

Synergy of HBO₂ and a local antibiotic carrier for experimental osteomyelitis due to staphylococcus aureus in rats.

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Mendel V, Simanowski H-J, Scholz H. Synergy of HBO₂ and a local antibiotic carrier for experimental osteomyelitis due to staphylococcus aureus in rats. Undersea Hyperb Med 2004; 31(4):407-416. A standard rat model of *Staphylococcus aureus*-induced osteomyelitis was used to compare the effect of HBO₂, a local antibiotic carrier (gentamicin-containing collagen sponge) and the combination of HBO₂ with a local antibiotic carrier. For the induction of osteomyelitis, a defined *Staphylococcus aureus* suspension was inoculated into the medullary cavity. Arachidonic acid was used as sclerosing agent. With that procedure an infection rate of more than 95 percent was attained. Prior to the treatment interval surgical debridement of the soft-tissue infection was performed. In the control group the extent of infection was 4.9×10^6 CFU x g⁻¹ of tibial bone three weeks following implantation of organisms. Subsequent to debridement of the soft tissue infection, the bone infection decreased slightly with a value of 3.7×10^6 CFU x g⁻¹ of tibial bone at the end of the experiment. HBO₂ as single-agent therapeutic reduced the infection to 1.7×10^5 CFU x g⁻¹ of tibial bone. Due to its high local antibiotic level, the gentamicin-collagen sponge achieved a reduction in organisms to 1.4×10^2 CFU x g⁻¹ of tibial bone. The effect was most marked using a 4-wk combination therapy with local application of the gentamicin-containing sponge and additional treatment with HBO₂. In 9 of 11 animals, bacteria were no longer detectable in the processed bone substance. Each of the treatment modalities resulted in a significant therapeutic effect. No complete healing of the infection was achieved with the flexible collagen sponge characterized by pronounced and rapid release of gentamicin. In combination with hyperbaric oxygen an additive effect was attained and thus a significant improvement of treatment.

INTRODUCTION

In spite of new surgical techniques and new antibiotics, there is no fully satisfactory treatment of chronic post-traumatic osteomyelitis. Chronic osteomyelitis often requires long-lasting and expensive therapy (1,2). The goal of modern treatment of post-traumatic osteomyelitis is to functionally and anatomically restore the affected extremity to the largest possible extent. Most important is the meticulous surgical debridement of the infected area by complete removal of necrotic bone and soft tissue (3) because bacteria which are located in the center of necrosis can neither be accessed and thus killed by antibiotics administered via the bloodstream nor by locally released antibiotic agents. Although many modern chemotherapeutic agents are available as adjuvant therapy, if administered systemically, the antibiotic is distributed via the bloodstream to the tissue compartments, but penetration is impeded by diffusion barriers. Hence, not even high and potentially toxic doses can guarantee adequate levels of tissue activity. If, however, the antibiotic is directly applied by implantation to the site of infection with use of a suitable carrier, high concentrations of active substance are attainable without toxic stress for the overall organism (4,5,6,7).

The use of hyperbaric oxygen has been referred to as adjuvant therapy (8,9,10), a therapeutic approach to increase the proportion of oxygen in tissues and thus to support the

cellular defense system. Based on the osteomyelitis model of rat tibia described by Zak and coworkers (11), we were able to perform quantitative infection analyses. HBO₂ achieved a significant reduction in bone infections and, in combination with a systemic antibiotic, led to an additive effect. Healing of the bone and soft-tissue infection, however, was not attained (12).

Due to varying operative techniques, different forms of adjuvant therapy and high relapse rates of surgically treated cases of chronic osteomyelitis, it is difficult to assess therapeutic methods and compare different studies (3,13,14,15,16,17). Clinical and experimental studies on the use of HBO₂ and locally released antibiotics are not available.

The aim of the study was to ascertain whether, subsequent to surgical debridement of infection, treatment with *hyperbaric oxygen* and use of a *locally applied antibiotic* may lead to better therapeutic results or even to healing of osteomyelitis.

MATERIALS AND METHODS

Animal model

The experiments were conducted in 104 female, approximately 6-month-old Wistar rats. At study onset, the animals weighed approximately 160-200 g. The animals were given water and food ad libitum. A constant room temperature of 20°C was maintained, and the atmospheric humidity varied between 60 and 70 percent. To facilitate identification, the ears of the animals were marked and the cages numbered. The sequence of figures cage/ per animal/ per day permitted a correct allocation to control and treatment groups before placement of the infection.

Chronic osteomyelitis of the rat tibia was induced as described by Zak et al. (11). After sterilizing and shaving the right hind leg, the skin was incised over a length of approximately 1.0 cm at the medial aspect of the proximal tibial segment. After displacement of the soft-tissue mass, a cortical-bone defect was produced by means of a twist drill (diameter 1 mm). Arachidonic acid (No. A-6523, Sigma Chemical Co., St. Louis, MO) was used as sclerosing substance, 5 µl of which was applied into the medullary cavity with a dispersion syringe, followed by infection with 5 µl of a defined *Staphylococcus aureus* suspension.

Culture of *Staphylococcus Aureus*

Staphylococcus aureus (No. 292113, American Type Culture Collection, Rockville, MD) was used for the induction of infection. The organism was sensitive to oxacillin, mezlocillin, tetracycline, gentamicin, co-trimoxazole, and cefazolin. Having been streaked and incubated on blood-agar plates, four or five colonies were transferred into a standard nutrient broth (Makrotube, No. 13419, Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. The overnight culture was washed twice with 5 ml of 0.9 % NaCl and centrifuged at 3000 rpm for 10 min. After decanting of the supernatant, the remaining organisms were transferred into 1 ml of 0.9 % NaCl. The concentration of organisms determined was 3.2 and 4.7 x 10⁶ colony-forming units (CFU) per ml.

Experimental trial

The experimental test lasted 7 weeks and was divided into: establishment of infection (3 weeks) and treatment interval (2 and 4 weeks).

Three weeks after induction of infection roentgenograms were performed. The roentgenograms were reviewed by a radiologist. The presence of periosteal elevation, architectural distortion, widening of the bone shaft, and new bone formation were determined for

each tibia. Animals with development of osteomyelitis got a number of identification. The animals' ears were marked and the cages numbered. By using a randomization table all animals were located to the control and treatment groups:

Control groups (A)

- A. 1. infection over 3 wk (n = 12)
- 2. infection over 3 wk (n = 12)
treatment with 0,9 % NaCl over 2 wk
- 3. infection over 3 wk (n = 11)
treatment with 0.9 % NaCl over 4 wk

Treatment groups (B, C, D)

- B. therapy with HBO₂
 - 1. treatment over 2 wk (n=11)
 - 2. treatment over 4 wk (n=12)
- C. therapy with gentamicin-collagen sponge
 - 1. treatment over 2 wk (n=12)
 - 2. treatment over 4 wk (n=12)
- D. therapy with HBO₂ and gentamicin-collagen sponge
 - 1. treatment over 2 wk (n=12)
 - 2. treatment over 4 wk (n=11)

Starting the treatment interval, surgical debridement of the infected soft tissue was performed. After anesthetization the skin was incised over a length of approximately 1.0 cm at the medial aspect of the proximal tibial segment. With use of telescopic spectacles the encapsulated pinhead-sized soft-tissue abscess was exposed and completely removed. The operative field was flushed with 0.9 % NaCl (A2,A3). Thereafter, the antibiotic-containing sponge was implanted into the animals of the treated groups C and D. Wound margins were adapted using interrupted sutures.

The animals of control group A1 were killed 3 weeks after the onset of infection, all the others 2 respectively 4 weeks after treatment.

Therapy with HBO₂ and gentamicin-collagen sponge

Treatment was performed in the hyperbaric chamber (Hyperbaro-Therapie-Kammer 1000", Dräger-Werke AG, Lübeck, Germany). The test animals (B,D) were exposed to a pressure of 3 atm abs and the chamber was flooded with 100 percent O₂ for one hour. The compression rate was 0.5 atm abs · min⁻¹. Accordingly, decompression was 0.5 atm abs · min⁻¹ and was begun 60 min after the compression phase. This was performed twice a day.

We used a gentamicin-collagen sponge, Sulmycin[®] (Essex-Pharma, Munich, Germany) for local therapy. The implanted collagen sponge measured 10 x 5 mm. The carrier contained 2 mg of gentamicin sulfate equivalent to 1.3 mg of gentamicin base.

Macropathological and bacteriological analysis

The macropathologic staging was performed in accordance with the method of Rissing and associates(18). The extent of new bone formation and bone loss was determined. In addition, abscess size and erythematous changes of the skin were examined.

For quantitative bacterial bone counts, muscle and connective tissue were first removed from the tibiae. Tibiae were aseptically cross-sectioned at both ends with a high-speed circular saw. Proximal sectioning was performed between the metaphysis and the epiphyseal plate; distal sectioning was performed approximately 8 mm from the distal articulating surface. The resulting bone segments were weighed, snap-frozen at -60°C, and crushed in a mortar with pestle. Subsequently, the bone powder was suspended in 20 ml of 0.9 % NaCl. 5 ml of the suspension were diluted at a ratio of 1:10. 100 µl of each dilution were streaked onto blood-agar plates and incubated at 37°C for 24 h, and the organisms were subsequently counted.

Statistical analysis

Data were analyzed using SAS software, version 6.12 for Windows. Deviation of data from a normal distribution was tested using a Kolmogorov-Smirnov test with a correction by Liliefors. Analysis of variance (ANOVA) was performed between the control groups. ANOVA was also performed for the treatment groups after 2 and 4 weeks with inclusion of the respective control group. Following an ANOVA between the control groups, a test by Tukey was employed to confirm which of the control groups, if any, did indeed significantly differ from each other. The significance level used for this test was 5%.

Dunnet's test was then used to confirm if any of the treatment groups at a particular week were significantly different (at the 5 % level) to their respective control, following an initial ANOVA between all groups. Being a "many-to-one" comparison, Dunnet's test allows for control of the Type I error (5%) when performing multiple comparisons ie. HBO₂, gentamicin and HBO₂ + gentamicin were compared to one control group.

RESULTS

Infection was induced in 125 animals and 4 animals died. In the remaining animals, primary wound healing occurred. In 113 rats, the roentgenographic follow-up revealed unequivocal signs of inflammation with a score > 5. To form similar groups, 108 animals with roentgenographically established bone infections were allocated to the control and treatment groups. Three weeks following organism implantation, soft-tissue debridement was performed in the animals of control groups A2, A3 and treatment groups B, C, D. Three animals died due to excessive depth of anesthesia: in the control group A3 and in the treatment groups B and D.

Macropathological findings:

After a 2-wk respectively 4-wk treatment interval, all animals were killed. After the skin had been incised, a pinhead-sized encapsulated abscess was found in the proximal tibial segment of the animals of control groups A2 and A3, whereas in the treatment groups only one animal of the gentamicin-collagen group C had formed an abscess. After removal of the abscess and soft-tissue mass, exact staging was possible on the basis of macroscopic bone evaluation (Table 1). More than 50 percent of the control group A1 had category 4 changes. The control groups A2 and A3 were allocated to stages 2 and 3. Eighty percent of the therapy group receiving hyperbaric oxygen were allocated to stage 2. Only a few animals had developed an abscess (Table 2).

Table 1:Categories of osteomyelitis changes (control groups)

Ratings, %	A1 (n=12)	A2 (n=12)	A3 (n=11)
0. Absence of abscess, sequestrum, active bone formation and erythema			
1. Absence of abscess or erythema, new bone formation, minimal bone destruction			
2. Absence of abscess or erythema, distinct new bone formation minimal bone destruction		50.00%	45.45%
3. Abscess, new bone formation, distinct bone destruction	33.30%	50.00%	54.50%
4. Abscess, severe bone resorption, diaphyseal or total tibial involvement	66.70%		
Macropathological findings, control group			
A1	3 wk after implantation of infection		
A2	3 wk after implantation of infection and adjuvant therapy with 0.9 % NaCl over 2 wk		
A3	3 wk after implantation of infection and adjuvant therapy with 0.9 % NaCl over 4 wk		

Table 2: Categories of osteomyelitis changes (treatment groups)

Ratings, %	B (n=12) (n=11)*	C (n=12) (n=12)*	D (n=11) (n=12)*
0. Absence of abscess, sequestrum, active bone formation and erythema			
1. Absence of abscess or erythema, new bone formation, minimal bone destruction	75% (81 %)*	100 % (91.7 %)*	100 %
2. Absence of abscess or erythema, distinct new bone formation, minimal bone destruction			
3. Abscess, new bone formation, distinct bone destruction	25% (19 %)*	(8.3 %)*	
4. Abscess, severe bone, resorption, diaphyseal or total tibial involvement			
Macropathological findings, treatment groups			
B	3 wk after implantation of infection and adjuvant therapy with HBO ₂ over 4 wk (over 2 wk*)		
C	3 wk after implantation of infection and adjuvant therapy with gentamicin collagen sponge over 4 wk (over 2 wk *)		
D	3 wk after implantation of infection and adjuvant therapy with HBO ₂ and gentamicin collagen sponge over 4 wk (over 2 wk*)		

Therapy groups C and D receiving gentamicin-collagen sponge and gentamicin-collagen sponge in combination with hyperbaric oxygen were mostly placed into category 1.

Quantitative analysis

The quantitative evidence of osteomyelitis of the control animals is shown in Table 3. Table 3 contains a synoptic quantitative assessment of chronic osteomyelitis under various treatment regimens. Three weeks after implantation of the organisms, the bacterial colony counts were 4.9×10^6 CFU \times g⁻¹ of tibial bone. After surgical debridement of the soft-tissue infection, the bone infection had decreased slightly until week 5 (A2) and after 7 weeks (A3) had reached 3.7×10^6 CFU \times g⁻¹ of tibial bone.

In relation to control values therapeutic effect became apparent after 2 and 4 weeks of treatment with HBO₂. 6.2×10^5 and 1.7×10^5 CFU \times g⁻¹ of tibial bone were found. After 2 weeks, 9.8×10^2 CFU \times g⁻¹ of tibial bone were attained with high, locally applied gentamicin concentrations, after 4 weeks 1.4×10^2 CFU \times g⁻¹ of tibial bone. An additive effect was achieved with the combination of gentamicin-collagen plus HBO₂. Organisms in the bone suspension were no longer detectable after 4 weeks in 9 of 11 animals. In animals No. 4 and No. 9 the infection observed was 0.43×10^1 and 0.28×10^1 CFU \times g⁻¹ of tibial bone.

Table 3: Quantitative Evaluation of Osteomyelitis, Experimental and Control Groups

Group Assignment	CFU * g ⁻¹ tibial bone, mean value \pm SD
A. Control Group	
1. 3 weeks after infection, n = 12	$4.9 \times 10^6 \pm 0.37$
2. therapy with 0.9 percent NaCl after 2 weeks, n = 12	$2.0 \times 10^6 \pm 0.17$
3. therapy with 0.9 percent NaCl after 4 weeks, n = 11	$3.7 \times 10^6 \pm 0.42$
B. Therapy with HBO ₂₂	
1. after 2 weeks, n = 11	$6.2 \times 10^5 \pm 0.06$
2. after 4 weeks, n = 12	$1.7 \times 10^5 \pm 0.03$
C. Therapy with gentamicin-collagen sponge	
1. after 2 weeks, n = 12	$9.8 \times 10^2 \pm 0.024$
2. after 4 weeks, n = 12	$1.4 \times 10^2 \pm 0.002$
D. Therapy with HBO ₂ + gentamicin-collagen sponge	
1. after 2 weeks, n = 12	$1.0 \times 10^2 \pm 0.002$
2. after 4 weeks, n = 11	0*
* Organisms in the bone suspension were no longer detectable after 4 weeks in 9 of 11 animals. Only in animal No. 4 and No. 9 infection was observed (0.43×10^1 and 0.28×10^1 CFU of tibial bone)	

Table 3 provides the mean and standard deviation of the four treatment groups at each week in units of 10⁶. No test of deviation from the normal distribution was performed because the methods used to analyse the data do not assume that the data is normally distributed. Further, with the low number of data available, there is little power to reject the hypothesis of normality.

A univariate analysis of variance (ANOVA) was performed on the data from the three control groups. The F-test was not significant indicating that the control groups did not differ from each other (Tukey's test).

The analysis of variance was then carried out on the four treatment groups at week 2 and 4. In each case the F-test was significant ($p < 0.001$). Dunnet's test was then performed comparing each treatment group with its respective control. The comparisons were significant at a level of $p < 0.05$.

The second ANOVA tells us that each of our treatment groups are different to the respective control groups, both at week 2 and week 4. All the treatments significantly lower the bone infection with respect to their control.

The treatment groups were found to be significantly different from each other both at week 2 and week 4. The F-test was significant ($p < 0.001$).

DISCUSSION

The osteomyelitis model in rat tibia described by Zak was the base for our studies. The level of infections observed in the control groups is constant between week 3 and 7 so that the various treatment modalities may be reviewed. Interactions between bone and surrounding soft tissue mass are discernible. Irrespective of surgical debridement, the control animals presented soft-tissue infections at the end of the so-called "treatment interval" after 2 and 4 weeks.

Different adjuvant treatment forms following surgical debridement of bone and soft tissue were studied and evaluated for their combined effects.

1. Therapy with hyperbaric oxygen.
2. Local antibiotic therapy with direct application of the active substance to the site of infection.
3. Local antibiotic therapy in combination with hyperbaric oxygen

Our studies confirm experimental results and positive clinical experience with hyperbaric oxygen and local antibiotic therapy. HBO₂ leads to significant colony-count reduction and thus to better results than in the study conducted without soft-tissue debridement (12).

The local antibiotic carrier acts clearly. As expected, gentamicin, released from the collagen sponge, causes marked colony-count reduction. Complete healing, however, is not attained. Release kinetics of the porous carrier is discernible already after 2 weeks and attributable to the high discharge of gentamicin from the collagen sponge. The advantage of the sponge is good plasticity and thus short diffusion distance from the carrier to the area of infection. Due to the implantation of collagen sponge, concentrations are attained many times above the minimum inhibitory concentration (MIC) of the etiologically most relevant organisms (14). Hence, organisms are covered that are rated resistant to systemic therapy (19). Ledge and coworkers (16) used this absorbable antibiotic carrier clinically and found, in the first days of therapy high gentamicin concentrations in serum, urine and soft-tissue secretions, while toxic levels were never observed. High local levels of active substance were also observed by von Hasselbach in release kinetics in the first 24 hours (14). As shown in our studies even these high antibiotic concentrations alone do not guarantee the healing of an infection. Despite surgical debridement border areas between vital and devitalized tissue persist as barriers. This specific topographic feature of the infective focus has to be regarded as a problematic issue in our therapeutic efforts. Bone tissue has the possibility to interrupt the host defense mechanisms. Architecture and biochemical composition of bone tissue permit the separation of pathogens from blood flow. The defense system has hardly a chance to surmount these barriers. Bony

structures foster bacteria. Bacteria are able to survive radical debridement even in perifocal pathogen nests. Therefore, if the pathogen is able to escape both: local surgical intervention and systemic defense mechanisms, it is necessary to incorporate other adjuvant therapeutic options into existing treatment modalities.

The colony-count reduction is most remarkable with the combination of HBO₂ and local antibiotic application. In 9 of 11 animals, organisms were no longer detectable in the processed bone suspension.

Hyperbaric oxygenation is achieved by breathing 100 % oxygen at an elevated atmospheric pressure. Physiologically, this produces a directly proportional increase in the plasma volume fraction of transported oxygen which is readily available for cellular metabolism. Under these conditions, O₂ has access to cells located far away from capillaries. Hence, for instance, capillary rarefaction or edema may be counterbalanced (20). The action of numerous cellular systems active in the endogenous defense system is oxygen-dependent. In hypoxic tissues such as those seen in chronic osteomyelitis, supply of oxygen is a limiting factor. Distribution of available oxygen to different carrier molecules (hemoglobin, myoglobin) or consumer molecules (cytochrome oxidase) follows the principle of affinity and is subject to the rules of diffusion and enzyme kinetics. In hypoxic wounds where microcirculation is impaired, competition for available oxygen ensues. The effect of HBO₂ on neovascularization and wound healing is well known. The migration of the cell population occurs along the concentration gradients of O₂ (high → low) and lactate (low → high) between wound margin and wound base (21,22). Directed vascular sprouting is based on the same principle (23). Cell division hardly occurs in heavily hypoxic wound areas (24) and wound healing is reduced. Studies have demonstrated that fibroblast proliferation can be stimulated depending on the dose of HBO₂ (24). Therefore, adequate O₂ supply leads to increased collagen production, improved cross-linkage and elevated synthesis rate of cells. This was measured by a rise in the RNA/DNA quotient (25). Also osteoblasts and osteoclasts are susceptible to hypoxic metabolic functions. When minimal residual perfusion is still persisting, hypoxia may be minimized and neutralized under the influence of hyperbaric oxygen. In this case osteoblasts and osteoclasts become activated again.

In addition, the positive influence on the immune system and on bacteria must be taken into account. The phagocytic activity of granulocytes is oxygen-dependent. Hence, phagocytic activity in hypoxic tissues is diminished and infective defense reduced (26). By neutralizing hypoxia, phagocytosis of granulocytes is stimulated and necrotic zones are significantly reduced (22,27,28). The bactericidal influence of HBO₂ on gas-forming clostridia is well documented (29,30). In addition to this bactericidal action, a bacteriostatic effect on *Escherichia coli*, *Staphylococci* and *Pseudomonas* strains is known (31)

The treatment of the chronic osteomyelitis has five therapeutic steps. The initial surgery includes full debridement of all sinus tracts, sclerotic bone and sequestra. Any residual necrotic bone serves as a focus of infection, and therefore, leads to persistent infection or late recurrences long after an apparently good result. All internal fixation or other foreign material should be removed, and external fixation devices applied to maintain bone stability. Large wounds may require autogen cancellous bone grafting and muscle transposition flaps or free muscle flaps with microvascular techniques. Modern antibiotics are available as adjuvant therapy.

Our studies have again demonstrated the positive influence of hyperbaric oxygen and, in particular, the synergistic effect when used in combination with local antibiotic therapy (12). The colony-count reduction was most remarkable and in 9 of 11 animals the organisms were no longer detectable in the processed bone suspension. This positive effect of hyperbaric oxygen is

understandable because in chronic infective foci are usually located in avascular or other poorly perfused bone areas and are characterized by hypoxic metabolic conditions. We recommend the use of HBO₂ as adjuvant therapy in the setting of the five classical principles of surgical osteomyelitis therapy.

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