Photodynamic Therapy Using Toluidine Blue O (TBO) Dye as a Photosensitizer against *Leishmania major*

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**Abstract**

**Background:** Photodynamic therapy (PDT) is alternative treatment of cutaneous leishmaniasis (CL), and phenothiazine dyes such as Toluidine Blue O (TBO) have the potential role in PDT and notably affect parasites inactivation. This study aimed to evaluate the effectiveness of PDT by using TBO and a light-emitting diode (LED) in the treatment of zoonotic CL (ZCL).

**Methods:** The study was conducted in Iran University of Medical Sciences, Tehran, Iran in 2018-2020. Different concentration (7.8 µg/mL up to 500 µg/mL) of TBO as a photosensitizer and a 630 nm LED light as a source of light were used for antileishmanial activity against both forms of *Leishmania major* promastigotes and intracellular amastigotes. Effective concentration (EC₅₀) and cell cytotoxicity (CC₅₀) were calculated in both infected and non-infected J774.A1 macrophages, respectively. As well as inhibitory concentration (IC₅₀) was quantified in *L. major* promastigotes for 2 h, 24 h, and 48 h after incubation using a MTT colorimetric assay.

**Results:** TBO dye in combination with the PDT significantly decreases the *L. major* promastigotes and intracellular amastigotes viability when compared with TBO alone. Both TBO dye in combination with the PDT and TBO alone had no toxic effects on the mice macrophages; however, it significantly killed the entered parasites inside the cells. Our results in the current study established satisfactory findings in clearing intracellular *L. major* parasites in in-vitro conditions.

**Conclusion:** TBO dye in combination with the PDT can be considered as a harmless, effective and importantly perfect treatment against *L. major*, causative agent of ZCL, in an in-vitro situation without any negative toxicity to the mice macrophages.

**Keywords:** *Leishmania major*; In vitro; Photodynamic therapy (PDT); Toluidine blue O (TBO)
Introduction

Cutaneous leishmaniasis (CL) is a parasitic destructive disease caused by protozoan parasite of the genus *Leishmania*, transmitted to humans through the bite of a female sandfly (1). Over the past 5 years, there were more than one million new cases of the disease, distributed on several continents with an incidence of 95% of cases in the Americas, the Mediterranean basin, the Near East and Central Asia especially in Afghanistan, Algeria, Iran and Syria (2).

Physical treatment such as thermotherapy, surgery and cryotherapy have been used as one of the therapeutic options to reduce the various side effects of CL, which often cause disfiguring scars and the recurrence of *Leishmania* infection (3-6). In addition, low-cost and easy-to-use treatment for CL that can be administered conveniently is still novel topic in the field of biomedical research (3-6). Importantly, treatment failure or resistance of *Leishmania* parasites to chemotherapy particularly for first-line anti-*Leishmania* drugs such as pentavalent antimonial compounds have been reported (7-9). These limitations have led to the search for various effective alternative treatments.

Photodynamic therapy (PDT) has emerged as a new and effective alternative therapeutic modality in the clinical management of CL (10-12). PDT is based on the use of a photosensitizer (PS) compound that, excited by light of a suitable wavelength, releases the generation of cytolytic reactive oxygen species (ROS) in the presence of oxygen that can destroy the microorganism or the target cell (13, 14). Moreover, the energy densities and high optical power may cause thermal damage by increasing the temperature in the exposed tissue (15, 16). Light-emitting diode (LED) is a PDT light applicator that can induce high power light of desired wavelengths and assemble in a range of geometries and sizes (17).

To date, there are no documented published data on the effectiveness of combination photosensitizers and LED against causative agent of ZCL (*L. major*) in an in-vitro model of mice macrophages.

Methods

This study was approved by the Research Committee of Iran University of Medical Sciences (Ethics Committee Code: IR.IUMS.FMD.REC.1397.007). The study was done in Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran in 2018-2020.

Parasite and macrophage cultures

Iranian pathogenic strain of *L. major* (MRHO/IR/75/ER) promastigotes provided as a gift from Prof. Sima Rafati (Department of Immunotherapy and *Leishmania* Vaccine Research, Pasteur Institute of Iran, Tehran, Iran) was used in the current study. *L. major* promastigotes were cultured at 26 °C in RPMI-1640 medium (Sigma-Aldrich Chemicals; Germany) with 10% heat-inactivated fetal calf serum (FCS) and supplemented with 100µg/ml penicillin and 100µg/ml streptomycin (pH=7.4). The mice macrophage cell line J774A.1 (ATCCCTIB-67TM) was purchased from Pasteur Institute of Iran and was applied in the current experiment. Macrophage was also cultured in RPMI 1640 medium with 10% FCS and antibiotics (100µg/ml penicillin and 100µg/ml streptomycin). Cells were incubated under standard conditions at 37 °C and 5% CO₂ incubator (Memert; USA). The passage of the mice macrophage cell line was performed every three days.

Photosensitizer and light source

TBO (Sigma-Aldrich, Steinheim, Germany) as a photosensitizer was prepared in sterile 0.9% (wt/vol) NaCl to obtain a concentration of 0.4 mg/ml. Then this solution was sterilized by a 0.22µm syringe filter and subsequently kept under dark conditions before use (18). Irradiation
treatment was carried out using a LED system (FotoSan 630 nm LAD, CMS dental, Copenhagen, Denmark) with a wave length of maximum emission at 630 ± 10 nm with an average output intensity of 3000 mW/cm² (19). The output power density was measured by a power meter (Laser Point s.r.l, Milano, Italy). The distance between the light source and each well surface was fixed at 1 mm (20).

Five experimental groups were selected: 1) LED group (cells with no photosensitizer and irradiated with LED light); 2) dark group (cells incubated with TBO in all concentrations, not irradiated); 3) PDT (cells incubated with TBO in all concentrations which irradiated with LED light); 4) positive control (cells incubated with glucantime); 5) negative control (cells without photosensitizer and light). Serial dilution (1:2) for TBO was performed as following: 7.8 µg/ml, 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, and 500 µg/ml. In dark group, all cells were incubated with TBO for 1 h.

**Promastigote growth inhibition (IC₅₀)**

The effects of several concentrations of TBO (7.8 µg/ml up to 500 µg/ml) on the stationary phase (5 d) of *L. major* promastigotes (1×10⁶ parasites) were assessed. The inhibitory concentration that caused a 50% decrease in survival (IC₅₀) of promastigotes was determined (22).

**Cytotoxicity assay on the Leishmania infected macrophages (EC₅₀)**

The ability of the TBO to kill intracellular amastigotes of *L. major* was measured based on the viability of the parasites evaluated by the MTT assay as described previously (21). Results are reported as effective concentrations that kill 50% of intracellular parasites (EC₅₀) calculated by the linear regression equation.

**Selectivity Index (SI) determination**

In this investigation, the ratio of the obtained CC₅₀ value of the cytotoxic concentrations to the obtained EC₅₀ value of the antileishmanial activity was determined to calculate the TBO selectivity index (SI) (23). Moreover, SI was calculated for promastigote forms of parasite (SI= CC₅₀ Macrophages/IC₅₀ Promastigotes) (24). At a time that the SI value is under 10, that compound ideal antileishmanial activity. On the other hand, the ideal TBO compound would be cytotoxic slowly at very high concentrations and have antileishmanial activity at very low concentrations (higher reported values=greater TBO activity).

**Statistical analysis**

All statistical analyses were performed using Graph Pad Prism software, ver. 8.0. The differences between control and treatment groups were measured using analysis of variance (ANOVA), and differences with p values of less than 0.05 were considered statistically significant.

**Results**

**In vitro cytotoxicity of TBO and PDT (CC₅₀)**

To examine the TBO effects on mice J774A.1 cell line, CC₅₀ (µg/mL) was measured. As illus-
trated in Fig. 1 and Table 1, raising concentration of TBO with and without light stimulation (PDT and dark groups) decreases cells viability. For instance, TBO in both PDT and dark groups and light stimulation with 500µg/ml had the highest and TBO with 7.8 µg/ml had the lowest toxicity for macrophages. Both TBO with and without illumination have been only toxic for J774A.1 macrophages at high concentrations. The best results were achieved in 48 h. There was a significant difference in cell viability between macrophages treated by TBO with irradiation (PDT) and without irradiation ($P<0.05$). Moreover, there was no significant difference between macrophages growth in the control group and the groups treated with laser without TBO. The lowest survival rates of macrophage cells were observed when cells were irradiated with 10 J/cm² at 500µg/ml concentration of TBO dye in 48 h after incubation (Fig. 1 and Table 1).

**Fig. 1:** Cytotoxicity assay ($CC_{50}$) of several concentrations (7.8 µg/ml up to 500 µg/ml) of TBO on J774A.1 macrophages cell line with and without illumination after 2 h (A), 24 h (B), and 48 h (C) using MTT method. All data have been reported as the mean ± SD of triple repeated experiments. $CC_{50}$ µg/ml was calculated for each groups by using dose response curve (Prism 8 software).
Table 1: CC50, IC50, EC50 and SI values of the TBO with and without illumination (LED) for 2 h, 24 h, and 48 h after treatment

| Groups         | Time | CC50 (µg/ml) for Non-infected MQ | IC50 (µg/ml) for parasite (Promastigotes) | EC50 (µg/ml) for infected MQ (Amastigotes) | SI (CC50/IC50) |
|----------------|------|---------------------------------|------------------------------------------|-------------------------------------------|---------------|
| No illumination| 2h   | 329 (CI:205.8,548.1)            | 178.8 (CI:58.71,586)                     | 172.5 (CI:65.97,486.2)                    | 1.84          |
|                | 24h  | 150.5 (CI:44.64,538.8)          | 69.23 (CI:24.08,197)                     | 102.9 (CI:35.37,304.5)                    | 2.17          |
|                | 48h  | 123.3 (CI:30.77,514.1)          | 27.37 (CI:10.31,69.2)                    | 73.71 (CI:23.60,229)                      | 4.50          |
| After illumination | 2h   | 174.4 (CI:89.84,347.1)          | 94.95 (CI:30.46,287)                     | 58.33 (CI:19.53,167.8)                    | 1.83          |
|                | 24h  | 77.95 (CI:21.49,276.3)          | 27.58 (CI:12.53,59)                      | 37.12 (CI:15.36,87.12)                    | 2.82          |
|                | 48h  | 67.77 (CI:19.16,234.4)          | 11.71 (CI:6.46,20)                       | 21.41 (CI:9.72,45.14)                     | 5.78          |

Antileishmanial effects of TBO and PDT on promastigotes (IC50)

To examine the TBO effects on L. major promastigotes, IC50 (µg/ml) was assessed by using MTT method (Fig. 2). The viability of L. major promastigotes treated with TBO with illumination had the highest effect on promastigotes and killed parasite effectively in comparison with the other groups (P<0.05). There was no significant difference between LED alone (in the absence of TBO) and negative control group (Fig. 2 and Table 1).

Fig 2: Inhibitory concentration (IC50) of TBO (7.8 µg/ml up to 500 µg/ml) on promastigotes of L. major parasite with and without illumination after 2 h (A), 24 h (B), and 48 h (C) using MTT method. All data have been reported.
as the mean ± SD of triple repeated experiments. IC$_{50}$ μg/ml was calculated for each groups by using dose response curve (Prism 8 software).

**Antileishmanial effects of TBO and PDT on infected macrophages by Leishmania (EC$_{50}$)**

To examine the TBO with and without PDT effects on *L. major* amastigotes in infected J774A.1 macrophages, the EC$_{50}$ (μg/ml) was assessed using MTT method (Fig. 3). As indicated in Fig. 3, the TBO with and without PDT could inhibit the growth of *Leishmania* amastigotes. TBO with and without PDT did not show any toxic effects to J774A.1, but it could kill the parasite effectively. Infected macrophages with parasites with concentration of 500 μg/ml TBO showed the highest decrease in the total number of *Leishmania* parasites. Moreover, antileishmanial activity increases after light exposure. The EC$_{50}$ in infected cells treated with TBO with and without light were calculated 172.5 μg/ml and 58.33 μg/ml for 2 h; 102.9 μg/ml and 37.12 μg/ml for 24 h; and 73.71 μg/ml and 21.41 μg/ml for 48h, respectively (Table 1). There was a statistically significant difference between TBO (with and without light) and positive control (glucantime) (*P*<0.01) (Fig. 3 and Table 1).

**TBO and selectivity index (SI)**

TBO photosensitizer with LED light was active against the amastigotes of *L. major* parasite inside the J774A.1 macrophages with and without illumination after 2 h (A), 24 h (B), and 48 h (C) using MTT method. All data have been reported as the mean ± SD of triple repeated experiments. IC$_{50}$ μg/ml was calculated for each groups by using dose response curve (Prism 8 software).

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Fig. 3: Effective concentration (EC$_{50}$) of several concentrations (7.8 μg/ml up to 500 μg/ml) of TBO on amastigotes of *L. major* parasite inside the J774A.1 macrophages with and without illumination after 2 h (A), 24 h (B), and 48 h (C) using MTT method. All data have been reported as the mean ± SD of triple repeated experiments.

IC$_{50}$ μg/ml was calculated for each groups by using dose response curve (Prism 8 software).

Furthermore, as shown in Table 1, the cytotoxicity determination showed that TBO photosensitizer with LED light was strongly selective against *L. major* promastigotes, compared to J774A.1 cell line with an SI of (1.83 μg/ml, 2.82
μg/ml, and 5.78μg/ml) for 2h, 24h, and 48h, respectively. Therefore, TBO was highly active against both forms of L. major promastigotes and amastigotes in comparison with macrophages.

Discussion

To date, the only effective approach for Leishmania parasite control is the application of chemotherapy. Increased anti-parasites resistance and importantly high negative effects and toxicity to both first and second-line chemotherapies against leishmaniasis have obliged scientists to discover effective chemicals and other alternatives with less toxicity. In addition, some chemicals compounds like miltefosine are limited due to high price and are not cost-effective (25, 26). Moreover, both first and second-line chemotherapies against leishmaniasis may cause bone marrow suppression, hepatotoxicity as well as myalgia, fever, nausea and severe pain at the site of drug application (27-29). Therefore, finding out distinct chemical and non-chemical compounds are vital for reduction drugs complications. In this respect, using other methods such as different dyes and PDT are extremely attractive alternatives with promising antileishmanial activity used in treatment of disease. Several studies have been conducted on the PDT with application of different dyes and photosensitizers which had strong effects against old-world CL (30). The current study is the first comprehensive study conducted on the therapeutic effects of TBO and or PDT as an antileishmanial compound without excessive negative toxicity to the host cells with direct antileishmanial activity in vitro condition.

TBO is a phenothiazine dye that interacts with biological molecules and mediators. TBO molecules have the high ability to bind to DNA and could aggregate alone DNA surface (31). This dye is playing an important role in the light-mediated killing off the cells of the microorganism that may be due to generation of reactive oxygen species (ROS) particularly singlet oxygen (13, 14). Although the action mechanism throughout which PDT kills Leishmania is not completely identified yet, studies has illustrated that PDT using a photosensitizing compound and visible light can promote the production of oxygen species (superoxide anion and hydroxyl radical) and nitrogen-oxygen species (RNS). These species damage cells, and finally the cell membrane destruct. In addition, photosensitization reactions in biological tissues can also induce immune-stimulatory reactions (13).

Our results showed that TBO in dark group has no cytotoxic to J774.A1 macrophages. Cells viability decreases when the TBO in high concentrations was illuminated for 2h, 24h, and 48h after incubation, respectively. On the other hand, TBO dye showed to have less toxicity to macrophages compared to glucantime as positive control. In the current study, leishmanicidal activity of both illuminated and no illuminated TBO dye is probably due to the specific inhibition of spermidine synthase, which may result in decreased trypanothione but increased glutathione levels inside promastigotes (32).

Taylor et al. (10) had used the MTT test to determine the viability of L. amazonensis after treatment with Dimethyl and Diethyl Carbaporphyrin Ketals as photosensitizers. They have observed toxicity of these compounds in the dark group. Our investigation was in agreement with those of Taylor et al. We showed that after treatment with high concentrations of TBO significant reduction of parasite viability occurred in dark group. The possible action mechanism of parasite cell death is related to apoptosis mediated by photosensitizing action on Leishmania mitochondria (33). Overall, 10 μg/ml TBO significantly reduced L. braziliensis parasite viability and no lethality was observed on groups treated with laser (PDT). Moreover, they did not find statistical differences between the dyes but reported that the best result was observed with TBO (11).

In our study, L. major promastigotes in the PDT group were more sensitive to the toxic effects of TBO that these effects depend on factors such as toluidine blue o type, molecular charges and concentration. Molecular charges and structure were important effective factors in PDT against Leishmania (34). PDT with aluminum phthalocyanine
tetra sulfonate as the photosensitizer effectively eliminated *L. major* and *L. braziliensis* in-vitro (35). Aluminum phthalocyanine was also phototoxic to *L. major* when irradiated with 10.0µM concentration and 10 J/cm² irradiation. Using methylene blue (MB) as the photosensitizer, parasites viability in the dark decreased up to 50% in the higher concentration of MB while the PDT decrease viability by more than 70% (36, 37). Our experiment provide more evidence for other studies (36, 37). TBO dye in the highest concentrations had intermediate toxicity in the dark group (no PDT), while the PDT indicated decrease in viability in more than 70% especially, for the three highest concentrations used after 24 h.

A suggested mechanism for this activity is that TBO is excited by LED light (630nm), reacts with biomolecules, and increases the availability of transition electrons and substrates for nitric oxide synthase; thereby, increasing the oxidative in the *L. major* promastigotes (36, 37). In this in-vitro study, PDT using TBO dye in J774.A1 macrophages infected with *L. major* was dependent on the cell type, concentration of dye as well as laser strength(J/cm²). We evaluated therapeutic effects of PDT in infected macrophages by using TBO. Moreover, the therapeutic activity of TBO dye may be improved by enhancing such uptake by conjugating them to ligands for high-capacity endocytosis via scavenger receptors of macrophages (38).

In this study, SI data demonstrated that TBO dye with LED light was approximately 3.16 µg/ml and 5.78 µg/ml more toxic to *L. major* amastigotes and promastigotes in comparison with macrophage cells after 48 h of incubation, respectively. Moreover, TBO compound with LED light can decrease the viability rate of intracellular parasites at nontoxic concentrations for the host macrophages. TBO compound is a dye-photosensitizer useful for PDT, which has advantages such as low cost, easy to purchase, antibacterial and antifungal (39). Moreover, the use of LED may be affordable even for low incoming people. In our study, one of the main limitations was clearing the blue color of TBO from the culture media and another was also constructed an easy-to-use light emission tool.

Further studies especially in the mice model are vital to decipher the TBO with and without PDT functions in the treatment of CL. Therefore, it is suggested to assess TBO/PDT in the infected BALB/c mice with *L. major*. Moreover, it is suggested to use combination of methylene blue (MB) and TBO in the treatment *L. major* promastigotes in-vitro and in-vivo. Moreover, further studies are needed to test the efficiency of TBO with and without PDT in the human and population of CL patients.

**Conclusion**

The low-cost PDT protocol using TBO under LED light has strong activity against *L. major* in an in-vitro situation. Thus, the TBO dye is promising candidate as photosensitizer beside PDT which was a potential contributor or to the combinatorial therapy, in the clearing of *L. major* as a causative agent of CL in-vitro situation. Combinatorial therapy with TBO/PDT could be an ideal candidate for production of novel, safe and low-cost compounds against *L. major* in infected macrophages and will have a favorable perspective for future studies.

**Ethical Considerations**

All ethical issues including informed consent, plagiarism, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by all authors.

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Conflict of interest

The authors declare that they have no competing interests.

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