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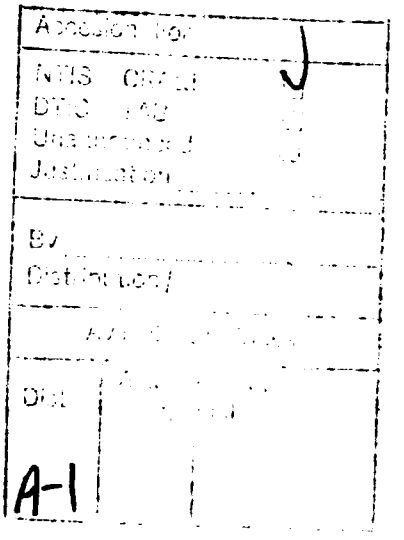
ABSTRACT

13. This study examined the role of oxygen in the development of neuromotor dysfunction generated by temporary aortic occlusion in awake and anesthetized rabbit models of experimental spinal cord ischemia. Animals underwent 30 minutes of infrarenal aortic occlusion, via a pre-implanted aortic snare, followed by 15 minutes of reperfusion prior to treatment with one of three inhaled gas compositions: air (control), 100% oxygen (sea level oxygen), and 100% oxygen at 2.8 atmospheres pressure (hyperbaric oxygen). After the 90 minute treatment and at specific times thereafter, the animals hindlimb motor function was graded on a five point neuromotor index (4 = normal, 0 = total paralysis). Control animals were paralysed after reperfusion but regained hindlimb neuromotor function within six hours after reperfusion and retained substantial hindlimb movement at 24 hours. However, both the sea level oxygen and hyperbaric oxygen groups failed to regain hindlimb neuromotor function within six hour and were totally paralyzed at 24 hours. Histopathological examination of the animals' spinal cord revealed good correlation between spinal cord damage and the clinical neurological outcome. In an alternative anesthetized model, hyperbaric oxygen treatment appeared to temporarily retard the post reperfusion improvement in neuromotor function. This phenomenon was not accompanied by an increase in tissue lipid peroxidation but was accompanied by post treatment hyperemia of the affected spinal cord segments. These data support the notion that the degree of inspired oxygen tension in the immediate reperfusion period may play a role in the development of spinal cord reperfusion injury.



INTRODUCTION

1. Objectives

This study examined the effect of hyperbaric oxygen therapy (HBOT) on the neurological, biochemical and histopathological outcome in a rabbit model of spinal cord ischemia/reperfusion injury. The specific objectives of this study were as follows:

a) to determine whether the application of HBOT following ischemia attenuates or accelerates delayed neurological deterioration.

b) to determine whether the effect of HBOT is dose dependent.

c) to gauge the degree of lipid peroxidation in the post-ischemic period.

d) to examine spinal cord blood flow in the post-ischemic period.

It was the intention of the original protocol to measure the production of prostaglandins in the post-ischemic/reperfused spinal cord by the technique of gas chromatography/mass spectroscopy. This could not be accomplished due to the departure of the investigator assigned to the task and due to the budgetary shut-down of the Institute's mass spectrometer. Instead, methods to gauge the degree of lipid peroxidation, which often accompanies prostaglandin production, were used as stipulated in the appendices.

2. Findings

A cumulative, substantive and comprehensive statement and discussion of research background, rationale, material, methods and scientific significance can be found in the appendices. The major findings of this study are summarized as follows:

1) Following 30 min of infrarenal aortic occlusion, control rabbits (treated with room air) in this study displayed complete paralysis of their hindlimbs and regained substantial hindlimb motor function over the ensuing six hr, only to lose a portion of motor function by 24 hr after reperfusion. In contrast, animals treated shortly after reperfusion with 100% oxygen or 100% oxygen at 2.8 atmospheres pressure failed to regain any significant motor function and were completely paralysed by the 24 hr time point. This finding supports the notion that inspired oxygen tension may be a factor in the development of reperfusion injury in the post-ischemic spinal cord.

2) Histopathological examination of the spinal cord indicates that the degree and location of tissue injury is closely associated with the degree of clinical neuromotor deficit.

3) Interestingly, hyperbaric oxygen treatment did not appear to be associated with extensive lipid peroxidation of the post-ischemic/reperfused rabbit spinal cord as determined by the thiobarbituric acid assay.

4) Animals treated with hyperbaric oxygen appeared to experience a hyperemia of the post-ischemic/reperfused spinal cord shortly after treatment that was not present in control

animals. Blood flow was determined by the radiolabeled microsphere injection technique.

3. Presentations and Publications

6 June 1990 - Submission of Research Paper (Appendix A) and Slide presentation to the Walter Reed Army Institute of Research as a requirement for participation by the principal investigator (C.M.H.) in the Medical Research Fellowship Program, conducted under the auspices of the Department of Medicine, Walter Reed Army Institute of Research.

14 August 1990 - Publication of Abstract (Appendix B) and Slide Presentation delivered before the Joint Meeting on Diving & Hyperbaric Medicine of the Undersea and Hyperbaric Medical Society (UHMS) and the European Undersea Biomedical Society (EUBS) held in Amsterdam, The Netherlands, 12-17 August 1990. Abstract citation: Harrison CM, Mehm WJ, Criswell D., Anderson LH. "Effect of hyperbaric oxygen on neuromotor function after temporary aortic occlusion in the awake rabbit". Undersea Biomedical Research 1990; Suppl.17:66-67.

16 September 1990 - Poster presentation: Plemons TJ, Harrison CM. "Three techniques of temporary aortic occlusion in the rabbit model". Presented at the Annual Meeting Of the National Capital Area Branch, American Association of Laboratory Animal Scientists, Ellicott City, Maryland, 15-16 September 1990.

30 November 1990 - Draft research paper (Appendix C) planned for later submission to the journal Undersea Biomedical Research. Title and authors as stated in the appendix. A second research paper is planned to be submitted to Undersea Biomedical Research under the title "Effect of hyperbaric oxygen on spinal cord lipid peroxidation and blood flow after temporary aortic occlusion in the rabbit".

4. Appendices

Appendix A - This paper was presented at the Walter Reed Army Institute of Research on 6 June 1990 in partial fulfillment of the requirements for participation in the Medical Research Fellowship Program by the principal investigator (C.M.H.).

Appendix B - This abstract accompanied a slide presentation delivered to the UHMS meeting, as mentioned above, in August 1990.

Appendix C - Draft of research paper to be submitted to the journal Undersea Biomedical Research. Please note that the photomicrographs (Figures 6 thru 8) refered to in the "Results" section are in preparation at this time at the Department of Scientific Illustration, Armed Forces Institute of Pathology, and will be submitted at a later time.

Appendix D - These are unpublished data that deal with blood flow to the post-ischemic/reperfused spinal cord and will be included in a second paper, in preparation, to be submitted to Undersea Biomedical Research. These data were derived from

rabbits which underwent anesthetized aortic occlusion for 30 min, were allowed to reperfuse for 30 min and then were treated for 30 min with either air or 100% oxygen at 2.8 atmospheres pressure (this model is described in detail in Appendix A). Spinal cord blood flow measurements were made at three points: prior to occlusion, 30 min after reperfusion, and shortly after the 30 min treatment. The radiolabeled microsphere-injection reference-organ technique was used. This technique is described in detail in Haymann MA and Payne BD, Blood flow measurement with radionuclide-labeled particles. Progress in Cardiovascular Disease Jul/Aug 1977;Vol XX No. 1. These data indicate the immediate post-HBOT period is accompanied by a hyperemia of the post-ischemic/reperfused lumbo-sacral spinal cord that is seen in air-treated control animals.

APPENDIX A

EFFECT OF HYPERBARIC OXYGEN ON THE RECOVERY OF NEUROMOTOR FUNCTION AFTER EXPERIMENTAL SPINAL CORD ISCHEMIA

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Running Head:
HBO and Spinal Cord Ischemia

HBO and Spinal Cord Ischemia

ABSTRACT

This study examines the role of inspired oxygen tension in the development of neuromotor dysfunction generated by temporary aortic occlusion in an experimental model of spinal cord ischemia in the rabbit. Under general anesthesia, animals were implanted with an infrarenal aortic snare and allowed to recover for 24 hours. After recovery, the awake animal underwent a 30 minute period of aortic occlusion followed by a 15 minute period of re-perfusion prior to treatment. Animals were then randomly assigned to one of three treatments: air (control), pure oxygen at 1.0 ATA (sea level oxygen) and pure oxygen at 2.8 ATA (hyperbaric oxygen), conducted contemporaneously in three identical hyperbaric chambers. After a 90 minute exposure and at specific times thereafter, the animal's hindlimb motor function was graded on a five point neuromotor index (0 = total paralysis, 4 = normal) by an individual blind to the animal's chamber atmosphere. All animals had normal hindlimb motor function prior to temporary aortic occlusion. All animals became paraplegic within one minute after occlusion and remained paraplegic after 15 minutes when they were placed in their respective treatment chambers. After six hours of re-perfusion, the control group regained hindlimb motor function to a median score of 3 (hopping), whereas the median scores of both treatment groups were 0 (total paralysis). After 24 hours of re-perfusion, the control group deteriorated to a median score of 2 (moving, not hopping), whereas both treatment groups remained at median scores

of 0. Histopathological examination of the animal's lumbosacral spinal cord revealed that the degree of damage correlated well with the animal's clinical examination. In an alternative model using anesthetized animals, we found that early exposure to hyperbaric oxygen tended to retard the neuromotor recovery of rabbits that underwent aortic occlusion while anesthetized, although we could not demonstrate increases in lipid peroxide formation in the affected cord. These data support the involvement of oxygen in the development of re-perfusion injury and suggest that exposure to an hyperbaric oxygen atmosphere shortly after re-perfusion may amplify the injurious process.

INTRODUCTION

Hyperbaric Oxygen Therapy (HBO) has had mixed results when employed to ameliorate neurological deterioration seen after ischemic insult to the central nervous system (CNS)[1]. The rationale behind the use of HBO assumes that the deterioration seen in the post-ischemic re-perfused CNS can be attributed to a vicious cycle. This cycle is initiated by uncorrected tissue hypoxia that leads to tissue edema and decreased blood flow, which, in turn, exacerbates tissue hypoxia. It is reasoned that intermittent exposure to hyperbaric oxygen tensions is sufficient to restore physiological oxygen tensions in damaged tissue and thereby interrupt the vicious cycle[2]. In recent years; however, evidence

has accumulated to suggest that oxygen metabolism in post-ischemic tissue is fundamentally altered. It now appears that a significant portion of the tissue damage may actually occur during the re-perfusion phase and may be caused by oxygen-derived free radicals[3]. Although it is obvious that restoration of oxygen supply to ischemic tissue is necessary to restore normal function, it must be recognized that oxygen may simultaneously participate in certain deleterious chemical reactions. The evolving concept of "re-perfusion injury" questions the wisdom of immediate exposure of post-ischemic tissue to hyperoxic blood flow and it is reasonable to surmise that indiscriminate use of HBO may amplify, rather than attenuate, CNS injury.

In order to investigate this possibility, we examined the effects of a single hyperbaric oxygen exposure on the biochemical changes and course of clinical recovery seen in the rabbit aortic occlusion re-perfusion model of spinal cord ischemia.

MATERIALS AND METHODS

Male New Zealand White rabbits (2.5-3.5 kg) were randomized in blocks of three in the conscious occlusion model and in pairs in the anesthetized occlusion model.

HBO and Spinal Cord Ischemia

Conscious occlusion model

We employed the model of aortic occlusion-induced spinal cord ischemia in the conscious New Zealand White rabbit originally developed by Zivin et al.[4] which exploits the segmental arterial blood supply of the rabbit spinal cord. Under general anesthesia (Ketamine 50 mg/kg i.m. and Xylazine 10 mg/kg i.m.) an endotracheal tube was placed and the animal was artificially ventilated with air. The animal's abdomen was shaved and prepared with iodine soap. The abdomen was entered through a midline incision and the retroperitoneal space was bluntly dissected to expose the abdominal aorta just distal to the left renal artery. A ligature, which consisted of 3-0 suture threaded through a small button, was looped around the infrarenal aorta and passed through a vinyl guide tube. This guide tube was then sutured to the abdominal wall to close the wound and exteriorize the ligature. The animal was allowed to recover for twenty-four hours. On the day after surgery, the ligature was pulled to occlude the aorta and secured with a small hemostat. Aortic occlusion was confirmed by loss of femoral Doppler signal and by rapid (within one minute) development of complete hindlimb paralysis. Any animal that did not develop complete paralysis within a minute of occlusion was excluded from the study. After thirty minutes the ligature was released and the animal was allowed to re-perfuse for fifteen minutes. Animals were then randomized to one of three hyperbaric chambers and exposed to

one of the following atmospheres: air (control), 1 ATA O_2 (100% oxygen) and 2.8 ATA O_2 (hyperbaric oxygen) for ninety minutes. The clinical recovery of each animal was followed for twenty-four hours after re-perfusion and neuromotor scoring was performed at 2, 4, 6, and 24 hours. Certain animals were selected prior to scoring for extensive histopathological study.

Anesthetized Occlusion Model

Animals were anesthetized as above and the infrarenal aorta was exposed as previously described. In this situation, a small rubber-shod Debakey clamp was used to occlude the aorta while the animal was still anesthetized and artificially ventilated. Occlusion was confirmed by the amelioration of pulsatile flow as monitored by a femoral artery catheter. After a thirty minute occlusion, the clamp was removed and flow restored. The abdomen was closed and the femoral artery catheter was removed. After one hour re-perfusion, each of a pair was scored and then treated with air (control) or 2.8 ATA O_2 (hyperbaric oxygen) for ninety minutes. Shortly after exiting the chamber, the animals were scored again, euthanized, and spinal cords were removed for biochemical analysis.

HBO and Spinal Cord Ischemia

Hyperbaric Oxygen Treatment

Windowed, cylindrical metal chambers measuring 15 X 30 inches comfortably accommodated individual animals. For animals in the hyperbaric treatment group, the chamber pressure was increased at a rate of 1 foot of sea water (fsw)/sec or as the animal tolerated. Animals were observed continuously during pressurization and upon reaching treatment pressure, continuous ventilation was employed to stabilize the temperature and gas composition of the chamber atmosphere. Temperature, pressure and oxygen tension of the chamber atmosphere were measured continuously while carbon dioxide tension was measured by sampling every thirty minutes. After the ninety minute treatment period, chamber pressure was decreased at a rate of 1 fsw/sec. When chamber pressure was equal to room atmospheric pressure, the animal was removed from the chamber. Control animals were placed in identical chambers for the ninety minute period and ventilated with the appropriate gases but were not pressurized.

Neuromotor Assessment

Hindlimb motor function was graded using a five point neuromotor index: 0 = total paralysis, 1 = minimal movement; severe paresis, 2 = functional movement present; cannot hop, 3 = ataxic hopping; supports own weight, 4 = normal. Animals in the conscious

occlusion model were examined at 0.25, 2, 4, 6, and 24 hours after re-perfusion. Animals used in the anesthetized occlusion model required at least a thirty minute recovery from the general anesthetic to render the clinical examination meaningful. Therefore, they were examined 1 and 3 hours after re-perfusion. The examiners were blind to the animals' treatment atmosphere.

Histopathology

After 24 hours of re-perfusion, animals in the conscious occlusion group were selected prior to neuromotor scoring and underwent perfusion-fixation and histopathological examination as follows: After induction of general anesthesia (Ketamine 50 mg/kg i.m. and Xylazine 10 mg/kg i.m.) subphrenic thoracotomy was performed and the heart was dissected free from the pericardium. The left ventricle was punctured and perfused with 500 ml saline followed by at least one liter of 10% formalin solution. Clear drainage from the right atrium indicated adequate perfusion-fixation. Spinal cords were removed, dehydrated in graded alcohols and embedded in paraffin. Beginning at the conus medularis, a 0.5 cm block of tissue was taken at 1.5 cm intervals and 7 micron sections were taken from each block. Sections were stained with hemotoxylin and eosin. The sections were graded by a veterinary pathologist who was unaware of the individual animal's treatment. Grading was based upon: 1) Percent necrotic neurons, 2) Percent vacuolation of

the grey matter, 3) Number of spheroids per high power field, and 4) Number of dilated axon sheaths per high power field.

Biochemical Assessment

Three hours after re-perfusion, spinal cords were harvested from animals occluded under general anesthesia. Spinal cord sections were subjected to thiobarbituric acid assay[5] to gauge the degree of lipid peroxidation in various spinal cord segments.

In this procedure, animals were euthanized via T-61 i.v., immersed in water, removed, and then packed in ice while a 4-5 cm segment of lumbosacral cord and an equivalent length of thoracic cord were removed within 15 min. Meningeal material was dissected free, the tissue weighed, and then homogenized in 2.0 ml ice cold 7% trichloroacetic acid (TCA). The homogenate was reacted with 11.25 mg 2-thiobarbituric acid in a 0.25 N HCl/15% TCA buffer by heating in a boiling water bath for 15 minutes. The mixture was cooled and centrifuged to a clear solution and then read spectrophotometrically at 532 nm against a concurrently generated standard curve.

Data Analysis

We used the Kruskal-Wallis test to analyze differences among the cumulative motor scores from the three awake occlusion groups.

Thereafter, pair-wise comparisons were made using the Student-Newmann-Keuls test. Data from the two anesthetized occlusion groups were compared with the Mann-Whitney U test. Results were considered significant at $p < 0.05$.

RESULTS

Conscious occlusion - motor score

Illustrated in Figure #1 are the results of serial hindlimb neuromotor examination performed on animals subjected to 30 min aortic occlusion and re-perfusion and then treated as air control ($n = 6$), 100% oxygen exposure ($n = 6$) and hyperbaric oxygen exposure ($n = 7$). Considerable animal mortality was encountered due to immediate or delayed aortic disruption attributable to aortic shearing by the ligature guide-tube device. The animals included in the figure; however, met the following conditions: 1) All animals displayed complete hindlimb paralysis by one minute of occlusion (most were paralysed within 30 seconds), 2) No animal displayed evidence of hypovolemic shock during the course of evaluation, and 3) At necropsy, no animal displayed evidence of retroperitoneal hematoma or thrombosis of the aorta or the femoral arteries. Control animals displayed a pattern of improvement followed by loss of function similar to that seen by others who employ this model. There was a significant ($p < 0.05$) difference

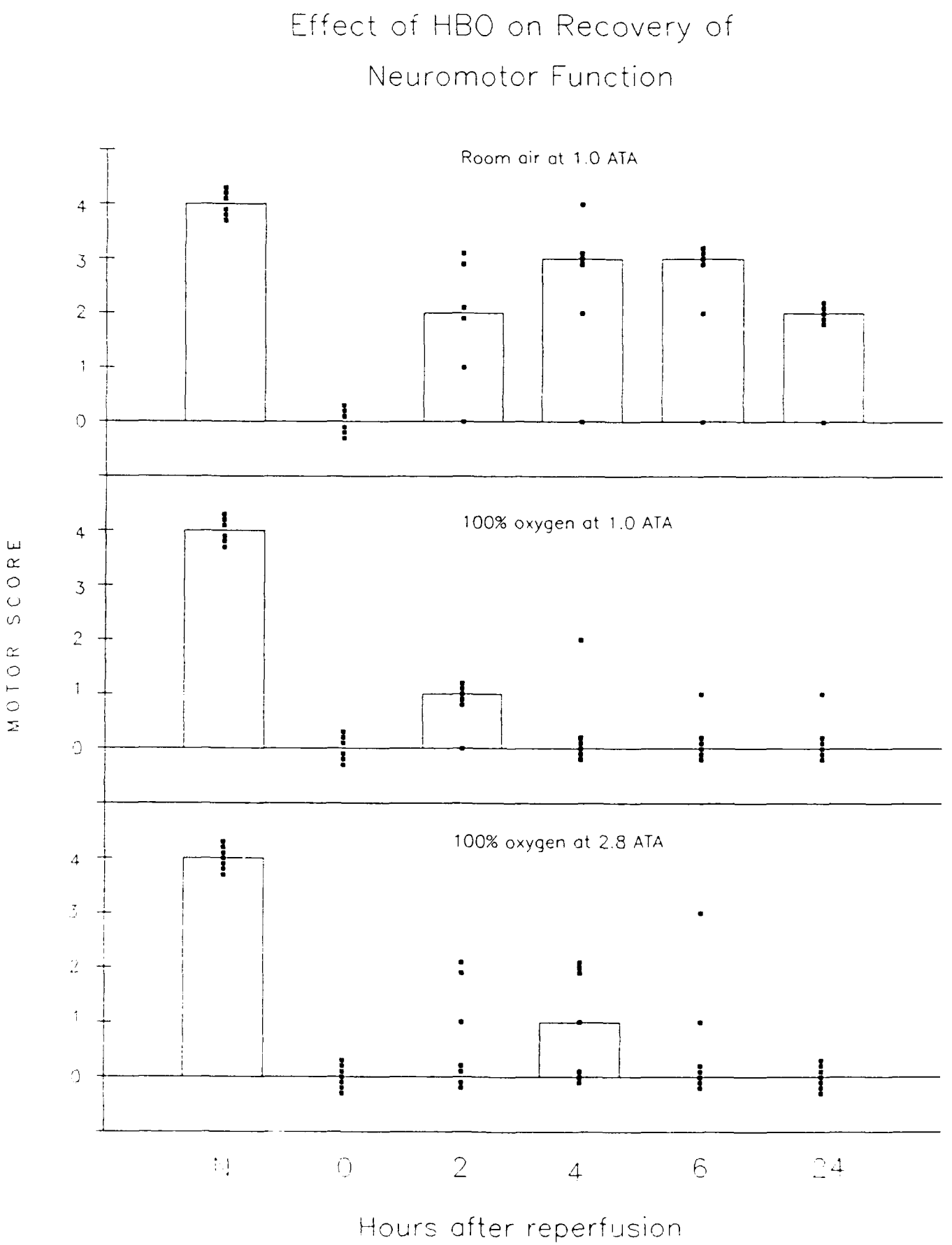
Effect of HBO on Recovery of
Neuromotor Function
Room air at 1.0 ATA
100% oxygen at 1.0 ATA
100% oxygen at 2.8 ATA
MOTOR SCORE
0
1
2
3
4
0
2
4
6
24
Hours after reperfusion

Figure 1

between the cumulative motor score of the control group and the oxygen-treated groups: the recovery of the oxygen-treated groups was not as prompt nor as complete as the control group. The difference between the oxygen treated groups themselves was not statistically significant.

Conscious occlusion - histopathology

Histopathology from three control animals, two animals treated with 100% oxygen and one animal treated with hyperbaric oxygen is summarized in Figure #2. In all cases, there was good correlation among the following variables: the spinal segment in which pathology was found, the severity of the lesion and the animal's clinical course. In all cases, the lesion appeared less severe in the air control group than in the oxygen-treated animals. In one case, a hemispinal lesion was noted both on clinical exam and in the tissue section.

Anesthetized occlusion - neuromotor function

Figure #3 displays results of neuromotor scoring after 30 minute occlusion during general anesthesia (Ketamine 50 mg/kg and Xylazine 10 mg/kg). Scores were taken one and three hours after re-perfusion (before and shortly after chamber treatment). In pilot studies, we observed that all animals that underwent thirty minute

CONSCIOUS OCCLUSION - HISTOPATHOLOGY

SITE	1	2	3	4	5	6	7	8	ANIMAL #
vac grey %	2	10	35	40					43937
neuronal necro %		1	90	90					1 Atm 02
swollen sheaths/HPF		1							
spheroids/HPF		1	1	1					
vac grey %	100	100	100	100	100				48694
neuronal necro %	100	100	100	100	100				1 Atm 02
swollen sheaths/HPF	100	100	100	100	100				
spheroids/HPF	100	100	100	100	100				
vac grey %		10	30	5					47721
neuronal necro %			50	15	2				2.8 Atm O2
swollen sheaths/HPF									
spheroids/HPF									
vac grey %			5	20	5				46455
neuronal necro %			1	5					rm air
swollen sheaths/HPF			1	2	1				
spheroids/HPF			1	1					
vac grey %			50	50					43969
neuronal necro %			50	50					rm air
swollen sheaths/HPF									(unilateral lesion)
spheroids/HPF									
vac grey %									47812
neuronal necro %									rm air
swollen sheaths/HPF				4					
spheroids/HPF				2	1				

Figure 2

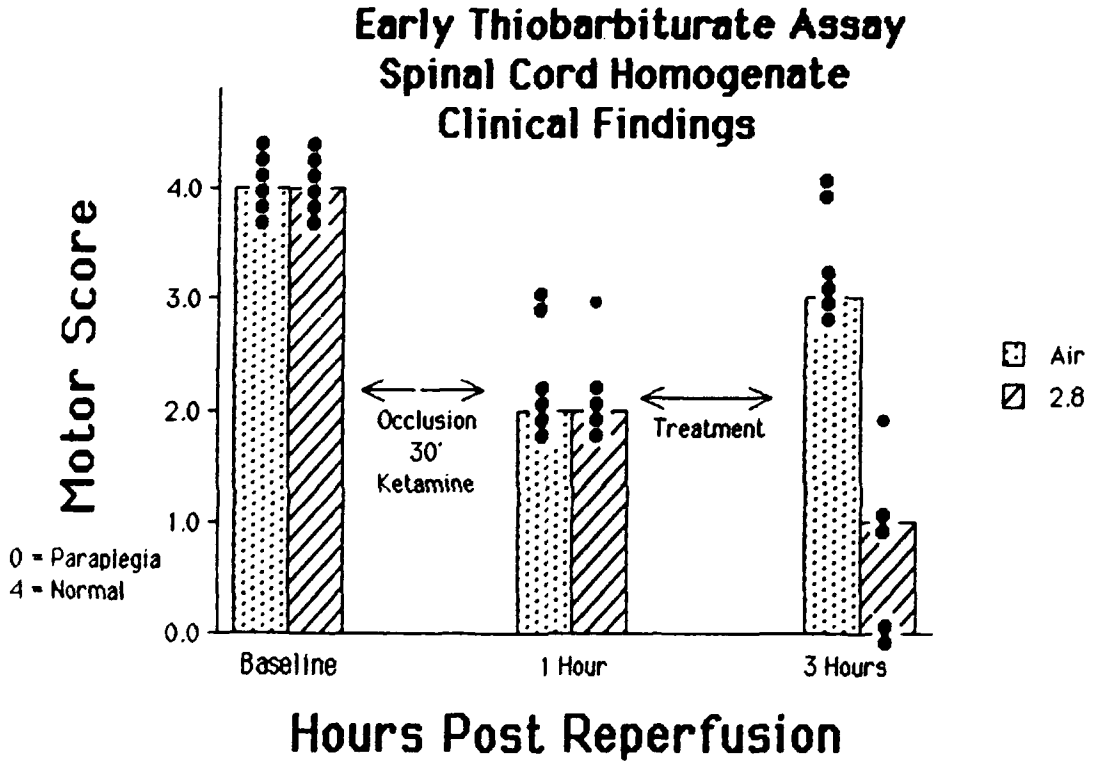
Early Thiobarbiturate Assay
Spinal Cord Homogenate
Clinical Findings
Motor Score
0 = Paraplegia
4 = Normal
4.0
3.0
2.0
1.0
0.0
Occlusion
30'
Ketamine
Treatment
Baseline
1 Hour
3 Hours
Hours Post Reperfusion
Air
2.8

Figure 3

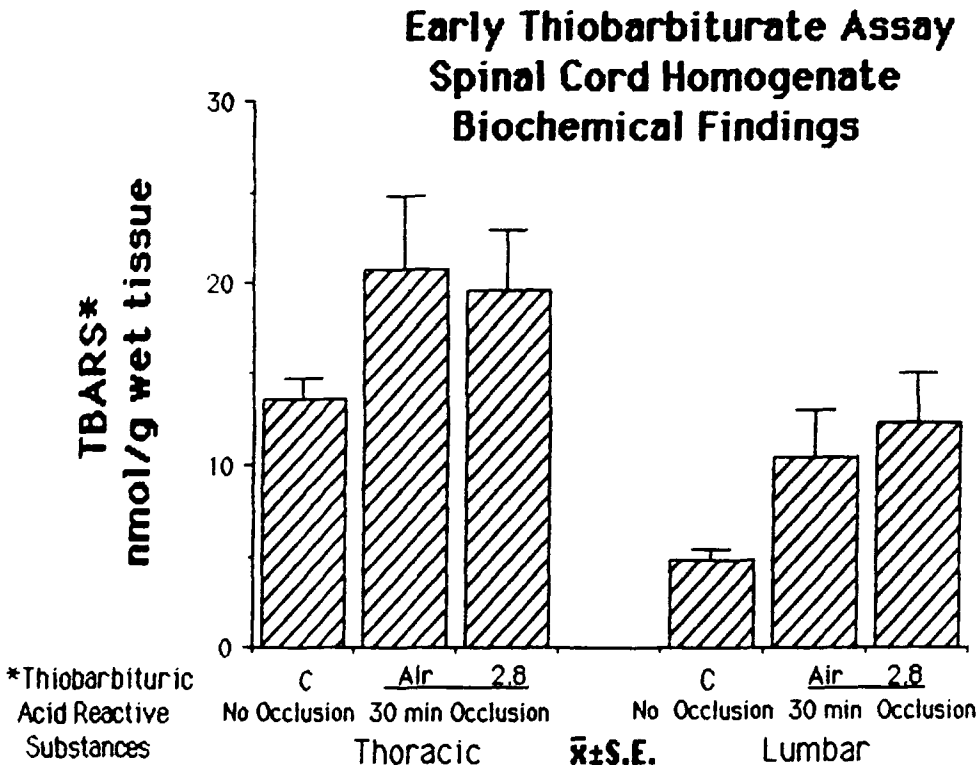
Early Thiobarbiturate Assay
Spinal Cord Homogenate
Biochemical Findings
TBARS*
nmol/g wet tissue
30
20
10
0
*Thiobarbituric
Acid Reactive
Substances
C
Air
2.8
No Occlusion
30 min Occlusion
Thoracic
X̄±S.E.
Lumbar

Figure 4

occlusion under ketamine/xylazine anesthesia regained significant hindlimb function by six hours and had normal hindlimb motor function by twenty-four hours. There was no significant difference in hindlimb motor function between the control ($n = 6$) and hyperbaric oxygen ($n = 5$) groups prior to treatment. However, there was a significant difference ($p < 0.05$) between these groups shortly after treatment.

Anesthetized occlusion - thiobarbituric acid assay

Figure #4 displays the results of thiobarbituric acid assay performed on the lumbosacral and thoracic spinal cords of the animals used in the previous neuromotor evaluation and spinal cords of animals not subjected to aortic occlusion of any kind.
Results are expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) per gram wet tissue. There is no significant difference between air treated and hyperbaric oxygen treated animals in the thoracic or lumbosacral cord. It appears, though, that TBARS are in greater abundance in the thoracic cord across all groups than in the lumbosacral cord across all groups.

DISCUSSION

Animal Model

The awake rabbit model of spinal cord ischemia was introduced

by Zivin et al. to develop a model of reproducible, graded degrees of CNS ischemia that resulted in an objective predictable clinical deficit. With such a model, the underlying mechanisms of neurological deterioration could be investigated and the efficacy of pharmacological interventions could be evaluated. For example, Martinez-Arizala et al.[6] have employed this model to develop a dose response curve for MK-801, a non-competitive glutamate receptor antagonist reputed to have neuro-protective properties. They found that animals treated with a specific dose regained a greater portion of their neuromotor function when compared to saline dosed controls. The use of a pharmacological paradigm to examine hyperbaric oxygen therapy (oxygen as a drug) is especially helpful in that HBO is meaningfully described in terms of dose-response: a quantifiable dose can be classified as ineffective, effective, or toxic.

Oxygen and Re-perfusion Injury

Restoration of adequate tissue perfusion and oxygenation is the principal goal of medical intervention for situations in which tissue has been rendered ischemic. Although prolonged tissue hypoxia is never desirable, simple restoration or amplification of tissue oxygen tension is not the entire answer and may lead to problems of its own. There now exists a substantial body of data supporting the notion that the post-ischemic CNS may be rendered

particularly sensitive to the toxic aspects of high oxygen tension environments[7,8]. Ischemia and hypoxia have been noted by many to deplete CNS tissue of its endogenous antioxidants such as ascorbate, superoxide dismutase and reduced glutathione[9]. In addition to antioxidant depletion, ischemia and hypoxia may cause intracellular conditions which foster the generation of activated oxygen species once physiological oxygen tensions have been restored: iron and other transition metals are dislocated from their protein bound sites and are free to catalyze single electron reduction of oxygen, various enzyme systems known to generate oxygen radical species such as the xanthine oxidase and the cyclo-oxygenase complexes are activated, and lastly, free fatty acids and catecholamines tend to accumulate and form substrates for oxygen radical generation[10,11].

Under a pharmacological model, there exists a dosage above which oxygen is clearly toxic. Indeed, in whole animal models of spinal cord decompression sickness, Leitch and Hallenbeck[12] have demonstrated that no additional recovery of spinal cord evoked potentials is gained with oxygen exposures above 2 ATA O_2. This is consistent with the work of Holbach[13] who reported that after CNS injury, patients improved on hyperbaric regimens of 1.5 ATA O_2 and tended to deteriorate once oxygen exposures climbed above 2.0 ATA O_2. Our data indicate that early intervention with relatively high doses of oxygen hindered the course of neuromotor recovery normally seen with this model. Demonstrable evidence of segmental

spinal cord tissue damage noticed on histopathological evaluation locates this loss of function at the level of the cord itself. The comparable results obtained in animals treated with either 100% oxygen or hyperbaric oxygen indicate that simple oxygen tension alone may not be the sole toxic factor, rather, duration of exposure at a particular tension may play a significant role. These findings are consistent with those of Marsala et al.[14] who used a similar anesthetized rabbit aortic occlusion model to demonstrate that the spinal cord energy charge (concentrations of ATP and phosphocreatine), neuropathology, and clinical outcome were all improved when the animal's early post re-perfusion oxygen exposure was graded, i.e., allowed to progress from a slightly hypoxic to a normoxic inspired gas mixture, in contrast to immediate exposure to hyperoxic inspired concentrations.

Anesthetized Model

An unacceptable rate of animal loss prompted us to look for an alternative model. The ketamine/xylazine anesthetized model offered such an alternative in that our particular anesthetic regimen allowed for minimal hemodynamic interference and fast emergence, which permitted meaningful early neurological evaluation. Curiously, pilot studies demonstrated that in animals so anesthetized and subjected to 30 minute occlusion, the pattern of slow recovery and eventual deterioration was replaced by a

pattern of fast recovery and sustained near-normal hindlimb neuromotor function. This suggests that the anesthetic regimen was neuroprotective. Nevertheless, we were able to demonstrate that a vigorous hyperbaric oxygen treatment one hour after re-perfusion significantly retarded the animals' recovery when compared to the recovery of control animals.

TBA Assay

In seeking evidence for oxygen free-radical damage as an etiology for this occurrence, we could not demonstrate increased levels of lipid peroxides in the most likely affected segment of the spinal cord when homogenates of cord sections were heated in the presence of acid-buffered thiobarbiturate (TBA). The TBA assay we employed, although touted for its simplicity and sensitivity, is frequently criticized due to its lack of specificity (it can react to a variety of cell carbohydrates and proteins as well as lipid peroxides) and due to the number of variables that could confound the results such as the presence or absence of ionic iron, the presence or absence of oxygen during heating, and the antioxidant state of the assayed tissue[15]. Nevertheless, Hall and Braughler[16] were able to demonstrate a four to fivefold increase in TBARS within an hour after spinal cord contusion in the cat. Purely ischemic injury may not be severe enough to generate a lipid peroxide abundance necessary for detection. Alternatively, lipid

peroxidation may be a fleeting phenomenon and cord assay at three hours post re-perfusion may miss the appropriate window. Although we could not demonstrate increased lipid peroxidation after hyperbaric oxygen treatment, we did describe a consistent and significant difference in the concentration of TBARS between the thoracic cord and the lumbosacral cord on a per gram wet tissue basis in all animals assayed. To our knowledge, this has never been reported and we speculate that it may relate to differing lipid constituents or differing antioxidant states normally found in the rabbit spinal cord.

Conclusions and Future Studies

As an alternative to actual tissue damage, the retarded recovery seen after hyperbaric oxygen treatment may represent a hemodynamic perturbation which may render the tissue ischemic a second time. We suspect this change is reversible because preliminary data indicate that recovery-retarded animals can "catch up" to control animals when recovery time is extended to 24 hours. We plan to study the spinal cord blood flow to determine its contribution to retarded recovery.

In conclusion, we find that simple exposure to high oxygen environments early in the course of post-ischemic re-perfusion appears to harm the rabbit spinal ischemia model. We would caution against casual treatment of CNS injury with hyperbaric oxygen and

we would direct greater effort toward restoring adequate antioxidant capabilities to post-ischemic tissues.

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APPENDIX B

Indicate below the numbers and topics of sessions in which your abstract might be programed (see list of suggested topics.)

1st Choice: # 11 Title HBO, Basic Sciences

2nd Choice: # ..... Title .....

PRESENTATION PREFERENCE (scientific sessions only)
Preferred choice (CHECK ONE ONLY)

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1990 JOINT MEETING ON DIVING AND HYPERBARIC MEDICINE

EFFECT OF HYPERBARIC OXYGEN ON NEUROMOTOR FUNCTION AFTER TEMPORARY AORTIC OCCLUSION IN THE AWAKE RABBIT

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This study examines the role of oxygen in the development of neuromotor dysfunction generated by temporary aortic occlusion in an awake rabbit model of spinal cord ischemia. Under general anesthesia, animals were implanted with an infrarenal aortic snare and allowed to recover for 24 hr. After recovery, the awake animal underwent a 30 min period of aortic occlusion followed by a 15 min period of reperfusion prior to treatment. Animals were then randomly assigned to one of three treatments: air (control), pure oxygen at 1.0 ATA (sea level oxygen) and pure oxygen at 2.8 ATA (hyperbaric oxygen), conducted contemporaneously in three identical hyperbaric chambers. After 90 min exposure and at specific times thereafter, the animal's hindlimb motor function was graded on a five point neuromotor index (0 = total paraplegia 4= normal) by an individual blind to the animal's chamber atmosphere. All animals had normal hindlimb function prior to temporary aortic occlusion. All animals became paraplegic within one minute after occlusion and were found to be paraplegic after 15 min of reperfusion when they were placed in their respective chambers. After 6 hr of reperfusion, the control group regained hindlimb motor function to a median score of 3 (hopping), whereas the median scores of both treatment groups were 0 (total paraplegia). After 24 hrs of reperfusion, the control group deteriorated to a median score of 2 (moving, not hopping), whereas both treatment groups remained at median scores of 0. These data support the involvement of oxygen in the development of reperfusion injury and suggest that exposure to a hyperbaric oxygen atmosphere shortly after reperfusion may amplify the injurious process.

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APPENDIX C

EFFECT OF HYPERBARIC OXYGEN ON THE RECOVERY OF NEUROMOTOR FUNCTION AFTER TEMPORARY AORTIC OCCLUSION IN THE AWAKE RABBIT

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Running Head:
HBO and Spinal Cord Ischemia

ABSTRACT

The role of inspired oxygen tension in recovery from neuromotor dysfunction generated by temporary aortic occlusion was examined in an experimental model of spinal cord ischemia in the rabbit. Under general anesthesia, an infrarenal aortic snare was implanted in each animal. Twenty-four hours after surgery, each awake animal underwent a 30 minute period of aortic occlusion followed by a 15 minute period of reperfusion prior to treatment. Animals were then randomly assigned to one of three treatment groups: air (control), 100% oxygen at 101.3 kPa (1.0 ATA, sea level oxygen) and 100% oxygen at 283.7 kPa (2.8 ATA, hyperbaric oxygen). Treatments were conducted contemporaneously in three identical hyperbaric chambers. After the 90 minute treatment and at specific times thereafter, the animal's hindlimb motor function was graded according to a five point neuromotor index (0 = total paralysis, 4 = normal) by an individual blind to the animal's chamber atmosphere. All animals had normal hindlimb motor function prior to temporary aortic occlusion. All animals became paraplegic within one minute after occlusion and remained paraplegic for 15 minutes when they were placed in their respective treatment chambers. After six hours of reperfusion, the control group regained hindlimb motor function to a median score of 3 (hopping), whereas the median scores of both treatment groups were 0 (total paralysis). After 24 hours of reperfusion, the control group deteriorated to a median score of 2 (moving, not hopping), whereas both treatment groups remained at median

scores of 0. Histopathological examination of the animal's lumbosacral spinal cord revealed that the degree of damage correlated well with the animal's clinical examination.

These data support the involvement of oxygen in the development of reperfusion injury and suggest that exposure to high inspired oxygen concentrations shortly after reperfusion may amplfy the injurious process.

INTRODUCTION

Hyperbaric Oxygen Therapy (HBOT) has had mixed results when employed to ameliorate neurological deterioration seen after ischemic insult to the central nervous system (CNS) (1). The rationale behind the use of HBOT assumes that the neurological deterioration seen in the post-ischemic reperfused CNS can be attributed to uncorrected tissue hypoxia. Tissue hypoxia leads to tissue edema and decreased blood flow, which, in turn, exacerbate tissue hypoxia. Intermittent exposure to hyperbaric oxygen tensions is sufficient to restore physiological oxygen tensions in damaged tissue and thereby interrupt the chain of events that exacerbate hypoxia (2). However, in recent years, evidence has accumulated to suggest that oxygen metabolism in post-ischemic tissue is fundamentally altered (3,4,5). It now appears that a significant portion of the tissue damage may actually occur during the reperfusion phase and may be caused by oxygen-derived free radicals (3). Although the restoration of

oxygen supply to ischemic tissue is necessary to restore normal function, oxygen may simultaneously participate in certain deleterious chemical reactions. The evolving concept of reperfusion injury questions the benefit of immediate exposure of post-ischemic tissue to hyperoxic blood flow; indiscriminate use of HBO may amplify, rather than attenuate, CNS injury.

To investigate this possibility , we used the awake rabbit model of spinal cord ischemia induced by temporary aortic occlusion originally introduced by Zivin et al. (6). This model exploits the segmental arterial blood supply and poor collateral circulation of the rabbit spinal cord. A predictible and reproducible ischemic injury results. With such a model, the underlying mechanisms of neurological deterioration could be investigated and the efficacy of pharmacological interventions could be evaluated. Martinez-Arizala et al. (7) have employed this model to develop a dose response curve for MK-801, a non-competitive glutamate receptor antagonist reputed to have neuro-protective properties. Animals treated with a specific dose of MK-801 regained a greater portion of their neuromotor function when compared to saline treated controls.

The use of a pharmacological paradigm to examine hyperbaric oxygen therapy (oxygen regarded as a drug) is especially helpful in that HBOT is meaningfully described in terms of dose-response: a quantifiable dose can be classified as ineffective, effective, or toxic. To this end, we examined the effect of prompt treatment with hyperbaric oxygen on the animal's course of

clinical neuromotor recovery after ischemia and on the subsequent histopathological changes in the animal's spinal cord.

MATERIALS AND METHODS

Animal Model and Preparation

Forty-two male, New Zealand White rabbits (2.5-3.5 kg, Hazelton Laboratories, Denver, PA) were used. All animal work was done in accordance with HHS/NIH Pub. No. 85-23 Guide for the Care and Use of Laboratory Animals. Treatment randomization was accomplished prior to surgical preparation. The animals were housed in individual cages throughout the study, allowed food (Purina Rabbit Chow #5326, Ralston-Purina, Inc.) and water ad libitum, and underwent 12 hr light-dark cycles. Surgical preparation followed the procedure described in Zivin et al. (6). Under general anesthesia (Ketamine (Fort Dobbs Labs, Fort Dobbs, IA) 50 mg/kg i.m. and Xylazine (Mobay Corp., Shawnee, KS) 10 mg/kg i.m.) an endotracheal tube was placed and the animal was mechanically ventilated with air. The animal's abdomen was shaved and prepared with iodine soap. The abdomen was entered through a midline incision and the retroperitoneal space was bluntly dissected to expose the abdominal aorta just distal to the left renal artery. A ligature of our own construction, which consisted of 3-0 suture threaded through a small button, was looped around the infrarenal aorta and passed through a vinyl guide tube as described in Zivin et al. (9). The guide tube was then sutured to the abdominal wall, the ligature exteriorized,

and the wound closed. The animal was allowed to recover from surgery for twenty-four hours. On the day after surgery, the ligature was pulled to a tension sufficient to occlude the aorta and then secured with a small hemostat. Aortic occlusion was confirmed by loss of the femoral Doppler signal and by rapid (within one minute) development of complete hindlimb paralysis. Any animal that did not develop complete paralysis within a minute of occlusion was excluded from the study. After thirty minutes, the ligature was released and the animal was allowed to reperfuse for fifteen minutes. Prior to surgical preparation, the animals were randomly assigned to one of three hyperbaric chambers and after reperfusion were exposed to one of the following atmospheres: air (control), pure oxygen at 101.3 kPa (1 ATA O_2) and pure oxygen at 283.7 kPa (2.8 ATA O_2) for ninety minutes. The clinical recovery of each animal was followed for twenty-four hours after re-perfusion and neuromotor scoring was performed at 2, 4, 6, and 24 hours. The animals included in the study had to meet the following conditions: 1) All animals displayed complete hindlimb paralysis by one minute of occlusion (most were paralyzed within 30 seconds), 2) No animal displayed evidence of hypovolemic shock during the course of evaluation, and 3) At necropsy, no animal displayed evidence of retroperitoneal hematoma or thrombosis of the aorta or the femoral arteries. Animals were selected at random, prior to scoring, for extensive histopathological study.

Hyperbaric Oxygen Treatment

Individual animals were comfortably accommodated in windowed, cylindrical metal chambers of our own construction that measured 38 X 76 cm. For animals in the hyperbaric treatment group, the chamber pressure was increased at a rate of 3.07 kPa/sec (1 foot of sea water (fsw)/sec). Animals were observed continuously during pressurization and upon reaching treatment pressure. Continuous ventilation was employed to stabilize the temperature and gas composition of the chamber atmosphere. Temperature, pressure and oxygen tension of the chamber atmosphere were measured continuously while carbon dioxide tension was measured by sampling chamber gas every thirty minutes. After the ninety minute treatment period, chamber pressure was decreased at a rate of 3.07 kPa/sec (1 fsw/sec). When chamber pressure was equal to room atmospheric pressure, the animal was removed from the chamber. Control animals and animals treated with pure oxygen at 101.3 kPa (1 ATA) were placed in identical chambers for the 90 min period and ventilated with the appropriate gases, but were not pressurized.

Neuromotor Assessment

Hindlimb motor function was graded using a five point neuromotor index: 0 = total paralysis, 1 = minimal movement; severe paresis, 2 = functional movement present; cannot hop, 3 =

ataxic hopping; supports own weight, 4 = normal. Animals were examined at 0.25 hr (15 min after reperfusion, immediately prior to chamber treatment), 2 hr (shortly after chamber treatment), and thereafter at 4, 6, and 24 hr after reperfusion. The examiners were blind to the animals' treatment atmosphere.

Histopathology

After 24 hours of reperfusion, animals selected at random prior to neuromotor scoring underwent perfusion-fixation and histopathological examination as follows: Following induction of general anesthesia (Ketamine 50 mg/kg i.m. and Xylazine 10 mg/kg i.m.) a transphrenic thoracotomy was performed and the heart was dissected free from the pericardium. The left ventricle was punctured and perfused with 500 ml saline followed by at least one liter of neutral-buffered 10% formalin solution. Clear drainage from the right atrium indicated adequate perfusion. Spinal cords were removed and stored in 10% formalin for 14 days, after which, they were dehydrated in graded alcohols and embedded in paraffin. Beginning at the conus medularis, a 0.5 cm block of tissue was taken at 1.5 cm intervals and 7 micron sections were cut from each block. Sections were stained with hematoxylin and eosin. The sections were graded by one of the authors (M.V.S) who was unaware of the individual animal's treatment or clinical course. Grading was based upon: 1) Percent necrotic neurons, 2) Percent vacuolation of the grey matter, 3) Number of spheroids

per high power field, and 4) Number of dilated axon sheaths per high power field.

Data Analysis

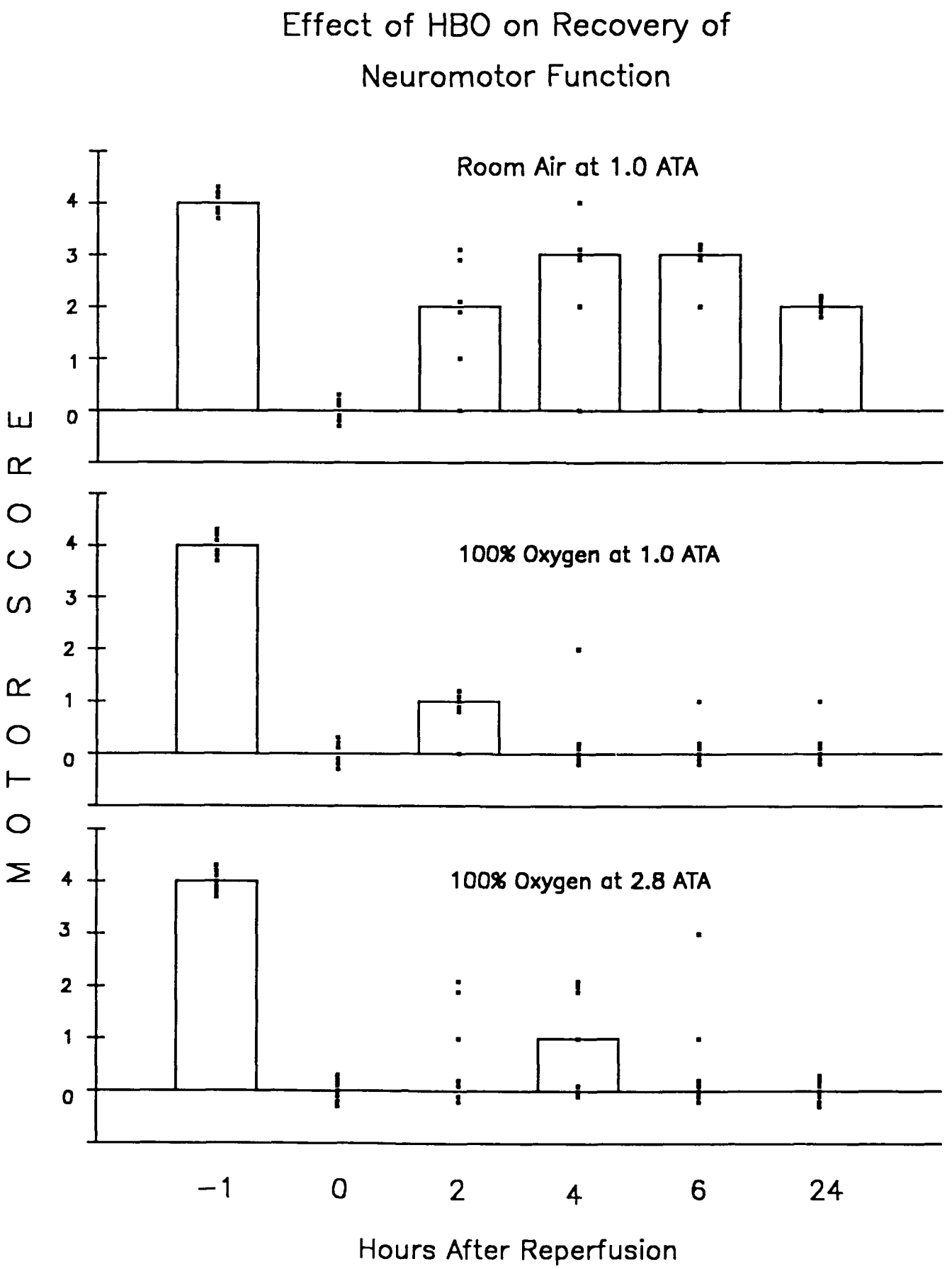
We used the Kruskal-Wallis test to analyze differences among the cumulative motor scores from the three awake-occlusion groups. Thereafter, pair-wise comparisons were made using the Student-Newmann-Keuls test. Results were considered significant at $p < 0.05$.

RESULTS

Motor Score

Figure #1 illustrates the results of serial hindlimb neuromotor examination performed on animals subjected to 30 min aortic occlusion and reperfusion and then treated with air (controls) (n = 6), 100% oxygen (n = 6) and hyperbaric oxygen exposure (n = 7). All animals had normal hindlimb motor function prior to temporary aortic occlusion. All animals became paraplegic within one minute after occlusion and remained paraplegic for 15 minutes when they were placed in their respective treatment chambers. After six hours of reperfusion, the control group regained hindlimb motor function to a median score of 3 (hopping), whereas the median scores of both treatment

FIGURE 1


Effect of HBO on Recovery of
Neuromotor Function
Room Air at 1.0 ATA
100% Oxygen at 1.0 ATA
100% Oxygen at 2.8 ATA
MOTOR SCORE
0
1
2
3
4
−1
0
2
4
6
24
Hours After Reperfusion

groups were 0 (total paralysis). After 24 hours of reperfusion, the control group deteriorated to a median score of 2 (moving, not hopping), whereas both treatment groups remained at median scores of 0. There was a significant ($p < 0.05$) difference between the cumulative motor score of the control group and the oxygen-treated groups: the recovery of the oxygen-treated groups was not as prompt nor as complete as the control group. The difference between the oxygen treated groups themselves was not statistically significant. Control animals displayed a pattern of improvement followed by loss of function similar to that seen by other investigators who have employed this model (7,8). Considerable animal mortality was encountered due to immediate or delayed aortic disruption attributable to aortic shearing by the ligature guide-tube device.

Histopathology

Due to random mortality in each group, spinal cord sections taken from three control animals, two animals treated with 100% oxygen and one animal treated with hyperbaric oxygen were studied. Of this group, data from two animals could not be described meaningfully by our evaluation scheme: one animal, treated with 100% oxygen, showed complete liquifaction necrosis of the lumbosacral cord and and the other animal, treated with air, showed distinctly hemispinal lesions. Data from the remaining four animals are displayed in figures 2 thru 5.

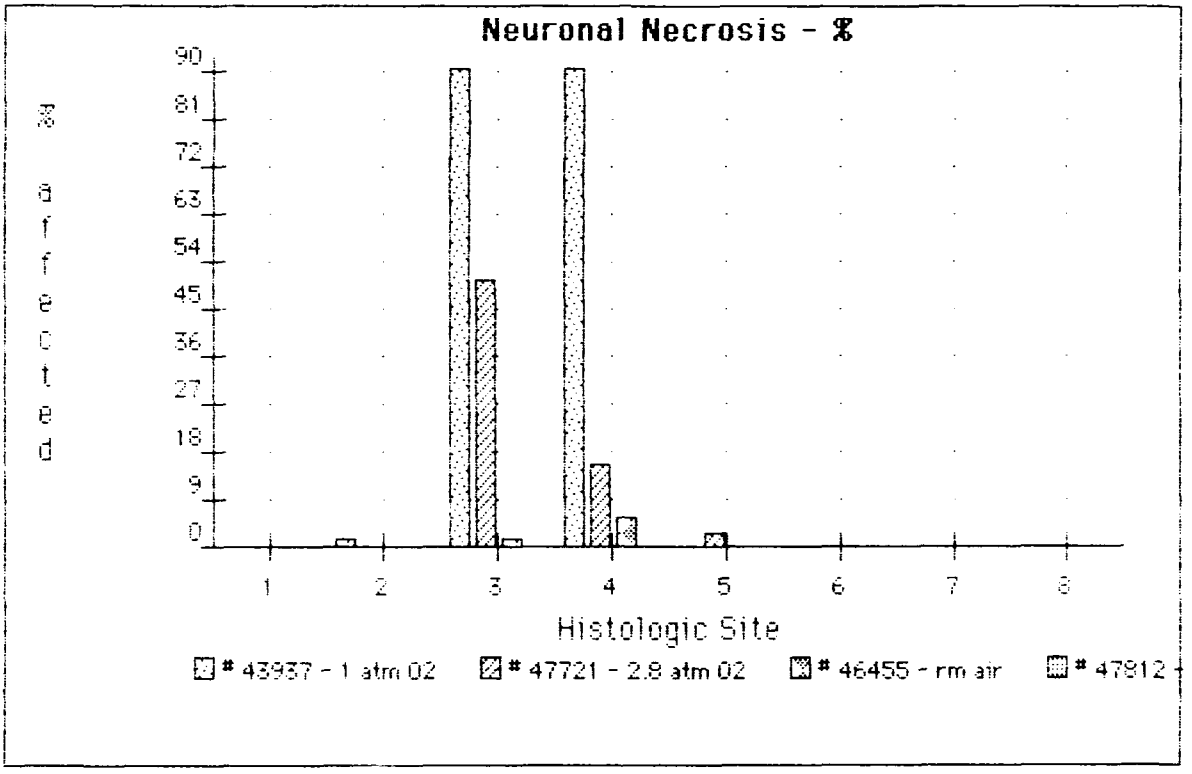


Fig. 2 The percentage of necrotic neurons in the spinal cord grey matter ranged from zero (#47812) to 90% (#43937).

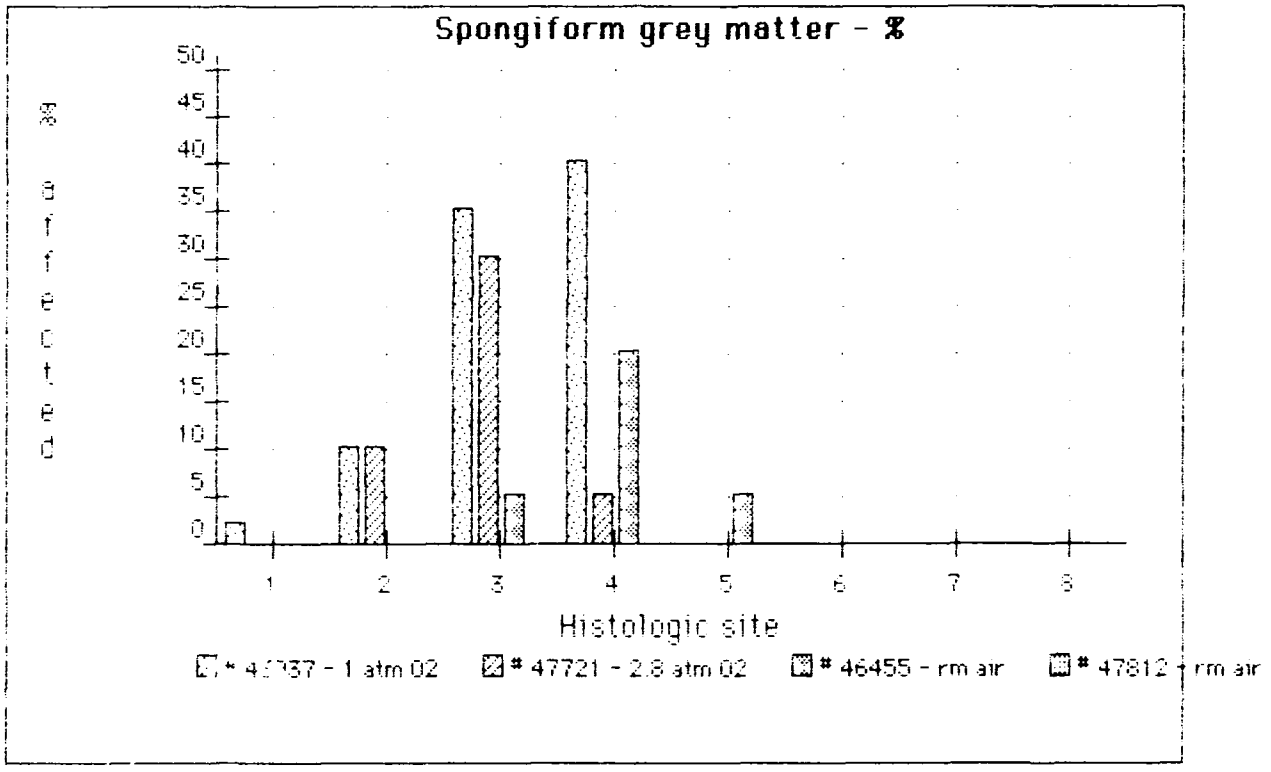


Fig. 3 The percentage of spinal cord grey matter exhibiting sponge form change (vacuolation) is depicted and ranged from zero (#47812) to 40% (#43937) for the four animals depicted. Note the abscence of change in #47812 (room air).

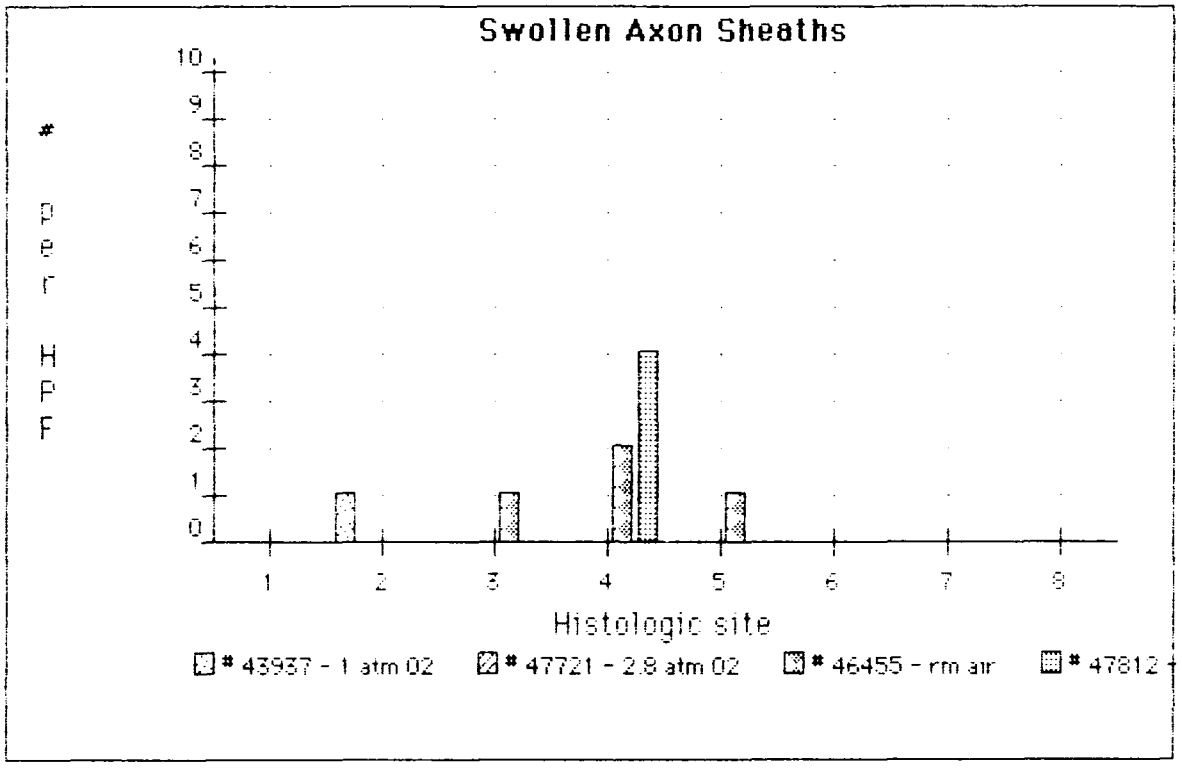


Fig. 4 The number of swollen axon sheaths per high power field ranged from zero to four and roughly paralleled the number of swollen axon in fig. 5.

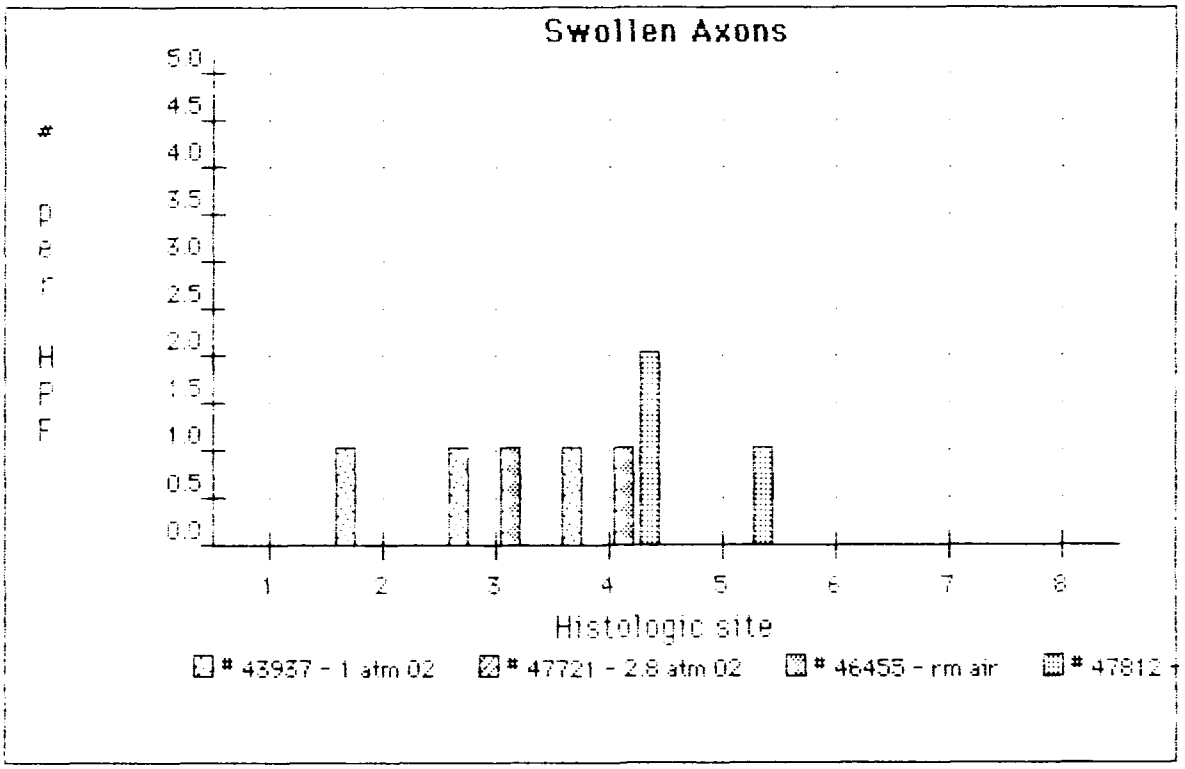


Fig. 5 The number of swollen axon per high power field (HPF) ranged from zero to two for the four animals depicted.

Lesions appeared to begin abruptly at the T12-L1 level (site 6), progressed to maximum severity at the mid to lower lumbar dilation (site 3), and thereafter tapered in severity until little or no damage was noticed at the S4-S5 level (site 1). In all six cases, there was good correlation among the following variables: the spinal segment in which pathology was found, the severity of the lesion and the animal's clinical course. Figure #6 is a hematoxylin and eosin stained section taken from the lumbar dilation of an air treated animal with a motor score of "2" at 24 hrs. post-reperfusion. We note significant loss of staining in the ventral horns, which is consistant with a reduced number of motor neurons compared to normal tissue. This impression is confirmed by Figure #7, the same section viewed under high power. High power reveals the spongiotic degeneration of the neuropil, the necrotic neurons and the swollen axons and axon sheaths that are consistant with early ischemic damage and that have been reported by others who have used this model. Figure #8 is taken from the lumbar dilation of an animal treated with hyperbaric oxygen that was noted to have a motor score of "0" at 24 hrs. post-reperfusion. In addition to the spongiotic degeneration of the ventral horns, we note white matter necrosis and dramatic cavitation of the neuropil. Both findings are consistant with a far more severe ischemic injury. In all cases, the lesion appeared less severe in the air control group than in the oxygen-treated animals. In one case, a hemispinal lesion was noted both on clinical exam and in the tissue section.

DISCUSSION

In this study, we could not demonstrate any benefit to neuromotor recovery offered by prompt treatment with 1.0 and 2.8 ATA inspired oxygen. Moreover, the failure of the experimental groups to match the neuromotor recovery seen in controls, coupled with the greater degree of tissue damage ascertained by light microscopy suggests that high concentrations of inspired oxygen may be deleterious to the post-ischemic spinal cord in the early reperfusion period.

Functional deterioration in the HBO treated post-ischemic CNS is not unprecedented. For example, in whole animal models of spinal cord decompression sickness, Leitch and Hallenbeck (8) have demonstrated that no additional recovery of spinal cord evoked potentials is gained with oxygen exposures above 2 ATA O_2. This is consistent with the work of Holbach (9) who reported that after CNS injury, patients improved on hyperbaric regimens of 1.5 ATA O_2 and tended to deteriorate once oxygen exposures exceeded 2.0 ATA O_2. More recently, Marsala et al. (10) used an anesthetized rabbit aortic-occlusion model, similar to ours, to demonstrate that the spinal cord energy charge (concentrations of ATP and phosphocreatine), neuropathology, and clinical outcome were all improved when the animal's early post reperfusion oxygen exposure was graded, i.e., allowed to progress from a slightly hypoxic to a normoxic inspired gas mixture, in contrast to

immediate exposure to hyperoxic inspired concentrations. Similarly, Cerchiari et al. (11) found that oxygen free-radical scavengers administered during brief anoxic ventilation yielded superior cerebral resusitation when compared to normoxic ventilated controls in a canine cardiac arrest model. Taken together, our data and these reports support the notion that the reperfused CNS may be vulnerable to oxidative stress.

Hyperoxia alone tends to increase the oxidative stress, i.e., the production of reactive oxygen intermediates, in a variety of in vivo and in vitro models (12). Ischemia alters CNS tissue chemistry in a manner would amplify the formation of oxygen radicals during hyperoxia, namely, the conversion of xanthine dehydrogenase to xanthine oxidase in addition to the accumulation of arachidonate in the presence of prostaglandin synthase leads to a "burst" of superoxide production during tissue reperfusion (3,13). Superoxide is thought to promote the release of ferritin bound iron which catalyzes the production of the destructive hydroxyl radical (11,14). It would follow that the oxidative stress placed on the post-ischemic CNS by hyperoxia would be poorly tolerated.

DeGirolami and Zivin (15) noted in the rabbit aortic-occlusion model, that as the duration of occlusion was lengthened, complete hindlimb paralysis was more common and spinal cord injury was more widespread. By comparison, our oxygen-treated animals, although occluded for the same duration as controls, appeared to have a degree of injury consistent with

a longer duration of occlusion. We may infer from our data that high inspired oxygen tensions do not render a pattern of injury specifically attributable to hyperoxia per se. Rather, high inspired oxygen tensions appeared to amplify the natural history of ischemic infarction in this setting. This suggests that the focus of injury in our oxygen treated animals may be vascular in nature.

The notion that hyperoxia might heighten reperfusion injury to the vasculature is consistant with the theory that ischemia-reperfusion is accompanied by oxygen free-radical production in the CNS and that the vasculature is a target for free-radical damage (13,16,17,18). Indeed, the CNS vascular endothelium, rich in the free-radical producing enzyme xanthine oxidase (19) appears to be adversly affected by free radical generation (13,20).

A large number of our animals were excluded due to large vessel vascular damage resulting in delayed large vessel hemorrhage of various degrees. This suggests that macroscopic vascular occlusion, missed at necropsy, might explain much of our results. We would argue that large vessel occlusion would result in areas of infarction randomly distributed among the organs supplied by the abdominal aorta. To the contrary, infarction of the bowel was conspicuously absent and spinal lesions were discrete, segmental and predictable.

In conclusion, we believe our data is consistent with prevailing theories of reperfusion injury and that high inspired

oxygen consentrations, a mainstay of resuscitation, may concomitantly participate in untoward processes at certain stages of recovery from spinal cord ischemia. Presently, we are examining this model for evidence of spinal cord lipid peroxidation and for alterations in spinal cord blood flow.

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APPENDIX D

BLOOD FLOW DATA

	Controls (n=3)		100% oxygen at 2.8 ATA[a] (n=5)	
	Thoracic[b]	Lumbosacral	Thoracic	Lumbosacral
Baseline	11.37 (6.90)[c]	20.17 (7.46)	16.08 (10.74)	29.10 (16.38)
	30 minute aortic occlusion followed by 30 minutes of reperfusion		30 minute aortic occlusion followed by 30 minutes of reperfusion	
Pre-Treatment	15.17 (2.77)	30.51 (12.07)	13.37 (4.75)	55.12 (18.63)
	30 minute of breathing air		30 minute HBO treatment	
Post-Treatment	13.24 (2.26)	28.64 (8.50)	16.09 (4.64)	107.04 (14.02)

a atmospheres absolute

b portion of spinal cord which was sampled

c mean ± standard deviation, expressed as milliliters of blood flow/min/100 g wet tissue