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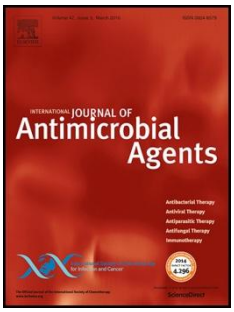

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PII: S0924-8579(17)30240-6
DOI: http://dx.doi.org/doi: 10.1016/j.ijantimicag.2017.04.025
Reference: ANTAGE 5180

To appear in: *International Journal of Antimicrobial Agents*

Received date: 13-1-2017
Accepted date: 5-4-2017

Please cite this article as: Lerche CJ, Christophersen LJ, Kolpen M, Nielsen PR, Trøstrup H, Thomsen K, Hyldegaard O, Bundgaard H, Jensen PØ, Høiby N, Moser C, Hyperbaric oxygen treatment augments tobramycin efficacy in experimental *staphylococcus aureus* endocarditis, *International Journal of Antimicrobial Agents* (2017), http://dx.doi.org/doi: 10.1016/j.ijantimicag.2017.04.025.

Hyperbaric Oxygen Treatment Augments Tobramycin Efficacy in Experimental *Staphylococcus aureus* Endocarditis

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Running title: Hyperbaric oxygen treatment in *S. aureus* endocarditis

Highlights

- *Staphylococcus aureus* infective endocarditis (IE) is a serious acute infectious disease with reported mortality rate up to 40%
- Proposed oxygen dependent bactericidal effect of aminoglycosides
- Hyperbaric oxygen treatment (HBOT) augments the efficacy of tobramycin (aminoglycoside) in cardiac valve vegetations
- The host response in experimental *S. aureus* IE was evaluated by key inflammatory markers in IE
- Proof of concept using adjunctive hyperbaric oxygen treatment in severe *S. aureus* IE

ABSTRACT

Background. *S. aureus* infective endocarditis (IE) is a serious disease an in-hospital mortality of up to 40%. Improvements of effects of antibiotics and host responses could potentially benefit outcomes. Hyperbaric oxygen treatment (HBOT) represents an adjunctive therapeutic option. We evaluated the efficacy of HBOT in combination with tobramycin in *S. aureus* IE.

Methods. A rat model of *S. aureus* IE mimicking the bacterial load in humans was used. Infected rats treated with tobramycin were randomized into two groups, 1) HBOT (b.i.d) or 2) normobaric

air breathing (non-HBOT). Quantitative bacteriology, cytokine expression, valve vegetation size and clinical status were assessed 4 days post infection.

Results. Adjunctive HBOT (n=13) reduced bacterial load in the aortic valves, myocardium and spleen compared to the non-HBOT group (n=17) (*p=.04, p<.001, and p =.01, respectively)* and improved the clinical score *(p* <.0003). Photoplanimetric analysis and weight of valve vegetations showed significantly reduced vegetations in the HBOT group (*p <.001*). Key pro-inflammatory cytokines (IL-1b, IL-6, KC and VEGF) were significantly reduced in valves from the HBOT group compared to the non-HBOT group.

Conclusion. HBOT augmented tobramycin efficacy as assessed by several parameters. The present findings suggest the potential use of adjunctive therapy in severe *S. aureus* IE.

Keywords: Hyperbaric Oxygen Therapy; Host Response; Oxidative Stress; Hydrogen Peroxide; Biofilm; Small Colony Variants; Interleukin 10; Hypoxia; Neutrophils; Platelets; Inflammation.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) infective endocarditis (IE) is a serious acute infectious disease involving colonization of the cardiac valves, endocardium or prosthetic material in the heart. The *S. aureus* IE incidence is increasing and has a reported mortality of 40% (1-year mortality), which has not changed during the last 5 decades [1]. New therapeutic approaches, interventions and particularly optimization of the initial treatment are required [2].

S. aureus is a facultative aerobe Gram-positive, versatile and ubiquitous pathogen, which can infect any tissue in humans. *S. aureus* causing endovascular infections are generally highly virulent and triggers a prompt inflammatory host response. To optimize treatment, expanded knowledge of the bacterial-host interplay in IE is needed. This complex interplay activates cascades of consecutive processes recruiting host defence cells and the release of multiple inflammatory cytokines. The pathogenesis of the host and pathogen interactions has been intensively studied for decades, mainly focusing on the pathogen virulence factors [3,4], and with less attention on influences of the host response in relation to infection and treatment response.

The crucial key point in treating *S. aureus* IE, like any other severe infectious disease, is early [5,6] and sufficient high dose antibiotic combination therapy [7] to retain infection control and minimize complications. Unfortunately, diagnostic delay and inadequate antibiotic treatment, especially in the initial phase of the IE course are seen in a considerable fraction of the patients. A serious infection like *S. aureus* IE involving endothelium damage, bacteraemia and septic dissemination to vital organs triggers an exaggerated host response activating platelets and immune cells to the site of inflammation, which may lead to additional tissue and organ damage [8].

Polymorphonuclear leukocytes (PMNs) are first line of defence against *S. aureus* [9]. Increased tissue oxygen consumption is a major component of the response in severe sepsis [10], osteomyelitis [11] infected wounds [12], biofilm infections [13] and is expected to be like-wise for left-sided native *S. aureus* IE. Although, PMNs are primarily dependent on glycolysis rather than oxidative metabolism for ATP generation, they need O_2 supply to maintain the NADPH oxidase-driven respiratory burst, which generates reactive oxygen species (ROS) required for killing pathogens. Several studies have shown that infected sites are severely depleted of oxygen and hypoxia has been shown to inhibit the ability of PMNs to kill *S. aureus* [14,15].

Accumulating evidence has shown that several bactericidal antibiotics like beta-lactams, fluoroquinolones and aminoglycosides partly depends on bacterial aerobic respiration in addition to their target-specific killing mechanisms [16,17]. Therefore, the efficacy of these antibiotics is to some extent dependent on the availability of O_2 and the metabolic status of the bacteria [17,18]. The purpose of hyperbaric oxygen treatment (HBOT) as adjunctive therapy is to stimulate the aerobic respiration of pathogens and to reoxygenate the infected and O_2-depleted tissue and hereby increasing the pathogens susceptibility. Additionally, HBOT may increase the capacity of PMNs respiratory burst against *S. aureus* as well as impairing exotoxin production, the latter being O_2 sensitive and can be inhibited at tissue partial pressures achievable with HBOT [11,19] .

Therefore, we hypothesized that HBOT may augment the efficacy of tobramycin treatment in *S. aureus* experimental IE, especially in the early course of IE. HBOT as adjunctive therapy in the early course of IE may be of beneficial value by some of above mentioned reasons. HBOT has already proven beneficial in a variety of infectious diseases [20] mostly in deep-seated and

recalcitrant infections like necrotizing fasciitis [21], osteomyelitis [22] and chronic wounds [23]. Investigations of adjunctive HBOT in IE are sparse [24]. In a recent study [25], we showed poor efficacy of functional monotherapy with tobramycin (aminoglycoside) in *S. aureus* experimental IE. The clinical consideration behind this study is that a substantial fraction of patients with methicillin susceptible *S. aureus* (MSSA) as well as methicillin resistant *S. aureus* (MRSA) IE may be treated insufficient (functional monotherapy or single agent therapy) in the early phase of the disease.

The objective of the study was to evaluate the potential effects of HBOT in *S. aureus* IE, why we intentionally used at suboptimal antibiotic treatment to be able to measure any potential effects of adjunctive HBOT in our experimental *S. aureus* IE model.

MATERIAL AND METHODS

S. aureus strain

A penicillin- and methicillin-susceptible *S. aureus* (MSSA) strain (NCTC 8325-4) was used in the present study. Inoculum was made from an overnight culture of *S. aureus* at 37°C, resuspended in fresh Luria-Bertani (LB) media and grown to logarithmic phase (OD 0.5, λ 600 nm), centrifuged at 5000 rpm at 5° C and washed in saline (0.9 %) and diluted to the desired inoculum size of $0.5x10^7$ CFU, as described previously [25].

Experimental endocarditis in rats

All experiments were approved by The National Authority (License no. 2013-15-2934-00952) and the rats were maintained and handled in accordance with guidelines for animal research. High-grade aortic valve IE was produced in rats as described previously [25]. In brief, male Wistar rats (225-250 g of body weight) were anesthetized with a mixture of hypnorm (fentanyl,

0.315 mg/ml; fluanisone, 10 mg/ml), sterile water and midazolam (5 mg/ml) in 1:2:1 dilution. The tip of a sterile polyethylene catheter (Portex Ltd., Hythe, Kent, UK) was surgically placed at the aortic valve in the left ventricle of each animal via the right carotid artery and verified by pulsation. Catheters were kept in-situ for 24 hours in order to damage the valve and then surgically removed in general anaesthesia as outlined above. Subsequently, rats were injected with an intravenously bacterial suspension of $0.5x10^7$ CFU inducing left-sided high-grade aortic valve IE.

Study design

Effect of HBOT has been shown to be dose-dependent [10,26]. In extensive pilot studies, we have found that HBOT given once a day in combination with tobramycin is insufficient to reduce bacterial load significantly in some rats in our high-grade IE model (data not shown). For this reason, we intensified the HBOT to twice a day (b.i.d.). Infected rats were randomized into two groups, one group receiving HBOT (b.i.d. for 90 minutes with 8 hours interval) in combination with tobramycin (n=13) and another group receiving tobramycin under normobaric oxygen breathing (non-HBOT, n=17). Six catheter inflicted non-infected sham control rats were included - three receiving HBOT and three kept at normobaric conditions. A arm of untreated rats was not included in this study, but has been performed in previous work [25]. All animals were evaluated at 4 days post infection (DPI) (**Fig. 1**).

Tobramycin treatment in the experimental model of endocarditis

Rats were treated with tobramycin (Nebcina®, Eurocept International) 20 mg/kg/day subcutaneously (s.c.) initiated 1 DPI, as described [25].

Hyperbaric oxygen treatment (HBOT)

After IE induction, the rats were treated HBO twice a day (b.i.d.) with 8 hours interval and received a pressure of 280 kPa (2.8 bar) at room temperature in a hyperbaric oxygen chamber (OXYCOM 250 ARC; Hypcom Oy, Tampere, Finland). At 280 kPa, no air-breaks were applied and rats breathed 100% oxygen throughout. Rats received a total of 6 series HBOT. The HBOT profile consisted of a 12 minute pressurization and depressurization before and after the 90 minutes of treatment at 280 kPa. Rats breathed 100% oxygen during pressurization and depressurization given a total of 114 minutes of oxygen breathing. The first HBOT was initiated 24 hours (1 DPI) after induction of IE.

Clinical status of rats

Clinical scores of the appearance of the rats were: 0, no clinical signs; 1, slightly ruffled fur, but clinically unaffected; 2, ruffled fur and signs of sepsis, clinically affected; 3, lethargy, signs of severe sepsis and neurological deficits; 4, severe neurological deficits and moribund.

Photoplanimetric evaluation

All rats were autopsied immediately after lethal i.p. injection of pentobarbital/lidocaine (200 mg/ml and 20mg/ml) and hearts were aseptically dissected for photographic imaging of the aortic valves (Sony Cyber-shot DSC-RX100). Valve vegetations size (mm^2) was measured by digital photoplanimetric evaluation (ImageJ, vers. 1.49m) by a pathologist blinded to treatment regimens.

Measurement of serum and tissue cytokines

Expression of interleukin (IL)-1b, IL-6, keratinocyte-derived chemokine (KC - rat analogue to human IL-8), G-CSF, IFN-γ, TNF-α, IL-10, IL-17, and vascular endothelial growth factor (VEGF), was measured in supernatants from serum and tissue homogenates harvested at time of evaluation (4 DPI) and stored at -80° C until analysis. For multiplex analysis we used the rat cytokine assay (Bio-Rad, Hercules, CA, USA) on the LUMINEX® 200™ platform (Luminex Corporation, Austin, TX, USA), according to the manufacturer's instructions.

Statistical analysis

Statistical calculations were performed using GraphPad Prism (version 7.2, GraphPad Software, Inc., San Diego, USA). Quantitative bacteriology measurements were verified by D'Agostino & Pearson omnibus normality test for parametric data and compared by two-tailed parametric T-test for two-group comparison. Cytokine measurements were compared by two-tailed nonparametric Mann–Whitney U-test for two-group comparison. $P \leq 0.05$ was considered significant. Bacterial tissue densities were calculated as $\log_{10}$ CFU/g or organ ± mean standard deviation.

RESULTS

Clinical evaluation

Rats were clinically scored (scale, 0-4) at 4 DPI before being sacrificed. The HBOT group had significantly improved clinical status as assessed by the clinical score ($p < 0.0001$). The non-HBOT group was more septic and some rats showed obvious signs of neurologic deficits indicating cerebral affection.

Hyperbaric oxygen treatment (HBOT) in *S. aureus* IE

Adjunctive HBOT (n=13) significantly reduced bacterial load in the aortic valves compared to non-HBOT group (n=17) (5.2 ± 2.68 vs. 7.01 ± 1.73 $\log_{10}$ CFU/g) (p = 0.04). A corresponding reductions was seen for the myocardium (3.36 ± 2.09 vs. 5.29 ± 1.58 $\log_{10}$ CFU/g) (p <0.001) and in the spleen (1.79 ± 1.22 vs. 3.38 ± 1.80 $\log_{10}$ CFU/spleen) (p = 0.01) (**Fig. 2**). No rats died during the treatment period in either group. In the HBOT group 3/13 (23%) was blood culture positive at 4 DPI as compared to 9/17 (52%) in the non-HBOT (p = 0.10). No adverse effects of were seen in rats treated with HBO. All catheter inflicted sham controls had sterile tissue cultures (n=6).

Another important observation was the occurrence of *small-colony variants* (SCV) in both treatments groups. The characteristic of SCV were reduced size of a one CFU compared to wild-type CFU size, pigment loss and they preserved catalase activity as the wild-type. Subanalysis revealed that 7/13 (53%) rats in the HBOT group and 3/17 (18%) in the non-HBOT group had SCV in the valve vegetations. The susceptibility for SCV found in both groups showed a 50-fold increase of MIC for tobramycin and gentamicin from the wild-type (0.125 to 6 µl/ml). The SCVs continued to be susceptible as the wild-type to methicillin, penicillin, vancomycin, rifampicin, clindamycin, linezolid and fucidic acid.

Valve vegetation evaluation

To investigate the severity of the primary site of infection we used photoplanimetric evaluation and weight measurement of the dissected valves. The weight (mg) of valves including vegetations was reduced in the HBOT group as compared to the non-HBOT group (0.085 ± 0.045 vs. 0.165 ± 0.073, respectively) (p < 0.001). Similar observations were obtained by

photoplanimetric evaluation of the macroscopic valve vegetation size (mm^2) (2.05 ± 1.1 vs. 3.73 ± 1.77, respectively) ($p < 0.001$) (**Fig. 3b and 3c**).

Cytokine measurement in tissue homogenates and serum

Valves. Pro-inflammatory cytokines expression in the aortic valve vegetations are shown in **Fig. 4**. The pro-inflammatory cytokines IL-1b, IL-6 and KC are indicators for disease progression and they were all significantly reduced in the HBOT compared to non-HBOT group ($p = 0.04$, $p = 0.005$ and $p = 0.005$, respectively). Vascular endothelial growth factor (VEGF) is produced by and also stimulates macrophages, monocytes and endothelial cells, pivotal in regulating the host inflammatory response in sepsis. VEGF has pro-coagulant activity and is expressed in high concentrations in platelets intracellularly. We found that the level of VEGF was significantly reduced in valve vegetations in the HBOT compared to non-HBOT ($p = 0.002$), indicating a decrease of platelets aggregation in the valve vegetation. We found no significant differences in the expression of G-CSF, IFN- γ, TNF-α, IL-17 or IL-10 in valves between the two groups (data not shown). To investigate the pro- and anti-inflammatory effects of HBOT we compared the ratio between KC/IL-10 (pro-/anti-inflammatory) and VEGF/IL-10 (pro-/anti-inflammatory). The HBOT group showed a significantly lower ratio score compared to non-HBOT group (mean 0.45 vs. 2.18, $p < 0.0002$ and mean 0.09 vs. 0.21, $p < 0.0003$, respectively) (**Fig. 4**).

***Myocardium*.** IL-10 increased significantly in the HBOT as compared to the non-HBOT group (924 ± 375 vs. 717.2 ± 269 pg/ml) ($p = 0.04$) indicating anti-inflammatory response in the myocardium. No other significant differences in cytokines levels were seen between the two groups. The KC/IL-10 ratio was significantly reduced in the HBOT group (0.27 ± 0.17 vs. 0.79 ± 0.86) ($p = 0.02$) (**Fig. 4**).

***Serum*.** The pro-inflammatory markers IL-1b, IL-6, IL-10 and KC (a murine IL-8 homologue[25]) were highly elevated compared to healthy controls. All measured cytokines showed no significance difference between the HBOT and non-HBOT group (data not shown).

DISCUSSION

The present study revealed that adjunctive HBOT (b.i.d) significantly reduced the bacterial load in the valves, myocardium and reduced the vegetation size by augmented efficacy of tobramycin. Additionally, the inflammatory host response was reduced and the rats' clinical condition was significantly improved in the HBOT group compared to non-HBOT group.

A few studies have shown that HBOT is dose-dependent [10,26]. Our experience was similar in our high-grade *S. aureus* IE model. In extensive pilot studies, using HBOT once daily in combination with tobramycin was not sufficient to cause a significant reduction of the bacterial load in valves for all rats. However, rats with a low-grade IE responded already 3 DPI, by means of quantitative valve bacteriology, whereas this effect was not observed until 4 DPI in a subgroup of rats with a high-grade IE. In account of tobramycin monotherapy insufficiency in high-grade IE this observation is not unexpected. Based on these results, we intensified the HBOT to twice a day (b.i.d.) and demonstrated a marked effect resulting in significant reductions of the bacterial load in valves, myocardium and spleen. A solid indication of adjunctive HBOT augments tobramycin activity in our high-grade *S. aureus* IE model. To verify that HBOT could augment tobramycin activity without host cells, we developed an *in vitro S. aureus* biofilm model (see

supplemental material). By direct exposure of the bacteria in microplates with one single HBOT for 90 minutes at 280 kPa, we could demonstrate increased bacterial susceptibility of *S. aureus* in anoxic biofilm exposed to different tobramycin concentrations.

We suggest that HBOT stimulates aerobic respiration leading to increased drug uptake resulting in antibiotic-induced formation of bactericidal amounts of hydroxyl radicals [16]. Although this classic pathway of killing bacteria has been questioned [27] our observations are in accordance with Collins et al.[28]. One possible explanation for this apparent contradiction could be the pathogen specific tolerance against hydroxyl radicals [29,30]. The confirmation of pathogen and antibiotic specific significance of hydroxyl radicals needs further studies.

There was a remarkably difference in the clinical score between the two groups, although some rats in the HBOT group had comparable high bacterial load. Rats exposed to HBOT were in significantly better clinical condition compared to non-HBOT animals. Whether this was due to an immune modulated effect within the host or shift in virulence and toxin production in *S. aureus,* or a combination, is not fully known. However, in septic hypodynamic rats the addition of HBO may also improve tissue microcirculation by inhibition of neutrophil β_2 integrin adhesion without compromising neutrophil antibacterial functions [31]. Furthermore, several studies have shown that HBO attenuates pro-inflammatory cytokine production and reduce sepsis mortality in *in vivo* animal models of systemic inflammation, in accordance with our observations [26,32,33].

Subpopulation of aminoglycoside-resistant SCVs has been shown previously in various animal models treated with aminoglycosides alone [34]. SCVs and persister cells has garnered increasing attention in recent years due to the awareness to persisting and relapsing infections [35].

S. aureus is well known for its adaptation to oxidative stress, where catalase ability in *S. aureus* is a key virulence factor. Hydrogen peroxide (H_2O_2) and most antibiotics can induce the generation of SCVs and H_2O_2 can select for SCV phenotype with increased stability and frequency, reducing the rate of reversion to wild-type. Gentamicin-resistant SCVs has also been shown to display a greater catalase activity than wild-type [36]. Taking these *in vitro* observations in consideration, the increased frequency of SCVs in HBOT is logical. HBOT increases the intensity of the respiratory burst by the PMNs [11,37], which may lead to increased oxidative pressure of *S. aureus in vivo*, making the wild-type alteration into SCVs, changing its metabolism (oxygen independent). Furthermore, the increased oxidative stress may in part result from treatment with aminoglycoside [30].

The inability of HBOT to increase the frequency of SVCs during tobramycin treatment of the *in vitro* biofilm may be due to the relatively short exposure time (90 minutes). However, it also indicates, that the host's own defence mechanisms are able to induce SVCs *in vivo* under exposure of HBOT in combination with aminoglycoside. We observed cross-resistance for the tobramycin and gentamicin with a 50-fold increase, but not for other antibiotics. Unfortunately, we were not able to quantify the frequency in different tissue compartments between wild-type and SCV due to the ability of SCV to revert back to wild-type when not exposed to oxidative stress during the incubation period.

If adjuvant HBOT is to be used we suggest combination with two different targeting antibiotics. Optimally, one of them should be an antibiotic with intracellularly activity and independently of bacterial aerobic respiration, due to SCVs ability to survive intracellularly in host cells and change its metabolism. SCVs can be prevented by combination therapy [38], but further studies

323 needs to clarify if combination or single agent therapy is the most beneficial treatment of *S.*
324 *aureus* left-sided IE in terms of outcome and prevention of recurrent infection.
325
326
327 *S. aureus* IE can be characterized as an acutely devolving biofilm infection, where the
328 aggregation of platelets play a pivotal role in the progression and development of IE [39,40].
329 VEGF has been shown to be a key player for inflammatory cytokine production in murine sepsis
330 [41]. We measured VEGF in the valve vegetations and found it to be significantly decreased in
331 HBOT treated rats compared to non-HBOT. VEGF is highly expressed in platelets, thus
332 indicating that adjuvant HBOT was able to reduce the inflammation and aggregation of platelets
333 in the valves. Hence, indicating VEGF as an important inflammatory mediator in *S. aureus* IE.
334 This was also observed by the reduced weight and size of vegetations in the HBOT group.
335 Another explanation could be that VEGF is tightly regulated by hypoxia-inducible factor (HIF)-
336 1α and HBOT might also modulate the expression of VEGF in platelets and endothelial cells
337 decreasing the activation of the cells [42]. In some models, HBOT have been demonstrated to
338 ameliorate post ischemic injuries by downregulation of HIF-1α expression [19]. Lastly, the
339 reduced VEGF could be due to a more efficient treatment response reducing the bacterial load,
340 inflammation and diminishing the aggregation of additional platelets [43].
341
342 As shown in a previous study, the progression of *S. aureus* IE correlated to the pro-inflammatory
343 cytokines IL-1b, IL-6, KC measured in valve tissue, which therefore provides a potential
344 indicator for treatment response [25]. Adjunctive HBOT reduced these markers significantly in
345 valve tissue correlating to the enhanced efficacy of tobramycin. However, no differences in levels

of serum cytokines were seen, making it difficult to use systemic cytokine biomarkers for diagnosis, treatment efficacy and prognosis in the early stage of IE.

Anti-inflammatory marker IL-10 inversely correlated with the bacterial load. In our model, adjunctive HBOT induced an anti-inflammatory response by means of increased IL-10 expression in valve tissue. This observation is in accordance with other studies showing dose-dependent effect of HBOT and protection from sepsis mortality via an IL-10 dependent mechanism [26]. Macrophages are a major source of IL-10 during infection and important contributor for resolution of infection [44]. Several *in vitro* studies have shown HBOT affects these cell lines by transiently suppressing stimulus-induced pro-inflammatory cytokine production and enhance PMNs apoptosis and clearance by the macrophages, which is essential for tissue healing. These mechanisms are extremely important in a systemic infection like *S. aureus* IE, where large numbers of PMNs migrate to site of infection and contributing to severe oxygen depletion affecting bacterial growth, antimicrobial activity and PMN function [14][15].

Our study shows the delicate regulation of the immune response in relation to the severity of infection and the dynamic changes of expression of pro- and anti-inflammatory markers in response to adjuvant HBOT.

Further studies evaluating adjunctive HBOT together with penicillin or β-lactam antibiotics alone or in combination with other effective anti-staphylococci agents are of high importance in a clinical perspective.

In conclusion, we have shown beneficial effect of adjunctive HBOT in combination with tobramycin by reduced bacterial load in *S. aureus* IE paralleled by an increased anti-

inflammatory host response. Therefore, the present proof of concept suggests adjuvant HBOT in handling of *S. aureus* IE.

ACKNOWLEDGEMENTS

The valuable technical assistance and maintenance by Senior Hyperbaric Supervisor, Michael Bering Sifakis, Rigshospitalet, has been greatly appreciated. We thank Pia Meincke and colleagues at Institute for Inflammation Research, Copenhagen University Hospital Rigshospitalet for collaboration and usage of their LUMINEX® 100/200™ instrument. Finally, thanks to Peter Lerche for critical proofreading prior to submission.

DECLARATIONS

Funding: This work was supported by fellowship grants to C. Lerche from The Danish Heart Association (Hjerteforeningen), Helene and Georg Jensens, Ethel Merethe and Christian Pontoppidans Foundations (grants 15-R99-A5982).

Competing Interests: None

Ethical Approval: All experiments were approved by The National Authority (License no. 2013-15-2934-00952) and the rats were maintained and handled in accordance with guidelines for animal research.

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Figure 1. Schematic timeline representation experimental rat model of high-grade *S. aureus* IE receiving adjunctive HBOT with 100% O_2 at 280 kPa (2.8 bar) for 90 minutes in combination with Tobramycin (20mg/kg/day, s.c.) performed in current study.

Abbreviations*: S. aureus, Staphylococcus aureus,* IE, infective endocarditis, HBOT, hyperbaric oxygen treatment; non-HBOT, normobaric air breathing, DPI, days post infection, h, hours. †, sacrificed.

Figure 2. Quantitative bacteriology of homogenized valves (a), myocardium (b), and spleen (c) at 4 days post infection (DPI). All rats were treated with tobramycin 20mg/kg/day subcutaneously. Two groups was compared, one group receiving adjunctive hyperbaric oxygen treatment (HBOT) (+) for 90 minutes twice a day (b.i.d.) the other receiving normobaric air breathing (non-HBOT) (-). The bacterial load after 4 DPI was significantly reduced in HBOT (+) compared to non-HBOT (-). $*p < 0.05$ and $**p < 0.01$.

Figure 3. Clinical score (a), and relative valve vegetation weight (b) and valve vegetations size (c) in animals 4 days post infection (DPI) . All rats were treated with tobramycin 20mg/kg/day subcutaneously. The clinical symptom score was significantly reduced in the hyperbaric oxygen treatment (HBOT) group (+) compared to non-HBOT (-) ($**p < 0.0003$) Both the relative valve vegetation weight and valve vegetations size (mm^2) measured by digital photoplanimetric evaluation (ImageJ, vers. 1.49m) were significant reduced in the HBOT group compared to the non-HBOT group ($*p < 0.001$). Pooled data was obtained from tree independent experiments. Error bars are means ± SD.

Figure 4. The expression of pro-inflammatory cytokines in the aortic valve vegetations, correlated to disease progression are shown **(a, b, c, d)** in adjunctive hyperbaric oxygen treatment (+) compared to normobaric conditions (-), all treated animals received tobramycin (20mg/kg/day) with exception of sham controls. Catheter inflicted sham controls (non-infected) receiving ± HBOT. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. IL, Interleukin, KC, *keratinocyte-derived chemokine*. VEGF, *vascular endothelial growth factor*. Pooled data was obtained from three independent experiments. Data represented by box plot with mean ± SD including min to max.

Figure 5. Expression of pro-inflammatory cytokine KC and VEGF and anti-inflammatory cytokine IL-10 ratio in aortic valves **(a, b).** The expression of anti-inflammatory marker IL-10 and the pro-inflammatory marker KC (IL-8)/IL-10 ratio in the myocardium (c, d). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. IL, Interleukin, KC, *keratinocyte-derived chemokine*. VEGF, *vascular endothelial growth factor*. Pooled data was obtained from three independent experiments. Data represented by box plot with mean ± SD including min to max.