

Isoflurane Preserves Spatial Working Memory in Adult Mice After Moderate Hypoxia

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Perioperative hypoxia may contribute to postoperative cognitive impairment. It is unknown, however, whether anesthetics exacerbate or protect against hypoxia-related central nervous system impairment. We sought to determine whether hypoxia alone or in combination with isoflurane disrupts working memory in mice. To this extent, we assigned adult mice to one of four treatments for 1 h: oxygen 21%, oxygen 21% + isoflurane 1.2%, oxygen 8%, or oxygen 8% + isoflurane 1.2%. Mice breathed spontaneously throughout the experiment. Body temperature was maintained at 37°C + 0.5°C. Mice were allowed to recover for 24 h to avoid the confounding influence of residual anesthetics on neurobehavioral performance. Working memory was assessed by use of a Y maze modified for mice. For the training trial, entry to one arm was blocked and mice were permitted to run between the two open arms for 15 min and inspect the objects outside. For the test trial, carried out 1 h later, all arms were open. Time spent in

each arm was automatically recorded by a camera and associated software. Mice were tested 1, 4, and 7 days after anesthesia. A different arm was used as the novel arm for each test. Performance was analyzed with repeated-measurements analysis of variance, followed by analysis of simple main effects and by *post hoc* comparison using Newman-Keuls test when appropriate. *P* values <0.05 were considered significant. Animals subjected to hypoxia (8% oxygen for 1 h) spent significantly less time in the novel arm 1 day after the insult. The impairment, however, was transient. Hypoxic mice performance improved to the level of the control animals on the fourth post-treatment day. Mice subjected to hypoxia plus isoflurane exhibited no impairment and were comparable to the control group at all time points. Hypoxia transiently impairs performance in a spatial memory task. It appears that isoflurane protects against this deleterious effect of hypoxia.

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Perioperative hypoxemic episodes resulting from altered pulmonary mechanics, drug-induced physiological changes, or technical errors are common during administration of anesthesia (1,2). Mild-to-moderate hypoxemia in the perioperative period has been implicated as one of the factors contributing to postoperative central nervous system (CNS) impairment (3,4). It is unknown whether anesthetics exacerbate or protect against hypoxia-related postoperative cognitive deficit.

A large clinical trial failed to demonstrate conclusive evidence of an association between hypoxemia and cognitive impairment (5). Several smaller studies, however, showed a correlation between oxygen saturation during the first 2 days after surgery and postoperative brain dysfunction (6,7). Moreover, postoperative delirium was

successfully treated with supplemental oxygen in at least two studies (6,8). Hence, the role of perioperative hypoxemia in the development of postoperative CNS impairment remains unknown. There is no agreement about the extent or duration of hypoxemia that may produce lasting cognitive dysfunction. Concurrent administration of anesthetics further complicates the issue.

Brain energy metabolism is not affected by moderate hypoxia (9). In contrast, animal studies showed that moderate hypoxia decreases production of cerebral neurotransmitters, particularly acetylcholine (ACh), which, in turn, may lead to impaired cognitive function (10,11). The resultant impairment of anesthetized animals that have been subjected to moderate hypoxia has not been investigated. It is unclear if anesthetics exacerbate or protect against the deleterious effects of hypoxia.

The aim of our study was to investigate the effect of moderate hypoxia with and without anesthetic on the CNS. Moderate hypoxia does not result in obvious neuronal damage, but may lead to cognitive dysfunction. This issue has received little attention

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in anesthesia research, despite the numerous investigations examining the mechanism of cerebral infarction/ischemia. We, therefore, examined impairment in spatial memory and psychomotor skills in mice subjected to moderate hypoxia for 1 h with and without anesthesia. The effects of hypoxia on cognitive performance were evaluated using a simple two-trial test for working memory that exploits the innate tendency of rodents to explore novel environments (12).

Methods

This study was approved by the Institutional Animal Care and Use Committee of the New York University Medical Center. Swiss-Webster male mice (35–45 g, 7–10 wk old) were used in all experiments. The animals were housed in groups of 5 per cage in a temperature controlled room with 12-h light/dark cycle. Mice had access to food and water ad lib.

The Y maze is constructed from Plexiglas. Each arm is 15" long and 3.5" wide with 3" high walls. The maze is painted flat gray throughout except for the top 1" of the walls, which allowed mice to see the outside of the maze. Five objects (2 on each side and 1 at each end) were placed outside of each of the 3 arms in such a position that mice could visually inspect them but could not touch them. The procedure consisted of 2 phases; an information trial and a test trial 1 h later. During the information trial, 15 min in duration, one arm was blocked (varied for each animal) and mice were permitted to run between the 2 open arms and inspect the objects outside. During the 3-min test trial, all arms were open. The position of the animal in the maze was automatically recorded by a tracking system (SMART, San Diego Instruments, San Diego, CA). The following data were recorded on the test trial; first arm entered, number of entries into each arm, and time spent in each arm. Mice that had intact spatial memory made the first turn into the previously blocked (novel) arm and spent approximately 60%–70% of the time in that arm inspecting the objects. Mice with spatial memory deficits did not exhibit a significant difference in arm entries, presumably as a consequence of impaired ability to discriminate between previously seen and novel objects. This is an ethological valid test for mice that does not require food deprivation, foot shock, or any other stressor to motivate performance.

Experimental groups were as follows: 1) oxygen 21% ($n = 20$), 2) oxygen 21% + isoflurane ($n = 15$), 3) oxygen 8% ($n = 14$), and 4) oxygen 8% + isoflurane. Five animals at a time were placed in a transparent, sealed, flow-through chamber (40 cm \times 25 cm \times 25 cm) during each experiment to control oxygen exposure. Data from the experimental sets were combined

for analysis. Two animals (1 from Group 3 and 1 from Group 4) escaped during the testing, one animal died during hypoxic exposure (Group 4), and one animal never explored the Y-Maze (Group 4). Normal atmosphere was purged from the chamber and rapidly replaced with the experimental hypoxic mixture of N₂-O₂. The temperature of anesthetized mice was maintained at 37°C + 0.5°C using a warming blanket. Experimental groups included control (21% oxygen) and 8% hypoxia. The same experiments were repeated with the addition of isoflurane 1.2%. Mice were exposed to the experimental gas mixture for 1 h. The hypoxia variables were determined from a series of pilot experiments optimizing hypoxic exposure while minimizing associated mortality. Animal exposed to 6% oxygen died within 10 min. Oxygen and isoflurane concentrations were measured continuously (Datex-Ohmeda, Helsinki, Finland). The experiment was terminated by discontinuing the anesthetic and flushing the chamber with 21% oxygen for 10 min. Mice were allowed 24 h to recover from the experimental treatment and then were tested in the maze on days 1, 4, and 7.

The dependent variable was time spent in the novel arm of the Y-maze. The experiment was designed as a 2 \times 2 \times 3 factorial with oxygen level (8% or 21%) and isoflurane level (isoflurane or room air) as the between-group factors and test day (1, 4, and 7 days) as the within-group factor. Comparisons of the session and treatment effects were made using analysis of variance, followed by analysis of simple main effects and by *post hoc* comparisons using Newman-Keuls test as appropriate. Statistics were performed with STATISTICA (StatSoft, Tulsa, OK).

Results

A 2 \times 2 \times 3 fixed effects analysis of variance performed on these data revealed no significant main effects, i.e., neither oxygen level, presence or absence of isoflurane, nor test day were significant sources of variance. However, spatial working memory was impaired on the first test day in the 8% oxygen group that did not receive isoflurane (Fig. 1). A one-way analysis of variance computed for the first day data revealed a significant difference among the 4 treatment groups ($F(3,66) = 9.29$; $P < 0.0001$). *Post hoc* comparisons using Newman-Keuls tests showed that the 8% group exhibited significantly ($P < 0.05$) poorer working memory than all the other groups. This memory impairment, however, was transient as performance returned to normal 3 days later.

Examination of the interaction effects reveals additional details of the effects of isoflurane and hypoxia on spatial working memory (Table 1). The significant interaction between level of oxygen and the presence

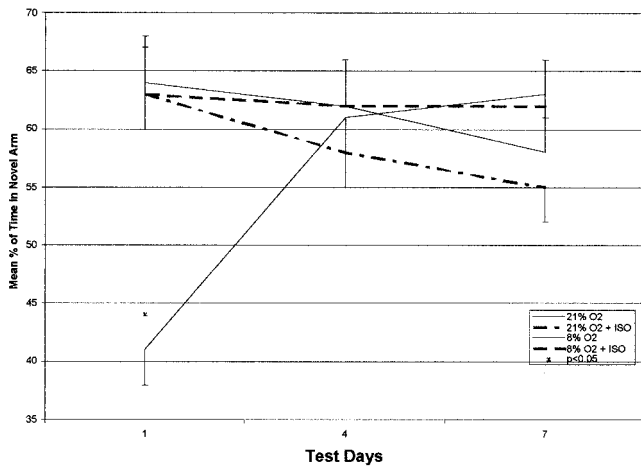


Figure 1. Mean time that mice spent in the novel arm. A one-way analysis of variance computed for the first day data reveals a significant difference among four treatment groups ($F = 9.29$; $P < 0.0001$). Data expressed as mean \pm SEM.

Table 1. Summary of Statistical Interaction Effects (Analysis of Variance)

Effect	Df effect	MS effect	Df error	MS error	F-statistics	P value
1	1	.0092	62	.0245	0.3784	.5407
2	1	.0325	62	.0245	1.3261	0.2539
3	2	.0152	124	.0164	0.9261	0.3988
12	1	.1116	62	.0245	4.5549	0.0368
13	2	.1389	124	.0164	8.4520	0.0004
23	2	.0939	124	.0164	5.1063	0.0074
123	2	.0569	124	.0164	3.4660	0.0343

1 = oxygen, 2 = isoflurane, 3 = test day.
Df = degree of freedom; MS = mean squares.

or absence of isoflurane (interaction 1×2) shows that memory performance under isoflurane may depend on the level of oxygen. Under normal room air conditions, mice not treated with isoflurane performed better than isoflurane-treated mice, but under hypoxic conditions this difference was reversed. The significant interaction between level of oxygen and test day shows that under room air (21% oxygen) conditions, working memory declined with increasing test days. Hypoxic mice showed the opposite effect; poor memory on test day 1 and good memory on test days 4 and 7. The significant interaction between test day and presence or absence of isoflurane shows that the effects of isoflurane on memory also depend on the test days. In the absence of isoflurane the performance was poor on test day 1 but recovered on days 4 and 7. For isoflurane-treated mice, the trend was in the opposite direction; memory was worse on days 4 and 7 than on test day 1.

Discussion

The main questions being addressed by this investigation were whether hypoxic conditions that typically

are not associated with overt neuronal damage produce cognitive changes and whether isoflurane ameliorates or exacerbates the effect of hypoxia on CNS. We demonstrated that mice subjected to hypoxia at an oxygen concentration of 8% developed transient impairment of spatial working memory (24 hours after the insult). When tested 4 and 7 days after treatment the same mice exhibited normal performance. It appears that isoflurane protects against this deleterious effect of hypoxia.

The Y maze test is based on the natural drive of rodents to explore novel environments. It reliably investigates spatial memory performance with results equivalent to those obtained with the classical Morris water maze (12). This test requires neither food deprivation (as opposed to the radial maze) nor electrical foot-shock (as opposed to light/dark box), which could modify the motivational and emotional status of the animal. It is less stressful than the Morris water maze (13). The Y maze test was successfully applied to study the effect of age, administration of various drugs and food supplements, as well as various stressors on spatial memory performances (14–16). Perhaps the short duration of memory retention in this task (4 to 8 hours) allows its repeated use within a few days. Our results, however, disagreed with this assertion.

Control animals in our study spent significantly less time in the novel arm on day 7 compared with day 1. The Y-maze test studies various components of exploratory behavior at once. Exploratory behavior includes cognitive and motivational factors that may be affected by neophobia and anxiety. Perhaps repetitive testing affected the motivational component of the exploration; animals were maintained in normal conditions between tests and had no stressors or other factors that could have affected their working memory. The other possibility is retention of information (object recognition) from previous testing. This observation is important for future studies using the Y-maze. The amount of time that mice spent in the novel arm progressively decreased despite an intact working memory. Hence, the corrected time should be used when one studies the progressive effect of various treatments on mouse behavior in time.

One hour exposure to isoflurane 1.2% administered with room air marginally impaired mice's performance in our investigation ($P = 0.04$). Studies on the effect of inhaled anesthetics on rodent's spatial performance have produced inconsistent results. Exposure to inhaled anesthetics may impair acquisition of a spatial memory as well as attenuate performance on already learned spatial memory task in aged rats (17,18). Other investigators, however, could not corroborate these results (19,20). Because experiments are conducted under slightly different conditions (duration of anesthesia, multiple exposure versus single

exposure to anesthetics, variable time of testing), the difference in results is not surprising.

Animals subjected to moderate hypoxia display a combination of cognitive and neurochemical changes. Behaviorally, they are less responsive to their environment, explore less, cannot learn as readily, and appear debilitated (9). Whether (and at what level) hypoxia affects animal performance after return to a normal level of oxygen is not clear. Exposure to hypobaric hypoxia at a simulated altitude of 5950 m (approximately 10% oxygen) for 8 hours produced decrements in working, but not reference, memory (21). Animals were tested in the Morris water maze 24 hours after exposure. Post-hypoxic animals required a longer time to initially locate the position of the submerged platform by using spatial cues located around the pool (reference memory). Rats exposed to 5% O₂-95% N₂ atmosphere for 40 min/day for 3 consecutive days did not show behavioral impairment 5 days after insult as measured by Morris water maze or passive avoidance tasks (22). Our results are consistent with these observations. The animals' performance was impaired 1 day after insult but returned to baseline on the fourth day.

The cellular mechanism of transient deterioration of spatial memory in the current study and its preservation by isoflurane is unknown. There is a marked difference between conditions in which the supply of oxygen is abolished and conditions in which it is only impaired. In anoxia, levels of energy substrate decrease within seconds, ionic gradients fail, and cell death follows promptly. By contrast, hypoxia, in which the supply of oxygen is reduced, but not abolished, impairs neural function without any demonstrable changes in the levels of adenosine triphosphate (9). Mild to moderate hypoxia may produce cognitive deficit because it impairs the metabolism of central neurotransmitters, including ACh (23). Although synthesis of all neurotransmitters (i.e., norepinephrine, dopamine, 5-hydroxytryptamine) depends on the energy state of the brain, the cholinergic system is one of the most important modulatory neurotransmitter systems in the brain that controls activities that depend on selective attention and memory (24). This mechanism may explain the transient change of cognitive function in our experiments.

The neuroprotective effects of isoflurane after focal ischemia, at least within a few days after an insult, are well documented (25). Isoflurane produces a dose-dependent decrease in metabolic rate (i.e., oxygen demand) that is linked to antagonism of excitatory neurotransmission or enhancement of inhibitory neurotransmission. This mechanism, however, cannot explain protection from the transient changes associated with the moderate hypoxia observed in our experiments.

ACh appears to play a central role in the pathophysiology of hypoxia-associated CNS impairment. ACh is

formed in cholinergic neurons from the co-substrate choline and acetyl-coenzyme A (Ac-CoA). Ac-CoA is produced from pyruvate by oxidative decarboxylation catalyzed by pyruvate dehydrogenase, which may be injured in the energy-deficient states (9). In addition, the pyruvate level in mitochondria decreases by cytosolic reduction to lactate under hypoxic conditions. Decreased pyruvate, in turn, will lead to decreased Ac-CoA production (27). Isoflurane may retard both processes by reducing the requirement for pyruvate oxidation, thus increasing the amount of pyruvate available for the ACh synthesis. There is no direct experimental evidence of the proposed hypothesis at the present time.

Although our objective was to study hypoxia-produced cognitive changes, which are not associated with the overt neuronal death, we cannot exclude the possibility of permanent neuronal injury without histological evaluation. Hypoxia (10% oxygen for 96 hours) produces hippocampal morphological changes, as demonstrated by histological studies (26). The damage in the CA1 region was more pronounced when animals were killed 6 days after exposure compared with 3 days after exposure, indicating delayed neuronal death. The hippocampus (especially the CA1 region) is intimately involved in memory formation. Hence, it is expected that animal performance would worsen with time. We did not observe deterioration in mouse performance tested on day 7 versus day 4. It is possible, however, that the Y-maze test could not detect subtle changes in working memory formation.

Our study is limited by a lack of physiologic measurements in the experimental animals. Unfortunately, there is no commercially available noninvasive equipment adapted for mice. Invasive arterial blood pressure monitoring, arterial blood gas sampling, or electroencephalographic recordings could have introduced confounding variables by limiting animals' movement and/or eliciting stress responses.

Hypoxia produces a plethora of physiologic responses, including hypocapnic alkalosis (secondary to dyspnea and increased ventilation during hypoxia), which produces cerebral vasoconstriction and subsequent reduction of cerebral blood flow. It is possible that the 'neuroprotective' effect of isoflurane in our experiments was related to cerebral vasodilation rather than alteration in ACh metabolism. In addition, isoflurane depresses ventilation and suppresses ventilatory response to hypoxia. These effects further complicate the interpretation of our results.

The experiments described above indicate that administration of isoflurane ameliorates transient changes in working memory in a mouse model of moderate hypoxia. Isoflurane did not cause any serious behavioral or working memory impairment in

untreated mice as measured by the Y-maze test. Isoflurane may have protective effects on cognitive deficits after a moderate level of hypoxia.

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