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Effect of the variation in the extracellular concentration of L-arginine in the physiology of *Leishmania (Viannia) braziliensis* and its susceptibility to some antileishmanial drugs

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ABSTRACT

The knowledge about amino acid metabolism in trypanosomatids is a valuable source of new therapeutic targets. L-arginine is an essential amino acid for Leishmania parasites, and it participates in the synthesis of polyamines, a group of essential nutrients used for nucleic acids, proteins biosynthesis, and redox modulation necessary for proliferation. In the present study, we evaluated the effect of changes in the availability of this amino acid on promastigotes and intracellular amastigotes on U937 macrophages and showed that the absence of L-arginine in culture medium negatively influences the growth and infectivity of *Leishmania (Viannia) braziliensis*, causing a decrease in the percentage of the infected cells and parasite load tested through light microscopy. In addition, the absence of L-arginine resulted in the parasite's inability to regulate its reactive oxygen species (ROS) production, which persisted for up to 24 h by flow cytometry following the probe H2DCF-DA dye. Moreover, the differentiation of promastigote to amastigote in axenic culture was more significant at low concentrations of L-arginine suggesting that this depletion induces a stress environment to increase this transformation under axenic conditions. No association was established between the availability of L-arginine and the effectiveness of antileishmanial drugs. All these results confirm the importance of L-arginine in *L. braziliensis* life cycle vital processes, such as its replication and infectivity, as documented in other *Leishmania* species. Based on these results, we proposed that the L-arginine uptake/metabolism route is possible in exploring new antileishmanial drugs.

1. Introduction

Leishmaniasis is an infectious disease caused by parasites of the genus *Leishmania*. The main clinical manifestations of this infection (cutaneous, mucous, and visceral leishmaniasis) depend on several inherent factors in the infected patient and the parasite species causing the disease (World Health Organization, 2010). In Colombia, the most prevalent species are *L. panamensis* and *L. braziliensis* (Herrera et al., 2020) whose sensitivity to meglumine antimoniate (MA) is lower than other *Leishmania* species (Fernandez Pimentel et al., 2011).

Leishmania has a dimorphic life cycle, with the promastigote form developing in the phlebotomine vector while the amastigote forms developing in the mammalian host (Wheeler et al., 2011). The knowledge about the different forms of the parasite's adaptation to its hosts is the basis of the disease pathogenesis and the new therapies proposal. Therefore, developing better treatments is mandatory because available medicines have multiple disadvantages, such as difficult access, handling, and high toxicity resulting from antimonial-based therapies (Wheeler et al., 2011; Singh et al., 2012).

Worldwide, pentavalent antimonials are the standard drugs for treating CL. However, these drugs are often poorly tolerated because of common and sometimes severe adverse effects, development of drug resistance, and parenteral administration. Other treatment options include miltefosine (MIL), amphotericin B (AMB), pentamidine (PEN), paromomycin, and some azoles such as ketoconazole, fluconazole, and itraconazole) but also local therapies, including thermotherapy and cryotherapy (Aronson and Joya, 2019).

One of the most used approaches in searching for new drugs is rational development, where candidate drugs are selected based on a molecular target in the parasite. In *Leishmania* spp, numerous molecular

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targets have been explored in diverse metabolic pathways, allowing novel candidate drugs to be generated; some are already in advanced stages of development. However, none of these candidates have been introduced into the market. Therefore, searching for molecules with antileishmanial activity is still needed.

The L-arginine is the precursor of polyamine and trypanothione synthesis. Both are molecules with high importance in the *Leishmania* species' biological processes of growth and infectivity *in vitro*. L-arginine participates in the leishmanicidal action of the macrophage through nitric oxide (NO) production, which has been linked to the death of the parasite due to oxidative stress (Wanasen and Soong, 2008; Mandal et al., 2016; Colotti and Ilari, 2011; Nayak et al., 2018; Muxel et al., 2018). Based on these findings and others not reviewed here, we proposed the metabolism of L-arginine as a potential source in the search for new therapeutic targets for the leishmaniasis treatment.

This work evaluated the effects of changes in the extracellular concentration of l-Arg in different *L. braziliensis* physiological processes such as replication, stage differentiation, and infectivity. The effect of L-arginine concentrations on the sensitivity of *L. braziliensis* to antileishmanial drugs MA, PEN, MIL, and AMB, and reactive oxygen species (ROS) generation was also determined to identify a link between L-arginine metabolism and the mode of action of antileishmanial drugs and, therefore, to determine the potential of L-arginine as a therapeutic target.

2. Materials and methods

2.1. L. braziliensis culture conditions and maintenance

Promastigotes of L. *braziliensis* (MHOM/CO/88/UA301) (both wild type (wt) and transfected with green fluorescence protein (EGFP)), were incubated in complete RPMI 1640 medium (supplemented with 5% fetal bovine serum (FBS), 25 mM HEPES, 4 mM $NaHCO_3$ and 1% antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin) (Sigma-Aldrich, USA)), pH 6.9, at 26 °C. The medium was changed every four days until promastigotes use for each experiment.

2.2. L. braziliensis (UA 301-EGFP) growth curves

Growth curves were constructed in L-arginine and l-lysine-free medium, supplemented with 5% FBS, 32 mg/mL l-lysine (Sigma-Aldrich, USA), 25 mM HEPES, 4 mM $NaHCO_3$, 1% antibiotics. For different experiments, L-arginine (Sigma-Aldrich, USA) was added to the medium in different concentrations (0, 50, 100, 200, and 300 μg/mL). The L-arginine at 200 μg/mL was considered the control for all the experiments, as the same concentration was present in the conventional RPMI 1640 medium. Promastigotes (3-days old) were adjusted to 5×10^5 parasites/mL in 1.5 mL of medium in each concentration of L-arginine described previously. Later they were deposited in each well of 24-well plate (SPL Life Science, USA) and were kept for 5 days at 26 °C. The daily number of parasites was determined using Resazurin from a calibration curve (Walzl et al., 2014). The reaction was read using a spectrofluorometer (Varioskan, Thermo Scientific, USA) at 560/590 nm for excitation/emission wavelength. The acquired data was used for the construction of parasite growth curves under different culture conditions.

The formula used to determine the doubling time of the parasite population in each experimental condition was:

DT = T*Ln2/Ln(Xe/Xb), where:

T is the incubation time in hours.

Xb is the parasite's number at the beginning of the incubation time.

Xe is the parasite number at the end of the incubation time.

2.3. Evaluation of the L. braziliensis (UA 301-EGFP) differentiation

The evaluation was based on the methodology proposed by Ruben t'Kindt et al. for obtaining axenic amastigotes (t'Kindt et al., 2010). In this case, *L. braziliensis* (UA301-GFP) promastigotes were grown at an initial 1.5×10^6 parasites/mL concentration in 5 mL of free L-arginine and l-lysine RPMI 1640 medium supplemented with different concentrations of L-arginine as previously described. After four days in culture, promastigotes were centrifuged at 600 *g* for 5 min and transferred to 5 ml of the same medium, pH 5.4 and cultivated at 34 °C, respectively, for four additional days (Teixeira et al., 2002). On days two and four, the number of promastigotes and amastigotes/mL was determined by counting in a Neubauer chamber under a light microscope. The morphological criteria used to identify the amastigote form in the culture were a decrease in size, an ovoid form, and reduction or even loss of flagellum. The experiment was performed in two independent experiments, each in duplicate (n = 4).

2.4. Quantification of L-arginine by HPLC-MS

Dionex 3000 chromatograph with Reprosil-Pur Basic-C18 reversed-phase column (4.6 mm i.d x 150 mm) was used for HPLC-MS analyses. A 5 min isocratic elution program with a flow rate of 400 μL/min and mobile phase composition of 95:5 in water: acetonitrile with 0.1% formic acid was used for the separation. Calibration curves for L-arginine (Sigma-Aldrich, USA) at 0–300 μg/ml were constructed with 6 points by dissolving each L-arginine standard in acetonitrile (Nemkov et al., 2015). The L-arginine uptake by *L. braziliensis* promastigotes was evaluated as follows: The parasites cultured in the media previously described with L-arginine at 0, 200, and 300 μg/mL were evaluated. Each day 80 μL of spent media were taken, quenched in an ethanol bath at 0 °C for 20 s, then 240 μL methanol (V/V 1:3 water: methanol) were added and mixed vigorously for 10 s. Subsequently, samples were centrifuged (15,000 × g, 5 min, 1 °C). The supernatant was collected and filtered through a 0.22 μm nylon, then exposed to liquid nitrogen for 60 s. Later, it was evaluated by HPLC-MS. The evaluation was performed on day 0 (starting point), on days 3 and 5, to determine the changes in the L-arginine concentration in the exometabolome. HR-MS data were obtained using Bruker Impact II UHR-QqTOF equipped with an electrospray ionization source. The desolvation gas was dry nitrogen, and gas flow was 8 L/min. The source temperature was 200 °C, the capillary voltage was set to 3000 V in positive ionization mode, and the collision energy was 4 eV. Scanning values ranging from 50 m/z to 1200 m/z were collected and processed through data analysis 4.3 (Bruker Daltonik).

2.5. Measurement of ROS

The variation in the generation of ROS under different stimuli was measured with a 2′, 7′dihydrofluorescein diacetate (H_2DCF-DA) - (Invitrogen, USA) probe, using the protocol described by Carvalho et al., 2011 (Carvalho et al., 2011). The L. *braziliensis* (wt) was used in ROS production analysis to avoid interference between GFP emission and the H_2DCF-DA probe (λ Excitation/Emission: ~492–495/517–527 nm). Parasites of 3 days old (exponential phase) were adjusted to 1×10^6 cells/mL and were incubated at 26 °C in the free L-arginine and l-lysine RPMI 1640 medium, supplemented as was previously described. ROS production was evaluated at 1, 4, and 24 h of culture. After incubation, 200 μL of the parasite solution were taken from the culture, washed with Phosphate-Buffered Saline (PBS), 0.47 (% w/v) Na_2HPO_4, 1% (w/v) NaCl, and 0.45% KH_2PO_4 (w/v) by centrifugation at 1000×g/min for 10 min. Later, the pellet was resuspended in 100 μL of PBS at 0.4 μg/mL H_2DCF-DA, then incubated for 1 h at 26 °C in the dark. Dichlorofluorescein resulting from the oxidation of H_2DCF-DA by ROS was measured through flow cytometry (Beckman Coulter Cytomics, FC500). Rotenone at 50 μg/mL was used as the ROS generation positive control.

2.6. Cell line and culture conditions

U-937 promonocytes (CRL1593.2™) (ATCC, USA) were grown in

RPMI 1640, pH 7.2, with 10% SFB and 1% antibiotics at 37 °C and 5% CO_2. The medium was changed every three days until use.

2.7. Evaluation of macrophage infection

U937 promonocytes were differentiated into macrophages according to the methodology proposed by Palacios et al. (2017). For the infectivity assay, the cells were infected with promastigotes in the early stationary phase (4-days old) in a ratio of 15 parasites per cell, corresponding to the number of parasites needed to infect 50% of macrophages. The parasites were resuspended in 500 μL of RPMI 1640 medium free of SFB and at different concentrations of L-arginine (0, 50, 100, 200, and 300 μg/mL). After incubation for 3 h at 34 °C and 5% CO_2, the extracellular parasites were removed by washing three times with PB. 1 mL of fresh medium was added, maintaining the different concentrations of L-arginine supplemented with 5% FBS, and incubated again for 24 h at 34 °C and 5% CO_2. Next, the cells were washed and prepared to evaluate infection through optical microscopy with Giemsa staining. The number of infected cells and the number of amastigotes per cell (parasitic load) were evaluated by counting 200 cells in total. The experiment was performed in two independent experiments, each in triplicate (n = 6).

2.8. In vitro antileishmanial activity against intracellular amastigotes

The cells were infected following the same procedure described in the infectivity assay. Once the cells were washed after 24 h of infection at 34 °C in conventional RPMI 1640 + FBS 5%, the medium was changed to RPMI 1640 L-arginine free medium + FBS 5%, modified at the different concentrations of L-arginine (0, 50, 100, 200, and 300 μg/mL) and the half-effective concentration (EC_{50}) of each antileishmanial drug, as follows: MA (56.25 μg/mL), pentamidine (PEN) (27.8 μg/mL), Miltefosine (MIL) (37 μg/mL) and Amphotericin B (AMB) (0.039 μg/mL), as previously described for *L. braziliensis*-EGFP (Palacios et al., 2017). Next, they were incubated for 72 h at 34 °C. After this time, the cells were prepared for flow cytometry reading according to the methodology described previously. The antileishmanial activity was determined according to the reduction of infected cells percentages obtained for each experimental condition.

2.9. Statistical analysis

Each experiment was performed twice in triplicates. Differences between experimental groups were analyzed using ANOVA with *posthoc* Tukey's test and were considered statistically significant with $p < 0.05$. The normality, independence, and homoscedasticity were verified using GraphPad Prism 8.0.2 software (GraphPad Software, USA).

3. Results

3.1. The L-arginine depletion affects the L. braziliensis (UA301-EGFP) in vitro replication

It was observed that the absence of L-arginine generated a reduction in the replication ratio, increasing the amount of parasite twice its initial concentration until the fourth day when all experimental groups reached the maximum peak of the exponential phase. On the fifth day, a decrease in the parasites per mL was observed in all the conditions (Fig. 1A). The amount of parasites increase at L-arginine concentrations from 50 to 100 μg/mL, even though it did not reach the growth rate in the control group (200 μg/mL). Unlike the parasite's growth in the absence of L-arginine, the control group and the highest L-arginine evaluated concentration (300 μg/mL) evidenced an increase in the number of parasites of ~23 and ~28 times, respectively, their initial concentrations. Although more parasites were observed in the 300 μg/mL concentration, no statistically significant difference was found between them (Fig. 1A). The parasite's growth was directly proportional to the concentration of L-arginine in the medium ($R^2 = 0.9326$) at four days. No statistically significant differences were found in the growth between those cultivated at 200 and 300 μg/mL of L-arginine. However, statistically, significant differences were observed in the number of parasites per mL per hour of culture in all treatments compared to the treatment in the absence of L-arginine (Fig. 1B).

3.2. L-arginine is not essential but can improve the promastigote-amastigote differentiation in a dose-response manner

After observing that the parasite's replication was affected by the absence of L-arginine, we decided to evaluate its effect on the promastigote-amastigote differentiation. Promastigotes' density at 48 h was similar in all the experimental groups, except in the 300 μg/mL

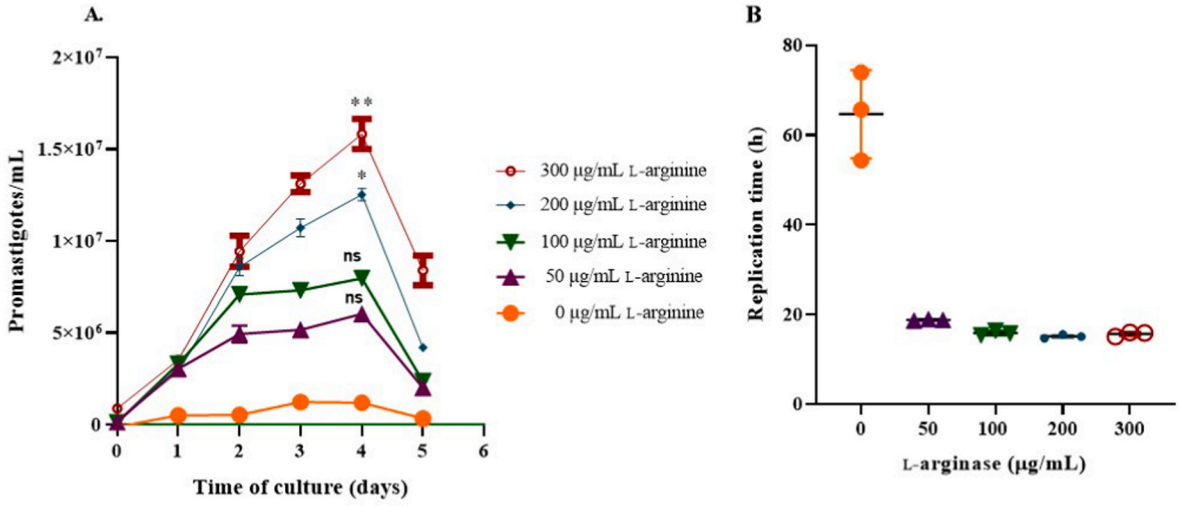

Fig. 1. Effect of different extracellular L-arginine **concentrations on the *L. braziliensis in vitro* growth.** A growth curve was performed using Rezasurin method for 5 days, under different concentrations of L-arginine (from 300 μg/mL to 0 μg/mL). Error bars for SD of six values, three replicates in two experiments (n = 6) (A). Moreover, the parasite's replication was calculated per hour of culture, under each L-arginine levels availability mentioned above and compared to the absence of the L-arginine experimental group (B). Statistically significant differences between L-arginine 0 μg/mL and the other groups was represented $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (****). NS: No statistical differences were observed.

treatment, where there was an increase in the number of promastigotes (Fig. 2A). However, at 96 h, a dramatic decrease in the promastigote's density was observed since these are sensitive to heat shock. Furthermore, it was evidenced that the L-arginine depletion did not affect promastigote/amastigote differentiation. However, the depletion increased de transformation process, increasing the number of amastigotes obtained in lower concentrations (50 and 100 μg/mL), for 96 h, evidenced by the presence of 15% of promastigotes and 85% of amastigotes in the cultures with 50 and 100 μg/mL of L-arginine ($p < 0.0001$ and $p < 0.001$, respectively (Fig. 2B). These results suggest that L-arginine concentration can modulate the *in vitro* differentiation efficiency.

3.3. L-arginine is depleted by L. braziliensis-EGFP promastigotes as they replicate

To explain the growth curve and parasite's differentiation findings, we attempted to elucidate how the L-arginine uptake was during the parasite's replication. A gradual decrease in the L-arginine concentration in the exometabolome was observed as the number of parasites in the culture medium increased (Fig. 3A; 3B). Furthermore, it was found that an increment in the L-arginine concentration (300 μg/mL) generated an increase in the amino acid uptake (Fig. 3A). In all the experimental groups, L-arginine is consumed until approximately 50 μg/mL, indicating that it is the equilibrium level needed biologically (See Figures in the Supplementary Mass spectra Material). The condition of L-arginine absence was demonstrated when the amino acid was not detected. A statistically significant replication was evidenced in parasites cultured with 200 and 300 μg/mL of L-arginine between days 0 and 5 of culture ($p < 0.0001$). In the absence of L-arginine, no statistically significant differences were observed in the parasite's replication at the evaluated times, which agrees with the results previously shown in the growth curve (Fig. 1A).

3.4. ROS production is not efficiently regulated in the absence or low concentrations of L-arginine

Due to the ROS relation with cellular death, we proposed to evaluate its production under the previously tested culture conditions. All L-arginine evaluated concentrations showed ROS production at 4 h of culture (Fig. 4). Parasites grown in the presence of 200 μg/mL and 300 μg/mL of L-arginine showed a reduction of ROS levels at 4 h, with ($p < 0.001$ and $p < 0.0005$, respectively) (Fig. 4). These results demonstrate that the absence or low concentrations of the amino acid do not allow efficient regulation of ROS production.

3.5. The absence of L-arginine reduces L. braziliensis-EGFP infectivity and parasitic load in vitro

Because no effect was observed with the absence of L-arginine on the promastigote to axenic amastigote differentiation, we attempted to investigate whether L-arginine availability would affect the *in vitro* macrophage infection. The parasitic load was 5.9 ± 0.6, 6.7 ± 1.0, 7.0 ± 0.6, 8.2 ± 0.5, 9.1 ± 0.9 amastigotes per cell for 0, 50, 100, 200 and 300 μg/mL L-arginine respectively. *L. braziliensis* infectivity and parasitic load showed a statistically significant decrease in the absence of L-arginine compared to the control group (200 μg/mL) ($p < 0.001$) and the treatment with 300 μg/mL ($p < 0.0001$). This decrease was evidenced by ~23% fewer amastigotes per cell and 6–10% fewer infected cells, respectively. Nevertheless, there were no statistically significant differences between the groups with 50 and 100 μg/mL of L-arginine compared to the absence of the L-arginine group in neither of the two experiments.

3.6. L-arginine availability does not alter the effectiveness of the evaluated antileishmanial drugs

L-arginine metabolism products are related to the detoxification of some antileishmanial drugs. Therefore, we decided to test the effect of amino acid availability on the effectiveness of some antileishmanial compounds. It was found that the effectiveness of the evaluated compounds is independent of the L-arginine concentration. The average infection percentage of experimental groups was: 60.67 ± 3.95 for MA (Fig. 5A), 50.33 ± 3.32 in the presence of PEN (Figs. 5B), 57.27 ± 4.27 for MIL (Figs. 5C), and 19.18 ± 2.96 for AMB (Fig. 5D), reflecting a similar behavior on sensibility in all the different L-arginine availability levels.

4. Discussion

L-arginine is an amino acid with an essential role in the parasite, like in the host cell. *Leishmania* spp, through the L-arginine catabolism by the action of arginase (ARG), produces polyamines and trypanothione, which are fundamentals for the parasite's replication and infectivity, and for the defense against oxidative stress, respectively. On the other hand, the host cell metabolizes the L-arginine by employing Nitric oxide synthase 2 (NOS2) to produce nitric oxide and induce the parasite's death by oxidative stress (Munder et al., 2009). The L-arginine importance for *in vitro* growth of some *Leishmania* species such as *L. donovani* and *L. tarentolae* has been demonstrated (Mandal et al., 2016; Nayak et al., 2018). However, its importance in *L. braziliensis* has not yet been evaluated. The present work demonstrated that the absence of L-arginine

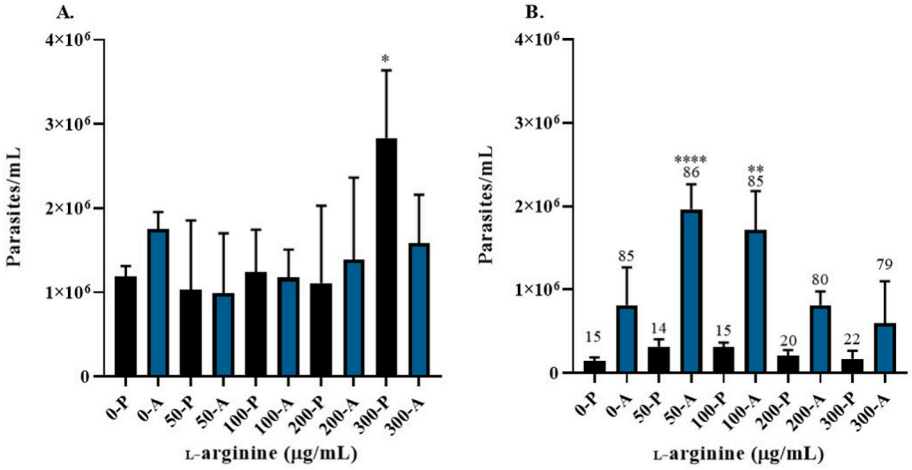

Fig. 2. *In vitro* promastigotes *L. braziliensis* differentiation to axenic amastigotes in response to variations in the extracellular L-arginine concentrations. *L. braziliensis* promastigotes were grown for 4 days in a RPMI medium supplemented with different L-arginine concentrations. Post culture, they were subjected to changes in pH and temperature, inducing a differentiation to axenic amastigotes, as is specified in the materials and methods. The density on the proportion of promastigotes-P and amastigotes-A were evaluated according to the L-arginine availability at 48 h (A) and 96 h of culture (B). The number of amastigotes was compared between all experimental groups at 96h of culture. Statistically significant differences $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (****). NS: No statistical differences were observed. Data are expressed as the mean ± SD of two independent experiments, each in duplicate (n = 4).

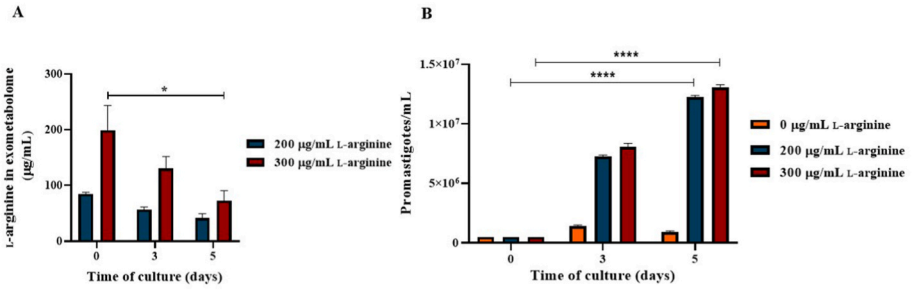

Fig. 3. Effect of L-arginine uptake on the *Leishmania braziliensis*-EGFP growth. *L. braziliensis* promastigotes were cultured in RPMI medium and supplemented as mentioned in materials and methods. The L-arginine uptake was evaluated over the culture time on the third and fifth day through quantification of L-arginine by HPLC-MS (A) and its relation with the parasite's growth curve in the different concentrations at the same time (B). Statistically significant differences $p < 0.05$ (*), $p < 0.0001$ (****). No statistical differences were observed (NS). Error bars for SD of two independent assays, each one for triplicate (n = 6).

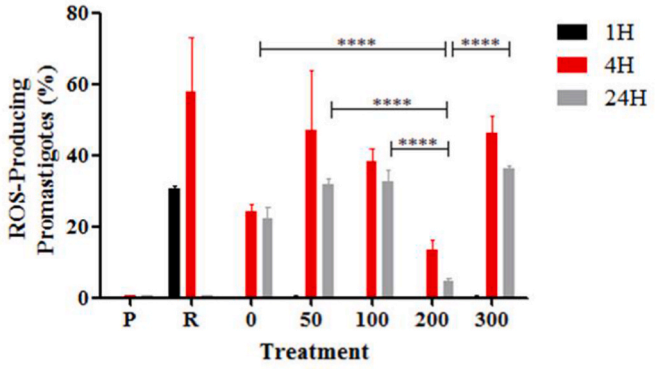

Fig. 4. Effect of L-arginine availability on the ROS production in promastigotes of *L. braziliensis*.
ROS production in the different culture conditions was evaluated at 1, 4 and 24 h using H_2DCF-DA dye stain by flow cytometry. The *x*-axis corresponds to treatments: untreated non-stained cells or plain (P), Rotenone at 50 µg/mL as positive control (R), and the concentrations of L-arginine (0, 50, 100, 300 µg/mL) and the control 200 µg/mL. The *y* axis corresponds to the percentage of promastigotes producing ROS. Statistically significant differences $p < 0.0001$ (****). Error bars for SD of two independent assays, each one for triplicate (n = 6).

affects the *in vitro L. braziliensis* growth (Fig. 1A). The addition of extracellular L-arginine seems to have a directly proportional relationship with *in vitro* parasite replication. A previous study reported similar results for *L. donovani*, but the effect of L-arginine excess was not evaluated (Mandal et al., 2016). A study conducted by Badirzadeh et al. (2017) demonstrated that in *L. major* and *L. tropica*, the expression and enzymatic activity of ARG were higher in promastigotes during their exponential growth than in the stationary phase (Badirzadeh et al., 2017). This finding could explain that the difference in growth curves was observed mainly in the logarithmic phase, where the slope decreases as the concentration of L-arginine in the medium diminish. Additionally, ARG deficient *L. mexicana* and *L. donovani* promastigotes were auxotrophic for polyamines and required supplementation of ornithine or putrescine, products of the L-arginine metabolism to grow (Badirzadeh et al., 2017).

According to the parasite replication findings, we evaluated the effect of L-arginine availability changes on the promastigote to amastigote differentiation. It was evidenced that the lower concentrations evaluated (50 and 100 µg/mL) improved amastigote survival or replication in the long term (96 h). It is known that low L-arginine concentration simulates the stress conditions to which the parasite is subjected in the phagolysosome (Goldman-Pinkovich et al., 2016) and may improve the methodology to produce axenic amastigotes by including the nutritional stress.

The L-arginine transport by *Leishmania* species was evaluated indirectly by measuring the amino acid in the exometabolome because the parasite is auxotrophic for the L-arginine and, therefore, must take the amino acid from the external medium through membrane transporters as the *Ld*AAP3 (Shaked-Mishan et al., 2006). A depletion in the exometabolome of L-arginine to approximately 50 µg/mL on the fifth day of growth was observed, which corresponds to the death phase. These findings suggest that these concentrations are not enough to support the parasite's replication.

Another explanation for the decrease in parasite proliferation in the absence of L-arginine could be ROS accumulation. A decrease in the levels of thiol groups such as trypanothione, an antioxidant synthesized from the L-arginine or ornithine and polyamines (putrescine and spermidine) (Munder et al., 2009) may result in an inability to regulate ROS production (Mandal et al., 2016). This observation is consistent with the results found in the present study for *L. braziliensis*, where the absence of L-arginine and all the concentrations increased ROS levels at 4 h. Also, it was observed that despite the increase of ROS production at 4 h by the L-arginine excess (300 µg/mL), it could regulate the production at 24 h as the control. The other experimental groups could not efficiently regulate the ROS production, which agrees with the lowest replication levels observed in the growth curve. However, the specific modification of this amino acid generates an imbalance in the ROS production, allowing us to propose that the availability of other nutrients such as Glucose and some amino acids is insufficient to make an adequate redox balance (Filomeni et al., 2015).

Also, this finding agrees with previous studies, where the induction of ROS production by molecules as the hydrogen peroxide generates the most significant effect on the promastigote-amastigote *in vitro* differentiation compared to the effect of changes in pH and temperature (Khan et al., 2018). In this work, at low levels of L-arginine (50 and 100 µg/mL) was observed, an increment in the ROS production at 4 h, remaining until 24 h, favoring the promastigote-amastigote *in vitro* differentiation until the fourth day of culture.

On the other hand, we found a decrease in the percentage of macrophage infection and parasite load in the absence of L-arginine. Iniesta et al. (2001) suggested the participation of Nv-hydroxy- L-arginine (LOHA), a physiological inhibitor of ARG, evaluated in *L. major* and *L. infantum,* which is used by the macrophages as a defense mechanism against *Leishmania* infection (Shaked-Mishan et al., 2006). This inhibitor leads to a dramatic decrease in both the number of intracellular amastigotes and the percentage of infected cells. This finding can be explained because ARG metabolizes L-arginine as the only substrate for polyamines essential for intracellular parasite replication (Iniesta et al., 2001). Moreover, the authors proved that the antiparasitic effect of LOHA was not due to the production of NO by the macrophage, and the observed effects indicate the mere inhibition of ARG in the parasite. Thus, the absence of L-arginine may be causing the same effects as LOHA by a related mechanism. In addition, the specific activity of ARG has a direct relationship with the replication of amastigotes (Marchese et al., 2018), emphasizing the importance of the enzyme and, therefore of the amino acid in infectivity and intracellular division (Muxel et al., 2018; Marchese et al., 2018).

Because the products of L-arginine metabolism are related to the detoxification of antileishmanial (Purkait et al., 2012), the impact of

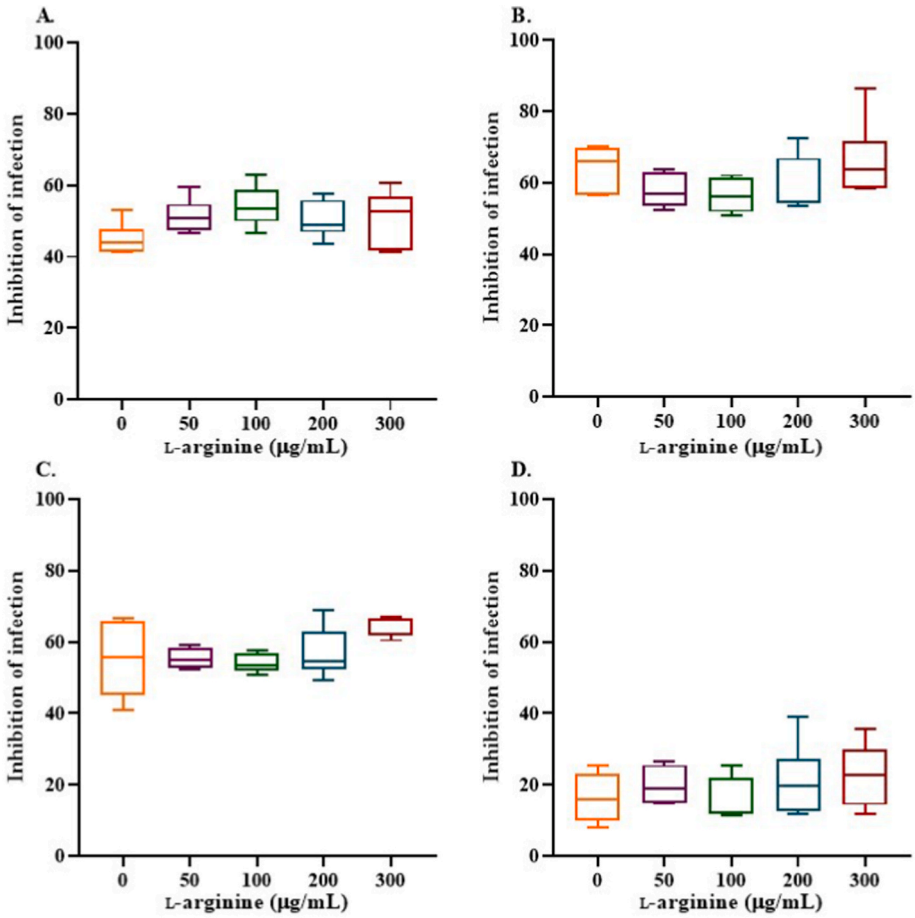

Fig. 5. Effect of the L-arginine concentration on the infection inhibition by antileishmanial agents. The graph shows the % of uninfected cells after administration of EC_{50} (y-axis) of MA: meglumine antimoniate (A); PEN: pentamidine (B); MIL: Miltefosine (C); AMB: Amphotericin B (D) and different L-arginine availability levels (x-axis). No statistical differences were observed (NS). Error bars represent the standard deviation of two independent experiments, each in triplicate (n = 6).

variation in L-arginine availability on the effectiveness of antileishmanial drugs (MA, PEN, MIL, and AMB) was also evaluated. Although our results showed no statistical differences in the drug's effectiveness, it has been reported that isolates of *L. tropica* resistant to MA present an overexpression of the specific L-arginine transporter (*Ld*AAP3) (Kazemi-Rad et al., 2013). L-arginine contributes to the increase in the levels of trypanothione and drug detoxification. Similar to AMB has been reported in resistant parasites, which efficiently eliminates ROS produced by the compound autoxidation through the biosynthesis of polyamines and thiol groups (Mandal et al., 2016; Purkait et al., 2012). Moreover, some authors proposed the PEN mechanism of action as competitive and non-competitive inhibition of L-arginine and polyamines transporter in *Leishmania*, respectively (Singh et al., 2012). Even though there is a relation with the amino acid, we concluded that the extracellular concentration of the amino acid does not matter since its mode of action is to prevent its transport, which is even effective in conditions of excess of the amino acid (300 µg/mL). Because the infectivity of newly transformed amastigotes with low concentrations of L-arginine was not evaluated in the present study, further studies are needed to determine if the increase in the number of amastigotes is related to high infectivity to macrophages. Nonetheless, this study lays the foundations for developing future projects that make it possible to contribute to understanding resistance phenomena in *Leishmania* species and thus improve the currently available therapies or propose new therapeutic targets.

5. Conclusions

Our findings support that the absence of L-arginine affects the *in vitro* growth and infectivity of *L. braziliensis*. In addition, the limited L-arginine concentrations favor the *in vitro* differentiation from promastigote to amastigote. Despite the importance of L-arginine availability in the parasite, its depletion in the medium did not alter the *in vitro* sensitivity to four drugs tested, including the first line antileishmanial drug. In addition, L-arginine metabolism is a promising set of therapeutic targets for new treatments for infections caused by *L. braziliensis* and other *Leishmania* species where similar effects have been demonstrated.

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Author statement

The authors declare that this work did not include animals or human subjects.

Declaration of competing interest

No conflict of interest is declared.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2022.108395.

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