Hyperbaric Oxygen Exposures at 3 and 4 Atmospheres Absolute Pressure for Experimental Gas Gangrene: Succinate Protection Against Oxygen Toxicity

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Received for publication 14 April 1972

The concurrent effect of succinate administration to protect against oxygen toxicity and of hyperbaric oxygen (HBO) exposures to treat model gas gangrene in mice was tested to determine whether succinate would interfere with the therapeutic efficacy of HBO. HBO (seven 90-min exposures) at 3 atmospheres absolute pressure (ATA) had been shown to reduce significantly the mortality of mice injected with Clostridium perfringens suspended in 10 μg of Adrenalin. When succinate was tested with this system, mortality of HBO-exposed infected animals was again significantly reduced (79% control mortality versus 17% HBO-exposed mortality), indicating that succinate does not interfere with the action of HBO. Exposures to 4 ATA of O2 were evaluated in the same model clostridial infection with succinate used to prevent oxygen toxicity. Five 30-min exposures at 4 ATA reduced the mortality of infected animals (62% control versus 6% HBO-exposed mortality). Intraperitoneal succinate injections (10 mmoles/kg) were given 20 to 25 min prior to four of the seven 3-ATA exposures and before three of the five 4-ATA exposures. The intermittent succinate injections gave significant protection against the development of oxygen toxicity in infected and noninfected mice at both O2 pressures. These studies support the potential clinical use of succinate or other oxygen-protective agents (i) to shorten HBO exposure times by using higher pressures to deliver the necessary O2 dose, (ii) to increase the O2 dose for difficult clinical situations by using maximal exposures at 4 ATA or more prolonged exposures at 2 to 3 ATA, and (iii) to continue HBO exposures in patients who require treatment but develop symptoms of oxygen toxicity.

Experimental and clinical data indicate that hyperbaric oxygen (HBO) is therapeutically valuable in treatment of gas gangrene (clostridial myonecrosis; 1, 3–6, 8–10, 16, 17). The medical application of high pressures of oxygen is limited by the toxicity of oxygen, however, and short intermittent exposures are used to remain within a symptom-free latent period. Sanders and colleagues demonstrated that succinate injections significantly protect rats and dogs against the development of oxygen toxicity as measured by convolution times and various biochemical parameters (13–15). In the future, this protective agent may be used clinically to protect patients from possible adverse effects of high oxygen tensions. Since a primary medical use of HBO is for the treatment of gas gangrene, it is important to ascertain whether the concurrent administration of succinate might interfere with the action of HBO on the clostridial infection and perhaps negate a therapeutic effect. Moreover, higher oxygen dosages could be advantageous in certain clinical situations, and oxygen-protective agents might allow longer exposures at the currently used...
pressures of 2 to 3 atmospheres absolute pressure (ATA) or exposures to higher pressures such as 4ATA or even 5ATA. Presently, oxygen is not utilized clinically at pressures greater than 3ATA because of the increasingly rapid onset of oxygen toxicity with increased oxygen pressure.

In previous studies (6), HBO exposures at 2 and 3ATA markedly reduced mortality among mice injected with a mixture of Clostridium perfringens and Adrenalin. However, in a similar model infection in which 3% calcium chloride was injected with the clostridia, HBO exposures at 3ATA did not significantly reduce the overall mortality among infected mice although the survival time was significantly lengthened (6).

The primary purpose of the present study was to test the combined effect of succinate injections given concurrently with HBO exposures at 3ATA for control of the standardized infection initiated with clostridia plus Adrenalin. A secondary purpose was to investigate whether higher pressures of oxygen could safely be used and, if so, whether the mortality among mice given clostridia plus calcium chloride would be reduced.

MATERIALS AND METHODS

Bacterial culture. A clinical isolate of C. perfringens was lyophilized to provide a constant stock of bacteria. For each experiment, the bacteria were cultured from this source and harvested at log phase for preparation of a suspension for injection into mice. The details of culture and titration of these bacteria have been described previously (5, 6).

Experimental animals. Male, inbred A/J mice (The Jackson Laboratory, Bar Harbor, Me.) were used at an age of 11 to 13 weeks. Previous studies (7) had shown that these mice possess a relatively high tolerance to oxygen at 3ATA. Mice were rested for 2 weeks to recover from the stress of shipping and to stabilize their health before use in any experiments. They were fed Purina Laboratory Chow and water ad libitum except during hyperbaric exposures or fasting periods prior to succinate injection.

Model gas gangrene infection. The model infection has been described in detail elsewhere (6). Briefly, 0.1 ml of washed, log-phase C. perfringens cells contained in 10µg of Adrenalin (Parke, Davis & Co.) was inoculated into the right gastrocnemius and hamstring muscles of mice. An alternate infection model was produced by injection of the clostridia suspended in 3% calcium chloride instead of Adrenalin. The pathology of these model infections was similar to that seen in human gas gangrene infection except that the disease in mice progressed more rapidly. The number of deaths among infected animals was dependent on the challenge dose. In all experiments, an equal number of infected control and HBO test mice were inoculated. A mortality of 50 to 85% in the infected control animals was considered optimal. Differences in overall mortality at 5 days after infection were evaluated by a chi-square analysis, and the significance of differences in survival time was determined by the Kolmogorov-Smirnov statistical test.

HBO. Mice were given oxygen exposures in a pressure chamber which possessed viewing ports and a temperature control device. The method of oxygen exposure has been described previously (6). In the present studies, various HBO exposure schedules were tested (Table 1). Schedule A was the same pattern of HBO exposures that had reduced mortality among mice given clostridia plus Adrenalin (6) in the absence of any succinate injections. Schedule B was similar to A in that the exposures were begun at the same point in time relative to the bacterial challenge, but each exposure at 4ATA lasted only 30 min instead of 90 min.

Succinate injections. Sodium succinate (0.4 m, pH 6.4) was prepared from succinic acid (Sigma Chemical Co.), and 10 mmoles/kg was injected intraperitoneally into mice 20 to 25 min prior to an HBO exposure. The HBO-exposed and control infected animals received the same injections of succinate. Two to four noninfected HBO control mice not given succinate were included in each experiment. Also, noninfected HBO control animals given succinate were usually included. Mice were fasted a minimum of 16 hr prior to a succinate injection.

RESULTS

Protection by succinate of mice exposed to HBO. Initial experiments were performed to establish the protective capacity of succinate in mice. Con-

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<th>Table 1. HBO exposure schedules tested with varying pressure, duration, and pattern of exposurea</th>
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a HBO = 100% O₂ at designated pressure, 21 ± 1°C.
b Exposure number.
c No exposure 6 for one trial.
vulsion times in oxygen at 4 and 5 ATA were determined for saline-injected control mice and mice pretreated intraperitoneally with succinate. Preliminary experiments indicated that succinate gave more consistent protection when the mice were fasted for 18 hr (method of Sanders et al.) as opposed to shorter periods without food. However, a 50-min time lapse between a single injection of succinate and compression in oxygen, as used for rats (13–15), did not always give protection from oxygen toxicity in the present study with mice, so various succinate incubation periods (from 15 to 30 min) were tested. Greatest protection was observed with a 20- to 25-min incubation time. The average time in 5 ATA of O_2 to grand mal-type convulsions was 29.4 ± 4.5 min for control mice and 49.2 ± 10 min in mice given succinate (four experiments, total of 25 mice/group).

It was necessary to give HBO exposures at frequent intervals within the first 24 hr to combat the rapidly progressive nature of the clostridial infection (6), but, when succinate was injected prior to each HBO exposure on schedule A, the mice became ill and weak. Therefore, tests were performed with noninfected mice to determine the frequency with which succinate could safely be administered. A period of approximately 6 hr between injections was required to avoid adverse reactions in the animals. Thus, succinate was used only for a portion of the total number of HBO exposures in each type of schedule.

**Succinate injections concurrent with HBO at 3 ATA for infection.** The combination of HBO exposures on schedule A and succinate injections was tested on mice inoculated with clostridia plus Adrenalin. Succinate was injected into infected control and HBO-exposed mice prior to HBO exposures 1, 3, 4, and 6. The reduction in overall mortality of infected animals given HBO exposures and succinate (Fig. 1) was approximately the same as had been previously observed without the use of succinate (6), and indicated that succinate injections did not interfere with HBO therapy of this infection.

A benefit derived from the use of succinate was the total absence of oxygen toxicity symptoms among the HBO-exposed infected animals. In previous experiments (6) with HBO schedule A, central nervous system toxicity was observed in a few animals beginning at about the fifth HBO exposure. The seventh exposure was often shortened, or even deleted, to prevent severe oxygen damage when toxicity continued to develop. This toxicity was eliminated in the present series with the use of intermittent succinate injections.

Uninfected HBO control mice given succinate were compared to mice without succinate for their reaction on this HBO schedule. If all overt symptoms of central nervous system toxicity in these controls were counted, 78% of the non-succinate HBO animals (25 of 32) showed toxicity compared to 24% of the succinate animals (6 of 25). The reactions ranged from a hyperexcited state which could be readily identified to severe grand mal-type convulsions. The nonsuccinate HBO control mice generally demonstrated toxic symptoms earlier (i.e., during the third, fourth, or fifth HBO exposure) and had more serious grand mal-type convulsions than the succinate-injected animals. The succinate-injected controls that showed toxic symptoms, in general, had mild symptoms such as hyperexcitability or short seizures without complete loss of consciousness. These symptoms began only during the final HBO exposures. Nine mice among the 32 non-succinate controls died, but no deaths occurred among the HBO control animals given succinate.

**Succinate injections concurrent with HBO at 4 ATA for infection initiated with clostridia suspended in Adrenalin.** On the basis of the results obtained when succinate was used in conjunction with HBO exposures at 3 ATA, the effect on clostridial infection of HBO at 4 ATA was tested. Succinate injections were administered prior to HBO exposures 1, 3, and 4 on schedule B. To avoid oxygen toxicity, the number and duration of exposures were reduced. Again, a significant reduction in the mortality of infected mice given HBO exposures was observed (Fig. 2). Protection from the development of oxygen toxicity was also apparent even though succinate was only used for three of five exposures. Only 23% of the infected mice demonstrated mild symptoms of oxygen toxicity during the HBO exposures.
Uninfected mice with and without succinate were again compared for their reactions in HBO. Protection from the development of oxygen toxicity followed the same general pattern as had been observed at 3 ATA. Noninfected mice given succinate had no or only mild symptoms of oxygen toxicity (two of seven), whereas the mice without succinate developed earlier and more severe symptoms (seven of seven).

**Succinate injections concurrent with HBO at 4 ATA for infection initiated with clostridia suspended in calcium chloride.** Exposures to 4 ATA of O₂ were tested for control of the infection initiated with clostridia suspended in CaCl₂. Mice with this model infection survived slightly longer than when the clostridia were given with Adrenalin, so three different exposure schedules were tested (schedules B, C, and D, Table 1). Succinate was injected prior to exposures 1, 3, and 4 for schedules B and C and only for exposures 1 and 3 on schedule D. With schedules B and C, no differences were observed between HBO-exposed and control infected mice insofar as survival time and overall mortality were concerned (Table 2). Schedule D appeared to be a better match between the delivery of HBO and the time course of the infection, but again no differences were found except in a single experiment. In one of four experiments, the HBO-exposed mice had a significantly prolonged survival time compared to the infected controls (Kolmogorov-Smirnov statistical test, P > 0.01), but the overall mortality at 5 days was the same (Table 2). The trials in which the mortality among infected control mice was higher than anticipated are included in Table 2 as additional information.

Only a single infected mouse demonstrated mild symptoms of oxygen toxicity (1 of 110). Even though no generalized oxygen toxicity was encountered in the infected mice, there were indications (response after HBO exposure, mortality rates, and final mortality) that HBO might have had an added deleterious effect when given to mice already very toxic from advanced clostridial gangrene. Of 26 uninfected mice without succinate, 23 had central nervous system toxicity. This occasionally developed during the first HBO exposure, but usually began during the third or fourth exposure. Four of these mice died. Only one succinate-injected control (1 of 12) had mild symptoms of toxicity. On schedule D, the third succinate injection (given prior to exposure 4) could be deleted without development of oxygen toxicity in the infected mice. However, it was necessary to give the uninfected succinate controls the third injection, or they would develop oxygen toxicity. In this series, it was again observed that the infected mice showed less tendency to develop oxygen toxicity than the uninfected HBO control mice.

**DISCUSSION**

The administration of sodium succinate as a protective agent against the development of oxygen toxicity not only did not interfere with the therapeutic usefulness of HBO in controlling clostridial infection, but also improved the method by preventing any development of oxygen toxicity at 3 ATA. Even though each HBO exposure given at 3 ATA (or 4 ATA) fell within a period normally symptom-free, unprotected animals must have retained some toxic effects of oxygen after return to ambient conditions. This
toxicity evidently accumulated with the repetitive, closely spaced oxygen exposures and finally became sufficient to give overt oxygen toxicity symptoms.

Sanders and co-workers have theorized that succinate protects rats and dogs against the toxic effects of oxygen by maintaining tissue adenosine triphosphate (ATP) concentrations within normal limits (13–15). It was unlikely that succinate would have protected the anaerobe *C. perfringens*, since these bacteria do not possess a cytochrome system which can readily utilize succinate as a substrate for ATP production. In vitro inactivation experiments done by methods previously described (5) demonstrated no in vitro protection by succinate (0.1 mM in Trypticase soy agar pour plates) of *C. perfringens* against inactivation by 100% O₂ at 3 ATA.

Very sick animals with advanced toxicity due to infection sometimes appeared to worsen considerably when given an HBO exposure. This may have been a general response to an added stress or a specific enhancement of oxygen toxicity. However, fewer symptoms of overt oxygen toxicity were observed in animals with either model infection as compared to the uninjected HBO control animals. One possible explanation is that the uninjected animals were more alert and therefore excited and fearful during the HBO exposures, which might have potentiated the development of oxygen toxicity. Also, the infected animals may have been hypoxic, as suggested by Bullen (2), owing to the gas gangrene infection, and thus did not respond to the increased O₂ tensions to the same degree.

The basis for the resistance to control by HBO of infection initiated with clostridia plus CaCl₂ is not understood, but may be related to the known potentiation of the alpha-toxin of *C. perfringens* by calcium ions (11, 12, 18). Reported results of clostridial infections in animals in which CaCl₂ was used have ranged from no improvement to significant reduction of mortality via HBO exposures (3, 6, 9). These differences may have depended on the toxigenic capabilities of each clostridial strain used in relation to the presence of added calcium ions. In the present series with with CaCl₂, the infected mice appeared especially toxic.

The similar reduction in mortality among infected mice given O₂ at 4 ATA compared with 3 ATA indicates that application of HBO at 4 ATA may be practical for clinical use if an oxygen-protective agent is utilized. These experiments suggest the possible use of pressures higher than 3 ATA either to reduce the HBO exposure time necessary to obtain an equivalent dose of O₂ or to increase the dose greatly by using maximal exposure at high pressure. Previous in vitro work has shown that the inactivation of *C. perfringens* in 100% O₂ at 2, 3, 4, and 5 ATA follows a typical dose-response curve; the rate of bacterial inactivation increases as the pressure of O₂ is increased (5). It is logical to assume that the response of clostridial infection to oxygen is also dose-dependent. Although experiments at 3 ATA (6) demonstrated that prompt and intensive (closely spaced) HBO exposures were necessary to halt the spread of this infection, no attempt has been made to ascertain the exact time requirements for each exposure either at 3 or 4 ATA. The oxygen dose requirements for effective therapy are probably somewhat different, depending on variables such as the strain of clostridia involved and, certainly, on the host, the anatomical site, and the extent of infection. Chemical protection from oxygen toxicity would allow greater flexibility in the administration of HBO which may be a valuable asset to the method.

ACKNOWLEDGMENTS

The valuable technical assistance of Ouida Ayers is gratefully acknowledged.

This investigation was supported by Public Health Service grant AI 08226 from the National Institute of Allergy and Infectious Diseases and by contract N00014-67-A-0251-002, NR102-682 from the Office of Naval Research, Department of the Navy.

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