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Photodynamic therapy for *Schistosoma mansoni*: Promising outcomes

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ABSTRACT

The purpose of this study was to assess, for the very first time, the effects of photodynamic therapy (PDT) on *Schistosoma mansoni in vitro* by measuring reactive oxygen species (ROS) generation throughout the treatment, as well as the behavior of the parasites (mating, motility and contraction/shortening), and damage to their tegument and excretory systems. The parasites were divided into 4 groups: control, photosensitizer, laser and PDT. Light irradiation was delivered with an InGaAlP low-level laser device operating at 660 nm, with 40 mW and 100 J/cm^2. For PDT, different toluidine blue dye (TBO) concentrations and times of exposure were utilized. Interestingly, TBO-mediated PDT was able to kill *S. mansoni* ($P < 0.001$) due to the significant amount of ROS released that inflicted damages in the tegument and excretory system, as well as contraction and cessation of motility. In addition, males of *S. mansoni* were shown to be more sensitive to PDT if compared to their corresponding females when the optimal TBO concentration of 31.2 μL was considered ($P = 0.0126$). PDT presents two major advantages: not inducing microbial resistance and also lacking adverse effects. Therefore, PDT may become a promising therapeutic alternative for schistosomiasis in the near future, especially for cases of allergy and resistance to praziquantel.

1. Introduction

The trematode *Schistosoma mansoni* is a long-living intravascular parasite considered to be the major causative specie of schistosomiasis, an acute and chronic disease that afflicts approximately 258 million people worldwide [1,2]. Schistosomiasis is endemic in 78 countries, especially in poor tropical and subtropical areas that lack access to potable water and adequate sanitation, such as in Africa, Asia and South America [2,3]. In the Sub-Saharan Africa, the mortality by schistosomiasis was estimated at 280,000 deaths/year [4]. In this context, control strategy recommendations by the World Health Organization (WHO) focuses on reducing the disease through periodic and targeted treatment of the affected populations with 40 mg/kg of praziquantel (PZQ), a preventive chemotherapy [5].

The mechanism of action of PZQ is still uncertain [6]; however, it is suggested that the key events relate to calcium influx to the intact parasites, as well as muscle contraction and tegument alterations [7,8]. It also known that although PZQ is effective against adult schistosomes [9], it is ineffective against juvenile parasites [10]. Besides, cases of allergy [11,12] and resistance [13] to PZQ have been reported and thus clearly point out the urgent necessity for the upsurge of new anti-schistosomal therapies [6].

Interestingly, recent studies showed that *S. mansoni* is very susceptible to oxidative stress [14,15], electing the generation of reactive oxygen species (ROS) or inhibition of endogenous antioxidant enzymes as possible new therapeutic approaches [16]. Suitably, photodynamic therapy (PDT) would then rise as a plausible alternative to *S. mansoni* killing, once PDT utilizes the combination of a photosensitizer (PS) and visible light within suitable wavelengths that, when in the presence of molecular oxygen, induce cytotoxicity [17]. Essentially, in a long-lived

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triplet state, the PS can interact with molecular oxygen by electron transfer (type I) or by energy transfer (type II); type I will lead to superoxide anions ($O_2\cdot^-$) and more reactive ROS such as hydroxyl radicals (·OH), whilst type II will form singlet oxygen ($^1O_2$) [18,19].

Hence, the purpose of this study was to assess the effects of PDT on *S. mansoni in vitro* for the very first time, by measuring the production of ROS, as well as the behavior of the parasites (mating, motility and contraction/shortening) and damage to their tegument and excretory system following PDT; the parameters sketched herein will possibly contribute to the future application of PDT to such a relevant disease.

2. Materials and Methods

2.1. Ethics Statement

This research was overseen and approved by the Commission on the Ethical Use of Animals from the Federal University of Alfenas (CEUA-UNIFAL), under the protocol registration No. 534/2013. All animal experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals [20].

2.2. Parasites

S. mansoni cercarie were obtained from infected *Biomphalaria glabrata* snails, as previously described by Smithers and Terry [21]. Swiss mice were infected by subcutaneous injection of 1 mL of water containing about 150 cercariae and then monitored and fed for about 45 days. Then, the animals were sacrificed using 3.0% pentobarbital sodium, which was administered intraperitoneally (± 0.3 mL/mouse) [22]. Following exposure of the heart and abdominal viscera, culture medium RPMI 1640, pH 7.4 (Sigma ChemicalCo., St. Louis, MO, USA) heparinized (0.2%) was injected to heart ventricles, thereby obtaining the adult parasites. Subsequently, the parasites were cultured in 6-well plates (4 couples per well) containing RPMI 1640 supplemented with 5.0% of heat inactivated fetal bovine serum (FBS) (Gibco Limited, Paisley, Scotland) and 1.0% penicillin (10,000 UI/mL)/streptomycin (10 mg/mL) (Sigma ChemicalCo., St. Louis, MO, USA), and incubated in an atmosphere of 5% CO_2 at 37 °C.

2.3. Photodynamic Therapy and Laser Irradiation

Parasites were randomly divided into 4 groups: Control group - culture medium only; PS group - TBO was added at different concentrations (3.7 µL, 7.8 µL, 15.6 µL, 31.2 µL, and 62.5 µL) to the medium; Laser group - wells were irradiated with red laser light (660 nm; 40 mW; 100 J/cm^2; 100 s per point and 1 point per parasite with the laser tip slightly touching the bottom surface of the well plates, giving 4 J of total energy per point and 1 W/cm^2 of irradiance) originated from an InGaAlP laser device (Twin Flex, MMO®, São Carlos, SP, Brazil); PDT group - TBO was delivered at the same concentrations of the PS group and then irradiated identically to the laser group. Laser irradiation and PDT were performed thrice.

2.4. Detection of ROS During PDT

The fluorescent probes (Molecular Probes, Life Technologies, NY, USA) 3′-p-(aminophenyl) fluorescein (APF - A36003), 3′-p-(hydroxyphenyl) fluorescein (HPF - H36004) and singlet oxygen sensor green (SOSG - S36002) were used for detection of ROS (·OH, $O_2\cdot^-$ and $^1O_2$) [23,24] during TBO-mediated PDT. The following groups were included on a 96-well plate (353072, BD Falcon): group 1, containing only solvent (acetonitrile 50/50%); group 2, containing only PS; group 3, containing solvent and PS; group 4, containing solvent and fluorescent probe (5.0 µM); and group 5, containing PS and fluorescent probe (5.0 µM). The plates were subsequently laser irradiated as already described and with increasing fluences of 6 J/cm^2 each, until fluences reached up to 132 J/cm^2 (laser irradiation took place at every 6 s, during a total of 132 s). The fluorescence emission was measured (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using excitation/emission wavelengths of 490/515 nm for APF and HPF probes, and 505/525 nm for SOSG probe.

2.5. Viability Assay

The viability of the parasites was assessed under a light microscope using an adaptation of the motility scale described by Ramirez et al. [25], attributing scores of 0 to 3 (0 = total absence of motility; 1 = minimal activity, occasional movement of head and tail; 2 = slow activity; and 3 = normally active); the criteria of scoring the motility and survival of worms in a viability scale of 0–3 was recently published [26]. The motility of parasites was evaluated in relation to the concentration of TBO and time of exposure to this PS and to PDT, separately for females and males. All groups were analyzed daily for 5 days, checking for recovery after treatment with PS, laser or PDT.

2.6. Evaluation of the Excretory Activity and Damage to the Tegument of S. mansoni by PDT

In order to evaluate the performance of TBO-mediated PDT on the excretory system activity and on the tegumental surface layer of *S. mansoni*, experiments were conducted with the probes Resorufin and Hoechst 33258 (Sigma Chemical Co., St. Louis, MO, USA), respectively [27]. The probe Resorufin is passively diffused through the tegument of *S. mansoni* and further excreted through a supposed P-glycoprotein expressed in excretory epithelium [28]. In contrast, the probe Hoechst 33258 fluoresces when binds to the DNA of cells; however, this probe is hydrophilic and is only able to diffuse to the interior of cells when lesions occur, acting as an indicator of membrane integrity [27], and especially used to evaluate schistosomicidal activity through tegument lesions [22,29]. In that way, 10 µL of either Resorufin or Hoechst 33258 were added to the wells of 96-well plate (each well contained 4 couples of *S. mansoni*). Then, the plates were incubated for 30 min concerning Resorufin and for 15 min concerning Hoechst 33258. Further, the parasites were divided into 4 groups (control, PS, laser, and PDT) and then incubated for 15 min more. The parasites were washed 5 times with RPMI 1640 and transferred to slides for fluorescence viewing (Nikon - Eclipse 80i). Rhodamine filter was used for Resorufin (excitation/emission maximum of 571/585 nm) and DAPI for Hoechst 33258 (excitation/emission maximum of 352/455 nm).

2.7. Statistical Analysis

All tests were performed in triplicate and analyzed in GraphPad Prism software version 5.0 for Windows (GraphPad Software Inc., San Diego, CA) with a significance level of 5% ($P < 0.05$). Experimental groups were compared using one-way analysis of variance (ANOVA) followed by Tukey and Scott-Knott test for multiple comparisons.

3. Results and Discussion

3.1. ROS Generation During PDT

APF and HPF are employed for detection of ·OH and $O_2\cdot^-$, differing in response to the hypochlorite ion (ClO^-), because the APF probe is converted to its fluorescent form in the presence of this ion, in contrast to the HPF probe, which is not converted [30]. On the other hand, SOSG probe is highly selective for $^1O_2$ [24].

The kinetic behavior of the APF probe during TBO-mediated PDT showed a maximum release point of ROS at around 50 J/cm^2 of energy density and was followed by a plateau and consequent slight decay (Fig. 1A). On the other hand, the HPF probe indicated that values close to 132 J/cm^2 of energy density are related to the highest peak of ˙OH