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

Ehab Kotb Elmahallawy [a,b], Aroa Jiménez-Aranda [c], Antonio Sampedro Martínez [d], Javier Rodriguez-Granger [d], Miguel Navarro-Alarcón [e], José Gutiérrez-Fernández [a], Ahmad Agil [c,*]

[a] Department of Microbiology, Faculty of Medicine, University of Granada, Granada, Spain
[b] Department of Zoonotic diseases, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt
[c] Department of Pharmacology and Neurosciences Institute (CIBM), Faculty of Medicine, University of Granada, Granada, Spain
[d] Service of Microbiology and Parasitology, University Hospital Virgen de las Nieves, Granada, Spain
[e] Department of Nutrition and Food Science, School of Pharmacy, University of Granada, Granada, Spain

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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.

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

* Corresponding author. Address: Department of Pharmacology and Neurosciences Institute (CIBM), School of Medicine, University of Granada, Avenida de Madrid s/n, 18012 Granada, Spain. Fax: +34 958243537.
E-mail address: aagil@ugr.es (A. Agil).

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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].

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

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