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EXPERIMENTAL THERAPEUTICS

Hyperbaric Oxygen Reduces *Aspergillus fumigatus* Proliferation *In Vitro* and Influences *In Vivo* Disease Outcomes

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ABSTRACT Recent estimates suggest that more than 3 million people have chronic or invasive fungal infections, causing more than 600,000 deaths every year. *Aspergillus fumigatus* causes invasive pulmonary aspergillosis (IPA) in patients with compromised immune systems and is a primary contributor to increases in human fungal infections. Thus, the development of new clinical modalities as stand-alone or adjunctive therapy for improving IPA patient outcomes is critically needed. Here we tested the *in vitro* and *in vivo* impacts of hyperbaric oxygen (HBO) (100% oxygen, >1 atmosphere absolute [ATA]) on *A. fumigatus* proliferation and murine IPA outcomes. Our findings indicate that HBO reduces established fungal biofilm proliferation *in vitro* by over 50%. The effect of HBO under the treatment conditions was transient and fungistatic, with *A. fumigatus* metabolic activity rebounding within 6 h of HBO treatment being removed. *In vivo*, daily HBO provides a dose-dependent but modest improvement in murine IPA disease outcomes as measured by survival analysis. Intriguingly, no synergy was observed between subtherapeutic voriconazole or amphotericin B and HBO *in vitro* or *in vivo* with daily HBO dosing, though the loss of fungal superoxide dismutase genes enhanced HBO antifungal activity. Further studies are needed to optimize the HBO treatment regimen and better understand the effects of HBO on both the host and the pathogen during a pulmonary invasive fungal infection.

KEYWORDS *Aspergillus fumigatus*, fungal biofilm, fungal growth, hyperbaric oxygen, invasive aspergillosis, oxygen radicals

Invasive pulmonary aspergillosis (IPA) is a life-threatening disease caused by the filamentous fungus *Aspergillus fumigatus*. Populations at particular risk include those with prolonged neutropenia and those undergoing high-dose corticosteroid therapy (1). While development of new triazole antifungal drugs (e.g., voriconazole, posaconazole, and isavuconazole) has markedly improved invasive aspergillosis treatment outcomes, mortality rates remain high (1–6). Multiple complex factors contribute to poor disease outcomes in the setting of antifungal drug therapy, including, but not limited to, differences in the underlying immune status of the patients, underlying primary disease, time to infection diagnosis, intraspecies heterogeneity in fungal virulence, and the complexity of the infection site microenvironment (1, 7–9).

One of the major factors governing the virulence of *A. fumigatus* is its ability to persist *in vivo* under low-oxygen conditions that arise during infection (7, 10–12). Oxygen perfusion is severely limited at the infection site due to tissue damage caused by the invading fungus and the high metabolic activity of recruited host immune cells such as neutrophils. Oxygen levels of 1.5% or lower have been observed at sites of *A. fumigatus* infection in both chemotherapy- and corticosteroid-mediated murine models of IPA (12). Moreover, low oxygen levels at the site of infection have been suggested

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to contribute to fungal tissue invasion (13). Thus, the ability of *A. fumigatus* to adapt to and tolerate various levels of oxygen *in vivo* is critical for pathogenesis and disease progression in multiple murine models of IPA (10, 14, 15).

How infection tissue microenvironment oxygen levels affect antifungal drug therapy is unknown, though low-oxygen conditions in tumors are known to inhibit cancer therapies (16, 17). For example, hypoxia may be associated with increased expression of drug efflux pumps in *A. fumigatus in vitro*, and this is also known to occur in cancer therapies (18). It is unclear if altered drug efflux pump expression or activity causes lower drug concentrations at the site of fungal infection. Also, angioinvasion and tissue necrosis caused by *A. fumigatus* limit the blood supply to infected tissue (19, 20), which may limit the penetration of antifungal drugs into the infection site (21). Thus, the effective drug concentration in serum might not be a true reflection of drug levels at the hypoxic site of infection, which is a major treatment challenge as high doses of antifungal drugs are associated with host toxicities (22, 23).

Hypoxia has also been directly linked to inflammation in cancer and infectious disease settings (24, 25). Previous research identified significant increases in interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-1 protein levels in serum and from isolated macrophages when mice were exposed to 5% O_2 for 2 h (26). As severe inflammation can be detrimental to the host, hypoxia has been associated with poor disease outcomes in IPA (12, 13). For example, the IL-1 receptor antagonist anakinra was shown to reduce pulmonary hypoxia and increase murine survival in a corticosteroid model of IPA (27). Taken together, data support the concept that, similar to the case for cancer pathogenesis, hypoxia at the site of fungal infections promotes poor disease outcomes through multiple mechanisms. Consequently, alleviating hypoxia at the site of infection is worth exploring as an approach to improve fungal infection outcomes.

Precedent exists for modulating infection site oxygen levels to improve infectious disease outcomes. Previous studies have shown that modulation of oxygen tension *in vivo* using hyperbaric oxygen (HBO) (100% oxygen at a pressure of $>$1 atmosphere absolute [ATA]) improves outcome for a variety of infectious diseases, including chronic wounds, osteomyelitis, and necrotizing fasciitis (28–30). HBO has been shown to augment the efficacy of tobramycin against the bacterial pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (31, 32). Limited retrospective, single-center studies report that HBO adjunctive therapy resulted in better disease outcomes for invasive aspergillosis and zygomycosis cases (33–36); however, controlled studies to evaluate the effects of HBO as stand-alone or adjunctive treatment options for IPA are lacking. Importantly, it has long been noted that HBO *in vitro* inhibits the growth of many microbes associated with human disease, including known human fungal pathogens (37–41).

Attractive aspects of HBO therapy for invasive fungal infections include its widespread clinical use for many indications, strong safety record, and ease of noninvasive delivery (42, 43). The aim of this study was to assess the effects of both oxygen and pressure against the growth and survival of *A. fumigatus* established biofilms *in vitro* and *in vivo*. As one of the major limitations of current antifungal drug usage against IPA is host toxicity (44, 45), we also explored a combination of subtherapeutic voriconazole and/or amphotericin B therapy with HBO. Our results show that HBO markedly reduces the proliferation of *A. fumigatus* colony biofilms *in vitro* through fungistatic mechanisms. Loss of fungal superoxide dismutase (SOD) genes increased the effect of HBO on fungal growth inhibition. However, no synergy was observed between HBO and subtherapeutic antifungal drugs *in vitro* or *in vivo* with the dosing regimen used, though HBO therapy alone in a chemotherapeutic murine model of IPA showed promise at slowing disease progression and extending murine survival.

RESULTS

HBO inhibits *A. fumigatus* colony biofilm proliferation in a dose-dependent manner. Hyperbaric oxygen (HBO) inhibits the germination and growth of fungal conidia and yeast (40, 41), but it is unclear if established mycelia consisting of

interconnected hyphae such as found at sites of infection are affected by HBO treatments. *A. fumigatus* mycelia, or colony biofilms, were generated by allowing conidia to germinate on solid medium for 12 h before starting an HBO treatment regimen (Fig. 1A). Colony biofilms were exposed to HBO treatments (3.5 ATA, 100% O_2) for up to 8 h, and then colonies were returned to normobaric conditions. The cycle was repeated over a 5-day time course and the proliferation of the colony biofilms monitored by quantifying radial growth. A strong inhibitory effect of HBO on fungal colony biofilm expansion was observed at each time point tested, with a striking ~50% growth reduction at days 2, 3, 4, and 5 (Fig. 1A and B).

As clinical indications determine the pressure regimen for HBO treatment, we studied the effects of different pressures on the growth of *A. fumigatus*. At 2.5 ATA, we observed a pattern of growth inhibition similar to that with 3.5 ATA but with reduced magnitude. At 2.5 ATA, on day 2, an ~35% growth inhibition was observed, compared to the ~50% inhibition observed at 3.5 ATA. On days 4 and 5 at 2.5 ATA, an ~40% growth reduction was observed, which is significantly lower than the ~50% observed at 3.5 ATA ($P < 0.01$) (Fig. 1B).

Because fungal growth inhibition showed a pressure-dependent response, we investigated the role of pressure alone by using hyperbaric air (~21% oxygen, ~79% nitrogen) at 3.5 ATA of pressure. Cultures grown under the hyperbaric air regimen did not show a significant growth difference compared to normobaric air cultures, suggesting that fungal growth inhibition is, at least in part, oxygen dependent (Fig. 1B). To assess the role of oxygen alone, we subjected the cultures to normobaric oxygen (100% oxygen, 1 ATA). Cultures grown with normobaric oxygen showed a statistically significant but biologically minimal (~10%) growth inhibition on days 4 and 5 (Fig. 1B). These observations indicate that the combination of pressure and oxygen used in HBO treatments results in growth inhibition of established *A. fumigatus* colony biofilms.

HBO is fungistatic against *A. fumigatus*. Previous reports suggested that pressure determines whether HBO antifungal activity is fungistatic or fungicidal (40). Thus, the observations with *A. fumigatus* colony biofilms prompted us to test the fungistatic versus fungicidal effect of HBO against *A. fumigatus* using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay as previously described (46). Consistent with the observed colony proliferation inhibition, at 3.5 ATA, a 75% reduction in fungal metabolic activity as measured by XTT reduction was observed 2 h after HBO treatment ($P < 0.0001$), and a striking 98% inhibition was observed after 8 h ($P < 0.0001$) (Fig. 2A). Given that colonies continued to proliferate over time even after HBO treatment, we hypothesized that this effect was fungistatic. To test this hypothesis, the metabolic activity of colony biofilms was monitored over time under normobaric conditions following HBO treatments (Fig. 2B). Consistent with the slow but continued growth of fungal colony biofilms after HBO treatments, fungal metabolic activity increased over time and reverted to untreated levels by 6 h after HBO treatment (3.5 ATA, 100% O_2), indicating a fungistatic and transient effect of HBO against *A. fumigatus* biofilms under the conditions tested (Fig. 2B).

mRNA levels of known ROS defense pathways in *A. fumigatus* are not induced by HBO, but loss of fungal superoxide dismutase genes increases HBO susceptibility. HBO is associated with increased reactive oxygen species (ROS) production and specifically with an increased generation of superoxide radicals (43, 47). ROS are an important deterrent to fungal infections *in vivo*, and we hypothesized that HBO-mediated ROS generation was playing a role in the observed growth inhibition of *A. fumigatus* (48–50). To test this hypothesis, we measured the mRNA levels of transcripts from genes known to increase in response to superoxide and ROS stress in *A. fumigatus*, i.e., the superoxide dismutase genes *sod1* to *-4* and the master oxidative stress response transcription factor gene *yap1* (51, 52). Interestingly, we did not observe an increase in the mRNA levels of any of the oxidative stress-associated genes examined in response to HBO treatment under the conditions examined (Fig. 3A). To examine whether other gene products are responsible for mitigating ROS stress induced by HBO, we tested *A. fumigatus* colony biofilm sensitivity to menadione in the presence of HBO (53). Con-

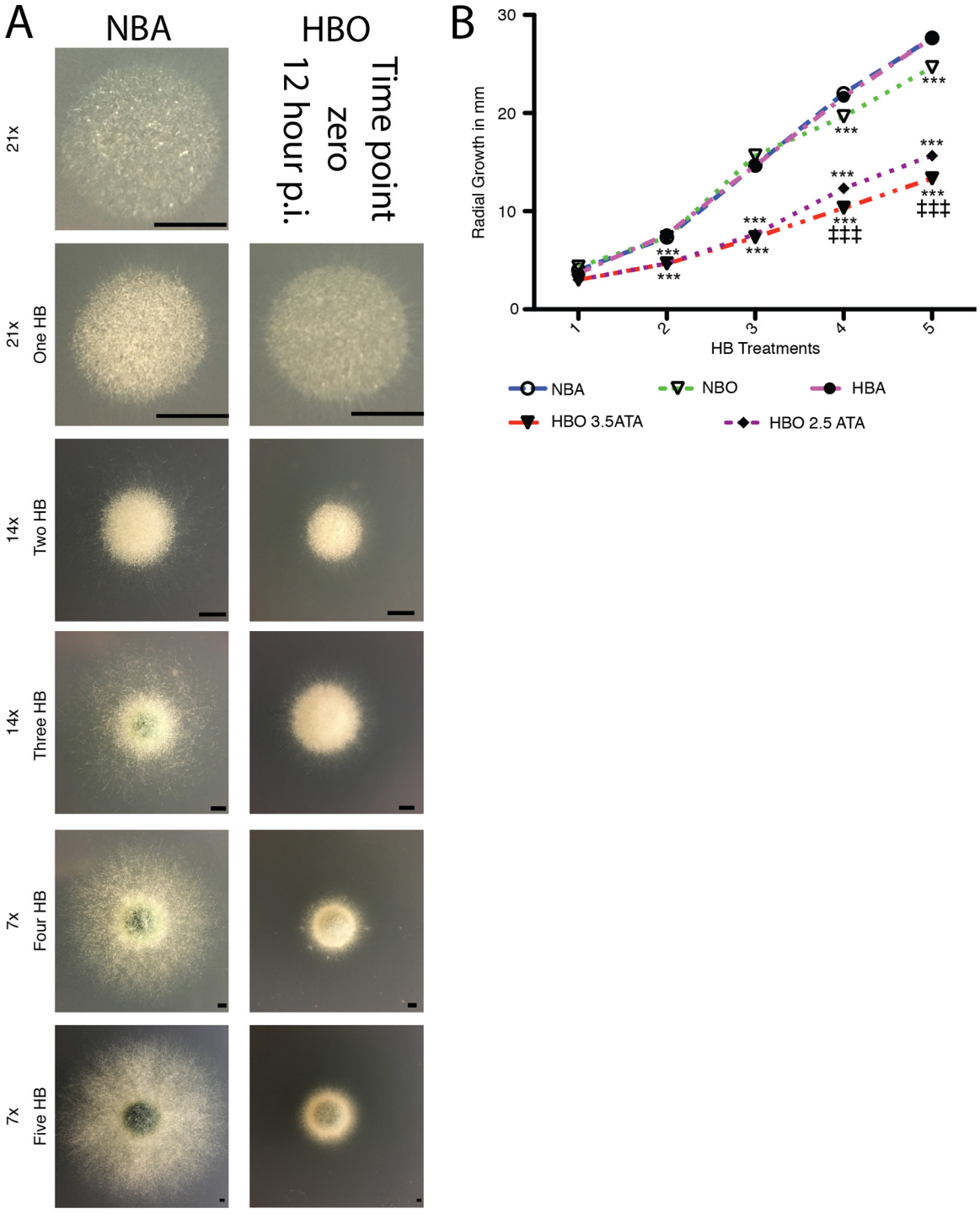

FIG 1 Hyperbaric oxygen (HBO) inhibits *Aspergillus fumigatus* colony biofilm expansion. (A) Conidia (10^4) of *A. fumigatus* strain CEA10 were point inoculated on glucose minimal medium (GMM) agar. Conidia were allowed to germinate for 12 h at 37°C to establish a colony biofilm (mycelium) before the first HBO treatment. HBO treatments were given every 24 h, and each lasted 8 h at 36.7 lb/in^2 (3.5 ATA) at ambient temperature. Images were captured at the time points indicated and at the magnifications indicated using the Macro Pro lens set (Olloclip) for iPhone 6s. Scale bar, 1 mm. (B) Radial growth rate of *A. fumigatus* colonies as grown for panel A. Data presented are means and standard errors of the means for three biological replicates. ***, statistically significant difference compared to NBA using two-way analysis of variance ($P < 0.001$). ‡‡‡, statistically significant difference between the HBO 3.5 ATA and HBO 2.5 ATA groups as calculated using two-way analysis of variance. NBA, normobaric air (1 ATA, 21% O_2); NBO, normobaric oxygen (1 ATA, 100% O_2); HBA, hyperbaric air (3.5 ATA, 21% O_2); and HBO, hyperbaric oxygen (2.5 or 3.5 ATA, 100% O_2).

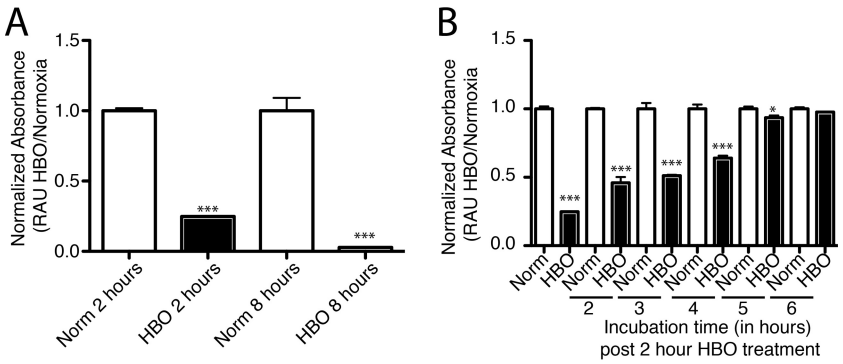

FIG 2 Hyperbaric oxygen inhibits *Aspergillus fumigatus* metabolism. Conidia of *A. fumigatus* strain CEA10 were allowed to germinate for 12 h in liquid glucose minimal medium (GMM) in 6-well plates and treated with HBO for 2 or 8 h. (A) The XTT assay was performed as described in Materials and Methods to measure metabolic activity. ***, statistically significant difference ($P < 0.001$). (B) Temporal fungal adaptation to HBO stress. *A. fumigatus* was treated with HBO for 2 h as described above. Postincubation, plates were further incubated at ambient temperature for the indicated amounts of time, and fungal metabolic activity was measured at each time point with XTT. Data are means and standard errors from three biological replicates. *** and *, statistically significant differences ($P < 0.01$ and $P < 0.05$, respectively). RAU, relative absorbance units. Open bars, normobaric normoxia (1 ATA, 21% O_2); closed bars, hyperbaric oxygen (3.5 ATA, 100% O_2).

sistent with the mRNA levels of the *sod* genes and *yap1* transcription factor, we did not see increased sensitivity to HBO in the presence of menadione (Fig. 3B).

To further explore the role of HBO antifungal activity, we next examined the requirement for fungal superoxide dismutase activity in mediating HBO tolerance. We inoculated plates with 100 conidia of a wild-type (WT) strain (*akuB*^{ku80}) or a genetic null mutant with 3 of the 4 *A. fumigatus* superoxide dismutase genes deleted (Δ*sod1* Δ*sod2* Δ*sod3*) and allowed conidia to germinate for 12 h, followed by HBO treatment for 4 h. Following HBO treatment, cultures were placed back in standard laboratory conditions and monitored for fungal colony development. While HBO did not affect the ability of the wild-type strain to form colonies after 24 h, the Δ*sod1* Δ*sod2* Δ*sod3* strain showed a significant reduction in colony formation upon exposure to HBO (Fig. 4) ($P = 0.0008$,

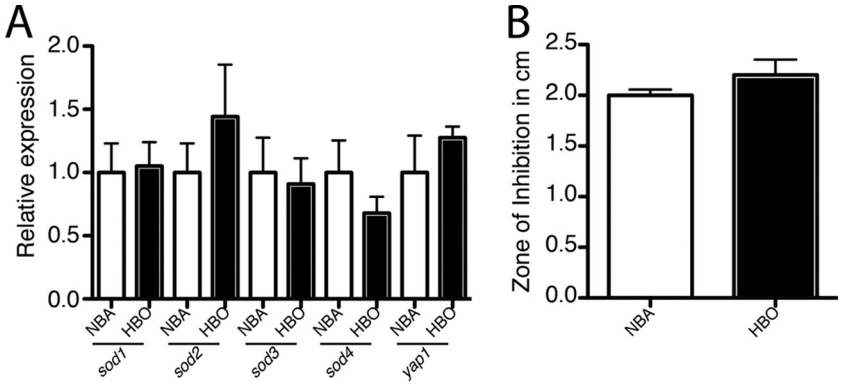

FIG 3 Hyperbaric oxygen does not induce mRNA level increases from oxidative stress-associated genes in planktonic culture and does not increase menadione sensitivity in biofilm cultures of *Aspergillus fumigatus*. (A) Conidia of *A. fumigatus* were allowed to germinate in liquid GMM cultures for 16 h with constant shaking at 250 rpm and were treated with HBO (3.5 ATA, 100% O_2) for 4 h. mRNA levels of ROS response genes *sod1* to *-4* and *yap1* were measured by quantitative RT-PCR. Data represent the means and standard errors from three biological replicates. No significant difference was observed. (B) *A. fumigatus* conidia (10^5) were overlaid on GMM plates in top agar and allowed to germinate for 12 h. After germination, a hole was punched in the center of the plate, and either DMSO (GMM) or 2 mM menadione was added. HBO treatment was given for 8 h (3.5 ATA, 100% O_2) every 24 h. When not subjected to HBO, plates were incubated at ambient temperature. After 72 h, the diameter of the zone of inhibition was measured. No significant difference was observed ($P = 0.28$, two-tailed *t* test). NBA, normobaric air; HBO, hyperbaric oxygen.

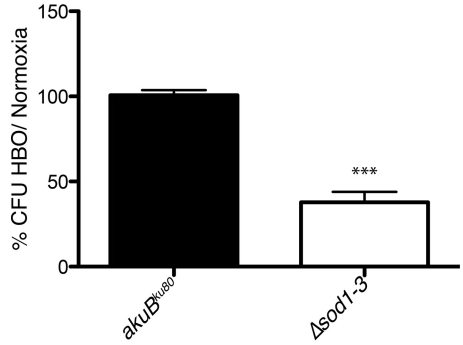

FIG 4 Fungal superoxide dismutases are critical to protect young hyphae from HBO. Conidia (100) of the WT strain (*akuB*^{ku80}) and a genetic null mutant missing three superoxide dismutases (Δ*sod1* Δ*sod2* Δ*sod3*) were overlaid on GMM plates and allowed to incubate for 12 h. After initial germination, strains were subjected to HBO or ambient air for 4 h. Plates were further incubated at 37°C in normoxia for 24 h, and colonies were counted manually. The bar graph represents percent CFU in HBO compared to normoxia. Data represent means for three biological replicates, and error bars represent standard errors of the means. ***, $P = 0.0008$.

two-tailed Student *t* test). Thus, while ROS may not be the causative factor in HBO fungal growth inhibition, fungal superoxide dismutases are critical for fungal conidium viability when exposed to HBO, suggesting that some superoxide is produced by HBO treatments.

HBO increases median survival time in a chemotherapy murine model of IPA. As HBO reduced fungal colony biofilm proliferation *in vitro* and prior research hinted at the usefulness of HBO as an adjunctive modality against invasive fungal infections, we first investigated the dose-dependent role of HBO as a stand-alone treatment in a chemotherapy murine model of IPA. As HBO under 3.5 ATA gave the strongest *in vitro* fungal growth inhibition, we first examined the effect of this treatment on murine survival using Kaplan-Meier survival analysis (Fig. 5A). A statistically significant increase in murine survival was observed in mice treated with HBO daily for 2 h compared to the untreated control group, with 25% of mice surviving the infection in the HBO group compared to 4% in the untreated group (Fig. 5A) ($P = 0.019$, log rank test). Moreover, HBO treatment significantly prolonged the median survival time of the treated animals to 10 days, as opposed to 8 days in the untreated group (Fig. 5A) ($P = 0.019$, $n = 25$ for fungus-challenged groups and $n = 10$ for phosphate-buffered saline [PBS]-challenged [uninfected] groups). Two mice in the HBO group developed unexplained neurological symptoms following HBO treatment and were immediately euthanized; these were included in the final analysis as censored objects.

To probe further into the potential mechanism underlying the improved survival of the animals under HBO treatment, we analyzed the *in vivo* fungal burdens at days 4 and 5 after fungal challenge (after 3 and 4 HBO treatments, respectively). No difference in fungal burden was detected between the control untreated group and HBO-treated groups as measured by levels of fungal 18S DNA (Fig. 5B). Consistent with the quantitative PCR (qPCR) fungal burden data, histopathology of the mice treated with HBO did not reveal any substantial difference in fungal growth at the time points examined. However, HBO-treated animals had reduced lung parenchymal inflammation with inflammation largely centered in the major airways compared to the untreated control group, where inflammation and fungal invasion spread into the lung parenchyma (Fig. 5C). These data suggest that HBO may alter fungal disease progression in part through an effect on the host inflammatory response and/or physiology of the invading fungus that warrants further investigation.

Given that HBO therapy in the clinic is most commonly given under 2.5 ATA and we saw fungal growth inhibition under these conditions *in vitro*, we tested whether HBO at 2.5 ATA would yield a therapeutic benefit similar to that observed under 3.5 ATA. While a slight delay in disease progression as monitored by host survival and animal

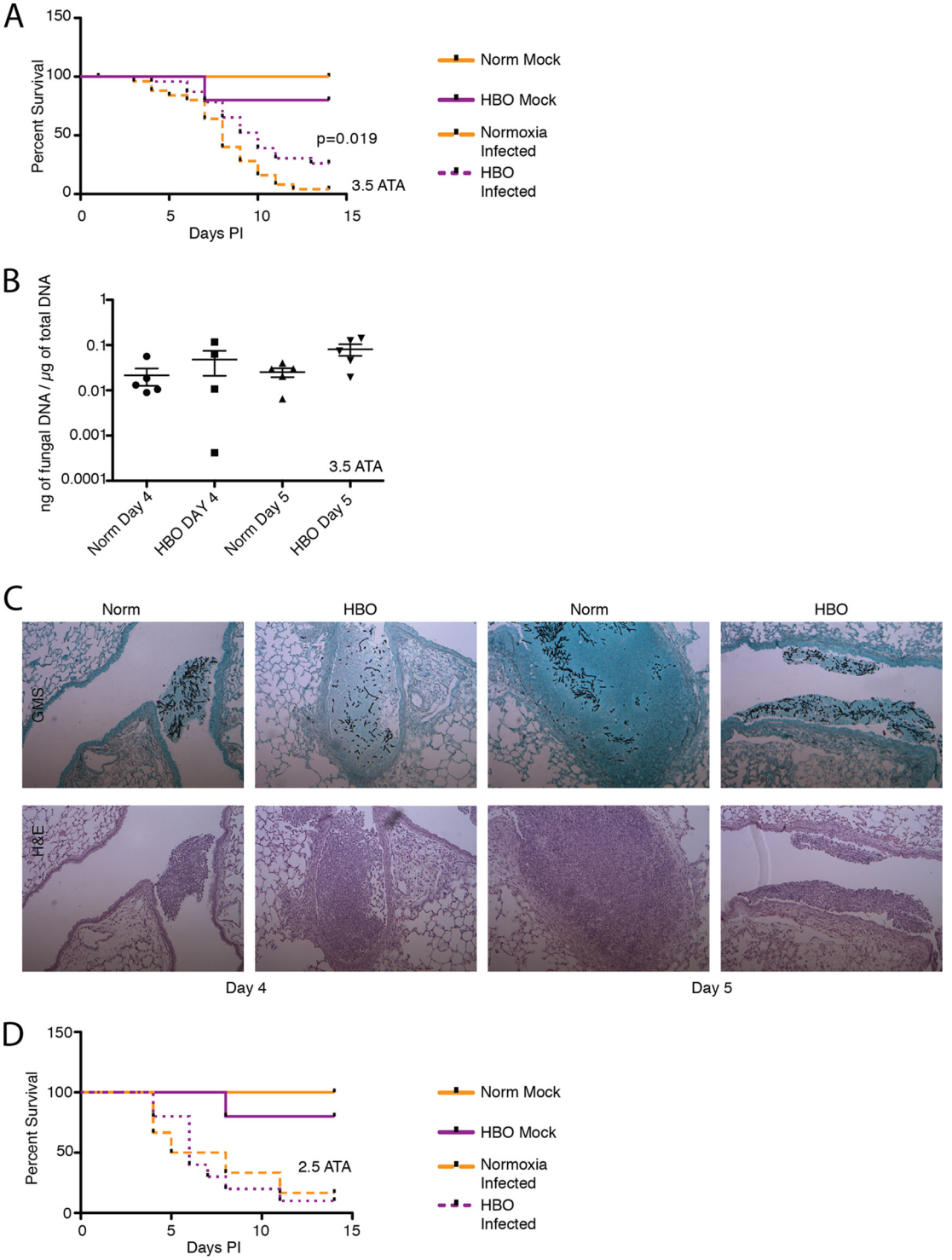

FIG 5 Hyperbaric oxygen promotes increased murine survival in a chemotherapeutic murine model of invasive pulmonary aspergillosis. Outbred CD-1 mice (Charles River) were immune suppressed with cyclophosphamide (175 mg/kg) i.p. on days −2 and +3 and kenalog-10 s.c. on days −1 and +6. Mice were inoculated with 10^4 conidia of *A. fumigatus* strain CEA10 in 40 μl of PBS. (A) Mouse survival was significantly different in the HBO-treated versus untreated groups ($P = 0.016$, log rank test) at 3.5 ATA ($n = 25$ for infected groups and 10 for mock-treated groups). (B) HBO does not affect pulmonary fungal burden. Lungs were collected from HBO-treated and untreated mice on days 4 and 5 postinoculation and total DNA extracted. The fungal burden in the lungs was measured by measuring 18S fungal DNA using a TaqMan probe. Data represent the ratio of fungal DNA to total isolated DNA. (C) Representative histopathology of HBO-treated and untreated animals. Lungs were collected from infected HBO-treated or untreated animals on days 4 and 5, fixed in 10% formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) and Gomori methanamine silver stain (GMS). No significant differences were observed. (D) However, no significant difference in survival was observed at 2.5 ATA ($P > 0.05$, log rank test) ($n = 10$ for infected group and 5 for mock-infected group).

symptoms was observed when HBO was delivered at 2.5 ATA, the overall median time to survival (6.5 days for the normoxia group versus 7 days for the HBO group) and overall survival were not affected, with all animals succumbing to the infection (Fig. 5D).

HBO does not increase antifungal drug efficacy *in vitro* or *in vivo* in a murine model of IPA. Voriconazole is the primary antifungal drug used to treat IPA (54); however, outcomes are still less than optimal. Combination therapies targeting different pathways have been recommended to improve disease outcomes (55). Given the fungistatic nature of HBO and voriconazole against *A. fumigatus*, we explored the combined activity of HBO and voriconazole treatment. First we tested our hypothesis *in vitro* using Etest strips with or without HBO treatment; however, no significant change in susceptibility to voriconazole was recorded using this assay (MIC of 0.190 to 0.250 μg/ml in the normobaric oxygen group versus 0.125 to 0.190 μg/ml in the HBO group) (Fig. 6A). Given the limited case series on success of voriconazole and HBO combination therapy in patients with invasive aspergillosis and the complexity of the *in vivo* microenvironment (56), we next tested a combination therapy in the chemotherapy murine model of IPA.

To test the effects of combination therapy *in vivo*, we first sought to determine a suboptimal dose of voriconazole against IPA in the chemotherapy murine model of IPA. To determine the suboptimal dose of voriconazole, we tested 7.5 mg, 10 mg, and 20 mg voriconazole per kg of body weight given orally for 5 days to animals with *ad libitum* access to the 50% grapefruit-water required for voriconazole efficacy in mice (57, 58). Doses of 10 mg and 20 mg protected the mice (90% survival) against *A. fumigatus* infection and thus were not considered for use in assessing combination therapy efficacy. However, mice given 7.5 mg/kg of voriconazole showed delayed but still significant disease progression with 100% mortality 8 days after fungal challenge, and this dose was thus considered suboptimal and was selected for the HBO combination therapy study (Fig. 6B). The pulmonary fungal burden was measured on day 3 postinoculation (p.i.), and no significant difference was detected in mice treated with normoxia plus voriconazole versus HBO plus voriconazole at this time point. However, there was a slight but statistically significant decrease in pulmonary fungal burden in the group given combined HBO and voriconazole treatments compared to the group given HBO alone (Fig. 6C) ($P < 0.05$, Kruskal-Wallis test).

Amphotericin B and its derivatives are recommended for therapy when treatment with voriconazole fails or if azole drugs are not tolerated (54). HBO may augment the activity of amphotericin B by correcting lactic acidosis (59), and thus we aimed to determine the efficacy of HBO and amphotericin B combination therapy *in vitro* and *in vivo*. Similar to the results with voriconazole, with Etest strip assays, we did not see a difference in the MIC of amphotericin B against *A. fumigatus* under the conditions tested (Fig. 7A). However, given case reports on amphotericin B and HBO treatments and the slight beneficial effect of HBO with voriconazole treatment *in vivo*, we examined the amphotericin B-HBO combination *in vivo* in the chemotherapeutic murine model. Previously it was reported that a dose of 1 mg/kg/day was suboptimal for treatment of IPA in murine models, and thus we selected this dose for these experiments (60). Mice were inoculated with *A. fumigatus* and treated with amphotericin B at 1 mg/kg/day for 5 days after fungal challenge. No survival benefit of the combination therapy versus drug alone under the conditions and with the concentrations tested in murine models of IPA was observed (Fig. 7B).

DISCUSSION

Hyperbaric oxygen has been used as an adjunctive treatment modality for some infections; however, it is not commonly used for invasive fungal infections (28–30, 61). Retrospective studies have reported clinical efficacy of HBO in invasive fungal infections, though no prospective studies have been reported (35). The present study revealed that HBO reduces proliferation of established *A. fumigatus* colony biofilms *in vitro* in a dose-dependent manner (i.e., we saw a greater reduction in fungal proliferation at 3.5 ATA than at 2.5 ATA pressure). Previous reports have indicated that the

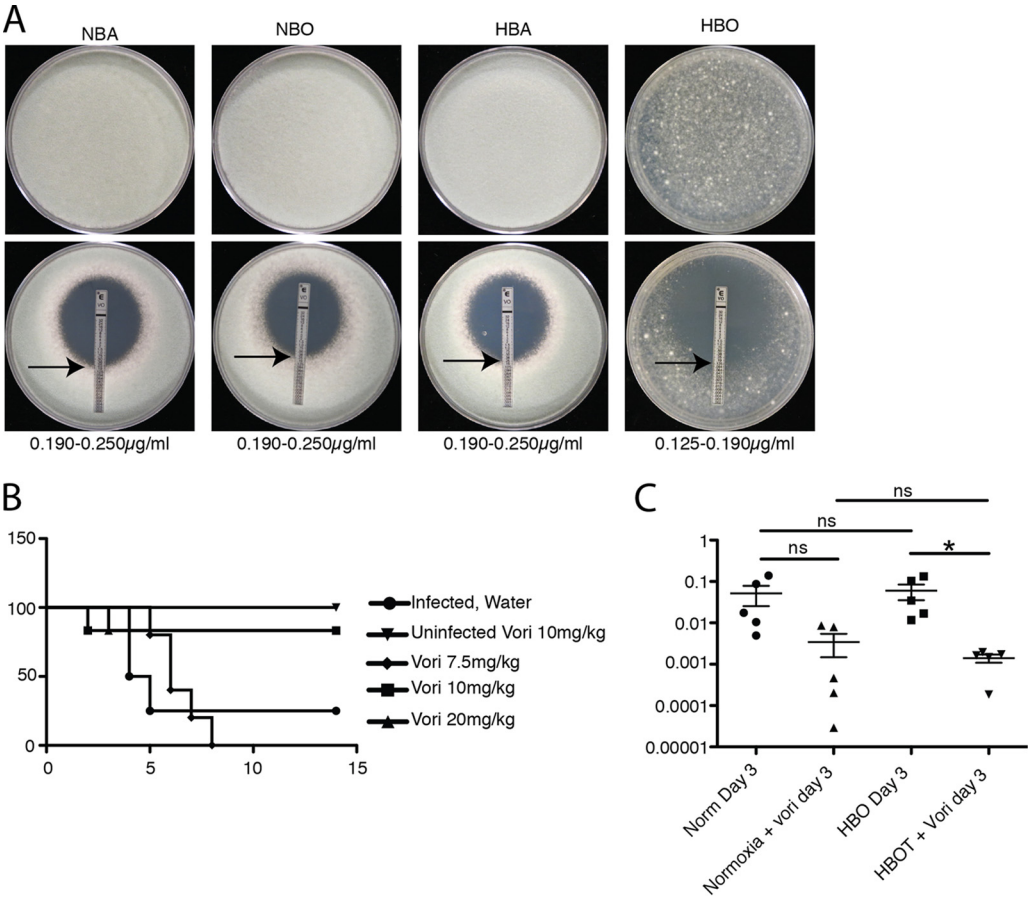

FIG 6 Combination therapy with hyperbaric oxygen and voriconazole does not promote increased fungal growth inhibition *in vitro* or murine survival *in vivo*. *A. fumigatus* conidia (10^5) were overlaid on GMM plates in top agar and allowed to germinate for 12 h. After germination, voriconazole Etest strips were placed in the center of the plate treated with conditions as indicated for 8 h every 24 h. After 48 h of incubation, plates were further incubated at 37°C for 12 h and photographed. No difference in MIC was observed. NBA, normobaric air (1 ATA, 21% O_2); NBO, normobaric oxygen (1 ATA, 100% O_2); HBA, hyperbaric air (3.5 ATA, 21% O_2); and HBO, hyperbaric oxygen (3.5 ATA, 100% O_2). (B) To determine the suboptimal dose of voriconazole for combination therapy, outbred CD-1 mice (Charles River) mice were started on grapefruit juice 4 days before infection. Mice were immune suppressed with cyclophosphamide (175 mg/kg) i.p. on days −2 and +3 and kenalog-10 s.c. on days −1 and +6. Mice were inoculated with 10^4 conidia of *A. fumigatus* in 40 μl of PBS. Conidia were allowed to germinate for 12 h, and mice were subsequently treated with the indicated amounts of voriconazole once daily via oral gavage. Mock-infected mice were also injected with voriconazole to determine the adverse effects of repeated oral gavage and drug alone. Mice were also inoculated with drinking water alone as a control. No survival benefit was observed with the 7.5-mg/kg dose of voriconazole, and this dose was chosen for combination analysis. (C) Combination therapy reduces fungal burden compared to HBO alone. Lungs were collected from HBO-treated and untreated mice on day 3 postinoculation and total DNA extracted. The fungal burden in the lungs was measured by measuring 18S fungal DNA using a TaqMan probe. Data represent the ratio of fungal DNA to total isolated DNA. *, statistically significant difference ($P < 0.05$, Kruskal-Wallis test, $n = 5$ in each group); ns, not significant. Vori, voriconazole.

effects of HBO are dose dependent in *Candida* infections (40), and our studies confirm the dose dependency of HBO therapy in *A. fumigatus* growth inhibition.

Under the conditions examined, however, HBO treatments were fungistatic. HBO-exposed cultures continued to grow radially during the course of study. Thus, *A. fumigatus* has mechanisms that allow recovery from HBO-mediated stress, at least in the time frame and under the conditions examined here. Consistent with this interpretation, a temporal XTT analysis of fungal metabolic activity demonstrated recovery of fungal metabolic activity after HBO treatment, which reached untreated levels after 6 h after HBO treatment (Fig. 2B). We chose the XTT assay because it has been shown to follow a linear curve as a function of biomass for the closely related species *Aspergillus nidulans* (62) and is able to detect exponential and death phases of growth, whereas dry weight comparison does not differentiate dead mycelia from live mycelia.

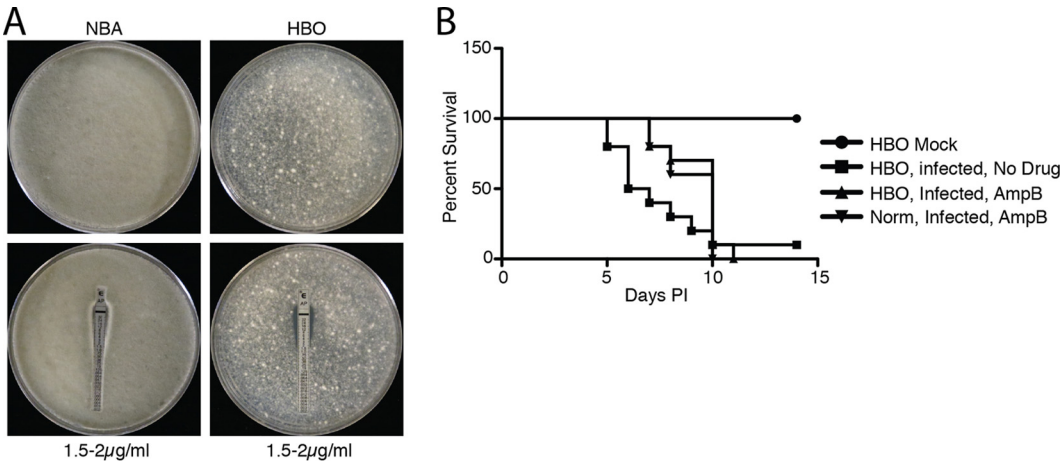

FIG 7 Combination therapy with hyperbaric oxygen and amphotericin B does not promote increased fungal growth inhibition *in vitro* or murine survival *in vivo*. *A. fumigatus* conidia (10^5) were overlaid on GMM plates in top agar and allowed to germinate for 12 h. After germination, amphotericin B Etest strips were placed in the center of the plate treated with HBO for 8 h every 24 h. After 48 h of incubation, plates were further incubated at 37°C for 12 h and photographed. No difference in MIC was observed. (B) Combination therapy does not increase survival in the chemotherapy murine model of IPA compared to drug alone. Outbred CD-1 mice (Charles River) were immune suppressed with cyclophosphamide (175 mg/kg) i.p. on days −2 and +3 and kenalog-10 s.c. on days −1 and +6. Mice were inoculated with 10^4 conidia of *A. fumigatus* in 40 μl of PBS. Mice were treated with HBO alone or HBO plus amphotericin B given as a 1-mg/kg/day i.p. injection right after the HBO treatment. No significant survival difference was observed in the HBO-alone versus combination therapy group ($P > 0.05$, log rank test) (n = 10 for infected groups and 5 for mock-infected groups).

The marked reduction in the ability of *A. fumigatus* to reduce XTT following HBO treatment strongly suggests that HBO inhibits a fundamental aspect(s) of fungal physiology. We also cannot rule out that some hyphae in the biofilm are in fact killed by HBO and persistent hyphae within the biofilm are able to resume growth and metabolic activity. More studies are needed to define the effects of HBO on fungal physiology.

These *in vitro* data potentially explain the lack of major decreases in fungal burden *in vivo* observed in our animal model experiments, given that mice recovered for 22 h between HBO treatments that lasted 2 h. During this time, fungal growth has adequate time to resume. This once-a-day HBO regimen was employed since it mirrors existing HBO regimens often used clinically, but more frequent HBO treatment (i.e., two or three times a day) is possible and has been used for other indications. Ultimately, our studies reveal important information on the time course of the inhibitory effect of HBO, which needs to be taken into account when designing future studies of HBO as a therapeutic agent for invasive fungal infections.

To this end, defining the mechanism(s) of HBO-mediated fungal growth inhibition would be a significant advance in overcoming the observed fungal ability to recover from HBO stress and the limited time a patient can be kept under HBO conditions. HBO has been correlated with increases in ROS production (63). *A. fumigatus* is known to respond to ROS via increased expression of superoxide dismutase genes (*sod1* to *-4*) and the global ROS response transcriptional regulator gene *yap1* (51, 52). Surprisingly, our initial analyses of the ROS defense response in *A. fumigatus* through quantitation of the mRNA levels of *sod1* to *-4 and yap1* and biofilm sensitivity to menadione under HBO conditions (Fig. 3A and B) suggest that HBO-mediated ROS generation may not be the primary mechanism of action. However, while mRNA levels of known fungal ROS oxidative stress genes did not increase in response to HBO, young hyphae (germlings) from a triple SOD genetic null mutant displayed increased susceptibility to HBO growth inhibition (Fig. 5). These data suggest that HBO treatments do generate increased ROS in the fungus that require the presence of SODs. Additional experiments are needed to examine the role of fungal SODs in mediating HBO resistance in established fungal biofilms. Finally, ROS response mutants, including the triple SOD null mutant Δ*sod1*

Δ*sod2* Δ*sod3* and Δ*yap*1 strains, are not affected in their virulence in murine models of IPA (51, 52), indicating that in these models ROS stress is potentially not significant or that other cryptic noncanonical pathways may tackle ROS stress *in vivo*. Studies to examine the whole-genome transcriptomic changes in response to HBO in *A. fumigatus* are ongoing to identify potential candidate pathways and mechanisms.

The remarkable *in vitro* fungal proliferation inhibition observed was not directly transferable *in vivo* using once-daily HBO treatment. As suggested by the *in vitro* experiments and discussed above, *A. fumigatus* rebounds rather quickly from the HBO treatments under the conditions examined *in vitro*. Thus, our data suggest that a 2-hour HBO treatment once per day may not be sufficient to achieve robust fungal growth inhibition *in vivo*. Nevertheless, our results indicated that there was a significant benefit in the median survival rate of the HBO-treated group versus the untreated group. One important note in addition to the survival data is that HBO-treated mice inoculated with *A. fumigatus* displayed less severe symptoms of IPA (reduced ruffled fur, labored breathing, and hunched posture) than untreated fungus-challenged mice throughout the course of the experiment, even though no difference was observed in pulmonary fungal burden as measured by qPCR and Gomori methanamine silver (GMS) staining. Histopathology analyses of the HBO treatment suggested qualitative and potentially quantitative differences in inflammation between the experimental groups that remain to be further examined. It is also plausible that HBO treatments impact exposure and/or expression of fungus-associated molecular patterns that induce inflammation. Therefore, additional studies focused on the immune response during HBO IPA treatment are warranted.

As noted in our experimental design, we utilized the upper limit of HBO regimens clinically used in humans to minimize the risk of oxygen toxicity. Oxygen toxicity can manifest as grand mal seizures, and intracranial fungal dissemination may further lower the seizure threshold (64). Although it seems that no animals had seizures during treatment, two animals experienced unexplained neurological symptoms during the decompression stage of HBO treatment. These events were unlikely to be oxygen seizures, since these seizures occur while the animals are receiving the high-pressure oxygen rather than afterwards. The seizures are due to the effects of the high oxygen tension on the central nervous system. A return to atmospheric pressure typically stops the seizures, and these seizures do not lead to long-term neurological consequences such as paralysis. These two animals, however, experienced twitching of the hind limbs and what appeared to be a motor paralysis event even after returning to atmospheric pressure. Currently it is unclear what caused the unexplained neurological symptoms in the 2 mice in our studies.

Given that HBO therapy for an invasive fungal infection would clinically be applied in combination with an antifungal drug, we examined the potential benefits of HBO combined with antifungal drug treatment in our murine model. Initial *in vitro* combination studies did not reveal significant synergy between HBO treatment and either voriconazole or amphotericin B. These assays, however, should be interpreted with caution given that they were complicated by the severe fungal growth inhibition caused by HBO treatments alone. It is likely that more precise assays are needed to critically assess the impact of HBO combined with antifungal drugs on fungal physiology. Given that limitation of the *in vitro* studies and likely clinical deployment of HBO therapy, we treated animals with suboptimal doses of voriconazole and HBO (2.5 ATA), and we did see a small but significant reduction in pulmonary fungal burden in the combined-treatment group compared to the group treated with HBO alone (2.5 ATA) ($P < 0.05$). This is significant, as 2.5 ATA by itself did not provide a survival benefit or a difference in fungal burden (Fig. 4B and 5C).

However, with amphotericin B, we did not observe any survival benefit when HBO was included in the treatment regimen. Based on success of combination HBO and amphotericin B therapy against invasive fungal infections reported in the clinical literature (35), Barratt et al. applied the same principle for treatment of zygomycoses in a murine model (64). Even though no survival benefit was detected when comparing

amphotericin B alone with HBO and amphotericin B in that study, the combination therapy is still recommended as an adjunctive modality for treatment of zygomycoses (33, 34, 64). Thus, further studies are needed to optimize and explore voriconazole and amphotericin B dosing and HBO regimens both *in vitro* and *in vivo*. Considering additional experimental limitations in using a murine model for these studies, another important potential limitation is the higher resting VO_2 (amount of oxygen consumed while sitting at rest) in mice than in humans. The standard human resting VO_2 is 3.5 ml/min/kg (65); however, in mice an ~15-fold-higher resting VO_2 has been reported (66). Presumably, mice utilize O_2 much faster than humans, limiting the duration of high oxygen partial pressure in their lungs, which could minimize HBO's effect on fungal growth *in vivo*. Further studies are needed to optimize the duration and interval of HBO treatment, infection route, and pathogen dosing in this murine model to potentially maximize the beneficial effects of HBO while considering the potential limitations in immunocompromised patients with pulmonary tissue damage.

In conclusion, we observed a strong HBO-mediated inhibition of *A. fumigatus* biofilm proliferation *in vitro*. HBO was found to be fungistatic (not fungicidal), and the inhibitory metabolic effect lasted approximately 6 h. Loss of fungal superoxide dismutase genes in young hyphae dramatically increased the efficacy of HBO in inhibiting fungal proliferation. In a murine model of IPA, daily HBO therapy showed a modest and surprising survival benefit, but the mechanism remains unclear. A direct effect of HBO on the host immune response cannot be ruled out and warrants further study. No significant synergy was observed with currently approved antifungal drugs used at suboptimal doses in combination with a common clinical HBO dosing regimen (daily treatment at 2.5 ATA), though a small benefit of the combination of voriconazole and HBO was observed as evidenced by a small decrease in fungal burden. Though no synergy was observed in these murine studies, additional experimental design parameters could be tested to optimize the drug-HBO combinations. Given the existing clinical data for patients and clinical score benefits observed in this study with mice, future studies are warranted to understand and optimize the potential of HBO to inhibit fungal growth and extend therapeutic windows. Investigating the mechanism(s) of HBO-mediated fungal growth inhibition and the resulting fungal recovery responses seems particularly promising and may reveal novel antifungal drug targets that could be exploited pharmacologically.

MATERIALS AND METHODS

Strain and growth conditions. *Aspergillus fumigatus* strain CEA10 (CBS144.89) was used in this study and was routinely grown on glucose minimal medium (GMM) unless otherwise noted. All cultures were incubated at 37°C or at ambient temperature as specified.

Hyperbaric chamber. All experiments were performed in a Reimers dual-control hypobaric-hyperbaric chamber (model 20-48) with 100% oxygen or 21% oxygen at 2.5 ATA (22 lb/in^2) or 3.5 ATA (36.7 lb/in^2) absolute as specified. Pressure and temperature were stable during all experiments and were monitored routinely.

***In vitro* hyperbaric oxygen cultivation.** Approximately 10^4 conidia of *A. fumigatus* strain CEA10 were point inoculated on GMM plates and allowed to germinate for 12 h at 37°C. After initial germination for 12 h, plates were subjected to five cycles of 8 h of hyperbaric oxygen (3.5 ATA, 100% oxygen) and 16 h of normobaric oxygen (1 ATA, 21% oxygen) at ambient temperature. To assess the individual roles of pressure and oxygen, plates were also subjected to five cycles of 8 h of hyperbaric normoxia (3.5 ATA, 21% oxygen) or normobaric oxygen (1 ATA, 100% oxygen) followed by 16 h of normobaric normoxia at ambient temperature. Colony growth was measured and photographed at the end of each 8-hour hyperbaric cycle for each specific condition. Colony diameter was measured and compared to that for cultures grown in normobaric normoxia at each time point.

To assess the effect of HBO on colony formation, 5 ml of top agar containing approximately 100 conidia of the WT (*akuB*^{ku80}) or Δ*sod1* Δ*sod2* Δ*sod3* (*akuB*^{ku80}) strain was overlaid on GMM plates and allowed to germinate for 12 h at 37°C. After initial germination, plates were subjected to normoxia or HBO for 4 h at ambient temperature. Plates were further incubated at 37°C for 24 h, and colonies were manually counted.

XTT assay. *A. fumigatus* CEA10 conidial suspensions were diluted to 10^6 conidia/ml, and 3 ml was used to inoculate each well of a six well plate. Conidia were allowed to germinate for 12 h at 37°C and were subjected to hyperbaric oxygen (3.5 ATA, 100% O_2) for either 2 h or 8 h at ambient temperature. After 2 h of hyperbaric oxygen, plates were further incubated at normobaric normoxia for 2, 3, 4, 5, or 6 h. The XTT assay was performed at each time point as described previously (46).

RNA extraction and quantitative RT-PCR. *A. fumigatus* CEA10 conidia (10^6 per ml) were inoculated in 250 ml of GMM and incubated at 37°C with shaking for 16 h. Flasks were then transferred to HBO or normoxia for 4 h. Mycelia were collected and frozen in liquid nitrogen. RNA extraction, cDNA synthesis, and qPCR analysis of *sod1* (forward primer, 5′-TTGCTGTCCTCCGTGGTGAC-3′; reverse primer, 5′-GGTCG TTGCCCTTGATGTTCC-3′), *sod2* (forward primer, 5′-GGTGCTCTGGAGCCTTCGATC-3′; reverse primer, 5′-GGCGATCTGAGACGCAATGTC-3′), *sod3* (forward primer, 5′-GCCCTCCAACCCTACATCTCCC-3′; reverse primer, 5′-CTTCCGCCGCTTTCTTCTGC-3′), *sod4* (forward primer, 5′-CGACAAGTTCGCCAAGGACATTATCG-3′; reverse primer, 5′-CGCTCAAGGTTCTTAGCCAGCCATAC-3′); and *yap1* (forward primer, 5′-TCGACCCTG TCCTGTTCGGTG-3′; reverse primer, 5′-TCGCCAGGCACAACCTCCTC-3′) were measured by quantitative reverse transcription-PCR (RT-PCR) as described previously (12). mRNA levels of tubulin (forward primer, 5′-ATAATGTTCAGACCGCCCTCTGCT-3′; reverse primer, 5′-GACGATGTGGAATTGCCCACAAA-3′) were used as an internal control.

Sensitivity against ROS. Top agar containing 10^5 spores of *A. fumigatus* CEA10 was overlaid on a GMM plate, and spores were allowed to germinate for 12 h at 37°C. After 12 h of incubation, a hole was punched in the center of the plate with a 200-μl pipette tip and filled with either 100 μl of dimethyl sulfoxide (DMSO) or 100 μl of 2 mM menadione as described previously (53). Plates were subjected to HBO (3.5 ATA, 100% O_2) for 8 h every 24 h. When not subjected to HBO, plates were incubated at ambient temperature. After 72 h, the diameter of the zone of inhibition was measured.

Murine infection and hyperbaric oxygen treatment. All mice were housed in autoclaved cages at 5 per cage with HEPA-filtered air and autoclaved water and food *ad libitum*. Immune suppression and fungal challenge were done as described previously with some modifications using a chemotherapy murine model of invasive pulmonary aspergillosis (IPA) (10). Briefly, CD-1 female mice (Charles River, Raleigh, NC) weighing between 20 and 24 g were immunosuppressed with 175 mg/kg cyclophosphamide (Baxter Healthcare Corporation, Deerfield, IL) on days −2 and +3 intraperitoneally (i.p.) and kenalog-10 (triamcinolone acetonide; Bristol-Myer Squibb, Princeton, NJ) (40 mg/kg) on days −1 and +6 subcutaneously (s.c.). Immunosuppressed mice were intranasally inoculated with either endotoxin-free phosphate-buffered saline (PBS) alone (mock) or 10^4 conidia of *A. fumigatus* in 40 μl PBS. Conidia were allowed to germinate *in vivo* for 12 h before the first hyperbaric oxygen treatment.

Twelve hours after fungal challenge; mice were treated with hyperbaric oxygen (3.5 ATA, 100% oxygen) (dive and climb rate, ±1 lb/in^2/min) for 2 h with a 10-min air break between the hours. Mice were continuously monitored during the treatment. The treatment was given once per day for 5 days. Control mice were housed under normobaric normoxic conditions. A log rank test of the associated Kaplan-Meier curve was conducted using GraphPad Prism software (version 5.0) as described previously (10).

Histopathology. Outbred CD-1 mice were immunosuppressed, infected, and treated with hyperbaric oxygen (3.5 ATA, 100% O_2) as described above. Five mice per group were sacrificed at 4 days postinoculation and their lungs perfused with and stored in 10% buffered formalin until embedding. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and Gomori methanamine silver (GMS). Slides were analyzed microscopically with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) fitted with a QImaging Retiga-SRV Fast 1394 RGB camera.

***In vitro* susceptibility to antifungal drugs.** Etest strips of voriconazole and amphotericin B (BioMérieux) were used to determine the MICs as described previously with some modifications (10). Briefly, top agar containing 10^5 spores of *A. fumigatus* strain CEA10 was overlaid in GMM plates, and spores were allowed to germinate for 12 h at 37°C. After 12 h of incubation, test strips were placed in the centers of the plates and were subjected to either HBO or normoxia at ambient temperature for 8 h every 24 h. When not subjected to HBO, plates were incubated at ambient temperature. After 48 h, plates were further incubated in normoxia at 37°C for 16 h and photographed.

***In vivo* voriconazole treatment to determine the suboptimal dose.** All mice were housed in autoclaved cages at 5 per cage with HEPA-filtered air and autoclaved water and food *ad libitum*. Four days before infection, the water was replaced with 50% grapefruit juice and was provided *ad libitum* to increase the serum voriconazole levels as described previously (57, 58). Mice were immunosuppressed and challenged with *A. fumigatus* as described above. Conidia were allowed to germinate for 12 h, and voriconazole was orally gavaged at 7.5 mg, 10 mg, and 20 mg per kg body weight every day for 5 days. Control infected mice were gavaged with drinking water alone. Mice were monitored three times daily and their survival recorded for 14 days. A log rank test of the associated Kaplan-Meier curve was conducted as described previously (10).

Hyperbaric oxygen and drug treatment in murine model of IPA. For determination of the combined treatment effect, mice were immunosuppressed and challenged with *A. fumigatus* as described above. Voriconazole combined treatment was studied by orally gavaging voriconazole at a concentration of 7.5 mg/kg of body weight at the end of hyperbaric oxygen (2.5 ATA, 100% oxygen) for 5 days as described above. Amphotericin B combined treatment was studied by injecting amphotericin B in 10% dextrose solution at a concentration of 1 mg/kg body weight at the end of each hyperbaric oxygen treatment (2.5 ATA, 100% oxygen) for 5 days.

Ethics statement. All animal studies were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (67). The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College.

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S.D., J.C.B., and R.A.C. designed the experiments. S.D. conducted the experiments and acquired the data. S.D. and R.A.C. analyzed the data. J.C.B. and R.A.C. provided the reagents. S.D. and R.A.C. wrote the manuscript with input from J.C.B.

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