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**Carbon monoxide attenuates vasospasm and improves neurobehavioral function after subarachnoid hemorrhage.**

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**Abstract**

Subarachnoid hemorrhage (SAH) is a devastating form of hemorrhagic stroke and is a serious medical condition caused by bleeding usually due to a ruptured aneurysm. Oxidative stress and inflammation from hemoglobin and heme released from lysed red blood cells are some postulated causes of vasospasm during SAH, which could lead to delayed cerebral ischemia. At low amounts, carbon monoxide (CO) gas may be neuroprotective through anti-inflammation, anti-cell death, and by restoring normal blood flow. This study focuses on a noninvasive strategy to treat SAH by using CO as therapeutic medical gas. Mice were treated with 250ppm CO or air 2 h after SAH and we monitored various anatomical and functional outcomes over time. CO decreased neurological deficit score (47.4±10.5%), and increased activity (30.0±9.1%) and stereotypic counts (261.5±62.1%) at day 7. In CO-treated SAH mice, lumen area/wall thickness ratio in the middle cerebral artery increased significantly (173.5±19.3%) and tended to increase in the anterior cerebral artery as well (25.5±4.3%) at day 7. This is a first report to demonstrate that CO prevents delayed, SAH-induced neurobehavioral deficits, which suggests that, post-treatment, CO gas or CO donors should be considered a therapy against SAH.

**Keywords:** Carbon monoxide; Heme oxygenase; Hemorrhagic stroke; Medical gas; Neurologic functions; Vasospasm

**Abbreviations:** ACA, Anterior cerebral artery; CO, Carbon monoxide; CV, Cerebral vasospasm; HO1, Heme oxygenase 1; MCA, Middle cerebral artery; NDS, Neurological deficit score; SAH, Subarachnoid hemorrhage.

**1. Introduction**

 Subarachnoid hemorrhage (SAH) is a type of hemorrhagic stroke that involves bleeding in the subarachnoid space because of an aneurysmal rupture [1–3]. This bleeding is a common complication that occurs after an aneurysm. [4–6]. In addition, SAH induces vasospasm, which leads to neuroinflammation, oxidative stress, and neuronal injury; SAH is also considered a major cause of mortality and morbidity [7]. Ongoing neurologic deterioration from vasospasm remains the greatest cause of death and disability after SAH [8,9]. Cerebral vasospasms occur in more than one-half of all patients with SAH, and it is recognized as the main cause of delayed-presentation cerebral ischemia after SAH [10–12], which itself normally happens between 4 and 14 days after SAH. The most severe conditions of delayed-presentation cerebral ischemia are observed between 7 and 9 days after SAH [13]

 Carbon monoxide (CO) can potentially impact pathophysiological conditions such as neuronal death, neuroinflammation, cell metabolism, neuromodulation, and vasomodulation [14–16]. CO is produced endogenously through the enzyme heme oxygenase (HO) as it breaks down heme. Studies have also concluded that most of the endogenously generated CO is HO dependent [17,18]. Other evidence shows that CO in the body is also formed by photo-oxidation, lipid peroxidation, and xenobiotic metabolism [19]. We and others have previously shown that constitutive HO2 and inducible HO1 have a protective role in the brain when the substrate remains within physiological levels [20]. Furthermore, studies have also reported that HO-derived CO, CO-releasing molecules [21,22], and low concentrations of inhaled CO protect against various insults through the activation of anti-inflammatory, anti-cell death, and vasodilatory effects [23,24]. While in most scenarios, the free heme substrate can be the limiting factor for the production of large amounts of CO, our hypothesis is that exogenous CO might provide protection against SAH-induced vasospasm and neurobehavioral deficits. The levels we examined are still safe and can be reached within the environment. We performed an observational study to examine the effect of CO inhalation compared to air delivered at the same flow rate to describe the functional outcome of experimental SAH.

 We report for the first time that CO is neuroprotective in an endoperforation model of SAH. CO has beneficial effects on neurobehavioral function and vasospasm. This study will help us to investigate the neuroprotective mechanisms of CO in SAH and how CO or CO-donors can be used as a potential therapeutic candidate for SAH treatment. When we can understand the etiopathology of SAH, i.e., delayed vasospasm, delayed cell death, and delayed cerebral ischemia, SAH then becomes an appropriate target to evaluate for the potential of CO as a treatment.

**2. Materials and Methods**

*2.1. Animals and induction of SAH by endoperforation*

 We created SAH in male C57BL/6, 8- to 10-week-old mice by endoperforating microvessels along the circle of Willis using a 5–0 nylon monofilament [25]. . Sham control mice were subjected to the same surgical procedure without endoperforation. All postoperative care was given by the experimenter and UF veterinarian technicians. All experiments were performed following the University of Florida Institutional Animal Care and Use Committee recommendations and the ARRIVE guidelines [26]. All personnel performing the surgery, functional assessments, histology, and histological assessments were blinded to the experimental conditions.

*2.2. CO treatment in SAH-induced mice*

 We used one time point for CO exposure that started at day 1 immediately after 2 h of SAH (endoperforation) when mice were exposed to CO (SAH+CO) or medical-grade air (SAH+air). This took place once every day for 7 d. Mice were placed inside a Plexiglas chamber at room temperature and exposed to CO (250ppm) at 1 L/min flow. We adopted the dose of CO and treatment time point from our previous study [27]. The CO level in the chamber was monitored by a Single Gas Analyzer (CO91, Universal Enterprises, Beaverton, OR). Sham control (Sham+air) mice received air for 1 h at the same flow rate as SAH mice. CO at 250ppm was administered daily for 1 h. After 1 h of CO/air exposure, mice were removed from the chamber and placed in their original cages.

 After the surgery, mice were assessed for neurobehavioral functions such as motor function, locomotor activity, and neurological deficit at 24 h and 7 d. After the behavioral assessments, mice were perfused and their brains were harvested and kept at −80oC for further processing. Ten-micrometer brain sections were obtained using a cryostat and were stained with hematoxylin and eosin to analyze vasospasm in the middle and (MCA) anterior cerebral arteries (ACA).

*2.3. Functional assessments*

 We blind tested functional outcomes in wildtype mice after SAH. For open field activity and rotarod testing, each group received pretesting before surgery followed by 24-h and 7-d post-surgery neurobehavioral assessments. All neurobehavioral and functional tasks were done at the same time of the day and mice were given 30 to 45 min in between each task.

*2.3.1. Neurological deficit score (NDS)*

 We used a 24-point scoring system originally introduced by Clark *et al.* [28] and previously reported by us [29,30] to evaluate the neurologic deficits induced by SAH The NDS scoring system includes body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling. Each test score is graded from 0 to 4, thus establishing a maximum deficit of 24 points.

*2.3.2. Open field locomotor activity test*

 This test is a sensitive method used to assess gross and fine locomotor activity, including ambulatory and stereotypic counts. We monitored open field activity with an automated MED Associates (Med Associates, Inc., St. Albans, VT) video tracking interface system following the procedure we have reported previously [29]. Mice were pretested for 3 d before surgery to obtain their baseline activity. Thereafter, these activities were observed 1 and 7 d after SAH. In brief, mice were individually housed in four transparent acrylic cages and their simultaneous activities were recorded for a period of 30 min.

*2.3.3. Rotarod test*

 This test is used for the analysis of motor coordination and sensorimotor function. Mice were pretested for 3 d before surgery to obtain their baseline functions. Thereafter, mice were tested 1 and 7 d after SAH. The pre- and post-surgery rotarod test used a speed ranging from 5 to 30 rpm as we previously described [29,31]. Each session had a 5-min maximum duration, and each group of mice underwent three trials with a 30-min interval. The total time of retention on the rod for each mouse was recorded by Rotamex computer software (Columbus Instrument, Columbus, OH).

*2.4. Body weight*

 We measured body weight to find a change in weight loss after SAH surgery or an effect of CO treatment on weight loss. To monitor such changes, we observed body weight 1 and 7 d after SAH.

*2.5. Mortality*

 Mortality rate is a measure of the number of animals that died during the course of experiment. We observed mortality throughout the experiment up to 7 d after SAH.

*2.6. Hematoxylin and eosin staining and assessment of vasospasm*

 At the terminal end point (7 d after SAH), we anesthetized mice with isoflurane and transcardially perfused them with phosphate-buffered saline and 4% paraformaldehyde. We removed the brain and post-fixed it in 4% paraformaldehyde for 24 h, after which we transferred it to 30% sucrose until it settled down at the bottom of a tube. The brains were later kept in a small plastic container that was filled with optimal cutting temperature compound and then snap frozen. We sliced the brains into 10-μm sections using a cryostat and stained them with hematoxylin and eosin. We took representative digital images of eight consecutive MCA and ACA cross-sections from each animal and then quantified the lumen area/wall thickness ratio and the lumen circumference/wall thickness ratio to assess vasospasm [32,33]. All slides were scanned using an Aperio ScanScope CS and analyzed with Image Scope software (Leica Biosystems, Cincinnati, OH).

*2.7. Statistical analysis*

 To estimate statistical significance, we performed Student’s t-test for unpaired observations between two groups by using GraphPad prism 5. Results are considered statistically significant at *p* < 0.05. All data are presented as mean±SEM.

**3. Results**

*3.1. Mortality rate*

 There was no mortality in the sham group. However, there was 30% mortality in SAH+air group and 37% mortality in the SAH+CO group. This range of mortality was similar to that previously reported by others using the endoperforation model of SAH [25,34].

*3.2. SAH induces vasospasm*

 To study the time-dependent changes in vasospasm, we performed 24-h, and 3- 5-, and 7-d survival studies after SAH. We induced SAH by endoperforation in WT mice that were sacrificed 24 h, and 3, 5, and 7 d after SAH to study vasospasm (Fig. 1). We determined vasospasm by finding the vessel lumen area/wall thickness ratio. Interestingly, we found significant vasospasm by comparing the lumen area/wall thickness ratio in mice brain after SAH at 24 h (313.3±45.7), 3 d (497.0±110.0), 5 d (918.3±118.0), and 7 d (1508.0±180.5) compared to sham (2078.0±133.2). We also separately measured the lumen area and wall thickness to correlate any significant changes in the lumen wall and lumen area, and we expressed the results as the percent difference compared to the sham. The lumen area at 24 h was 25.6±2.9, at 3 d was 38.6±8.0, at 5 d was 64.0±6.1, and at 7 d was 78.12±5.9 compared to 100.0±4.1 sham. Wall thickness also changed: at 24 h, it was 178.6±9.1, at 3 d it was 182.2±10.9, at 5 d it was 167.0±10.8, and at 7 d it was 119.5±6.7 compared to 100.0±3.8 in sham (Fig. 2A-E).

*3.3. Effect of SAH on neurological deficit*

 Neurological deficit is an important neurofunctional outcome after SAH, so we observed them in mice. SAH-induced mice showed significant neurological deficit at 24 h, and on days 3, 5, and 7 compared to sham (Fig. 2F).

*3.4. CO treatment attenuates SAH-induced neurological deficit*

 We observed NDS in the different groups of mice at d 1 and 7 after SAH and found that SAH in mice caused significant neurological deficits (17.0±1.8 vs 9.5±0.6, respectively). However, treatment with 250 ppm CO on d 7 significantly improved NDS (13.3±2.3 vs 6.0±1.0, respectively) but no significant changes were observed between the SAH+air and SAH+CO groups at d 1 (Fig. 3A).

*3.5. CO treatment improves ambulatory distance and stereotypic counts in SAH-induced mice*

 We measuredambulatory distance using an automated open field activity monitor and the a video tracking interface system (Med Associate). We found that SAH led to a significantly smaller distance travelled compared to Sham on d 1. However, there was no significant difference in ambulatory distance and stereotypic counts between the SAH+air (236.2±41.2) and SAH+CO (271.7±39.9) groups. Interestingly, treatment with CO significantly improved animal activity on d 7 (SAH+air, 499.4±37.2 vs SAH+CO, 649.2±45.7) but resulted in a non-significant effect on d 1. In addition, we also found that CO treatment in SAH+CO (2458.0±422.5) significantly improved the stereotypic counts in SAH mice on d 7 compared to SAH+Air (680.0±231.0) (Fig. 3B-3C).

*3.6. Effect of CO treatment on motor function after SAH*

 When mice were subjected to the rotarod test, we found that there was a significant change in motor function (latency to fall from a rotating rotarod) in SAH+air and SAH+CO mice compared to sham+air; however, latency to fall did not improve with CO treatment in SAH mice on d 1 (57.8±15.0) or 7 (92.2±33.7), although a trend of lower latency to fall was observed in SAH+CO at d 7 (Fig. 3D).

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*3.7. CO treatment attenuates SAH-induced vasospasm*

 We induced SAH by endoperforation along the circle of Willis in wildtype mice to study vasospasm. After 2 h of SAH, we exposed mice to 250ppm CO or air followed by a single exposure to CO or air for 1 h daily for 7 d. Interestingly, we found significantly improved lumen/wall ratio and in the ratio at 7 d in the SAH+CO group compared to the SAH+air group. CO significantly decreased vasospasm as noted by a higher lumen area/wall thickness ratio in the right MCA in SAH+CO-treated group (944.8±66.7) to SAH+air--treated (345.4±55.3) group on d 7. We observed the trend toward a higher lumen area/wall thickness ratio in the right ACA in the SAH+CO-treated group (2073.0±117.5) compared to SAH+air--treated group (1918.0±246.8) on d 7. We also separately measured the lumen area and wall thickness to correlate any significant changes in the lumen wall and lumen area and we expressed the results as the percent difference compared to the SAH+air group. We found a significantly higher lumen area in the SAH+CO-treated group (139.7±8.4) compared to the SAH+air-treated group (100.0±15.8) and lower wall thickness in the SAH+CO (50.9±1.6) group compared to SAH+air-treated mice (100.0±4.0) in the MCA region. Additionally, in the ACA, we also found a significant change in the lumen area in the SAH+air group (100.0±15.8) compared to the SAH+CO-treated group (189.7±8.4). There was also a significant decrease in wall thickness in the SAH+air group (100.0±3.7) compared to the SAH+CO-treated group (68.7±2.4). We also analyzed vasospasm by measuring the lumen circumference/wall thickness ratio. CO significantly decreased the vasospasm as noted by a higher lumen circumference/wall thickness ratio in the right MCA on d 7 (SAH+air, 8.3±1.9 vs SAH+CO, 18.7±2.4) and in the right ACA on d 7 (SAH+air, 15.4±2.3 vs SAH+CO, 20.7±1.2). We also measured the lumen circumference and expressed the results as the percent difference compared with the SAH+air mice. We found a significantly higher lumen circumference (100.0±18.9 vs 166.7±10.0) in the MCA region in the SAH+CO mice compared to SAH+air mice. However, we found no significant change in lumen circumference (100.0±11.2 vs 91.7±5.0) in the ACA region (Fig. 4A-F).

**4. Discussion**

 SAH is caused by bleeding in the subarachnoid space of the brain and is considered the most life-threatening type of stroke due to its high mortality and morbidity [7,35]. Low doses of exogenous and endogenous physiological concentrations of CO have shown protection against various types of stroke and hypoxia [27,36,37] but have not yet been tested in an endoperforation model of SAH as we have done here. Reports have established that neurobehavioral alterations and vasospasm are serious complications after SAH [13,38,39]. The endogenous bioactive gas CO is produced all over the body, including the brain, by HO enzymes in response to various stresses. There is still active debate regarding where the free heme pool would be accessible to produce – when needed – sufficient molar concentrations of CO that would match its exogenous-associated beneficial effects. However, determining how the exogenous supply of relatively low levels of CO improved neurological function and vasospasm after SAH is still a goal. We should state that such amount keep levels of carboxyhemoglobin within the safe range.

 Several experimental models are used to recapitulate the SAH pathology, and here, we used the endoperforation model of SAH as it mimics some clinical features of SAH [40]. However, the amount of bleeding after SAH may be challenging to control; consequently, the magnitude of vasospasm is proportional to the amount of SAH. The effects of CO after SAH are not well explained or well-described in mouse preclinical endoperforation SAH models, although they are reported in a blood injection model [41,42]. Cerebral vasospasm is considered an important cause of morbidity in patients after SAH, when bleeding results in the generation of various cells, bioactive molecules, and toxins in the nervous system. Therefore, we diligently looked at the time-dependent effects of SAH on vasospasm in mice by analyzing blood vessel lumen area and thickness. We first investigated vasospasm in mice at different time points and found that vasospasm was consistent after 24 h and up to 7 days. We also found significant neurological deficits at day 7 compared to sham controls.

We designed this study to characterize the time-dependent changes in vasospasm in SAH-induced by endoperforation of the circle of Willis. We found a significant increase in vasospasm at 24 h, and 3, 5, and 7 d as evaluated by lumen area/wall thickness ratio using Image J software to assess the lumen area and wall thickness, which we then used to measure vasospasm. This method would correct any potential issues that came from vessel deformity. We then evaluated the effect of CO treatment on SAH-induced vasospasm, neurological deficit, ambulatory distance, stereotypic counts, and motor function and found that there were significant benefits offered by CO in SAH-delayed vasospasm/vasoconstriction. In additional, CO improved neurological deficit and ambulatory function. Although CO has been shown to have benefits in other acute brain injury protocols, we are the first to document that CO prevents SAH-induced neurological deficit, animal activity (by showing the ambulatory distance travelled), stereotypic counts, and motor function.

We then tested the effect of CO on vasospasm and neurological function. We choose the 7-d time point because maximum vasospasm is observed at day 7 in clinical settings [13]. In a separate cohort, after the confirmation of the SAH time point, we investigated the effect of CO on SAH-induced vasospasm and neurological function by using three neurobehavioral tests with a high sensitivity for detecting deficits after SAH. Our laboratory and others have used the NDS successfully in ischemic and hemorrhagic stroke pathology [28,29,43]. The present study demonstrated that there was significant impairment in motor function after SAH. In additional, we also monitored animal activity by measuring ambulatory distance and stereotypic counts. We found that SAH causes a significant loss in ambulatory distance and stereotypic counts. Interestingly, CO treatment significantly improves motor function and animal activity.

 Cerebral vasospasm after aneurysmal SAH is a well-established phenomenon that is caused by narrowing of large and medium intracranial arteries [44]. Clinically, vasopspsm affects the anterior circulation that is supplied by the internal carotid arteries, and vasospasm after SAH causes delayed cerebral ischemia, which continues to be a major complication and source of morbidity in cases of aneurysmal SAH [45,46]. Furthermore, we have demonstrated the effect of CO on vasospasm by estimating lumen area, lumen circumference, wall thickness, and the ratio of lumen area to wall thickness and lumen circumference to wall thickness to confirm the degree of vasospasm. Although lumen circumference to wall thickness ratio is one way to show vasospasm, we have also measured vasospasm by a lumen area to wall thickness ratio that provides an accurate measure of vasospasm and would correct any potential error due to vessel deformity [47]. Interestingly, we found that a 250ppm CO treatment significantly improved vasospasm in mice by increasing the lumen area to wall thickness ratio and lumen circumference to wall thickness ratio in the MCA region. Although there was no significant difference in the lumen area to wall thickness ratio in the ACA region, there was a significant difference in lumen circumference to wall thickness ratio in the MCA region. Apart from calculating the ratio to confirm the significant difference in lumen area and lumen circumference from the SAH+Air- to the SAH+CO-treated group, we also estimated value individually and found that there was a significantly increased lumen area and lumen circumference, and decreased wall thickness in the MCA region of CO-treated mice compared to treated SAH mice. However, there was no significant difference observed in lumen circumference between air-treated SAH and CO-treated SAH mice, although there was significant reduction in wall thickness and lumen area in the ACA region in CO-treated SAH mice. These data indicate the potential direct or indirect vasoprotective mechanism of CO on SAH-induced vasospasm. Further work is ongoing to pinpoint the cascade of events leading to such outcomes.

 In conclusion, using this endoperforation model, we demonstrated that SAH induces vasospasm, neurological deficit, motor function, and ambulatory activity. Interestingly, intermittent treatment with 250ppm CO daily 7 d significantly protects mice from SAH-induced vasospasm and neurobehavioral function. These data establish the therapeutic potential of CO treatment to limit vasospasm, functional deficit, and brain damage; additional studies are needed to unravel the mechanism of CO-mediated neuroprotection. Considering the particular etiopathology of SAH, we think it is theclinical target of choice to test the optimal therapeutic delivery of CO.

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**Conflict of Interest**

 The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Figure legends**

**Fig. 1.** Schematic representation of experiment procedure. In this figure we have shown that we have started CO (250ppm) treatment after 2 h of SAH and we monitored behavioral function at 24 h and at day 7.

**Fig. 2.** Time dependent changes in vasospasm after SAH. There was significant less lumen area/wall thickness ratio at 24 h, 3 d , 5 d, and 7 d in comparison to sham. There was also less lumen area/wall thickness ratio at 7 d but not significant. However, there was significant reduction in area at day 7 of SAH in comparison to sham control (n=3-4). NDS Score: There was a significant increase in NDS score at 24 h, 3 d, 5 d and 7 d as compared to sham after SAH. (n=5-6). Data presented as mean±SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 as compared with sham.

**Fig. 3.** Demonstration of effect of CO on deficit in neurological deficit score (NDS), in male C57BL/6 mice after SAH. There was significant increased NDS at day 1 and day 7 in SAH induced mice and SAH+CO treated mice as compared to sham, whereas treatment with CO (250ppm) significantly decreases the NDS score in SAH mice at day 7. However no significant difference observed in NDS between SAH and SAH+CO treated group at day 1 (n=5-6). Fig. 3B-3C: Effect of CO on open field activity in mice after SAH. 3A. in open field activity, a significant decrease was found in activity in a SAH and SAH+CO treated group in comparison to sham at day 1 and day 7. However, CO treatment significantly improves the activity at day 7 but no difference observed at day 1. Additionally, there was significant decrease in stereotypic counts in SAH mice at day 1 and day 7, however, CO treatment significantly improve the stereotypic counts at day 1 and day 7 (3C), (n=4-5).Fig. 3D: Effect of CO on motor function and body weight. Significant motor function (latency of fall from rod) was found in the SAH and SAH+CO treated groups in comparison to sham. However, no significantly change in motor function was observed in CO treated mice as compared to SAH at either day 1 or day 7 (n=5-6). A significant decrease in body weight was observed in SAH and SAH+CO treated group in comparison to sham at day 1 and day 7 (n=5-6). Data presented as mean±SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 as compared with sham; #p<0.05 compare to sham vs SAH+air; and SAH+air vs SAH+CO at day 7 in fig. 3B; ##p<0.01 compare to sham at day 1 and compare to SAH+air at day 7 in fig. 3C; ###p<0.001 as compared to sham at day 7 in fig. 3A.

**Fig. 4.** Effect of CO on vasospasm. There was significant improved area/wall thickness in MCA region; and lumen circumference/wall thickness ratio in MCA and ACA at day 7 in CO (250ppm) treated mice in comparison to SAH+air. There was also increased in percentage difference of lumen area and lumen circumference and decreased wall thickness by CO treatment in SAH mice at day 7 in MCA region. However, no percent difference observed in lumen area/wall thickness observed in ACA region though wall thickness was significantly reduced and lumen area was significantly increased by CO treatment as compare to SAH at day 7. (n=4-6). Data presented as mean±SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 as compared with sham

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