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Original article

Promastigote parasites cultured from the lesions of patients with mucosal leishmaniasis are more resistant to oxidative stress than promastigotes from a cutaneous lesion

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ABSTRACT

Human leishmaniasis caused by *Leishmania (Viannia) braziliensis* can be presented as localized cutaneous leishmaniasis (LCL) or mucosal leishmaniasis (ML). Macrophages kill parasites using nitric oxide (NO) and reactive oxygen species (ROS). The aim of this study was to evaluate the ability of parasites obtained from patients with LCL or ML to produce and resist NO or ROS. Promastigotes and amastigotes from LCL or ML isolates produced similar amounts of NO in culture. Promastigotes from ML isolates were more resistant to NO and H_2O_2 than LCL parasites in a stationary phase, whereas amastigotes from LCL isolates were more resistant to NO. In addition, in the stationary phase, promastigote isolates from patients with ML expressed more thiol-specific antioxidant protein (TSA) than LCL isolates. Therefore it is suggested that infective promastigotes from ML isolates are more resistant to microbicidal mechanisms in the initial phase of infection. Subsequently, amastigotes lose this resistance. This behavior of ML parasites can decrease the number of parasites capable of stimulating the host immune response shortly after the infection establishment.

1. Introduction

Leishmaniasis are endemic neglected diseases present in approximately 88 countries, including Europe and the Mediterranean, South America, Central Asia, and Africa [1,2]. The natural infection starts when a sand fly vector inoculates metacyclic promastigotes into the host skin. Promastigotes rapidly invade resident phagocytes in the dermis and become intracellular amastigotes [3–5]. Several species cause this disease, *Leishmania (Viannia) braziliensis*, which is the major one responsible for localized cutaneous (LCL), mucosal (ML), or mucocutaneous leishmaniasis (MCL) in Brazil [2,6,7].

Most of the *Leishmania* species are killed in murine models, mainly because of nitric oxide (NO) production by the inducible isoform of the NO synthase (iNOS) enzyme, which is expressed after the activation of the macrophage by cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). Cytokine-activated macrophages can produce reactive oxygen species (ROS), which are also induced by the phagocytosis of parasites [8–10].

In humans, LCL appears as ulcerated skin lesions that typically develop at the infection site and often heal spontaneously. Despite the cure of primary lesions, approximately 3–10% of *L. (V.) braziliensis*-infected individuals develop ML after several months or years, indicating that the parasites were not completely eradicated from the lesions [7,11]. ML is a severe form of the disease which shows destructive lesions located mainly in the mucosal tissue of nose and mouth [12,13]. Despite the high production of and IFN-γ TNF-α, parasites causing ML are not efficiently killed, and the strong inflammatory response leads to an increase in the lesion size [14–16]. The reason for the occurrence of ML in only a small number of individuals is not completely known. Furthermore some factors present in the host, such as

Abbreviations: TSA, thiol-specific antioxidant protein; NO, nitric oxide; ROS, reactive oxygen species; LCL, localized cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; ML, mucosa leishmaniasis; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; iNOS, inducible nitric oxide synthase, FCS, fetal calf serum; MTT, 3-(4,5-dimethyl-2-thiazolyl) − 2,5-diphenyl-2H-tetrazolium bromide; SC, subcutaneously; SNP, sodium nitroprusside

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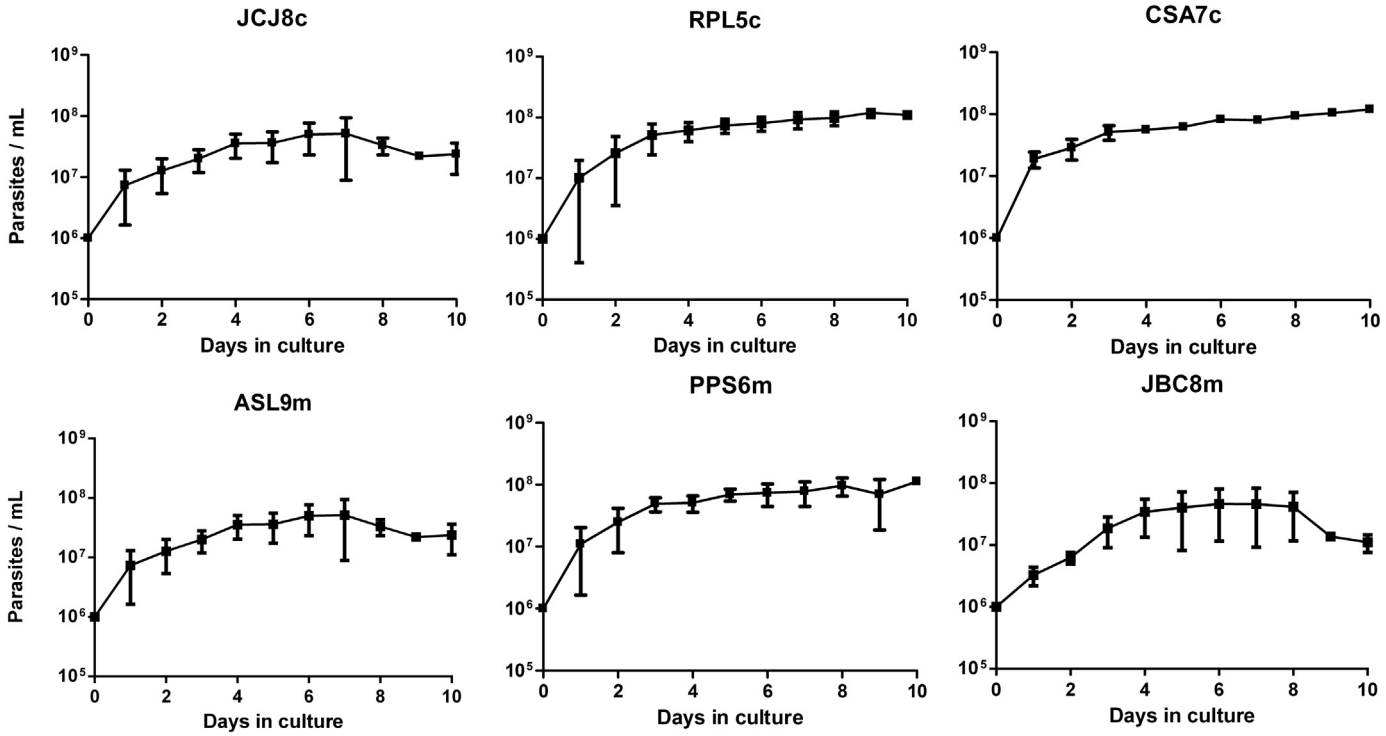

Fig. 1. *In vitro* growth curves of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. The parasites from LCL (JCJ8c, RPL5c and CSA7c) or ML (ASL9m, JBC8m and PPS6m) isolates were cultured in complete Grace's insect medium for ten days. The data represent mean ± SEM of the number of promastigotes in three independent experiments performed in triplicate.

polymorphisms in encoding proinflammatory cytokines and chemokines genes have been described as the possible causes [17–20]. In contrast, recent studies revealed that isolates from patients with ML had increased arginase and ectonucleotidase activity and decreased expression of prostaglandin F2 α-synthase compared those from patients with LCL [16,21,22]. In addition, our previous study showed that amastigotes from ML isolates are more susceptible to NO than those from LCL ones and have high ability to disseminate [23]. These data suggest that ML and LCL isolates behave differently when infecting a mammalian host, which probably contributes to the pathogenesis of the disease.

Here we employed amastigotes and promastigotes of *L. (V.) braziliensis* isolates obtained from patients with ML and LCL to investigate the differences in the parasite susceptibility to microbicidal products from macrophages. A changes in the resistance ability of ML and LCL isolates against NO or ROS in during vertebrate infection may play a crucial role in the leishmaniasis pathogenesis.

2. Materials and methods

2.1. Mice

Male or female C57BL/6 mice with disrupted IFN-γ genes (IFN-γ KO mice) and female BALB/c mice were bred at the animal facility of the Federal University of Goiás/IPTSP, Brazil. IFN-γ KO mice were originally purchased from Jackson Laboratories, ME/USA (B6.129S7-ifng^{tm1Ts}). The mice used in this study were 6–12 weeks old and maintained in a clean conventional mouse facility with ad libitum access to water and food. All the experimental procedures were performed according to the guidelines from the Ethics Committee on the Animal Use of the Federal University of Goiás, approved under the protocol number 062/2010.

2.2. Parasites

In this study, the isolates obtained from the mucosal lesions of patients with ML (MHOM/BR/2009/ASL9m, MHOM/ BR/2006/PPS6m, MHOM/BR/2008/JBC8m, MHOM/BR/2010/NFS10m and MHOM/BR/2009/ILM9m), cutaneous lesions of patients with LCL (MHOM/BR/2005/RPL5c, MHOM/BR/2008/JCJ8c, MHOM/BR/2007/CSA7c, MHOM/BR/2005/WSS5c, MHOM/BR/2005/EFSF5c, MHOM/BR/2006/HPV6c and MHOM/BR/2005/UFA5c) and cutaneous lesions of patients with MCL (MHOM/BR/2007/SMB7mc) were used. Our previous study has characterized parasites as *L. (V.) braziliensis* [24,25]. In this study, ML was considered when the patient did not show any sign of concomitant cutaneous lesions; when the patient show mucosal and cutaneous lesions simultaneously MCL was considered, however, the parasites were isolated from the cutaneous lesion.

To study parasites as amastigotes, 1×10^6 amastigotes obtained from the lesions of the patient were inoculated into the paw of IFN-γ KO mice. When the footpad lesion reached 3–4 mm thickness, mice were euthanized, and the footpad was carefully minced. The amastigotes were separated by centrifugation through a 40% and 90% Percoll® gradient (GE Healthcare, São Paulo, Brazil) as described previously [25].

To obtain promastigotes, the amastigotes were inoculated into Grace's insect medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 20% inactivated fetal calf serum (FCS- Cripion, Andradina, São Paulo, Brazil), 2 mM L-glutamine, 100 U/ML penicillin and 100 µg/ML streptomycin (all purchased from Sigma-Aldrich) in 24-well culture plates (TPP, Switzerland). In five days, after amastigotes transformed into promastigotes, 1×10^6 parasites were transferred to a new medium and split every two days. The parasites from logarithmic and stationary growth phases were harvested in three and ten days, respectively.

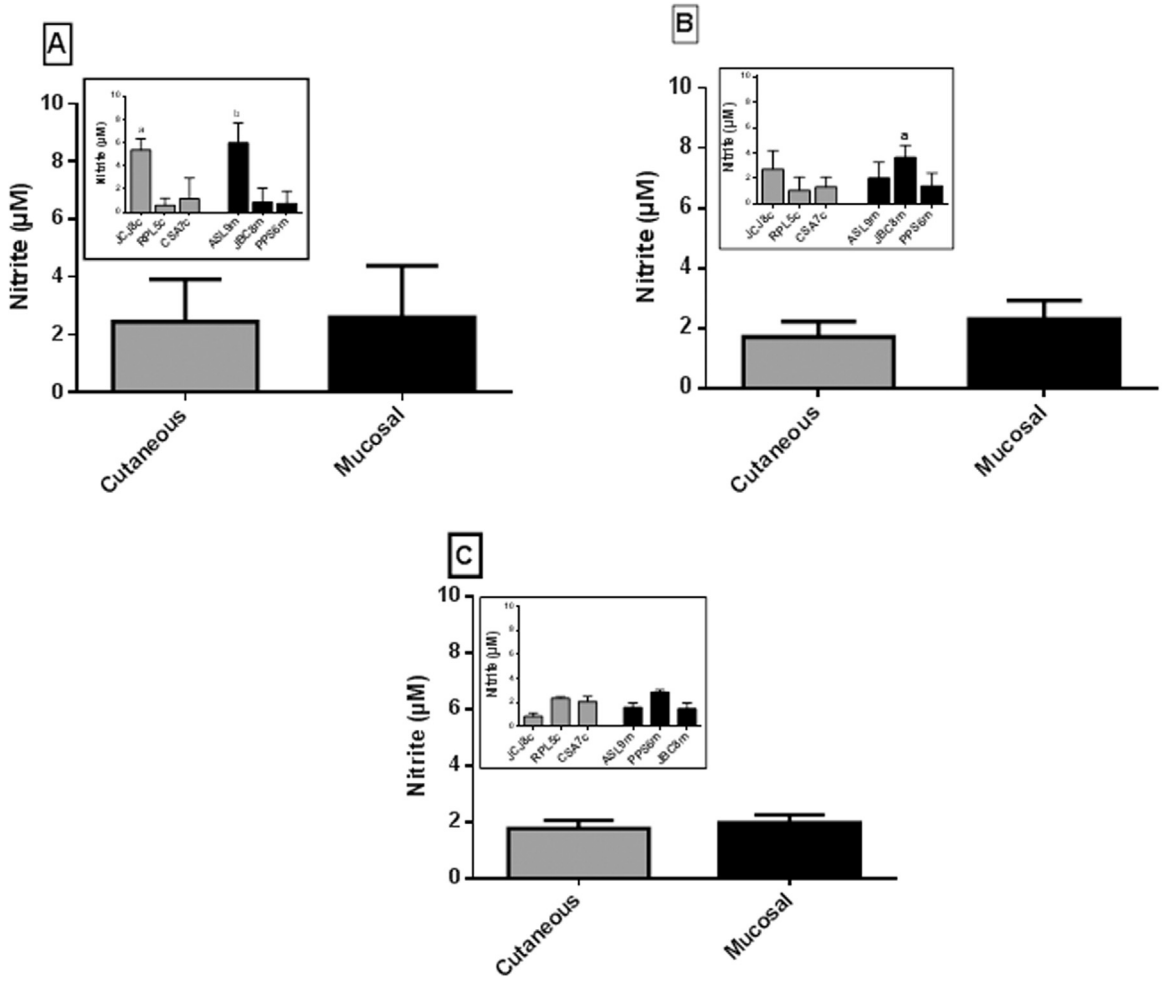

Fig. 2. Nitrite measurement on the supernatants from the cultures of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. Promastigotes in logarithmic-phase (A), stationary-phase (B) or amastigotes (C) of *L.(V.) braziliensis* isolates from LCL (gray bars) ML (black bars) patients were maintained in the culture medium during 24 h at 26 °C (promastigotes) or 48 h at 32 °C (amastigotes). The bars represent the mean ± SD of the nitrite produced per group from four independent experiments performed in triplicate. Insets present mean ± SD of nitrite produced by each isolate. The different letters indicate statistical difference by One-Way ANOVA followed by the Bonferroni test. $p < 0.05$.

2.3. In vitro susceptibility of Leishmania

Promastigotes in the logarithmic or stationary growth phases (1×10^7 parasites/well) and amastigotes (5×10^6 parasites/well) were suspended in 0.1 ML of Grace's insect medium and cultured at 26 °C and 32 °C, respectively, in the presence or absence of various concentrations of H_2O_2 (Merck, São Paulo, SP, Brazil – 0.04; 0.2; 1.0; 5.0; 25 or 100 mM) or sodium nitroprusside (SNP; Sigma-Aldrich – 0.2; 1.0; 5.0; 25, 100 or 500 mM), used as NO donor, for 1 h or 2 h, respectively.

Parasites were washed three times with phosphate buffered saline (PBS), transferred to a 96-well plate and incubated with sterile MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich] at a final concentration of 1 mg/ML in PBS in each well at 26 °C for 4 h (promastigotes) or at 32 °C for 20 h (amastigotes) in supplemented Grace's medium to produce formazan crystals. The reaction was stopped by the addition of 100 μL of aqueous solution containing 50% dimethyl sulfoxide and 10% sodium dodecyl sulfate to each well. The plate was incubated at 36 °C for 12 h, and the absorbance was determined at 550 nm in a microplate reader (Multiskan, Thermo Labsystems, Finland). Assays were performed in triplicate, and the results were expressed as the mean percentage reduction of parasite numbers compared with the untreated control wells calculated for five independent experiments.

2.4. Arginase activity

Leishmania isolates were washed twice with PBS and then the cell pellets (1×10^6 cells) were resuspended in 50 μL of lysis buffer (Tris–HCl 500 mM pH 7.5 and Triton 1%). The arginase activity was determined by the urea production as described previously [26] with some modifications. Briefly, the cell lysate (50 μL) was added to 50 μL of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MnCl_2$ (Vetec, Brazil). These samples were activated by heating the mixture at 56 °C for 10 min. The hydrolysis reaction of L-arginine by arginase was carried out by incubating the lysate with 50 μL of 0.5 M L-arginine (pH 9.7; Sigma Chemical Co., USA) at 37 °C for 1 h. The reaction was stopped by the addition of 200 μL of H_2SO_4:H_4PO_4:H_2O (1:3:7). Subsequently, 25 μL of 9.0% α-isonitropropiophenone solution in absolute ethanol was added and incubated at 100 °C for 45 min for color development, and 120 μL solution from each sample (in duplicate) was transferred to a microplate and read in the ELISA reader on the 550 nm filter. The standard curve was made using the assay detection limit for 0.015 mg/ML of urea.

2.5. Determination of nitric oxide production

Promastigotes (1×10^8) and amastigotes (1×10^6) were incubated at 26 °C for 24 h or at 32 °C for 48 h, respectively. Subsequently, the

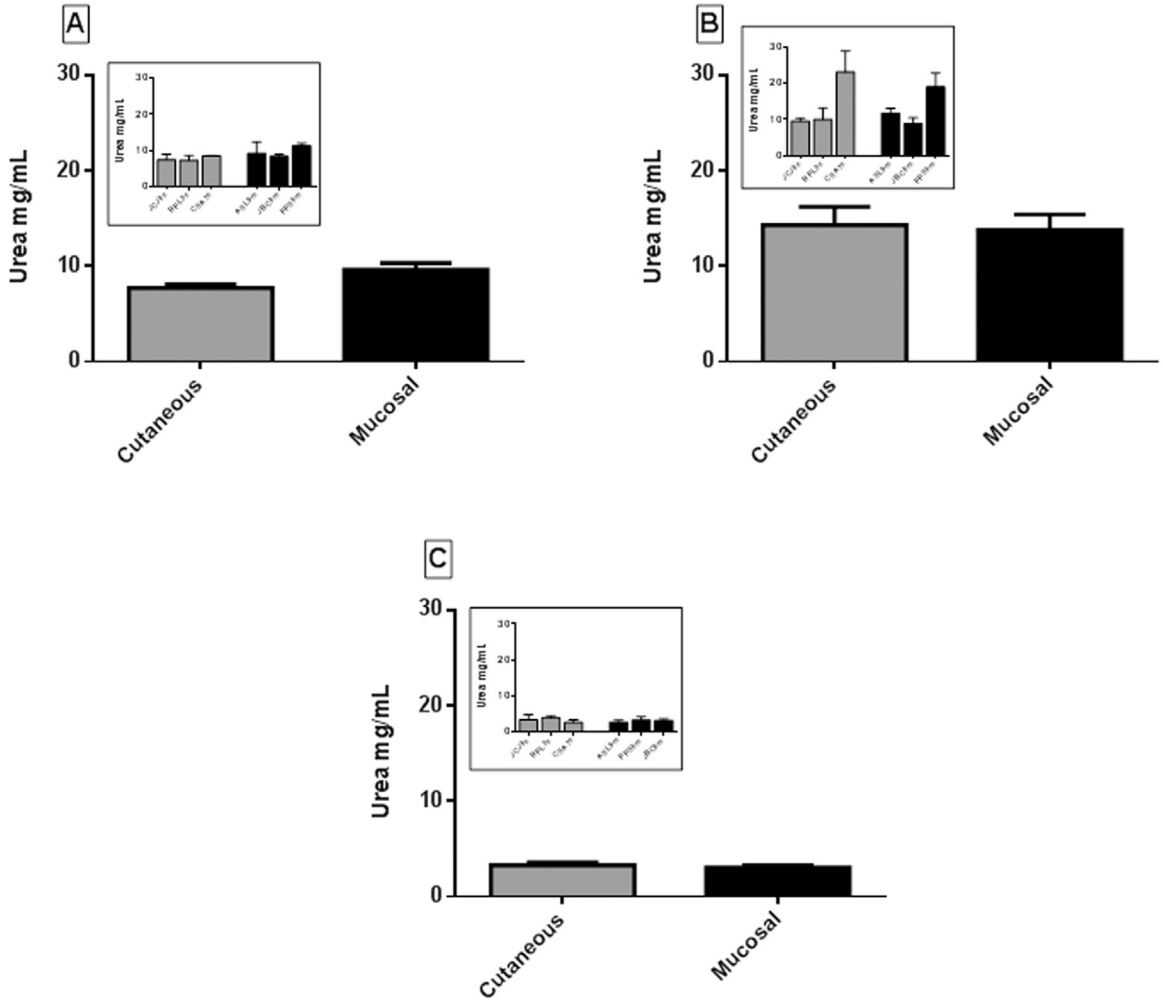

Fig. 3. Arginase activity of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. Promastigotes in logarithmic-phase (A), stationary-phase (B) or amastigotes (C) of *L. (V.) braziliensis* isolates from LCL (gray bars) or ML (black bars) patients were lysed and arginase activity was obtained by the measurement of urea production as described in material and methods. The bars represent the mean ± SD of the urea produced per group from four independent experiments performed in triplicate. The insets present mean ± SD urea produced by each isolate.

supernatant from the *Leishmania* culture was collected and the NO production was estimated using the Griess method [27]. This method is based on a colorimetric reaction in which the culture supernatant (50 µL) is incubated with an equal volume of the Griess reagent (0.5% sulfanilamide, 0.05% N-1-naphthyl-ethylenediamine dihydrochloride, and 2.5% ortho-phosphoric acid) for 10 min at room temperature. The absorbance was determined at 550 nm using a microplate reader. The results were expressed as micromoles of nitrite on the basis of a standard curve established by the known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich) dissolved in the culture medium with a detection limit of 1.5 µM.

2.6. Antibodies against thiol-specific antioxidant protein

Recombinant TSA proteins were obtained as previously described in details [28]. Purified TSA protein was resuspended in PBS. BALB/c mice were injected subcutaneously with 10 µg of recombinant TSA dissolved in 50 µL of PBS plus 50 µL of complete Freund's adjuvant (Sigma-Aldrich). After 30, 60 and 90 days, the mice were injected subcutaneously with protein dissolved in 50 µL PBS plus 50 µL of incomplete Freund's adjuvant (Sigma-Aldrich). After 100 days, mice were euthanized and the sera obtained were used in western blotting or purified in protein G-agarose column (Sigma- Aldrich) followed by biotinylation with Biotin-N-hydroxysuccinimide ester (Sigma-Aldrich) to be used in flow cytometry. Control serum or antibody was obtained from non-immunized mice.

2.7. FACS analysis

Before labeling, parasites were spun, and 1×10^7 cells were resuspended in 250 µL of 3% FCS and 2% paraformaldehyde (Merck) in PBS for 15 min. Parasites were washed twice with 3% FCS in PBS and, resuspended in 0.1% saponin (Sigma-Aldrich) in PBS for 15 min. After washing the parasites twice, they were incubated for 15 min with 2 µg/ML of biotinylated anti-TSA or mouse control antibody in 3% FCS in PBS. The streptavidin-FITC conjugate (Serotec, UK) was added for 20 min, followed by washings with 3% FCS in PBS. A total of 5000 events were collected for each sample in a flow cytometer (C6, Accuri, BD). The percentage of labeled cells and the increase in fluorescence were analyzed using software FCS 4.0 express (De novo software, Los Angeles, CA, USA). The increase in fluorescence was calculated by comparing the average fluorescence values obtained from labeled cells and that obtained from control cells.

2.8. Immunoblot

The extracts from *L.* (*V.*) *braziliensis* promastigotes and amastigotes were obtained after incubation of 1×10^9 parasites in 100 µL of lysis

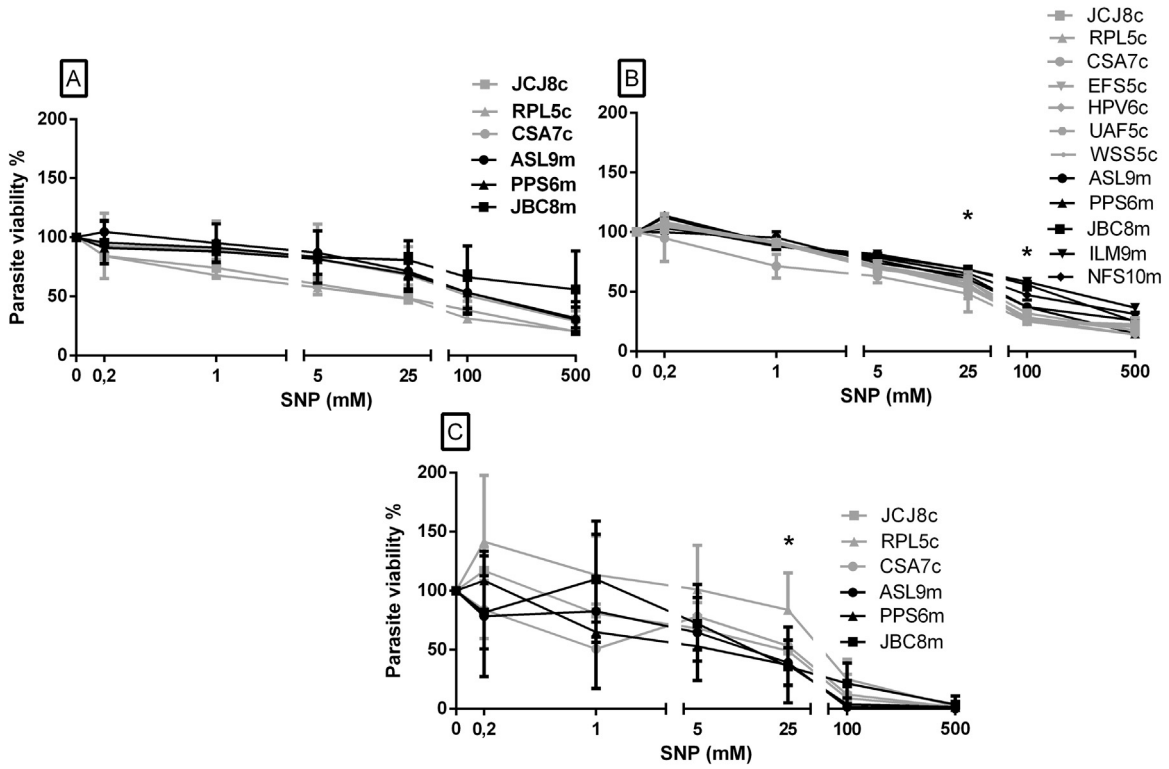

Fig. 4. Effect of sodium nitroprusside (SNP) on the killing of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. Promastigotes in logarithmic-phase (A), stationary-phase (B) or amastigotes (C) of *L. (V.) braziliensis* isolates were cultured for 2 h in complete Grace's insect medium with SNP at the different concentration (0; 0.2; 1.5; 25; 100 and 500 mM) at 26 °C (promastigotes) or at 32 °C (amastigotes). The viability was measured by MTT assay as described in material and methods. The lines represent mean ± SD of the relative sensitivities to the SNP-induced toxicity of five independent experiments performed in triplicate. * indicates statistical difference between LCL and ML parasites by One-Way Student´s *t*-test ($p < 0.05$). # indicates p value for statistical comparison between LCL and ML parasites.

buffer (10 mM Tris-HCl pH 7.6; 150 mM NaCl; 2% SDS; and 1% proteinase inhibitor cocktail; all from Sigma-Aldrich). Furthermore, 10 µL of suspension contained 2×10^7 lysed parasites were suspended in sample buffer (62.5 mM Tris pH 6.8; 2.5% SDS; 10% glycerol; 2.5% β-mercaptoethanol; 1% EDTA; and 0.015% bromophenol blue) and injected into 12% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% powdered skim milk in PBS and incubated for 1 h with sera diluted at 1:400 from immunized BALB/c mice or anti-GAPDH diluted at 1:3000 (Sigma-Aldrich). After washing with 0.05% Tween 20 in PBS, the nitrocellulose strips were incubated with peroxidase-conjugated anti-mouse IgG and then developed with SuperSignal West Pico substrate (Pierce Biochemicals Inc., Rockford, IL, USA).

2.9. Ethical considerations

The Ethics Committee for Human and Animal research of the "*Hospital das Clínicas*" of the Federal University of Goiás approved all procedures reported in this study (Protocol number 062/2010).

2.10. Statistical analysis

Data were presented as mean ± standard deviation and compared for significance by Student's *t*-test or ANOVA followed by Bonferroni's test using the Graph-Pad Prism Software 5.0 (Inc. San Diego, CA, USA). The value of $p < 0.05$ was considered significant.

3. Results

3.1. Parasites from patients with localized cutaneous or mucosal leishmaniasis share characteristics in culture

L. (V.) braziliensis isolates obtained from patients with LCL (JCJ8c, RPL5c, and CSA7c) or ML (ASL9m, PPS6m, and JBC8m) were cultured as promastigotes for ten days to evaluate their growth kinetics. The proliferation of the parasites was similar, reaching the stationary phase between the third and fifth-day of culture without loss of viability until the tenth day (Fig. 1).

The ability of promastigotes and amastigotes to produce NO (Fig. 2A-C) and urea (Fig. 3A-B) was evaluated in culture supernatants and cell lysate respectively. The amount of NO and urea produced by promastigotes (logarithmic or stationary phase) and amastigotes was similar when comparing LCL and ML isolates. An individual analysis of the isolates showed that some of them produced more NO in the logarithmic or stationary phase of culture (Fig. 2A and B; inset). Regarding the urea production, differences were observed between individual isolates only in the stationary phase (Fig. 3B; inset). It was not possible to associate differences in the NO or urea production with different clinical forms of the disease.

3.2. Stationary phase mucosal leishmaniasis promastigotes are more resistant to nitric oxide and H_2O_2 than localized cutaneous leishmaniasis promastigotes

ROS and NO are the major compounds produced by macrophages that can kill most *Leishmania* parasites, despite their ability to produce NO [10,29–31]. To evaluate the resistance of the isolates to these molecules, LCL and ML parasites were incubated at different

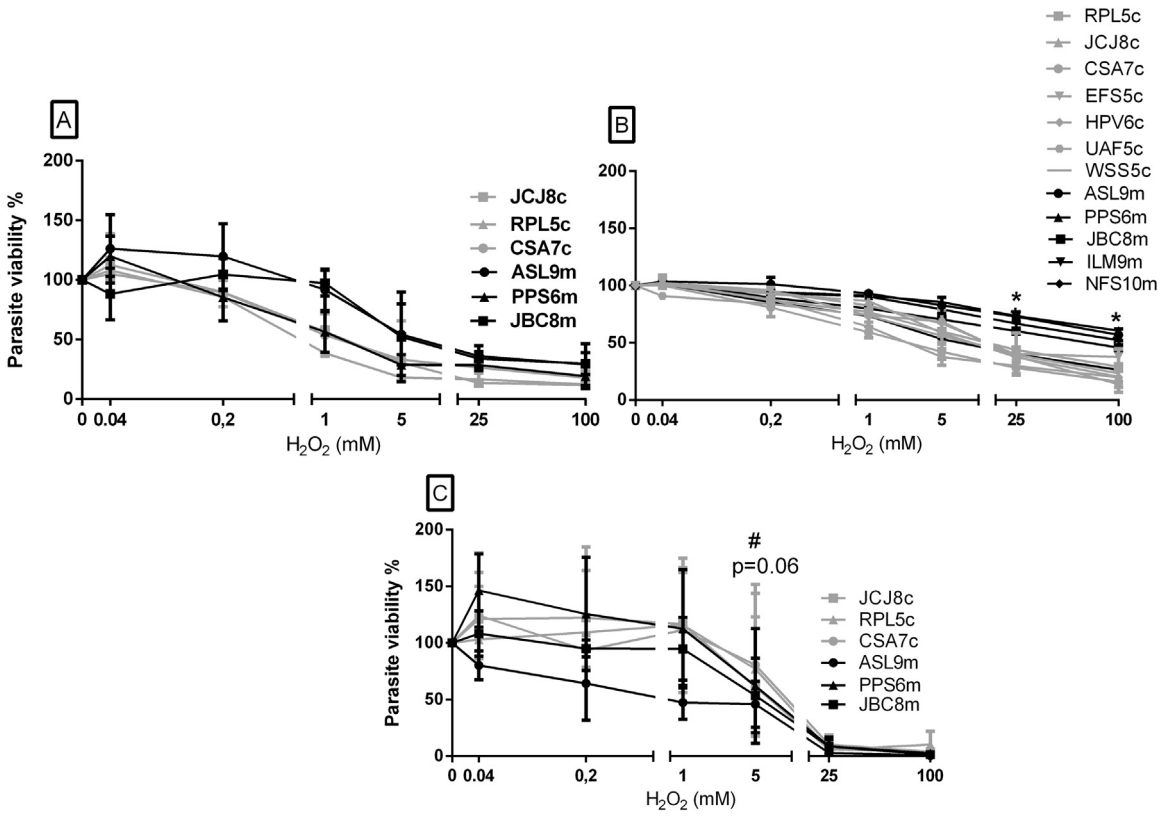

Fig. 5. Effect of H_2O_2 on the killing of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. Promastigotes in logarithmic-phase (A), stationary-phase (B) or amastigotes (C) of *L. (V.) braziliensis* isolates were cultured for 1 h in complete Grace's insect medium with H_2O_2 at different concentrations (0; 0.04; 0.2; 1.0; 5.0; 25, 100 mM) at 26 °C (promastigotes) or at 32 °C (amastigotes). The viability was measured by MTT assay as described in materials and methods. The lines represent the relative sensitivities to H_2O_2-induced toxicity from five independent experiments performed in triplicate. * indicates statistical difference between LCL and ML parasites by One-Way ANOVA followed by the Bonferroni test ($p < 0.05$).

Table 1
Viability of LCL and ML promastigotes in logarithmic growth phase cultured in the presence of SNP.

L.(V.) braziliensis	IC50 ± SE (mM)	95% IC
Isolates		
JCJ8c	16.36 ± 5.67	5.44–49.1
RPL5c	15.21 ± 6.61	4.07–56.8
CSA7c	56.47 ± 15.47	14.3–223.0
Mean	**29.35 ± 13.57**	
ASL9m	43.43 ± 9.39	14.2–132.6
JBC8m	51.37 ± 12.79	3.74–705.7
PPS6m	65.10 ± 7.87	25.97–163.2
Mean	**53.30 ± 6.33**	

LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.
SNP sodium nitroprusside.

Table 2
Viability of LCL and ML promastigotes in in stationary growth phase cultured in the presence of SNP.

L. (V.) braziliensis	IC_{50} ± SE (mM)	95% IC
Isolates		
JCJ8c	17.53 ± 4.78	9.29–33.09
RPL5c	15.44 ± 2.97	10.36–23.01
CSA7c	17.18 ± 5.30	7.34–40.21
UAF5c	14.20 ± 3.89	8.25–24.44
HPV6c	18.24 ± 3.62	11.72–28.41
EFS5c	13.70 ± 3.65	8.47–22.18
WSS5c	14.20 ± 3.89	8.25–24.44
Mean	**15.78 ± 0.70**	
ASL9m	21.26 ± 5.01	10.52–42.97
JBC8m	31.23 ± 5.28	18.03–40.00
PPS6m	23.12 ± 4.41	7.11–25.44
ILM9m	24.97 ± 4.33	28.61–46.61
NFS10m	14.88 ± 4.16	27.89–45.18
Mean	**23.09 ± 2.65***	

LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.
SNP, sodium nitroprusside.
* indicates statistical difference between LCL and ML parasites by student´s *t*-test ($p < 0.05$).

concentrations of NO donor (SNP) or ROS compound H_2O_2 (Figs. 4 and 5; Tables 1–6). SNP and H_2O_2 showed dose-dependent effects when incubated with parasites (Figs. 4 and 5). Stationary phase ML promastigotes were more resistant than LCL promastigotes when incubated with SNP as observed from IC_{50} values (15.78 mM - LCL x 23.09 mM - ML; $p = 0.01$; Table 2). This resistance was observed mainly at 25 and 100 mM SNP (Fig. 4B). Resistance to SNP was not observed by promastigotes in the logarithmic phase (IC_{50} = 29.35 mM - LCL × 53.30 mM – ML, $p = 0.82$; Fig. 4A, Table 1). In contrast, the amastigote of LCL isolates were more resistant to NO as observed from IC_{50} values (IC_{50} = 37.63 mM - LCL x 13.91 mM - ML; $p = 0.03$; Fig. 4C, Table 3). The difference was observed among the same six isolates tested for promastigotes or amastigotes in the stationary phase. However, to obtain more robust results, the number of isolates at stationary phase was increased.

Stationary phase promastigotes from ML isolates were more resistant to H_2O_2 than LCL parasites as observed from IC_{50} values (3.98 mM - LCL x 9.68 mM - ML; $p = 0.04$; Table 5), and the difference was mainly observed at the dose of 25 mM ($p < 0.05$; Fig. 5B). No difference was observed for promastigotes in the logarithmic phase

Table 3
Viability of LCL and ML amastigotes cultured in the presence of SNP.

L. braziliensis	IC50 ± SE (mM)	95% IC
Isolates		
JCJ8c	15.31 ± 13.01	4.486–52.26
RPL5c	40.03 ± 15.34	10.80–148.3
CSA7c	57.54 ± 11.60	13.23–250.2
Mean	**37.63 ± 12.25**	
ASL9m	20.66 ± 22.06	2.066–248.6
JBC8m	16.64 ± 5.72	2.980–92.97
PPS6m	4.425 ± 7.62	1.025–19.10
Mean	**13.91 ± 4.89***	

LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.
SNP, sodium nitroprusside.
* indicates statistical difference between LCL and ML parasites by student´s *t*-test ($p < 0.05$).

Table 4
Viability of LCL and ML promastigotes in logarithmic growth phase cultured in the presence of H_2O_2.

L. braziliensis	IC50 ± SE (mM)	95% IC
Isolates		
JCJ8c	0.75 ± 1.16	0.23–2.4
RPL5c	0.37 ± 0.33	0.19–0.71
CSA7c	0.61 ± 0.72	0.33–1.12
Mean	**0.58**	
ASL9m	2.80 ± 2.60	0.60–5.08
JBC8m	4.29 ± 3.73	1.09–16.9
PPS6m	0.35 ± 0.18	0.17–0.71
Mean	**2.48**	

LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.

Table 5
Viability of LCL and ML promastigotes in in stationary growth phase cultured in the presence of H_2O_2.

L. braziliensis	IC50 ± SE (mM)	95% IC
Isolates		
JCJ8c	5.28 ± 4.04	3.16–8.82
RPL5c	3.25 ± 5.91	1.17–9.02
CSA7c	1.04 ± 2.95	0.48–1.81
UAF5c	8.35 ± 3.94	3,67–19,06
HPV6c	1.06 ± 2.62	0,67–1,69
EFS5c	1.39 ± 2.13	0,91–2,16
WSS5c	7.47 ± 4.80	4,18–13,39
Mean	**3,98 ± 1.17**	
ASL9m	18.68 ± 16.19	5,05–69,11
JBC8m	2,56 ± 3,23	1135–5,77
PPS6m	15.18 ± 5.89	4,88–47,16
ILM9m	9.82 ± 3.45	5,11–18,87
NFS10m	2.17 ± 3.05	1,23–3,80
Mean	**9.68 ± 3.31***	

[a]indicates statistical difference between LCL and ML parasites by student´s *t*-test ($p < 0.05$).
LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.

(0.58 mM - LCL x 2.48 mM - ML; $p = 0.34$; Table 4). For amastigotes, LCL parasites were observed to be more resistant to ROS than ML parasites on the basis of IC_{50} values (8.31 mM - LCL × 5.37 mM - ML; $p = 0.08$; Table 6). In addition, this tendency was observed when resistance to ROS was analyzed with 5 mM-dose of H_2O_2 (6.12 mM - LCL × 1.43 mM - ML; $p = 0.06$).

Table 6
Viability of LCL and ML amastigotes cultured in the presence of H_2O_2.

L. braziliensis	IC50 ± SE (mM)	95% IC
Isolates		
JCJ8c	5.49 ± 21.23	1.12–26.9
RPL5c	9.15 ± 36.24	0.67–123.6
CSA7c	10.28 ± 25.58	1.86–56.7
Mean	**8.31**	
ASL9m	6.30 ± 11.77	1.36–29.1
JBC8m	5.82 ± 19.0	2.36–14.2
PPS6m	4.00 ± 10.33	0.93–17.1
Mean	**5.37**	

LCL, localized cutaneous leishmaniasis.
ML, mucosal leishmaniasis.

3.3. Mucosal leishmaniasis promastigotes express more thiol-specific antioxidant protein than localized cutaneous leishmaniasis promastigotes

As ML promastigotes had higher resistance to ROS, we decided to investigate whether these parasites express an increased amount of enzymes that are capable of inactivating ROS. Among these enzymes, the TSA protein was reported to protect against oxidative damage of macrophages in fungal infection [32]. TSA was highly expressed in stationary phase promastigotes from LM isolates (Fig. 6A and B). No significant differences were observed in the expression of TSA between LCL and ML isolates when analyzing amastigotes (Fig. 7A and B).

The highest expression of the TSA enzyme in ML promastigotes was confirmed using the western blotting technique (Supplementary Figure 1). In addition, this technique confirmed the homogeneous expression of TSA in ML and LCL amastigotes. SMB7mc obtained from a cutaneous lesion of a patient with MCL disease was used in the western blotting assay. These data suggest that all promastigote parasites obtained from cutaneous lesions have similar TSA expression, although the patient also presented mucosal lesion (relative fluorescence intensity: 0.75 - SMB7mc × 0.44 - JCJ8c × 0.86 - RPL5c).

4. Discussion

The reason for the development of mucosal lesions in patients infected with *L. (V.) braziliensis* is poorly understood. In this study, the isolates of *L. (V.) braziliensis* obtained from patients with ML or LCL were used, and it was observed that they shared similar characteristics, such as the ability to grow in in vitro culture. The comparison of arginase activity and NO production between LCL and ML parasites showed similar results, however, some isolates showed an increased ability to produce NO or urea. Despite this, it was not possible to associate this ability with clinical characteristics. The arginase enzyme hydrolyzes the amino acid L-arginine producing L-ornithine and urea. L-ornithine is used as a substrate for polyamine production and, is a crucial molecule for nucleic acid synthesis and parasite proliferation. *Leishmania* expresses the arginase enzyme located within the parasite glycosome [33,34]. Vendrame *et. al.* 2010 evaluated the arginase activity from different isolates of *L. (V.) braziliensis* promastigotes in the stationary phase of growth and showed that ML isolates had a higher enzymatic activity than LCL isolates. In addition, this higher arginase activity was suggested to contribute to the survival and proliferation of the parasite within a hostile environment [21]. We did not find differences in the arginase activity between ML and LCL promastigotes; this may be because of the greater variability observed in the arginase activity among the isolates used in this study. When comparing the arginase activity among amastigote isolates, we observed that the enzyme activity was extremely homogenous, suggesting that arginase can help in parasite infection only in the early phase.

In this study, NO production by LCL and ML isolates was similar, although some isolates produced higher amounts of this molecule than

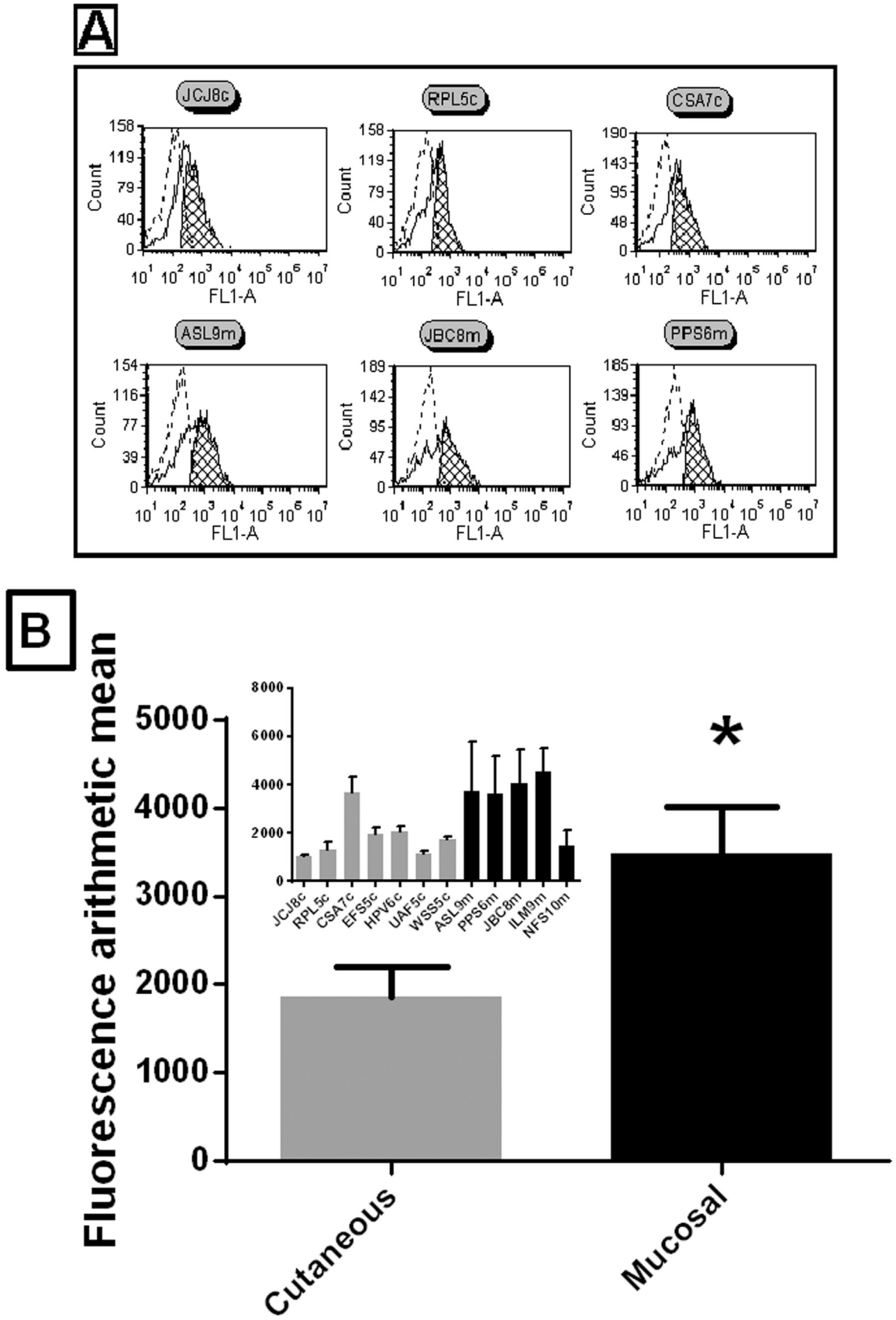

Fig. 6. TSA expression on stationary-phase promastigotes *of Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. (A) promastigote isolates from LCL or ML patients in stationary-phase were assayed by flow cytometry. Histograms represent fluorescence intensity of promastigotes labeled with anti-TSA antibodies (continuous line) or control antibodies (dashed lines). The hashed area represents subtraction between control and TSA-labeled parasites. (B) Bars showed the arithmetic mean of fluorescence ± SD per group of five independent experiments performed in triplicate. Insets present fluorescence for each isolate.* indicates statistical difference between LCL and ML parasites by Student´s *t*-test ($p < 0.05$).

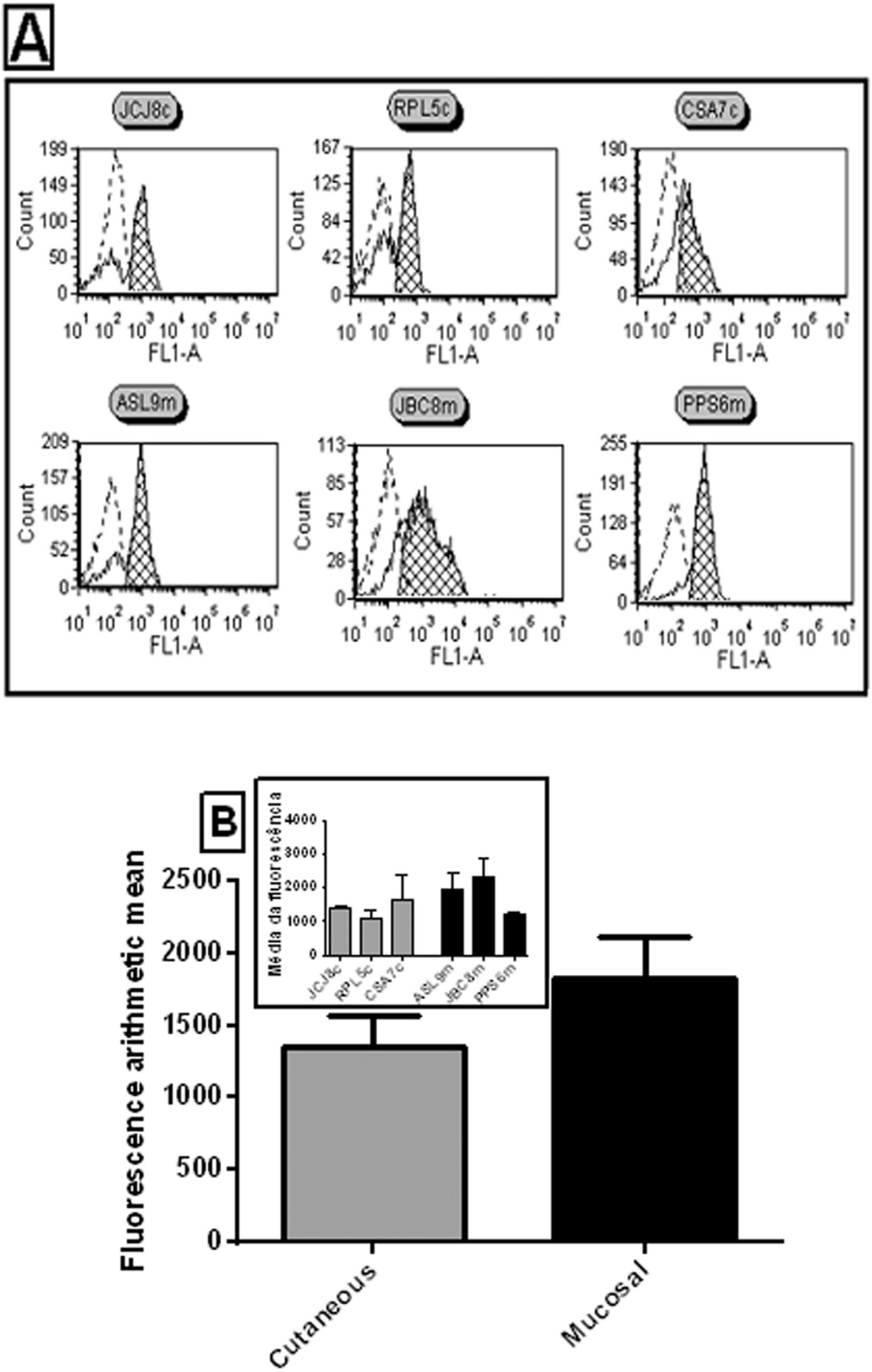

Fig. 7. TSA expression of amastigotes of *Leishmania (V.) braziliensis* isolates from patients with localized cutaneous (LCL) or mucosal (ML) leishmaniasis. (A) amastigote isolates from LCL or ML patients were assayed by flow cytometry. Histograms represent fluorescence intensity of promastigotes labeled with anti-TSA antibodies (continuous line) or control antibodies (dashed lines). The hashed area represents subtraction between control and TSA-labeled parasites. (B) The bars represent arithmetic mean of fluorescence ± SD per group from five independent experiments performed in triplicate. Insets represent fluorescence for each isolate. * indicates statistical difference between LCL and ML parasites by One-Way Student´s *t*-test ($p < 0.05$).

others. In mammals, NO production depends on the family of enzymes called nitric oxide synthases (NOS), which catalyze the oxidation of L-arginine to L-citrulline and NO [35,36]. NO has several functions, including blood pressure regulation, neurotransmission, cellular differentiation, immune response, among others [37,38]. NO production by a constitutive NOS present in the parasite cytoplasm has already been described in some species of *Leishmania*, including *L. (V.) braziliensis* [39]. In this study, it was shown that NO is secreted by promastigotes and amastigotes of all isolates. Despite NO production, no association of this ability was observed with different clinical forms of the disease. The secretion of NO by promastigotes favors metacyclogenesis in vitro and may be related to metacyclogenesis in the vector [39]. In addition, our data suggest that amastigotes produce more NO than promastigotes as reported previously [40], therefore, we used 100 times more promastigotes than amastigotes in culture to obtain similar levels of NO. It is difficult to achieve a proper comparison between promastigotes and amastigotes because amastigotes were cultured for a longer than promastigotes. The function of NO produced by amastigote is to be determined.

Although *Leishmania* parasites secret NO, this molecule is also known as the major microbicidal compound that can kill most *Leishmania* species in murine cells [8,41]. The resistance to NO by *L. (V.) braziliensis* and *L. (L.) amazonensis* promastigote isolates was associated with the lesion size and ML promastigotes tend to be more resistant than LCL ones [42]. In addition, it has been reported that the amastigotes of *L. (L.) amazonensis* have a higher resistance to NO than *L. (V.) braziliensis*, which contributes to the virulence of the parasite [43]. In this study, NO donor SNP was used and stationary phase promastigotes from ML isolates were observed to be re more resistant than those from LCL isolates at 25 mM dose. In contrast, our previous study has revealed that amastigote parasites from ML patients are less resistant to NO [23]. In addition, a lower resistance of ML amastigotes to NO was observed. These data altogether suggest that when ML parasites are transmitted by vectors as promastigote, they are more resistant to NO than LCL parasites, which facilitates their survival at the beginning of infection. However, they become less resistant to NO as they turn into amastigotes, i.e., as the infection progresses. Typically, the mucosal lesion appears years after the primary cutaneous lesion is cured. In this study, we suggested that a weaker immune response stimulated by ML amastigotes at the site of infection should facilitates the dissemination of parasites to the mucosal sites. In contrast, LCL amastigotes activate stronger immune response at the site of infection that keeps the parasite at the initial site of infection. It remains unclear why the immune response becomes stronger at the mucosal site at the later time, however, one of the reasons may be the constant stimulus by disseminating-parasite.

In addition, ROS are also important to kill *Leishmania* parasites. In macrophages, ROS can be produced by NADPH oxidase, a multi-enzymatic complex that reduces extracellular oxygen to superoxide anion that forms several reactive molecules such as hydrogen peroxide, oxidized halogens, oxygen singlet, and other free radicals [44,45]. The Inhibition of respiratory burst may contribute to the survival of pathogens in the phagosomes of macrophages [46,47]. In this study, the incubation of stationary phase promastigotes with H_2O_2 showed that ML parasites are more resistant to ROS than LCL. The role of ROS as a crucial molecule to kill some *Leishmania* species was shown previously in murine and human infection [29,30,48]. Resistance to ROS is a strategy developed by pathogens to increase survival in macrophages [49]. To increase resistance, parasites have developed an complex antioxidant defense mechanism consisting of enzymes that regulate or eliminate O_2^- and H_2O_2 [49–51]. These systems are based on low molecular weight thiols, such as glutathione and trypanothione, which play major roles in antioxidant defense mechanisms by regulating redox homeostasis in parasites [49,51,52]. In this study, it was demonstrated that stationary phase ML promastigotes, but not amastigotes, express more TSA than LCL promastigotes. TSA was described initially in *Saccharomyces cerevisiae* that can remove ROS and increase fungal survival in the macrophage [32]. The high expression of TSA in ML stationary phase promastigotes is in agreement with our results, showing that these parasites have increased resistance to H_2O_2 when compared with LCL parasites. The high expression of TSA is not observed in ML amastigotes, explaining their lower resistance to H_2O_2. These data favor the hypothesis that ML parasites are more adapted to ingress in macrophages at the beginning of infection, despite losing their ability after becoming amastigotes. Notably, the O_2 production is higher after the interaction of macrophages with promastigotes than that with amastigotes [53–56] suggesting that the increased resistance to ROS is crucial to ensure infection by promastigotes.

In this study, ML promastigotes in the stationary phase, representing the infective form of parasite transmitted by vectors, were more resistant to microbicidal mechanisms than LCL parasites, which in turn are less resistant to NO and ROS and express less TSA protein. Afterwards, ML amastigotes become lesser resistant than LCL ones, which is in agreement with our previous study [23]. This behavior should facilitates the infection with a low parasite burden inoculated by the vector and keeps amastigotes in low number within the cells. This can avoid the rapid proliferation of parasites, killing of the host cells, and activation of strong immune response at the site of infection. Interestingly, we showed early that lesion development in murine challenged with ML amastigotes were delayed, and ML amastigotes had increased ability to disseminate when compared to LCL ones [23]. Notably, the analyses of the *Leishmania*-infected mucosal sites in patients typically show fewer parasites whereas cutaneous lesions in a patient with LCL present more parasites [57] which is in agreement with lower proliferation and increased dissemination ability. However, the spread of few parasites to other sites may provide a prolonged and continuous stimulation of the immune system, which should booster the immune response afterwards. Notably, patients with chronic ML present a strong immune response against *Leishmania* antigens which is highly inflammatory and with high levels of IFN-γ and TNF-α [15,58].

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Conflict of interest

The authors declare no conflicts of interest

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.09.005.

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