Efficacy of hyperbaric oxygen therapy in bacterial biofilm eradication

Objective: Chronic wounds typically require several concurrent therapies, such as debridement, pressure offloading, and systemic and/or topical antibiotics. The aim of this study was to examine the efficacy of hyperbaric oxygen therapy (HBOT) towards reducing or eliminating bacterial biofilms *in vitro* and *in vivo*.
Method: Efficacy was determined using *in vitro* grown biofilms subjected directly to HBOT for 30, 60 and 90 minutes, followed by cell viability determination using propidium monoazide-polymerase chain reaction (PMA-PCR). The efficacy of HBOT *in vivo* was studied by searching our chronic patient wound database and comparing time-to- healing between patients who did and did not receive HBOT as part of their treatment.
Results: *In vitro* data showed small but significant decreases in cell viability at the 30- and 90-minute time points in the HBOT group. The *in vivo* data showed reductions in bacterial load for patients who underwent HBOT, and ~1 week shorter treatment durations. Additionally, in patients' chronic wounds there was a considerable emergence of anaerobic bacteria and fungi between intermittent HBOT treatments.
Conclusion: The data demonstrate that HBOT does possess a certain degree of biofilm killing capability. Moreover, as an adjuvant to standard treatment, more favourable patient outcomes are achieved through a quicker time-to-healing which reduces the chance of complications. Furthermore, the data provided insights into biofilm adaptations to challenges presented by this treatment strategy which should be kept in mind when treating chronic wounds. Further studies will be necessary to evaluate the benefits and mechanisms of HBOT, not only for patients with chronic wounds but other chronic infections caused by bacterial biofilms.
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biofilm • chronic wounds • hyperbaric oxygen therapy • HBOT

Historically, the cause of chronic non-healing wounds has been attributed to diabetes, arterial and venous disease, and burn and radiation exposure wounds.[1,2] However, greater attention has been paid to wound microbiota, propagating mainly as biofilms, and their contribution to the chronicity of wounds.[3–8] Bacterial biofilms have been recognised as the primary cause of wound chronicity.[9–11] Several definitions for bacterial biofilms have been proposed in the literature, but the most widely accepted definition is 'a coherent cluster of bacterial cells embedded in a matrix, which is more tolerant to most antimicrobials and the host defence than planktonic bacterial cells.'[12]

Increased resistance to antimicrobials and host defence systems results from physical factors such as the extracellular polymeric substance (EPS) matrix. The EPS encloses a multitude of bacteria in the biofilm superstructure, protecting the bacteria from environmental factors, such as ultra violet (UV) radiation and desiccation.[13] Additionally, the EPS and sheer size of the biofilm superstructure may hinder the host immune recognition and phagocytosis, respectively.[14] The EPS also influences diffusion of oxygen and antibiotics into the biofilm, factoring into the durability of biofilms. Genetic factors are also responsible for bacterial biofilm resilience. The close association of bacteria of the same or different species allows the fast and organised sharing of resistance plasmids, and enables efficient cell-to-cell communication throughout the entire biofilm community.[15–17] This communication system facilitates the dynamic existence of bacteria in either the planktonic or biofilm mode of growth by signalling for recruitment of bacteria in favourable conditions and, alternatively, signalling for dispersal in the planktonic form in unfavourable conditions.[18] These factors make bacterial biofilms difficult to eradicate.

The principal method for biofilm eradication from wounds is aggressive and frequent debridement. Unfortunately, complete removal of the biofilm in a clinical setting is imperfect and, even with local anaesthetic, can be very painful for the patient, and allows the biofilm to return within 24–48 hours.[6,16] Hyperbaric oxygen therapy (HBOT) has been proposed and, to some extent, researched as an adjuvant therapy for chronic wound healing.[17,19–21] While there are conflicting reports in the literature, there is limited evidence contradicting the benefits of HBOT in the practice of wound care and healing.

For nearly five decades, HBOT has been used as an oxygen delivery system to ameliorate oxygen deficiencies in the blood and ischaemia by diffusing oxygen into the plasma, allowing cellular production of appropriate signalling molecules and metabolites

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necessary for cell migration and mitosis.[22] At a tissue level, HBOT enhances vasculogenesis and angiogenesis, helping to sustain the area of the wound after healing.[21,23–25] Furthermore, HBOT stimulates the immune system, via white blood cell (WBC) activation, and enhances phagocytosis.[26] In traditional HBOT, the entire body is placed in a pressurised chamber at 100% oxygen. At 3 atmospheres of pressure there are enough oxygen molecules dissolved in the plasma that no red blood cells are needed to adequately oxygenate the tissues and keep them viable. While the mechanisms of wound healing at the host tissue level by HBOT are moderately well understood, the effects of HBOT on wound microbiota are underrepresented in the literature.

HBOT is lethal for anaerobic organisms and can retard bacterial growth at pressures greater than 1.3 atmospheres absolute (ATA).[26] However, the ability of oxygen to diffuse into biofilms is lower, because the EPS and high cell density impede convective flow of the bulk fluid, substantially increasing the diffusion distance.[27] Increased diffusion time prevents oxygen from reaching the hypoxic core of a bacterial biofilm, leaving anaerobic bacteria unharmed.[24] Moreover, bacterial growth rates are much slower within biofilms compared with their planktonic counterparts, attenuating the beneficial effects of HBOT.[28]

Nonetheless, HBOT is an excellent adjuvant therapy for healing chronic wounds, which raises the following question: what is the bactericidal capacity toward the chronic wound biofilm of HBOT? A low number of studies of HBOT regarding chronic wound biofilm have limited its use as an adjuvant therapy in wound care, due to denials for reimbursement by insurance companies and the Centers for Medicare and Medicaid Services (CMS). It is our hope that renewing interest in HBOT as an adjuvant treatment for chronic wounds will supply the necessary data to make the technology more accessible to patients.

Methods

Patients who participated in this study provided consent under a protocol that was approved by the Western Institutional Review Board (WIRB PRO NUM: 20062425). All elements of this study were considered to pose less than minimal risk to the patients, and each patient was fully informed and educated through the consenting process. All patient identifiers were removed from all study data, and only the clinical research coordinator securely retained the documentation linking an individual patient to study data.

Study patients were chosen from our patient database. Patients presenting with wounds that had persisted for at least 30 days with no considerable signs of healing were eligible for HBOT. The control group comprised of patients with similar wounds who do not receive HBOT. Reasons for omission of HBOT include the clinician not deeming HBOT necessary for wound care, the patient responded to our standard of care (SOC), the patient was claustrophobic and declined HBOT, the patient could not afford the prescribed number of treatments, or the patient could not come in often enough for HBOT to contribute to wound healing. A second criterion for study inclusion was that the patient had molecular diagnostic testing on wound samples at the initial visit, during the course of treatment, and on the final treatment.

Wound sampling

The study wound was cleansed with normal saline as part of our usual SOC. Next, the patient's wound was biopsied under local anaesthesia then subjected to sharp debridement using sterile curette, scissors, and/or scalpel to remove slough and devitalised tissue from its surface. The slough and devitalised tissue were then transferred to a sterile 2ml tube and stored at room temperature for no more than two hours before laboratory analysis. Samples of patient wounds were collected at the beginning of treatment and after completion of the HBOT treatment regimen.

Patient HBOT treatments

All patients completed a minimum of 30 HBOT treatments. Chambers were pressurised to 2.0ATA at a rate of 1 pound-force per square inch (psi) per minute. Once a pressure of 2.0ATA was attained, a timer was set for 90 minutes. Sechrist 3200 monoplace hyperbaric

Table 1. Patient demographics

	Number in study	Average age	Age range	Sex Male/female	Caucasian/ hispanic	Diabetic
SOC	10	56	34–90	5/5	7/3	4/10
SOC + HBOT	11	63	49–83	6/5	5/6	8/11
		Wound type				
	Time to healing (weeks)	**DFU**	**NHSW**	**DU**	**VLU**	**CW**
SOC	6.6	3	0	1	3	3
SOC + HBOT	5.8	7	1	1	1	1

SOC—standard of care; HBOT—hyperbaric oxygen therpay; DFU—diabetic foot ulcer; NHSW—non-healing surgical wound; DU—diabetic ulcer; VLU—venous leg ulcer; CW—chronic wound

chambers (Sechrist, Anaheim, CA, US) at the Southwest Regional Wound Care Center were used for the study.

Cell culture

The Lubbock chronic wound biofilm (LCWB) was grown as described by Sun et al.[29] The biofilm contained *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Cells were grown overnight at 37ºC in tryptic soy broth (TSB) (Sigma Aldrich, St. Louis, MO, US) to produce planktonic cultures, after which *Pseudomonas aeruginosa* culture (100µl), *Enterococcus faecalis* culture (150µl), and *Staphylococcus aureus (*200µl) cultures were added to Bolton broth (Oxoid Ltd., Basingstoke, Hampshire, UK) containing 50% (v/v) bovine plasma (Innovative Research Inc., Novi, MI, US) and incubated for 48 hours at 37ºC with rotational shaking at 200rpm. A pipette tip was used as the scaffold for biofilm formation. Once biofilms were formed and tip-attached, the biofilm was transferred from the tip to tryptic soy agar (TSA) plates (Sigma Aldrich).

HBOT treatment of *in vitro* biofilms

Open TSA plates containing the LCWB were placed into Sechrist 3200 monoplace hyperbaric oxygen chambers. Chambers were pressurised to 2.0ATA at a rate of 1psi per minute. Once a pressure of 2.0ATA was attained, a timer was set for 30, 60 and 90 minutes. After HBOT treatment, samples were split for cell viability determination.

Cell viability

In vitro biofilms were divided in two groups, propidium monoazide (PMA)-treated and untreated, for performing the live-dead assay. Samples were added to 0.65ml microtubes, resuspended in 1×phosphate-buffered saline (PBS), and sonicated in ice using a Bioruptor for 12 minutes (Diagenode, Denville, NJ, US). After sonication, 400µM PMA was added to the PMA-treated group. Both treated and untreated samples were incubated in the dark at 4°C for 10 minutes with frequent vortexing. Samples were exposed to light for 15 minutes using a PMA-Lite LED photolysis device (Biotium, Hayward, CA, US) to cross-link the PMA dye to DNA. Percentage viability was determined by averaging the inverse of the threshold cycle (Ct) values of the treated and non-treated groups, and dividing the resulting values of the HBOT group by the non-treated group.

The *in vitro* LCWB was used to assess HBOT bactericidal activity. In our study, *in vitro* biofilms on TSA were subjected to HBOT, and control biofilms on TSA were placed in an inactive chamber to account for any chamber effects. Reductions in the amount of bacterial genomic DNA were determined using the PMA cell viability assay and quantitative polymerase chain reaction (qPCR) of the universal 16S rDNA (ribosomal DNA). PMA binds irreversibly to any extracellular DNA, and DNA in cells with damaged cell walls and plasma membranes which are considered dead. The covalent binding of PMA to DNA inhibits the polymerase enzyme from binding to and amplifying the target nucleic acid sequence during qPCR, resulting in different Ct values for PMA-treated and untreated samples at the same time point. The untreated Ct value represents the total DNA in the sample, which includes extracellular DNA of the biofilm and DNA from cells with damaged membranes (considered unviable). The PMA-treated Ct value represents the fraction of DNA within the sample coming only from viable cells.

Genomic DNA extraction and quantitative polymerase chain reaction (qPCR)

Genomic DNA was extracted from wound samples and *in vitro* biofilms using the Roche High Pure PCR Template Preparation kit (Roche Life Sciences, Indianapolis, IN, US) according to manufacturer specifications. Sample lysates for DNA extraction were produced using the Qiagen TissueLyser (Qiagen Inc., Valencia, CA, US) and 0.5mm zirconium oxide beads (Next Advance, Averill Park, NY, US). Semi-quantitative determination of bacterial load using the universal 16S rDNA was performed using the LightCycler 480 (Roche Life Sciences). Forward (5′-CCATGAAGTCGGAATCGCTAG-3′) and reverse (5′-GCTTGACGGGCGGTGT-3′) 16S rDNA primers (20µM each) were used with a 16S rDNA probe (5′-TACAAGGCCCGGGAACGTATTCACCG-3′) in Quanta PerfeCTa qPCR ToughMix (Quanta Biosciences, Beverly, MA, US). The template DNA (2.5µl) was added to the master mix containing the primers and probe (10µl each), and the reaction was run with the following thermal cycling profile: 50ºC for two minutes, 95ºC for 10 minutes, 35 cycles at 95ºC for 15 seconds, 60ºC for one minute, and 40ºC for 30 seconds. 16S rDNA quantification cycle (Cq) values were used for pre- and post-HBOT comparisons of bacterial load. *Escherichia coli* c600 (ATCC 23724, Manassas, VA, US) genomic DNA was used as a positive 16S rDNA control and molecular grade water (Phenix Research Products, Chandler, NC, US) was used as a no template control.

Sequencing

Samples were amplified for semiconductor sequencing using a forward and reverse fusion primer. The forward primer was constructed with the Ion A linker (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′), an 8–10 base pair (bp) barcode, and the 28F primer (5′-GAGTTTGATCNTGGCTCAG-3′). The reverse fusion primer was constructed with a biotin molecule, the Ion P5 linker (5′-CCTCTCTATGGGCAGTCGGTGAT-3′), and the 388R primer (5′-TGCTGCCTCCCGTAGGAGT-3′). Amplifications were performed in 25µl reactions with Qiagen HotStarTaq master mix (Qiagen Inc.), 1µl of each primer (5µM), and 1µl of template. Samples were amplified with the ABI Veriti thermocycler (Applied Biosystems, Carlsbad, CA, US) under the following thermal profile: 95°C for five minutes, 35 cycles at 94°C for 30 seconds, 54°C for 40 second, 72°C for one minute, 72°C for 10 minutes, and 4°C hold.

Amplification products were visualised with eGels (Life Technologies, Grand Island, NY, US). Products were pooled into equimolar mixtures. Each pool was size-selected using Agencourt AMPure XP (Beckman Coulter, Indianapolis, IN, US) following Life Technologies protocols. Size-selected pools were quantified using the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay Kit (Life Technologies), and diluted to 23pM. Diluted pools were subjected to emulsion PCR (emPCR), enriched using the OneTouch 2 System (Life Technologies), and sequenced using the Ion Torrent Personal Genome Machine (PGM) (Life Technologies), following the manufacturer protocols.

Bioinformatics

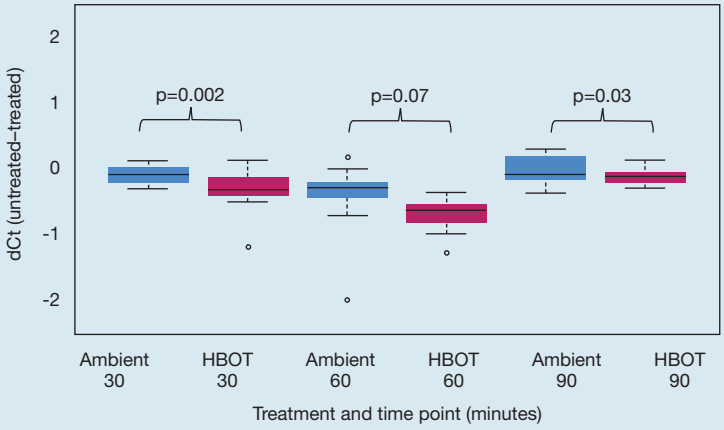
The sequence data were analysed at RTLGenomics (Lubbock, TX, US) using its standard microbial diversity analysis pipeline. The data analysis pipeline consisted of two major stages, the de-noising and chimera detection stage, and the microbial diversity analysis stage. De-noising was performed by various techniques to remove short sequences, singleton sequences, and noisy reads. Once the low-quality reads were removed, chimera detection was performed to aid the removal of chimeric sequences. Finally, the remaining sequences were corrected, base by base, to remove noise from within each sequence. During the diversity analysis stage, each sample was run through the analysis pipeline to cluster reads into operational taxonomic units (OTUs), which went through taxonomic classification down to species-level identification.

Results

Important considerations when analysing clinical HBOT data are patient demographics (Table 1) and the nature of treated wounds, which are characteristically quite diverse and unique. In this retrospective study comprised of patients who underwent SOC alone and SOC with HBOT as an adjuvant, the HBOT group was, on average, seven years older than the SOC group. Additionally, patients undergoing HBOT typically have increased comorbidities, such as diabetes mellitus, neuropathy, and/or arterial and venous insufficiencies, compared with patients who successfully heal with SOC alone. In our patient population, sex- and age-matched acute wounds (controls) are not readily available. These factors can easily confound experimental data, suggesting that HBOT only works by contributing to host healing mechanisms rather than having any bactericidal activity.

The trends of each treatment over time were very similar, with both control and HBOT group viability dropping at the 60-minute time point (Fig 1). Cell viability of treated biofilms, relative to control, was significantly different at the 30- and 90-minute time points and approached significance (p=0.07) at the 60-minute time point. While these differences are minor, and perhaps clinically insignificant, the data suggest that HBOT does bear a degree of biocidal activity towards bacterial biofilms. The differences observed may possibly be amplified with additional age/sex-matched samples and/or different bacterial species included in the biofilms. One caveat of the *in vitro* data is that the biofilms did not have to contend with the host immune system, were only subjected to a single treatment of 30, 60 and 90 minutes, and did not receive any SOC therapy, such as antibiotics or antimicrobial dressings.

Fig 1. Response of *in vitro* biofilms to hyperbaric oxygen therapy (HBOT). The *in vitro* Lubbock chronic wound biofilm model contained three bacterial species. Biofilms were exposed to HBOT for 30, 60, and 90 minutes. Control biofilms (ambient) were placed in inactive closed chambers to account for any chamber effects. Decreases on the Y-axis correspond to decreases in cell viability. Cell viability was determined using propidium monoazide (PMA)-PCR. Statistical significance was determined using the Welch 2 sample t-test



The rebound in cell viability, observed in both the control and treatment groups at the 90-minute time point, may be due to a reorganisation of the community structure of the biofilm by decreasing the competition in the biofilm superstructure or a reversion of some or all species to the faster growing planktonic phenotype (Fig 1). The rebound that was observed in the *in vitro* biofilms was interesting and gave rise to the question of whether a similar trend is observed in patients with chronic wounds who have undergone HBOT. Retrospective data from 2011 to 2016 from patients who had received molecular diagnostics before and after their prescribed treatment regimens were selected from the patient database. To refine the list, patients who had HBOT during their treatment regimen were selected. Patients who did not undergo HBOT but had similar lengths of treatment and wound type were selected, attempting to balance each group for sex, age, and race. The patient demographics (Table 1) show the relevant metric and diabetic status of the patients included in this study.

To assess if and how HBOT as adjuvant therapy contributes to wound healing clinically, bacterial burden determined via qPCR of patient wounds from initial and final visits were compared (Fig 2). Both the HBOT and the SOC group showed reductions in bacterial burden, as expected for healing wounds.

Fig 2. Microbial response to hyperbaric oxygen therpay (HBOT) *in vivo*. The reduction in bacterial load for the treatment and control group was determined by comparing the initial and final molecular diagnostics. Reductions in bacterial load are evident but not significant for the HBOT and standard of care (SOC) groups (p=0.06 and p=0.1641 respectively). Statistical significance was determined using the Welch 2 sample t-test

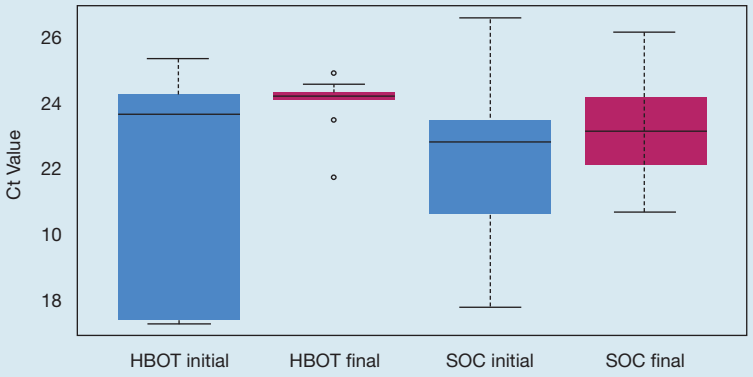
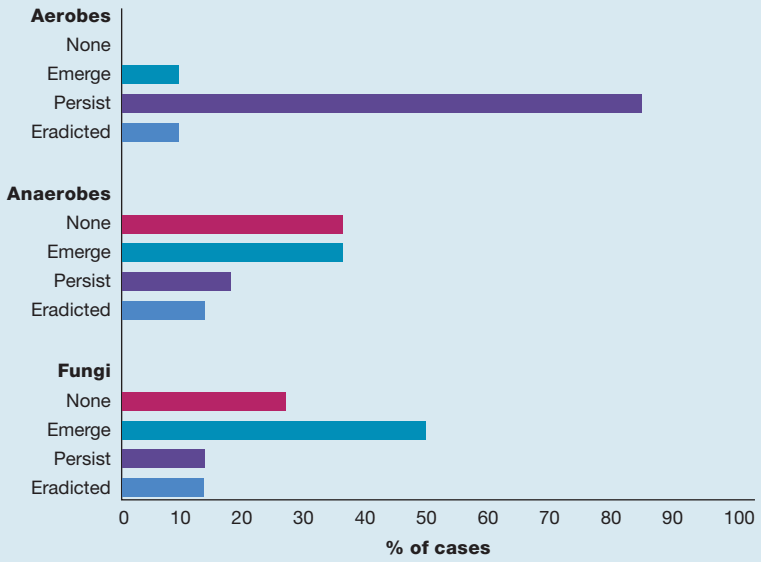


Fig 3. Response of wound microbiota to hyperbaric oxygen therapy (HBOT). 16S rDNA sequencing of patient samples at the initial and final visits revealed how biofilm communities were altered after standard of care (SOC) treatment protocols with adjuvant HBOT therapy. On the Y-axis, 'none' indicates that no microbes of the specified type were detected at either sequencing event. 'Emerge' indicates that microbes not present in the initial sample were detected at the second sequencing event at the end of the treatment protocol. 'Persist' indicates that microbes were present at both sequencing time points. 'Eradicated' indicates microbes that were present in the first sequencing sample but not in the second. Bacteria were grouped according to oxygen-dependence, and fungi were categorised separately



However, the data for the HBOT group showed an appreciably smaller spread and higher median Ct at the final visit, indicating a greater reduction in bacterial burden for the HBOT group than for the SOC group. This, coupled with the time-to-healing information shown in Table 1 (~1 week shorter for HBOT patients) provides additional evidence that HBOT not only contributes to host healing processes, but may also facilitate reduction of bacterial burden.

The molecular diagnostics not only take into account bacterial load but also microbial diversity. Analysis of the microbial subpopulations (aerobe versus anaerobe versus fungi) in the HBOT group, upon completion of the treatment regimen, revealed peculiarities about these biofilm communities. Fig 3 shows how the microbial diversity shifted after completion of treatment. Unsurprisingly, aerobes were the most persistent subpopulation of the wound microbiota and, during treatment, some of the original species were eradicated, leaving a niche for others to emerge as more dominant members of the community. It is important to note that commensal aerobes likely make up for the majority of the detected microbiota in this analysis.

In approximately 35% of cases, anaerobes were not present before or after treatment with HBOT. However, in another approximately 35% of cases, anaerobes emerged as dominant members of the wound microbiota after the treatment regimen. Lastly, in approximately 17% of cases, anaerobes persisted throughout HBOT, while only approximately 13% were eradicated by the treatment. A similar outcome was observed for fungal species within the wound microbiota, with an increased prevalence of fungal emergence after treatment (48% of cases), likely due to the use of antibiotics decreasing bacterial competition. These data suggest that, while HBOT does have bactericidal effects against microbial biofilms, a collapse of the dominant species in the biofilm community may take place. This allows the expansion of rarer and less competitive species which may not be as recalcitrant as the original dominant species, allowing SOC treatment practices, such as debridement and antibiotics (systemic and topical), to be more effective in promoting wound healing.

To better understand how the microbial diversity of the biofilms shifted in this particular cohort with higher resolution, exemplar patients were chosen for further analysis (Figs 4 and 5). The patient described in Fig 4 (control group) showed a significant reduction in bacterial load and complete wound closure using SOC treatment alone within four months. Fig 5 describes a patient with a chronic wound that underwent SOC+HBOT, and had complete wound closure in less than three months. However, at the last molecular testing, the patient had many more microbial species compared with the initial testing event, suggesting a massive community disruption and reorganisation that led to an increase in microbial diversity perhaps more susceptible to SOC methods. These data suggest that HBOT is beneficial as an adjuvant therapy by disrupting the microbiota in the biofilm phenotype, likely interfering with many processes that are finely tuned for certain groups of microbiota within the biofilm. Disruption appears to play a major, if not primary, role in eradicating biofilms, providing a window of susceptibility to SOC treatments.

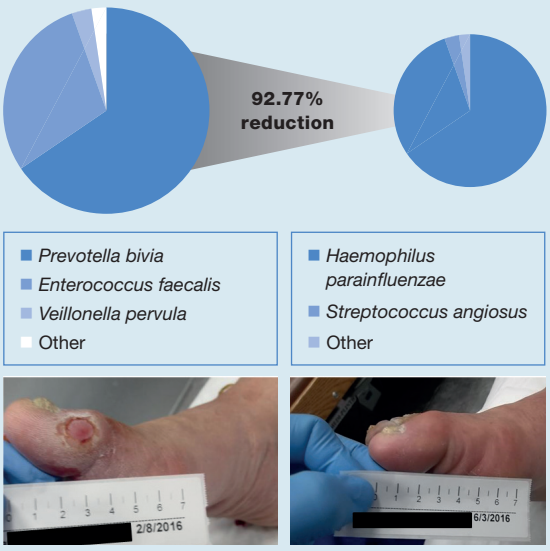


Fig 4. Patient progression with standard-of-care (SOC) protocol. A 51-year-old male with a diabetic foot ulcer (DFU) who underwent SOC alone. The initial bacterial load was quite high for this patient (threshold cycle (Ct)=17.39) but was able to be effectively treated with SOC alone (final Ct=21.78). The sequencing data showed that the biofilm community in this wound was completely disrupted, having changed species composition by the time of wound closure. While the 92.77% reduction in bacterial load was very important, this patient spent approximately four months in treatment

Discussion

The efficacy of HBOT on angiogenesis, bone formation, and skin rejuvenation has been well documented.[24–27,30,31] However, few studies focused on the bactericidal activity relative to the bacterial biofilm phenotype, despite many reports that described HBOT expediting healing of chronic wounds.[24–27,30,31] Recent research in our laboratory demonstrated that wound microbiota was the primary cause of pathogenesis in chronic wounds.[11] That finding raised the question: does HBOT have bactericidal activity against wound microbiota-forming biofilm?

Typically, patients undergoing SOC alone have acute infections inhabited by microbiota, which can be treated quickly and efficiently. To qualify for HBOT, patients must have had a persistent wound being treated for at least 30 days, indicative of a chronic wound inhabited by recalcitrant multispecies bacterial biofilms.

Biofilm recalcitrance to antibiotics is compounded by low diffusion rates into biofilms. Low diffusion rates may contribute to lessened antibiotic delivery and limits oxygenation of the biofilm which suppresses bacterial metabolism, further limiting antibiotic targets.[32,33] HBOT drastically increases oxygenation of host cells and very likely the biofilm as well. This oxygenation probably occurs at the host/biofilm interface, as well as at the biofilm/environment interface, which would increase oxygen diffusion into the biofilm. This scenario could possibly lead to upregulation of metabolic genes and proteins enhancing antibiotic susceptibility. Moreover, increased oxygenation and metabolism could potentially trigger a detachment and dispersion event within the biofilm, reverting biofilm cells to the more susceptible planktonic phenotype. While this explanation is attractive, much work remains to be done to fully elucidate the mechanisms involved.

Taken together, the data from this study provide compelling evidence that HBOT possesses bactericidal activity towards wound biofilms *in vitro* and *in vivo*. The efficacy of HBOT on wound healing is documented;[24–27,30,31] however, the reimbursement for treatment of chronic wounds is severely limited to patients with only a few indications, such as diabetic foot wounds or osteomyelitis. It is the hope of the authors that this study will spark interest in the community to conduct further research on the efficacy of HBOT, which may hopefully lead to a more widespread use of the technology in wound care.

Interestingly, sequencing of wound microbiota before and after HBOT revealed that anaerobes and fungi become more prevalent in wounds after HBOT. This may be due to the combination of targeted treatments for common wound microbiota such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, serial debridement, and HBOT decreasing competition in the wound for less prevalent microbes. This finding suggests that molecular diagnostics should be used in conjunction with HBOT to determine if certain antibiotics/antifungal treatments are necessary during and post-HBOT for wound healing and closure. It is also reasonable to speculate that such increased oxygen concentrations within the capillary may diffuse out of the host, potentiating antibiotics and host counter-measures to aid in removal of the wound microbiota.

While the exact mechanism of bactericidal action has not yet been elucidated, it can be speculated that cellular responses to hyperoxygenation are involved. Hyperoxygenation leads to increased accumulation of reactive oxygen species (ROS) in cells which, at certain thresholds, overwhelm the antioxidant defense and repair systems of the cell.[34] The biological targets of ROS are widespread, including DNA, RNA, proteins, and lipids. Immune cells exploit ROS production via the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme as a weapon during invasion of pathogenic bacteria.[35] Increased ROS production under hyperbaric conditions may produce the same effect and serve to temporarily destabilise the biofilm community, allowing a window for improved efficacy of SOC treatment protocols. It is clear that if there is any significant planktonic load, 3ATA of pure oxygen will have bactericidal activity regardless of the species; however, it is less certain how hyperoxygenation affects bacterial biofilms. The results presented here demonstrate in real patient wounds that total wound microbiota decreased when using HBOT as an adjuvant relative to that using SOC alone.

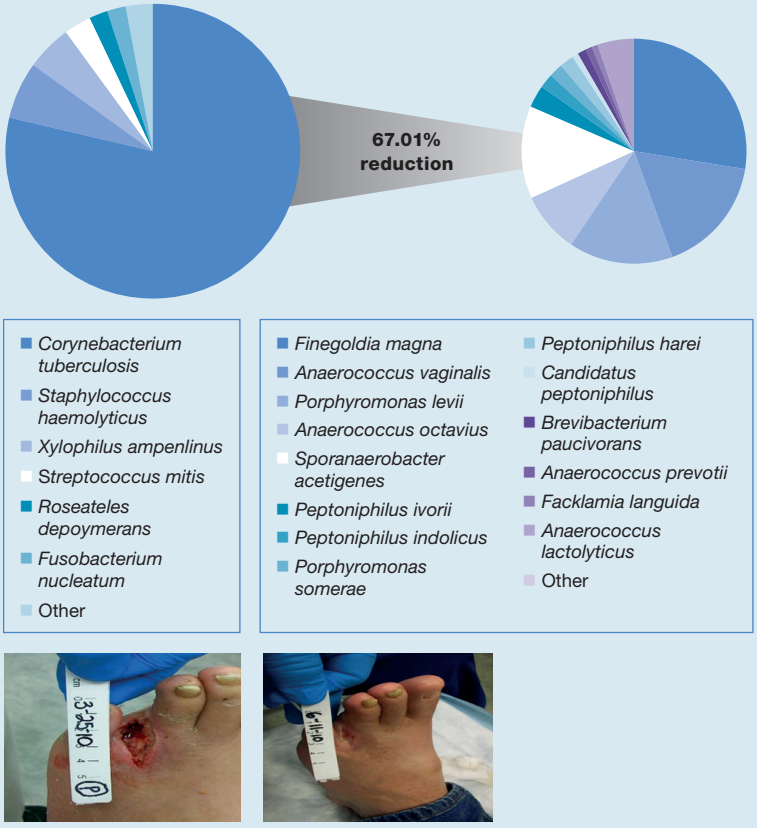
Limitations of the study

The *in vitro* biofilms used in this study were grown in broth media and transferred to TSA plates upon maturation to undergo the HBOT treatment. In such an experimental setup, metabolic waste products may accumulate and nutrient shortage within the biofilm may signal a dispersal event and cause bacterial cells to revert to the planktonic phenotype. Additionally, handling the *in vitro* biofilms excessively will damage the cells resulting in misleading cell viability data. This prevented *in vitro* biofilms from undergoing multiple HBOT treatments over the course of several days. It is possible that additional HBOT treatments may have produced more robust reductions in viable bacterial cells strengthening the evidence presented. The *in vivo* data was collected retrospectively from the patient database which limited the ability to match HBOT patients and SOC patients by sex, age and wound type. Retrospective data collection was deemed appropriate for the purpose of this study because patients at our facility receive targeted treatments based on severity of the wound, various comorbidities, etc. which would prohibitively increase time needed to collect an appropriate number of samples for the study.

Conclusions

Previous works on the efficacy of HBOT as an adjuvant therapy has produced varying evidence regarding its use as an adjuvant therapy in treating chronic wounds.[36,37] This may result from differences in choice of experimental systems, antibiotic usage, and/or duration of HBOT treatments. Recently, Jorgensen et al.[37] studied the efficacy of daptomycin and rifampicin with intermittent HBOT to treat *Staphylococcus aureus* biofilms and did not observe any significant benefit in treating implant-associated osteomyelitis in a murine model. Kurt et al.,[37] also using a murine model, observed significant reductions in bacterial load using vancomycin or tigecycline in combination with HBOT compared with using antibiotic alone in post-sternotomy mediastinitis. In addition, HBOT as an adjuvant to ciprofloxacin resulted in increased bacterial killing *in vitro Pseudomonas aeruginosa* biofilms and in humans with malignant otitis externa.[38,39] The evidence here supports the hypothesis that hyperoxygenation of wound biofilms using HBOT as an adjuvant to SOC treatment contributes to the reduction of total bacterial load. CMS only covers HBOT for a very small subset of patients suffering from chronic wounds. This is likely due to a lack of research on the topic and conflicting results reported from different groups working on disparate models. It is our hope that the literature on HBOT and its use as an adjuvant therapy for chronic wound healing will continue to grow, so that this technology will be better understood and can potentially be more widely available to patients with chronic non-healing wounds and other afflictions, which HBOT may be able to relieve. **JWC**

Fig 5. Patient progression – standard of care (SOC) + hyperbaric oxygen therapy (HBOT). A 74-year-old male with a diabetic foot ulcer who underwent HBOT. This patient began treatment with a medium-high bacterial load (threshold cycle (Ct)=23.82) and opted for SOC with adjuvant HBOT (final Ct=26.18). The sequencing data for this patient showed a complex polymicrobial community at the initial sequencing event, which became more diverse as treatment progressed to the final sequencing event. Interestingly, the initial biofilm community was composed of aerobic microbes, and the final sequencing event contained several anaerobic species. This suggested that the original wound biofilm was successfully disrupted by SOC with adjuvant HBOT, promoting successful wound healing within approximately 2.5 months of treatment



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Reflective questions

- What are the methods used by microbiota that cause wound chronicity?
- What are the molecular mechanisms involved in the biocidal activity of Hyperbaric oxygen therapy (HBOT)?
- Does HBOT trigger a dispersion event of the biofilm, reverting biofilm cells to the planktonic phenotype, which may be easier to treat with standard care therapies?

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