing the oviposition and the number of adult worms in *S. mansoni* samples obtained from the perfusion of the hepatic portal system (Mourão et al., 2005; Frezza et al., 2007; Frezza et al., 2013).

The encapsulation of PZQ in liposomes has been used to improve the treatment of schistosomiasis. Another method involves the use of hyperbaric oxygen (HBO), alone or in combination, with free PZQ and lip.PZQ. The "Undersea and Hyperbaric Medical Society" defines HBO therapy as a treatment in which the patient breathes 100% pressurized oxygen in an atmosphere greater than sea level (1 ATA). Indeed, O_2 plays an important role in the development of infections, and there is extensive literature discussing the toxicity of this element and the effects of HBO on different microorganisms (Widyanti, 2011; Kaide and Khandewal, 2008). It has been reported that both hyperoxia and HBO exert anti-microbiological effects by increasing the intracellular flow of Reactive Oxygen Species (ROS). For example, in some species of bacteria, ROS induces DNA breaks, RNA degradation, amino acid biosynthesis inhibition and protein transport inactivation in membranes (Park et al., 1992). One study, evaluating the effect of HBO on *S. mansoni* showed that exposure between 12 and 20 weeks post-infection reduces the number of worms collected from the hepatic portal system (Kuntz et al., 1980).

In hyperbaric oxygenation, hemoglobin becomes saturated with oxygen, and the blood-carrying capacity of this molecule increases with increasing dissolved of O_2 in the plasma; thus, hyperoxia increases the activity of antimicrobial drugs. Both hyperoxia and HBO enhance tissue PO_2 to levels that affect microbial growth, antimicrobial activity and leukocyte function *in vitro* (Park et al., 1992).

For the treatment of schistosomiasis mansoni, HBO could act synergistically with PZQ and lip.PZQ, increasing the effectiveness of this drug. The aim of the present study was to analyze the efficacy of HBO, in combination with PZQ and lip.PZQ, in the treatment of schistosomiasis mansoni in mice infected with *S. mansoni*, BH strain, using numbers of adult worms and oviposition as outcome measures.

2. Materials and methods

2.1. Preparation of liposome-encapsulated PZQ

Soybean phosphatidylcholine (PC) liposomes were prepared using the sonication method of Cinto et al. (2009). A lipid film was made by evaporation of a PC chloroform solution under vacuum in a rotary evaporator. Final traces of solvent were eliminated in a vacuum desiccator overnight. Subsequently, 5 mL of Tris/HCl buffer (20 mM, pH 7.5) was added to the lipid film for hydration, followed by incubation at 25 °C for 40 min. The PC liposomes were obtained through sonication in an ice bath for 25 min. To prepare the PZQ-loaded liposomes, PZQ was dissolved in the PC-chloroform solution at a PZQ/PC molar ratio of 1:6, as the loading capacity of PZQ, without precipitation, in PC liposomes is approximately 1:5 PZQ/PC (molar ratio; Mourão et al., 2005).

2.2. S. mansoni *strain*

The *S. mansoni* BH strain (from Belo Horizonte, Minas Gerais, Brazil) was obtained from the Department of Animal Biology of the Institute of Biology of Unicamp, maintained in *Biomphalaria glabrata* molluscs (Pellegrino and Katz, 1968).

2.3. Intermediate and definitive hosts

B. glabrata specimens, sympatric with the *S. mansoni* strain, were infected with miracidia collected from the feces of mice solely used for the infection of the intermediate host at 45–60 days prior to experimental use (Souza et al., 1987). After 40 days, the molluscs were subjected to illumination at 28 °C to expose the cercariae. The cercariae were subsequently used to infect 30-day-old female Swiss mice (*Mus musculus*), weighing 20 g, through tail immersion with a cercarial suspension at 70 cercariae per animal (Olivier and Stirewalt, 1952; Pellegrino and Katz, 1968). The protocol for these infection experiments (1117-1) was approved through the Ethics Commission for Animal Experimentation (CEEA) of the Institute of Biology of Unicamp, in accordance with the ethical principles of the Brazilian Association of Animal Experimentation (COBEA).

2.4. Animal groups

We examined the effects of both PZQ and lip.PZQ at concentrations of 60 and 100 mg/kg in groups of ten mice each. One group received PZQ, the other group received lip.PZQ and the remaining ten received an oral administration of Tris–HCl buffer (20 mM, pH 7.5) in a single dose of 0.3 mL/mouse at 50 days after infection. Subsequently, the mice caged in groups of ten were exposed to 100% O_2 at a pressure of 3 ATA for 1 h in a small animal hyperbaric chamber (Research Chamber, model HB 1300B, Sechrist, CA, USA). The chamber was pressurized and decompressed at a rate of 0.5 ATA/min (Arrais-Silva et al., 2006). Three groups of ten mice each were not exposed to HBO, receiving treatment with only PZQ, lip.PZQ or Tris–HCl buffer (Table 1).

2.5. Treatment analysis

Fifteen days after treatment, the animals were euthanized through cervical dislocation to prevent the displacement of the worms to other organs (Delgado et al., 1992). The worms were collected through perfusion of the hepatic portal system (Yolles et al., 1947), and males, females and couples were counted. The liver was removed and compressed between glass plates to observe the worms (Delgado et al., 1992). After removing 1-cm^2 fragments from the ascending colon, oogram analyses were performed at various stages of egg maturation, namely immature (1st to 4th stage), mature (5th stage), and dead eggs (Hermeto et al., 1994). The oogram was considered to have been changed when one or more stages of the immature eggs were missing (Pellegrino et al., 1962). Kato-Katz exam (Kato and Miura, 1954; Katz et al., 1972)