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Photodynamic effect of zinc porphyrin on the promastigote and amastigote forms of *Leishmania braziliensis*†

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Leishmaniasis is a neglected disease present in more than 88 countries. The currently adopted chemotherapy faces challenges related to side effects and the development of resistance. Photodynamic therapy (PDT) is emerging as a therapeutic modality for cutaneous leishmaniasis. Zn(II) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (ZnTE-2-PyP^{4+}, ZnP) is a cationic, water-soluble, zinc porphyrin-based photosensitizer whose photodynamic effect on *Leishmania braziliensis* was analyzed by evaluating the number of visibly undamaged and motile cells, cell membrane integrity, mitochondrial membrane potential, and ultrastructural damage. Treatment of parasites with ZnP and light induced damage in up to 90% of *L. braziliensis* promastigote cells. Propidium iodide labeling suggested the loss of plasma membrane integrity. In samples treated with ZnP and light, a hyperpolarization of the mitochondrial membrane potential was also observed. Ultrastructural evaluation of promastigotes after photodynamic treatment indicated a loss of cytoplasmic material and the presence of vacuoles. Scanning electron microscopy showed wrinkling of the plasma membrane and a reduced cell volume. Additionally, the number of amastigotes per macrophage was reduced by about 40% after photodynamic application. The treatment showed no considerable toxicity against mammalian cells. Therefore, the results indicated that PDT associated with ZnTE-2-PyP^{4+} represents a promising alternative to cutaneous leishmaniasis treatment.

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1. Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease, with clinical presentation of dermal manifestations. It represents up to 75% of all new and severe cases caused by *Leishmania major*, *L. tropica*, *L. amazonensis*, or *L. braziliensis*. The life cycle of the parasite involves two different forms: the promastigote, an extracellular form that is found in the vector, and the amastigote, an intracellular form found in mammalian host cells.[1] Although rarely fatal, the course of the disease is often accompanied by psychological and social repercussions, stigmatization, and disfiguration.[2]

Over the years, several drugs have been used to treat CL. The current treatment is based on pentavalent antimonials, amphotericin B, pentamidine, and paromomycin. However, the use of these drugs has some limitations, such as high levels of toxicity in humans, and the development of drug-resistant parasites has also been reported.[3]

Photodynamic therapy (PDT) appears as a promising option for leishmaniasis treatment, as it can offer rapid and localized destruction of the lesions, without affecting adjacent normal tissue.[4] This therapeutic modality is based on the use of non-toxic dyes with photosensitizing properties, followed by light irradiation, to cause damage to the target tissue.[5]

The success of PDT depends, among other factors, on the choice of appropriate photosensitizer (PS).[6] Porphyrins belong to a group of tetrapyrrole compounds that may possess the

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relevant chemical and physical properties required for PDT, such as a high affinity for cell membranes (in particular the cationic ones), synthetic versatility, and efficiency to generate intracellular reactive oxygen species (ROS).[7,8] These properties allowed the clinical application of porphyrins in PDT for inhibiting viral agents in blood, and as inactivators in antimicrobial therapy, as well as in the treatment of hospital wastewater.[9,10] Despite being widely applied in cancer treatment, the use of porphyrin-based PDT for leishmaniasis treatment remains little explored.[11]

Zn(II) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (ZnTE-2-PyP^{4+}, ZnP) is a cationic, water-soluble, zinc porphyrin-based PS. The use of this porphyrin has been considered promising for microbial photodynamic inactivation of *Escherichia coli* and *Candida albicans*.[9,12–14]

Although cationic porphyrins have been previously applied in the inactivation of other species of Leishmania,[15] there is no literature showing the action of this type of PS in *L. braziliensis* cells. There is still a lack of studies exploring the biological mechanisms involved in the damage caused by PDT. In addition, zinc porphyrins have the potential to be more efficient PSs than their metal-free analogs, as the presence of zinc increases the PS interactions with cell membranes.[16,17]

Owing to the limitations of conventional therapy for leishmaniasis, the search for new drugs and novel forms of treatment are increasingly necessary. Considering the growing interest in the search for new PDT protocols, the current study evaluated the photodynamic effect of ZnTE-2-PyP^{4+} on the promastigotes and amastigotes of *L. braziliensis*—a main etiological agent for CL.

2. Materials and methods

2.1 Parasites

Promastigote forms of *L. braziliensis* (MHOM/BR/1975/M2903) were maintained in Schneider's medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin/streptomycin (v/v) (Sigma-Aldrich) at 26 °C, and used during the phase of exponential growth. Amastigotes were obtained from *L. braziliensis*-infected BALB/c peritoneal macrophages. Macrophages (1×10^5 cells per mL) were seeded in a 24-well plate and cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS for 24 h at 37 °C under 5% CO_2. Peritoneal macrophages were infected (14 h) with promastigote forms at a ratio of 10 : 1 (parasites : macrophages).

2.2 Photodynamic effect

ZnP was synthesized by alkylation of the precursor Zn(II)*meso*-tetrakis(2-pyridyl)porphyrin as previously reported by Viana *et al.*[14] Aliquots of 100 μL of promastigote cell suspension (1×10^7 cells per mL) were transferred to a 96-well plate. To each well, 100 μL was added of either PBS (control) or PBS containing ZnP (PS) to reach the final concentration of 5 or 10 μM. Experimental groups were divided in: (1) control – no treatment; (2) light – treatment with irradiation only, (3) 5 μM ZnP – treatment with 5 μM ZnP in the dark; (4) ZnP 5 μM + light – treatment with 5 μM ZnP followed by irradiation; (5) 10 μM ZnP – treatment with 10 μM ZnP in the dark; and (6) ZnP 10 μM + light – treatment with 10 μM ZnP followed by irradiation. For all ZnP-containing samples, cells were incubated with the PS for 10 min. As a light source, a 455 ± 20 nm LED (LEC photopolymerizer, MMOptics, Brazil) was used. Light and photodynamically treated groups received an irradiance of *ca.* 300 mW cm^{-2} during 5 or 10 min (corresponding to light doses of 90 or 180 J cm^{-2}, respectively). These systems were irradiated from top (at the sample level) to bottom covering the entire well area in unlidded plates. Immediately after irradiation, cell counts were performed using a Neubauer chamber, considering only the cells that were morphologically undamaged and motile.[18] The percentage of visibly damaged cells was calculated in comparison with the number of control cells, which was considered as 100%. The experiments were performed in triplicate in two different assays.

To determine whether the parasites were able to recover and multiply after photodynamic treatment, cells were reincubated in Schneider's medium supplemented with 10% FBS and 1% antibiotic (v/v) (penicillin/streptomycin) at 26 °C. Then, parasite growth was evaluated 24 h after treatment by cell counts as described above.

For amastigotes, experiments were performed in 24-well microplates, and 500 μL of each treatment system was added to each well containing the *L. braziliensis*-infected macrophages. The treatment groups were identical to those described for the promastigotes experimental design. The microplate wells used for the amastigote assay had a larger area than the ones used for the promastigotes; therefore, in order to irradiate the entire area of each well, the LED guide tip was removed, and the light power was increased to maintain irradiance and a light dose similar to that used to treat the promastigote forms (*ca.* 300 mW cm^{-2}, 90 J cm^{-2}). Light, ZnP 5 μM + light, and ZnP 10 μM + light samples were individually irradiated for 5 min, following the same protocol described for the promastigotes. After, the cells were fixed in Bouin's fixative and stained with Giemsa stain solution (Sigma-Aldrich) for 15 min. The number of amastigotes was determined by analyzing a hundred infected host cells distributed in randomly chosen microscopic fields. The experiments were performed in triplicate in two independent assays.

2.3 Flow cytometry

After treatment, parasites were washed with PBS and incubated with 30 μg mL^{-1} propidium iodide (PI; Sigma-Aldrich) for 15 min, and with 10 μg mL^{-1} rhodamine 123 (Rh 123; Sigma-Aldrich) for 15 min. Cells were washed with PBS, resuspended, and analyzed using an Accuri C6 flow cytometer (Becton-Dickinson, USA). A total of 20 000 events were acquired for each sample using detectors 533/30 nm for Rh 123, and 582/42 nm for PI. Median fluorescence intensities for Rh 123 were evalu-

ated by flow cytometry for control and treated samples, and the variation index (VI) was quantified by the equation: VI = (TM − CM)/CM, where TM is the median of fluorescence for treated parasites and CM is that of the negative control (non-treated).[19] VI indicates the relative increase/decrease of median fluorescence of the treated samples relative to the control. The mitochondrial membrane depolarization induces a decrease of Rh 123 median of fluorescence (TM) compared to control (CM) and a negative VI value is observed, whereas hyperpolarization induces the opposite, with an increase in TM relative to CM, and a positive VI value is observed.

2.4 Ultrastructural assays

To evaluate possible ultrastructural damage in the treated-promastigotes, electron microscopy analyses were performed. After treatment, cells were processed as previously described by Aliança *et al.*[18] For transmission electron microscopy (TEM), samples were observed using a Zeiss EM109 transmission electron microscope (Zeiss, Germany), and for scanning electron microscopy (SEM), cells were observed using a JEOL Scanning Electron Microscope (JEOL USA, Inc., USA).

2.5 Cytotoxicity against mammalian cells

The cytotoxic effect of the treatments on J774.A1 macrophages (ATCC® TIB-67™), Vero cells (ATCC® CCL-81™), and peritoneal macrophages obtained from BALB/C mice was evaluated by MTT colorimetric assay. Briefly, 100 μL of the cell suspension (1×10^6 cells per mL) was added per well in 96-well plates and cultivated in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic (v/v) (penicillin/streptomycin), at 37 °C and 5% CO_2 for 24 h. After this, ZnP was added to reach final concentrations of 5 μM or 10 μM per well and the cells were subjected to irradiation as described previously (section 2.2). After treatment, cells were immediately washed and incubated in RPMI-1640 containing 5 mg mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 3 h at 37 °C under 5% CO_2. Next, the supernatant was discarded and 100 μL of dimethylsulfoxide was added per well to solubilize the formazan crystals formed. The optical density (OD) was analyzed at 490 nm in a multiplate reader (Bio-Rad®, USA). Two independent experiments were performed in quadruplicate.

2.6 Statistical analyses

Statistical analyses were carried out with the Student's *t*-test for independent samples using GraphPad Prism 5 (Graphpad, USA). Differences were considered significant at $p < 0.05$.

2.7 Ethical standards

This study was performed in compliance with relevant national laws and institutional guidelines for laboratory animals. The protocol was approved by the Ethical Committee for Animal Research of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA-FIOCRUZ 39/2012).

3. Results and discussion

3.1 Photodynamic effect of ZnP on promastigotes

The ZnP concentrations used for photodynamic treatment were lower than the IC_{50} (concentration that inhibited cell growth by 50%) of ZnP in the dark (see ESI – Fig. 1†). Therefore, cytotoxic effects in the absence of light were expected to be negligible (Fig. 1). About 25% of damaged cells were observed in samples subjected only to light treatment compared to the control. This finding may have been due to the interaction of light with endogenous biomolecules.[20] The increased irradiation time (10 min) had no significant effect on the amount of cells visibly photodynamically damaged compared to cells irradiated for 5 min (data not shown). Thus, all subsequent assays were carried out by applying 5 min of light irradiation. Treatment with 5 μM ZnP + light (5 min) increased the number of visibly damaged cells to 65% compared to the control cells. Compared to the control (visibly undamaged cells), the highest percentage of damaged cells was observed when the parasite suspension was treated with 10 μM ZnP + light, which resulted in only about 10% of the cells remaining visibly undamaged. Therefore, the treatment with 10 μM ZnP followed by irradiation caused a drastic effect on the parasites, probably by decreasing the cell viability of promastigote forms (Fig. 1). In PDT, it is important that the emission of the light source overlaps with the PS absorbance. Herein, the ZnP absorption[14] did not completely overlap with the emission of the LED device. If an optimal light source had been used, shorter exposure times could have been applied and similar results could have been obtained. Moreover, cells in this study were unable to replicate, when cell growth was evaluated 24 h after photodynamic inactivation (see ESI – Fig. 2†).

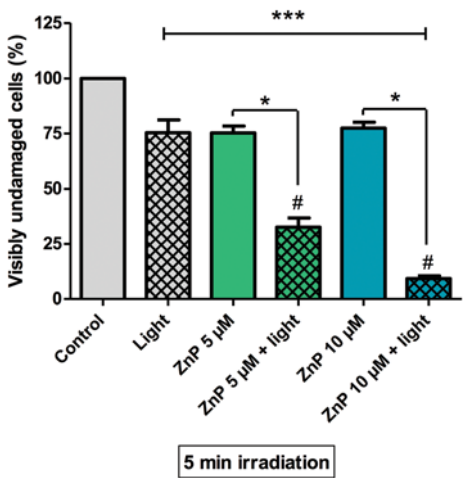

Fig. 1 Survival of *L. braziliensis* promastigotes determined by cell counts of morphologically sound and motile cells immediately after exposure to ZnP-associated photodynamic treatment (90 J cm^{-2}). Notes: Groups statistically significant ($p < 0.05$) when compared to the control (***), to the treatment with light (#), and to ZnP in the dark at the corresponding concentration (*).

Several compounds have already been used as PS for CL treatment, including methylene blue, 5-aminolevulinic acid (ALA), aluminium and zinc phthalocyanines, and chloroaluminum phthalocyanine.[21–23] However, the investigation of new substances for the treatment of CL remains important as the "ideal protocol" has not been reported.

Song *et al.* subjected the promastigote forms of *L. amazonensis* to PDT using methylene blue as the PS.[23] When incubated with 50 μM methylene blue for 1 h, cells had a survival rate of about 60%. To achieve *ca.* 10% of survival percentage, the cells required irradiation for 30 min (9 J cm^{-2}). Pinto *et al.* performed a PDT study using aluminum tetrasulfonated phthalocyanine and observed that this agent was able to reduce the viability of the promastigote forms of *L. braziliensis* by 99% (10 J cm^{-2}).[24] However, for this, an incubation time of 1 h with the PS was required, and the cytotoxic effect of the treatment was not evaluated on mammalian cells. Nevertheless, in this work, the described protocol induced cell damage in up to 90% (Fig. 1) applying a low PS concentration, shorter PS incubation time, and shorter irradiation time than in the studies mentioned above.[23,24] This is an important issue considering that decreases in irradiation time and PS incubation time may reduce the phototoxicity of PDT on non-target tissues.[25]

3.2 Photodynamic effect on parasite plasma membrane permeability

Promastigotes that received ZnP-based photodynamic treatment yielded considerable cell damage immediately after treatment and demonstrated drastic inhibition of parasite growth at 24 h post-treatment. Therefore, further investigations were performed in order to identify whether the observed effects were due to the loss of cell viability related to the lack of membrane integrity or to a cytostatic action, for example. A defect in the plasma membrane of cells can be determined using the PI fluorescence.[26]

As expected, most of the negative control cells remained unlabeled with PI (Fig. 2A). On the other hand, cells treated with H_2O_2 (positive control) were totally positive (Fig. 2B). When cells were treated with light only, or ZnP (5 or 10 μM) in the dark, the percentages of PI-positive promastigotes (Fig. 2C–E) were similar to the negative control. Groups treated with ZnP + light presented more than half the cells being labeled with PI (Fig. 2F and G), indicating a considerable increase in plasma membrane permeability, and confirming that ZnP-mediated photodynamic treatment led to a loss of cell viability. Regardless, the region of intermediary fluorescence observed in Fig. 2F may have been related to a

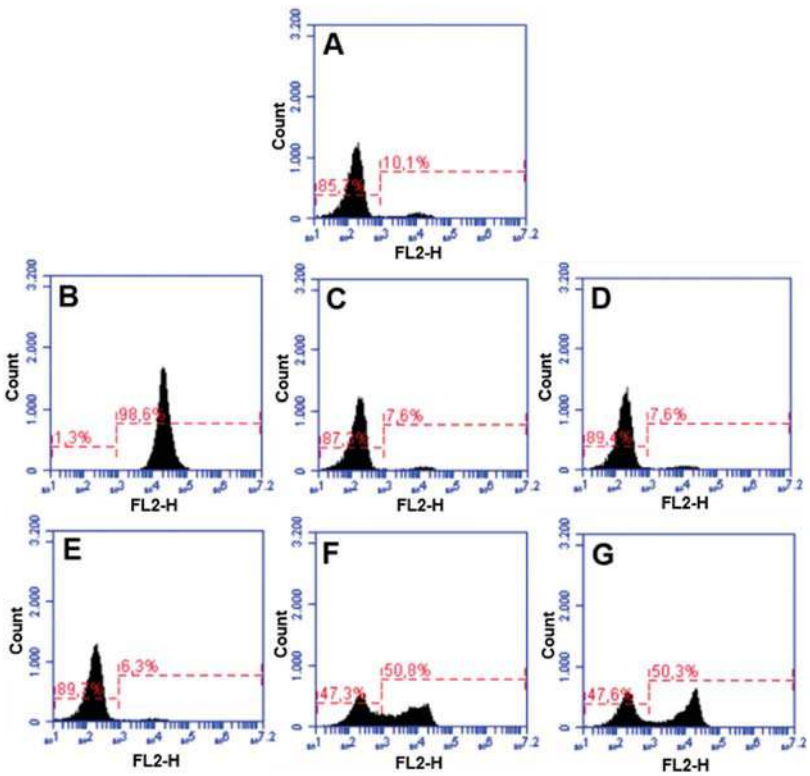

Fig. 2 Fluorescence histograms of *L. braziliensis* promastigotes labeled with PI immediately after ZnP-associated photodynamic treatment (90 J cm^{-2}). Percentage levels indicated in the left and right sides of the histograms refer to unlabeled and labeled cells, respectively. Notes: A – Untreated cells: negative control; B – cells treated with H_2O_2 (positive control); C – treatment with light; D – treatment with 5 μM ZnP in the dark; E – treatment with 10 μM ZnP in the dark; F – treatment with 5 μM ZnP + light; G – treatment with 10 μM ZnP + light.

gradual enhancement in plasma membrane permeability associated with the cell susceptibility to the PS concentrations applied during the photodynamic treatment. PI positive cells are considered to be necrotic cells or a result of late apoptosis. However, it is possible that other mechanisms, with no commitment of membrane integrity, may also have occurred. For example, early apoptosis or autophagy can evolve into late apoptosis along with a gradual loss of membrane integrity, and ultimately lead to necrosis.

3.3 Photodynamic effect on parasite mitochondrial membrane potential

To evaluate the possible changes in the mitochondrial membrane potential ($\Delta\Psi_M$) of the promastigote forms subjected to photodynamic treatment, Rh 123 was used. It is well established that the movement of protons through the internal mitochondrial membrane can induce, directly or indirectly, alterations in the $\Delta\Psi_M$ during oxidative phosphorylation. Under physiological conditions, there is an active extrusion of protons through respiration, and a passive entry of protons through the ATP-synthase pathway during ATP synthesis. Thus, membrane depolarization induces a loss of Rh 123 fluorescence, whereas hyperpolarization induces an increase in fluorescence.[27] Values for the median fluorescence of Rh 123 are shown in the ESI (Fig. 3†).

Flow cytometry analysis showed that the $\Delta\Psi_M$ of cells treated with 5 μM or 10 μM ZnP in the absence of light remained essentially unchanged, with VI values of 0.00 and +0.04, respectively, which were similar to those found in the negative control. A discrete hyperpolarization on the mitochondrial membrane (VI = +0.13) was noticed in the samples treated only with light. However, a noteworthy enhancement of VI was observed in the samples treated with ZnP + light, especially in the ZnP 10 μM + light (VI = +0.45). Conversely, an intense depolarization for the cells treated with H_2O_2 (VI = −0.88) was evident, as indicated by the negative VI value. This latter event may be related to increased mitochondrial permeability, and subsequent death through apoptosis of the eukaryotic cells.[28]

Previous studies have attributed the collapse of $\Delta\Psi_M$ after PDT to the opening of the mitochondrial permeability transition pores.[29] In some cases, mitochondrial depolarization is preceded by a transient hyperpolarization that has often been considered as a final attempt by the cells to prevent death.[30] Thus, both hyperpolarization and the loss of $\Delta\Psi_M$ may result in the apoptotic death of promastigote forms, demonstrating the importance of maintaining $\Delta\Psi_M$ suitable for parasite survival.[31,32]

3.4 Ultrastructural analysis

TEM analysis showed that the control cells presented a typical morphology. In Fig. 3A, it is observed that the parasite has its characteristic elongated shape with an evident nucleus. In these cells, a well-preserved mitochondrion, kinetoplast, and endoplasmic reticulum (ER) could be observed. No substantial changes in this profile were observed in the parasites treated

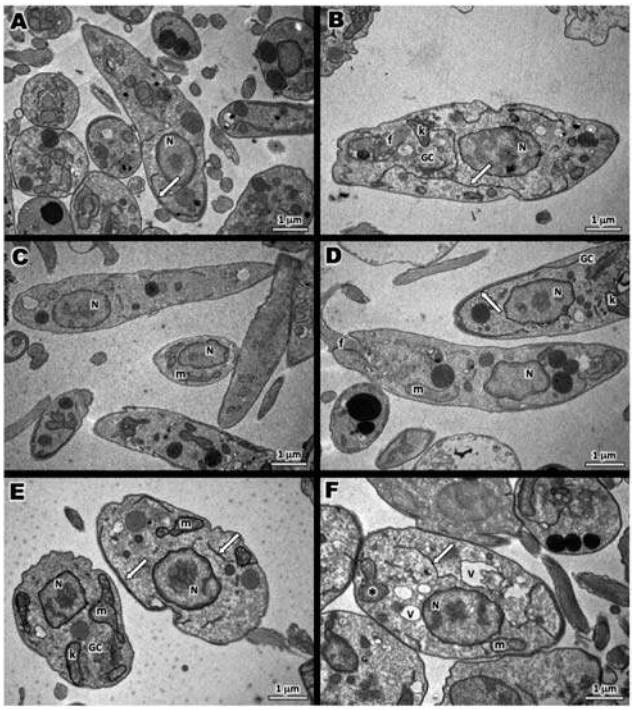

Fig. 3 Representative transmission electron micrographs of *L. braziliensis* promastigote cells processed for TEM immediately after photodynamic treatment (90 J cm^{-2}). Notes: A – Control (without treatment); B – treatment with light; C – treatment with 5 μM ZnP in the dark; D – treatment with 10 μM ZnP in the dark; E – treatment with 5 μM ZnP + light; F – treatment with 10 μM ZnP + light. (N): Nucleus; (m): mitochondrion; (K): kinetoplast; (f): flagellum; (GC): golgi complex; (V): vacuolization; (*): Ruptured mitochondrion; arrows: endoplasmic reticulum.

with 5 μM or 10 μM ZnP in the dark (Fig. 3C and D). Slight changes in the electron density of the ER, mitochondrion and nuclear membrane were observed in the cells treated with light without ZnP (Fig. 3B). In contrast, morphological changes were observed in the promastigotes treated with ZnP + light. Both concentrations of ZnP tested in photodynamic inactivation induced a wrinkled plasma membrane, rounding, and shortening of the cell body (Fig. 3E and F). Under these conditions, an enhancement of ER, mitochondrial, and nuclear membrane electron density became more pronounced than in the cells treated with only light or ZnP. Compared to control cells, in photodynamically treated-samples, the swelling of the mitochondrion and in some cells the rupture of the organelle outer membrane are also observed (Fig. 3F). Cytoplasmic vacuolization, mainly in cells treated with the higher ZnP concentration, was also observed after photodynamic treatment (Fig. 3F). The process of vacuolization was previously described after the use of drugs with leishmanicidal action, and is one of the first signs of increased autophagic activity in *Leishmania*.[33] Autophagy is considered a normal recycling process, and usually is used as a defensive and repair pathway in response to toxic compounds. However, ROS released by PDT, and mitochondrial dysfunction may overwhelm the capacity of cells to

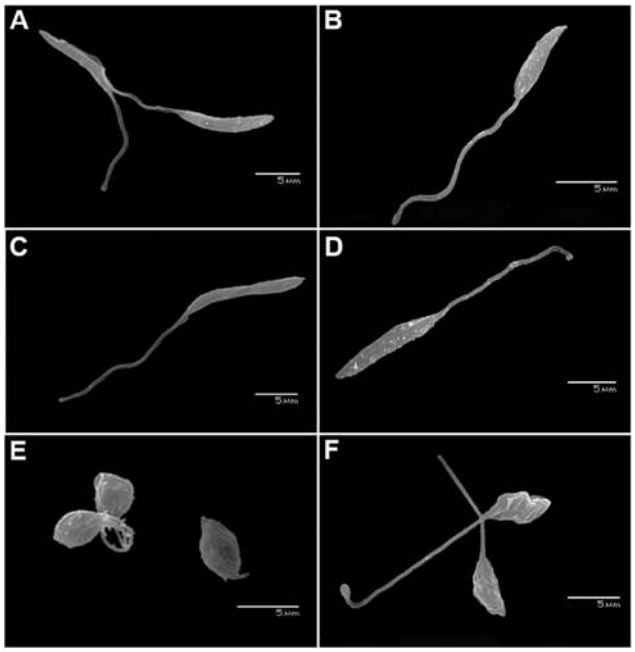

Fig. 4 Representative images of the photodynamic effect on the ultrastructure of the promastigote forms of *L. braziliensis* observed by scanning electron microscopy immediately after photodynamic treatment (90 J cm^{-2}) using ZnP as the photosensitizer. Notes: A – Control (without treatment); B – treatment with light; C – treatment with 5 μM ZnP in the dark; D – treatment with 10 μM ZnP in the dark; E – treatment with 5 μM ZnP + light; F – treatment with 10 μM ZnP + light.

recycle damaged structures by autophagy, leading to cell death in the parasite through apoptosis/necrosis.[34]

The findings from SEM analysis corroborated the TEM data. Control promastigotes showed spindle-shaped bodies and smooth cell membranes (Fig. 4A). Treatment with light only (Fig. 4B), 5 μM ZnP in the dark (Fig. 4C), or 10 μM ZnP in the dark (Fig. 4D) did not cause any apparent changes in the parasites' cell morphology. On the other hand, parasites demonstrated shortening of the cell body in photodynamically treated samples, which also induced a roughened and corrugated cell membrane (Fig. 4E and F). Fig. 4E and F show changes in the membrane morphology and the cell volume, compatible with alterations in the permeability of the plasma membrane noticed before by PI labeling.

The subcellular localization of PS is one of the most important factors to be considered to evaluate the PDT efficiency.[35] As described in the literature, ZnTE-2-PyP^{4+} is a PS that tends to accumulate mainly in the lysosomes and/or has diffuse distribution in the cytoplasm.[12,16] The activation of ZnP in PDT could lead to a local and concentrated production of ROS within the lysosomes and the adjacent cytoplasmic areas, resulting in damage to organelles and other structures, as observed by TEM and SEM.

3.5 Cytotoxicity against mammalian cells

When samples were treated with light only, or 5 μM or 10 μM ZnP solutions in the dark, at least 80% cells remained viable for all mammalian cell lines evaluated (Fig. 5). Vero cells and J774.A1 macrophages presented about 70% viability after photodynamic treatment. Peritoneal macrophages were however more susceptible to photodynamic treatment, with viability reduced to *ca.* 50%. The cause of this effect remains uncertain, but it is known that primary cultures are usually more sensitive to treatments and cellular stress.[36]

Dutta *et al.* observed slight lysis of J774.A1 macrophages immediately after PDT with aluminum phthalocyanine chloride.[37] In contrast, some of the four cationic porphyrins tested by Bristow *et al.*, although affecting the viability of *L. major* promastigotes, were shown to be toxic to macrophages (U937) and/or keratinocytes.[15] The low cytotoxicity of ZnP to Vero cells and J774.A1 macrophages also suggests the use of ZnP as a potential candidate for application as a PS in PDT. The action of PDT can be restricted to the site of PS administration and

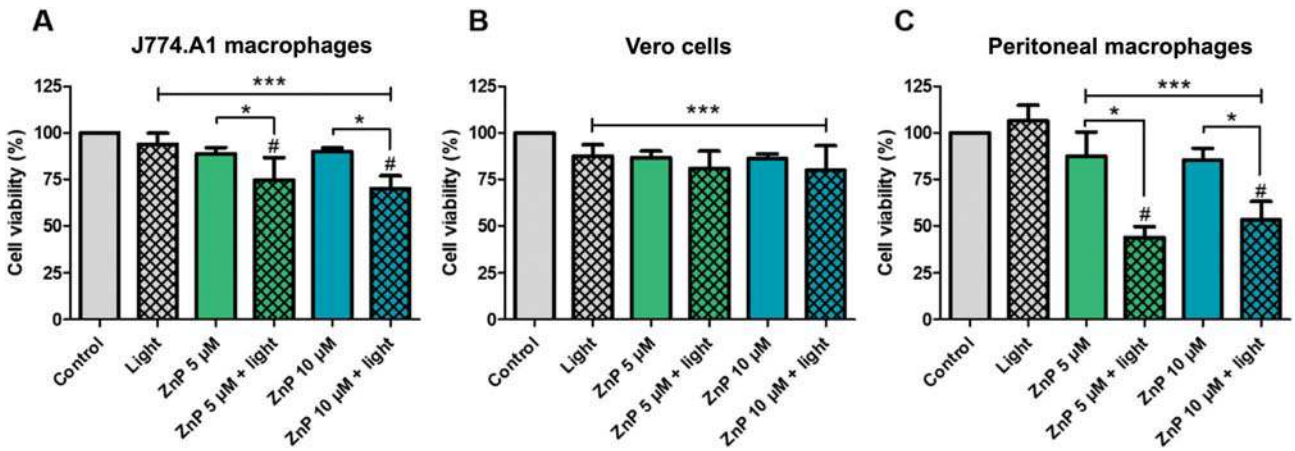

Fig. 5 Cell viability of mammalian cells after ZnP-associated photodynamic treatment (90 J cm^{-2}): J774.A1 macrophages (A), Vero cells (B) and peritoneal macrophages from BALB/c mice (C). Photodynamic effect was evaluated immediately after treatment. Notes: Groups statistically significant ($p < 0.05$) when compared to the control (***), to the treatment with light (#), and to ZnP in the dark at the corresponding concentration (*).

light irradiation, thus controlling the photodynamic effect. Therefore, in the case of cutaneous lesions caused by leishmaniasis in which PDT is locally applied, it may be possible to limit damage to adjacent healthy tissue.

3.6 Photodynamic effect on amastigotes

Samples treated with light only decreased an average of 6 amastigotes per macrophage (Fig. 6). However, the greatest reduction in the number of parasites within the macrophages was observed when the infected cells were treated with ZnP followed by light exposure, which resulted in a decrease of infection by 40%. As there were no significant differences among the untreated control and samples incubated with ZnP in the dark, this result suggests that the photodynamic effect was responsible for the observed decrease in the number of amastigotes. However, additional studies are required to further improve the described protocol and better understand the photodynamic inactivation of the amastigote form of the parasite. Fig. 7 shows an untreated infected macrophage (A) and an infected macrophage that received a ZnP-associated photodynamic treatment (B). It is also worth mentioning that the incubation time with ZnP and the irradiation time used in our protocol were considerably less than most of those reported in the literature.[5,37,38] In addition, macrophage infection was performed using a ratio of 10 : 1 (promastigotes : macrophages), resulting in an average number of amastigotes per macrophage of *ca.* 20, which is higher than the average of 5–7 amastigotes per macrophage described in the literature.[38]

Although studies such as those by Enk *et al.*,[39] Gardner *et al.*[40] and, Peloi *et al.*[22] have reported encouraging results from clinical trials regarding the eradication of *Leishmania*, and good healing after PDT, it is agreed that an optimal treatment protocol for cutaneous leishmaniasis using PDT has not yet been established.[41]

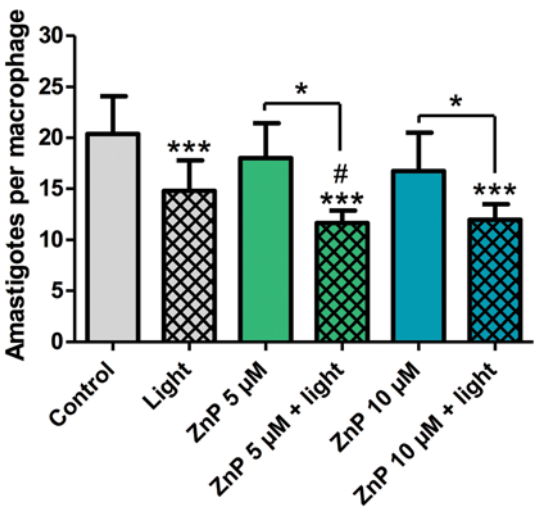

Fig. 6 Number of *L. braziliensis* amastigotes per macrophage immediately after ZnP-associated photodynamic treatment (90 J cm^{-2}). Notes: Groups statistically significant ($p < 0.05$) when compared to the control (***), to the treatment with light (#) and to ZnP in the dark at the corresponding concentration (*).

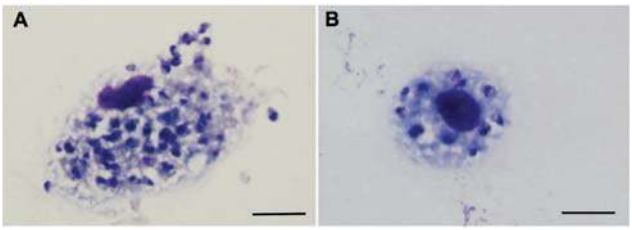

Fig. 7 Light microscopy images of Giemsa-stained infected macrophages: A – control, and B – treated with ZnP 10 μM + light. Scale bar: 10 μm.

4. Conclusions

Since leishmaniasis represents a serious public health problem, efforts to develop alternative and effective therapies should be valued and encouraged. There is still much to be learned in the context of a better understanding of parasite eradication using ZnP-mediated PDT. It is worth noting, however, that the results described in this study allow ZnTE-2-PyP^{4+} to be recognized as a photoactive experimental therapeutic and promising drug for leishmaniasis therapy.

Conflicts of interest

There are no conflicts to declare.

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