Effects of nitrous oxide exposure on behavioral changes in mice

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Abstract

This study was designed to examine the effects of continuous exposure to low levels of nitrous oxide on several behavioral paradigms and the occipital cortex cells of mice. Different groups of mice were exposed to air or two different levels of nitrous oxide (1000 ppm or 2000 ppm) 8 hr/day for eight consecutive days. The exposure to nitrous oxide was achieved by placing animals in a specially designed, enclosed chamber. At the end of the exposure period, all mice were tested for motor coordination, locomotor activity, stereotypic behavior and anxiety level. Cellular examination of the occipital cortex was conducted by counting both the larger neural cells and the smaller neuroglial cells in a specific region. Our results indicated that animals showed no deficit in motor coordination or anxiety level. Histological examination indicated no significant difference in the number of neural cells, neuroglial cells, or total cells counted in the control tissue, as compared to the neural tissue from mice exposed to nitrous oxide. Nitrous oxide-exposed mice showed reduced locomotor activity compared to control animals; however, with the exception of one time period, this decrease was not statistically significant. Animals exposed to nitrous oxide showed a dose-dependent reduction in stereotypic behavior. Our results suggest that short-term exposure to trace levels of nitrous oxide might alter central dopaminergic neuronal activities in striatal and mesolimbic regions. Further research in this area is needed to provide more information regarding the potential effects of repeated exposure to low levels of nitrous oxide. (Pediatr Dent 15:93–98, 1993)

Introduction

Nitrous oxide is used in dentistry for its analgesic and anxiolytic properties. During use, small quantities of nitrous oxide leak into the dentist's breathing zone. This waste nitrous oxide is measured in parts per million (ppm). Levels of nitrous oxide in the dentist's breathing zone have been found to be quite variable. Studies have reported levels of waste nitrous oxide from several hundred to several thousand ppm. Ambient levels of nitrous oxide in the dentist's breathing zone have been greater than 1,000 ppm, even in the presence of scavenging equipment. The United States National Institute of Safety and Health (NIOSH) recommends a maximum atmospheric exposure to nitrous oxide concentration of 25 ppm, 8 hr/day, 40 hr/week.

Chronic exposure to nitrous oxide is a concern to dental personnel, in part, because the gas is administered so frequently. A recent study indicated that 28.7% of pediatric dentists use nitrous oxide-oxygen for more than 75% of their patients; 58.9% use nitrous oxide-oxygen on selected patients; and only 12.4% never use nitrous oxide-oxygen for patients. Therefore, a total of 87.6% of pediatric dentists employ nitrous oxide-oxygen in their practices. This is a dramatic increase from only 35% using nitrous oxide oxygen in 1971 and 65% in 1980.

The pediatric dentist is especially vulnerable to ambient nitrous oxide because of the frequency with which the gas is used, and because an increased amount of ambient nitrous oxide accumulates as patient behavior deteriorates. Dental personnel are exposed to concentrations of nitrous oxide two or three times greater than operating room personnel. Previous studies reported levels in the dental operatory to be as high as 90,000 ppm. Other investigators have found levels as high as 7000 ppm, though lower nitrous oxide levels, in the hundreds of ppm, also have been encountered. Maximum nitrous oxide levels ranging from 132–815 ppm were measured in the breathing zone of dentists. One study used an infrared spectrophotometer with a sensitivity of 1–250 ppm. This study found that the analyzer frequently measured levels at its 250 ppm limit, especially during care of uncooperative children whose activity increased nitrous oxide leakage into the environment. Precise levels were, however, undetermined due to the limits of the sensor. In another study, nine dental operatories showed a mean nitrous oxide concentration of 4304.4 ppm at the head of the dental chair during conscious sedation. Another study found high levels of nitrous oxide present in all dental offices (n = 23), regardless of size or the presence of scavenging equipment. Nitrous oxide peaks were often very high and beyond the calibration range of the infrared spectrophotometer (greater than 1000 ppm).

Since nitrous oxide is used so frequently, many dentists are exposed to the gas several hours a week and possibly have been for several years. The systemic effects of this repeated exposure to nitrous oxide remain unknown. However, the systemic effects of abusing nitrous oxide are well documented, including ataxia, paresthesias, headaches, memory deficit, altered mood, impotence, and an increase in miscarriages among female dental personnel and wives of male dentists. An earlier study showed that exposure of rats to 40% nitrous oxide-oxygen induced cell damage to the oc-
cipital cortex. The potential systemic and behavioral effects of chronic exposure to low levels of nitrous oxide (1000 ppm or 2000 ppm) remain unclear.

Locomotor activity and the total number of stereotypies, which include sniffing, grooming, licking, and biting are natural animal behavior. It has been suggested that dopaminergic activity in the nucleus accumbens is associated with the mediation of locomotor activity, while that in the striatum is involved with the initiation of stereotyped behavior. Abnormality in these brain regions have been implicated in the pathophysiology of psychomotor disorders such as Parkinson’s disease, Huntington’s chorea, and schizophrenia. Other behavioral changes such as motor coordination and anxiety changes are more complex and may involve other neuronal systems.

A human study to evaluate the behavioral effects of repeated exposure to low levels of nitrous oxide would be difficult. This animal research project creates an animal model feasible to evaluate several behavioral changes due to short-term exposure to trace levels of nitrous oxide, including changes in motor coordination, locomotor activity, stereotyped behavior, and anxiety level. Changes in the cellular counts of the occipital cortex of the mouse also will be measured. This study will determine if exposure of mice to low levels of nitrous oxide (levels comparable to those breathed by dentists) causes a detectable change in their behavior and cortical cell count as compared to control animals.

**Methods and materials**

**Animal treatment**

Male adult Swiss mice (Dominion Laboratories, Omaha, NE) weighing 19–21 g were housed in plastic cages (four mice per cage) in a light- and temperature- controlled environment with a 12-hr light-dark cycle and free access to water and food (Purina Lab Chow, St. Louis, MO). Animals were weighed and assigned randomly to control and experimental groups. They were allowed a minimum of three days to adapt to the housing environment prior to experimentation. A total of 22 animals were used; eight controls, seven in N₂O- (1000 ppm) exposed group and seven in N₂O- (2000 ppm) exposed group. Animals in control group (N = 8/group) were exposed to air, while those in experimental groups (N = 7/group) were exposed to nitrous oxide (1000 ppm or 2000 ppm) for 8 hr/day (9 AM to 5 PM) for eight consecutive days. All neurobehavioral testing was conducted on day nine between 8 AM and 4 PM. Both experimental and control mice were tested in different apparatus at the same time.

**Exposure of mice to nitrous oxide**

Nitrous oxide (Joliet, IL) was supplied in five-foot cylinders, premixed to either 1000 ppm or 2000 ppm of nitrous oxide with air. During nitrous oxide exposure, animals were put in a closed chamber, with a volume of 327 liters and ports for entry and exhaust of the nitrous oxide mixture to a fume hood. Ports were pvc pipes with holes extending deep into the chamber to create a homogeneous mixture of gas and to prevent stratification of the chamber air during the 8-hr exposure period. The gas coming from the cylinder entered a regulator, was down-regulated to a pressure of approximately 50 psi, and entered a flowmeter to allow for the accurate delivery of the required liters per minute.

The animals to be exposed to nitrous oxide were placed in the chamber, which was then flushed with the nitrous oxide mixture to allow the gas within the chamber to rapidly approximate the experimental concentration of 1000 ppm for the initial group and 2000 ppm for the final group. The nitrous oxide/air from the premixed cylinders flowed into the chamber at a rate of 16 L/min for 30 min. For the next 8 hr, the nitrous oxide/air was set at a steady flow of 1.5 L/min, for adequate ventilation to meet the animals’ respiratory demands. This routine of flushing followed by the flow at a steady rate, was done for both of the experimental exposure periods. Control animals were placed in a separate chamber and exposed to room air.

**Analysis of nitrous oxide concentration**

The level of nitrous oxide within the chamber was analyzed at five different times: time zero; 30 min after the chamber had been flushed with 480 L of the nitrous oxide/air gas (1000 ppm for the initial experiment and 2000 ppm for the final experiment); 2 hr; 4 hr; and 6 hr respectively. The samples of nitrous oxide within the chamber were gathered in 13 ml Venaject® vacutainer tubes. The chamber air samples were taken by needle puncture of a latex port on the side of the chamber. Nitrous oxide was drawn into the vacutainer tube for 1 min. The chamber air samples were subsequently analyzed by gas chromatography.

**Analysis of carbon dioxide concentration**

The carbon dioxide within the chamber was tested for the ppm concentration at 2-hr intervals during days one and two of both the initial experiment (1000 ppm) and the final experiment (2000 ppm). The air was again tested for carbon dioxide content at 4-hr intervals on day three of both the initial and final experiments. The analysis was made by using a Precision Gas Detector, model #400 Kitagowa Precision Gas Detector Tubes of a 0.05–1.0% (500–10,000 ppm) sensitivity were used for the detection of the carbon dioxide. The purpose of this was to ensure that the carbon dioxide did not reach the unsafe level of 10,000 ppm (NIOSH 1991).

**Assessment of motor coordination using the rolling-roller performance test**

Each mouse was tested for its ability to stay on a rotat- ing rod, rotating 10 revolutions per min. Each animal was allowed to adapt to this roller for 60 sec and was allowed a maximum of three trials to stay on the roller for
Histological study

Assessment of horizontal locomotor activity and stereotyped behavior

Following the assessment of motor coordination, mice were used to determine locomotor activity and stereotypic behavior, which include sniffing, grooming, licking, and biting with a Digiscan® animal activity monitor (Omnitech Electronic, Columbus, OH). Each animal was allowed to adapt to the monitor for 30 min. Locomotor activity and stereotypic behavior were measured every 30 min for 120 min. The monitor’s horizontal movement sensors direct 16 beams from front to back (x-axis) and 16 beams from side to side (y-axis). Interrupting several of these beams during a certain time period generated data recorded by an analyzer. The results were printed automatically at the end of each time period.

Assessment of the level of anxiety

The purpose of the anxiety test was to evaluate the effect of nitrous oxide on the mouse’s anxiety. The method used was similar to that as described by Crawley, based on the natural tendency of rodents to explore a novel environment balanced against the aversive properties of a brightly lit area. Thus, if mice were taken from a dark adapting environment and placed into a two-compartment box (one side dark and one side bright of equal dimension), they would normally spend more time in the dark area since the brightly lit area is an averse environment. Therefore, measuring the time the mice spent in the dark area of this two-compartment model enabled the detection of anxiolytic and anxiogenic effects of nitrous oxide exposure. Animals treated with a compound such as diazepam, which has an anxiolytic action, will spend more time in the bright area.

A closed black-colored Plexiglass® box (41 x 21 x 25 cm) with an opening (13 x 5 cm) at the center of the box was placed in the left side of a clear plexiglass chamber (42 x 42 x 30 cm) in a quiet, darkened room illuminated with a 30 Watt red light. The right side of the clear Plexiglass box was brightly illuminated with a 60 Watt light source. The entire clear chamber was placed inside a Digiscan animal activity monitor RXVZCM-16 (Omnitech Electronic Inc., Columbus, OH) equipped with photocells to detect behavioral changes. The data generated was collected by an analyzer and the results printed automatically. Each mouse was given 60 min to acclimatize to a quiet, darkened room. The animal then was placed in the center of the bright area of the testing chamber. The following behaviors which included the total number of side changes and the amount of time each mouse spent in the dark and bright areas were recorded for a period of 30 min.

Statistical analysis

All results from the behavioral tests and the histology examination were expressed as the mean ± standard error of the mean. All groups were subjected to one-way analysis of variance (ANOVA), followed by Newman-Keuls test. A P-value of < 0.05 was considered to be statistically significant.

Results

Concentration of nitrous oxide and carbon dioxide

The results of the analysis showed that the concentration of nitrous oxide rapidly approximated the level in the pre-mixed cylinder (1000 ppm or 2000 ppm) and remained at this level throughout the 8-hr exposure period. At no
time did the carbon dioxide reach this level. The steady flow of 1.5 L/min maintained the levels of carbon dioxide in the 4500–5500-ppm range.

Effect of nitrous oxide exposure on body weight of mice

The control group and the two nitrous oxide-exposed groups (1000 ppm and 2000 ppm) showed a similar body weight gain during the study. There was a time-dependent gain in body weight among all groups of animals. However, the gain in body weight of air and nitrous oxide-exposed mice was at the same rate ($P > 0.05$, data not shown).

Rolling-roller performance test

All of the mice passed the rolling-roller performance test ($P > 0.05$). It appears that the exposure to nitrous oxide for this time period did not induce any motor incoordination in these animals.

Effect of nitrous oxide exposure on horizontal activity and stereotypic behavior

The results are presented in Figs 1 and 2. Mice exposed to nitrous oxide showed a consistent reduction in their horizontal activity though the data was significant at only one time point when compared to the control group (Fig 1). Animals exposed to nitrous oxide showed a dose-dependent reduction in the numbers of stereotypies counts ($P < 0.05$).

Effect of nitrous oxide exposure on anxiety level in mice

The results are presented in Table 1. Our study showed that there was no significant change in the anxiety level of the nitrous oxide-exposed mice when compared to the control mice.

Results of histological study

Table 2 shows the results of the counts of the large cells, the small cells, and the total number of cells for the control and nitrous-oxide exposed animals. We could not detect any significant changes between the control and the nitrous oxide-exposed mice in cortical cell counts ($P > 0.05$). However, the large cell counts, representing the neural cells, for the exposed animals (those mice exposed to 2000 ppm nitrous oxide) were less than the large cell counts of the control mice. This reduction in the number of large cells was not statistically significant ($P > 0.05$). The number of neuroglial cells counted was virtually equal between the control and the nitrous oxide exposed mice. The total cell count was also very similar.

Based on these results, it was not visibly apparent in the occipital cortex that repeated exposure to 2000 ppm nitrous oxide caused an increase in neuroglial (small) cells, nor a statistically significant decrease in the neural (large)
Table 2. Effect of nitrous oxide on cell counts of mouse occipital lobe

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neural Cells</th>
<th>Neuroglial Cells</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>206 ± 8.0</td>
<td>84 ± 6.2</td>
<td>290 ± 14.1</td>
</tr>
<tr>
<td>Nitrous oxide</td>
<td>186 ± 7.2</td>
<td>88 ± 2.4</td>
<td>274 ± 7.4</td>
</tr>
</tbody>
</table>

Coronal sections of the occipital lobe of control and animals exposed to 2000 ppm nitrous oxide were examined under 500x magnification light microscopy. The large cells (neural cells), and small cells (neuroglial cells) were counted in a 0.03-mm² area. The examined area was consistently the same for each slide as explained in Methods. The results showed that exposure to nitrous oxide did not significantly change the cell counts for the large or the small cortical cells. The total cell counts also were not significantly changed. Although the number of neural cells (large cells) was less in the nitrous oxide-exposed mice, this was not statistically significant (P > 0.05).

cells. The total cell counts were not statistically significant when the nitrous oxide-exposed group was compared to the control.

Discussion

This study was designed to evaluate the effects of short-term exposure to trace levels of nitrous oxide on several behavioral paradigms of the mouse. The experimental design involved exposing mice to trace levels of nitrous oxide. These levels are comparable to levels inhaled by dentists in their working environment.

Our results showed that mice exposed to nitrous oxide showed no deficit in motor coordination and no change in anxiety level. Specifically, these mice showed a reduction in locomotor activity and stereotypic behavior. Locomotor activity and stereotypy are thought to be mediated in the brain primarily via the mesolimbic and nigrostriatal dopaminergic pathways, respectively. The measurement for horizontal activity indicated that the animals exposed to nitrous oxide exhibited a reduction in horizontal activity as compared to control animals. However, this aberration did not uniformly reach statistical significance (P > 0.05). Only the 1000 ppm group, at the 120-min, showed a statistically significant reduction in horizontal activity (P < 0.05). Thus, it appears that higher levels of nitrous oxide may depress dopaminergic activity in the nucleus accumbens of the mouse. The behavioral test for the number of stereotypy showed a generalized statistically significant difference between control and exposed mice (P < 0.05). The exposed animals expressed fewer stereotypies than control mice. Thus, it appears that low concentrations of nitrous oxide, such as those used in this experiment, cause a depression of activity in the dopaminergic neural pathway of the mouse striatum as manifested in a decrease in stereotypic behavior.

As an adjunct to evaluate behavioral effects of nitrous oxide exposure, this project also examined the neural tissue of the cerebral cortex. In previous research, high levels of nitrous oxide caused a decrease in the number of neural cells of the occipital cortex of the rat, and an increase in the number of neuroglial cells. The histology examination revealed that those animals repeatedly exposed to a 2000 ppm level of nitrous oxide had decreased numbers of neural cells as compared to control mice. The aberration in neural cell numbers between control and nitrous-oxide exposed animals was not statistically significant (P < 0.05). The number of neuroglial cells was virtually the same among control and treated animals. Therefore, it appears that, in the mouse, trace levels of nitrous oxide do not significantly decrease the number of neural cells observed in the occipital cortex, and have even less of an effect on the number of neuroglial cells.

In conclusion, this study provided some information regarding the potential effects of occupational exposure to nitrous oxide on behavior and the cells of the occipital cortex. Further animal research examining the impact of nitrous oxide on the striatal and mesolimbic dopaminergic regions is warranted. To safeguard the health of dentists and dental staff, we recommend routine monitoring for possible nitrous oxide contamination in the dental operatory in addition to using a scavenger system.

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14. Davis MJ: Conscious sedation practices in pediatric dentistry: a


