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FINAL REPORT

THE EFFECT OF HYPERBARIC OXYGEN ON MICROORGANISMS *IN VITRO* AND *IN VIVO*

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The Effect of Hyperbaric Oxygen on Microorganisms *In Vitro* and *In Vivo*

OCD Work Unit 2421B

Prepared by

The Department of Medicine, Cornell University Medical College

for

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through

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ANIMAL CARE STATEMENT

This research was conducted in accordance with the "Principles of Laboratory Animal Care" of the National Society for Medical Research.

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There have been reports of the value of hyperbaric oxygen therapy in treatment of infection in man. Boerema and Brummelkamp (Lancet 2:990, 1962), Smith et al. (Lancet 2:756-757, 1962), Brummelkamp et al. (Lancet 1:235-238, 1963), and an editorial (M. World News Sept. 14, 1962, p. 79) have reported cases of gas gangrene and tetanus which have been successfully treated with hyperbaric oxygen. None of these were controlled studies.

The evidence for efficacy of hyperbaric oxygen therapy of infection is sparse and in fact the possibility exists that oxygen therapy may have a deleterious effect in some infections. Furthermore, hyperbaric oxygen has serious toxicities in man. In view of the widespread enthusiasm concerning hyperbaric oxygen therapy, it is essential to obtain adequately controlled laboratory observations on the effect of hyperbaric oxygen on experimental infection before proceeding to widespread clinical trials.

The purpose of this research was to study the effect of oxygen under pressure on microorganisms *in vitro* and to investigate the influence of oxygen therapy on infections in animals.

MATERIALS AND METHODS

Bacteria - One strain each of *Clostridium perfringens*, *Clostridium tetani*, *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis*, *Diplococcus pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes* were studied. Stock cultures were maintained by storing aliquots of an 18-hour culture at -20° C. The liquid culture medium for *D. pneumoniae* was beef heart infusion broth with 5% sheep blood; trypticase soy broth without blood was used for the other bacteria unless otherwise specified. Cultures of *C. perfringens* or *C. tetani* were incubated anaerobically. For each

experiment an aliquot of stock culture was subcultured to broth and incubated for 4 or 24 hours.

The number of D. pneumoniae was determined by serial dilution in trypticase soy broth and streaking 0.1 ml aliquots on the surfaces of trypticase soy agar plates with 5% sheep blood. Numbers of bacteria other than D. pneumoniae were determined by serial dilution in trypticase soy broth and making pour plates with trypticase soy agar. All plates were read after incubation for 48 hours at 37° C. Clostridia were incubated anaerobically.

In vitro experiments - In vitro experiments were performed in Torbal-B.T.L. Anaerobic Jars (Torsion Balance Co., Clifton, N.J.) which are fitted with a pressure gauge and can be pressurized to 15 pounds per square inch (2 atmospheres absolute). Cultures of bacteria in broth in covered petri dishes were incubated in air or oxygen at 15 pounds per square inch absolute (p.s.i.a.) or 30 p.s.i.a. In addition anaerobic bacteria were incubated in nitrogen at 15 or 30 p.s.i.a. These provided respectively environments with oxygen at about 150 mm Hg, 300 mm Hg, 760 mm Hg and 1520 mm Hg and anaerobic environments at atmospheric pressure and twice atmospheric pressure. The broth was only 1.5-2.5 mm deep to allow good diffusion of gas; trypticase soy broth was used for all bacteria except D. pneumoniae for which beef heart infusion broth with 5% sheep blood was used.

Periodically aliquots of culture medium were removed and the numbers of viable microorganisms determined.

In vivo studies - Following infection, half of the mice in each group were exposed to 100 per cent oxygen at 30 or 45 p.s.i.a. in a Vicker's Tank Type Hyperbaric Oxygen Chamber; the other half of each group was not exposed to oxygen and served as controls. With each exposure 10 minutes were allowed for compression and 10 minutes for decompression.

RESULTS

In vitro experiments

Anaerobic bacteria - Exposure of C. perfringens in trypticase soy broth to air or oxygen at 15 pounds per square inch absolute (15 p.s.i.a.) or to oxygen at 30 p.s.i.a. all resulted in a marked bactericidal effect. Four hour cultures were more susceptible to the toxic action of oxygen than 24 hour cultures. Figure 1 demonstrates the results of a typical experiment in which 2 x 10^5 C. perfringens from a 4 hour culture and 8 x 10^5 C. perfringens from a 24 hour culture were incubated anaerobically in nitrogen or exposed to air or oxygen. There was significant multiplication of the microorganisms in the anaerobic cultures. However, there was a marked decrease in the number of microorganisms in the petri dishes exposed to air or oxygen and all were sterile after 24 hours. In this and other experiments exposure to air or oxygen resulted in more rapid sterilization of 4 hour cultures than 24 hour cultures. There was no significant difference. between the rate of sterilization in air at 15 p.s.i.a., oxygen at 15 p.s.i.a., or oxygen at 30 p.s.i.a. C. perfringens multiplied equally well in nitrogen at 15 p.s.i.a. or 30 p.s.i.a.

The rate of inactivation of C. perfringens by oxygen was temperature dependent. As shown in Figure 2 the number of culturable clostridia decreased at a more rapid rate at 37° than at 25° C.

The presence of 10% sheep, human, rabbit or mouse blood inhibited the bactericidal activity of oxygen.

Figure 3 demonstrates an experiment in which 4 or 24 hour cultures of C. perfringens were suspended in trypticase soy broth or trypticase soy broth containing 10% sheep blood. During a 4 hour exposure to air or oxygen at 15 or 30 p.s.i.a. there was a significant decrease in the number of viable

clostridia in all of the cultures in broth without blood. However, in cultures containing blood, there was no significant decrease in number of viable bacteria.

Ten per cent rabbit or mouse muscle suspended in trypticase soy broth also inhibited the bactericidal activity of oxygen. Figure 4 demonstrates an experiment in which a 10 per cent suspension of rabbit muscle in broth markedly inhibited the bactericidal activity of oxygen at 30 p.s.i.a. for C. perfringens.

Exposure of C. perfringens on the surface of agar plates to air or oxygen also resulted in inhibition of growth. Four hour cultures were more susceptible to the toxic activity of oxygen than 24 hour cultures and presence of blood in the agar inhibited the activity of oxygen.

Oxygen was also bactericidal for C. tetani. Exposure of broth cultures to air at atmospheric pressure or oxygen at 15 or 30 p.s.i.a. resulted in a marked bactericidal effect similar to that observed with C. perfringens. Furthermore, 4 hour cultures were more susceptible to the bactericidal action of oxygen than were 24 hour cultures.

Aerobic bacteria. - Four hour and 24 hour broth cultures of strains of E. coli, A. aerogenes, P. aeruginosa, S. typhimurium, P. mirabilis, D. pneumoniae, S. aureus, and Strep. pyogenes were incubated for 24 hours at 37° C in oxygen at 30 p.s.i.a. or air at atmospheric pressure (15 p.s.i.a.). All of these bacteria multiplied in oxygen and air. Multiplication was never more rapid in oxygen than in air. However, the rate of growth and the peak titer achieved was frequently inhibited in oxygen as compared with air. The inhibitory effect of oxygen was inconsistent and minimal with P. mirabilis, A. aerogenes, and E. coli; more consistent with P. aeruginosa and Strep. pyogenes; and consistent and marked with S. typhimurium, D. pneumoniae and

S. aureus. In general, the 24 hour cultures were inhibited by oxygen more often than the 4 hour cultures. Fig. 5 demonstrates typical experiments with E. coli, S. typhimurium, D. pneumoniae, and S. aureus.

In vivo experiments

C. perfringens. - Mice could be protected from death following infection with C. perfringens. The hamstring muscles of mice were crushed with padded hemostats for one minute and 4×10^6 to 3×10^7 C. perfringens mixed with infusorial earth in 0.2 ml. saline solution was injected into the crushed area. Half of the mice were exposed to oxygen at 45 p.s.i.a. for 30 minutes every 12 hours for 3-6 treatments; the rest of the mice were not exposed to oxygen and served as controls. The first exposure to oxygen was started within 90 minutes after injection of C. perfringens. With each oxygen treatment 10 minutes was allowed for compression and 10 minutes for decompression; therefore, total time in the chamber was 50 minutes for each exposure. Death usually occurred within 72 hours after infection and mortality was calculated 14 days following infection. As shown in Table I, the mice exposed to oxygen were significantly protected from mortality ($P < .01$) and 3 exposures to oxygen seemed to be as effective as 6 exposures. Crushing the muscles with or without injection of infusorial earth resulted in no mortality.

In contrast to these studies, exposure to oxygen did not significantly protect mice from death following injection into normal muscle of C. perfringens mixed with infusorial earth, C. perfringens suspended in 5% calcium chloride solution, or C. perfringens alone. Similarly there was no significant protection following injection into crushed muscle of C. perfringens suspended in 5% calcium chloride solution or C. perfringens alone. Table II demonstrates

the results of these experiments in which C. perfringens suspended in 0.2 ml saline solution with or without infusorial earth or 5% calcium chloride solution was injected intramuscularly in normal hamstring muscles of mice or hamstring muscles that had been crushed for one minute. It is clear that oxygen did not protect against mortality. Exposure to the various regimens of oxygen therapy listed in Table II did not result in any deaths in groups of normal mice.

In these experiments, death was related to multiplication of C. perfringens in the muscle. Mice were injected intramuscularly with lethal inocula of C. perfringens with or without 5% calcium chloride. Groups of mice were sacrificed immediately after the injection and 6 hours after injection. The injected leg was resected, skinned and homogenized. Determination of the number of bacteria present revealed at least a 10-fold increase in the titer of clostridia during the 6 hour interval.

In additional experiments C. perfringens or C. tetani with or without calcium chloride solution were injected intramuscularly in mice. Immediately after infection, half of each group was exposed to oxygen at 45 p.s.i.a. for 2 hours followed by 1 hour out of the chamber followed by another 2 hours in oxygen at 45 p.s.i.a. The same procedure was repeated the following day. Exposure to oxygen did not protect any of the groups against mortality as compared with control groups.

Infection with aerobic bacteria. - Mice were injected intravenously with varying inocula sizes of D. pneumoniae, S. aureus or S. typhimurium. In different experiments groups of mice were exposed to oxygen at 30 or 45 p.s.i.a. for 4-8 treatment periods of one half to one and a half hours twice a day starting within 1 hour after infection. In all of the experiments performed there was no difference in rate of mortality between groups exposed to oxygen and control groups.

DISCUSSION AND CONCLUSIONS

These studies indicate that oxygen is effective in killing C. perfringens and C. tetani in vitro. However, oxygen under pressure (at 30 p.s.i.a.) is no more bactericidal than air at atmospheric pressure (15 p.s.i.a.). Furthermore, blood and muscle markedly inhibit the anticlostridial effect of oxygen.

Protection of mice against death from infection with C. perfringens can be demonstrated only if the model is carefully chosen. Oxygen will not protect mice infected by intramuscular injection in normal muscle of C. perfringens, C. perfringens with calcium chloride, or C. perfringens with infusorial earth. Nor was protection observed when mice were infected by intramuscular injection of C. perfringens or C. perfringens with calcium chloride into crushed muscle. Protection by oxygen was observed only when mice were infected by injection of C. perfringens with infusorial earth into crushed muscle.

Oxygen at 30 p.s.i.a. was inhibitory for some aerobic bacteria including strains of S. typhimurium, D. pneumoniae and S. aureus. However, mortality rates were not altered by exposure of mice to oxygen at 45 p.s.i.a. following intravenous injection of S. typhimurium, D. pneumoniae or S. aureus. Whereas no protection was afforded by oxygen therapy, it is also of significance that no increase in mortality from infection occurred in mice exposed to oxygen.

Further investigation is warranted on the effect of oxygen therapy on infection due to C. perfringens. Study of additional strains of C. perfringens in mice and in other species of animals would also be of value. Similarly, as only one strain of each aerobic bacterium was studied in the present investigation, it would be of interest to study additional strains in vitro and in vivo.

TABLE I

Results following injection of C. perfringens and infusorial earth in crushed muscle

Number of exposures to oxygen	Inoculum	Exposed to Oxygen: Mice alive/Mice infected* (%)		Not exposed to Oxygen: Mice alive/Mice infected* (%)	
3	4×10^6	10/11	(91)	4/11	(36)
4	1.5×10^7	21/22	(95)	14/22	(64)
5	1×10^7	11/14	(79)	8/15	(53)
6	7×10^6	11/11	(100)	8/11	(73)
6	2.8×10^7	9/10	(90)	7/10	(70)
	Totals	62/68+	(91)	41/69+	(59)

* Calculated 14 days after infection

+ $P < .01$ by chi square analysis

TABLE II

Results following injection of C. perfringens with or without calcium chloride or infusorial earth in normal or crushed muscle

Procedure			Inoculum of C. perfringens	Number of exposures to oxygen	Exposed to Oxygen		Not exposed to Oxygen	
Muscle crushed	Calcium Chloride	Infusorial earth			Mice alive/Mice infected*	(%)	Mice alive/Mice infected*	(%)
No	No	No	8×10^7	6	24/30	(80)	19/30	(63)
No	No	Yes	6×10^7	4	21/25	(84)	20/24	(83)
No	Yes	No	3×10^7–9×10^7	3-5	24/55	(44)	22/55	(40)
No	Yes	No	2×10^5–8×10^5	3-5	85/135	(63)	87/135	(64)
Yes	Yes	No	3×10^7–9×10^7	3-5	33/50	(66)	30/50	(60)
Yes	Yes	No	8×10^5	4	26/31	(84)	29/31	(94)
Yes	No	No	8×10^7	4	7/11	(64)	6/11	(55)

* Calculated 14 days after infection

Figure 1

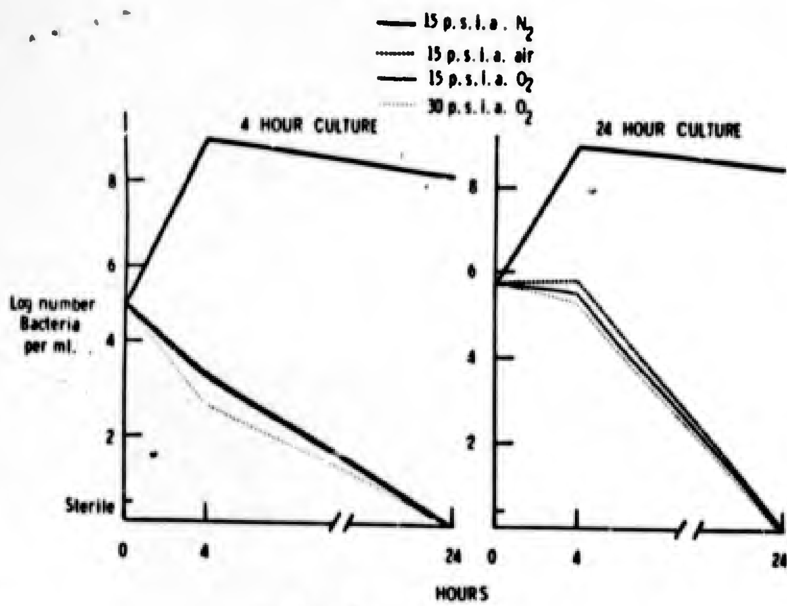

Figure 2

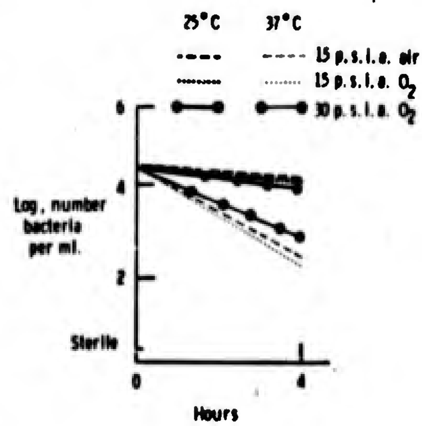

Figure 3

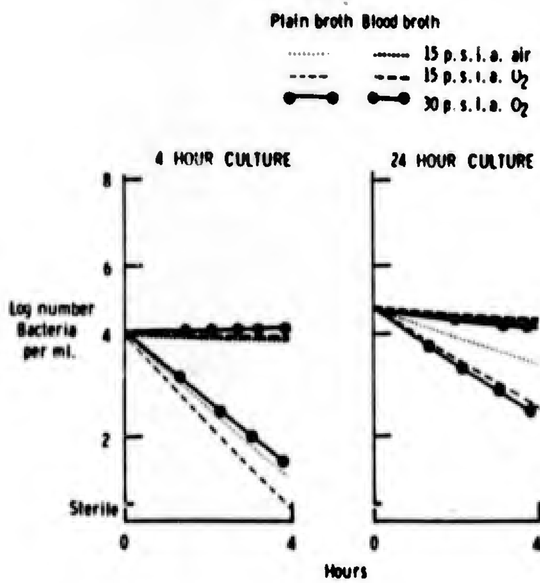

Figure 4

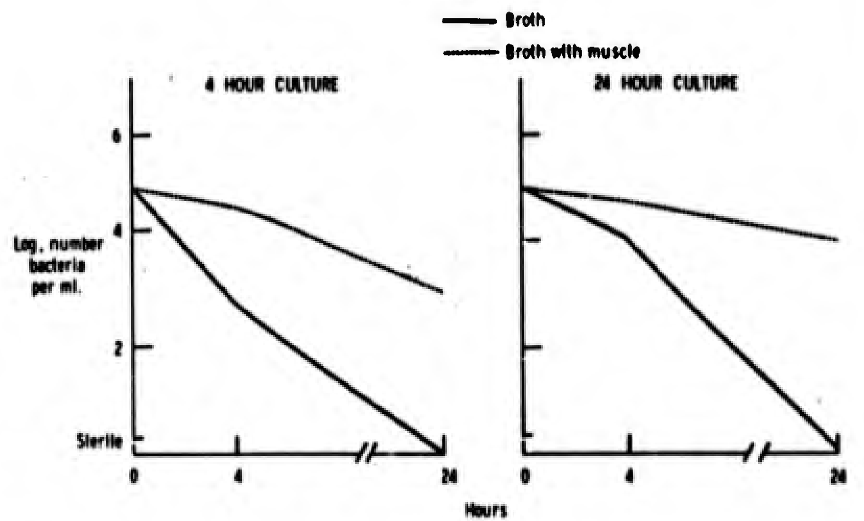

Figure 5