Evaluation of ultrastructural changes and cell death on *Leishmania amazonensis* promastigote forms induced by a new coordinated complex Co (II)

Avaliação de alterações ultraestruturais e morte celular em formas promastigotas de *Leishmania amazonensis* induzidas por um novo complexo coordenação Co (II)

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ABSTRACT
Leishmaniasis are diseases caused by protozoa of the genus *Leishmania* and transmitted by vectors of the phlebotomine subfamily. In Brazil, the species *Leishmania amazonensis* is the etiological agent of the diffuse cutaneous form. Currently, the treatment employed is associated with several side effects, which stimulated the development of new alternatives for the treatment of this important neglected disease. Thus, metallocomplexes appear as a new alternative for antiparasitic therapy. Its action has been evaluated on different species of parasites of the *Trypanosomatidae* family, including species of the genus *Leishmania*. In this work, we evaluated the effect of a new Co (II) complex, against promastigotes of *L. amazonensis* of strain WHOM / BR / 75 / Josefa. Transmission electron microscopy (TEM) was performed to evaluate the parasite's ultrastructure and confocal laser microscopy to check for death by autophagy of the parasite. The complex was able to induce ultrastructural changes in the parasite, such as the formation of autophagic vacuoles near the pouch region flagellar and myelin figure formations. Finally, tests with anti-LC3B labeling indicated the possible death of the parasite by autophagy.

Keywords: *Leishmania amazonensis*, Co(II) complex, TEM, autophagy.

RESUMO
As leishmanioses são doenças provocadas por protozoários do gênero *Leishmania* e transmitidas por vetores da subfamília dos flebotomíneos. No Brasil, a espécie *Leishmania amazonensis* é o agente etiológico da forma cutânea difusa. Atualmente, o tratamento empregado é associado a diversos efeitos colaterais, o que estimulou o desenvolvimento de novas alternativas para o tratamento desta importante doença negligenciada. Assim, os metalocomplexos surgem como uma nova alternativa de terapia antiparasitária. Sua ação tem sido avaliada em diferentes espécies de parasitas da família *Trypanosomatidae*, incluindo espécies do gênero *Leishmania*. Neste trabalho, avaliamos

o efeito de um novo complexo de Co (II), contra promastigotas de *L. amazonensis* da cepa WHOM / BR / 75 / Josefa. Foram realizadas microscopia eletrônica de transmissão (MET) para avaliação da ultraestrutura do parasite e microscopia confocal a laser para verificar a morte por autofagia do parasita. Os resultados mostraram que o complexo foi capaz de induzir alterações ultraestruturais no parasita, como como formação de vacúolos autofágicos próximos a região da bolsa flagelar e formações de figura de mielina. Por fim, ensaios com marcação anti-LC3B indicaram a possível morte do parasita por autofagia.

Palavras-chave: *Leishmania amazonensis*, complexo Co (II), MET, autofagia.

1 INTRODUCTION

Leishmaniasis are infectious and parasitic diseases that affect humans, caused by several species of protozoa of the genus *Leishmania* (ASHFORD, 2000; WHO 2020). According to the World Health Organization, they are considered an important public health problem, as well as a neglected tropical disease (WHO, 2020).

Parasites belonging to the genus *Leishmania* have a digenetic life cycle, living alternately in vertebrate hosts and vector insects, the latter being responsible for the transmission of parasites from one mammal to another (TEIXEIRA, 2013). In mammalian hosts, represented in nature by various orders and species, the parasites assume the amastigote shape, rounded and immobile, which multiplies in cells that may be hematopoietic or not, such as keratinocytes, Langerhans cells, neutrophils, macrophages and fibroblasts, however, the macrophage is considered the main host cell in which the parasite can survive and multiply (MOUGNEAU, BIHL and GLAICHENHAUS, 2011; KAYE and SCOTT, 2011; ASHOK and ARCHA-ORBEA, 2014). In invertebrate hosts, leishmanias inhabit the lumen of the intestine of the female insect. There, the amastigote forms, ingested during the blood meal, differ in flagellate forms, morphologically and biochemically distinct from the amastigotes, being subsequently inoculated in the promastigote form in the skin of mammals during the bite (TEIXEIRA, 2013).

Leishmaniasis can present different clinical manifestations. In Brazil, the species *Leishmania amazonensis* is one of the species responsible for the cutaneous form of the disease; however, in some individuals the disease can evolve, leading to clinical manifestations of diffuse cutaneous leishmaniasis (DCL) (MARSDEN and JONES, 1985). DCL was first described in the Brazilian Amazon, in the state of Pará, by Silva (1958), who reported the first clinical findings in a rare form of cutaneous leishmaniasis,

highlighting the nature of keloid skin lesions and bone tissue lesions in extremities (hands and feet) (SILVEIRA, 2009).

The current treatment for leishmaniasis is the pentavalent antimonial drugs Glucantime® (meglumine antimoniate) and Amphotericin B, used in the first and second lines, respectively, being applied intramuscularly or intravenously. However, these drugs often cause side effects, such as arthralgia, myalgia, nausea, tachycardia, fever and vomiting, in addition to being expensive drugs (NEVES, 2007; BRASIL, 2009; DE SOUZA et al., 2013), thus, it is necessary to develop new drugs to be used in the treatment of this disease of great impact on public health.

Metallocomplexes are an excellent alternative. These compounds are made up of transition metals, coordinated with drugs, and have possibilities of coordination and geometries with the ligand (drug) (VAN RIJT and SADLER, 2009). This coordination allows to interact with the specific molecular target, mainly with biological molecules (FRICKER, 2007) allowing the increase of lipophilicity (AHMAD, 2006; BRUIJNINCX and SADLER, 2008). Several studies have already shown that metal-coordinated compounds can be an alternative in antiparasitic therapy. Compounds coordinated with lanthanides have shown antiparasitic potential against *L. infantum*, *L. brasiliensis* and *Trypanosoma cruzi*. Its *in vitro* activity was similar to or significantly exceeded those demonstrated by two of the most commonly used drugs for the treatment of leishmaniasis and Chagas disease, Glucantime and benznidazole, respectively (CABALLERO et al., 2014).

Studies by our group showed that coordination compounds with iron (III), Zn (II) and Cu (II) can control the growth of *Toxoplasma gondii*, the causative agent of toxoplasmosis, and even promote the death of the parasite (PORTES et al., 2018). The complexes reduced the growth of T. gondii and, at the same time, caused low cytotoxicity in the host cells. In addition, one of the complexes used induced distinct morphological and ultrastructural changes in the parasites (PORTES et al., 2017; 2018). Knowing that coordination compounds can be applied as a good antiparasitic strategy, this work aimed to evaluate the leishmanicidal effect of the new Co (II) complex, against the extracellular (promastigote) form of *L. amazonensis*, through the analysis of ultrastructure by Transmission Electron Microscopy (TEM), as well as evaluating the induction of parasite death by autophagy, using Confocal Laser Microscopy.

2 METHODS AND METHODS

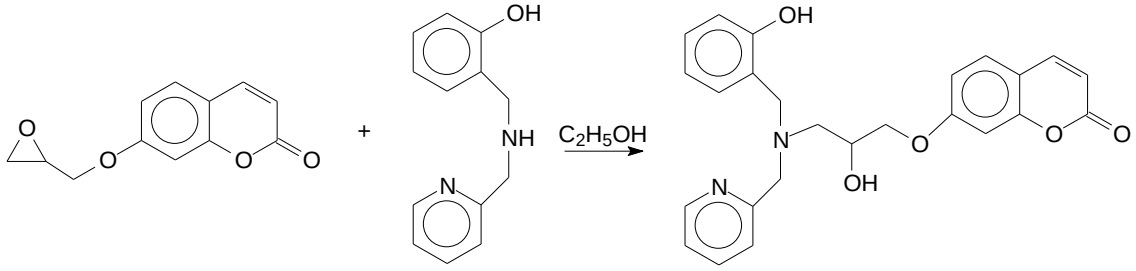
2.1 CELL CULTURE OF *LEISHMANIA AMAZONENSIS*

The promastigote forms of *L. amazonensis* were obtained from the differentiation of amastigotes isolated from lesions in Balb/C mice and grown in Warren's medium, (BHI - Brain Heart Infusion - Sigma®) plus 20 µg / L hemin - Sigma® and 10 µg / mL folic acid - Sigma®, in inoculum of 5% of volume plus 10% of fetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific®) in bottles for sterile cultures and kept at temperature of 25° C ± 1ºC. The samples were maintained through weekly passages up to a total of six passages, ensuring that the parasites used in the experiments remained infectious.

2.2 SYNTHESIS OF THE LIGAND H2L

The ligand was obtained as described in Scheme 1, by the reaction between equimolar amount of the epoxide (4g, 18 mmol) and HBPA (3g), in 100 mL of ethanol, under reflux, for 5 days. The reaction was follow by TLC, using ethanol as eluent. Subsequently, the solvent was removed under reduced pressure and the residue was to 50 cm^3 of water. The compound was extracted with five 50 cm^3 portion of $CHCl_3$ and the extracts were combined, washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. A orange oil was obtained.

Scheme 1: Synthesis of the ligand H2L.

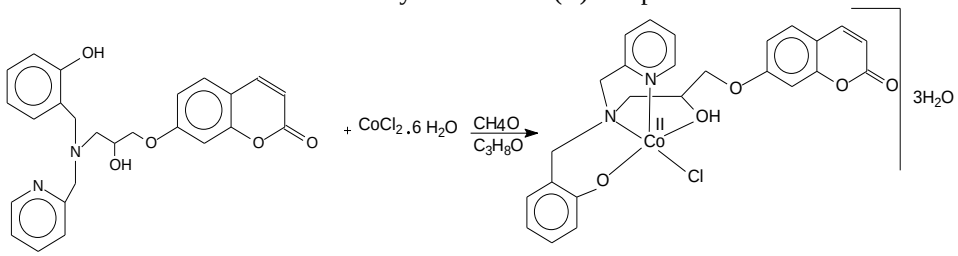


2.3 SYNTHESIS OF $[Co(HL)C\ell](H_2O)_3$

The cited complex (Scheme 2) was prepared by the reaction between the ligand H2L (1 mmol, 432 mg) and $CoC\ell_2.6H_2O$ (1 mmol, 237 mg), in methanol/propan-2-ol (1:1), at room temperature. After allowing the purple solution to stand for a few days, purple crystals were filtered off, washed with cold propan-2-ol and dried in a desiccator. Yield: 70 mg (7%). m.p.: 245°C. Anal. Calcd. for $C_{25}H_{29}ClCoN_2O_8$; MW= 579.90 g mol^{-1}: C, 51.78; H, 5.04; N, 4.83. Found: C, 52.84; H, 5.15; N, 4.79. The IR spectrum exhibits bands at 3412 (ν OH), 3068 (aromatic CH), 2970-2864 (aliphatic CH), 1506, 1477, 1444, 1400 (ν C=N and C=C), and at 779 cm^{-1} (δ C-H), ESI(+)-MS spectrum shows a peak with

m/z of 490, attributed to the Co(III) complex: $[Co(III)(HL)]^+$, as a result of desprotonation of the ligand H2L with the releasing of a HCL molecule from the starting complex. Electronic spectrum of Co(II) complex, in DMSO, presents two d-d transitions at 596 nm (122 $dm^3.mol^{-1}.cm^{-1}$) e outra em 689 nm (46 $dm^3.mol^{-1}.cm^{-1}$).

Scheme 2: Synthesis of Co(II) complex.



2.4 TRANSMISSION ELECTRON MICROSCOPY

To evaluate the ultrastructure of *L. amazonensis* promastigotes in the presence of the complex, the parasites were kept in the presence (10 µM) and absence of the complex for 24 and 48 h, and then they were washed with serum-free medium and was fixed with 2.5% glutaldehyde and 4% recently prepared formaldehyde in sodium cacodilate buffer (0.1M, pH 7.2) for 2 h. Cells were washed with sodium cacodilate buffer and post-fixed with 1% osmium tetroxide, 1.6% potassium ferrocyanide and 5 mM calcium chloride in sodium cacodilate buffer for 1 h. Cells were dehydrated with acetone serial concentrations of 30%, 40%, 50%, 70% and 100%. Inclusion was performed with epoxy resin. Ultrafine sections were contrasted with uranyl acetate and lead citrate and observed in a Transmission Electron Microscope Zeiss®900, using voltage acceleration of 30 kv.

2.5 CONFOCAL LASER MICROSCOPY

In order to evaluate the induction of the parasite's death by autophagy, cultures of the *L. amazonensis* promastigote forms were treated with the complex at concentrations of 5 µM and 10 µM. The control used was culture without treatment. These concentration values were stipulated according to the results of the growth curve tests and evaluation of the antiproliferative effects. For such experiments, the parasites were first fixed in PHEM buffer (60mM Pipes, 20mM Hepes, 10mM EGTA, and 5mM magnesium chloride - Sigma® pH 7.2) containing 4% (v/v) of paraformaldehyde (Merck®), for 30 min at room temperature (RT ≅ 36 ° C). Then, the parasites were adhered for 20 min to RT in coverslips previously coated with 0.1% poly-L-lysine. After fixation and adhesion, the

coverslips were washed, permeabilized in PHEM containing 0.3% Triton X-100 (Sigma®) for 5 min, and incubated with 50 mM Ammonium chloride (NH_4Cl) for 30 min., then with Serum Albumin Bovine (SAB) 3% in PHEM buffer (PHEM-SAB) for 30 min at RT (room temperature). After incubation with the blocks, the coverslips were incubated with rabbit polyclonal antibody LC3B (dilution 1: 1000) for 1 h at RT. Subsequently, incubation was performed with secondary goat anti-rabbit antibody conjugated to Alexa-546 (dilution 1: 100) (Molecular Probes) for 1 h at RT. After labeling, the cells were washed with PBS and the coverslips were mounted with the "ProLong® Gold antifade" reagent (Invitrogen) with 4 ', 6-diamidino-2-phenyl- indole (DAPI) and observed using a confocal laser scanning microscope (LSM-710, Zeiss).

3 RESULTS

The Co(II) complex was analyzed for its antiproliferative activity against the promastigote form of *L. amazonensis*. Co(II) complex, were evaluated using TEM. Promastigote forms were treated with this complex at a concentration of 10 μM in the periods of 24 and 48 h. Control parasites, untreated, were also observed by MET and showed a normal ultrastructure, where the nucleus (N) was with its entire structure and decompressed chromatin, kinetoplast (k) in its characteristic compaction, flagellum (f) emerging from the flagellar pouch (fp) and subpellicular microtubules (sm) in the membrane (sm) (Figures 1a and 1b). After treatment with the complex, several changes were observed, among them: formation of autophagic vacuoles near the region of the flagellar pocket (Figures 1 and 2) myelin figure (mf) formations (Figure 1).

Figure 1: **Transmission electron micrograph of *L. amazonensis* after 24 hours of treatment with 100 µM of the compound Co7HCHBPA.** (a, b) Untreated cells with characteristic parasite structures. (c) Altered flagellar pouch, indicating death by autophagy (1). (d) Altered flagellar pouch (1) and formation of myelin figures (2). (e) Alteration of the flagellar pouch (1). (f) Myelin figure - Image d enlargement (2).

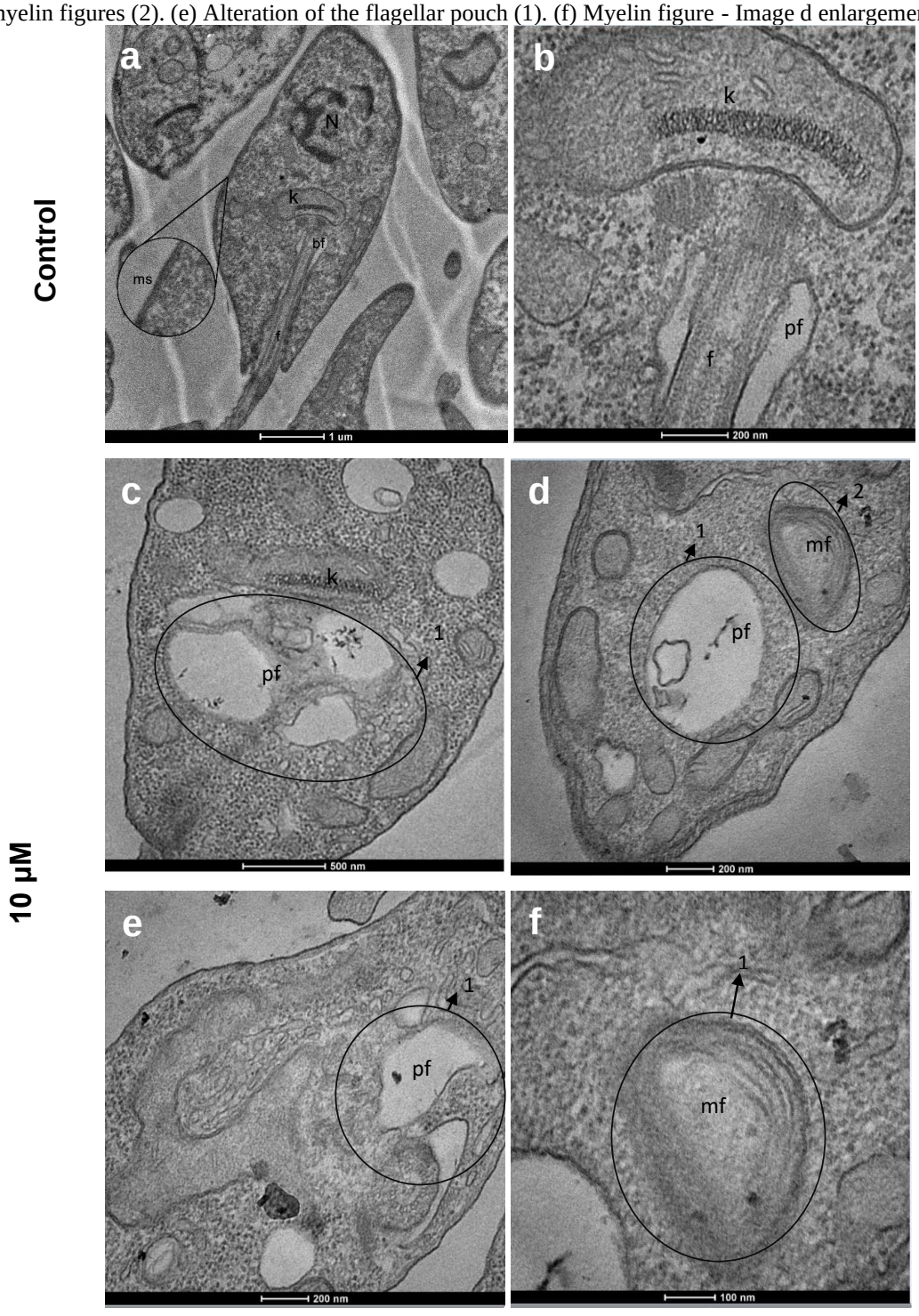
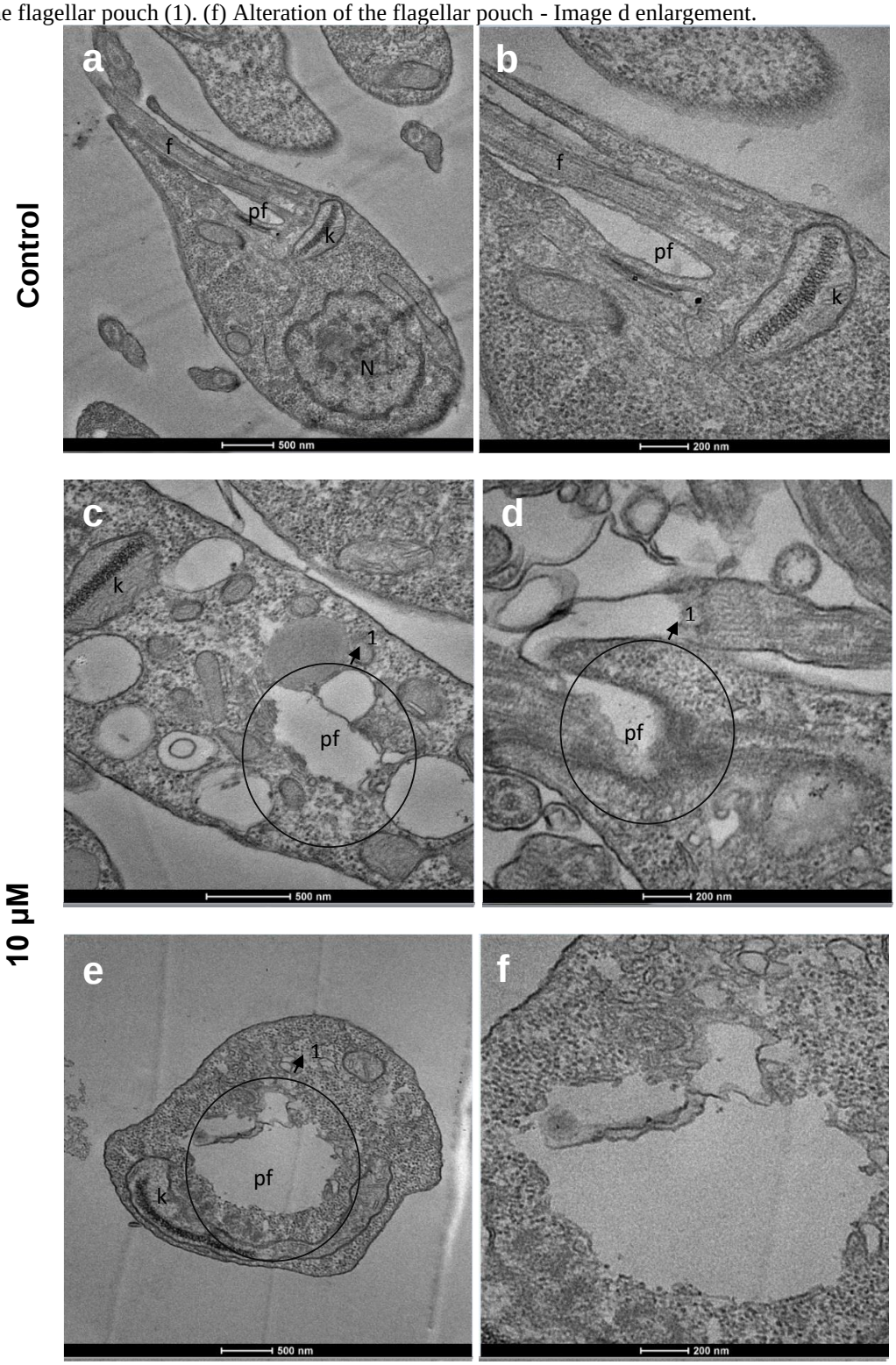


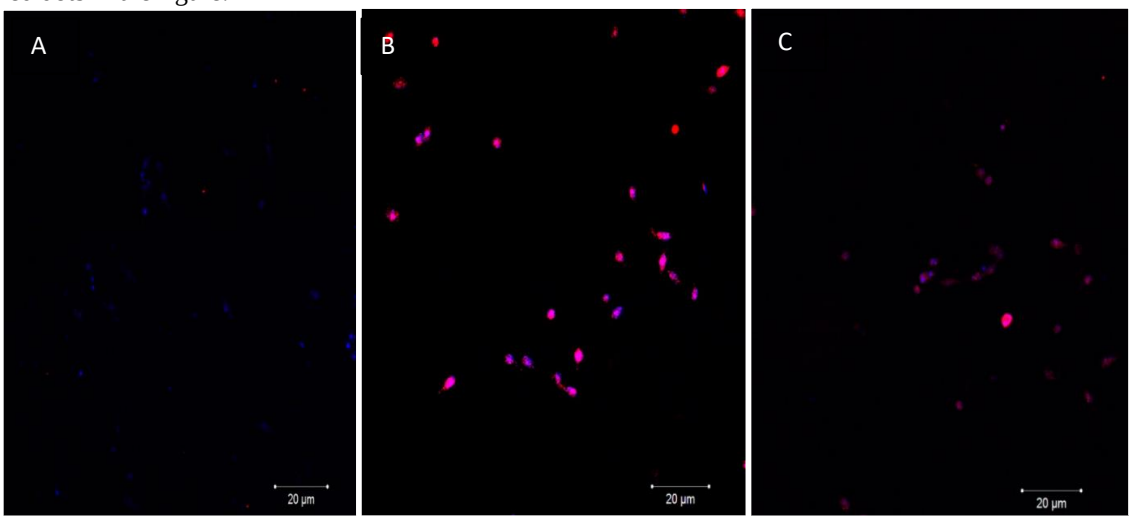
Figure 2: **Transmission electron micrograph of *L. amazonensis* after 48 hours of treatment with 10 µM of the Co7HCHBPA compound.** (a, b) Untreated cells with characteristic parasite structures. (c) Altered flagellar pouch, indicating death by autophagy (1). (d) Altered flagellar pouch (1). (e) Alteration of the flagellar pouch (1). (f) Alteration of the flagellar pouch - Image d enlargement.



Since the ultrastructure analysis of *L. amazoensis* promastigotes treated with the Co(II) complex presented the formation of autophagic vacuoles (in the region of the flagellar pocket), it was investigated whether this current treatment is inducing autophagy

in these promastigotes. For this, the parasites treated at 5 µM and 10 µM of Co(II) complex were incubated with an antibody against the LC3B protein and observed under an immunofluorescence microscope. In the control cells, there were no markings for autophagy, as expected (Figure 3a). The cells treated with 5 µM showed positive staining for the LC3B protein (Figure 3b), which indicates that the complex is inducing the parasite to autophagy. Subsequently, cells treated with 10 µM were subjected to the same evaluation (Figure 3c), also showing positive marking for anti-LC3B indicating autophagy.

Figure 3: **Laser confocal microscopy showing cell death by *L. amazonensis* autophagy after treatment with the Co7HCHBPA compound.** (a) Control cells in blue, with autophagy marking on cells treated with 5 µM (b) and 10 µM (c) of the compound Co7HCHBPA. The anti-LC3B marking corresponds to the pinkish red dots in the figure.



4 DISCUSSION

In this work we evaluated the effects of a new Co(II) complex on the promastigote forms of *L. amzonensis in vitro*. Throughout this study, we observed, by transmission electron microscopy, that there are drastic ultrastructural changes in the promastigote form of *L. amazonensis* caused by the Co(II) complex. Regarding the ultrastructure, changes were observed in the periods of 24 and 48 h, where several changes were seen, among them: formation of autophagic vacuoles close to the region of the flagellar pocket, myelin figure formations (Figure 1 and 2), indicating cell death. Other complexes had an effect on the parasite's energy metabolism, also causing degradation in the membranes of organelles and cell death (RAMÍRES-MACÍAS, 2011; CABALLERO et al., 2014; COSTA et al., 2017; HUBIN et al., 2019). Investigations carried out with Fe(III) complexes already demonstrated morphological and ultrastructural changes in *T. gondii* (PORTES et al., 2015 and 2017).

Finally, considering that the ultrastructure analysis of *L. amazoensis* promastigotes treated with this Co(II) complex indicated the formation of autophagic vacuoles (in the region of the flagellar pouch), it was investigated whether these promastigotes could have cell death due to autophagy triggered by this treatment. The cells treated with 5 μM showed positive staining for the LC3B protein (Figure 3), indicating that the Co(II) complex induced the parasite to autophagy. Subsequently, cells treated with 10 μM were subjected to the same evaluation (Figure 3), also showing positive marking for anti-LC3B indicating autophagy.

5 CONCLUSION

In conclusion, all the results obtained from this work, strongly suggest that the tested metallocomplex has a leishmanicidal action, presenting significant effects on the promastigote form with a concentration (in μM) that does not affect macrophages. However, in the future, tests on intracellular amastigotes should be developed so that we can evidence its effect, and in this way, indicate it as a good candidate for future tests, whether used alone or coordinated with other compounds.

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