LONG-TERM INHALATION REPRODUCTIVE AND TERATOGENIC TOXICITY EVALUATION OF NITROUS OXIDE PLUS HALOTHANE

FINAL REPORT

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MATERIAL: Nitrous Oxide and Halothane

SUBJECT: FINAL REPORT
Long-Term Inhalation Reproductive and Teratogenic Toxicity Evaluation of Nitrous Oxide plus Halothane
Project No. 785-200

I. OBJECTIVE

The purpose of this study was to evaluate the effects of long-term exposure to nitrous oxide (N_2O) plus halothane on the reproductive performance of female rats and the developmental processes in their offspring.

II. MATERIALS AND METHODS

A. Animals and Animal Groups

Two hundred forty sexually mature Sprague-Dawley strain rats (Charles River COBS), 120 males and 120 females, were used for this study. The animals were divided by random selection from a larger pool into three groups of 40 males and 40 females each. The groups are identified in Table 1.

Table 1 - Group designations for male and female rats exposed to filtered air or to mixtures of nitrous oxide and halothane.

Group No.	No. of Animals m	No. of Animals f	Treatment
1	40	40	Air Control
2	40	40	50 ppm N_2O + 1 ppm halothane
3	40	40	500 ppm N_2O + 10 ppm halothane

B. Exposure Conditions

The animals in the treatment groups were exposed under dynamic conditions at 1200 liters/minute airflow to the respective N_2O and halothane mixtures in a 6000-liter glass and stainless steel Rochester-type inhalation chamber. Exposures were conducted for seven hours per day, five days a week, for 12 weeks. The control males and females were exposed to filtered room air in a similar chamber with flow characteristics identical to that of the treatment groups.

The animals were individually numbered with ear tags and housed in groups of five of a sex in stainless steel mesh cages with stainless steel top loading feeders and glass water bottles. The cages were arranged on one layer during exposure. Water and basal laboratory diets (Purina Rat Chow) were available ad libitum. The cages were changed and washed weekly and the water bottles were changed and sterilized twice weekly.

C. Exposure Method

Input of chamber air was delivered via a tangential pipe into a cylindrical turret at the apex of the pyramidal top and the chamber exhausted via a goose-necked duct at the bottom above the drain pipe.

Nitrous oxide (N_2O) was supplied from cylinders containing certified 98% pure nitrous oxide anhydride. The gas was passed under positive pressure through a flowrator and critical orifice into a 3-necked mixing flask prior to being inserted via the input duct into the chamber turret.

The haolthane used was the Fluothane® brand of 2-bromo-2-chloro-

1,1,1,-trifluorothane. Nitrogen was passed via a flowrator and critical orifice through the headspace of a glass flask containing liquid halothane and then passed into the mixing flask to mix with the N_2O prior to delivery into the chamber input duct.

D. Chamber Monitoring

Chamber concentrations of N_2O and halothane were determined from samples pulled via a standard Teflon® probe located just above the middle cage in the chamber prior to exposure, at hourly intervals during the first five days of exposure, and at least daily during the remainder of the exposure period after chamber equilibration (T_{99} = 23 minutes). Analyses of chamber concentrations of nitrous oxide were determined by on-line infrared spectrometry validated against gas chromatography (1). Halothane concentrations were determined by peak height analysis on a gas chromatograph with an electron capture detector. Gas samples were taken by syringe from a septum in the sample line and injected into a vacutainer containing a measured volume of 2,2,4 trimethyl pentane, and then an aliquot of the solution was injected into the gas chromatograph.

E. Breeding

The animals from the respective groups were mated after the 12-week exposure period by placing one male and one female in an elevated wire mesh breeding cage outside the chamber until a sign of mating (vaginal sperm) was observed or 21 days of cohabitation had been completed. Unmated females were rotated to proven males after one and two weeks. A vaginal examination of each female

(vaginal douche method) was performed daily and slides prepared and examined for the presence and viability of spermatozoa. On the day evidence of mating was observed (designated as Day 0 of gestation), the male and female animals were again housed in sex segregated cages.

The first 20 females in each group in which sperm were observed were designated for cesarean delivery. These animals were re-exposed to their respective gas-vapor concentrations from Day 6 through Day 15 of their gestation. The remaining females in each group with found vaginal sperm were designated for natural delivery. These animals were re-exposed to their respective gas-vapor concentrations from Day 1 through Day 15 of gestation. Those females in which sperm was never observed were discarded after 21 days in the breeding cages.

Following the 21-day mating period, the males were returned to their exposure cages and maintained on the appropriate control or exposure level for use on a cytogenetic study to follow.

F. Observations

All females were observed daily for mortality. Body weights, appearance, and behavior were recorded on gestation Days 0, 5, 16, and 20 for all mated females.

G. Cesarean Delivery

1. Sacrifice

The 20 females per group re-exposed to treatment from Day 6 through 15 of pregnancy were sacrificed on Day 20 of gestation by chloroform overdose. Cesarean sections were performed and the following observations recorded for each sacrificed female and her litter:

number of copora lutea, number and placement of uterine implantation and resorption sites, number and placement of live and dead fetuses, individual fetal weight to the nearest 10 mg, individual fetal length (crown-rump distance) to the nearest 0.1 cm, and external fetal anatomical abnormalities. Necropsies were performed on all sacrificed females. The uterus and ovaries and all abnormal tissues of each maternal female were described and preserved in 10% neutral buffered formalin. All of these tissues were stored for possible future reference.

2. Visceral Examination of Fetuses

Upon completion of external observations, approximately one-third of the fetuses from each litter were fixed in Bouin's solution and examined for visceral changes by the method of Wilson and Warkany (2). Whole body transverse sections of the nasal, orbital, cervical, thoracic, and abdominal regions were examined for abnormalities under a dissecting microscope. Sectioned fetuses were preserved in 70% alcohol and stored for possible future reference.

3. Skeletal Examination of Fetuses

The remaining two-thirds of the fetuses were placed in distilled water for an hour, eviscerated, replaced in distilled water for two hours, and subsequently fixed in 95% ethyl alcohol for a minimum of 72 hours. The fetuses were then placed in 2.0% potassium hydroxide for about 24 hours, stained for approximately 24 hours in a solution of alizarin red S and potassium hydroxide,

and then rinsed with distilled water. The stain was then extracted from the soft tissue for a minimum of 24 hours with one part benzyl alcohol, one part glycerin, and two parts 70% alcohol. The fetuses were then cleared with 75% aqueous glycerin for approximately 24 hours. Lamps were kept over the specimen containers throughout the staining and extraction procedures. Each skeleton was evaluated for relative differences in size, location of normal or abnormal bone structure, degree of ossification, and the presence or absence of bone structure in the skull, ribs, sternebrae, vertebrae, pelvis, forepaws, and hindpaws. The skeletons were then stored in 100% glycerin.

H. Natural Delivery

The remaining mated females in each group which were re-exposed to treatment from Day 1 through 15 of gestation were then removed from the exposure chambers and allowed to deliver normally and carry their litters to weaning at three weeks. Within 24 hours following birth, the litters were examined for the number and sex of live and dead pups, the litter weight by sex recorded, and the pups examined for evidence of external anatomical abnormalities. The litters were arbitrarily reduced to a maximum of eight pups (equally divided by sex when possible) to be carried through a 21-day lactation period to weaning. Litter weights by sex were recorded again at four days post-partum and at weaning (21 days post-partum). Mortality, appearance, and behavior of the offspring were also recorded at these intervals during lactation.

At weaning (Day 21), all surviving maternal females were sacrificed and gross necropsies were performed, which included examination of the viscera and recording of the number of ovarian corpora lutea and evidence of implantation sites. Necropsies were also performed on any females which died and on all surviving pups at weaning. The uterus and ovaries and all abnormal tissues of each maternal female were preserved in 10% neutral buffered formalin and stored for possible future reference. All abnormal pups were similarly preserved.

I. Statistical Analysis

Statistical analysis of the difference between each experimental group and the control group on each of the following parameters was performed by Student's t-test with significance evaluated at the 0.05 probability level (3): for pregnant parental females (cesarean and natural deliveries evaluated separately) - body weight, number of corpora lutea, and number of implantation sites; for cesarean deliveries - resorption sites, live fetuses, fetal body weight, and fetal length; for natural deliveries - pup body weights by sex at 24 hours, four days, and 21 days post-partum and number of live and dead births.

III. RESULTS

A. Chamber Analyses

The grand means ± S.D. of N_2O and halothane generated for all analytical determinations during the 12-week exposure period were 48.8 ppm N_2O ± 5.8 plus 1.2 ppm halothane ± .3 and 499.1 ppm N_2O ± 17.9 plus 11.8 ppm halothane ± 2.5 for Groups 2 and 3, respectively. N_2O concentration ranges were 28.5 ppm - 60.5 ppm and 430 ppm - 540 ppm for Groups 2 and 3, respectively. Halothane concentration ranges were .5 ppm - 2.2 ppm and 5.1 ppm - 17.0 ppm for Groups 2 and 3, respectively.

B. Clinical Observations and Survival

All non-pregnant and pregnant females from the control and treated groups exhibited comparable appearance and behavior.

One female rat from Group 2 (48.8 ppm N_2O + 1.2 ppm halothane) was found dead during Week 8 of the exposure period (prior to mating).

No deaths occurred among the females scheduled for cesarean delivery. Survival among the females scheduled for natural delivery was 100% for Groups 1 and 2 and 89% (2/19) for Group 3. One of the Group 3 animals which died was found not to be pregnant. No premortem signs of toxic or pharmacologic effects were observed in either of these animals.

C. Body Weights

Group mean values ± (S.D.) for body weights for all mated females are presented in Table 2. Body weights were similar among all groups of non-pregnant females. Body weights for the treated animals selected for cesarean delivery were comparable to or greater

Table 2 - Group mean body weights ± (S.D.) for mated females.

Cesarean Delivery

Group No.	Non-Pregnant Day 0	Non-Pregnant Day 5	Non-Pregnant Day 16	Non-Pregnant Day 20	Pregnant Day 0	Pregnant Day 5	Pregnant Day 16	Pregnant Day 20
1	282.1 (16.2)	289.9 (26.0)	294.1 (22.1)	298.0 (18.9)	282.5 (12.3)	299.0 (15.0)	351.4 (21.8)	420.5 (27.2)
2	282.0 (4.1)	275.3 (19.0)	298.5 (15.3)	297.5 (12.7)	290.4 (18.9)	308.7 (20.6)	373.3* (25.9)	422.0 (46.2)
3	292.3 (6.7)	295.0 (13.2)	295.4 (12.4)	302.9 (10.0)	279.9 (15.4)	285.2 (23.5)	356.7 (18.9)	401.8 (37.2)

Natural Delivery

Group No.	Non-Pregnant Day 0	Non-Pregnant Day 5	Non-Pregnant Day 16	Non-Pregnant Day 20	Pregnant Day 0	Pregnant Day 5	Pregnant Day 16	Pregnant Day 20
1	293.0 (18.4)	297.0 (17.0)	298.0 (19.8)	323.0 (18.4)	290.3 (15.6)	308.0 (17.4)	355.0 (24.8)	420.8 (38.0)
2	289.8 (24.8)	308.6 (18.7)	326.8 (38.3)	315.6 (11.3)	283.3 (16.1)	309.2 (17.0)	356.1 (29.8)	386.2** (49.9)
3	290.8 (16.4)	311.8 (20.8)	314.5 (32.3)	309.8 (23.4)	281.6 (11.6)	305.8 (20.4)	348.0 (22.4)	380.0** (37.8)

* Significantly higher than pregnant controls (cesarean delivery) at $p \leq 0.05$.

** Significantly lower than pregnant controls (natural delivery) at $p \leq 0.05$.

than those of the cesarean controls at all recorded intervals during gestation. Body weights for the animals selected for natural delivery were comparable between the control and treated groups at Days 0, 5, and 16 of gestation. However, the weights for both of these treated groups were significantly lower than the natural delivery controls at Day 20 of gestation.

D. Fertility Index

The fertility indices (number of females pregnant*/number of females mated** x 100) for the females designated for cesarean delivery were 65% (13/20), 80% (16/20), and 65% (13/20) for Groups 1, 2, and 3, respectively. The fertility indices for the females scheduled for natural delivery were 89.4% (17/19), 72.2% (13/18), and 36.8% (7/19) for Groups 1, 2, and 3, respectively. The fertility index for Group 3 was significantly lower than the index for the natural delivery controls (Chi-square = 11.31, d.f. = 1).

E. Cesarean Data

Group mean values for ovarian, uterine, and litter data as well as the indices of pre-implantation loss, implantation efficiency, and post-implantation loss are presented in Table 3. With the exceptions of significantly lower mean fetal weight and mean fetal length for Group 3, all values for the treated females selected for cesarean delivery were comparable to the cesarean control group.

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* Pregnancy was based on the presence of uterine implantation sites.

** Mated females were those in which sperm were observed in vaginal washings.

Table 3 - Group mean ovarian, uterine, and litter data ± (S.D.) and group means for reproductive indices for pregnant females.

Cesarean Delivery

Observation	Group No.		
	1	2	3
Ovarian Corpora Lutea	18.5 (3.1)	15.2 (3.2)	14.8 (2.3)
Uterine Implantation Sites	13.4 (1.7)	11.9 (3.4)	11.9 (3.0)
Resorption Sites	0.5 (.8)	0.8 (.7)	0.8 (1.2)
Live Fetuses	12.8 (1.9)	11.1 (3.5)	11.2 (2.7)
Dead Fetuses	0 (0)	.06 (.30)	0 (0)
Mean Fetal Weight (g)	4.13 (.25)	4.01 (.66)	3.66* (.43)
Mean Fetal Length (cm)	4.1 (.1)	4.0 (.2)	3.9* (.2)
Pre-implantation Loss	5.1	3.3	2.9
Implantation Efficiency (%)	72.5	78.6	80.8
Post-implantation Loss Index (%)	4.0	6.8	7.7

* Significantly lower than cesarean controls at $p \leq 0.05$.

Findings observed at necropsy included a number of incidental changes which were considered unrelated to treatment and were observed at comparable incidences in the control and treated groups.

F. Fetal Examinations

Gross examinations of fetuses for external anatomical abnormalities and soft tissue examinations by Wilson's technique revealed no consistent unusual findings among the test or control fetuses. Major malformations consisted of ectopia of the right kidney in one Group 1 fetus. Fetal skeletal evaluations revealed a slight retardation in skeletal development in the test groups (Groups 2 and 3) when compared to the control group (Group 1). This retardation was indicated by significantly higher (Chi-square test) percent incidences of non-ossification in the sixth sternebrae, 25th - 26th caudal vertebrae, 3rd - 4th metacarpals of the forepaws, and 24th - 28th phalanges of the forepaws for the members of Groups 2 and 3.

G. Natural Delivery Data

A summary of the ovarian, uterine, and litter data and of the reproductive indices for mated females delivering naturally is presented in Table 4. One female in each group did not mate during the 21-day mating period.

The mean numbers of ovarian corpora lutea for Groups 2 and 3 and the number of uterine implantation sites for Group 3 were significantly lower than values obtained for the Group 1 control females. Implantation efficiency for Group 3 was significantly lower than the implantation efficiency for the control group (Chi-square = 41.44, d.f. = 1).

Table 4 - Group mean ovarian and uterine data ± (S.D.) and group means for reproductive indices and litter data for mated females.

Natural Delivery

Observation	Group No. 1	2	3
Ovarian Corpora Lutea	19.5 (3.8)	15.4* (5.9)	13.2* (5.2)
Uterine Implantation Sites	10.9 (5.5)	7.5 (6.2)	3.8* (6.2)
Pre-implantation Loss	8.6	7.9	9.4
Implantation Efficiency (%)	55.5	48.7	28.4*
Post-implantation Loss Index (%)	14.0	14.8	28.1**
Number of Pregnancies	17	13	7
Number of Full-Term Litters Born	16	12	4
Gestation Index (%)	94.1	92.3	57.1*
Number of Pups Born	178	115	46
Number of Pups Born Alive	177	110	44
Live Birth Index (%)	99.4	95.7	95.7

* Significantly lower than natural delivery controls at $p \leq 0.05$.

** Significantly higher than natural delivery controls at $p \leq 0.05$.

Table 4 - Continued.

Natural Delivery

Observation	Group No. 1	Group No. 2	Group No. 3
Number of Pups Left to Nurse	114	79	31
Number of Pups Weaned	100	65	30
Lactation Index (%)	87.7	82.3	96.8
Mean Litter Size	11.1	9.6	11.5
Mean Pup Body Weight (g) - 24 Hours			
Males	6.6 (.9)	6.5 (1.2)	6.1 (1.1)
Females	6.0 (.7)	5.8 (1.1)	5.6 (1.3)
Mean Pup Body Weight (g) - 4 Days			
Males	9.9 (3.2)	10.3 (1.8)	9.7 (1.2)
Females	9.5 (3.0)	9.9 (1.5)	9.2 (1.9)
Mean Pup Body Weight (g) - Weaning			
Males	49.5 (7.2)	50.4 (4.3)	40.9* (5.5)
Females	47.1 (6.7)	44.8 (8.0)	41.4 (3.4)

* Significantly lower than natural delivery controls at $p \leq 0.05$.

A Chi-square analysis of the percentage of implants not accounted for in dead fetuses or live births observed showed that Group 3 had a significantly larger proportion unaccounted for than Group 1 (Chi-square = 5.13, d.f. = 1). These were either resorbed or the pups cannibilized at birth. The "effect" was largely due to the complete loss of 13 implantations in one of the seven pregnant females in Group 3. Since one female in Group 1 also lost 13 out of 13 implantations, it is not clear that a biological effect should be inferred.

Only one instance of dystocia or delayed or prolonged labor was observed among the control and test animals allowed to complete the gestation period with natural deliveries. One animal was found dead on Day 24 of gestation with one dead fetus in the vagina and 13 dead fetuses in the uterine horns. Gestation indices were 94.1, 92.3, and 57.1% for the control group, Group 2, and Group 3, respectively. This index for Group 3 was significantly lower than the index for the control group (Chi-square = 4.88, d.f. = 1). The biological significance of this decrease at the high treatment level was difficult to assess due to the relatively few pregnancies in this group. The mean duration of the gestation period was 22.7, 23.1, and 21.8 days for Groups 1, 2, and 3, respectively (excluding the one Group 3 pregnant female found dead on Day 24 of gestation).

There was no indication of a compound-related effect with regard to the incidence of stillbirths or of pups found dead or missing during lactation. One control, five Group 2, and two Group 3 pups were found dead within 24 hours post-delivery. Fourteen control, fourteen Group 2,

and one Group 3 pups were either found dead or found to be missing due to apparent cannibalization during lactation. Live birth and lactation indices were similar among the groups.

There was no indication of a treatment-related effect regarding the mean number of male and female offspring produced per litter in either of the test groups.

The appearance and behavior of offspring of the treated females revealed no evidence of a compound-related effect although the pups at the high treatment level were smaller than the controls.

Statistical analyses of group mean body weights of male and female offspring revealed no significant differences between the control and treated groups at 24 hours and 4 days post-delivery. At weaning, a significantly lower mean body weight was indicated for the Group 3 male pups when compared to the controls. The females were smaller but not significantly so.

Gross necropsy of females which delivered naturally and their offspring did not reveal any alterations in any organs or tissues attributable to the treatment program.

IV. SUMMARY AND CONCLUSIONS

Two hundred forty sexually mature albino rats, 120 males and 120 females, were subdivided into three groups of 40 males and 40 females each. Two groups were each exposed to a mixture of nitrous oxide (N_2O) and halothane seven hours per day, five days a week, for 12 weeks. The remaining group was exposed to room air under the same conditions. The animals from the respective groups were mated after the 12-week exposure period outside the chambers until a sign of mating (vaginal sperm) or 21 days of cohabitation had been completed. The mated females were re-exposed on Days 1 through 15 (natural delivery) or Days 6 through 15 (cesarean delivery) of gestation. Following the mating period, the males were returned to the exposure chambers for use on a cytogenetic study to follow. On Day 20 of gestation the first 20 mated females of each group were sacrificed; litters were obtained by cesarean sections and subsequent fetal examinations performed. The remaining mated females were allowed to deliver naturally and neonatal development was observed through weaning. Group designations for this study are listed below.

Group No.	No. of Animals		Treatment	Mean Analytical Concentration ± S.D.
	m	f		ppm
1	40	40	Air control	-
2	40	40	N_2O + halothane	48.8 ± 5.8 1.2 ± .3
3	40	40	N_2O + halothane	499.1 ± 17.9 11.8 ± 2.5

Patterns of appearance and behavior of all females from the control and treated groups were comparable. One female from Group 2 was found dead during Week 8 of the exposure period (prior to mating) and was thus excluded from subsequent evaluations of reproduction data. Survival during gestation was 100% in all groups except among the Group 3 females scheduled for natural delivery; two out of 19 females in this group died during gestation. One of these females was not pregnant and was excluded from calculations of reproductive indices; the second female was pregnant (14 dead fetuses) and was included in calculations of the fertility and gestation indices only. There were no findings in any of these animals which indicated a treatment-related effect as the cause of death.

Body weight data for females selected for cesarean delivery were similar for the control and test groups at all recorded intervals during gestation. Body weights for the females selected for natural delivery were comparable between the control and test groups until Day 20 of gestation when significantly lower mean body weights were recorded for both treated groups when compared to the mean control weight.

With the exceptions of a significantly lower mean fetal weight and mean fetal length for Group 3 and a slight retardation in skeletal development for Groups 2 and 3, all reproduction data for the treatment groups resulting from the cesarean deliveries were comparable to the cesarean control group.

Differences from control values for the Group 3 animals selected for natural delivery included (a) significantly lower fertility index;

(b) significantly lower gestation index; (c) significantly lower mean numbers of ovarian corpora lutea and uterine implantation sites; (d) significantly lower implantation efficiency; (e) significantly higher post-implantation loss index; and (f) significantly lower mean body weight for the male pups at weaning. The mean number of corpora lutea was also lower for Group 2. Because of the few number of pregnancies in Group 3 (thus, a small sample size) and the complete loss of 13 implantations in one of the seven pregnant females in Group 3 as well as the loss of 13 out of 13 implantations for a Group 1 female, the increased post-implantation loss for Group 3 perhaps should not be conclusively interpreted to be an effect of the exposure to N_2O and halothane. On the other hand, the large relative pre-implantation loss, as indicated by implantation efficiency, appeared to be a clear-cut effect of the high level exposure of the females during the first six days after mating since the cesarean-delivered animals did not show this effect.

In summary, exposure of mated female rats to the combinations of N_2O and halothane resulted in reduced ovulation and implantation efficiency at the higher level of exposure during the first six days after mating and a slightly retarded development of the fetuses at both levels of exposure. No clear indication of exposure-related post-implantation loss was observed; a significant loss was recorded for the high level exposure females exposed from Day 1 after mating but no loss was observed for those exposed only from Day 6 on. Thus, the effects seen in rats did not clearly parallel the epidemiological findings reported in humans. The relative increase in stillbirths and spontaneous abortions in humans

were not apparent in the rats. Whether this represents a species difference in response to exposures to these agents or some difference in exposure parameters cannot be answered at this time. Nonetheless, it can be concluded that exposure to 10 ppm of halothane plus 500 ppm of N_2O can cause toxic effects on reproduction in the rat and thus should be of concern to humans in the reproductive age bracket.

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