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RESEARCH

Molecular effects of photodynamic therapy with curcumin on *Leishmania major* promastigotes

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Abstract

Leishmaniasis is a neglected disease mainly affecting low-income populations. Conventional treatment involves several side effects, is expensive, and, in addition, protozoa can develop resistance. Photodynamic therapy (PDT) is a promising alternative in treating the disease. PDT involves applying light at a specific wavelength to activate a photosensitive compound (photosensitizer, PS), to produce reactive oxygen species (ROS). Curcumin and its photochemical characteristics make it a good candidate for photodynamic therapy. Studies evaluating gene expression can help to understand the molecular events involved in the cell death caused by PDT. In the present study, RNA was extracted from promastigotes from the control and treated groups after applying PDT. RT-qPCR was performed to verify the expression of the putative ATPase beta subunit (ATPS), ATP synthase subunit A (F0F1), argininosuccinate synthase 1 (ASS), ATP-binding cassette subfamily G member 2 (ABCG2), glycoprotein 63 (GP63), superoxide dismutase (FeSODA), and glucose-6-phosphate dehydrogenase (G6PDH) genes (QR). The results suggest that PDT altered the expression of genes that participate in oxidative stress and cell death pathways, such as ATPS, FeSODA, and G6PD. The ATP-F0F1, ASS, and GP63 genes did not have their expression altered. However, it is essential to highlight that other genes may be involved in the molecular mechanisms of oxidative stress and, consequently, in the death of parasites.

Keywords *Leishmania* · Photodynamic therapy · PDT · Curcumin · Gene expression

Introduction

Cutaneous leishmaniasis (CL) is a neglected disease that affects vulnerable populations, causing an economic and social impact on the individual. CL is caused by protozoa of the genus *Leishmania* and is considered an infectious, non-contagious disease that causes ulcerative lesions on the skin and mucous membranes. Conventional treatment is carried out with specific medications, which are toxic to the body and can cause parasite resistance (Azim et al. 2021).

Photodynamic therapy (PDT) appears as a therapeutic alternative for CL, enabling local treatment, reducing adverse effects, and having a low chance of providing the development of parasitic resistance. PDT is a therapy that consists of the association of a photosensitive compound (PS), a light source, and molecular oxygen, triggering the formation of reactive oxygen species (ROS) and inducing cell death (Kharkwal et al. 2011). Curcumin is a yellow-orange pigment extracted from the rhizome of *Curcuma longa* and has antimicrobial, antioxidant, and anti-inflammatory properties (Wikene et al. 2015). It has an absorption spectrum in the visible region, with an absorption peak between 400 and 475 nm (Szlasa et al. 2020). Studies have shown that curcumin can intercalate with DNA, accumulating in the nucleus and kinetoplast of *Leishmania*, causing irreversible cellular damage (Nafisi et al. 2009; Pinto et al. 2016). This study aimed to evaluate changes in gene expression of *Leishmania major* promastigotes subjected to photodynamic therapy with curcumin.

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In vitro experiments

Cultivation of parasites

The *Leishmania major* strain (LV39) was maintained in LIT (liver infusion tryptose) medium supplemented with 10% fetal bovine serum (FBS), 2.5 μg mL^{-1} of hemin, 2% of sterile urine, and 1% of penicillin/streptomycin solution, in a incubator at 26 °C.

Photosensitizer

Curcumin (PDT-PHARMA®, Cravinhos-SP) was initially diluted in dimethyl sulfoxide — 0.1% DMSO in a stock solution of 10 mg ml^{-1} and then diluted in PBS to carry out the study (Pinto et al. 2016). Dilution was carried out in PBS to a concentration of 1.35 mmol L^{-1} for the tests.

Photodynamic therapy

The promastigotes were incubated with curcumin diluted in PBS for 1 h away from light. Then, the samples were centrifuged and resuspended in PBS. For irradiation, a Biotable (Biopdi/Irrad-Led) was used at a wavelength of 450 nm, at an energy density of 8 J cm^{-2}, and an irradiance of 40 mW cm^{-2}. The groups were divided into the Control group, no treatment; the Dark group, with PS only; the LED group, light only; and the PDT group, incubated with PS and irradiated.

RNA extraction and cDNA synthesis

RNA extraction from promastigotes was performed immediately after applying the protocol established in the item photodynamic therapy using the ReliaPrep™ RNA Cell Miniprep System kit (Promega). Seven extractions were performed, using 1×10^8 cells/mL, to obtain sufficient RNA concentrations for gene expression analysis by real-time reverse transcriptase quantitative PCR (RT-qPCR) of each group. The RNA concentration was quantified and evaluated using ultraviolet absorption spectroscopy on the NanoDrop equipment (ND-1000 Spectrophotometer v.3.0.1, Labtrade). At the same time, the quality of the RNA was evaluated by agarose gel electrophoresis (1.5 %). DNA digestion was performed using the SV Total RNA Isolation System (Promega) protocol. We used the pre-amplification system for cDNA synthesis according to the ImProm-II™ Reverse Transcription System (Promega). The reactions were carried out in the thermocycler (Veriti 96 Well Thermal Cycler, Applied Biosystems), and samples were stored in the freezer at − 20 °C.

Genes analyzed

The target genes selected for this study have functions related to infectivity, glycoprotein 63 (GP63), and ATP-binding cassette subfamily G member 2 (ABCG2) (Olivier et al. 2012; Manzano et al. 2017); ATP syntheses, such as putative ATPase beta subunit (ATPS) and ATP synthase subunit A (ATPS-F0F1) (Goswami et al. 2006); and oxidative stress and resistance patterns, such as ABCG2, superoxide dismutase (FeSODA), argininosuccinate synthase 1 (ASS), and glucose-6-phosphate dehydrogenase (G6PD) (Sardar et al. 2016; Manzano et al. 2017; Mittra et al. 2017). In this study, the expression of genes in the promastigote form of *Leishmania* was evaluated, as they have better-established cultivation in culture medium, facilitating the identification and understanding of cellular and molecular changes related to the parasite's response to applied therapy and obtaining a higher concentration of RNA when compared to amastigote forms.

The primers for amplifying target genes and the endogenous rRNA45 gene (which presents constitutive and uniform expression) were designed using the Primer Express software (version 3.0) (PE Applied Biosystems, Foster City, CA, USA). Based on the literature, the endogenous ribosomal RNA (rRNA45) gene was chosen (Ouakad et al. 2007).

The RT-qPCR reactions for the studied genes were performed on an ABI Prism 7500 Sequence Detection System (Life Technologies, Foster City, CA, USA) using GoTaq® qPCR Master Mix (Promega). The results were analyzed by the Delta-Delta *Ct* ($\Delta\Delta Ct$) method (Pfaffl 2001). For each sample, the ΔCt value = mean *Ct* (threshold cycles) value of the target gene − mean *Ct* value of the endogenous gene. To determine $\Delta\Delta Ct$, the ΔCt value of each sample was subtracted from the mean ΔCt value of the control samples. This value was added to the $2 - \Delta\Delta Ct$ formula, and the fold change or relative quantification (QR) values were obtained. The QR consists of the normalized gene expression value ($2^{(-\Delta Ct)}$) in the experimental group samples divided by the normalized gene expression ($2^{(-\Delta Ct)}$) in the control group sample calculated for each gene. The formula gives this calculation: $2^{(-\Delta\Delta Ct)}$. QR values greater than two indicate increased expression for the target gene, compared to the control group and QR values less than 0.5 indicate decreased gene expression.

Results and discussion

PDT associated with curcumin accumulates in the nucleus and kinetoplast (single mitochondria that contain kDNA) of promastigotes and amastigotes of *Leishmania* sp., triggering several metabolic changes, and may cause the death of the protozoan (Ceccarelli et al. 2014; Pinto et al. 2016; Pereira

Table 1 QR values of the target genes for all samples from the four groups analyzed

Groups	Target genes						
	ATPS	*F0F1*	*ASS*	*ABCG2*	*GP63*	*FeSODA*	*G6PD*
Control	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Dark	3.87***	1.24	1.11	0.86	1.00	0.24*	1.38
LED	0.32*	0.93	0.86	0.69	0.69	0.43*	0.03*
PDT	0.15*	1.22	1.46	1.01	1.48	0.59**	0.04*

*Expression decrease (QR < 0.5); **tendency to diminished expression (QR ~ 0.50); ***expression increase (QR > 0.5)

et al. 2021). Based on the results of Marcolino et al. (2021), who observed that PDT with curcumin in *L. major* triggered changes in mitochondrial activity, increased ROS production, DNA fragmentation, reduced mitochondrial membrane potential, changes in cell morphology, and decreased viability, the following genes were selected: ATPS, F0F1, ASS, ABCG2, GP63, FeSODA, and G6PD genes, as all of them, except the GP63 gene, are involved with mitochondrial functions.

The gene expression profile helps in the molecular characterization of the cellular response, making it possible to verify whether its functions were affected after PDT. The results obtained in this study suggest that PDT interferes with the expression of genes involved in ATP synthesis, oxidative stress, glycolytic pathway, and defense against host cells due to their altered expression. When comparing the Control, Dark, LED, and PDT groups, a reduction in the expression of the ATPS (QR = 0.32 and 0.15) and G6PD (QR = 0.03 and 0.04) genes was observed in samples from the LED and PDT concerning Dark and Control groups. A reduction in the expression of the FeSODA gene was observed in the Dark group (QR = 0.24) and LED group (QR = 0.43) (Table 1).

The functions of these genes are related to the kinetoplast, identified as a promising target for the inactivation of protozoa as it is an organelle responsible for all respiratory activity of the parasite (Mondal et al. 2014; Giorgio et al. 2017). Hellemond and Tielens (1997) demonstrated that although parasites can survive in low oxygen conditions, *Leishmania* promastigotes depend on the respiratory chain for energy generation and survival. In the inner membrane of the kinetoplast, there is the electron transport chain coupled to the ATPase, where the synthesis of ATP occurs, which provides energy (ATP) to the protozoan and is responsible for controlling the cell death process, which can be triggered by a normal physiological process, or by some pathological process, initiated by the increase in the permeability of the cell membrane.

After PDT, low gene expression of the ATPS gene was observed, which encodes the enzyme ATP synthase, necessary to maintain mitochondria and cell survival, promoting ATP synthesis, enzymatic regulation, and mitochondrial membrane potential, a crucial phenomenon for the generation of ATP in the respiratory chain (de Melo Mendes et al.2019). It was also noted a reduction in the expression of the FeSODA gene, which encodes the production of the iron superoxide dismutase enzyme, which protects the parasite against oxidative stress in the host cell and protects against cell death events, such as DNA fragmentation and phosphatidylserine exposure. In the mitochondrial membrane (Veronica et al. 2019), the genes F0F1, related to ATP synthesis (Goswami et al. 2006); ASS and ABCG, both related to resistance to oxidative stress (Sardar et al. 2016; Manzano et al. 2017); and GP63, related to the virulence factor (Olivier et al. 2012) did not show significant differential expression when comparing groups.

This study presents the methodological limitation of using the promastigote form of *Leishmania* and not amastigotes. However, the choice of promastigotes was due to the difficulty in obtaining amastigote cultures through the amastigogenesis process and because these are initial studies that will require future analyses of changes in gene expression in amastigotes.

Conclusion

PDT with curcumin can interfere with the expression of genes that participate in the oxidative stress and cell death pathways since the expression of the ATPS, FeSODA, and G6PD genes was altered after applying LED and PDT.

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J.G.Pinto—PDT experimental support and manuscript writing.
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B.H.Godoi – Manuscript discussion.
R.A.Canevari – qRT-PCR experimental support and analysis of results.
J.Ferreira-Strixino – Supervision, Project writing, Financing.

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Declarations

Competing interests The authors declare no competing interests.

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