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Nitrous Oxide and Infertility

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Our laboratory has reported changes in luteinizing hormone releasing hormone (LHRH) from the hypothalamus following nitrous oxide (N_2O) exposure. LHRH augments LH release, which in turn causes ovulation. This study evaluated how N_2O disrupts ovulation and the possible resulting infertility. Adult virgin female rats (N = 64) were housed with a 12 h:12 h light cycle. Daily vaginal smears were taken and only rats exhibiting two consecutive normal 4-day ovulatory cycles were used. Thirty-two rats were placed in an environmental chamber and exposed to a mix of hydrated 30% N_2O and compressed air delivered at 1.6 L/min for 8 h/day for 4 days (one cycle); controls received compressed air. All rats exposed to N_2O exhibited disrupted cycles following the first day of the 4-day exposure. From a group of 12 N_2O-exposed rats, 11 went into constant proestrus (day of ovulatory surge) for up to 3 weeks. Control rats cycled normally. Following each exposure, eight rats were perfused, brains sectioned, and LHRH cells identified by immunocytochemistry. Eight control rats also underwent this procedure. A threefold increase in LHRH cells was noted in N_2O rats. In addition, 12 rats received 30% N_2O for 4 days, followed by mating with proven male breeders for 4 days, as were controls. Six of 12 N_2O rats and 12 of 12 control rats gave birth. Contrary to previous reports, no significant difference was noted in litter size or weight. The constant proestrus seen after N_2O exposure is due to disruption of LHRH cells in the hypothalamus (blocked LHRH release). It is this disruption of LHRH, and therefore ovulation, which results in infertility.

Since its first use in 1844, nitrous oxide (N_2O) has been considered one of the safest of anesthetics. However, subsequent studies have associated prolonged exposure to high concentrations of nitrous oxide with certain adverse effects, such as leukopenia and embryotoxicity.[1,2] Additional research showed an alteration in fetal death rate and teratogenic effect due to gaseous anesthetics.[3]

In recent years it has been suggested that operating room and dental staffs are being exposed to an occupational hazard from pollution of the air in operating theaters with anesthetic gases.[4] Dentists exposed to N_2O alone had higher rates of reproductive and health problems when compared with dentists not using gaseous anesthetics.[5] There is controversy as to the extent of the risk to dental staffs; however, low levels of nitrous oxide over time have been implicated in fertility problems in male and female rats,[6,7] decreased size and litter weight,[8] and increased risk of first trimester abortion.[3] Female Wistar rats exposed for 5 h/day for 15 days at 60% N_2O–O_2 exhibited absence of ovulation, progressive reduction and eventual disappearance of the proestrus phase and there is an absolute functional incapacity for procreation.[a]

Our laboratory has shown that female rats exposed to acute levels of N_2O (40%) for 8 h/day for 4 days (or one ovulatory cycle) exhibited abnormal ovulatory cycles determined by vaginal smears. These changes were correlated with changes in the number of luteinizing hormone releasing hormone (LHRH) cells in the hypothalamus.[10] LHRH plays a critical role in ovulation because its neurosecretion augments the secretion of LH from the pituitary, thereby bringing about a series of events that initiate ovulation and therefore allow for fertilization.

In 1977 there were at least 30,000 dental offices in the United States using N_2O.[11] This would mean that approximately 120,000 dental personnel (the majority being female) were chronically exposed to nitrous oxide. Since this 1977 publication, the use of N_2O has remained

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popular as competition for patients among dental offices increases.

In a study by Middendorf[12], ambient concentrations of N_2O were determined within the workers' breathing zones as well as throughout the suites of a number of dental offices. These results found levels in all operatories studied to be above the National Institute of Occupational Safety and Health (NIOSH) recommended level of 25–50 ppm. Waiting rooms and offices showed background levels above 225 ppm following N_2O administration. The authors noted that the major sources of nitrous oxide exposure to personnel was from leaks around the nasal mask and exhaled air from the patient's mouth. Of the offices studied, 44% had scavenger systems and yet all had levels greater than 25 ppm in the office. In the dentist's and chairside assistant's breathing zone, levels ranged from 132–880 ppm. In offices without scavenger systems, levels ranged from 152–815 ppm in the breathing zone. Our own studies have also shown ambient concentration of N_2O to be above the NIOSH recommended level. In a study done at the Tufts University oral surgery clinic, levels in closed operatories ranged between 400 and 2,000+ ppm for the dental breathing zone (DBZ) and 325 to 750 ppm at the dental chair foot area (DCF) when a scavenger system was not used. For scavenged procedures, levels ranged from 160–1,000 ppm in the DBZ and 100–320 ppm in the DCF.[13]

The purpose of this study was to determine if a short term 30% N_2O acute exposure would disrupt ovulation, how this disruption occurs, and if there is an effect on fertility.

METHODS

Sixty-four adult virgin female Sprague-Dawley rats weighing 180–200 g were housed in commercial polypropylene cages containing three rats per cage in a temperature-controlled room with a 12 h : 12 h light/dark cycle (lights on at 0600 h). Food and water were available ad libitum. Following shipment, all animals were left undisturbed for 2 weeks before placement in an environmental chamber (Figure 1). Once placed in the chamber, rats were acclimated for 1 week, at which time vaginal smears were initiated for both experimental and control rats. Using a vaginal lavage technique, smears were collected daily between 9 and 10 AM and dried and stained with toluidine blue. These stained smears were examined under the light microscope to evaluate the qualities of the epithelial cells and classified according to their stage in the ovulatory cycle. Once animals were determined to exhibit two normal 4-day ovulatory cycles, exposure was initiated.

A total of 32 female rats were exposed to N_2O in an environmental chamber. Twenty-two of these rats were exposed randomly (exposure started on any day of the ovulatory cycle), and 10 rats were exposed on proestrus (day of ovulatory surge) to determine any variations in the effect of N_2O exposure on different stages of the ovulatory cycle. Experimental animals in this study received 30% nitrous oxide for 8 hours/day for 4 days, or one ovulatory cycle.

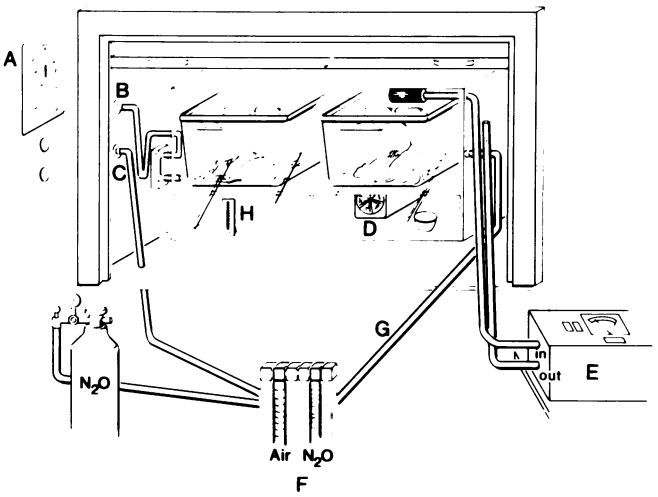

Figure 1. Nitrous oxide plexiglass environmental chamber in vacuum hood. A. 24-h timer. B. Vacuum outlet and meter. C. Compressed air hose. D. Circulation fan. E. MIRAN 101 N_2O gas analyzer. F. Anesthesia unit. G. Anesthetic gas input to chamber. H. Thermometer.

A total of 32 control animals at random stages of the ovulatory cycle were housed in identical environmental chambers and received a compressed air mix for the extent of the study. Also, exposed animals acted as their own controls by cycling normally for two cycles before N_2O exposure.

Animals received a mixture of N_2O and compressed air delivered at a flow rate of 1.6 L/min. Concentrations of nitrous oxide were confirmed throughout this study by random gas sampling using a MIRAN (Miniature Infra Red Analyzer) 101 Specific Vapor Analyzer (Foxboro Corporation, Foxboro, MA).

At termination of the exposure, eight of these 32 rats along with eight control rats were anesthetized with pentobarbital (40 mg/kg body wt) before fixation of their brains for study of hypothalamic LHRH-producing cells.

The brains were fixed via vascular perfusion. The descending aorta and ascending vena cava were clamped and the right atrium and the apex of the heart cut before inserting a cannula through the left ventricle and into the aorta. Physiological saline (approximately 8 ml) containing 10,000 U of heparin preceded the flow of 5% acrolein in the 0.1 M phosphate buffer, pH 7.2. Following removal from the cranium, the brains were placed in 0.1 M phosphate buffer.

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Fifty micrometer Vibratome (Lancer, St. Louis, MO) sections were cut and washed overnight in 0.05 M Tris (Sigma Chemical Co., St. Louis, MO) buffer, pH 7.6. As each brain was cut, sections were placed sequentially in several vials; each vial was treated with a specific primary antiserum for immunocytochemistry.

Details of the fixation and immunocytochemical procedures have been described.[14] Briefly, sections were treated with sodium metaperiodate and sodium borohydride to remove residual aldehydes. Incubations with antisera were as follows:

1) primary antisera #1076 and #419 (rabbit anti-LHRH) 1/500 in Tris buffered saline (TBS: pH 7.6) containing 0.4% Triton X-100, 0.1% gelatin and 0.02% sodium azide for 48–72 h at 4°C;
2) secondary goat anti-rabbit immunoglobulins (Antibodies, Inc., Davis, CA) 1/50 in TBS containing 0.1% gelatin, 0.02% Triton X-100 and 0.02% sodium azide for 2 h at room temperature;
3) tertiary antiserum (peroxidase-antiperoxidase, Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, MD) 1/100 in the same buffer, 1 h at room temperature.

These procedures result in the presence of a brown stain labeling the LHRH cells (Figure 2). Comparisons were made between the cell numbers for exposed and control rats.

A second group of 12 rats were cycled via vaginal smears during and following N_2O exposure until the cycles returned to normal. The ovulatory cycles for these rats were compared with those of 12 control animals. Disruption of the cycle along with the duration of the disruption were recorded.

A third group of 12 animals was mated with proven male breeders for a period of 4 days (one ovulatory cycle) following N_2O exposure. Twelve control animals were mated under the same conditions. Comparison was made between the number of pregnancies as well as litter size and weight. Females were checked for the presence of sperm by vaginal lavage to confirm that mating had occurred.

RESULTS

Of the first group of eight rats exposed to N_2O whose brains were perfused and stained using immunocytochemical procedures, those rats (N = 4) exposed on the morning of proestrus showed a threefold increase in total LHRH cell counts in the hypothalamus (Table 1). The four rats in which N_2O exposure started on the morning of metestrus (quiescent stage of ovulatory cycle) showed no difference when compared with control animals. Two

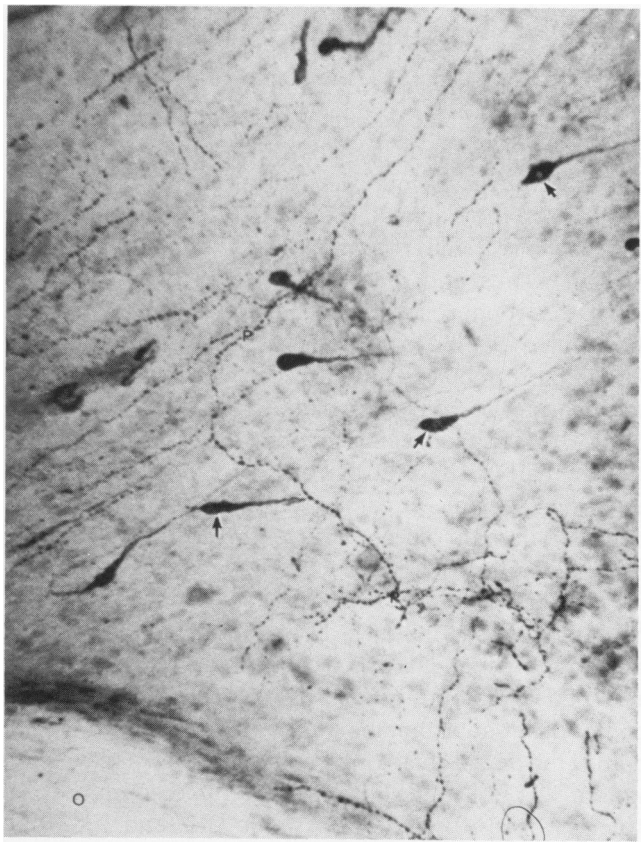

Figure 2. Light micrograph of immunoreactive perikarya (*arrows*) and neural processes (P) with beaded varicosities in the periventricular region caudal to the optic chiasm (O) ×425.

Table 1. Hypothalamic LHRH Total Cell Counts

		Nitrous Oxide	*Control*
Proestrus	(a)	723 cells	192 cells
	(b)	801 cells	176 cells
Metestrus	(a)	189 cells	210 cells
	(b)	393 cells	532 cells

a = LHRH antisera #419; b = LHRH antisera #1076.

Hypothalamic LHRH cell total counts of control and exposed animals at the stages of proestrus and metestrus. Cell counts for the two LHRH antisera used are shown.

different LHRH antisera were used to ensure that the differences in cell counts were not the result of nonspecific staining.

The second group of 12 rats exposed to N_2O who were cycled via vaginal smears showed disrupted cycles following the first day of exposure and 11 of the 12 rats went into constant proestrus. This dysfunction resolved itself after approximately 3 weeks. Control animals cycled normally throughout the experiment.

A third group of 12 animals, of which six had N_2O exposure start on proestrus and the remaining six on

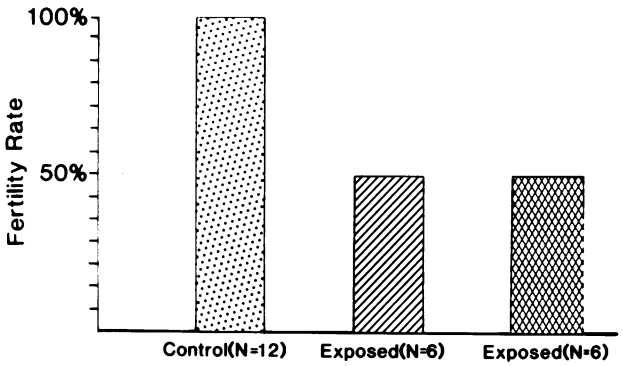

Figure 3. Fertility rates following 30% N_2O. Dotted bars = controls exposed to compressed air before mating. Hatched bars = Exposed to 30% N_2O starting on the day of proestrus and lasting 4 days (one ovulatory cycle) before mating. Crosshatched bars = Exposed to 30% N_2O starting on various days of the ovulatory cycle and lasting 4 days (one ovulatory cycle) before mating.

various days of the ovulatory cycle, were mated with proven male breeders. Vaginal smears demonstrated that mating had occurred with all animals. Following a 19–21-day gestation period, during which rats were checked daily, the number of animals giving birth was determined. Also recorded were the litter size and weight. Three of six exposed females in each group gave birth while 12 of 12 control rats came to term. This indicates a 50% decrease in fertility (Figure 3). No significant difference was noted in litter size and weight between exposed and control animals.

DISCUSSION

Thirty percent nitrous oxide, when given 8 h/day for 4 days, or one ovulatory cycle, decreases fertility in female rats. This is due to a block in the release of LHRH in the hypothalamus, as evidenced by the increased LHRH cell numbers found in exposed rats. This was interpreted as an increase in the number of cells that are positive for LHRH antisera due to decreased release and a subsequent increased intracellular content of LHRH, rather than an increase in the actual number of LHRH-producing cells. This hypothalamic disruption of LHRH prevents the pituitary LH surge necessary for ovulation and thus the resultant infertility. We hope to study the mechanism of this disruption in future investigations.

The animals are most sensitive to exposure in proestrus. Animals exposed on any day of the cycle became locked in a constant proestrus stage. This is important because it is in this phase that the LH surge occurs, producing the necessary stimulation for ovulation to take place.

The lack of LHRH release can be explained on the basis of an increase in opioids and substance P in areas of the brain where LHRH is synthesized. Previous studies have indicated an association between nitrous oxide as an addictive agent and endogenous opioids.[15] It has been proposed that nitrous oxide produces its analgesic and sedative effects through an increase of endogenous opioids,[16] which in turn have an inhibitory effect on the release of LHRH cells in the hypothalamus.

We are currently studying opioid and substance P levels in particular areas of the brain after 30% N_2O exposure, using the same protocol described in this experiment.

Our theory is further supported by the fact that no significant difference was noted in either litter size or weight between exposed and control animals, indicating that when ovulation took place a normal pregnancy was encountered and animals came to term, giving birth to normal pups. This contradicts previous reports that found decreased litter sized after N_2O exposure.[17]

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