Effects of hyperbaric oxygen on 
Pseudomonas aeruginosa susceptibility to 
imipenem and macrophages

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ABSTRACT Background: The seriousness to treat burn wounds infected with Pseudomonas aeruginosa led us to examine whether the effect of the carbapenem antibiotic imipenem is enhanced by hyperbaric oxygen (HBO). Materials & methods: The effects of HBO (100% O₂, 3 ATA, 5 h) in combination with imipenem on bacterial counts of six isolates of P. aeruginosa and bacterial ultrastructure were investigated. Infected macrophages were exposed to HBO (100% O₂, 3 ATA, 90 min) and the production of reactive oxygen species monitored. Results: HBO enhanced the effects of imipenem. HBO increased superoxide anion production by macrophages and likely kills bacteria by oxidative mechanisms. Conclusion: HBO in combination with imipenem can be used to kill P. aeruginosa in vitro and such treatment may be beneficial for the patients with injuries containing the P. aeruginosa.

KEYWORDS
• free radicals • hyperbaric oxygen • imipenem • macrophages • Pseudomonas aeruginosa • superoxide anion

Pseudomonas aeruginosa, a Gram-negative aerobic opportunistic bacterium, is associated with nosocomial infections and is the most common agent of burn injury infections [1,2]. Pseudomonas aeruginosa may be resistant to all clinically significant antibiotics [3], including group 2 carbapenems such as imipenem, which is the last choice for treating patients with serious hospital nosocomial infections [4]. In the search for new, alternative treatments, hyperbaric oxygen (HBO) therapy has been tested [5,6]. HBO is the application of pressure greater than 1 ATA to an environment of 100% O₂ and its therapeutic principles stems from increasing the partial pressure of oxygen in the tissues and the oxygen carrying capacity of blood plasma beyond those achievable under normobaric conditions [5–7]. HBO has been tested in animal models of P. aeruginosa osteomyelitis [5,8] and in vitro in combination with tobramycin [9] and streptomycin [10]. In addition, the interaction between P. aeruginosa and mononuclear phagocytes has been shown to be altered under long-term HBO exposure [11,12]. In this study we report for the first time the effect of HBO in combination with imipenem on P. aeruginosa counts and bacterial ultrastructure. We also evaluated the effect of HBO on infected macrophage cultures. Since HBO exposition induces oxidative stress both in vivo and in vitro [13–15], the production of superoxide anion Pseudomonas-infected macrophage cultures was monitored by a specific methodology.
Materials & methods

- Bacterial strains
The *P. aeruginosa* isolates PaHC1, PaHC2 and PaHC3 isolated from cystic fibrosis patients in Brazil were described previously [16]; isolates FC01 and 67eNM were a gift from Dr CE Levy (Hospital das Clinicas, Unicamp, Campinas, SP, Brazil) and the strain ATCC 27853 was a gift from Dr LG Rahme (Department of Surgery, Massachusetts General Hospital and Harvard Medical School, MA, USA).

- Macrophage cultures
Primary mouse macrophages were obtained from BALB/c mice by peritoneal lavage, cultured on 35 mm cell culture dishes (10^6 macrophages/dish) and 24-well plates (2 x 10^5 macrophages/well) containing 13-mm diameter glass...

Figure 1. Effect of hyperbaric oxygen exposition on *Pseudomonas aeruginosa*. Bacteria ATCC (A), 67eNM (B), FC01 (C), PaHC1 (D), PaHC2 (E) and PaHC3 (F), were exposed to normoxia (2), normoxia and imipenem (3), HBO (4), HBO and imipenem (5) for 5 h. (1) refers to CFU/ml measured immediately before treatments. Data are mean ± SD.

*p ≤ 0.05 (one-way ANOVA).
CFU: Colony-forming unit.
coverslips as described previously [17]. The experimental protocols were approved by the Institute of Biology/Universidade Estadual de Campinas Ethical Committee for Animal Research.

- HBO treatments
Inoculated bacteria on Mueller-Hinton broth agar contained or not antibiotic imipenem were exposed to normoxia (21% O_2, 5% CO_2 and balanced N_2) or HBO (100% O_2) in an HBO chamber (Research Chamber, model HB 1300B, Sechrist, CA, USA) under the pressure of 3 ATA at 37°C for 5 h. Since the isolates vary in their sensitivity to imipenem, different sub-inhibitory concentrations of this antibiotic were used to control _P. aeruginosa_. After treatments, an aliquot was sampled and plated for CFU/ml (colony-forming unit) determination during 18 h at 37°C in normoxia. The macrophage cultures were infected with _P. aeruginosa_ at different bacteria: macrophage ratios in RPMI medium (Sigma Chemical Co., MO, USA) without antibiotics for 90 min under HBO condition. The number of viable bacteria in the supernatants was determined by plating the samples and counting CFU/ml. The number of viable bacteria within macrophages was determined by washing cell cultures with saline and gentamicin 200 μg/ml for 30 min to kill extracellular bacteria. Macrophages were then washed with saline, lysed with 0.1% Triton X-100 in water and sampled for CFU/ml determination. Additional macrophages cultured on coverslips instead to be lysed were fixed with methanol and stained with Giemsa [17]. Macrophage viability was analyzed by the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye-reduction assay (Sigma Chemical) as described by Mosmann [18].

- Transmission electronic microscopy
For ultrastructural analyses, bacteria were washed with PBS, fixed during 12 h at 4°C in 3% glutaraldehyde (Electron Microscope Science, PA, USA) in 0.1 M sodium cacodylate (Electron Microscope Science) buffer at pH 7.4, centrifuged at 12,000 rpm (Eppendorf 5415R, rotor F45-24-11), resuspended in 0.1 M sodium cacodylate buffer, pH 7.4 and then post-fixed in 1% OsO_4 (Electron Microscope Science). Following dehydration in an ethanol gradient, bacteria were embedded in Epon 812 resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss LEO 906 (Leica, Oberkochen, Germany) transmission electron microscope operated at 60 kV A [19].

- Superoxide radical anion monitored by 2-hydroxyethidium (2-OH-E') production
Macrophages (1 × 10^6 cells) that were cultured overnight in 35-mm cell culture dishes were washed twice with PBS (pH 7.4), covered with 1 ml PBS/glucose (1 g/l)/diethylenetriaminepentaacetic acid (DTPA; 100 μM; Sigma Chemical Co., MO, USA), infected or not with _P. aeruginosa_ (1 × 10^7 bacteria), added with 50 μM dihydroethidium (DHE; Molecular Probes, OR, USA) and incubated in normoxia (5% CO_2/95% air) or HBO (100% O_2, 3 ATA) in the dark for 90 min at 37°C. After incubation, extracellular medium was collected, centrifuged and the supernatant was kept on dry ice. Cells were washed twice with PBS/DTPA, scraped in lysis buffer (PBS/DTPA/0.1% Triton X-100) and centrifuged. Cell protein level was measured in cell lysate supernatant by Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysate supernatant was treated with 0.2 M HClO_4 in methanol 1:1 (v:v) for 1 h on ice to precipitate proteins. After centrifugation, supernatant was treated with 1 M phosphate buffer pH 2.6 to eliminate excess of ClO_4^- and the supernatant
Figure 3. Effect of hyperbaric oxygen on Pseudomonas aeruginosa infected macrophages. Macrophages infected with P. aeruginosa at different bacteria:macrophage ratios 0.0002:1 (1), 0.002:1 (2), 0.2:1 (3), 2:1 (4), 20:1 (5) were exposed to normoxia or hyperbaric oxygen for 90 min, and supernatants (A) and the intracellular macrophage content (lysed macrophages) (B) evaluated for colony-forming unit/ml determination.

was kept on dry ice [20]. Supernatants from extracellular medium (extracellular sample) and cell lysate (intracellular sample) were analyzed for DHE and its derived oxidation products +, 2-OH-E’ and ethidium (E’) by HPLC using UV and fluorescence detection as described previously [20,21] with the following modifications. Chromatographic separation was carried out with the use of a Phenomenex Synergi 4 μm Polar-RP 80A column (150 × 4.60 mm) in a HPLC system (Shimadzu, Kyoto, Japan). Solutions A (water/60% acetonitrile with 0.1% TFA) and B (water/90% acetonitrile with 0.1% trifluoroacetic acid (TFA) were used as a mobile phase at a flow rate of 0.6 ml/min. Runs were started with 40% solution A (maintained for the initial 5 min), increased linearly to 100% solution A from 5 to 25 min, kept at this proportion for another 10 min, changed to 40% solution A for additional 5 min and kept at this proportion for another 6 min. DHE was monitored by ultraviolet absorption at 245 nm. 2-OH-E’ and E’ were monitored by fluorescence detection with excitation 480 nm and emission 580 nm. Quantification was performed by comparison of integrated peak areas between the obtained and standard solutions under identical chromatographic conditions and normalized against cell protein levels.

- Data analysis
All experiments were repeated at least twice in triplicate and the results are expressed as mean ± SD. Statistical analyses were performed using the one-way analysis of variance (ANOVA; p < 0.05) and the posteriori test two-sample t-test (p < 0.05).

Results
The sensitivities of six isolates of P. aeruginosa for HBO and imipenem are shown in Figure 1. In these experiments bacteria on agar surface were exposed for 5 h at 3 ATA to pure O₂, and then plated and tested for colonial growth in normoxia. Since these isolates vary in their sensitivity to imipenem different doses were used for each strain. The HBO alone reduced CFU/ml of ATCC strain significantly more than the treatment with imipenem; the combination of HBO and antibiotic was the most effective treatment (Figure 1A). HBO alone was more effective than imipenem against bacteria for the FCO1, PaHC1, PaHC2 and PaHC3 isolates and, the combination of HBO and antibiotic showed the most effective antimicrobial activity (Figure 1C–1F). The only exception was the 67eNM isolate; imipenem alone was more effective than HBO alone against this bacterium and the treatment with HBO and antibiotic was the most effective in reducing CFU/ml (Figure 1B).

The effect of HBO on the ultrastructure of P. aeruginosa was also examined in this study (Figure 2). Bacteria under normoxia condition exhibited typical nucleoid region characterized by a translucid area with fiber, the chromosome, normal ribosomes and multilayered cell wall in most cells examined (Figure 2A). Bacteria exposed to HBO have cell wall with normal appearance but the cytoplasm more finely granular and the absence of a defined nucleoid region (Figure 2B). The morphology of bacteria treated with imipenem showed the same pattern as those exposed to HBO, and extrusion of bacterial cytoplasm...
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occurring in about 20% of the cells (Figure 2C). Bacteria exposed to HBO and treated with antibiotic showed remarkable morphological changes including deformation and disorganization of cytoplasmic content (Figure 2D).

Next we evaluated whether HBO alters *P. aeruginosa* infection in macrophages. Immediately after bacteria were added, macrophage cultures were exposed to HBO for 90 min. Figure 3 shows that the supernatants of macrophage cultures exposed to HBO have a content of CFU/ml similar to that of supernatants of macrophage cultures exposed to normoxia. However, a reduction in CFU/ml was significant in the lysates of macrophage cultures exposed to HBO compared with CFU/ml in lysates from those in normoxia. Indeed, after *P. aeruginosa* infection macrophages under normoxia changed their morphological appearance and presented decreased viability with increasing bacteria: macrophage ratios (Figures 4 & 5). By contrast, macrophages exposed to HBO did not exhibit morphological changes and their viability was improved after 90 min exposition.

Next we examined whether the antibacterial effect of HBO in macrophages is related to free radical generation. To this end, we employed a methodology that permits detection of superoxide anion and other oxidants by the oxidation products of DHE, 2-OH-E' and E' ([20,21]). DHE was incubated with macrophages submitted to different conditions and DHE and its products quantified as described in materials and methods. Control experiments showed that DHE in buffer alone suffers considerable oxidation during the long incubation used in our experiments; therefore, only intracellular levels of DHE and its products in macrophages were quantified. The results showed that the intracellular levels of 2-OH-E' significantly change in macrophages submitted to HBO, whereas those of DHE and E' do not (Figure 6). The intracellular levels of 2-OH-E' in infected macrophages increased under HBO but not under normoxia as compared with noninfected macrophages under both conditions. Although the intracellular levels of 2-OH-E' did not change significantly with infection under normoxia, they were higher in infected macrophages than in noninfected macrophages under HBO. Therefore, HBO significantly increases the levels of superoxide anion produced in infected macrophages.

Discussion

*P. aeruginosa* is a Gram-negative obligate aerobic bacteria and a main cause of infection in

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**Figure 4.** Effect of hyperbaric oxygen on *Pseudomonas aeruginosa* infected macrophage cultures. Macrophages exposed normoxia (A) and hyperbaric oxygen (HBO) (B). Macrophages infected with *P. aeruginosa* at different bacteria: macrophage ratios (0.0002:1–20:1) were exposed normoxia (C–G) and HBO for 90 min (H–L). Note the difference in the number of macrophages in the coverslips maintained in normoxia and exposed to HBO. Photomicrographs were taken of cell cultures stained with Giemsa (×400).
Figure 5. Effect of hyperbaric oxygen on the viability of macrophages. (A) Cell cultures infected with *Pseudomonas aeruginosa* at different bacteria: macrophage ratios (2:1–20:1) were exposed to normoxia or hyperbaric oxygen for 90 min stained with Giemsa and the number of cells counted in 20 random fields. (B) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide production was determined in macrophages treated with H$_2$O$_2$ 800 uM (1), 400 uM (2) or exposed to normoxia (3) or hyperbaric oxygen (4).

hospitalized, immunocompromised and burned patients [1,2]. Since antibiotic resistance renders *P. aeruginosa* infection difficult to treat [3,22], HBO exposure is a valid therapy even for patients with lesions sustained by multidrug-resistant microorganisms [3]. In the present study 5 h HBO exposition was bacteriostatic for agar surface cultures of six isolates of *P. aeruginosa*. The adverse effects of HBO and hyperoxia on *P. aeruginosa* have been demonstrated in other...
Figure 6. Effect of hyperbaric oxygen in the intracellular levels of dihydroethidium and its oxidation products generated by macrophages submitted to different conditions. Macrophages were incubated in PBS/glucose (1 g/l) containing diethylenetriaminepentaacetic acid (100 μM); after the addition of dihydroethidium (50 μM) and infection or not with Pseudomonas aeruginosa, the incubations were exposed to normoxia or hyperbaric oxygen in the dark for 90 min at 37°C. The intracellular levels of dihydroethidium and its oxidation products (2-OH-E+ and E+) were analyzed and quantified by HPLC as described in materials and methods; quantification is shown in the left side and representative chromatograms of the sample exposed to hyperbaric oxygen are shown in the right side. The values shown correspond to the mean ± SD; n = 3.

*p ≤ 0.05 (one-way ANOVA).
research [9–10, 23–26]. For example, Pakman demonstrated that HBO (3 ATA for 24 h) was largely bactericidal for *P. aeruginosa* 268–3 cultures [23]. Bornside et al. noted the bactericidal effects on *P. aeruginosa* after exposition to pure O₂ at 3 ATA was higher than at 1 ATA, demonstrating that O₂ high-pressure is required for the observed effect [25] (also our data not shown). These investigators exposed bacteria to HBO for longer time periods (18–24 h) than those that can be tolerated by humans. Here, to observe microorganism killing, we monitored growth inhibition after 5 h HBO. Although the exact mechanism by which HBO affects bacteria cells is not known, it has been proposed that the delay in cell growth was associated with a decrease in protein synthesis [27]. Indeed, it has been shown that [35S] methionine incorporation was reduced in *P. aeruginosa* exposed to HBO [9]. HBO can inhibit growth also by blocking RNA transcription and DNA synthesis or via injury to DNA [28]. In fact, the changes in bacteria ultrastructure observed here and previously [29] indicated nucleoid disorganization of *P. aeruginosa* cells exposed to HBO.

The seriousness and difficulties to treat burn wounds infected with *P. aeruginosa*, which frequently does not respond to antibiotics [10, 30], led to a number of studies exploring the possibility that the effect of antibiotics is enhanced by HBO [9–10, 23, 27, 31]. Indeed, the efficacy against *P. aeruginosa* of aminoglycoside antibiotics, such as tobramycin, streptomycin and kanamycin and sulfonamides, such as mafenide and sulfisoxazole, was increased by HBO [9–10, 23, 28]. Hind and Attwell reported that the inhibitory effect of rifamycin against *P. aeruginosa* could be enhanced by HBO [31]. Our study showing the in vitro enhancement of imipenem antibacterial activity by HBO provides further support for the combination therapy. Currently, there is no report in the literature on the effect of HBO in combination with imipenem against *P. aeruginosa* and other bacterial species. The carbapenem β-lactam antibiotic imipenem exerts its antimicrobial action by inhibiting bacterial cell wall synthesis and by binding with high affinity to penicillin-binding proteins [32]. The manner by which HBO potentiates the activity of antimicrobials is not completely understood, some antibiotics are less effective in hypoxic environments and hyperoxia achieved during HBO has been suggested to increase the effectiveness of antibiotics [33]. An alternative mechanism suggested for enhancing susceptibility to antibiotics is that an inhibition of aminoacids synthesis by HBO leads to decreasing of membrane transport protein altering membrane permeability and no selecting transportation [5, 28, 31].

Exposition to HBO affects inflammatory and immune cells functions including antimicrobial activities of macrophages and neutrophils [34–36]. In fact, the data present here show macrophage death during *P. aeruginosa* infection as a process that is inhibited by HBO. It should be noted that while the time exposition for HBO potentiates the antimicrobial function of macrophages was 90 min the time exposition for HBO and antibiotic to inhibit bacteria growth was longer 5 h. The reason for the difference in the kinetics of HBO effect is not known at present. More important, we demonstrated a higher production of superoxide anion radical by infected macrophages under HBO. Therefore, we provide evidence for a role of oxygen radicals in the amplifying effect of HBO on the bactericidal activity of macrophages. Accordingly, it is well known that reactive oxygen metabolites plays a role in the bactericidal activity of phagocytic cells [37–39] and that HBO provides optimal oxygen tension for the oxidative burst in different cells [38–41].

**Conclusion & future perspective**

*Pseudomonas aeruginosa* is a Gram-negative obligate aerobic bacteria and a main cause of infection in hospitalized, immunocompromised and burned patients. Since *P. aeruginosa* may be resistant to all clinically significant antibiotics including group 2 carbapenens such as imipenem, the last choice for treating patients with serious hospital nosocomial infections, a number of studies has explored the hypothesis that the effect of antibiotics could be enhanced by HBO. The results indicate that HBO exposed enhanced the effects of imipenem in *P. aeruginosa*. This study adds new information on the effects of HBO in *P. aeruginosa*-infected macrophages. Furthermore, HBO increases the production of superoxide anion by macrophages and likely contributes to the killing of the bacteria by oxidative mechanisms. The findings argue for further research on time-dependent HBO treatment of soft tissues infection in animal models inoculated with *P. aeruginosa*. Developing HBO protocols (e.g., optimal partial pressure of oxygen and safe time) and determining the animal species differences. It is also our premise that the
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Treatment with HBO may be beneficial for the patients with injuries containing *P. aeruginosa* justifying clinical studies.

**Financial & competing interests disclosure**

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**Ethical conduct**

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

**EXECUTIVE SUMMARY**

**Sensitivity of six isolates of *Pseudomonas aeruginosa* for HBO and imipenem**

- Hyperbaric oxygen (HBO) alone was more effective against bacteria than imipenem for FCO1, PaHC1, PaHC2 and PaHC3 isolates and, the combination of HBO and antibiotic showed the most effective antimicrobial activity; the only exception was 67eNM isolate.

- HBO alone reduced colony-forming unit/ml of ATCC strain significantly more than the treatment with imipenem.

**The effect of HBO on the ultrastructure of *P. aeruginosa* determined by transmission electronic microscopy**

- Bacteria exposed to HBO exhibited cytoplasm more finely granular and absence of a defined nucleoid region.

- Bacteria treated with imipenem showed the same pattern of ultrastructure modifications as those exposed to HBO, and the extrusion of bacterial cytoplasm occurring in circa 20% of cells.

- Bacteria exposed to HBO and treated with imipenem showed the most remarkable morphological changes.

**HBO alters *P. aeruginosa* infection in macrophages**

- HBO reduced *P. aeruginosa* infection in murine macrophages under high bacteria: macrophage ratio.

- HBO appear to protect macrophages against *P. aeruginosa* and subsequent bacteria-mediated death.

**The antibacterial effect of HBO in macrophages is related to free radical generation**

- HBO significantly increases the levels of superoxide anion produced in *P. aeruginosa* infected-macrophages.

**Conclusion**

- This study demonstrates that HBO enhanced the effects of imipenem to six different strains of *P. aeruginosa*.

- HBO increased production of superoxide anion by macrophages and likely kills bacteria by oxidative mechanisms.

- This work offer new perspectives on the use of HBO in the treatment of patients with of tissue injuries infected by *P. aeruginosa*.

**References**

Papers of special note have been highlighted as:

• of interest; •• of considerable interest


• Review of current knowledge of hyperbaric oxygen (HBO) effects in the treatments of certain infectious diseases.


• Review of clinical and mechanistic data of HBO therapy.
• Investigation of hyperoxia effect on aminoglycoside activity against *Pseudomonas aeruginosa*.


• Description of hyperoxia effect on *Pseudomonas aeruginosa*.


• Investigation of the interaction between a hyperbaric environment and selected antibiotic-resistant microorganisms.


• Investigation of detrimental effect of enhanced antimicrobial activity induced by hyperbaric oxygen in the HL60 cell line


• Review of studies on the role that reactive species of oxygen and nitrogen play in therapeutic mechanisms of hyperbaric oxygen.


41 Barstow A, Ajello M,ammendola S, Superti F, Rotilio G, Valenti P. Involvement of
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**Research Article**

- Investigation of superoxide generation by a NAD(P)H oxidase-like complex and the limit of bacterial survival within epithelial cells.