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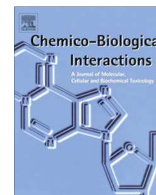

Activity of melatonin against *Leishmania infantum* promastigotes by mitochondrial dependent pathway

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ABSTRACT

Visceral leishmaniasis, a potentially fatal disease, remains a major international health problem. Only a limited number of effective antileishmanial agents are available for chemotherapy, and many of them are expensive with severe side effects or have a markedly reduced effectiveness due to the development of drug resistance. Hence, there is a genuine need to develop a novel effective and less toxic antileishmanial drug.

Melatonin, a neurohormone found in animals, plants, and microbes, can participate in various biological and physiological functions. Several *in vitro* or *in vivo* studies have reported the inhibitory effect of melatonin against many parasites via various mechanisms, including modulation of intracellular concentrations of calcium in the parasite and/or any other suggested mechanism. Importantly, many of available antileishmanial drugs have been reported to exert their effects by disrupting calcium homeostasis in the parasite.

The objective of the present study was to test the efficacy of exogenous melatonin against *Leishmania infantum* promastigotes *in vitro*. Interestingly, melatonin not only demonstrated a significant antileishmanial activity of against promastigote viability in tested cultures but was also accompanied by an alteration of the calcium homeostasis of parasite mitochondrion, represented by earlier mitochondrial permeability transition pore opening, and by changes in some mitochondrial parameters are critical to parasite survival.

These pioneering findings suggest that melatonin may be a candidate for the development of novel effective antileishmanial agents either alone or in associations with other drugs.

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1. Introduction

Leishmaniasis refers to a group of diseases caused by kinetoplastid protozoan parasites of genus *Leishmania*, which is endemic in rural and periurban areas of tropical and subtropical countries [1]. Its impact on public health has been increased by the rapid expansion of endemic zones, in part due to increases in global travel. More than 350 million people in 98 countries around the world are considered to be at risk of this disease, with an annual mortality rate of more than 60,000 [2].

There are three general classifications of human Leishmaniasis: (i) Cutaneous Leishmaniasis (CL), (ii) Mucocutaneous Leishmaniasis (MCL), and (iii) Visceral Leishmaniasis (VL), also known as kala-azar [3–5]. VL caused by *Leishmania donovani* (*L. donovani*) complex in Africa, India, and Asia, by *Leishmania chagasi* in America, and by *Leishmania infantum* in Europe [4,6]. This visceral form is frequently associated with HIV infection, and its effects range from potentially disfiguring cutaneous infection to visceral diseases that are often fatal in the absence of treatment [1,5,7].

Despite innumerable studies on leishmaniasis, many questions have not yet been answered. One of the main challenges is the presence of different causative *Leishmania* species and various clinical manifestations, complicating the therapeutic approach. Over the past decade, new formulations of standard drugs have become

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available and registered for use in many countries. However, around 25 compounds and formulations are available to treat leishmaniasis in humans [8,9], all are associated with toxicity and/or drug resistance problems, and there is an urgent need to develop an effective drug against all forms of leishmaniasis [10,11].

Pentavalent antimonials have been the first line of treatment since the early 20th century, especially for VL [11]. In recent years, however, there has been an alarming increase in reports of primary resistance, irregular effectiveness, serious side effects, and relapse [12,13]. The second line of treatment includes liposomal amphotericin B and miltefosine [11,14]. Liposomal amphotericin B is a highly effective antiparasitic agent but is very costly and associated with serious adverse effects such as renal tubular damage [15,16]. Miltefosine was the first oral drug approved against VL, but it is associated with teratogenicity and severe gastrointestinal side effects, and its efficacy is highly dependent on the *Leishmania* species or strain infecting the patient [2,14].

Leishmania is a member of the trypanosomatidae family which possess a single mitochondrion occupies 12% of the total volume of the parasite and can accumulate large amounts of Ca^{2+}, in common with many eukaryotic cells [17]. Importantly, mitochondrial function is supported by electron transport chain (ETC) which represents the main component, driving operations of the mitochondrial respiratory chain through a complex process termed oxidative phosphorylation [18].

In fact, many available antileishmanial agents exert their effects through the disruption of Ca^{2+} homeostasis in the parasite and/or through changes in different mitochondrial parameters [19,20].

Melatonin, N-acetyl-5-methoxytryptamine, is an indoleamine synthesized and released by the pineal gland during darkness. This hormone is thought to participate in regulation of circadian rhythms in many eukaryotes, including vertebrates, invertebrates, higher plants and dinoflagellates [21].

Several studies have been conducted on the relationship between melatonin and many parasitic or viral diseases [22–24]. Among parasites, melatonin has been reported to be effective against many parasites such as *Toxoplasma gondii* (*T. gondii*) [22], *Plasmodium* [25], *Entamoeba histolytica* [26], and *Trypanosoma cruzi* (*T. cruzi*) [27], controlling the parasite population and their life cycle through one or more of the following mechanisms: modulation of intracellular calcium (Ca^{2+}) concentrations [28]; strong modulation of the immune system; and/or reduction of nitric oxide synthase activity [iNOS] as in the case of mice infected with *T. gondii* or *T. cruzi* [22,29,30].

To our knowledge, there has been no previous study on the relationship between melatonin and *Leishmania*. The objective of the present investigation was to determine the effects of exogenous melatonin on *L. infantum* promastigote *in vitro*, analyzing the changes in parasite viability and some mitochondrial parameters in treated and untreated promastigote cultures.

2. Materials and methods

2.1. General

Melatonin, amphotericin B, and Dimethyl Sulfoxide (DMSO) were obtained from Sigma Chemicals (Madrid, Spain), while Alamar blue was obtained from Invitrogen™ of Life Technologies (Catalog N. DAL1025). Minimum Essential culture Medium (MEM; Sigma–Aldrich Corporation, St Louis, MO, USA), supplemented with 10% fetal calf serum (GIBCO, Invitrogen, NY, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich Corporation) was used in a growth inhibition assay to test the effect of melatonin on parasites. The required materials for following subcellular fractionation techniques such as Oregon green were obtained from Panreac (Madrid, Spain) and Invitrogen (Madrid, Spain). All the other remaining chemicals and reagents were purchased from Sigma–Aldrich (Madrid, Spain). All materials were of analytical grade.

2.2. Leishmania cells and culture conditions

L. infantum MHOM/Fr/LEM75 (PB75) strain was used in a preliminary screening test of the effects of melatonin. *L. infantum* Promastigotes were grown in 25-mL culture flasks using MEM at 26 °C supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin [31,32]. The parasites were maintained by weekly transfer from previous culture into new medium.

2.3. Drug assessment on promastigote growth inhibition assay

The susceptibility of promastigote to melatonin was assessed using the method described by Carrio and co-authors in three independent assays [33]. Briefly, a 5-day-old culture (log phase) was centrifuged at 1500–2000 × *g* for 10 min at 4 °C. The pellet was then resuspended in fresh MEM supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 26 °C, and the promastigote count was adjusted to 1×10^6 cells/mL.

Tested agents were: melatonin at different concentrations (1, 10, 25, and 50 nanomolar [nM]), and 0.1 μM amphotericin B dissolved in 0.1% DMSO. DMSO at this concentration is known to have no inhibitory effects on parasite growth [34,35]. 100 μL of log-phase promastigotes of *L. infantum* (1×10^6 cells/mL) were seeded in 96-well culture plate (Nunc, Denmark) containing 100 μL of MEM medium per well treated with melatonin (1, 10, 25, or 50 nM) or 0.1 μM amphotericin B (reference drug) for 72 h at 26 °C.

Parasite survival and viability were monitored by direct counting of parasites using Neubauer hemocytometer. The results were expressed as the percentage of parasite inhibition (PPI) for each concentration used while the number of parasites counted in wells without drug was set as 100% parasite survival (control negative) [8].

$$PPI = \frac{CFC - CFP}{CFC} \times 100$$

where CFC is the final concentration of control culture (cells/mL), and CFP is the final concentration of treated culture (cells/mL). The leishmanicidal effect was expressed as the concentration inhibiting parasite growth by 50% (IC_{50}) which was calculated from the sigmoidal dose response curve.

Results were verified by using the Alamar blue colorimetric assay, which has proven to be more accurate for estimation of cell growth and viability [36,37]. The assay was carried out on microtiter plates by adding 20 μL Alamar blue 8 h before the end of the incubation period and then measuring absorbance of the treated samples and medium blank with a spectrophotometer at 570 nm and 600 nm. The inhibition assays were repeated three times in triplicate wells.

For mitochondrial and subcellular fractionation, a parallel technique for incubation of promastigote cultures was carried out in 25 mL culture flasks under the same treatment and incubation conditions. The experiment was repeated at least three times in three independent assays.

2.4. Mitochondrial isolation

The mitochondrion of treated and untreated promastigote cultures was isolated according to a previously reported protocol [38,39] with slight modifications. Cells were harvested by centrifugation at 1000 × *g* for 10 min, washed three times in saline-sodium citrate buffer (SSC), and then resuspended in Sodium Chloride–

Tris–EDTA (STE) buffer, homogenized by passage through a 26 gauge needle, and centrifuged at 700 × *g* for 10 min. This centrifugation step was repeated until all cells were removed from the supernatant, which was then centrifuged at 8000 × *g* for 10 min. The pellet obtained was resuspended in STE buffer and incubated with 200 mg/mL of DNase I at 37 °C for 30 min in the presence of 7-mM $MgCl_2$. DNase I was then removed by washing thrice with 40-mM EDTA. The resulting fractions were then resuspended in 0.15-M NaCl, 0.1-M EDTA, and 0.05-M Na_2HPO_4.

2.5. Protein concentration measurement

Protein concentration of the isolated mitochondrion was measured according to Bradford method [40] using bovine serum albumin (BSA) as reference. Protein quantification was performed in mitochondrial suspension volumes of equal weight and volume (0.2 mL) to standardize the procedure.

2.6. Calcium retention capacity

The sensitivity of the mitochondrial Permeability Transition Pore (mPTP) opening to calcium ions and the calcium retention capacity of isolated mitochondria from treated and untreated cultures were assessed in the presence of 0.25 μM Oregon green using a fluorimetric assay. Pulses of 250 μM Ca^{2+} were injected until pore opening. One μM Cyclosporine A (CsA), the standard inhibitor of PTP, was added to evaluate the specificity [41]. mPTP opening was calculated from the area under the curve (AUC) of fluorescence signals over time.

2.7. Determination of mitochondrial nitrites

Determination of mitochondrial nitrites was performed using the Griess diazotization reaction, in which nitric oxide (NO) is spontaneously oxidized under physiological conditions and the resulting nitrate is measured spectrophotometrically [42,43]. Measurements were performed at 548 nm on isolated mitochondria using a Griess Reagent Kit [Molecular Probes, G-7921], according to the manufacturer's instructions.

2.8. Mitochondrial superoxide dismutase activity

Manganese (Mn) and Copper/Zinc (Cu/Zn) superoxide Dismutase (SOD) activities of isolated mitochondria were determined using a SOD Assay Kit-WST [Sigma–Aldrich, 19160, Switzerland], according to the manufacturer's instructions. Results were expressed as a percentage of inhibition of SOD activity.

2.9. Spectrophotometric assays of individual respiratory chain complexes

Subsequent assays of respiratory enzymes activity were performed as described below, expressing the specific activity as nmol min/mg protein.

2.9.1. Complex I

Submitochondrial fractions were incubated for 5 min in a medium containing 250 mM sucrose, 50 mM potassium-phosphate, 1 mM potassium cyanide (KCN), and 0.5 mM decylubiquinone, pH 7.4. The reaction was initiated by the addition of NADH. The specific activity was determined by the decrease in absorbance as a result of the oxidation of NADH at 340 nm [44].

2.9.2. Complex II

Submitochondrial fractions were added to the reaction buffer containing 1 M potassium phosphate, 1 M succinate, 0.1% DCIP, 1 mM EDTA and Triton x-100, pH 7.4. The reaction was initiated by adding 0.5% decylubiquinone (in ethanol) was added. The specific activity represented by the reduction in 2,6-dichlorophenolindophenol was determined at 600 nm [45].

2.9.3. Complex III

Specific activity of isolated submitochondrial fractions was measured by monitoring the reduction in cytochrome *c* at 550 nm, through mixing of a submitochondrial fractions [0.03 mg/mL], with a reaction medium of 0.1 M potassium phosphate, 15 mM decylubiquinone, 0.1 M sodium azide (NaN_3) and 10% BSA [pH 7.5]. The reaction was initiated by adding 1% oxidized cytochrome *c* [46].

2.10. Statistical analysis

Values were expressed as means ± SEM. The data were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 17.0 (IBM Inc., Michigan, IL, USA). Mean values were compared between treated and untreated promastigote cultures by using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The significance level was $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Inhibitory effect of melatonin on promastigotes and determination of IC_{50}

Fig. 1 depicts the inhibitory effects of melatonin against *L. infantum* promastigotes as a function of the concentration used. The percentage of parasite inhibition (PPI) with respect to controls values was 13.9%, 18.9%, 32.9%, and 58.3% at concentrations of 1, 10, 25, and 50 nM melatonin, respectively (IC_{50}/72 h = 42.8 ± 0.45 nM). Amphotericin B achieved a PPI value of 70.9%.

3.2. Alteration of calcium retention capacity and sensitivity of mPTP opening

Fig. 2 shows the effects of treatment with melatonin (at 1, 10, 25, or 50 nM) or with amphotericin on calcium-induced mPTP opening of the isolated mitochondrial fractions in comparison to negative and positive (CsA-treated) controls. Addition of Ca^{2+} or melatonin stimulated the release of accumulated Ca^{2+} in all isolated mitochondrial fractions, and this release was hindered by CsA. The AUC value was significantly higher for treatments with 25 or 50 nM melatonin or amphotericin than for treatments with lower melatonin concentrations or for controls, indicating a greater mPTP opening, given that the mPTP opens earlier with lower Ca^{2+} retention capacity and vice versa.

3.3. Melatonin increased mitochondrial nitrites levels

As shown in Fig. 3A, the level of mitochondrial nitrites was significantly higher in the cultures treated with the highest melatonin concentrations (25 or 50 nM) than in the untreated cultures, reaching level nearly similar to that in the amphotericin-treated culture.

3.4. Melatonin non-significantly reduced SOD activity

Fig. 3B depicts the mitochondrial SOD activity in treated and untreated cultures. Although SOD activity was lower in cultures treated with melatonin (especially 50 nM) than in controls, no significant difference was found with any melatonin concentration, whereas a significant reduction was observed with amphotericin.

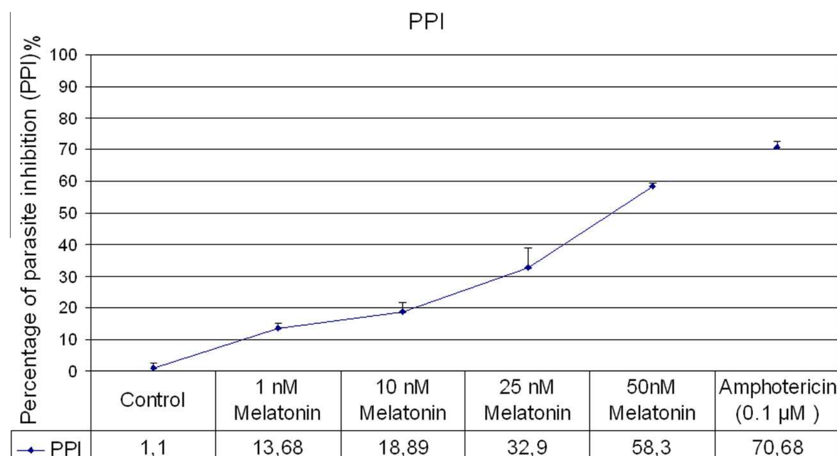

Fig. 1. Percentage of parasite growth inhibition against tested agents after 72 h of co-incubation. Values are means ± S.E.M.

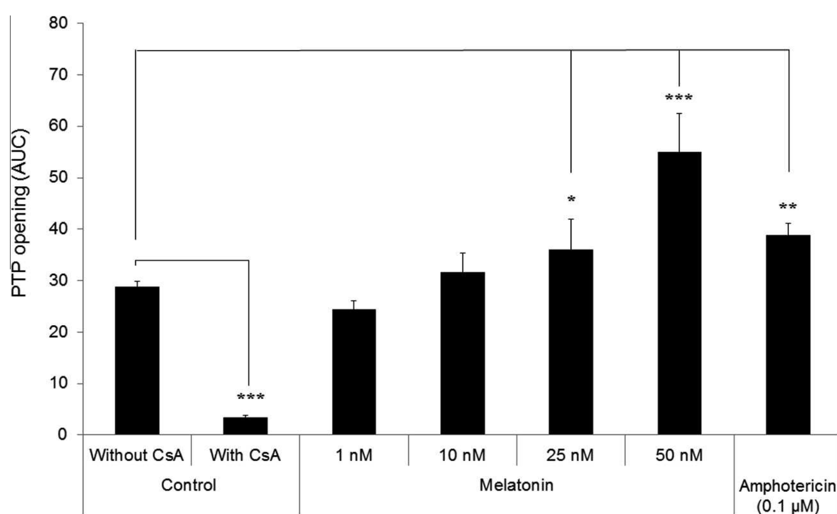

Fig. 2. Calcium-induced permeability transition pore (PTP) opening of control (untreated) and amphotericin- and melatonin-treated promastigote cultures (1–50 nM). Pulses of 250 µM Ca^{2+} were injected in the presence of 0.25 µM Oregon green until pore opening. 1 µM Cyclosporine A (CsA) was added *in vitro* to the isolated mitochondria. PTP opening was calculated from the area under the curve (AUC) of fluorescence signals versus time. Values are means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post hoc test. ($^{*}P < 0.05$ in 25 nM melatonin-treated cultures; $^{**}P < 0.01$ in amphotericin-treated cultures; and $^{***}P < 0.001$ in CSA-treated mitochondria and 50 nM melatonin-treated cultures, all versus control cultures.

3.5. Melatonin impaired activities of respiratory complexes I, II and III

Clearly, the promastigote cultures treated with 25 or 50 nM melatonin or with amphotericin showed significantly impaired complex I, II, and III activities in comparison to controls (Fig. 4A–C).

4. Discussion

This study shows that exogenous melatonin reduces the number of viable *L. infantum* promastigote accompanied by alteration of some mitochondrial parameters that are target for parasite viability. This antileishmanial effect was associated with enhanced mPTP opening, suggesting alterations in intracellular Ca^{2+} distribution, and also an increase in mitochondrial nitrite levels and respiratory chain complex impairment. All of these effects may indirectly compromise parasite survival, leading to cell death.

To our knowledge, no published data are available on the potential biocidal activity of melatonin against *Leishmania*. Melatonin is known to regulate important physiological processes, including pubertal development, sleep wake cycle and seasonal adaptation [47]. Various studies have shown that melatonin has an antioxidant effect, a good pharmacological safety profile, and represents a potentially useful tool for stand-alone or adjuvant therapy for many degenerative and infectious diseases, including Chagas disease and several immune disorders [30,48–58]. Among other properties, melatonin has also shown biocidal activity against a wide range of pathogenic agents including parasites [23,25,26]. Two mammalian subtypes of G protein coupled melatonin receptors, MT1 (Mel_{1a}) and MT2 (Mel_{1b}), have been characterized [59]. The signal transduction mechanisms of melatonin for its receptors appear to vary among different tissues and cell types [59]. It has been reported that melatonin exert its effects against some pathogenic agents through interaction through its interaction with intracellular proteins such as calreticulin [60], tubulin [61], or Calmodulin (CaM), antagonizing the binding of Ca^{2+} to CaM [29,62]. Thus, MT1 and MT2 are transmembrane G-protein-coupled heterodimers whose signaling pathways lead to downstream effects on Ca^{2+} channels, Ca^{2+} signaling, and changes in mitogen-activated protein kinases and extracellular-signal-regulated kinases [63], explaining the role of melatonin in the regulation of Ca^{2+} ion fluxes in cells. However, it should be borne in mind that

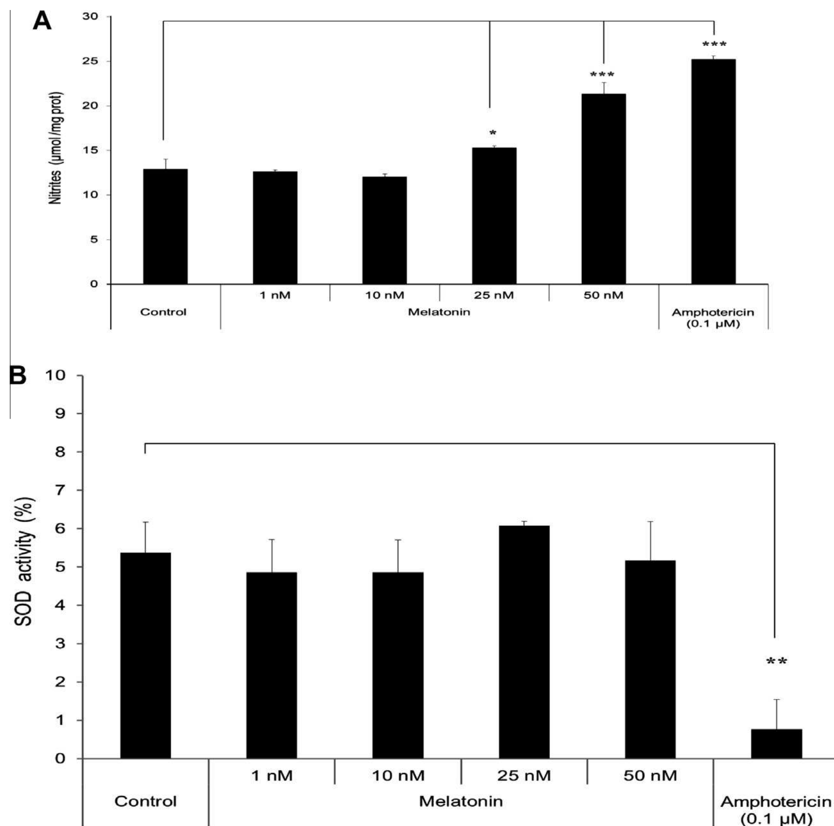

Fig. 3. (A) Nitrites concentration in mitochondria isolated from control (untreated), amphotericin, and melatonin-treated cultures. Values are means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post hoc test ($^{*}P < 0.05$ in 25 nM melatonin-treated cultures compared with control; $^{***}P < 0.001$ amphotericin and 50 nM melatonin-treated cultures versus control cultures). (B) Superoxide dismutase (SOD) activity in mitochondria isolated from control (untreated), amphotericin-, and melatonin-treated cultures. Values are means ± S.E.M. Superscript letters refer to significant difference measured using one-way ANOVA followed by the Tukey post hoc test ($^{**}P < 0.01$ amphotericin-treated cultures versus control cultures).

melatonin is also a very powerful free radical scavenger and antioxidant, and these actions do not require a receptor [30,44]. In plasmodium (*Plasmodium falciparum* and *Plasmodium chabaudi*), melatonin can modulate the cell cycle of human malaria parasite *in vivo* and *in vitro* by inducing an increase in cytosolic free Ca^{2+}. This is achieved by the mobilization of Ca^{2+} from internal Ca^{2+} pools of parasite trophozoite, augmenting the proportion of schizonts and increasing cytosolic free Ca^{2+} which modulates the parasite cell [64].

As noted above, trypanosomatids possess a single mitochondrion that occupies 12% of the total volume of the parasite and has been recognized as drug target in many of these parasites [17]. In such cases, the antiparasitic agents target mitochondria through one or more of the following programmed cell death mechanisms: intracellular Ca^{2+} levels alteration [19,65]; loss of membrane potential and ATP levels [66]; and increase of hydrogen peroxide (H_2O_2) and superoxide radical generation (O_2^-) [67].

The mitochondrion is known to be an essential organelle involved to be operative in the apoptotic pathway *in Leishmania*, but the main mechanisms by which the protozoal mitochondrion is involved in parasite apoptosis are not yet precisely understood [65]. Evidences have been published linking many leishmanicidal drugs with mitochondrial dysfunction, and the mitochondrion has become established as a target for antileishmanial drugs as in miltefosine and pentamidine [68,69], which exert their effects via multiple pathways.

Ca^{2+} homeostasis is a crucial matter in all organisms [70]. In common with most eukaryotes, cell function in parasites is coordinated using second messenger signaling cascades involving cyclic adenosine monophosphate (cAMP) and Ca^{2+} [71,72]. Moreover, in the family trypanosomatidae, Ca^{2+} is also involved in microtubule assembly, and differentiation processes, among numerous other functions [73].

Calcium antagonists, such as miltefosine, have shown antileishmanial activity by inducing a large increase in intracellular Ca^{2+}, thereby disrupting the parasite's intracellular Ca^{2+} homeostasis [68]. Other effective antileishmanial drugs against promastigote and amastigote phase, such as tafenoquine and amiodarone, were found to be partly localized in acidocalcisomes, important Ca^{2+} reservoirs in *Leishmania*; increasing the free cytosolic Ca^{2+} level mobilized from intracellular stores; disrupting Ca^{2+} homeostasis, resulting in mitochondrial damage [19,20].

Thus, fluctuations of cytosolic free Ca^{2+} concentrations may regulate a various cellular functions in all eukaryotes. The lytic effect of trypanocidal drugs against *Leishmania* and African trypanosomiasis was attributed to the alteration of calcium homeostasis produced by Ca^{2+} mobilization from intracellular stores during the progression of apoptosis [72,74].

It has also been proposed that cell damage results from the formation of reactive oxygen species (ROS) induced by Ca^{2+} alterations [75]. The oxidative phosphorylation process produces an accumulation of ROS such as H_2O_2 and O_2^- which are considered toxic products of mitochondrial respiration and have been implicated in altered Ca^{2+} homeostasis in parasites [76,77]. Accumulation of these products in the mitochondrion mediates the oxidation of critical thiol groups of adenine nucleotide translocase. This results in opening of the mPTP, which causes collapse of mitochondrial membrane potential ($\Delta\Psi m$), loss of matrix solute, mitochondrial membrane swelling, and indirectly enhance the release of death factors into the cytosol, leading to cell death [78–80].

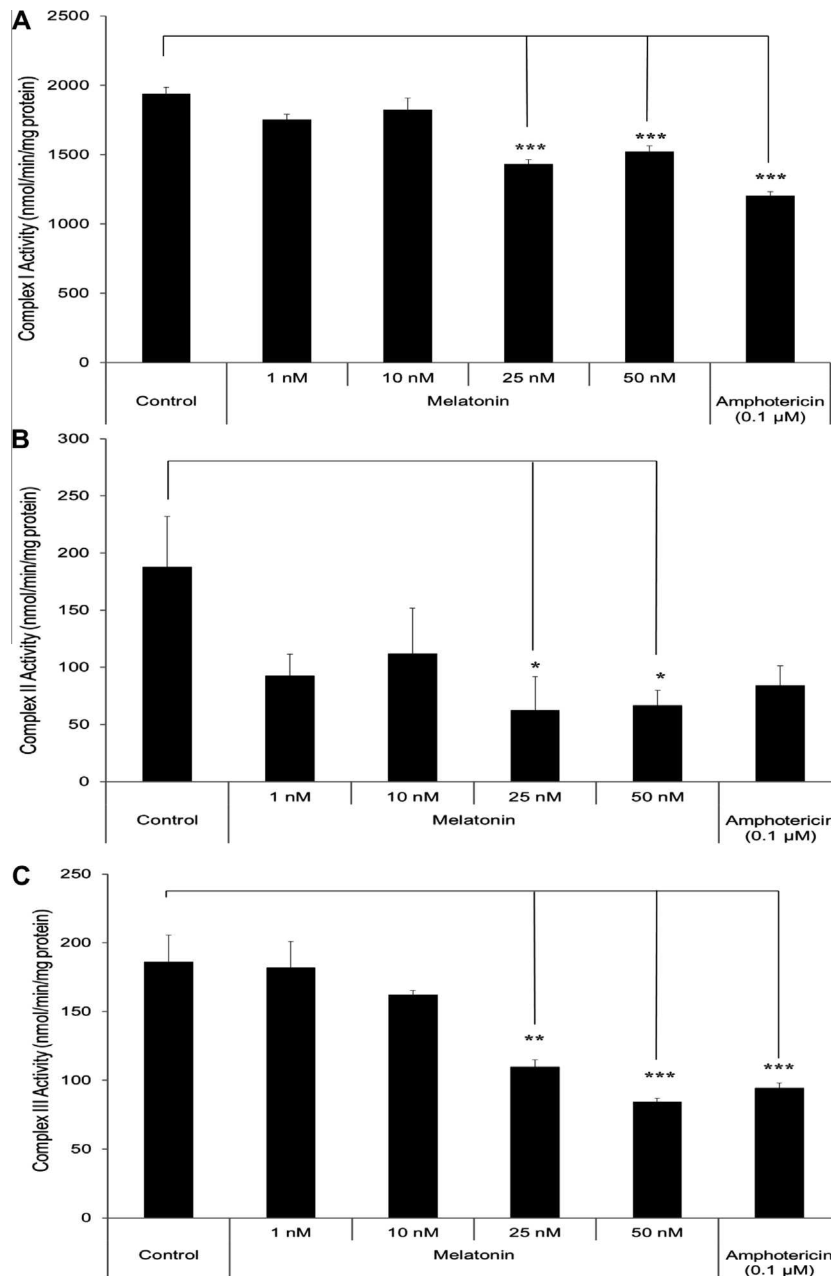

Fig. 4. (A)–(C) Activities of the electron transport chain complexes I, II, and III respectively, in mitochondria isolated from untreated and treated promastigote cultures. Values are the means ± S.E.M. Superscript letters refer to significant differences measured using one-way ANOVA followed by the Tukey post hoc test (Complex I: $^{***}P < 0.001$ for amphotericin- and 25 and 50 nM melatonin-treated cultures versus control cultures; complex II: $^{*}P < 0.05$ melatonin treated cultures at concentrations of 25 and 50 nM compared with control cultures; and complex III: $^{**}P < 0.01$ for 25 nM melatonin-treated cultures; $^{***}P < 0.001$ for amphotericin- and 50 nM melatonin-treated cultures versus control cultures).

As shown in our results, a significantly higher mPTP opening was observed in melatonin-treated cultures of *L. infantum* promastigotes, especially at a dose of 50 nM, than in control or amphotericin-treated cultures, signifying a reduced Ca^{2+} retention capacity with an earlier PTP opening. As expected; CsA had a blocking effect on mPTP opening. Interestingly, these findings confirms the phenomenon stats that mPTP opening involved in respiratory chain inhibitor-induced dissipation of $\Delta\Psi$m and corroborates with previous observations that PTP opening induces $\Delta\Psi$m collapse through a proton dissipation pathway [81]. Clearly, our data also support the hypothesis that *Leishmania* parasite utilizes intracellular Ca^{2+} based signaling during maturation and suggests that melatonin may modulate the cell cycle of *Leishmania* through its action on mPTP opening, calcium retention capacity, and the release of intracellular Ca^{2+}, disrupting the Ca^{2+} homeostasis of the parasite [73]. This is considered the most striking effect of melatonin treatment in our work as stimulation of the Ca^{2+}-induced mPTP in mitochondria isolated from melatonin-treated cultures, which either alone or in association with the other mitochondrial parameters, indirectly acts as an initiator of induced cell death [80,82–85]. Since mPTP is widely accepted be the most notorious of all the inner membrane megachannels and is considered the gatekeeper of apoptotic and necrotic cell death [80,81,85–89].

A previous study reported that increased calcium permeability is not responsible for the rapid lethal effects of amphotericin on *Leishmania* [90]; however, a more recent study concluded that amphotericin produces osmotic cell lysis by formation of aqueous pores in the membrane of promastigotes [91], establishing the role

of Ca^{2+} in amphotericin-induced apoptosis through osmotic alteration, consistent with our results [15].

In accordance with mitochondrial nitrites (NO_2^-) level, results for the level of NO_2^- suggest a potential role for the mitochondrion in regulating nitrite concentrations, which are considered an indicator for the stable breakdown product of NO in mitochondria. Several studies have revealed a major role of NO in controlling *Leishmania major* and *T. cruzi* multiplication *in vivo* and *in vitro* [92,93]. Melatonin administered to *T. cruzi*-infected animals was found to increase NO production, inhibiting parasite propagation or killing the parasite [29]. NO accumulation was also reported to inhibit the mitochondrial respiratory chain, resulting in inhibition of ATP production, greater ROS production, and increased the susceptibility to cell death. This cytotoxicity was preceded by a decrease in $\Delta\Psi$m followed by an increase in O_2^- and peroxynitrite levels [94].

Our results are in the same line, with a significant increase in nitrate level in cultures treated with 25 or 50 nM melatonin or amphotericin versus the control cultures. At 1 or 10 nM melatonin, however, the nitrite levels were non-significantly higher than those of control of control cultures. These non-significant variations may be attributable to the antioxidant effect of melatonin at these lower concentrations, hindering free radical production, given reports that melatonin and its metabolites are effective direct scavengers of nitric oxide free radical ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^-$) [95]. It should also be taken into account that the induced nitrite levels in the previous studies were *in vivo* and therefore mainly based on the immune system, in which the activated macrophages were reported to synthesize NO, considered the major effector molecule of *T. cruzi* intracellular amastigote killing [92,96].

Several studies have reported that *Leishmania* species are susceptible to ROS and reactive nitrogen species [97]. Three types of SOD have been identified: Cu/Zn-SOD, Mn-SOD, and Fe-SOD [98]. They are considered one of defense enzymes, protecting the parasites from mitochondrial-derived ROS damage and programmed cell death by detoxifying superoxide into hydrogen peroxide and oxygen, thereby maintaining the integrity of the mitochondrion [99]. Mitochondrial Fe-SOD has been shown to protect the *Leishmania* parasite from oxidative stress, and inhibit programmed cell death, as observed in miltefosine-induced cytotoxicity in *L. donovani* [99,100].

However, we found non-significantly lower Cu/Zn-SOD activities in melatonin-treated cultures (all concentrations) than in control cultures. This lack of a significant difference may be attributable to the modest effect of Cu/Zn-SOD in *Leishmania*, the antioxidant or protective effect of melatonin at lower concentrations, the parasite strain, among other possibilities. Consistent with these findings, a recent study reported the negligible inhibition of human Cu/Zn-SOD with pyrazole-based benzo[g]phthalazine derivatives on *L. infantum* and *Leishmania braziliensis* [101].

It is noteworthy that many available antileishmanial agents act by impairing of mitochondrial complexes. Complexes I, III and IV are known to function as H^+ ions (protons) pumps, generating a proton electrochemical gradient that drives ATP synthesis, explaining why mitochondrial complexes are the primary source of endogenous ROS [102]. Thus, inhibition of these complexes by antileishmanial agents interferes with electron transport processes, resulting in increased ROS from diverted electron flow, as in the case of antimycin A and tafenoquine against *L. donovani* [19,103].

In the present study, melatonin produced a significant inhibition of mitochondrial complexes I, II and III, which was marked at the highest concentrations of melatonin (25 and 50 mM). However, the activity of complex I is very low in *Leishmania*, and only the enzyme corresponding to rotenone-insensitive NADH: quinone oxidoreductase has been observed in *Trypanosoma* and *Leishmania* [104]. Therefore, these parasites possess only one NADH dehydrogenase gene in their total DNA that may be essential for parasite survival; explaining its appeal as a drug target [104]. Inhibition of complex I would result in generation of ROS and therefore leads to mitochondrial dysfunction, which in turn triggers the apoptotic mitochondrial pathway [19,104,105]. The present results are in agreement with the previous findings on the antileishmanial effect of luteolin resulted from reduction in the activities of electron transport chain complexes I, II, III, and IV [106].

According to results, a drastically lowered activity of complex II on melatonin-treated cultures versus control cultures was observed. This result correlates with report of induced death in *Leishmania* using new Benzophenone-Derived Bisphosphonium Salts, which inhibit complex II [107].

Several studies have described complex III as an attractive target for antiprotozoal drugs [106]. Tafenoquine was found to cause a mitochondrial dysfunction in *Leishmania* by complex III (cytochrome *c* reductase) inhibition [19]. In these cases, the stoppage of electron transfer may reduce the oxygen consumption rate, causing an imbalance of Ca^{2+} homeostasis and dissipating the $\Delta\Psi$m that is accompanied by production of O_2^-, triggering death of the parasite [19,97,103,108]. It should be stressed that inhibition of Complexes I, II and III results in an intracellular increase of Ca^{2+} that intimately associated with ROS generation, while the inhibition of complex II markedly alters Ca^{2+}[97,107].

These are highly promising findings, given that complex I inhibition may result on mitochondrial hyperpolarization that is usually preceded by increased O_2^- production, while inhibition of complexes II and III might cause dissipation of $\Delta\Psi$m. Both hyperpolarization and loss of $\Delta\Psi$m might target the viability of the promastigotes [109,110]. The inhibitory effect of melatonin on these mitochondrial complexes support the proposition that *Leishmania* mitochondria respond strongly to the inhibition of respiratory chain complexes [111].

Interestingly, the role of mitochondria in our findings may be initiated by the effects of melatonin on mPTP, a key implication, through disruption of Ca^{2+} homeostasis which indirectly drives to lethal effects resulting in cell death [85]. As shown in the results, melatonin-treated cultures depleted some respiratory components, probably due to the opening of mPTP. Remarkably, impairment of activity of mitochondrial respiratory enzymes in treated cultures may push the mitochondria towards mitochondrial oxidative stress expressed by inhibition of electron transfer, together with inhibition of SOD and the resulted nitrosative stress which may act collectively as a major inducer of mPTP opening, thereby indirectly drives to lethal effects by apoptosis or necrosis [75,112].

All of the above impressive changes in melatonin-treated cultures are in part attributable to alterations in mPTP opening, accompanied by higher mitochondrial nitrite levels and inhibition of the activity of mitochondrial complexes especially II and III, and these events may indirectly trigger death of the parasite.

In conclusion, the present results indicate that melatonin exerts *in vitro* activity on promastigote phase of *L. infantum*, producing mitochondrial alterations in Ca^{2+} distribution, nitrite concentrations, and complexes I, II and III, thereby targeting the parasite survival. Further research is warranted to elucidate the *in vivo* effects of melatonin alone and in association with other antileishmanial drugs in order to develop a novel chemotherapeutic strategy against the disease. There is also a need to examine the mechanisms underlying these effects and the role of melatonin receptors.

Ethical standards

The experiments comply with current Spanish legislation on research.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Conflict of interest

The authors declare that they have no conflict of interest.

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