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The bactericidal effect of 470 nm light and hyperbaric oxygen on methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

It has been shown that, *in vitro*, hyperbaric oxygen (HBO) suppresses 28% bacterial growth, while 470 nm blue light alone suppresses up to 92% methicillin resistant *Staphylococcus aureus* (MRSA) in one application *in vitro*. Therefore, we determined if combined 470 nm light (55 J/cm²) and HBO will yield 100% bacterial suppression in experimental simulation of mild, moderate or severe MRSA infection. We cultured MRSA at 3×10^6 , 5×10^6 , 7×10^6 , 8×10^6 or 12×10^6 CFU/ml and treated each concentration in four groups as follows: (1) Control (no treatment) (2) photo-irradiation only, (3) photo-irradiation then HBO, (4) HBO only, and (5) HBO then photo-irradiation. Bacteria colonies were then quantified. The results showed that at each bacterial concentration, HBO alone was significantly less effective in suppressing MRSA than photo-irradiation or combined HBO and photo-irradiation ($p < 0.0001$). Similarly, at no bacterial

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Ethical standards

The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors declare that they have no conflict of interest.

concentration did combined HBO and 470 nm light treatment yield a statistically better result than 470 nm light alone ($p > 0.05$), neither did HBO treatment either before or after irradiation make a difference. Furthermore, at no bacterial concentration was 100% MRSA suppression achieved. Indeed, the maximum bacterial suppression attained was in the mild infection model (3×10^6 CFU/ml), with blue light producing $97.3 \pm 0.2\%$ suppression and HBO +55 J/cm² yielding $97.5 \pm 2.5\%$ suppression. We conclude that: (1) HBO and 470 nm light individually suppress MRSA growth; (2) 470nm blue light is more effective in suppressing MRSA than HBO; and (3) HBO did not act synergistically to heighten the bactericidal effect of 470 nm light.

Keywords

Methicillin-resistant; *Staphylococcus aureus*; Hyperbaric oxygen therapy; Antimicrobial activity; Phototherapy; Low-level light therapy; Blue light

Introduction

To date, treatment with antibiotics remains the standard form of therapy for persons with methicillin-resistant *Staphylococcus aureus* (MRSA) infection, even though less than 5% of staphylococci strains are susceptible to penicillin related drugs [1, 2]. Outbreaks of MRSA infection continue to be rampant [3-8], necessitating intense efforts to find effective alternative therapies [9]. Recently documented alternative therapies include blue light [13-16], treatment with antibacterial clays [17], combination of honey and antibiotics [18], and hyperbaric oxygen (HBO) therapy [19, 20].

Previous reports from our laboratory indicate that one shot irradiation with 55 J/cm² fluence of either 405 nm or 470 nm light suppresses as much as 92% of two strains of MRSA colonies *in vitro* [10-12]. However, the remaining bacterial colonies not suppressed by blue light re-colonize and develop into larger viable colonies. Moreover, when more concentrated cultures designed to mimic severe bacterial infection are irradiated, for example, 7×10^6 CFU/ml to 12×10^6 CFU/ml, more MRSA colonies survive, prompting repeated irradiation at significantly higher irradiance in order to suppress the remaining colonies [15]. The latter finding suggests a need to explore alternative protocols that may yield 100% bacterial suppression.

HBO has been shown to have antimicrobial properties against MRSA [19], improve type 2 diabetics related hyperglycemia [21], foster healing of diabetic wounds [22], minimize acute focal cerebral ischemia [23] and cerebral vascular insult [24]. Moreover, it inhibits astrocyte activation and inflammatory response [25]. HBO therapy involves the administration of 100% of oxygen at a pressure higher than 1 atmosphere (atm) at sea level [26] for times not shorter than 60 minutes as delineated by the European Consensus Conference on Hyperbaric Medicine held in Lille, France in 1994 [27]. Studies have demonstrated that a 90-min exposure to hyperbaric hyperoxia (2.0 atm, 100% O₂) significantly inhibited growth of both MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA). HBO treatment alone has been shown to suppress 22– 28% of 240 ± 24 CFU/plate of MRSA *in vitro*, and the observed growth inhibition does not occur with normobaric normoxia (1.0 atm, 21% O₂), normobaric hyperoxia (1.0 atm, 100% O₂) or hyperbaric normoxia (2.0 atm, 21% O₂) [19].

The fact that a relatively brief 90-minute treatment with hyperbaric hyperoxia is sufficient to produce significant bacterial growth inhibition suggests that MRSA is susceptible to oxidative stress, and that hyperbaric hyperoxia may be useful in the treatment of serious staphylococcal infections. In the case of osteomyelitis with MRSA infection for example, the effect of HBO is attributed to increased availability of oxygen used by neutrophils for killing microorganisms through oxidative burst [28].

Considering the possibility that repeated dosing with blue light may not yield 100% suppression, or that the fluence needed to achieve 100% suppression may require light intensities that may be inimical to human cells, we were prompted to explore an alternative approach: that is, combining hyperbaric oxygen synergistically with 470 nm light with the goal of obtaining complete suppression of mild, moderate and severe MRSA infections *in vitro*. Since 405 nm or 470 nm blue light has the capacity to suppress as much as 92% of MRSA colonies in a standard bacterial culture and HBO alone suppresses 22–28% in plates with 240 ± 24 CFU of MRSA, it seems plausible that combined blue light and HBO therapy will yield 100% suppression. Thus, the purpose of this study was to determine if the combination of 470 nm blue light and HBO will optimally suppress MRSA colonies *in vitro* at 100% success rate.

Materials and Methods

Bacterial culture

USA300 community associated MRSA (CA-MRSA) strain (ATCC®-BAA-1680) was obtained from American Type Culture Collection (ATCC), (Manassas, VA, USA), and identified by standard procedures. As detailed in our previous report [15], bacteria cultures were separately diluted to concentrations of 3×10^6 , 5×10^6 , 7×10^6 , 8×10^6 and 12×10^6 CFU/ml in 0.9% sterile saline, to mimic mild (3×10^6), regular (5×10^6), and severe (7×10^6) MRSA infection *in vitro*. Bacteria were then volumetrically streaked onto round 5.0 cm² Petri dishes with tryptic soy agar (TSA) before being treated with HBO and/or 470 nm light. Treated cultures were then incubated at 37°C for 24 hours. Control non-HBO treated and non-irradiated bacteria cultures were similarly incubated for comparison.

Study Design

To determine the effect of HBO on MRSA, the bacteria were plated in Petri dishes containing TSA. Each of the five Petri dishes containing the bacterial suspension was assigned to one of five treatment groups: Group One: Control (no irradiation, no HBO); Group Two: 55 J/cm² 470 nm light; Group Three: 55 J/cm² 470 nm light followed by exposure to HBO (hyperbaric hyperoxia 2.0atm, 100% O₂); Group Four: HBO only, 5) HBO exposure and then 55 J/cm² 470 nm light (Fig.1). Each set of experiments was conducted in triplicate, in separate Petri dishes with separate bacterial cultures. Upon completion of the HBO exposure and irradiation protocols, Petri dishes containing MRSA were incubated at 37°C for 24 hours, digital images taken and number of colonies computed.

Photo-irradiation

A Dynatron Solaris 708 (Dynatronics Corp., Salt Lake City, UT) fitted with a 470 nm light probe was used to irradiate the bacteria as detailed in our previous reports [10-12, 15]. Briefly, the irradiation protocol involved the use of a 5.0 cm² applicator, with a cluster of 32 LEDs. The applicator emits 470 nm blue light with a spectral width of 455–485 nm, and has a rating of 150 mW average power and 30 mWcm⁻² irradiance. At a distance of 0.3–0.5 cm from the culture plate, the power emitted was 25 mW, with 18 mW absorbed within the culture. The applicator is cooled with a built-in fan, thus eliminating thermal radiation. Petri dishes, the same size as the surface area of the applicator (5.0 cm²), were used to ensure even irradiation from the applicator which was clamped at a distance of 0.3–0.5 cm perpendicularly above the surface of each open culture plate. The computerized device automatically timed the duration of treatment needed to ensure dose precision.

HBO treatment

Petri dishes in the hyperbaric oxygen groups were placed in a sealed chamber (Dixie Manufacturing Company Inc., Baltimore, MD) at 2atm with 100% O₂ (hyperbaric hyperoxia). During the treatment period, a flow of “air” is maintained in the chamber. The oxygen tank is left on and the drain valve slightly opened to prevent complete use of oxygen and accumulation of emitted gases. Immediately following the 90 min exposure period, Petri dishes were either placed directly at 37°C for 24 hours, or irradiated with blue light and then incubated for the same period (Fig.1). The number of colonies, i.e. colony-forming units/plate (CFU), present at 24 hours after treatment and incubation was then determined. Control non-HBO treated and non-irradiated bacteria cultures were similarly incubated for comparison. Colonies were counted and percent bacterial survival determined.

Quantification of bacterial colonies and data analysis

Standardized digital images of the bacterial colonies were taken and colonies quantified with Sigma Scan Pro 5 software, (Systat Software, Inc., Point Richmond, CA, USA) as detailed in our previous paper [15]. The colony counts were automatically computed and subjected to statistical analysis. Descriptive data were generated, and analyzed with ANOVA followed by Bonferroni's post-hoc test, using GraphPad Prism 5.01 software (GraphPad Software Inc., USA). The level of statistical difference was set at p<0.05.

Results

Treatment of 3 × 10⁶ CFU/ml MRSA with 55 J/cm² and/or HBO

The bactericidal effect of combined blue light and HBO on MRSA was commensurate with the results for blue 470 nm light alone, with 470 nm light producing as high as 97.3±0.2% suppression in the mild infection model (3 × 10⁶ CFU/ml) (Fig. 2a) and the combined 55 J/cm²+HBO yielding approximately 91.8±0.9% and HBO+55 J/cm² 97.5±2.5% suppression. At this concentration, HBO alone produced significantly less bacterial suppression (43.3±0.81%) compared with 470 nm light alone or combined 470 nm and HBO (p<0.0001). Nonetheless, compared to the control group, HBO treatment alone resulted in significant suppression of MRSA in this 3 × 10⁶ CFU/ml mild infection model (p<0.0001; Fig. 2a).

Increasing bacteria concentration to mimic moderate and severe infection, i.e., (5×10^6 and 7×10^6 CFU/ml, respectively), highlighted the superiority of blue light or the combined therapy over HBO alone (Fig. 2b–e). At each bacterial concentration, blue light therapy or the combination of blue light and HBO significantly suppressed more MRSA than HBO alone or no treatment ($p < 0.0001$).

Treatment of 5×10^6 CFU/ml MRSA with 55 J/cm^2 and/or HBO

At the standard 5×10^6 CFU/ml concentration, similar results were observed as with the mild 3×10^6 CFU/ml concentration detailed above. Except for the HBO only treatment group, significant bacterial suppression was observed in each treatment group compared to the control group (Fig. 2b). Treatment with 55 J/cm^2 or any of the two combinations of light and HBO, i.e., $55 \text{ J/cm}^2 + \text{HBO}$, or $\text{HBO} + 55 \text{ J/cm}^2$ treatment, produced statistically significant MRSA suppression compared with HBO only ($p < 0.0001$). The bactericidal effect of light treatment alone and either combination of light and HBO did not differ (Fig. 2b).

Treatment of 7×10^6 , 8×10^6 , 12×10^6 CFU/ml MRSA with 55 J/cm^2 and/or HBO

The result trend remained the same as it was with the 3×10^6 and 5×10^6 CFU/ml cultures, when the higher concentrations of MRSA (7×10^6 , 8×10^6 , 12×10^6 CFU/ml) were treated. Again, with the exception of the HBO only treatment group, significant bacterial suppression was observed in each treatment group compared to controls (Fig. 2c–e). Treatment with 55 J/cm^2 , $55 \text{ J/cm}^2 + \text{HBO}$, or $\text{HBO} + 55 \text{ J/cm}^2$ produced statistically significant MRSA suppression compared with HBO only ($p < 0.0001$) at each bacterial concentration tested ($p < 0.0001$; Fig. 2c–e; Fig. 4). Therefore, whereas blue light treatment alone or a combination of blue light and HBO significantly suppressed bacterial growth at each concentration tested, HBO alone had moderate but statistically insignificant bacterial growth suppression. Moreover, the superiority of blue light compared with HBO is evidenced by the fact that at each concentration tested, blue 470 nm light therapy alone or a combination of 470 nm light and HBO suppressed significantly more MRSA colonies than HBO alone ($p < 0.0001$). There was no statistically significant difference in MRSA suppression between the three groups involved with blue light treatment, i.e., blue light alone and the two combinations of blue light and HBO (Fig. 2c–e), indicating that the addition of HBO to blue light therapy did not further suppress bacterial growth.

Percent survival of MRSA in different culture concentrations treated with HBO only

Compared to controls, HBO alone suppressed a significant amount of MRSA in the 3×10^6 CFU/ml culture ($p < 0.05$; Fig. 3a). The amounts suppressed at other bacterial concentrations were not statistically significant, being 10% or less at concentrations 7×10^6 CFU/ml. However, in terms of proportion, more bacterial colonies were suppressed in the 3×10^6 CFU/ml culture than the others. For example, approximately 43% of the 3×10^6 CFU/ml culture (i.e., 1.3×10^6 of the 3×10^6 CFU/ml) was suppressed versus 31% of the 5×10^6 CFU/ml concentration (i.e., 1.5×10^6 of the 5×10^6 CFU/ml) (Fig. 3a).

Percent survival of MRSA in different culture concentrations treated with 55 J/cm² blue light

Compared to controls, 55 J/cm² of 470 nm light alone suppressed a statistically significant amount of bacteria cultured at each of the five concentrations ($p < 0.0001$), and almost suppressed all MRSA colonies in the 3×10^6 CFU/ml culture (Fig. 3b). In general, more bacteria were suppressed at the lower culture concentrations (3×10^6 CFU/ml and 5×10^6 CFU/ml) than at the higher concentrations (7×10^6 CFU/ml). MRSA suppression at each of the higher concentrations, i.e., 7×10^6 , 8×10^6 and 12×10^6 CFU/ml, did not differ statistically (Fig. 3b).

Percent survival of MRSA in different culture concentrations treated with a combination of either 55 J/cm²+HBO or HBO+55 J/cm²

As shown in Figs. 3c and 3d, either combination of treatment resulted in significant MRSA suppression at each culture concentration ($p < 0.0001$) compared to the control. However, the percentage of bacteria suppressed did not differ between the two.

Percent survival of MRSA in different culture concentrations subjected to all treatment protocols

At each concentration tested, HBO only treatment was significantly less efficacious in suppressing MRSA than the other three protocols, i.e., light alone or the two combinations of light and HBO ($p < 0.0001$; Fig. 4). Administering HBO either before or after 470 nm light did not make any difference in MRSA suppression.

Discussion

The present study was designed to test the hypothesis that combined 470 nm light and HBO will act synergistically to yield 100% bacterial suppression. Our results clearly indicate that the addition of HBO to blue 470 nm light does not offer any additional bactericidal effect than blue light treatment alone. This result warrants outright rejection of our hypothesis in favor of treatment with 470 nm blue light alone. Our group and others have shown that 405 nm, 415 nm and 470 nm blue light suppress growth of *Staphylococcus aureus* (both MRSA and MSSA), *Escherichia coli* and other bacterial pathogens [10-15, 29]. In particular, we have shown repeatedly that 470 nm light suppresses as much as 92% of 5×10^6 CFU/ml MRSA culture [10-12]. And further studies from our laboratory indicate that although the remaining bacterial colonies re-colonize and develop into larger viable colonies, repeated irradiation with 470 nm light suppresses more colonies albeit at higher irradiance [15].

That HBO does not act synergistically with blue light to optimize MRSA suppression suggests a common biochemical pathway for the bactericidal effect of both forms of therapy. It is possible that blue light exploits the pathway more effectively than HBO, such that the addition of HBO either before or after 470 nm treatment does not result in any additional benefit. As observed, each treatment protocol investigated—HBO alone, or blue light therapy, or a combination of HBO and blue light—had beneficial antimicrobial effect compared to untreated controls. HBO only treatment was significantly less bactericidal than either 470 nm blue light or the combination of HBO and blue light. Treatment with HBO

only resulted in approximate 43% suppression in the 3×10^6 CFU/ml culture and as low as 12% suppression in more concentrated cultures, i.e. 7×10^6 CFU/ml. This finding is supported by reports from others in which 28% suppression of MRSA was observed in a culture with 240 ± 24 CFU/ml [19].

Other studies have documented the antibacterial effects of HBO [20, 30]. Turhan et al. [20], reported that HBO therapy alone or in combination with either linezolid, teicoplanin or vancomycin was effective in treating mediastinitis caused by MRSA. Although HBO therapy has been documented to have beneficial synergistic effects with some antibacterial agents, there are reports which indicate a lack of synergy between HBO and other therapies. For example, the finding that the efficacy of metronidazole decreases in hyperoxic or even normoxic conditions [31] evince that HBO does not always act synergistically to optimize therapy. And this supports our finding that its action does not synergize with that of 470 nm light.

Turhan [20] and others [32-34] associated the bacteriostatic and bactericidal effects of HBO to hyperoxic toxicity which leads to increased production of oxygen radicals such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) at a rate in excess of the capacity of the cellular defense mechanisms to inactivate such species. Although a similar pathway, i.e., the production of reactive oxygen species, has been suggested as the mechanism underlying the bactericidal effect of blue light [35-36], ongoing works in our laboratory suggest that another mechanism may be at play, i.e., that 470 nm light alters MRSA membrane potential. A full report of this finding is currently in preparation.

Finding an alternative solution to incessant outbreaks of MRSA, in particular, outbreaks of the USA300 strain is a worthwhile endeavor. Since the first case of community associated MRSA (CA-MRSA) was reported from a remote region of Western Australia in 1993 [37], pandemic strains of CA-MRSA have been found in schools, offices, athletic fields and other common locations where large numbers of people congregate [4, 6]. Recent reports indicate that as many as 92% of persons hospitalized for MRSA infection have CA-MRSA, and because the prevalence rate has been on the rise, health care facilities in the United States have heightened surveillance testing [38]. Clinical cases of CA-MRSA have increased while the proportion of patients with hospital associated MRSA (HA-MRSA) has decreased [38-40]. Moreover, the USA300 strain is the mostly common isolated during community outbreaks [7, 41]; hence we tested the bactericidal effect of combined HBO and 470 nm light on this strain as an alternative therapy to antibiotics to which MRSA has growing resistance.

We conclude that: (1) HBO and 470 nm light individually suppress MRSA growth, (2) Blue 470 nm light is significantly more effective in suppressing MRSA growth than HBO; (3) HBO does not act synergistically with blue light to optimize MRSA suppression. Thus, the addition of HBO to 470 nm blue light treatment does not augment the effect of blue light alone.

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Fig. 1.
 Schematic representation of the study design

Fig. 2.

Effect of treatment on different concentrations of MRSA: (a) 3×10^6 , (b) 5×10^6 , (c) 7×10^6 (d) 8×10^6 and (e) 12×10^6 CFU/ml. Results are presented as mean $\pm$ SEM (n=3).

*p<0.0001; ** p<0.05

Fig. 3. Effect of bacterial concentration on treatment: (a) HBO, (b) 55 J/cm^2 , (c) $55 \text{ J/cm}^2 + \text{HBO}$, (d) $\text{HBO} + 55 \text{ J/cm}^2$. Results are presented as mean $\pm$ SEM ($n=3$). *represents statistical difference from treatment groups compared to the control ($p < 0.0001$) and horizontal lines with * represent statistical difference between groups with $p < 0.0001$

Fig. 4.

Comparison of bactericidal effect of each treatment protocol. Results are presented as mean $\pm$ SEM (n=3). HBO only treatment was significantly less efficacious in suppressing MRSA at each bacterial concentration tested, comparing to other treatment groups (*p<0.0001).