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Effects of hyperbaric oxygen on *Leishmania amazonensis* promastigotes and amastigotes

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Abstract

In the present study, we evaluated the effects of hyperbaric oxygen (HBO) exposure in both *Leishmania amazonensis* life stages (promastigotes and amastigotes) and on macrophage cultures infected with the parasite. HBO treatment protocols, which can be tolerated by humans and animals, induced irreversible metabolic damage and affected parasite morphology, growth and ability to transform. The observation that the antioxidant *N*-acetylcysteine (NAC) prevents some of these deleterious effects indicated an involvement of oxidative stress during parasite HBO exposure. In addition, HBO exposed *L. amazonensis*-infected macrophage cultures showed reduction of the percentage of infected cells and of the number of intracellular parasites per cell. Thus, the demonstration that HBO, a therapy used in the management of different diseases, is toxic for both *L. amazonensis* life stages and can alter macrophage susceptibility to the infection encourages further studies of this therapy in animal models of *Leishmania* infection.

Keywords: *Leishmania amazonensis*; Macrophage; Hyperbaric oxygen

1. Introduction

Leishmaniasis is a parasitosis caused by protozoa of the genus *Leishmania* affecting more than 12 million people worldwide [1]. These parasites exist as motile flagellated promastigotes that live extracellulary in the alimentary tract of blood-sucking sand flies and as nonflagellate intracellular amastigotes within the macrophages of mammalian hosts. The severity of the disease produced by several *Leishmania* species varies widely, ranging from cutaneous or mucosal to visceral or diffuse cutaneous infection. The former is generally caused by *Leishmania amazonensis*, a species transmitted mainly in the Amazon region, which is associated with localized cutaneous lesions [1,2]. Chemotherapy remains the mainstay for the control of leishmaniasis, as effective vaccines still have to be developed [3]. Organic pentavalent antimonials, amphotericin B, allopurinol and aminosidine are used as clinical treatment, and their systemic toxicity and the emergence of drug resistance are becoming major concerns [3,4].

Several characteristics of leishmanial lesions such as microcirculation impairment, metabolic demand for leukocyte infiltration into infected tissues, parasite proliferation and secondary bacterial infection [2,5–7] are strong indications of a hypoxic microenvironment in the lesions which may play a role in the outcome of infection. Hyperbaric oxygen (HBO) therapy has been shown to increase both systemic and wound tissue oxygen levels and to assist as an adjuvant to surgery and antibiotics for numerous soft-tissue infections [8]. Exposure to elevated oxygen tensions affects the viability and proliferation of some bacteria and pathogenic fungi, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhosa*, *Corynebacterium diphtheriae*, *Candida albicans* and *Rhizopus oryzae* [9]. Only one study performed with *L. braziliensis*

Abbreviations: HBO, hyperbaric oxygen; NAC, *N*-acetylcysteine.

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panamensis promastigote forms demonstrated parasite killing after long term HBO exposure [10]. In this same study, no attempt was made to determine the susceptibility of amastigotes, the intracellular stage within macrophage, to HBO. In the present report, we compared the effect of hyperbaric oxygen treatment on the viability, morphology, proliferation and differentiation of amastigote and promastigote forms of *L. amazonensis*. We also determined the effect of HBO on macrophage cultures infected with *L. amazonensis*.

2. Materials and methods

2.1. Parasite

L. amazonensis (MHOM/BR/73/M2269) promastigotes were cultured at 26 °C in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil). Amastigotes were isolated from active skin lesions from BALB/c mice as described previously [11].

2.2. Macrophage cultures

Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage, cultured on 24-well plates containing 13-mm diameter glass coverslips and infected with a three-fold excess of amastigotes as described previously [11]. For the evaluation of the percentage of infected macrophages and the number of amastigotes per macrophage, cells on coverslips were stained with Giemsa [11].

2.3. HBO treatment

To each 25-cm^2 culture flask parasites (10^6 parasites/ml) suspended in RPMI+10% FCS were added to a depth of 3 mm to facilitate oxygen diffusion. The flasks containing parasites and 24-well culture plates containing infected macrophage monolayers were placed in an HBO chamber (Research Chamber, model HB 1300B, Sechrist, Anaheim, CA). The chamber was flushed with oxygen (100% O_2) and pressurized over a period of 5 min. The pressure (PO_2 253.3 kPa, 2.5 ATA) was maintained for the required duration of the experiment and then the chamber was decompressed over a period of 5 min. The temperature inside the HBO chamber was similar to room temperature. The oxygen tension in the culture medium was 700 mm Hg under HBO conditions and 150 mm Hg under normoxia conditions (O_2 analyzer YSI/53, Yellow Springs Instruments, Yellow Springs, OH). The pH of the medium was 7.2 and did not change significantly during the course of the experiments. In some experiments, parasites were treated for 90 min with the antioxidant *N*-acetylcysteine (NAC) (30 mM) (Sigma) [12] prior to HBO exposure.

2.4. Assessment of HBO effects on L. amazonensis promastigotes and amastigotes

Promastigote number and morphology were determined using a Neubauer haemocytometer. Amastigote viability was determined by erythrosine B staining [13]. The transformation of amastigotes into promastigotes was estimated microscopically as described previously [14]. Cytotoxicity was analyzed by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye-reduction assay (Sigma) [15]. For ultrastructural analyses, promastigotes were washed with PBS and fixed during 12 h at 4 °C in a fixative mixture consisting of 2% paraformadehyde plus 1.5% glutaraldehyde in 1% sucrose in 0.1 M phosphate buffer at pH 7.4. After fixation, they were encapsulated in 1% agar, post-fixed in 1% osmium tetroxide for 2 h, dehydrated in a graded ethanol series and acetone, and embedded in Epon 812 resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss LEO 906 transmission electron microscope operated at 60 kV. The TUNEL assay (terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling) was used to distinguish between apoptotic and necrotic promastigotes, according to the manufacturer's instructions (in situ cell death detection kit POD, Boehringer Mannheim, Germany). Sections were counterstained with methyl green and examined by light microscopy.

2.5. Results analyses

All experiments were performed at least three times. Differences between treatments were evaluated by the Student's *t*-test.

3. Results

3.1. Effect of HBO on L. amazonensis promastigotes

Cytotoxicity was evaluated when promastigotes were treated with HBO, using protocols that could be tolerated by animals and humans. As shown in Fig. 1A, the numbers of promastigotes were not changed after 2 or 4 h of HBO treatment. Adverse effects were observed when promastigotes were exposed to HBO for 6 h; parasites became non-mobile, round in shape and about 30% of mortality was observed (Fig. 1A). Using the TUNEL staining to detect the free ends of DNA after breakage, we observed a significantly higher TdT labeled nuclei in the promastigotes exposed to HBO compared with non-exposed HBO parasites (data not shown), suggesting that HBO induces apoptosis in this *Leishmania* form. The transmission electron microscopy showed morphological changes that varies from cytoplasmic organelles structurally well preserved though an increased in lipid bodies was frequently seen (Fig. 2B) to drastic derangements of organelles (Fig.

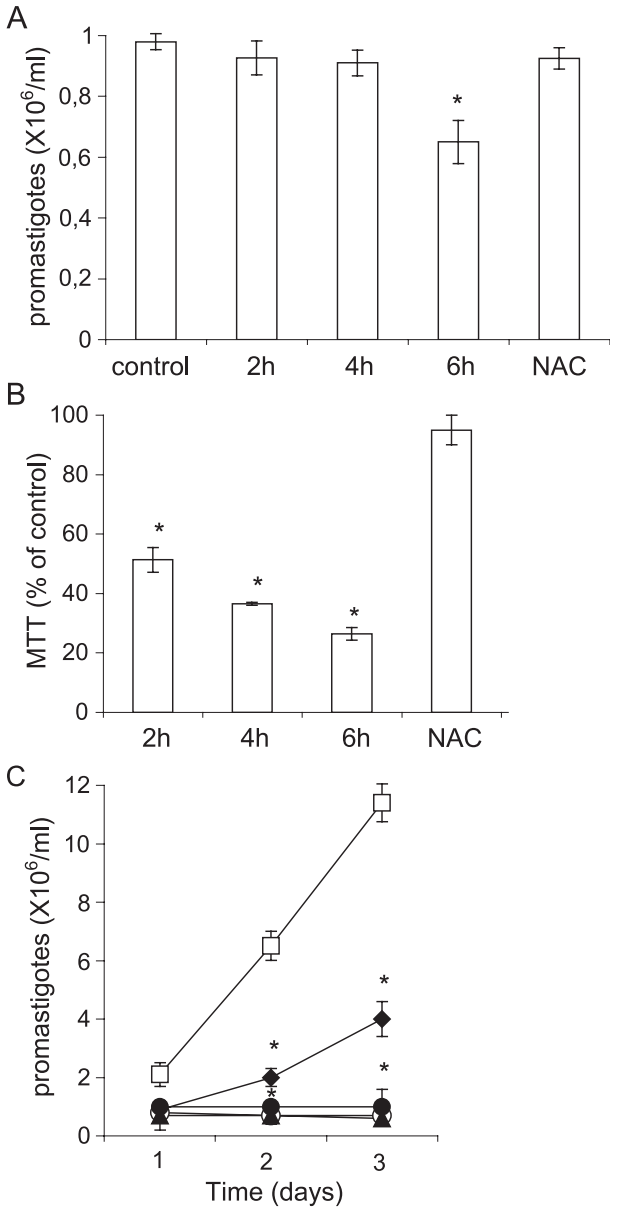

Fig. 1. Effect of HBO exposure on *L. amazonensis* promastigotes. (A) Numbers of viable promastigotes determined in cultures not exposed to HBO (control), exposed to HBO for 2, 4 and 6 h or pretreated with NAC and exposed to HBO for 6 h (NAC). (B) Promastigote MTT production determined in cultures exposed to HBO for 2, 4 and 6 h or pretreated with NAC and exposed to HBO for 6 h (NAC) compared to control (non-exposed promastigotes). (C) Growth of promastigotes not exposed to HBO (□), exposed to HBO for 2 (◆), 4 (▲) or 6 h (●) and treated with NAC before a 6-h exposure to HBO (○). The results represent the mean±S.D. of three experiments. The significance of the difference between treatments is indicated in the figure. *$P \leq 0.01$.

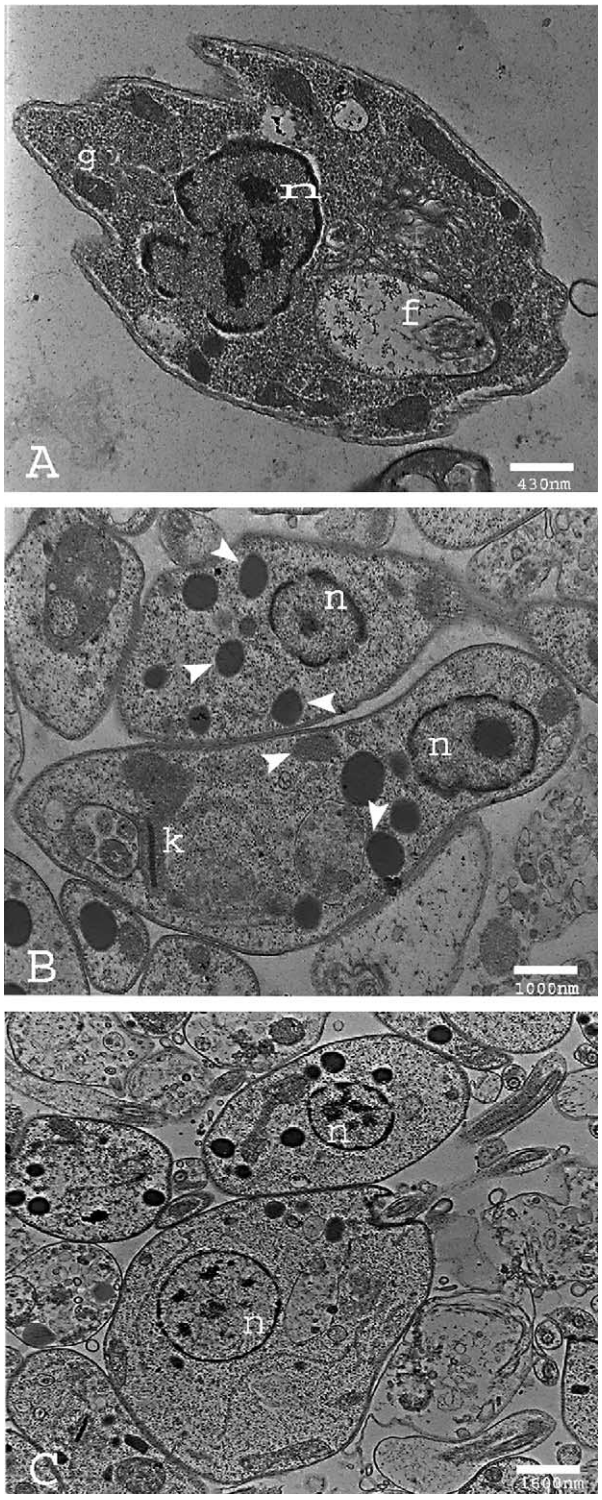

Fig. 2. Effect of HBO on *L. amazonensis* promastigote ultra structures. (A) Promastigote not exposed to HBO. Note the normal appearance of nucleus (n), flagellar pocket (f) and electron-dense granules (g). (B, C) Promastigotes exposed to HBO for 6 h. Panel B demonstrate the normal appearance of nucleus (n) and kinetoplast (k), and an atypical large amount of lipids bodies (arrow heads) distributed all over the cytoplasm. Panel C demonstrate HBO treated promastigotes with the most of the cytoplasmic organelles disintegrated and lipid bodies.

2C), after HBO treatment; which can represent a sequence of morphological changes occurring during HBO exposure. In contrast, HBO non-exposed promastigotes remained 98% viable (Fig. 1), highly mobile, spindle shaped and showed all the typical ultra structural characteristics of this parasite form (Fig. 2A). The effect of antioxidant NAC seems to be protective for promastigotes exposed to HBO, since the

number of parasites and their morphology did not change in cultures previously treated with NAC (Fig. 1A and data not shown).

The cytotoxicity of HBO was also estimated using the MTT assay. As shown in Fig. 1B, a decreased MTT metabolism was observed in promastigotes exposed to 2, 4 or 6 h of HBO, indicating a deleterious effect of elevated oxygen tension on the energy metabolism of this *Leishmania* form. The decreased metabolism of MTT persisted in these HBO exposed promastigotes cultured under standard parasite conditions for 24 h (data not shown), suggesting that HBO exposure can cause irreversible damage to the parasites. The deleterious effect of HBO on MTT metabolism was prevented by treatment with NAC (Fig. 1B).

To assess the effect of HBO on the ability of promastigotes to grow, culture flasks exposed to HBO and controls were incubated at 26 °C and the parasite numbers were determined over a period of 3 days. It is clear from the data shown in Fig. 1C that the fastest growth occurred in HBO-non-exposed promastigotes. Some growth occurred in promastigotes exposed to 2 h of HBO. In addition, parasites exposed to 4 or 6 h of HBO remained as a static cell culture (Fig. 1C) and then started to deteriorate after 4 days. Interestingly, promastigotes pre-treated with NAC and exposed to HBO lost their capacity to proliferate (Fig. 1C). Similar results were obtained with different concentrations of NAC and with the antioxidant catalase (data not shown).

For the experiments described above, promastigotes maintained in flasks with medium were exposed to HBO and immediately transferred to an incubator at 26 °C in air. The partial pressure of oxygen dissolved in the medium following application of 100% oxygen under hyperbaric conditions was high (700 mm Hg). Thus, to avoid interference of the hyperoxic environment with cell growth, promastigotes exposed to 6 h of HBO were transferred to fresh normoxic medium (150 mm Hg) and the parasite number was determined over a period of 72 h. Under these conditions, promastigotes were unable to proliferate (data not shown), indicating that the inhibitory effect of HBO on parasite proliferation was not simply the consequence of a hyperoxic environment and a treatment hyperbaric oxygen, was required to induce parasite growth stasis.

To test another HBO protocol that could be tolerated by animals and humans, promastigotes were exposed to HBO 1 h per day for seven days. As shown in Fig. 3, parasites did not proliferate during the period of HBO exposure and remained as a static cell culture at least 3 days after the HBO sessions (data not shown). HBO non-exposed promastigotes grew at a normal rate throughout the culture period (Fig. 3).

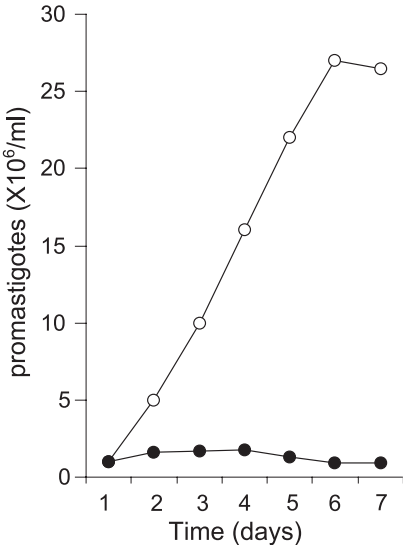

Fig. 3. Effect of HBO exposition on *L. amazonensis* promastigote growth. Numbers of promastigotes were determined daily in cultures not exposed to HBO (●) and in cultures exposed to HBO (1 h/day for 6 days) (○). The results are from one experiment and two other experiments yielded similar results.

3.2. Effects of HBO on L. amazonensis amastigotes

Exposure of amastigotes to 2, 4 or 6 h of HBO failed to affect parasite viability or morphology as evaluated by erythrosine-B staining (Fig. 4A and data not shown). Cytotoxicity evaluated by a decrease in MTT metabolism was only observed in amastigotes after 4 h of HBO treatment (Fig. 4B). Previous studies indicated that these parameters represent valid measures of parasite viability [14]. However, only healthy amastigotes (molecularly undamaged cells) are expected to be able to transform into promastigotes [14]. Thus, we examined the ability of HBO-exposed amastigotes to differentiate into promastigotes. As shown in Fig. 4C, HBO non-exposed amastigotes transformed into promastigotes and a time-dependent inhibition of amastigote transformation was observed when parasites were pre exposed to HBO. Exposure to HBO for 6 h hardly affected the ability of amastigotes to transform into promastigotes (Fig. 4C). The pre-incubation of amastigotes with NAC seems to be partially protective since the ability of transformation was not completely lost in parasites after 6 h of HBO treatment. As expected, inhibition of transformation was observed when parasites were exposed to HBO and then transferred to fresh normoxic medium for 3 days, indicating that this inhibitory effect of HBO on parasites was not simply the consequence of a hyperoxic environment and a treatment with hyperbaric oxygen, was required to induce parasite growth stasis (data not shown). In a separate experiment, amastigotes exposed to HBO (1 h/day for 3 days) transformed into promastigotes at a low rate (data not shown).

3.3. Effect of HBO on L. amazonensis-infected murine macrophages

Experiments were undertaken to study a possible effect of HBO treatment on *L. amazonensis* infected murine macrophages. As shown in Fig. 5A, macrophage cultures

exposed to HBO for 2 h showed a significant reduction of parasitized cells compared with cell cultures not exposed to HBO. There was also a significant reduction in the number of amastigotes per cell in HBO exposed macrophage cultures compared with control cell cultures (Fig. 5B). Comparable results were obtained in similar experiments using the U937 human cell line (data not shown). It should be noted, however, that on a morphological basis HBO treatment proved to be toxic to the macrophages after 4 h of exposure.

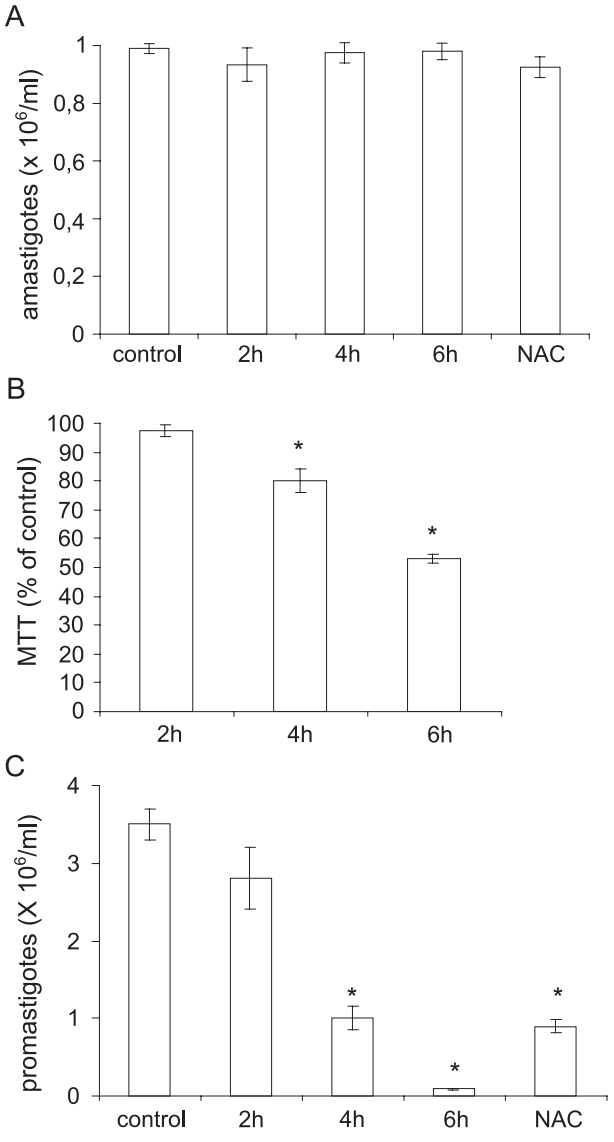

Fig. 4. Effect of HBO exposure on *L. amazonensis* amastigotes. (A) Numbers of viable amastigotes determined in cultures not exposed to HBO (control), exposed to HBO for 2, 4 or 6 h or pretreated with NAC and exposed to HBO for 6 h (NAC). (B) Amastigote MTT production determined in cultures exposed to HBO for 2, 4 and 6 h or pretreated with NAC and exposed to HBO for 6 h (NAC) compared to control (non-exposed amastigotes). (C) The transformation of amastigotes into promastigotes was estimated by counting promastigotes after a 72-h culture period in air. Amastigote cultures not exposed to HBO (control), exposed to HBO for 2, 4 or 6 h HBO, or treated with NAC before 6h HBO exposure. The results represent the mean±S.D. of three experiments. The significance of the difference between treatments is indicated in the figure. *$P \leq 0.01$.

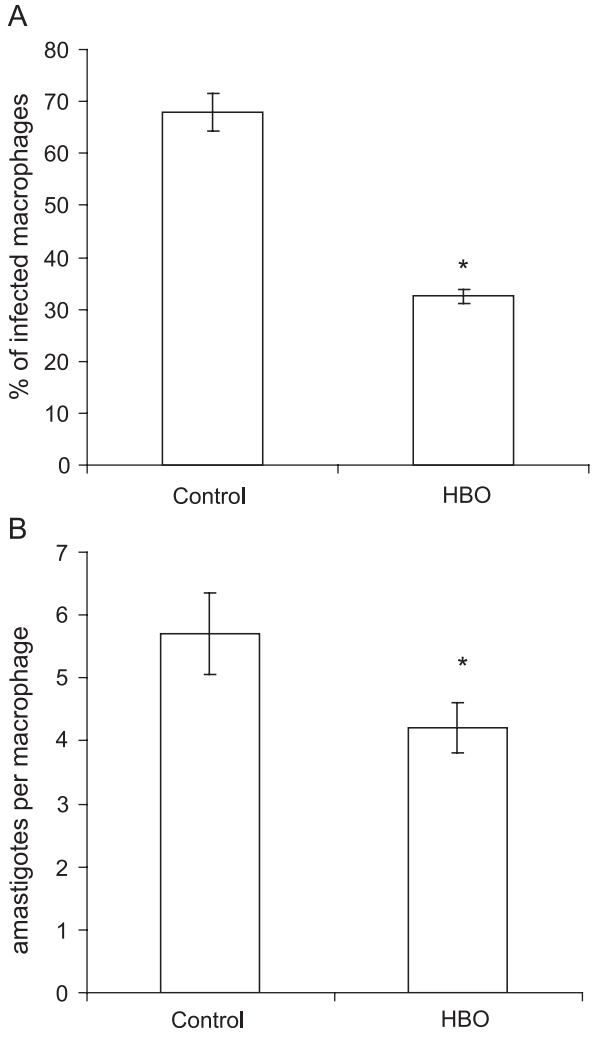

Fig. 5. Effect of HBO exposure on *L. amazonensis* infection of murine peritoneal macrophages. Macrophages previously infected with amastigotes were exposed to HBO for 2 h. Cell cultures were then immediately evaluated for the percentage of infected macrophages (A) and the number of amastigotes per macrophage (B). Control cultures were not exposed to HBO. The results represent the mean±S.D. of three experiments. The significance of the difference between treatments is indicated in the figure. *$P \leq 0.01$.

4. Discussion

This report provided direct evidence that HBO exerts toxic effects on both parasite forms of *Leishmania* and reduces the infection in macrophage cultures. Under our experimental conditions, we showed that an exposure time of 6 h induced morphological changes and death in approximately 30% of promastigotes, whereas HBO exposure failed to affect amastigote morphology or viability as measured by erythrosine B staining (Figs. 1, 2 and 4). Two hours of HBO exposure was sufficient to decrease the

energy metabolism and to reduce promastigote growth, whereas the inhibitory effect on metabolism and on the ability of transformation was pronounced in amastigotes after 4 h of HBO exposure (Figs. 1 and 4). Short-term exposure of parasitized macrophage cultures to HBO reduced the percentage of infected cells when compared with HBO-non-exposed infected cell cultures (Fig. 5). These results indicate a considerable potential for the control of *Leishmania* infection rate in macrophages by HBO.

The effect of HBO on *Leishmania* has only been evaluated on the promastigote forms of *L. braziliensis panamensis* [10]. The cited study concluded that 24 h of HBO exposure killed about 90% of promastigotes. The major difference from our study was that while Muhvich et al. [10] needed to expose promastigotes to HBO for a much longer time (24 h) than could be tolerated by humans to observe parasite killing, we detected irreversible parasite damage after 2 h of HBO (Fig. 1). Potentially, the HBO conditions needed to obtain irreversible metabolic injury could be considered therapeutically. More important, the anti-leishmanial effects observed in promastigotes were extended to amastigotes. Interestingly, our results indicated that amastigotes are less sensitive than promastigotes to HBO treatment since a longer time (4 h) was required to inhibit the metabolism and the ability of transformation (Fig. 4). Numerous reports have shown that amastigotes are less susceptible than promastigotes to reactive species such as hydrogen peroxide, superoxide and inorganic nitrogen oxide [14,16–18]. It has been suggested that amastigotes, which live exclusively in mammalian macrophages, have developed compensatory mechanisms to subvert a toxic macrophage environment [16–19]. The partial resistance could be associated with the differential expression of molecules implicated in antioxidant defenses, such as glutathione peroxidase and intracellular thiols [17,19].

It is generally assumed that HBO generates an excess of reactive oxygen species, including superoxide and hydrogen peroxide, which are, at least in part, involved in HBO-induced cell apoptosis [20,21]. Furthermore, there is apoptosis-like death in *L. donovani* promastigotes exposed to hydrogen peroxide [22]. Thus, the presence of apoptotic parasites as demonstrated by TUNEL labeling of DNA in promastigote cultures exposed to HBO, and the observation that pre-treatment of *Leishmania* with NAC, an antioxidant which scavenges reactive oxygen species [12] significantly inhibited morphological and metabolic changes induced by HBO confirms the involvement of the oxidative stress (Fig. 1A,B and data not shown). Interestingly, we also showed that NAC (Fig. 1C) and catalase did not prevent the cytostatic effect of HBO on promastigotes. These results are consistent with those reported by others [23–25]. For example, Padgaonkar et al. [24] demonstrated that the antioxidant glutathione is important to maintain epithelial cell viability after an extended exposure to HBO but is incapable to prevent inhibition of cell growth. It is possible that the marked increase in parasite oxidative stress induced by HBO leads to the accumulation of free radicals and other reactive oxygen-based molecules involved in signaling systems linked to proliferation and differentiation [24,26] which cannot be adequately detoxified by the antioxidants tested.

Exposure of *Leishmania*-infected macrophages to HBO for 2 h resulted in a reduced percentage of infected cells as well as a reduced number of parasites per cell without apparent damage to the host cells (Fig. 5). These results suggest that HBO led to macrophage destruction of intracellular *L. amazonensis* amastigotes. The parasite killing could be the result of a direct action of HBO treatment on the parasites within macrophages. Alternatively, the HBO may act by stimulating microbicidal processes in the host cells. Although our experiments have shown that amastigotes are sensitive to direct exposure to HBO, it is reasonable to assume that the parasite control is mediated at least in part by the macrophages. This notion is supported by the observations that the respiratory burst induced by bacterial and fungi was increased in macrophages after HBO exposure [27,28], but this still remains to be determined in the cell system used in our studies.

Hyperbaric oxygen treatments protocols such as those used in our investigation and in clinical therapy have proved to be nontoxic for human cutaneous cells and to stimulate fibroblast proliferation, collagen synthesis and epithelialization, factors involved in the repair process of cutaneous wound healing [29]. The results presented here show, for the first time, that HBO treatment (100% O_2, PO_2 253.3 kPa) can irreversibly affect the growth, metabolism and ability to differentiate of *L. amazonensis* and also reduce the percentage of infected macrophages in an in vitro test. On this basis, it would be of interest to determine whether controlled HBO therapy can be beneficial to animal models of cutaneous *Leishmania* infection. This question is currently under investigation in our laboratory.

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