Hyperbaric hyperoxia enhances the lethal effects of amphotericin B in *Leishmania braziliensis panamensis*  

K. H. MUHVICH, L. H. ANDERSON, D. W. CRISWELL, and W. J. MEHM  

Division of Altitude and Hyperbaric Physiology, Armed Forces Institute of Pathology, Washington, DC 20306-6000  

Muñich KH, Anderson LH, Criswell DW, Mehm WJ. Hyperbaric hyperoxia enhances the lethal effects of amphotericin B in *Leishmania braziliensis panamensis*. Undersea Biomed Res 1993; 20(4):321-328.— *Leishmania braziliensis panamensis* promastigotes were exposed in vitro to amphotericin B (AmB), menadione, or phenazine methosulfate under normoxic conditions. Promastigotes were also exposed to hyperoxia alone (100% O₂ at total pressures of 101.3 or 253.3 kPa), or combined with drugs. After incubation for 24 h at 27°C, viable promastigotes were stained with fluorescein diacetate and counted using epifluorescence microscopy. Hyperbaric hyperoxia alone (P₀₂ = 229.3 kPa) was as effective as AmB alone (0.2 μM); both reduced the number of viable promastigotes to approximately 13% of the original inoculum. In addition, AmB in a hyperbaric hyperoxic environment killed more promastigotes (97% of the original inoculum) than AmB in normoxic (P₀₂ = 21.1 kPa) or hyperoxic conditions (P₀₂ = 91.7 kPa). Finally, AmB in hyperbaric hyperoxia killed significantly more (75%) promastigotes than hyperbaric hyperoxia alone. High oxygen tensions did not significantly alter the lethal effects of either menadione or phenazine methosulfate. In conclusion, the lethal effects of low dose AmB in *Leishmania* promastigotes were augmented by hyperbaric hyperoxia in vitro, but only at oxygen doses too high to be tolerated by human patients.  

hyperbaric hyperoxia, oxidative stress, Leishmania, promastigotes, amphotericin B, phenazine methosulfate, menadione, redox cycling drugs  

*Leishmania* are protozoan parasites that cause debilitating disease in tropical regions of South America, Asia, Africa, and the Middle East. Leishmaniases are more abundant than was previously thought. Twelve million cases exist worldwide and 350 million people are estimated to be at risk for acquiring these infections (1). In endemic areas, leishmaniases are transmitted to human hosts through bites of phlebotamine sand flies infected with the extracellular promastigote form of the parasite. Recently, attention has been focused on this disease in the United States because seven cases of visceral leishmaniases were identified in military personnel returning from countries surrounding the Persian Gulf (M. Grogl, personal communication, 1991).  

321
Pentavalent antimony is the chief chemotherapeutic agent used to treat the clinical forms of leishmaniases: visceral, cutaneous, and mucocutaneous (2). Amphotericin B (AmB) is used as a second-line drug for treatment of nonresponsive leishmaniases (2, 3). AmB is a polycyclic antibiotic primarily used to treat fungal infections and its fungicidal effects are mediated by an oxygen-dependent mechanism (4–6). Because AmB autooxidizes in the presence of molecular oxygen (7, 8), reactive oxygen intermediates formed are thought to be responsible for the fungicidal effects of AmB. Pro-oxidants, such as ascorbic acid under high oxygen tensions (9) and hyperbaric hyperoxia (10), enhance the lethal effects of AmB against Candida albicans.

A proposed mechanism for the lethal effects of AmB is formation of superoxide anion when the drug undergoes autooxidation (8). Superoxide anion is also produced when oxygen accepts an electron from drug free radicals. Electron carriers, such as menadione (MDN) and phenazine methosulfate (PMS), generate superoxide anions during redox cycling reactions and induce lethal effects in Leishmania promastigotes (11). However, the combined effects of pro-oxidants such as hyperoxia and redox cycling agents against Leishmania have not been characterized.

Leishmania promastigotes may be more vulnerable than fungi to oxidative stress induced by hyperoxia combined with AmB, MDN, or PMS. The major antioxidant defense of Leishmania spp. promastigotes is superoxide dismutase. Superoxide anions formed during AmB auto-oxidation (7), during MDN or PMS redox-cycling reactions, or under hyperoxic conditions (12) undergo rapid dismutation catalyzed by superoxide dismutase to form hydrogen peroxide (H₂O₂). In most eukaryotic cells the H₂O₂ is then detoxified by catalase or glutathione peroxidase. However, low levels of these enzymes are present in Leishmania spp. promastigotes (13, 14). Thus, H₂O₂ may accumulate in a sufficient concentration in promastigotes under oxidative stress to cause toxic effects, such as enzyme inactivation. In fact, studies have shown that H₂O₂ is directly toxic to Leishmania spp. in vitro (14–16) and inactivates L. tropica superoxide dismutase (13). Therefore, it is likely that Leishmania promastigotes are vulnerable to even the low concentrations of H₂O₂ (nM) generated under hyperoxic conditions (17). The purpose of this study was to explore the oxygen tolerance of the free-living promastigotes in combination with drugs. Specifically we studied the tolerance of L. braziliensis panamensis promastigotes for high doses of oxygen and whether the drug-induced killing of promastigotes could be augmented by concomitant exposure to high oxygen tensions.

MATERIALS AND METHODS

Test organism

Leishmania braziliensis panamensis (WRAIR 676) promastigotes were obtained from Dr. Ronald Anthony, Department of Pathology, University of Maryland, Baltimore, MD. L. braziliensis panamensis promastigotes were maintained at 27°C (optimal temperature for growth) in Dulbecco's modified Eagle's medium (DMEM), formula no. 78-0176P, containing 10% fetal bovine serum (GIBCO, BRL, New York). Before each experiment, actively growing cell suspensions were diluted 1:10 and incubated as above for 72 h. Promastigotes were then pelleted by centrifugation at 250 × g for 10 min and the supernatant was discarded. The pellet was washed twice.
with phosphate buffered saline (PBS), adjusted to $10^7$ organisms/ml, and added to oxygenated media with or without drug such that the final concentration of promastigotes was $10^6$/ml.

**Drugs and oxygen exposures**

Amphotericin B, MDN bisulfite, and PMS were purchased from Sigma Chemical Co., St. Louis, MO. The formulation of AmB contained sodium deoxycholate (35%) to facilitate dispersion of AmB in water. AmB, MDN, and PMS were dissolved in distilled water to yield 1.0 M stock solutions. Stock solutions of drugs were filter sterilized and stored under nitrogen at -20°C to prevent auto-oxidation. Sodium deoxycholate used alone, in the concentration used to solubilize AmB, did not affect promastigote viability. The stock solutions of drugs were diluted in DMEM to yield the following concentrations for experiments: AmB (0.2 $\mu$M), MDN (9.4 $\mu$M), and PMS (6.3 $\mu$M). Preliminary results showed that these drug concentrations were sufficient to induce at least 20% killing of *L. braziliensis panamensis* promastigotes. These concentrations were low enough to enable detection of additional killing by oxygen.

Dissolved oxygen tensions of media with or without drugs were adjusted using vacuum-assisted diffusion (18). Each desired oxygen atmosphere (Table 1) was achieved in a controlled atmosphere glove box (Labconco Corp., Kansas City, MO) using an oxygen regulator, and confirmed using a model 1100 medical gas analyzer (Perkin-Elmer Corp., Pomona, CA). Media were pipetted into a 0.5-liter bottle, and dissolved gases were removed using a vacuum pump connected to the glove box. Each desired dissolved oxygen tension was achieved by swirling bottled media while under negative pressure, followed by venting the bottle into the glove box atmosphere. The vacuum/venting procedure was repeated several times over a 5-min period. Achievement of the target oxygen tension was confirmed by injecting oxygenated media into a model 170 pH/blood gas analyzer (Corning Medical, Medfield, MA).

**Promastigote viability**

After the target oxygen tension was achieved in the culture media, one part promastigote suspension was added to nine parts oxygenated media with or without a drug (AmB, MDN, or PMS). The final concentration of promastigotes was $1 \times 10^7$/ml.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percent $O_2$</th>
<th>Pressure*</th>
<th>Dissolved $Po_2$ $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>kPa</td>
<td>mmHg</td>
</tr>
<tr>
<td>Normoxia</td>
<td>21</td>
<td>101.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Normobaric hyperoxia</td>
<td>100</td>
<td>101.3</td>
<td>91.7</td>
</tr>
<tr>
<td>Hyperbaric hyperoxia</td>
<td>100</td>
<td>253.3</td>
<td>229.3</td>
</tr>
</tbody>
</table>

*Total pressure of gases expressed as kilopascals.

*Partial pressure of oxygen as measured in culture media.
Five-milliter aliquots were pipetted into 25-cm² tissue culture flasks. The culture media in the flasks was approximately 3-mm deep to facilitate oxygen diffusion. Flasks were incubated in air (normoxia, 21% O₂, 101.3 kPa), normobaric hyperoxia (100% O₂, 101.3 kPa), or hyperbaric hyperoxia (100% O₂, 253.3 kPa) at 27° ± 1°C for up to 24 h (Table 1). Normobaric hyperoxic cultures were maintained in controlled atmosphere culture chambers (Bellco Inc., Vineland, NJ). Hyperbaric hyperoxia was achieved and maintained in a model 615 HP hyperbaric chamber (Bethlehem Corp., Bethlehem, PA). After incubation, promastigote suspensions were removed from flasks, pelleted, and washed with PBS as above. Promastigotes were then stained with 5 µg/ml fluorescein diacetate (FDA) for 5 min. At least 100 viable organisms, which stained green with FDA, were counted on a hemacytometer using an epifluorescent microscope. All assays were conducted in a blinded manner.

Statistical analysis

Data were analyzed using a one-way analysis of variance. The Kruskal-Wallis test was used to determine if significant differences existed between experimental groups. Data were considered significantly different at P < 0.05.

RESULTS

Effects of oxygen on AmB-induced killing of promastigotes

As oxygen tensions in culture media increased, numbers of viable L. braziliensis panamensis promastigotes decreased (Fig. 1). Growth stasis was seen in hyperoxia-exposed promastigote cultures, whereas hyperbaric hyperoxia induced lethal effects. Normoxic control cultures (Po₂ = 21.1 kPa) grew 3-fold over 24 h. Numbers of viable promastigotes in normobaric hyperoxic control cultures (Po₂ = 91.7 kPa) were not significantly different from the original inoculum, yet the number of viable promastigotes decreased 87% from the original inoculum in hyperbaric hyperoxia (P < 0.05). The Po₂ in these cultures had been increased to 229.3 kPa via a hyperbaric chamber. Promastigote viability was decreased to the same extent in hyperbaric hyperoxia control cultures as in normoxic AmB-treated cultures.

Achievement of the maximal oxygen tension induced by hyperbaric oxygenation was necessary before interactive effects with AmB were observed. There was no decrease in viability when promastigotes were exposed to AmB under hyperoxic conditions as compared to normoxic AmB-treated cultures. However, AmB-induced killing of promastigotes was significantly increased by concomitant exposure to hyperbaric hyperoxia as compared to normoxic and hyperoxic conditions (75% more killing). Significant lethal effects for AmB under hyperbaric hyperoxic conditions were not found when promastigote cultures were exposed for less than 2 h.

Effects of oxygen on menadione and phenazine methosulfate-induced killing of promastigotes

As seen in Fig. 1, both MDN and PMS induced lethal effects in promastigotes which were slightly (NS) augmented by high oxygen tensions. Each redox cycling
HBO ENHANCES LETHAL EFFECTS OF AMB

![Graph showing viability of L. braziliensis panamensis promastigotes after 24-h exposure to AmB (0.2 μM), MDN (9.4 μM), and PMS (6.3 μM) in different oxygen environments. Initial concentration of promastigotes was 1 × 10^6/ml (broken line). Each vertical bar represents the mean number of viable promastigotes ± SD (n = 3). At least 100 promastigotes were counted for each condition in these experiments. Asterisk denotes significant difference from normoxic control (P < 0.05). Double asterisk denotes significant difference from AmB in normoxic and hyperoxic conditions and from hyperbaric hyperoxic control (P < 0.05).]

The results of this study showed that prolonged exposure to high oxygen tensions is toxic for L. braziliensis panamensis promastigotes. Normobaric hyperoxia inhibited growth of promastigotes, whereas hyperbaric hyperoxia actually killed them. It is probable that promastigotes suffered sublethal and lethal effects due to the increased flux of oxygen-derived free radicals under hyperoxic and hyperbaric hyperoxic conditions. The study also examined the combined effects of high oxygen tensions and drugs capable of generating oxygen free radicals. AmB-induced killing of promastigotes in normobaric hyperoxia was not different from AmB-induced killing in normoxic conditions. This comparison serves to underscore the conclusion that normobaric hyperoxia inhibited growth of promastigotes but was not synergistic with AmB-induced lethal effects. The combined effects of hyperbaric hyperoxia and AmB were

drug reduced the original inoculum by at least 64% in normoxic conditions over 24 h. Exposure of promastigotes to MDN and PMS under hyperoxic or hyperbaric hyperoxic conditions did not significantly decrease the numbers of viable organisms as compared to normoxic conditions.

DISCUSSION

The results of this study showed that prolonged exposure to high oxygen tensions is toxic for L. braziliensis panamensis promastigotes. Normobaric hyperoxia inhibited growth of promastigotes, whereas hyperbaric hyperoxia actually killed them. It is probable that promastigotes suffered sublethal and lethal effects due to the increased flux of oxygen-derived free radicals under hyperoxic and hyperbaric hyperoxic conditions. The study also examined the combined effects of high oxygen tensions and drugs capable of generating oxygen free radicals. AmB-induced killing of promastigotes in normobaric hyperoxia was not different from AmB-induced killing in normoxic conditions. This comparison serves to underscore the conclusion that normobaric hyperoxia inhibited growth of promastigotes but was not synergistic with AmB-induced lethal effects. The combined effects of hyperbaric hyperoxia and AmB were
additive in that they increased promastigote killing over that seen in normobaric hyperoxia and AmB. In contrast, neither normobaric hyperoxia nor hyperbaric hyperoxia significantly increased promastigote killing by MDN or PMS. The flux of oxygen-derived free radicals from AmB, MDN, and PMS may have been nearly maximal under normoxic conditions, so that no significant increase in promastigote killing was seen under normobaric hyperoxic conditions. Under hyperbaric hyperoxic conditions, the reducing equivalents necessary for continuous redox cycling of MDN and PMS may not have been available. The lethal effects observed with hyperbaric hyperoxia alone may have been due to lipid peroxidation. AmB auto-oxidation does not result in lipid peroxidation (7), but does result in the formation of carbon-centered free radicals (19). These carbon-centered radicals react with oxygen to eventually form superoxide anion (19). Dismutation of superoxide to H$_2$O$_2$ and formation of other reactive oxygen metabolites could result in lethal effects to Leishmania. We conclude that hyperbaric hyperoxia, AmB, and the redox cycling drugs MDN and PMS may kill Leishmania by different mechanisms.

It is known that oxygen is required for the lethal effects of AmB against C. albicans (5) and Trypanosoma cruzi (7), a protozoan parasite related to Leishmania. However, the mechanism(s) for the lethal effects of AmB have not been determined. Sokol-Anderson et al. (5) showed that by lowering the O$_2$ tension, the efficacy of AmB is reduced. In their work, AmB-induced lysis of C. albicans protoplasts was reduced by 80% in relatively hypoxic conditions (PO$_2$ = 40 mmHg) as compared to AmB in air. A PO$_2$ of 40 mmHg approximates normal host tissue oxygen tensions. However, in our study (20), AmB-induced killing of Leishmania promastigotes was not altered under the same conditions as compared to air.

The results of our current study are consistent with the work of Gudewicz et al. in C. albicans (10). They found that neither the minimal inhibitory concentration (MIC) nor the minimal candidacidal concentration (MCC) of AmB for C. albicans was altered by exposure to a PO$_2$ of 900 mmHg for 24 h. The MIC is the lowest concentration of an antifungal agent which will cause growth to cease in culture, and the MCC is the lowest concentration of an antifungal agent which will kill fungal cells. The lethal effects of short-term (90 min) hyperbaric hyperoxia (PO$_2$ = 1,800 mmHg) were additive to the lethal effects of AmB. Both the MIC and MCC of AmB for C. albicans were decreased from 0.39 μg/ml in air to 0.10 μg/ml in hyperbaric hyperoxia. In our study, an additive lethal effect was also seen when Leishmania promastigotes were exposed to AmB in hyperbaric hyperoxia (PO$_2$ = 1,720 mmHg). However, short-term lethal effects of hyperbaric hyperoxia were not observed. The results of these two studies indicate that PO$_2$ in excess of 1,700 mmHg may be required to enhance lethal effects of AmB in yeast cells and protozoan parasites. Such high oxygen tensions can be attained in wound tissue using hyperbaric oxygenation. Oxygen tensions ranging from 1,000 to 1,700 mmHg have been reported in wounds of human patients (21) during hyperbaric oxygen (HBO) treatment (100% O$_2$ at 2.4 atm abs, PO$_2$ = 243 kPa) using electrodes implanted in the wound. In this study, however, the duration of exposure of parasites to hyperbaric hyperoxia was much longer (24 h) than could be tolerated by a human patient. Interestingly, no significant detrimental effects were seen in promastigote cultures exposed to AmB under hyperbaric hyperoxic conditions for a single 2-h period.

Thus, it is clear that high partial pressures of oxygen (e.g., >240 kPa) might possibly exert detrimental effects in fungi and protozoan parasites treated with AmB.
in vivo. The effects of repeated intermittent HBO treatments on fungi and parasites treated with Amb in suitable animal models are yet to be determined.

We thank John Sacci, Jr. for helpful discussions. We also thank Patricia Schleiff for statistical support and Roderick Herrin, David Nelson, and Bernard Wilson for excellent technical support. This study was supported by grant 90-03177 from the Air Force Office of Scientific Research. Kenneth Muhlich was supported by the Callender-Brinfield Fellowship in pathology sponsored by the American Registry of Pathology, Washington, DC 20030-6000.

A portion of this study was published as paper no. 682 in the Program and Abstracts of the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy. The ICAAC meeting was held September 29–October 2, 1991 in Chicago, IL.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, the Department of the Air Force, or the Department of Defense. This manuscript was prepared by U.S. Government employees as part of their official duties and cannot be copyrighted and may be copied without restriction.

Address all correspondence to Major Anderson.—Manuscript received May 1992; accepted September 1993.

REFERENCES