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ORIGINAL ARTICLE

Single Versus Multiple Hyperbaric Sessions for Carbon Monoxide Poisoning in a Murine Model

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Abstract Hyperbaric oxygen (HBO) has been advocated for treatment of acute carbon monoxide (CO) poisoning. There exists considerable debate as to whether HBO prevents delayed neurologic sequelae (DNS) due to CO poisoning. Additionally, existing data in the literature supporting HBO efficacy do not identify an optimal number of HBO treatments. We sought to determine in a mouse model whether there is a difference between one versus multiple HBO sessions for the prevention of DNS. Fifty mice were randomized into five groups of ten mice each: (1) control, receiving no CO exposure or treatment; (2) CO poisoned, receiving no treatment (CO group); (3) CO poisoned, receiving normobaric oxygen for 58 min following the end of exposure (CO + NBO group); (4) CO poisoned, followed by one session of HBO(CO + HBO1); and (5) CO poisoned, followed by three HBO treatment sessions, one every 6 h (CO + HBO3). Prior to poisoning, all animals were trained in step-down latency (SDL) and step-up latency (SUL) tasks. One week after exposure and treatment, all five groups were retested to evaluate the retention of this training. There was no difference detected among groups in SDL (p = 0.67 among all groups) when evaluated using a Kruskal-Wallis test. There was a significant difference among groups in SUL (p = 0.027 among all groups) when evaluated using a Kruskal-Wallis test. When individual groups were compared using a Wilcoxon signed-rank test with Bonferroni correction, there were no statistically significant differences in either SDL or SUL. There was no difference between groups treated with either one or three HBO sessions. One possibility to explain this might be that HBO sessions administered some time after a CO exposure may enhance the lipid peroxidation cascade and worsen neurologic outcomes; alternatively, HBO may simply impart no benefit when compared to NBO.

Keywords Carbon monoxide · Hyperbaric oxygen therapy · Delayed neurologic sequelae · Rodent

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The views expressed herein are those of the authors and do not represent the official position of the US Navy, Department of Defense, or US Government.

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Introduction

Carbon monoxide (CO) poisoning results in approximately 50,000 visits to emergency departments in the USA each year [1]. CO may cause immediate and delayed neurologic sequelae (DNS). Hyperbaric oxygen (HBO) treatment has been advocated to prevent DNS, although human studies comparing normobaric oxygen (NBO) treatment and HBO have had varied results; Hampson et al. reviewed six trials of HBO for CO poisoning, four of which found better clinical outcomes in patients receiving HBO and two of which showed no treatment effect [2]. A study by Scheinkestel et al. suggested that

treatment with HBO may actually worsen neurologic outcomes [3]. A systematic review of the subject by Buckley et al. highlighted methodological shortcomings in many studies examining the subject and demonstrated conflicting evidence as to whether any benefit actually exists with HBO treatment [4].

The most convincing and cited trial in support of HBO treatment for CO poisoning was by Weaver et al., which showed approximately 21 and 15 % absolute reductions in cognitive sequelae at 6 weeks and 12 months, respectively, following a series of three HBO treatments when compared to a single course of normobaric oxygen [5]. In that study, three sessions of HBO were chosen since a prior retrospective study had suggested better outcomes with more than one session [6]. Another small prospective study did not demonstrate a statistically significant difference between two groups receiving a single treatment at either 2.4 atmospheres absolute (ATA) or 3.0 ATA [7]. The superiority of three treatments compared to a single treatment has not been prospectively validated, and the majority of facilities in the USA do not routinely give more than one hyperbaric treatment [8].

Prior animal studies have shown mixed results with HBO treatment. Thom et al. showed a decrease in inflammatory markers in the brain after HBO therapy with improved performance on maze testing [9–13]. Meanwhile, Gilmer et al. showed no benefit from one treatment in terms of cognitive outcome [14], but this study was subsequently criticized for excessive hypoxic insult to the animals, which may have inadvertently obscured a potential benefit to HBO therapy [15]. We sought to investigate whether three sessions of HBO would provide any benefit over a single treatment with HBO in preventing cognitive impairment in a murine model, using a previously established protocol [9, 10, 12, 13].

Materials and Methods

The protocol used for this study was reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC). Male Swiss-Webster mice (Harlan Laboratories, Inc.) weighing 30–35 g were fed a standard diet and water ad libitum. All animals were acclimated to the lab for 1 week in a standard 12:12 h light dark cycle.

Training As a marker of DNS, we sought to determine how well mice retained a task that they learned prior to poisoning. Neurobehavioral function was examined using passive avoidance testing. Prior to CO exposure, all mice underwent one training session of a passive avoidance learning task measuring step-down latency (SDL) and step-up latency (SUL) (Fig. 1). SDL assesses the memory of avoiding a noxious shock stimulus. These two tests can be used to measure the ability of an animal to retain and retrieve a consolidated memory. The training session occurred 24 h prior to CO exposure. The apparatus used consists of a grid floor with a rectangular acrylic glass wall (30 × 30 × 40 cm). At the center of the grid, a 4 × 4 × 4-cm wooden platform was affixed. Electric shock (1 Hz, 500 ms, 35 V DC, 0.3 mA) was delivered to the grid by a manual shock stimulator attached to a grid scrambler (MED Associates, Georgia, VT, USA). During the passive avoidance training, each mouse was placed on the wooden platform in the center of the grid. When the mouse stepped down, placing all four paws on the grid floor, a viewer blinded to the treatment group immediately delivered an electric shock to the grid for 15 continuous seconds, followed by the removal of the mouse. If upon initial introduction to the platform the mouse did not step down within 15 s, it was removed from the study. Previous studies using the passive avoidance retention model have shown that a SDL in this scenario of less than 15 s includes 95 % of the population [14, 16]. Passive avoidance training was repeated at 15-min intervals until either the mouse escaped from the grid floor onto the platform or it failed to step down from the platform within 90 s (SDL). After learning this task, the mice were placed back on the electrical grid and a shock was administered to verify that they again recognized and climbed back onto the block within 10 s (SUL). If the mouse did not climb onto the block within 10 s, it underwent additional training. If both of these criteria were met, the mouse was considered to have learned the tasks. The number of trials required to train each mouse was recorded. This enabled us to ensure that all groups were comparable at baseline in terms of learning ability.

Poisoning Ten animals were not exposed to CO and served as controls. All other animals were exposed to CO in a polyvinyl chloride (PVC) chamber according to a published protocol [9]. Mice were exposed (in batches of ten) to 1000 ppm CO for 40 min, then 3000 ppm for up to 20 min, or until they lost consciousness, whichever came first. Mice were removed individually from the PVC chamber via a small trapdoor when they lost consciousness and were subsequently allowed to breathe room air and regain consciousness. None of the mice died from this exposure regimen. Two additional mice were poisoned using the same method and were sacrificed immediately after poisoning in order to measure carboxyhemoglobin levels.

Treatment After poisoning, mice were randomized to four treatment groups of ten each: group 1 mice were CO poisoned and received no treatment (CO); group 2 mice were CO poisoned, then received normobaric oxygen in a sham HBO session in the hyperbaric chamber for 58 min following the end of exposure (CO + NBO); group 3 mice were CO poisoned, followed by one HBO treatment session (CO + HBO1); and group 4 mice were CO poisoned, followed by three HBO treatment sessions, once every 6 h (CO + HBO3). The initial

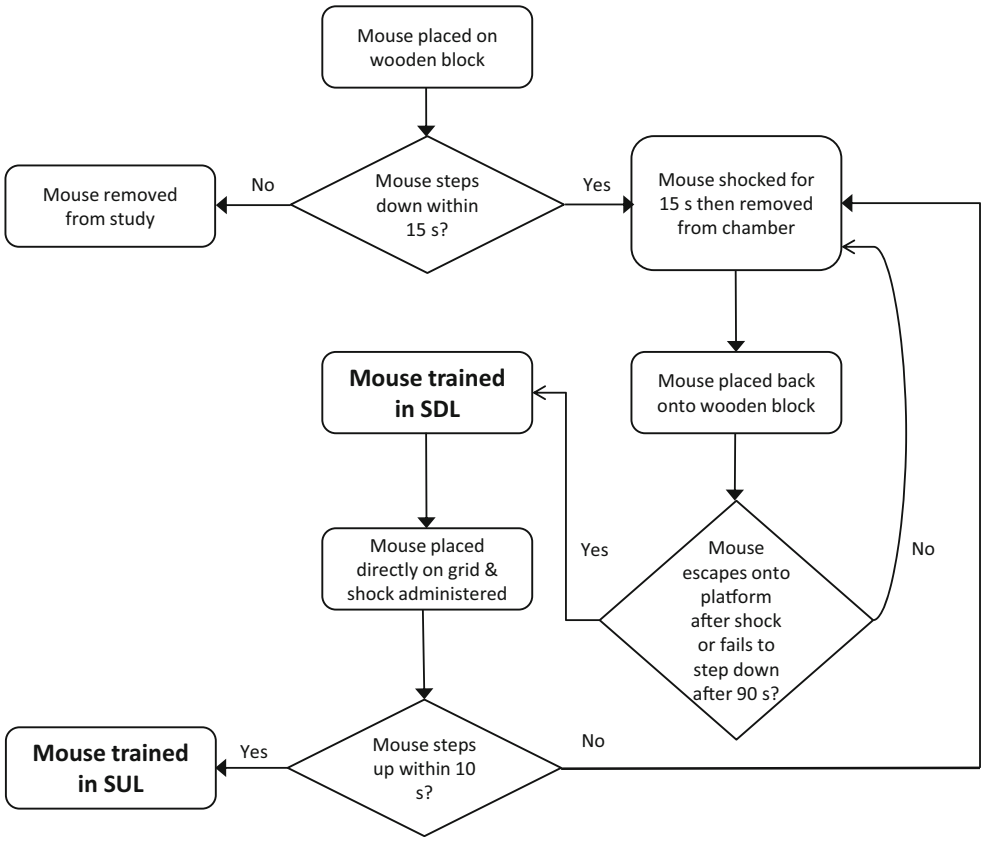

Fig. 1 Flowchart of passive avoidance testing in mice (*SDL* = step-down latency, *SUL* = step-up latency)

treatment sessions of groups 2, 3, and 4 occurred immediately after recovery from the poisoning session. The fifth unpoisoned group was not exposed to CO or treated and served as a control.

Mice in the CO + HBO groups were treated with HBO at 2.8 ATA for 45 min at depth starting 15 min after CO exposure following published methods [10]. Each group had a descent time of 8 min and an ascent of 5 min, for a total surface-to-surface time of 58 min. The CO + NBO group received 100 % O_2 at ambient pressure in the HBO chamber for 58 min. All animals in each group were treated in a single batch.

Outcome Measures One week after CO exposure and treatment, the control and treatment groups were tested to assess retention of the tasks. To assess task retention in the trained mice, testing was carried out in a manner similar to that of training, except that an electric shock was not delivered to the grid floor. Each mouse was placed on the platform, and SDL was recorded with an upper limit of 300 s. Immediately following SDL, SUL was measured by placing the mouse in the right corner of the chamber while applying the same shock until either the mouse climbed onto the block or until 10 s passed. A longer SDL demonstrates that the mouse remembers having received the shock and avoids it by not stepping off the platform, while a shorter SUL demonstrates the mouse's motility as well as its ability to remember that getting up onto the platform allows it to avoid the shock. Our primary outcome measure was SDL (in sec), with SUL (in sec) as a secondary outcome.

Statistical Analysis Our goal was to test the null hypothesis that the mean difference between groups was 0.00. With a two-tailed alpha of 0.05 and a power of 90 %, we calculated that nine animals per group would yield a statistically significant result. One subject per group was added to account for unforeseen events, for a total of ten animals per group.

Each subject's time for SDL and SUL (in sec) was recorded, and results within each group were reported with medians and interquartile ranges. SDL and SUL times among all groups were compared using a Kruskal-Wallis test, with the level of significance defined as $p < 0.05$ (Kruskal-Wallis was used instead of ANOVA because the smaller sample size resulted in non-normally distributed data). Comparisons between individual groups were performed using a Wilcoxon signed-rank test, with a Bonferroni correction used to account for multiple comparisons. For these comparisons, the level of significance was defined as $p < 0.005$ since ten individual comparisons were made. Statistical analysis was performed using the Stata 12.1 software (StataCorp LP, College Station, TX).

Table 1 Step-down latency (SDL) and step-up latency (SUL) in seconds. SDL: $p = 0.67$ among all groups using Kruskal-Wallis analysis. SUL: $p = 0.027$ among all groups using Kruskal-Wallis analysis

	Median	IQR		Median	IQR
Control	151	59–238	Control	4.3	1.9–5.4
CO	97	65–126	CO	7.6	6.4–10
NBO	114	95–198	NBO	9.7	8–10
HBO1	123	19–208	HBO1	6.3	3–10
HBO3	166	36–300	HBO3	10	6.9–10
SDL			SUL		

IQR interquartile range, *CO* carbon monoxide, *NBO* normobaric oxygen, *HBO1* 1 hyperbaric treatment, *HBO3* 3 hyperbaric treatments

Results

Fifty-two mice were trained in SDL and SUL passive avoidance tasks. Fifty of these mice were trained with an average of four sessions each. These mice underwent CO poisoning and were randomized to one of the five treatment groups. The remaining two mice were used for carboxyhemoglobin determination and were sacrificed immediately following CO exposure. All of the animals lost consciousness prior to the end of the 60-min exposure period and all survived the CO exposure. Carboxyhemoglobin concentrations were obtained from the two mice immediately following CO exposure and were 48.0 and 48.3 %.

Each mouse was timed in performing the SDL and SUL tasks; results are listed in Table 1. In the SDL task, there was no significant difference among groups ($p = 0.67$ among all groups) with Kruskal-Wallis analysis. In the SUL task, there was a significant difference among groups ($p = 0.027$ among all groups) with Kruskal-Wallis analysis. However, using the Wilcoxon signed-rank testing with the Bonferroni correction to account for multiple comparisons, no significant difference was noted between individual groups (Table 2).

Discussion

CO remains one of the biggest causes of non-medicinal poisoning-related fatalities in the USA, accounting for more than 400 deaths annually [17]. Those patients that survive poisoning by CO are at risk for developing DNS [18]. The mechanism by which cerebral injury occurs is likely multifactorial. CO causes release of excitatory amino acids such as glutamate, resulting in excessive calcium influx and free radical-mediated cell injury and death [19, 20]. Additionally, CO exposure results in neutrophil activation, which causes production of reactive oxygen species and ultimately leads to lipid peroxidation and CNS demyelination [21].

A number of therapies have been suggested and studied for the prevention of DNS; of these, HBO remains the best-studied. However, the efficacy of HBO for treating DNS is inconclusive. A recent Cochrane systematic review found no significant benefit of HBO therapy, but these results are limited by significant methodologic and statistical heterogeneity [22]. No studies to date have determined the optimal treatment regimen, including chamber depth and number of treatments.

We attempted to determine whether there was a difference in neurologic outcomes in CO-poisoned mice exposed to one or three hyperbaric treatments. In this study, there were no significant differences between HBO1 and HBO3 groups with respect to SUL and SDL. In the SUL task, the CO-poisoned subjects did worse compared to the controls, suggesting that CO exposure resulted in the development of DNS, although this difference was not statistically significant. Neither a single treatment nor three treatments prevented the development of DNS. It is possible that HBO administered once the cascade of events leading to delayed neurologic damage was initiated resulted in additional oxidative stress and damage [23, 24].

There was no difference between controls and CO-poisoned subjects in terms of SDL, again likely due to the wide variation in times between subjects. Previous studies have shown decrements in SDL with CO [14, 16]. However, in the Gilmer study, mice received 50,000 ppm rather than the 3000 ppm used in our study and may have also suffered a hypoxic injury.

Limitations

The main limitation in interpreting our data is the wide variability in measured SUL/SDL between subjects. In future studies, this could be reduced by a combination of reinforcement of passive avoidance training prior to the CO exposure

Table 2 *p* values of comparisons between groups for step-down latency (SDL) and step-up latency (SUL). Using the Bonferroni correction, the level of significance is <0.005

	Control	CO	NBO	HBO1		Control	CO	NBO	HBO1
CO	0.182	–	–	–	CO	0.029	–	–	–
NBO	0.248	0.477	–	–	NBO	0.026	0.321	–	–
HBO1	0.534	0.790	1.000		HBO1	0.182	0.321	0.087	–
HBO3	0.563	0.142	0.182	0.200	HBO3	0.011	0.264	0.241	0.098
SDL					SUL				

CO carbon monoxide, *NBO* normobaric oxygen, *HBO1* 1 hyperbaric treatment, *HBO3* 3 hyperbaric treatments

and increasing the CO exposure. Our study did not show any difference between HBO and NBO; this could mean that our study may have been underpowered to detect differences between one and three treatments of HBO, or it may reflect that there is actually no difference in outcomes between NBO and HBO therapy. Additionally, since we utilized a Kruskal-Wallis test to analyze our data given the small sample size, there was a loss of statistical power and this could have masked a difference between groups. It is also possible that the use of the Bonferroni correction was too conservative when comparing groups.

During the poisoning phase of the experiment, mice were removed individually as they lost consciousness, so it is possible that CO levels were not constant within the chamber. However, we think that the amount of time the trapdoor was open each instance was short enough and the influx of CO into the chamber was constant enough so as not to alter the CO concentration significantly. Finally, it is not always possible to extrapolate the results from animal studies to effects on human beings.

Conclusions

We were unable to detect differences in outcomes between mice exposed to one and three HBO sessions with respect to SUL or SDL. One possibility to explain this might be that HBO sessions administered some time after a CO exposure may enhance the lipid peroxidation cascade and worsen neurologic outcomes; alternatively, HBO may simply impart no benefit when compared to NBO.

Compliance with Ethical Standards

Conflicts of Interest None

Sources of Funding None

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