Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification: Dinitrogen oxide

EC Number: 233-032-0

CAS Number: 10024-97-2

Index Number: -

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Version number: 2 **Date: April 2022**

CONTENTS

1 PHYSICAL HAZARDS

Hazards not addressed in the CLH report.

2 HEALTH HAZARDS

2.1 Reproductive toxicity

2.1.1 Animal data

2.1.1.1 Holson et al., 1995

R.R. Holson, H.K.Bates, J.B. Laborde, DK. Hansen. Behavioral teratology and dominant lethal evaluation of nitrous oxide exposure in rats. *Neurotoxicology and Teratology* 17, n° 5 (1995): 583-92.

Detailed study summary and results

Test substance

- Dinitrogen oxide (checked for purity).

Test animals

- Crl:COBS CS (SD) BR outbred albino rats.
- Animals/group: see below
- Age and weight at the study initiation: 8-10 weeks of age, 225-300g for females, males were 10 and 14 weeks old at the onset of the study.

Administration/exposure

- Inhalation, $1m^3$ inhalation chambers, fitted with air-tight glass door in front. The animals lived in inhalation chambers during exposure but were returned to acrylic cages in the home vivarium on weekends.
- In all three studies, the animals exposed to 0, 1000, 5000 or 10,000 ppm dinitrogen oxide in air.
- Whole body exposure, (day of mating = gestation day 0), 6h per day. In the maternal study, dams were exposed for 20 consecutive days (GD1-20) and 3 hours on GD 21.
- Concentration levels: 0, 1000, 5000, 10,000 ppm in air
- Analytical verification of test atmosphere concentrations: yes, daily verification.

Description of test design:

Study 1: Dominant lethal study

- Males used in study 3 were mated with females. In addition 3 more replicates were used and mated with females. Thus, 24 potential males in each four groups were mated to 30 potential dams. In total there were 28, 26, 28 and 29 litters in 0, 1000, 5000 and 10,000 pm dinitrogen oxide exposure groups, respectively.
- Two weeks after end of male exposure, dams were sacrified and checked for uterine content. The number of live foetuses, dead foetuses and resorptions were recorded.

Study 2: Effect of exposure of pregnant rats on offspring behavior (maternal study)

- Female were mated with a single male. Three replicates, 4 litters per dose groups (total of 48 litters).
- Litter size was reduced to 8 pups (4/sex). Other pups were killed by exposure to carbon dioxide.
- Litters were weaned on PND21 (GD22 = PND1, day of plug=GD0). At this time, dams and 2 pups per litters were killed.
- The remaining 6 pups/litter were rehoused two per cage.
- Offspring were weighted at PND0, PND21, PND50 and PND100.
- Behavioral test (2 pups per groups): negative geotaxis on PND6-9; developmental activity on GD12, 16, 20 and 24; auditory startle on PND 18, 36, 55; Amphetamine challenge on PND42; 23h activity on 2 PND70, Barbiturate anesthesia on PND 59; complex maze (days not specified), Passive avoidance response at PND100

Table 1: Behavioral test order

Negative geotaxis	PNDs 6-9
23h activity	PND 68-73
Complex maze	PNDs 83-85, 90-92
Passive avoidance response	PNDs100-101
Developmental activity	PNDs 12, 16, 20, 24
Amphetamine challenge	PNDs 40-42 and 84-86
Auditory startle	PNDs 18, 36, 55
Barbiturate anaesthesia	PND 59

Study 3: Effect of exposure of male rats and behaviour of their offspring (paternal study).

- Proven male breeders were exposed to dinitrogen oxide for 5d/w for 9 weeks. Three replicates with 4 males per dose replicate. There were in total 10 control litters, 11 litters exposed to 1,000 ppm and 12 litters exposed to 5,000 and 10,000 ppm.
- Same behavioral assay as in study 2.

Results and discussion

Study 1: Dominant lethal assay.

- No statistically significant effect (chi-square test or one-way ANOVA). Dose-related trend in the number of resorptions at the highest dose slight increase lethality.

Table 2: Results of dominant lethal study per litters (mean ± SD)

Dose (no. rat)	Number of implants	Live foetuses	Resorptions	Dead
Control (n=28)	15.6±0.4	14.4±0.4	1.1±0.2	0.07±0.05
1000 ppm (n=26)	15.6±0.6	13.8±0.6	1.2±0.3	0.00±0.00
5000 ppm (n=28)	14.6±0.6	13.3±0.6	1.3±0.3	0.04±0.04
10,000 ppm (n=29)	14.8±0.6	13.0±0.6	1.8±0.5	0.03±0.03

Study 2 and 3: paternal and maternal study.

- No effect on weight of dams or offspring in the maternal or paternal studies. Maternal weight gain was unaffected by treatment.
- Maternal weight gain and pups weight were unaffected by treatment.
- No effect on litter size was noted in the paternal and maternal studies.
- In the behavioral studies with offspring, although few significant findings were noted in the assays (five statistically significant main effects), the authors concluded that overall there were no evidence for a treatment-related effect (differences between sexes, no clear dose-response or trend).

Table 3: Litter size (mean number of live pups per litter)

Variable	Exposed parent	Dose (ppm)			
		Control	1000	5000	10,000
Litter size	Dams	14.0±0.6	12.3±1.0	13.8±0.6	13.9±0.7
	Males	14.4±0.5	12.8±0.7	13.4±0.8	13.3±0.7

2.1.1.2 Fujinaga et al., 1991

M. Fujinaga, J.M. Baden, A; Suto, J.K. Mya, R.I. Mazze. Preventive effect of phenoxybenzamine in nitrous oxide induced reproductive toxicity in Sprague-Dawley rats. Teratology 43:151-157, 1991

Detailed study summary and results (only results with dinitrogen oxide are detailed in this report)

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group: 25 in dinitrogen oxide groups, 30 in control group
- Age and weight at the study initiation: 11-week old timed-pregnant females

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity.
- Whole body, 24 hours on GD8 of pregnancy (day of plug = day 0 of pregnancy)
- Concentration levels: 600,000 ppm dinitrogen oxide. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration. Total flow rate was 20-30l/min.
- In this study, groups of rats were exposed to folinic acid and halothane alone or in combination with dinitrogen oxide.
- Control group: compressed air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements

Description of test design:

- Two separate studies were performed using the same design.
- All rats were weighted before and after exposure and every 2-4 day during the study.
- Sacrifice on day 20 of pregnancy by carbon dioxide inhalation and caesarean sections were performed (The day of plug observed in the vagina was define as day 0 of pregnancy).
- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and all foetus were examined for external abnormalities. Every other foetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). All examinations were made without knowledge of the treatment group.
- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- Rats exposed 600,000 ppm dinitrogen oxide appeared mildly sedated and rested quietly during the exposure period.
- Treatment with 600,000 ppm dinitrogen oxide alone resulted in increased incidences of foetal resorptions, major and minor visceral malformations, and minor skeletal anomalies. Among the foetuses exposed to dinitrogen oxide alone, 28% showed altered body laterality.

Table 4: Summary of maternal and reproductive data (Fujinaga et al., 1991)

	Control	**600,000 ppm dinitrogen oxide**

No. rat studied/ examined on day 20	30/30	25/24
Mean bw of rats before exposure (g)	249	248
Mean bw after exposure (g)	226	217
Mean bw on GD 20	368	334
Death during exposure (no.)	0	1
Dams with resorptions only	0	1
Dams with live foetus	28	22
Dams with resorptions	14	21
Dams without resorptions	14	2
Mean number of live foetuses per litter	12.0±2.1	6.5±4.1*
Mean percent resorptions per litter	5.0±5.6	48.1±32.7*

*p<0.05

Table 5: Number of abnormalities in foetuses (litter)

	Control	**600,000 ppm dinitrogen oxide**
External examination		
No. examined	337 (28)	150 (22)
Runt	0	1(1)
Major malformations	1(1)	3(2)
Skeletal examinations		
No. examined	168(28)	78 (21)
Major malformations	2(2)	0
Minor anomalies - Cervical ribs	14(10)	46*(18)*
Developmental variants - 14th rudimentary rib		
Visceral examination		
No. examined	169 (28)	72 (21)
Major malformations - Situs inversus - Cardiac anomalies - Hydrocephalus - Others	 0 0 0 0	 20*(13)* 4*(3) 1(1) 3*(2)
Minor anomalies - Left sided umbilical artery	3(3)	18*(16)*

P<0.05 compare to control

2.1.1.3 Kugel et al., 1990

G.Kugel, C. Letelier, M.A. Zive and J.C. King. Nitrous Oxide and Infertility. Anesth Prog 37: 196-180, 1990

Detailed study summary and results:

Test type

Rats, non-guideline fertility study

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- 64 female Sprague-Dawley rats
- Age and weight at the study initiation: 180-200 g

Administration/exposure

- Inhalation: plexiglass environmental chamber in vacuum hood
- Whole body, 8 hours/day for 4 days or one ovulatory cycle.
- Concentration levels: 300,000 ppm, flow rate 1.6 L/min
- Control group: compressed air mix, identical environmental chamber. Exposed animals also acted as their own controls by cycling normally for two cycle before exposure.
- Vehicle: Compressed air
- Analytical verification of test atmosphere concentrations: yes throughout the study

Description of test design:

- Group 1: At termination of exposure, 8 exposed and 8 control rats were anesthetized with pentobarbital before fixation of their brain for study of hypothalamic LHRH-producing cells. Half of the rats were exposed on morning of proestrus and half on the morning of metestrus.
- Group 2: 12 rats were cycles via vaginal smears during and following exposure until the cycles returned to normal. 12 rats were used as controls.
- Group 3: 12 exposed and 12 treated rats were mated with proven male breeder for 4 days. Half were mated on the start of proestrus and half on various day of the cycle.

Results and discussion

- Group 1: increased in total LHRH cell count in animals treated on the morning of protestrus (~4-fold) but not in animals exposed in the morning of metestrus.
- Group 2: disrupted cycles following the first day of exposure and 11/12 exposed rats were into constant proestrus. The effect resolved after around 3 weeks. Normal cycle throughout the experiment in controls.
- Group 3: mating has occurred in all animals. Three of six exposed female gave birth compare to all controls giving birth (50% decrease fertility). No effect on the weight and on litter size was noted between exposed and control animals.
- No statistical analysis performed.

2.1.1.4 Fujinaga et al., 1990

M. Fujinaga, J.M. Baden T.H. Shepard and R.I. Mazze. Nitrous oxide body laterality in rats. Teratology 41:131-135, 1990.

Detailed study summary and results

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group: 35 in control group, 35 in treatment groups
- Age and weight at the study initiation: not stated

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity. The animals were place in the chambers in their cage without food and water.
- Whole body, 24 hours on day 8 of pregnancy (day of plug = day 0 of pregnancy)
- Concentration levels: 700,000-750-000 ppm dinitrogen oxide. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration. Total flow rate was 20-30l/min.
- Control group: compressed air

- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements

Description of test design:

- All rats were weighted before and after exposure and every 2-4 day during the study.
- Four to six rats from each groups were randomly killed by carbon dioxide inhalation on each days 11-16, 18 and 20 of gestation and laparotomy was performed.
- The viability of each embryo was ascertained. The side of tail flexion was also determined on day 11 and 12, the side from which the umbilical artery emerged from the body on days 13 and 14 and the side of body facing placenta on days 15 and 16. Foetuses obtained on days 18 and 20 were examined for viability and preserved in Bouin's solution. At a later date, the side to which the aortic arch curved was determined using a modification of the method described by Barrow and Taylor.

Results and discussion

- The overall embryofoetal mortality rate in the control group was 8.9 ±6.2% (mean ±S.D.). Within the dinitrogen oxide group, there was a stepwise increase in mortality on day 14 of gestation with the mean value increasing from 15.3 ± 2.1% on days 11-13 to 40.8 ± 3.3% on days 14-20 of gestation ($P < 0.05$). The increase was first observed on GD14 and then remained constant.
- Altered laterality was not detected in any of the control groups except on day 16 when 9% of control foetuses faced left. In contrast, dinitrogen oxide treatment resulted in high overall incidence of altered laterality (31.3% vs. 1.3%) as well as high incidences at all stages of development.

Table 6: Data from the dinitrogen oxide treated and control groups

Gestation day	**11**	**12**	**13**	**14**	**15**	**16**	**18**	**20**
Dinitrogen oxide								
Pregnant rats	4	4	4	4	4	5	4	4
Resorptions (litters with resorptions	7 (2)	8 (3)	4 (2)	23* (4)	18* (4)	23* (5)	14* (4)	20* (3)
Altered laterality (litters)	14* (3)	13* (4)	9* (3)	13* (3)	4* (2)	9* (4)	7* (3)	7* (4)
Controls								
Pregnant rats	4	3	3	4	4	4	3	4
Resorptions (litters with resorptions	5 (3)	2 (2)	1 (1)	1 (1)	8 (3)	3 (3)	4 (2)	3 (3)
Altered laterality (litters)	0	0	0	0	0	4(3)	0	0

Table 7: Number of embryos/foetuses with altered body laterality

Gestational day	**Endpoint**	**Control**	**Dinitrogen oxide**
11 and 12	Side of tail flexion	0/57 (0/7)	27/76* (7/7*)
13 and 14	Side of the body from which the umbilical artery emerged	0/91 (0/7)	22/57* (6/8*)
15 and 16	Side of the body that faced the placenta	4/85 (3/8)	13/55* (6/8)
18 and 20	Side to which the aortic arch curved	0/71 (0/7)	14/55* (7/8*)
Overall	All (pooled)	4/304 (3/29)	76/243* (26/31*)

*$p < 0.05$

2.1.1.5 Rice et al., 1990

Study reference

Rice SA. Effect of prenatal N_2O exposure on startle reflex reactivity. Teratology. 1990 Oct;42(4):373-81.

Detailed study summary and results

- Mice, postnatal developmental toxicity study

Test substance

- Dinitrogen oxide (no information on purity)

Test animals

- SW mice (Hla:[SW]BR), female and male
- 6-7 week-old virgin
- 10 litters examined per group

Administration/exposure

- Inhalation exposure in their home cage, Three 1000 L chambers through 2-inch i.d. plexiglass duct.
- Control: room air
- Concentration: 50,000, 150,000 ppm, 350,000 ppm for 4h per day on GD6-15.
- Dinitrogen oxide was mixed with oxygen and air
- Analytical verification of test atmosphere concentrations: yes

Description of test design:

- Dams were weight throughout the experiment.
- Pups: liveborn, stillborn, physical growth, maturation, brain weight in one male per litter
- Litters were culled on PND1 to 8 pups. No less than three pups of one gender were acceptable per litter. Any litters not meeting these criteria were eliminated from the study.
- Behavioral test: rotating rod (PND49), reactivity (PND60, PND95).

Results

- Ten litters per group were examined.
- Reproductive indices and postnatal surviva1 were not altered by exposure. Litters eliminated from the study due to insufficient numbers of pups or pups of one gender were evenly distributed among exposure groups.
- In the preweaning period, body weight of dinitrogen oxide-exposed groups showed a significant effect of exposure over days ($p<0.0l$). Exposure effects were isolated to PND 1, 4, 7, and 21 where the dinitrogen oxide-exposed groups tended to weigh more than the air-exposed group. There were no effects due to gender. Weights in the postweaning period showed significant effects due to gender ($p<0.001$) and a day by exposure effect ($p<0.03$).
- There were no developmental delays resulting from exposure: pinna detachment (range: PND 4.1-4.31), incisor eruption (range: PND 11-11.3), and eye-opening (range: PND 14.4-14.9).
- Brain weights measured on PND 126 or 127 were not different among groups or between genders.
- Rotating rod: The times for two successive trials on the rotating rod at PND **49** showed significant effects due to trial ($p<0.01$).
- Reactivity: The counts resulting from each stimulus have been averaged across the six stimuli for presentation purposes. Data were analysed nonparametrically by Kruskal-Wallis one-way ANOVA. There were significant exposure effects for both stimuli on both PND 60 ($P<0.02$) and PND 95 ($P<0.001$); there were no effects due to gender. Although the dinitrogen oxide-exposed groups appeared less responsive than the air exposed groups to both acoustic and tactile stimuli on PND 60, corrections for multiple comparisons to isolate specific differences between the air group, and the N20 groups prevented statistical significance. Comparisons of data from PND 95 revealed that all dinitrogen oxide exposure groups were significantly hyporeactive compared with the air control group ($P<0.05$ for each group). As is readily apparent, there is a significant age-related difference in startle response ($P<0.01$); control animals were significantly more reactive at 95 than at 60 days of

age. Of the dinitrogen oxide-exposed groups, only the 15% group showed a statistically significant increase in reactivity from 60 to 95 days of age.

2.1.1.6 Kugel et al., 1989

Study reference

G. Kugel, C. Letelier, H. Atallah, M.zive. Chronic low level Nitrous Oxide Exposure and Infertility. *J Dent Res* (1989) 68: 313

Abstract

The study was done to determine of chronic dinitrogen oxide exposure affects ovulation fertility and/or offspring. The study employed 24 female rats. 12 rats were placed in an environmental chamber with a mix of 500 ppm dinitrogen oxide in compressed air, 8h/day for 35 days. All exposed rats had disrupted ovulatory cycle consisting of constant proestrous and lasting 3 weeks. Ovulatory cycle gradually return to normal. Control rats had normal cycles. All rats were mated with control breeders. 6 of 12 dinitrogen oxide and 12 of 12 controls rats gave birth. No effect on litter size of weight of pups was noted.

2.1.1.7 Fujinaga et al., 1989

Study reference

M. Fujinaga, J.M. Baden, R. I. Mazze. Susceptible period of nitrous oxide teratogenicity in Sprague-Dawley rats. Teratology 40:439-44, 1989.

Detailed study summary and results

- Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group: 30 in control group, 20 in treatment groups
- Age and weight at the study initiation: 9-11 week-old

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity. The animals were place in the chambers in their cage without food and water.
- Whole body, 24 hours at GD 6, 7, 8, 9, 10, 11 or 12 of pregnancy
- Concentration levels: 600,000 ppm dinitrogen oxide. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration. Total flow rate was 20-30l/min.
- Control group: compressed air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements
- Temperature in the chamber range from 20-30 °, averaging 24°.

Description of test design:

- All rats were weighted before and after exposure and every 2-4 day during the study.
- Sacrifice on day 20 of pregnancy by carbon dioxide inhalation and caesarean sections were performed.
- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and all foetus were examined for external abnormalities. Every other foetus was fixed in 70% ethanol and

macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). Overall, half of the foetuses were examined for visceral anomalies and half of the foetuses were examined for skeletal abnormalities. All examinations were made without knowledge of the treatment group.

- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- A total of 15 rats among the 140 exposed to dinitrogen oxide died during the exposure. The mean maternal weight a caesarean section was decreased among all dinitrogen oxide treatment groups. The authors has no explanation on this high mortality rate.
- Dinitrogen oxide exposed rats appeared mildly sedated.
- There were no effects on the number of implantations, live foetuses, foetal weight, sex ratio, runts, and external abnormalities.
- An increase in foetal wastage was noted when rats were exposed on days 8 and 11 of gestation.
- Skeletal abnormalities of the ribs and vertebrae (including major malformations such as multiple vertebrae or multiple fusion of the ribs) were increase following exposure on gestational day 9.
- Cervical ribs (variations) only occurred following exposure on GD8.
- The incidence of right aortic arch and left-sided umbilical artery was increased when rats were exposed on GD8 of gestation. An increase incidence of hydrocephalus was also noted when rats were exposed on GD 9.
- The authors considered that the effects were not related to the high rate of mortality observed in the study as no such mortality rate was observed in their previous studies (1 among 200 animals exposed to 500,000 ppm dinitrogen oxide) and similar findings were noted.

Table 8: Summary of a selection of maternal variables and reproductive data

Gestation day	**Control**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
No rats that died during exposure	0	2	2	2	2	0	4	3
Mean body weight of pregnant rats (g)	352±26	309±33*	318±30*	321±28*	330±27**	322±20*	296±28*	313±18*
Mean % resorption per litter	3.4	18.9	5.5	36.8*	11.6	16.5	37.3*	20.1

Table 9: Selected major malformations and minor anomalies observed on GD 8 and 9 in foetuses (litter) in Fujinaga et al., 1989

Gestation day	Control	8	9	11
Skeletal examination				
Foetus examined	131	49	82	69
Major malformation - Ribs - Vertebrae	 0 0	 0 1(1)	 9*(3**) 7*(3**)	 0 0
Minor anomalies - Cervical ribs	 1(1)	 20*(9**)	 2(2)	 0
Visceral examination				
Foetuses examined	130	51	83	45
Major malformations - Right sided aortic arch - Hydrocephalus	 0 0	 11*(9*) 1(1)	 0 6*(4**)	 0 0
Minor anomalies - Left-sided umbilical artery	 3(3)	 9*(7*)	 4(4)	 1(1)

2.1.1.8 Mazze et al., 1988

R.I. Mazze, M. Fujinaga and J.M. Baden. Halothane prevents nitrous oxide teratogenicity in Sprague-Dawley rats; Folinic Acid does not.

Detailed study summary and results (only results with dinitrogen oxide are detailed in this report)

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group:
  - Experiment 1: 90 rats. 30 controls (air), 20 per exposed groups (Dinitrogen oxide, folinic acid, dinitrogen oxide + folinic acid groups).
  - Experiment 2: n=116 rats. N=37 controls (air), n=26 in dinitrogen oxide group, n= 27 in folinic acid group, n=26 in folinic acid and dinitrogen oxide group
  - Methionine synthase activity: 65 rats. 5 controls (air), 20 in exposed groups (Dinitrogen oxide, dinitrogen oxide + halothane, dinitrogen oxide + folinic acid groups). Only 5 per each groups subject to the assay.
- Age and weight at the study initiation: 11-week old timed-pregnant females

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity. The animals were place in the chambers in their cage without food and water.
- Whole body, 24 hours GD8 of pregnancy
- Concentration levels: 500,000 ppm dinitrogen oxide in experiment 1 and 750,000 ppm in experiment 2. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration. Total flow rate was 20-30l/min.
- In this study, groups of rats were exposed to folinic acid and halothane alone or in combination with dinitrogen oxide.

- Control group: compressed air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements

Description of test design:

- Two separate studies were performed using the same design.
- All rats were weighted before and after exposure and every 2-4 day during the study.
- Sacrifice on day 20 of pregnancy by carbon dioxide inhalation and caesarean sections were performed.
- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and all foetus were examined for external abnormalities. Every other foetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). Overall, half of the foetuses were examined for visceral anomalies and half of the foetuses were examined for skeletal abnormalities. All examinations were made without knowledge of the treatment group.
- In addition, five rats per group were killed immediately, 24, 48 and 72 hours after exposure and liver was removed for assay of methionine synthase activity.

Results and discussion

- Rats exposed 500,000-750,000 ppm dinitrogen oxide appeared mildly sedated and rested quietly during the exposure; they occasionally changed their posture and moved around the cage.
- Rats exposed to dinitrogen oxide alone weighed significantly less than control rats at caesarean section, but the fewer number of live foetuses that they carried can explain almost all of the difference.
- Treatment with dinitrogen oxide alone resulted in increased incidences of foetal wastage, major visceral malformations (primarily right-sided aortic arch), minor skeletal anomalies (primarily vertebral and rib anomalies), and skeletal developmental variants. No findings were noted for external examination by the study authors.

Table 10: Summary of foetal examination and reproduction indices (mean +/- SD)

	Control	**Dinitrogen oxide**
No. rat studied/ examined on day 20	67	46
Mean bw of rats on arrival GD4 (g)	205±20	206±17
Mean bw on GD 20	362±36	332±28*
No. of rats pregnant	58	37
No. live foetuses/rat	12±3.9	8.1±4.3*
Early resorptions/rat (%)	7.3±19	26.2*±26
Late resorptions/rats (%)	0.1±0.8	8.8*±14
Total foetal wastage/rat (%)	7.4±19	35±4.5
Mean foetal weight/rat	4.7±0.7	4.5±0.7
Female foetuses/rat (%)	46±19	52±22

Table 11: Foetal examination (Mazze et al., 1988)

	Control	Dinitrogen oxide
Visceral examination		
No. foetuses examined	348	149
Major malformations (%)	0.3±1.9	18.8*±32
Minor anomalies (%)	7.7±11.6	16±26
Skeletal examination		
No. of foetuses examined	346	152
Major malformations (%)	0.0±0.0	2.8±17
Minor anomalies (%)	27±29	59*±33

**p<0.05 vs control*

- Results of treatment with dinitrogen oxide plus folinic acid were essentially the same as treatment with dinitrogen oxide alone, including the type of anomalies that occurred. The only beneficial effect of folinic acid was a partial reduction in minor skeletal anomalies (P = .06 vs. dinitrogen oxide alone).
- In the methionine synthase experiment, folinic acid did not prevented the effects of dinitrogen oxide.

2.1.1.9 Fujinaga et al., 1987

M. Fujinaga, J.M. Bade, E.O.Yhap, R.I. Mazze. Reproductive and teratogenic effects of nitrous oxide, isoflurane, and their combination in Sprague-Dawley rats. 1987 Dec;67(6):960-4.

Detailed study summary and results (only results with dinitrogen oxide are detailed in this report)

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group: 40 in control group, 30 in treatment groups
- Age and weight at the study initiation: 9-11 week-old

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity. The animals were place in the chambers in their cage without food and water.
- Whole body, 24 hours GD-8 of pregnancy (plug day = day 0 of pregnancy)
- Concentration levels: 500,000 ppm dinitrogen oxide. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration. Total flow rate was 20-30l/min.
- Control group: compressed air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements
- Temperature in the chamber range from 20-30 °, averaging 24°.

Description of test design:

- Three separate studies were performed using the same design.
- All rats were weighted before and after exposure and every 2-4 day during the study.
- Sacrifice on day 20 of pregnancy by carbon dioxide inhalation and caesarean sections were performed.

- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and all foetus were examined for external abnormalities. Every other foetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). Overall, half of the foetuses were examined for visceral anomalies and half of the foetuses were examined for skeletal abnormalities. All examinations were made without knowledge of the treatment group.
- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- No rats died prior to the scheduled necropsy. Rat exposures to 500,000 ppm dinitrogen oxide were mildly sedated.
- Statistically significant weight loss was observed during exposure. Body weight was decrease compare to control on GD12 and 20.

Table 12: Selected maternal and foetal observations, (mean +/- SD)

Dinitrogen oxide	**Control**	**500,000 ppm**
No. of rats examined	37	26
Weigth (mean, g)		
- GD6 (on arrival)	211±15	210±12
- GD8 (before exposure)	233±15	231±13
- GD9 (after exposure)	212±15	208±11
- GD12	254±17	241±12*
- GD14	271±18	259±14
- GD16	290±21	279±24
- GD20 (at caesarean section)	348±32	328±24*
Weight loss during the exposure	21±4	24±5
Early resorptions/rat (%)	4.9 ±10.3	18±19.9*
Late resorptions/rat (%)	0	6.8±11.7*
Total foetal wastage/rat (%)	4.9±10.3	25.9±28*
Total live foetuses/rat	11.7±3.4	9.5±3.5

*p<0.05 vs control

- A statistically significant increase in early resorptions, late resorptions and total foetal wastage was noted in the dinitrogen oxide exposure group.
- A statistically significant increase in major visceral malformation and abnormalities was noted in the 500,000 ppm exposure group. The predominant lesion was a right-sided aortic arch in 5 out of 26 litters exposed to dinitrogen oxide.
- Skeletal abnormalities were also increased in the dinitrogen oxide group.

Table 13: Results of foetal examination (Fujinaga et al., 1987), mean +/- SD

	Control	500,000 ppm dinitrogen oxide
No. foetuses examined for visceral examination	216	123
Major visceral malformation	0±2.1	14.9±30.2*
Minor visceral anomalies	10±10.3	17.1±19.3
No. foetuses examined for skeletal examination	217	118
Minor skeletal anomalies	0.8±3.6	5.5±12.4
Developmental variants	15.2±20.8	33±28*

**p<0.05 vs control*

2.1.1.10 Mazze et al., 1987

R.I. Mazze, M. Fujinaga and J.M. Baden; Reproductive and teratogenic effects of nitrous oxide, fentanyl and their combination in Sprague-Dawley rats.

Detailed study summary and results (only results with dinitrogen oxide are detailed in this report)

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- Animals/group: 3 independant experiments : 114 in total in control (34 to 40 per study), 24 to 30 animals per groups in dinitrogen oxide exposed groups
- Age and weight at the study initiation: not stated

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000l capacity. The animals were place in the chambers in their cage without food and water.
- Whole body, 24 hours GD9 of pregnancy (plug day = day 1 of pregnancy; corresponding to GD8 for plug day = GD0 of pregnancy)
- Concentration levels: 350,000 ppm, 500,000 ppm dinitrogen oxide. Dinitrogen oxide was mixed with oxygen and room air to achieve the desired dinitrogen oxide and oxygen concentration.
- Control group: compressed air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, continuous measurements

Description of test design:

- Three separate studies were performed using the same design.
- All rats were weighted before and after exposure and every 2-4 day during the study.
- Sacrifice on day 21 of pregnancy by carbon dioxide inhalation and caesarean sections were performed.
- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and all foetus were examined for external abnormalities. Every other foetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). Overall, half of the foetuses were examined for visceral

anomalies and half of the foetuses were examined for skeletal abnormalities. All examinations were made without knowledge of the treatment group.

- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- The results of the three studies were combined since the design was identical and as analysed separately, no differences were noted in the three groups.
- Dams exposed to dinitrogen oxide appeared only mildly sedated. Weight loss was significant compare to control in the group exposed to 500,000 ppm dinitrogen oxide. Total weight gain was not affected by treatment in the 350,000 ppm group but was significantly decreased in the 500,000 ppm group.
- A statistically significant decrease in the number of live foetuses and a statistically significant increase in resorptions and post-implantation foetal losses were noted in the 500,000 ppm group.

Table 14: Maternal weight effects and reproductive indices (mean±SD) (Mazze et al., 1987)

	Control (group 1)	**350,000 ppm dinitrogen oxide (Group 3)**	**500,000 ppm dinitrogen oxide (group 5)**
Weight on arrival (GD6) (g)	213±3	212±2	212±3
Weight at GD21 (g)	351±32	357±23	321±26*
Weight loss during exposure (g)	23±3	24±5	29±6*
Weight gain during the experiment (g)	135±23	139±15	106±23*
Total live foetuses/rat	11.2±3.0	11.6±1.7	7.7±3.5*
Total resorptions/rat	0.5±1.0	0.6±0.9	4.1±3.5*
Post-implantation losses/rat (%)	5.8±14.6	5.4±7.6	34±28*

*p<0.05 (Student's t test)

- There were no increased in external abnormalities in any group.
- A statistically significant increase in skeletal anomalies was noted in the 500,000 ppm dinitrogen oxide group. Major and minor visceral malformations were also increased in this group.

Table 15: Summary of foetal examination (mean +/- SD)

	Control (group 1)	**350,000 ppm dinitrogen oxide (Group 3)**	**500,000 ppm dinitrogen oxide (group 5)**
Visceral examination			
No. Foetuses examined	474	241	153
Major malformations	0	0.3±2.2	6.1*±17.7
Minor anomalies	4.7±8.9	6.3±10.3	17.8*±29
Skeletal examination			
No. of foetuses examined	480	238	153

Major malformations	0±0	0.4±2.7	1.1±4.8
Minor anomalies	0.6±3.0	0.8 ±3.5	7.6*±14

*p<0.05

2.1.1.11 Mazze et al., 1986

R.I. Mazze, M. Fujinaga, S.A. Rice, S.B. Harris, J.M. Baden. (1986). Reproductive and teratogenic effects of nitrous oxide, halothane, isoflurane and enflurane in Sprague-Dawley rats. *Anesthesiology, 64*(3), 339-344.

Detailed study summary and results (only results with dinitrogen oxide are detailed in this report)

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (medical-grade)

Test animals

- Sprague-Dawley rats
- 19-50 animals/group
- Age and weight at the study initiation: no information

Administration/exposure

- Inhalation, gas-tight, plexiglass chambers, approximately 1000 L capacity. During treatment, rats remain in their cages, which were place in the chambers.
- Whole body, 6 hours on days 14-16 (Period I), 11-13 (Period II) and 8-10 (Period III) of pregnancy (Plug day = day 1 of pregnancy)
- Concentration levels: 750,000 ppm dinitrogen oxide mixed with oxygen.
- Control group: compressed air, simultaneously to treated groups in a similar chamber, positive control: retinoic acid (5 mg/kg by gavage in corn oil).
- Vehicle: air.
- Analytical verification of test atmosphere concentrations: yes, continuous measurements.

Description of test design:

- Sacrifice on day 21 of pregnancy by carbon dioxide inhalation and caesarean sections were performed.
- The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and each foetus was examined for external abnormalities. Every other foetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell (1964), cleared with glycerol and subsequently examined microscopically for skeletal abnormalities. The remainder of the foetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities as described by Barrow and Taylor (1967). All examinations were made without knowledge of the treatment group.
- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- All animals remain conscious throughout the experiment.
- Body weight gain of dams exposed to dinitrogen oxide was significantly decreased compare to controls (GD 14-16).
- A statistically significant increase in the number of foetal resorption was observed in the group exposed to dinitrogen oxide compare to control. This was also manifested by a decrease in the percentage of live foetuses/implantations for the dinitrogen oxide group (87.8%) compare to controls (96.7%). Rats with retinoic acid on days 11-13 also had an increased incidence of resorptions.
- A statistically significant reduction in foetal body weight was also noted in two of the three groups exposed to dinitrogen oxide.
- An increase in major anomalies was noted in the dinitrogen oxide group exposed during GD 8-10.

Table 16: Reproductive indices (mean +/- SD), Mazze et al., 1986

	Period	Control	Positive control	Dinitrogen oxide
No. dams	I	39	20	19
	II	48	21	29
	III	50	24	25
Dams weight gain (g)	I	84.9	81.7	68.5*
	II	95.1	91.1	90.9
	III	113	109	104
Foetal weight (g)	I	4.58	4.65	4.13*
	II	4.52	4.27	4.46
	III	4.49	4.38	4.29
Total foetal wastage[1]	I	0.49	0.30	1.37*
	II	0.63	2.05*	0.59
	III	0.62	0.38	0.32
Resorptions (no./dam)	I	0.46	0.25	1.32*
	II	0.63	2.05*	0.59
	III	0.56	0.29	0.24

Period I: 14-16, period II: 11-13 and Period III: exposure days 8-10; [1] number/dam; dead + resorbed

Table 17: Foetal examination (Mazze et al., 1986)

	Period	Control	Positive control	Dinitrogen oxide
External examination				
No. foetuses	I	429	224	198
	II	529	211	319
	III	557	249	272
Any external abnormalities	I	0.7±2.5	0.4±1.8	0.5±2.1
	II	0.4±1.8	1.7±7.4	0.0±0.0
	III	0.0±0.0	3.8±14	3.6 ±16
Major malformations	I	0.0±0.0	0.0±0.0	0.0±0.0
	II	0.0±0.0	0.0±0.0	0.0±0.0
	III	0.0±0.0	0.0±0.0	3.2±16
Minor anomaly	I	0.7±2.5	0.4±1.8	0.5±2.1
	II	0.1±1.0	1.7±7.4	0.0±0.0
	III	0.0±0.0	3.3±13.8	0.0±0.0
Skeletal examination				

Number of foetuses	I	213	111	98
	II	268	105	159
	III	285	127	135
Major malformations	I	0.0±0.0	0.0±0.0	1.1±4.6
	II	0.0±0.0	0.0±0.0	0.0±0.0
	III	0.0±0.0	4.2±20	0.0±0.0
Minor anomalies	I	0.5±3.2	0.0±0.0	1.9±5.9
	II	0.0±0.0	5.7±22	0.0±0.0
	III	0.0±0.0	7.7*±21	1.6±5.5
Developmental variants	I	0.0±0.0	0.0±0.0	2.6±11.5
	II	0.3±2.0	0.0±0.0	0.0±0.0
	III	0.0±0.0	1.5±5.2	0.7±3.4

*p<0.05

2.1.1.12 Mullenix et al., 1986

P. J. Mullenix, P.A. Moore, M.S. Tassinari. Behavioral toxicity of nitrous oxide in rats following prenatal exposure. Toxicology and Industrial Health 2, No.3, 1986.

Detailed study summary and results

Rats, pre/post-natal developmental toxicity study

Test substance

- Dinitrogen oxide

Test animals

- 40 Sprague-Dawley derived rats (Charles River)
- Age at the initiation of the study: not stated
- Number of animals per groups: 5 or 15 per groups

Administration/exposure

- Inhalation, chambers (5lx20x23 cm) fitted with a Plexiglass cover containing one inlet and two outlets.
- Whole body, 8h exposure on GD 15 in 15 females and on GD14 and 15 in 5 females (vaginal plug = day 1 of pregnancy)
- Concentration levels: 750,000 ppm dinitrogen oxide mixed with oxygen. Flow rate: 10l/min
- Control group: air only (n=15 on GD 15 and + 5 on GD 14-15)
- Analytical verification of test atmosphere concentrations: yes, but no further detailed.

Description of test design:

- At parturition, litters were reduced to 8 pups (4 per sex).
- Residential maze activity (n=4/sex/group)
- Time-lapse photography.

Results and discussion

- No effects of exposure to dinitrogen oxide on body weight in the mother during exposure or in the offspring on PND 1 to 21.
- *Residential maze activity*: no effect at 1 month old, hypoactivity by 5 month of age when dams were treated on GD15. In contrast male showed significant diurnal hyperactivity. Female pups exposed during GD14-15 had significant diurnal hyperactivity at both time point. In males, the effect was observed at the early age but not at 5 month. The effect was observed in 4 out of 5 litters.
- *Time-lapse photography.* No effect in one-month old male. Altered male behaviour was observed in rats exposed at GD14-15 at 5 months. In this group, a decreased frequency of standing and an

increase duration of rearing were noted. All-dinitrogen oxide treated males on GD 15 displayed effects on face washing behaviour (less washing of their face) whereas in rats treated doe two days face washing was more random over time. In females, hyperactivity was clear event at one month. The females exposed on day 14 and 15 had increased rearing and walking frequencies and significant shorter durations of standing and sitting. At 5 months of age, hyperactivity was even more evident in females. The effect was less severe in females exposed on GD 15 only and there was a tendency to hypoactivity at 5-month of age.

2.1.1.13 Koeter et al., 1986

Koëter HB, Rodier PM. Behavioral effects in mice exposed to nitrous oxide or halothane: prenatal vs. postnatal exposure. *Neurobehav Toxicol Teratol*. 1986 Mar-Apr;8(2):189-94.

Abstract: Mice exposed to four or six hours of dinitrogen oxide or halothane differed from controls on a variety of tests conducted before weaning. Whereas many agents that produce behavioral effects have very different effects at different stages of brain development, these inhalant anesthetics had similar effects, whether exposure occurred on the 14th day of gestation or two days after birth. Both treatment times and both agents were associated with delays in the appearance of developmental landmarks and delays in the appearance of righting reflexes and locomotion. The level of general activity just before weaning tended to be low in all treated groups and was significantly depressed in males exposed to dinitrogen oxide postnatally. The distribution of activity scores was shifted significantly in both postnatal groups compared to controls. The data are compatible with human studies suggesting that inhalants at parturition have an effect on early behavior. The persistence of effects over the first three weeks of life does not fit with the idea that the behavioral effects are mediated by continued presence of the drug. The similar effects of the two agents, which produce very different degrees of anaesthesia, supports earlier studies suggesting that the teratogenicity of inhalants is independent of the level of anaesthesia produced.

2.1.1.14 Keeling et al., 1986

P.A. Keeling, D.A. Rocke, J.F. Nunn, S.J. Monk, M.J. Lumb, M.J. Halsey. Folinic acid protection against nitrous oxide teratogenicity in the rat. Br. J. Anesth. 58:528-534, 1986.

Detailed study summary and results

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (purity not specified)

Test animals

- Sprague-Dawley rats (Charles River)
- Age at the initiation of the study: 9-10 weeks (weight: 240-300 g).
- Number of animals per groups: 23 in controls and 10 in the dinitrogen oxide exposed group.

Administration/exposure

- Inhalation (whole-body), in their own breeding box fitted with a gas-tight hood.

Table 18: Exposure group conditions

Group	No. of rats	Food on GD9 of pregnancy	Dinitrogen oxide	Folinic acid or water
1	6	+	-	-
2	6	+	-	W
3	6	-	-	W
4	5	+	-	F
5	10	+	+	W

6	7	+	+	F

- Whole body, 24h exposure on GD 9 (vaginal plug = day 1 of pregnancy; GD8 considering plug day = GD0)
- Concentration levels: 700-000-750,000 ppm mixed with oxygen. Total flow rate: 7l/min
- Control group: air only
- Analytical verification of test atmosphere concentrations: yes, at hourly interval.

Description of test design:

- Sacrifice: GD21 with carbon dioxide (plug day = GD1).
- Examination: number of live foetuses, dead, resorbed foetuses. Weight, crown-rump length and external malformation were examined. Skeletal examination was performed after fixation in 95% alcohol and then stain with alizarin red.
- Methyl folate was also measured.

Results and discussion

- As no differences were noted between the controls (groups 1 to 4), the results were pooled.
- Methyl folate was increased in the dinitrogen oxide group compare to control.
- There were no significant differences in numbers of implants, liver foetuses or resorptions per dam between the control and treated groups. There were no significant differences in sex ratios.
- A statistically significant decrease in foetal weight (3.55g vs 3.99g in controls) and placental weight (0.53g vs 0.73 in controls, $p<0.001$) was noted in the dinitrogen oxide exposure group compare to control. No effect on crown-rump length was noted.
- A statistically significant decrease in the mean number of sternebrae was noted in the study (79% of the controls, $p<0.001$).
- A statistically significant decrease in the mean number of caudal vertebrae was noted in the study (83% of the controls, $p<0.001$).

Table 19: Conception and resorption (+/- SEM) following exposure to dinitrogen oxide (Keeling et al., 1986)

	Pooled controls	**Dinitrogen oxide**	**Dinitrogen oxide + folinic acid**
Dams	23	10	7
Foetuses	243	131	74
Implantations	11.7 ± 0.95	14.9 ± 0.66	12.0 ± 1.6
Liver foetuses per dam	10.6 ± 0.99	13.1 ± 0.83	11.0 ± 1.84
Resorption per dams (%)	9.9	13	8.3
Male (%)	49	40	44

- A statistically significant increase in major skeletal malformation was noted in the dinitrogen oxide group (Cervical vertebral malformation, total malformations).
- Developmental effects were reduce in the combined dinitrogen oxide/folinic acid group.

Table 20: Foetal examination (Keeling et al., 1986)

	Control	Dinitrogen oxide	Dinitrogen oxide + folinic acid
Skeletal examination			
No. examined	17	57	15
Major malformation	8.4±3.9***	41.3±9.9***	19.1±8.5***
- Cervical vertebral malformation	5.1±3.3***	27.7±0.2***	13.3±6.6
- Thoracic vertebral malformations	1.0±0.7	4.4±1.8	1.6±1.7
- additional or absente vertebra	2.5±2.2	7.3±3.9	3.3±2.5
- Misaligned sternebrae	0	8.7±5.0	7.1±3.4
- bifid or fused ribs	0	3.7±1.7	0
- cranial abnormalities	0.3±0.3	2.0±1.4	0
Minor malformation and developmental variants	35.4±5.5	27.5±9.5	40.5±8.8

***p<0.001 vs control

2.1.1.15 Tassinari et al., 1986

M.S. Tassinari, P.J. Mullenix, P.A. Moore. The effects of nitrous oxide after exposure during middle and late gestation.

Detailed study summary and results

Rats, prenatal developmental toxicity study

Test substance

- Dinitrogen oxide (purity not specified)

Test animals

- Sprague-Dawley rats (Charles River)
- Number of animals per group: not clear seems to be 2 to 10 per groups
- Age and weight at the study initiation: no information

Administration/exposure

- Inhalation, polycarbonate cages (51x20x23cm) with plexiglass cover containing one gas inlet and two exhaust outlets.
- Whole body, 24h exposure on GD 11 through GD 15, 24h exposure on GD 16-20, 8h exposure on GD 9-13, 8h exposure on GD 11-15, 8h exposure on GD14-15, 8h exposure on GD15. Day of plug = GD1 of gestation. 24h exposure on GD10-14, 15-19, 8h exposure on GD8-12, 10-14 and 13-14 considering plug day = GD0. Additional females exposed during GD14-15, 8h per day, were allowed to delivered pups. Litters were reduced to 8 pups.
- Concentration levels: 750,000 ppm mixed with oxygen. Total flow rate: 10l/min
- Control group: air only
- Analytical verification of test atmosphere concentrations: yes, at least tree time during exposure using gas chromatography.

Description of test design:

<u>Prenatal</u>

- Maternal weight was record prior to and after exposure. Foetal weight was measured on GD21 and in one study on GD16 and 18.
- Sacrifice: GD21 with carbon dioxide.
- Examination: liver foetuses, resorptions, external examination. Skeletal examination: presence and number of ossification sites in the sternum, vertebrae, digits, limbs and cranium. Examination of brain and liver of foetuses at cellular levels (cell number and cell size).

Early postnatal development investigation

- Additional female allowed to deliver pups : postnatal weight was recorded on PND2, 4, 7, 14 and 21. Pups monitored for early postnatal growth were subject to a neurological developmental battery: reflex suspension, auditory startle and eye opening.

Results and discussion

- Exposure during 8h did not affect maternal body weight, weight of foetuses, resorptions, litter size.
- Exposure lasting 24 hours on gestational days 11-15 or 16-20 resulted in maternal and foetal reduced body weight. No effect on resorption or litter size was observed. No gross morphological, skeletal abnormalities or delayed ossification were noted in foetuses.

Table 21: Maternal and foetal weight after dinitrogen oxide exposure (24h on gestational days 16—20), Tassinari et al., 1986

Maternal weight	Dinitrogen oxide	Air
No. of litters	10	7
Maternal weight, Day 16 (g)	297	294
Maternal weight, Day 21 (g)	272 **	338
Mean foetal weight (g)	2.84**	3.86

- Exposure lasting 24h on gestational days 16-20, did not affect total protein and DNA levels in foetal liver and brain tissues.
- Pup weight at PND2, 4, 7, 14 or 21 in exposed and control groups did not differ. No effect on auditory startle or eye opening was noted in pups.
- Reflex suspension in the dinitrogen oxide treated group was generally decreased between the ages of 10 and 14 days. The decrease was significant in female only, who took longer to reach the time when 50% achieved the criterion (air-treated =11.6 days vs 13.4 days in exposed group, p=0.05).

2.1.1.16 Rice et al., 1985

S.A. Rice, R.I. Mazze, J.M Baden. Effect of subchronic intermittent exposure to nitrous oxide in Swiss Webster mice. Journal of environmental Pathology, Toxicology and Pathology 6(2): 271-282, 1985

Detailed study summary and results, as available on abstract and ECHA disseminated website

Test type

Mice, Subchronic inhalation toxicity: 90-day study

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Swiss Mice
- 15 animals/sex/group
- Age and weight at the study initiation: no information

Administration/exposure

- Inhalation
- Whole body, 4 hours/day, 5d/w for 14 weeks.
- Concentration levels: 0, 5000, 50,000, 500,000 ppm
- Control group: air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: no information

Description of test design:

- Body weight was measured twice weekly throughout the experiment. Liver, kidney, spleen and testis were weighed and histopathologically along with brain, stomach, seminal vesicle and ovary for evidence of drug induced damage. Blood smears were examined microscopically and complete blood count, differential white cell count, and reticulocyte and platelet counts were performed. In addition, liver microsomal cytochrome P-450 content and the rates of defluorination of enflurane and methoxyflurane were determined.

Results and discussion

- All animals survived;
- Treatment related decreases in body weight were observed in high dose group animals, with a depression of 77 and 63% in body weight gain in males and females respectively. This depression in weight gain was statistically significant ($p<0.025$ and $p<0.01$, respectively).
- Gross pathology: No treatment related changes were observed in any of the parameter measured.
- The study failed to demonstrate exposure related haematopoietic changes. There was no change in the white blood cell count nor was granulocytopenia or thrombocytopenia observed. The lack of effect suggests that either the strain of mouse was insensitive to dinitrogen oxide, or more likely that continuous exposure is necessary to induce leucocytopenia, as previous demonstrated following continuous exposure to dinitrogen oxide at high concentrations (20-80%).
- Furthermore, no treatment related changes in organ weights, biochemical or histopathological parameters were observed.
- Following dinitrogen oxide exposure, neither the hepatic microsomal cytochrome P-450 content nor the rates of aesthetic defluorination were increased; the rate of in vitro inorganic fluoride production was greater for methoxyflurane than for enflurane.

2.1.1.17 Mazze et al., 1984

R.I. Mazze, A.I. Wilson, S. A.Rice, J.M. Baden. Reproduction and foetal development in rats exposed to Nitrous oxide. Teratology 30:259-265, 1984.

Detailed study summary and results:

Test type

Rats, non-guideline reproductive toxicity

Test substance

- Dinitrogen oxide (degree of purity not specified but medical quality gas was used)

Test animals

- 323 Sprague-Dawley rats
- Age at the beginning of the study: 9 week-old
- Number of animals per groups: 78 rats in 3 groups in exp. I, 112 rats in 4 groups in experiment 2, 126 rats in four groups in experiment 3 and 149 rats in 4 groups in experiment IV.

Administration/exposure and test design

- Inhalation: plexiglass chambers approximately 1000l capacity
- Whole body, 24 hours on day 9 of pregnancy (plug day = day 1 of pregnancy, GD8 considering plug day = GD0
- Analytical verification of test atmosphere concentrations: yes, continuous monitoring

Experiment I

- Concentration levels: 750,000 ppm dinitrogen oxide in air. Total gas flow: 15-20l/min
- Control group: room air exposure (treatment control) and left untreated (colony control)

Experiment II: dose-response study

- Concentration levels: 7500, 75,000, 750,000 ppm dinitrogen oxide or air.

Experiment III: mated in-house

- Female were caged in pairs and mated nightly, one male to two females, for tow 3-day periods, 1 week apart.
- Animals were assigned to four groups: 750,000 ppm dinitrogen oxide, two air controls (treatment control), colony control.

Experiment IV: food deprivation and mechanism of teratogenicity

- Two groups exposed to 250,000 ppm dinitrogen oxide and two treatment controls
- One control and one treatment groups had food and water withheld during treatment on day 9 pregnancy and other groups had food and water ad lib.
- In this experiment, five rats from each group were killed immediately after exposure (day 10) and an additional five rats from each group were killed 24 hours later (day 11) to measure deoxyuridine (DU) suppression (bone marrow, embryonic cells).

Description of test design:

- All rats were weight at least weekly.
- Sacrifice: on gestational day 20, by carbon dioxide inhalation.
- The uterus was examined to determine the numbers and positions of live and dead foetuses and resorption. The sex and weight of each foetus were recorded and the presence of external abnormalities
- Skeletal and visceral examination was performed in foetuses.
- Abnormalities were classified as follows. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Foetuses weighing 25 % less than the mean weight of their litter were classified as runts.

Results and discussion

- Rats exposed to 7500, 75,000 and 250,000 ppm dinitrogen oxide showed no obvious changes in behaviour during treatment. During exposure to 750,000 ppm, rats appeared drowsy, their motor coordination was impaired and their food and water intake was decrease.
- Data for the four experiments were combined. The number of implantations per dam and the sex ratio of live foetuses were similar in all exposure groups. Exposure to 750,000 ppm dinitrogen oxide caused significant increases in early and late resorptions, with a resulting decrease in the number of live foetuses per litter. There were no significant differences in foetal weights between dinitrogen oxide-treated and control groups in any of the individual studies.

Table 22: Reproductive indices (mean ±SD), experiments I-IV (Mazze et al., 1984)

Dinitrogen oxide (ppm)	Control[1]	7500[2]	75,000[2]	250,000[3]	750,000[4]
Dams	160	27	25	49	62
Foetuses	1705	285	293	572	437
% Resorptions per dams	5.5±12.1	9.2±16	9.3±10	4.4±10	39±36*
% Early resorptions per dams	5.2±11.7	9.2±16	8.9±10	3.8±10	37±35*
% Late resorption in dams	0.3 ±2.3	0	0.3±1.5	0.6±2.3	2.5±7.1*
Live foetuses/dam	10.7±3.5	11±3	11.7±2.2	11.7±3.3	7±4.5*

[1] Combined data, experiments I-IV; [2] Data from experiment II, [3] Data from experiment IV, [4] Combined data, experiments I-III; *p<0.05.

- Examination of foetuses from the four experiments revealed a consistent teratogenic effect (e.g., runts, ocular malformations, limb deformities) at the 750,000 ppm dinitrogen oxide concentration only. In addition, extra lumbar rib, a developmental variant thought to be an early expression of developmental instability, occurred with increasing frequency as dinitrogen oxide concentration increased from 75,000 ppm to 750,000 ppm. However, no major effects were observed at concentrations up to 250,000 ppm.

Table 23: Foetal morphology, experiments I-IV (mean percent abnormal per litter, ± SD)

Dinitrogen oxide (ppm)	Control[1]	7500[2]	75,000[2]	250,000[3]	750,000[4]
External examination					
No. examined	1705	285	293	572	437
Any external abnormalities	2.3±4.8	2.8±6.6	1.0±3.0	1.4±3.4	15.4*±23.2
- runt	1.0±3.3	1.3±3.1	0.7±2.5	0.5±1.8	5.4*±11.9
- major malformation	0.2±2.6	0	0	0.3±1.7	10*±21.2
- minor anomalies	1.3±3.8	1.5±6.2	0.3±1.7	0.8 ±2.4	5.2±17.4
Skeletal examination					
No. Foetus examined	822	143	147	273	217
Major malformation	0.1±1.3	0	0	0	7.6*±21.3
- rib/vertebra	0.1±1.3	0	0	0	6.7*±21
Minor anomalies	2.8±10.6	0.6±3.3	0	0.7±4.8	32*±32.4
Variants	31±34	60*±34	68*±32	62*±32	62±36
- Rudimentary rib	30±33	55±36	58±33	52±28	29±31
- Extra lumbar rib	1.3±6.4	1.3±6.5	5.3*±8.0	9.7*±19	12±25
- Cervical rib	0.2±2.0	3.2±11	2.7±8.4	0	23*±35
- Sternum	0.7±3.4	2.4±7.1	3.2±8.2	0	22±31
Visceral examination					
No. foetuses examined	854	141	145	281	218
Major malformation	1.9±6.9±	2.4±12	0	4.4±15	14*±30
Minor anomalies	22±28	23±22	31±23	28±25	21±29
Variants[5]	76±27	75±32	76±22	97±7.3	55±36

[1]Combined data, experiments I-IV, [2]Data from experiment II, [3] data from experiment IV, [4] combined data exp. I-III, [5]increased renal pelvic cavitation, *p<0.05

- Immediately following exposure to 250,000 ppm dinitrogen oxide, DU suppression values in both maternal bone marrow and foetal cells were increased approximately 2.5-fold. Values returned toward normal in the next 24 hours. Neither control nor dinitrogen oxide treated rats showed a difference in DU suppression related to food deprivation.

- The authors did not determined whether food deprivation during the 24-hour exposure to 75% dinitrogen oxide acted synergistically with the anaesthetic to cause a teratogenic effect since rats given food while exposed to 750,000 ppm dinitrogen oxide did not eat. Withholding food and water during exposure to 250,000 ppm dinitrogen oxide did not result in an increased incidence of abnormalities.

2.1.1.18 Vieira et al., 1983a

E. Vieira, P. Cleaton-Jones and D. Moyes. Effects of intermittent 0.5% nitrous oxide/air (v/v) on the fertility of male rats and the post-natal growth of their offspring. *Anesthesia* 38: 319-323, 1983

Detailed study summary and results:

Test type

Rats, non-guideline fertility study

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Wistar albino rats
- 12 males per groups
- Age and weight at the study initiation: 250 g

Administration/exposure

- Inhalation: polypropylene cages.
- Whole body, 6 hours/day, 5d/w for 30 days.
- Concentration levels: 5,000 ppm in air
- Control group: oil free air
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes

Description of test design:

- At the end of the exposure period, male rats were mated overnight with three nulliparous female rats. Following the initial mating, the male rats were allowed a recovery period of 6 months during which they were exposed to room air temperature. Thereafter, each male was again mated with three nulliparous female rats. At birth the number in each litter was recorded and all litter mates were examined macroscopically for gross defect. The young rats were weighed and measured at weekly intervals for 8 weeks.

Results and discussion

- A total of 1014 rats were born: 382 controls, 252 from the initial group mated immediately after the male rats had been exposed and 380 from the group mated after the 6-month recovery period. The litter size was: 9-15 for the control group (mean = 12), 2-14 for the experimental group (mean = 7), and 8-14 for the group that was allowed the 6-month recovery period (mean = 11). The reduction in mean litter size in the female rats mated immediately after the male rats had been exposed was significantly different from that of the control group ($p < 0.001$). After the 6-month recovery period no statistically significant effect was noted compare to controls. Further examination of the litter size showed that in the control group one litter numbered 9 offsprings while the remaining 35 mothers had litters ranging from 11-15. This pattern was similar to that in the group following the recovery period, which showed one litter with eight off-spring and the remaining litters ranging from 10-14. In contrast, in the group mated immediately after exposure dinitrogen oxide, one litter comprised 14 offspring but the remaining 35 litters ranged between two and six offsprings.

- Mean body weight for all the groups was the same at week one. At week two the control group of offspring were heavier than the other two experimental groups. From 3 weeks onwards there was no significant difference between the control and the offspring of the group of males that were allowed a 6-month recovery period, but there was a significant difference in the offspring belonging to the male rats exposed to dinitrogen oxide.
- For body length there was no significant difference between the control group of offspring and the offspring of male rats that were permitted a 6-month recovery period. There was a significant difference in the group of offspring from the males who were exposed to dinitrogen oxide.
- Tail length followed a similar pattern and at 8 weeks the offspring of the male rats that had been exposed to the dinitrogen oxide had tail lengths approximately 10 mm shorter than those in the control group and the group of males which were allowed the 6 month recovery period.

Table 24: Postnatal growth of offspring for the eight postnatal weeks by group, mean ± SD (Vieira et al., 1983a)

Experimental group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Body weight (g)								
- Control group	12.9±2.7	20.1±7.6	28.8±8.4	37.9±7.5	48.2±6.9	60.8±7.4	78.2±15	97.3±19
- N_2O immediate	12.1±2.5	19.8±6.4	23.6±8.5	31.4±8.7	36.9±9.0	47.4±6.4	61.2±8.0	79.1±15
- N_2O 6-month recovery	12.7±2.6	19.8±5.1	27.6±8.3	36.9±8.4	48.5±5.6	58.6±6.9	77.1±7.4	97.3±15
Body length (mm)								
- Control group	62.4±9.8	88.5±15	103±16	114±16	127±12	139±22	151±26	164±26
- N_2O immediate	47.7±6.1	59.4±12	93.3±18	104±17	115±19	122±19	130±19	135±20
- N_2O 6-month recovery	60.4±8.9	86.9±13	101±16	114±14	126±12	136±17	150±19	164±26
Tail length (mm)								
- Control group	30.9±6.6	49.2±10	63.3±12	77.1±13	90.7±15	102±16	110±15	118±15
- N_2O immediate	27.9±6.0	37.0±9.2	58.2±12	70.5±12	78.1±15	91.7±16	99.8±15	107±14
- N_2O 6-month recovery	29.9±5.4	48.0±9.8	61.5±11	76.1±13	90.5±16	101±14	108±15	118±16

2.1.1.19 Vieira et al., 1983b

E. Vieira, P. Cleaton-Jones and D. Moyes. Effects of low intermittent concentrations of nitrous oxide on the developing foetus. Br. J. Anesth 55:67-69, 1983.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- 60 female Wistar albino Rats
- Number of rats per groups: 12
- Age and weight at the beginning of the study: 250g (South African Institute for Medical Research)

Administration/exposure

- Inhalation: animals were housed in plastic cages with mesh floors in two environmental chambers supplied with oil-free air from a diaphragm compressor.
- Whole body exposure
- Concentration levels: 250, 500, 1000 ppm dinitrogen oxide in air for 6h/day, 5d/w for 3 weeks.
- Control group: oil-free compressed air
- Analytical verification of test atmosphere concentrations: yes (not further specified)

Description of test design:

- Sacrifice: GD19 by carbon dioxide administration

- Examination : number of corporea lutea, placentation sites and embryonic remnants (foetal resorptions). Examination of all foetuses for external examination. The body cavity of foetuses was opened by a mid-ventral incision and a systematic investigation of internal organs was performed using a dissecting microscope. Each foetus was numbered, fixed in 10% buffered formal saline and cleared in 0.5% potassium hydroxide and then stained with alizarin red for examination of foetal skeleton. Crown-rump measurements were made and the foetuses were weight.

Results and discussion

- Statistically significant decreased in litter size at 5000 ppm ($p<0.001$).
- No evidence of foetal resorptions, skeletal malformations.
- Crown-rump length and body weight were similar in the groups.

Table 25: Developmental findings reported by Vieira et al., 1983b.

Dose levels (ppm)	No. of foetuses	Litter size (Mean ± SD)	Range per litter
0	120	11 ± 1.4	9-13
250	119	11 ±1.3	9-13
500	117	11 ±1.3	8-13
1,000	117	10 ± 1.2	8-13
5,000	98	7.0 ± 2.3 *	6-10

*$p<0.001$

2.1.1.20 Mazze et al., 1983

R.I. Mazze, S. A.Rice, A.J. Wyrobek, J.S. Felton, J.B. Brodsky, J.M. Baden. Germ cell Studies in mice after prolonged exposure to nitrous oxide. Toxicology and applied pharmacology 67:370-375, 1983.

Detailed study summary and results:

Test type

Mice, non-guideline reproductive toxicity

Test substance

- Dinitrogen oxide (degree of purity not specified but medical quality gas was used)

Test animals

- Hla(SW)BR mice
- Age at the beginning of the study: 13-14 week-old
- Number of animals per groups: 15/sex/groups

Administration/exposure

- Inhalation: Two gas-tight Plexiglass chambers, each of 1000 L capacity. All mice in the treated groups were exposed simultaneously.
- Whole body, 4 hours per day, 5 days per week, 14 weeks
- Concentration levels: 5000, 50,000, 500,000 ppm dinitrogen oxide in air. Total gas flow: 15-20l/min
- Control group: room air;
- Positive control for sperm investigation: methyl methanesulfonate (ip, 75 mg/kg) for 5 consecutive days, sacrifice 35 days later.
- Positive control for oocyte examination: Methylchloranthrene (ip, single injection 80 mg/kg). Sacrifice, 14 days after treatment.
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes. Concentration maintained always within 5% of the desired range values.

Description of test design:

- All mice were weight weekly and examined daily for signs of ill health.
- Necropsy: after 14-week exposure by carbon dioxide inhalation.
- Examination: caudae epididymides removal with sperm was examined for morphological abnormalities, sperm count. Weight and histopathological examination of testes (blind assessment).
- Ovary from 6 female mice from the control and 500,000 ppm group were examined for primordial oocyte count.

Results and discussion

- None of the dams appeared affected by treatment (no excitement or general anaesthesia).
- There were no significant differences among groups in testes weight, percentage of abnormally shaped sperm, sperm count and histopathological appearance. Statistically significant increase in abnormal sperm, decrease sperm count was observed in the positive control group.
- There were no significant difference between the group treated with dinitrogen oxide and control for the mean number of oocytes (33.3 +/- 14.4 in treated group vs 29.8 +/- 8 in controls). Significantly fewer primordial oocytes were noted in the positive control compare to the negative control group.

2.1.1.21 Mazze et al., 1982

R.I. Mazze, A.I. Wilson, A.Rice, J.M. Baden. Reproduction and foetal development in mice chronically exposed to nitrous oxide. Teratology 26: 11 – 16, 1982.

Detailed study summary and results:

Test type

Mice, non-guideline reproductive toxicity and developmental toxicity study

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Swiss/ICR mice

Administration/exposure

- Inhalation: Two gas-tight Plexiglass chambers, each of 1000l capacity. All mice in the treated groups were exposed simultaneously.
- Whole body exposure
- Concentration levels: 5000, 50,000, 500,000 ppm dinitrogen oxide in air.
- Control group: untreated (colony control), treated in an inhalation chamber with emprained room air (treatment control)
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes

Description of test design:

Experiment I: Exposure of female mice on days 6-15 of pregnancy

- Age at the beginning of the study: 9 weeks old
- Mating with untreated males, 2 females per males.
- Inhalation exposure 4h daily on gestation days 6-15 of pregnancy
- Positive control: retinoic acid by gavage at GD8
- Sacrifice at GD18 of pregnancy by cervical dislocation.
- Number of females per groups: 32 in colony control group, 34 in treatment control group, 24 to 27 in dinitrogen oxide treatment groups and 11 in the positive control group.
- Examination: numbers of live and dead foetuses, resorptions in uterus. The crown-rump length, weight and sex of each foetuses was determined and all foetuses were examined for gross

abnormalities. One-third of the foetuses were examined for visceral abnormalities, the other two-third were examined for skeletal abnormalities.

Experiment II: Exposure of male mice for 9 weeks prior to mating

- Age at the beginning of the study: 5-week old male mice
- Inhalation exposure: 4h per day, 5 days per week for 9 weeks prior to mating.
- Number of males per groups: 18 to 21
- Each male was then mated with two untreated females.
- Uterine and external foetal examination were done on day 18 of pregnancy as in Experiment I.

Results and discussion

Experiment I

- None of the dams appeared affected by treatment (no excitement or general anaesthesia).
- No differences between groups on maternal weight gain, litter size, foetal wastage, foetal size.
- The mean percentage of foetuses with minor internal abnormalities and the mean percentage of runts were higher in treated groups than in the colony control group. The positive control group had statistically significant increases in the percentage of foetuses with cleft palate, exencephalies, other external anomalies, skeletal anomalies, extra ribs and hydronephrosis and other kidney anomalies.
- There was no significant increase in abnormalities in any of the dinitrogen oxide treated groups.

Experiment II

- The male fertility study showed no differences among the groups in the ability of males to impregnate females or in litter size, foetal wastage or foetal size. Values were generally similar to experiment I.

2.1.1.22 Hardin et al., 1981

Hardin BD, Bond GP, Sikov MR, Andrew FD, Beliles RP, Niemeier RW. Testing of selected workplace chemicals for teratogenic potential. Scand J Work Environ Health. 1981;7 Suppl 4:66-75.

Abstract:

The reproductive toxicity and teratogenic potential of 19 industrial chemicals have been investigated during the past 3 a. Preliminary studies utilizing intraperitoneal treatments of rats on days 1-15 of gestation have been conducted on the following ten chemicals: allyl chloride, bisphenol A, copper naphthenate, ethylene dibromide, hexachlorobutadiene, 2-mercaptobenzothiazole, methyl styrene, naphthalene, 2-nitropropane, and 1,2,3-trichloropropane. Studies utilizing inhalation exposure of rats and rabbits on days 1-19 and 1-24, respectively, of gestation have been conducted on the following nine chemicals: butylene oxide, carbon disulfide, 2-ethoxyethanol, ethyl benzene, methyl bromide, dinitrogen oxide, styrene oxide, tetrachloroethylene, and trichloroethylene. In the preliminary studies, evidence of teratogenic potential was seen with allyl chloride and bisphenol A, and fetal toxicity was found in the absence of maternal toxicity with methyl styrene and 2-nitropropane. In the inhalation studies, 2-ethoxyethanol was strongly embryotoxic at the higher exposure levels employed and was teratogenic at the lower concentration.

2.1.1.23 Land et al., 1981

Land PC, Owen EL, Linde HW. Morphologic changes in mouse spermatozoa after exposure to inhalational anesthetics during early spermatogenesis. Anesthesiology. 1981 Jan;54(1):53-6.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Strain: (C57Bl xC3H)F1
- Age : 11week-old at study
- No. mice per group : 5

Administration/exposure

- Whole body inhalation (5l glass desiccators with fenestrated porcelain floors).
- Dinitrogen oxide in oxygen, 4h/d for 5 consecutive days. Concentration of 0.1 and 1 MAC (equivalent to 80,000, 800,000ppm
- Analysis: spermatozoa analysis
- Dinitrogen oxide concentration was checked during exposure

Results and discussion

- No increase in abnormal spermatozoa for dinitrogen oxide.

2.1.1.24 Lane et al., 1980

Lane GA, Nahrwold ML, Tait AR, Taylor-Busch M, Cohen PJ, Beaudoin AR. Anesthetics as teratogens: nitrous oxide is fetotoxic, xenon is not. Science. 1980 Nov 21;210(4472):899-901.

Abstract:

Exposure of pregnant rats to the anaesthetic dinitrogen oxide on the ninth day of gestation causes fetal resorption, skeletal anomalies, and macroscopic lesions including encephalocele, anophthalmia, microphthalmia, and gastroschisis. The inert gas xenon, which has anaesthetic properties similar to those of dinitrogen oxide, does not cause teratogenic effects under the same experimental conditions.

2.1.1.25 Vieira et al., 1980

E. Viera P., Cleaton-Jones, J.C. Austin, D.G. Moyes, R. Shaw. Effects of low concentrations of nitrous oxide on rat foetuses. Anesth Analg 59:175-177, 1980.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Wistar Rats
- Number of rats per groups: 12
- Age and weight at the beginning of the study: 150g

Administration/exposure

- Inhalation: animals were housed in plastic cages with mesh floors in two environmental chambers supplied with oil-free air from a diaphragm compressor.
- Whole body exposure
- Concentration levels: 1000, 500, 250 ppm dinitrogen oxide in air.
- Control group: air
- Analytical verification of test atmosphere concentrations: yes, three time during the exposure period

Description of test design:

- Sacrifice: GD19
- Examination of ovary and gravid uteri. Examination of foetal resorption, sex, crown-rump length.
- External examination and skeletal examination. Internal organ examination: Organs examined not specified.

Results and discussion

- Statistically significant decreased in litter size in the 1000 ppm dinitrogen oxide group and in resorptions.
- Statistically significant decreased in Crown-rump length.
- No effects on foetal body weight.
- No Effect on internal organs. Skeletal abnormalities were statistically significantly increased in the 1000 ppm dinitrogen oxide group (rib malformations, abnormal vertebrae columns).
- No effect on sex ratio.

Table 26: Litter size, crown-rump measurements *and foetal resorption in Vieira et al., 1980.*

	Number of litters	**Number of foetuses**	**Litter size (mean+/- SD)**	**Crow-rump** measurements **(mm, mean+/-SD)**	**Resorptions**
Control	12	120	11±1.4	44 ±1.4	None
Dinitrogen oxide, 1000 ppm	12	66	6.3±4**	35±1.6*	4**
Dinitrogen oxide, 500 ppm	12	118	11±1.4	43±1.3	None
Dinitrogen oxide, 250 ppm	12	120	11±1.3	43±1.4	None

**p<0.01, *p<0.05

2.1.1.26 Vieira et al., 1979

E. Vieira. Effect of the chronic administration of nitrous oxide 0.5% to gravid rats. Br. J. Anesth. 51:283-286, 1979.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Wistar Rats
- Number of rats per groups: 12
- Age and weight at the beginning of the study: 150g

Administration/exposure

- Inhalation: in house environmental chambers constructed to hold commercial polypropylene rat cages. The chambers, each of 245 l capacity, were supplied with compressed air at a flow rate of 35l/min.
- Whole body exposure (Vaginal plug = day 0 of gestation)
- Concentration levels: 5000 ppm (maintained within 4600-5000 ppm).
- Control group: compressed air
- Analytical verification of test atmosphere concentrations: yes

Description of test design:

- Sacrifice: GD19 by administration of carbon dioxide.
- Examination of ovary and gravid uteri. The number of corporea lutea of pregnancy was counted, foetal resorptions. Foetuses were counted, weighted and sexed. Each foetus was examined for external malformation. A systematic examination of internal organs were examined: head, palate, eyes, nasal cavities, thorax-oesophagus, lungs, diaphragm, liver, abdomen-alimentary canal, kidneys, bladder.
- The foetuses were fixed in 10% buffered formol saline and cleared in potassium hydroxide 0.5%, after which the foetal skeletons were stained with alizarin red. A systematic examination of the foetal skeletons was carried out in random order. The following were examined: skull, vertebral column, ribs, fore limbs, hindlimbs.
- Crown-rump measurements were performed on foetuses.

Results and discussion

- Significant decrease in the number of foetuses per females.
- Significant increased number of resorptions.
- No abnormalities in internal organs.

- Increased number of skeletal malformations in the dinitrogen oxide group.
- Significant reduction in the mean crown-rump length in the foetuses exposed to dinitrogen oxide.
- Significant decrease in the mean body weight of the foetuses in the dinitrogen oxide group compare to control.

Table 27: Foetal information (Vieira et al., 1979)

	Maternal rat number											
Dinitrogen oxide group												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	7	9			13			11	11	10	5	11
Foetal weight (g), mean ± SD	1.4 ±0.2	1.3 ±0.2			1.8 ±0.0			1.3 ±0.3	1.5 ±0.2	1.7 ±0.1	1.8 ±0.2	2.1 ±0.1
Resorption sites	4	1	11	12	2	10	12		1			
Crown-rump length (mm), mean ± SD	29± 0.2	28± 0.2			30± 0.2			29± 0.2	28± 0.2	28± 0.2	28± 0.2	29± 0.2
Live foetuses with abnormalities		1			2			1	1	2		2
No. of live foetuses without abnormality	7	8			11			10	10	8	5	11
Controls												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	12	12	10	10	10	13	12	12	9	13	12	10
Foetal weight (g) mean ± SD	2.0± 0.1	3.5± 0.2	3.1± 0.0	3.3± 0.6	3.1± 0.2	3.2± 0.3	2.6± 0.3	2.0± 0.1	2.0± 0.1	2.0± 0.2	2.3± 0.2	2.3± 0.2
Crown-rump length (mm), mean ± SD	44± 0.2	44± 0.2	44± 0.2	43± 0.2	42± 0.2	42± 0.2	44± 0.2	44± 0.2	42± 0.2	43± 0.2	44± 0.2	44± 0.2

2.1.1.27 Shah et al., 1979

Shah RM, Burdett DN, Donaldson D. The effects of nitrous oxide on the developing hamster embryos. Can J Physiol Pharmacol. 1979 Nov;57(11):1229-32.

Abstract:

Pregnant hamsters were exposed to different concentrations of dinitrogen oxide during the period of organogenesis. Teratogenic effects were observed in a small but significant number of fetuses. Types of malformations included cleft palate, limb defects, gut herniation, and fetal oedema. A dose-effect relationship was not observed. It is not clear from our observations whether the observed effect on the fetuses was due to the excess of dinitrogen oxide, hypoxia, or a combination of both. Comparison with published literature indicates that further studies on the effects of dinitrogen oxide in placental animals are needed.

2.1.1.28 Vieira et al., 1978

E. Vieira, P.E. Cleaton-Jones, J. Austin, P.L. Fatti. Inttermittent exposure of gravid rats to 1% nitrous oxide and effect on the postnatal growth of their offspring. S. Afr. Med. J. 53, 106-108, 1978.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Female Wistar albino Rats (South African Institute for Medical Research)

- Number of rats per groups: 8
- Age and weight at the beginning of the study: 12 week-old

Administration/exposure

- Inhalation: in house environmental chambers constructed to hold commercial popylpropylene rat cages.
- Whole body exposure (Vaginal plug = day 0 of gestation)
- Concentration levels: group 1 during entire gestation for 6h/d, 5d/w during 3 weeks, group 2 during first and second weeks and the third group was exposed for the first week of gestation only. Flow rate: 0.1l/min.
- Control group: three groups, atmosphere in the same room as exposed groups.
- Analytical verification of test atmosphere concentrations: not stated.

Description of test design:

- At birth all litter mates were examined for gross defects and the number of each litter was recorded. The young rats were weighted and tail and body length were measured.

Results and discussion

- Significant decrease in the number of rats per litter in the females exposed to dinitrogen oxide at 10,000 ppm.
- Significant decreased in the weight and body length of pups in the exposed group.

Table 28: Litter size (Vieira et al.1978)

Group	Exposure	Number of litters	Rats per litter (mean +/-SD)
Controls	None	8	10.4±2.3**
1	3 weeks	8	8.3±3.2**
2	1st and 2nd weeks	8	6.3±2.6**
3	1st week	8	5.7±1.6**

**p<0.001

2.1.1.29 Pope et al., 1978

W.D.B. Pope, M.J. Phil, A.B.G. Landdown, A. Simmonds, P.E. Bateman. Fetotoxicity in rats following chronic exposure to Halothane, nitrous oxide, or Methoxyflurane

Test type

Rats, non-guideline developmental toxicity study
Only results obtained with dinitrogen oxide alone are describe in this report.

Test substance

- Dinitrogen oxide

Test animals

- Inbred Sprague-Dawley rats
- Age and weight at the study initiation: 250-300g
- Controls: 8 to 10 animals,

Administration/exposure

- Inhalation: 170l Perspex chambers with external nylon tubing gas-circulating systems. Animal cages are directly inserted in the chamber.
- Whole body, 8 hours a day for whole gestation
- Concentration levels: 1 to 500,000 ppm dinitrogen oxide

- When an animals became excited, the animal was transfer in a stress group and exposed as the control group (control environmental chamber)
- Control group: control environmental chamber (air exposure)
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes, every 10 minutes

Description of test design:

- Sacrifice : in the morning of the last day of gestation (GD21, plug day= GD1 or GD20 considering plug day = GD0)
- Analysis: maternal liver and kidney weight, foetal weight and number, foetal loss (number of resorption and dead foetuses) and crown-rump length and sex ratio of foetuses.
- Skeletal examination in selected 5 or 6 foetuses per litters.
- During exposure, special care was taken to ensure that the animal's cages were consistently clean and metabolic wastes were not allowed to accumulate. Any handling or extraneous noise was kept to a minimum.

Results and discussion

- An increase in foetal loss was observed at 10,000 and at 100,000 ppm dinitrogen oxide compare to their respective control but the effect was not statistically significant when compared with the overall control levels and was within the normal spontaneous foetal loss of their breeding colony (< 10%). No increase in foetal loss was noted at 500,000 ppm.
- Foetal loss was dramatically increased in the "stress group" (65% to 100%), the variability was not related to the initial group of assignment.

Table 30: Litter sizes, percentage of foetal loss induced by dinitrogen oxide

	No. of pregnant rats	**Live/litter (SE)**	**% foetal loss (SE)**
Control Dinitrogen oxide, 10,000 ppm	8 7	14.3 (0.7) 12.7 (1.4)	0 8.2 (3.3)
Control Dinitrogen oxide, 100,000 ppm	8 7	11.3 (1.3) 14.3 (0.6)	1.1 (0.8) 8.3 (3.7)
Control Dinitrogen oxide, 500,000 ppm	10 10	13.7 (0.6) 12 (0.9)	8.1 (2.5) 10.4 (2.2)
Stress group	4	1.5(1.5)	91 (8.8)

- A decrease in foetal weight was noted at the top dose compare to control.
- The decrease was accompanied by a decrease in crown-rump lengths and slight delayed development (decreased number of centres of ossification in the distal parts of the limbs and in the vertebral column, delayed ossification of sternebrae).
- No gross skeletal anomaly related to treatment was noted. There were no effects on sex ratio

Table 31: Foetal and placental weight (Pope et al., 1978)

	Foetal weight (g)	**Placental weight (g)**
Control Dinitrogen oxide, 10,000 ppm	5.45 (0.04) 5.31 (0.07)	0.59 (0.01) 0.51 (0.01)**
Control Dinitrogen oxide, 100,000	5.0 (0.05) 4.22 (0.05)**	0.45 (0.01) 0.42 (0.01)*

ppm		
Control Dinitrogen oxide, 500,000 ppm	5.51 (0.04) 4.35 (0.07)**	0.47 (0.01) 0.43 (0.01)*
Stress group	3.25 (0.19)	0.31 (0.02)

*p<0.05, **p<0.01

- No effect on maternal food consumption or on body weight (final weight measured at post-mortem examination) was noted in the study.

Table 32: Maternal weight and food consumption

	Initial weight (g/g)	**g/g increase in weight**
Control Dinitrogen oxide, 10,000 ppm	1.40 1.39	3.56 3.90
Control Dinitrogen oxide, 100,000 ppm	1.69 1.72	3.31 3.34
Control Dinitrogen oxide, 500,000 ppm	1.52 1.74	3.19 3.76

*p<0.05, **p<0.01

2.1.1.30 Kripke et al., 1976

B.J. Kripke, A.D. Kelman, N.K. Shah, K. Balogh, A.H. Handler. Testicular reaction to prolonged exposure to nitrous oxide. Anesthesiology 44, No. 2, 1976

Detailed study summary and results:

Test type

Rats, non-guideline fertility study

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- 135 LEW/f Mai rats
- Age and weight at the study initiation: 125 g

Administration/exposure

- Inhalation: 532L clear plastic chambers containing 8 cages housing 3 rats each.
- Whole body, 8 hours or 24 hours daily for various periods.
- Concentration levels: 200,000 ppm dinitrogen oxide, 20% O_2 and 60% N_2. Flow rate: 200 ml O_2, 950 ml N_2O and 3600 ml of air per minutes
- Control group: similar cage, room air.
- Vehicle: air
- Analytical verification of test atmosphere concentrations: yes daily measurements

Description of test design:

- Sacrifice (4-6 rats) by cardiac puncture and exsanguination after 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 32 and 35 days of exposure.
- 15 rats were removed after 32 days of continuous exposure and 5 each sacrified after 3, 6 or 10 days along with an equal number of 35-, 38- and 42-day controls.

- Viscera, salivary glands, skeletal muscle, bones and bone marrow, thyroid and pituitary glands were sampled. Serum measurement of testosterone levels in blood at sacrifice.

Results and discussion

- Statistically significant decrease in testicular weight ($p < 0.05$) after dinitrogen oxide exposure for 28 days or 32 days exposure followed by 3-day recovery period. No effect observed after 32 days of exposure followed by 6-day recovery period (results not specified after 10-day recovery).
- No histopathological finding was reported in the testes of control rats.
- In experimental animals exposed continuously, various degree of injury of spermatogenic cells were noted in the seminiferous tubules. The lesion consisted of a slight depletion of spermatogenic cells. The severity and frequency increased with the duration of exposure. The pattern of injury was focal first, with eventual progression to a diffuse appearance. Continuous exposure to dinitrogen oxide for 28 to 35 days caused severe tubular damages.
- Intermittently exposed rats were affected less than after continuous exposure. Thirty-five day of exposure caused a reduction in the number of spermatogenic cells with some disorganization of the normal architecture.
- No findings were noted in the interstitial cells.
- There were no effect on serum testosterone levels between controls and experimental animals.

2.1.1.31 Corbett et al., 1973

T.H. Corbett, M.D. Cornell, J.L. Endres, R.I Millard; Effects of low concentration of nitrous oxide on rat pregnancy. Anesthesiology, V39, No. 3, 1973.

Test substance

- Dinitrogen oxide (degree of purity not specified)

Test animals

- Rats (strain not specified)
- Number per groups: 5-10
- Age and weight at the beginning of the study: not specified.

Administration/exposure

- Inhalation: homemade chambers constructed from metal shelving and plastic sheets. Rat cages were placed on the shelves and the plastic sheets secured to make the chambers as tight as possible.
- Whole body exposure
- Concentration levels: 1000, 15,000 ppm dinitrogen oxide mixed in oxygen and balance nitrogen for 24 hours per day from days 8 to 13 and days 12 to 19 of pregnancy (plug day = day 1 of pregnancy) (Groups 1 and 3). 24 hours/ from GD 7-12 and 11-18 considering plug day = GD0. Flow rate: 5l/min.
- 100 ppm, 1000 ppm dinitrogen oxide mixed with oxygen and balance nitrogen for 8 hours per day from days 10 to 13, days 14 to 19 or days 10 to 19 of pregnancy (Groups 5 and 10).
- Control group: two control groups exposed to oxygen (22%) with balance nitrogen for 24 hours from days 8 to 13 and days 12 to 19 of pregnancy (Group 2 and 4). One control group exposed to compressed air (group 11).
- Analytical verification of test atmosphere concentrations: yes, twice daily. Due to leakage from the homemade chamber, concentration were usually lower than the target concentration, ranging between 9000 and 12000 ppm with a maximum of 15000 ppm and a minimum of 100 ppm.

Description of test design:

- Sacrifice: GD 21 (method of sacrifice not specified)

- Uterus examination for foetal death. Foetal deaths include resorption spots, macerated foetuses and dead foetuses.

Results and discussion

Table 29: Study results as published by Corbett et al., 1973

	No. of pregnant rats	No. of pregnancies	Dinitrogen oxide (ppm)	Time exposure (h/d)	Days of pregnancy exposed	Implantations /rat	Foetal death rate
Group 1	12	72	15,000	24	8-13	6.0 **	11.1**
Group 2	10	112	0#	24	8-13	11.2	1.8
Group 3	6	53	1000	24	12-19	8.8*	18.9*
Group 4	9	100	0#	24	12-19	11.1	4.0
Group 5	10	109	1000	8	10-13	10.9	18.4**
Group 6	9	97	100	8	10-13	10.8	15.5*£
Group 7	7	76	1000	8	14-19	10.9	14.5*
Group 8	10	99	100	8	14-19	9.9	6.1
Group 9	7	82	1000	8	10-19¤	11.6	7.3
Group 10	7	80	100	8	10-19¤	11.4	5.0
Group 11	11	112	0$			10.2	5.4

Exposed to 22% O_2, 78% N_2; $ exposed to room air, ¤ Groups 9 and 10 were exposed from 2ppm until 10ppm; group 5-8 were exposed from 6am until 2 pm; *p<0.05, **p≤0.01; £ Significant only compare to group 2

- The non uniform number of rats per groups was the results of rats non being pregnant.
- Statistically significant increased foetal death after 24 hour/day exposure. Might have been due to total resorption.
- Significant increase in foetal death rate in rats exposed to 8 hours to dinitrogen oxide from 6am to 2 pm (group 5 and 7) at 1000 ppm. No increase was observed in group 9 exposed from 2pm to 10pm to a longer period (GD10-19).

2.1.2 Human data

2.1.2.1 Alhborg et al., 1996

Study reference: Ahlborg, G., G. Axelsson, et L. Bodin. « Shift Work, Nitrous Oxide Exposure and Subfertility among Swedish Midwives ». *International Journal of Epidemiology* 25, n° 4 (1996): 783-90.

Abstract:

Background: Shift work and dinitrogen oxide exposure have both been suspected of having adverse influence on the reproductive performance of health workers. Time to pregnancy has been suggested as a sensitive measure of fecundity in occupationally exposed groups. We investigated the effects of shift work and dinitrogen oxide exposure on the fertility of Swedish midwives.

Methods: A questionnaire was mailed to all members of the Swedish Midwives Association who were born 1940 or thereafter, 3985 in all. Eighty-four per cent responded. Detailed information on the number of menstrual cycles required to achieve pregnancy and the working conditions during that period were obtained concerning the most recent, planned pregnancy occurring after 1983. The per cycle probability of becoming pregnant was calculated for each exposure category, and the relation to the unexposed was expressed as fecundability ratios.

Results: Midwives who worked two-shift, three-shift rotas, or only nights had reduced fertility compared to those working in the day time. The fecundability ratios were 0.78 (95% confidence interval [CI]: 0.65-0.94),

0.77 (95% CI: 0.60-0.98), and 0.82 (95% CI: 0.64-1.03), respectively, after adjustment for covariates. No effect of dinitrogen oxide exposure was noted except in the small group reporting that they assisted at more than 30 deliveries per month when dinitrogen oxide was used (fecundability ratio = 0.64; 95% CI: 0.44-0.95).

Conclusion: Shift work and frequent, high occupational exposure to dinitrogen oxide may have a negative influence on the ability of women to become pregnant.

2.1.2.2 Rowland et al., 1992

Study reference: Rowland, A.S., DD. Barid, C.R. Weinberg, D.L. Shore, C.M. Shy, A.J, Wilcox. " Reduced fertility among women employed as dental assistants exposed to high levels of nitrous oxide. N Engl J Med. 1992 Oct 1;327(14):993-7

Abstract:

Background: Fertility is reduced in female rats exposed to levels of dinitrogen oxide similar to those found in some dental offices. Epidemiologic studies have suggested an association between exposure to mixed anesthetic gases and impaired fertility. We investigated the effects of occupational exposure to dinitrogen oxide on the fertility of female dental assistants.

Methods: Screening questionnaires were mailed to 7000 female dental assistants, ages 18 to 39, registered by the California Department of Consumer Affairs. Sixty-nine percent responded. Four hundred fifty-nine women were determined to be eligible, having become pregnant during the previous four years for reasons unrelated to the failure of birth control, and 91 percent of these women completed telephone interviews. Detailed information was collected on exposure to dinitrogen oxide and fertility (measured by the number of menstrual cycles without contraception that the women required to become pregnant).

Results: After controlling for covariates, we found that women exposed to high levels of dinitrogen oxide were significantly less fertile than women who were unexposed or exposed to lower levels of dinitrogen oxide. The effect was evident only in the 19 women with five or more hours of exposure per week. These women were only 41 percent (95 percent confidence interval, 23 to 74 percent; P less than 0.003) as likely as unexposed women to conceive during each menstrual cycle.

Conclusions: Occupational exposure to high levels of dinitrogen oxide may adversely affect women's ability to become pregnant.

2.2 Specific target organ toxicity – single exposure

2.2.1 Human data

2.2.1.1 Guylai et al., 1996

Study reference: Gyulai, F. E., L. L. Firestone, M. A. Mintun, and P. M. Winter. 'In Vivo Imaging of Human Limbic Responses to Nitrous Oxide Inhalation'. *Anesthesia and Analgesia* 83, no. 2 (August 1996): 291–98.

Detailed study summary and results:

Study design and population

- N= 8 healthy volunteers (21-46 y), 5 women and 3 men (21-46 years)
- Inclusion : No pregnant/No history of psychotic or mood disorder or substance abuse

Health outcome

- Changes in brain activity (regional Cerebral blood flow (rCBF) and regional metabolic rates (rCMR)), Cranial PET scans were performed
- Alteration of rCBF is interpretated as reflecting temporally related changes in neuronal activity

Exposure

- Inhaled gas mixtures were delivered through a conventional semiclosed anesthesia circuit system using a tight-fitting, soft plastic face mask and monitored during both inspiration and expiration.
- Two experimental conditions :
  - exposed to 200,000 ppm dinitrogen oxide and 30% atm O2, balance room air
  - control condition (air)
- Dinitrogen oxide administration began at least 15 min before PET scanning and brain concentrations, which were maintained during the entire scanning session.

Results

- Point analysis of rCBF and rCMR scans revealed no significant ($P < 0.05$) differences between the 20% dinitrogen oxide-induced percentage increases in randomly selected pixels in the anterior cingulate cortex images.
- No significant differences were found between the 20% dinitrogen oxide-induced percentage decreases in randomly selected pixels in the parahippocampal gyrus, hippocampus, and posterior cingulate cortex images.
- The cardiovascular and respiratory physiologic parameters were not significantly affected ($P < 0.05$) by administration of dinitrogen oxide.
- Comparison of the rCBF profiles obtained during control and dinitrogen oxide conditions revealed multiple areas of significant ($P < 0.005$) dinitrogen oxide-induced activation. These were localised by effect-size maps to the anterior cingulate cortex in both hemispheres. Comparison also revealed loci of significant bilateral deactivation ($P < 0.001$) in the posterior cingulate, hippocampus, parahippocampal gyrus, and visual association cortices.
- Although subjective experiences were not measured, every volunteer reported some degree of sedation and euphoria, as well as altered perception of time and self-image, during dinitrogen oxide inhalation.

2.2.1.2 Yajnik et al., 1996

Study reference:

Yajnik, S., J. P. Zacny, C. J. Young, J. L. Lichtor, G. Rupani, J. M. Klafta, D. W. Coalson, et J. L. Apfelbaum. « Lack of Acute Tolerance Development to the Subjective, Cognitive, and Psychomotor Effects of Nitrous Oxide in Healthy Volunteers ». *Pharmacology, Biochemistry, and Behavior* 54, n° 2 (1996): 501-8.

Detailed study summary and results:

Study design and population

- N=5 women and 6 men (mean age 22.8)
- Inclusion criteria: Although the majority of subjects had some experience with marijuana, their lifetime use of this drug and other illicit drugs was generally light.
- Exclusion criteria: history of significant psychiatric disorders or substance use disorder, taking prescription medications, history of neurology, cardiac, pulmonary, hepatic or renal disease

Health outcome: cognitive function

- subjective effects reported in questionnaires
- visual analogue scale
- Backward digit span test
- auditory reaction tume
- Eye-hand coordination

Exposure assessment:

- 5 sessions separated by at least 1 week, random cross-over design was used.
- Each session was approximately 190 min in duration.
- Each session consisted of three periods: baseline, inhalation and recovery
- Anaesthesia facial mask. Flow rate was 5l/min.
- 0, 100,000, 200,000, 300,000, 400,000 ppm dinitrogen oxide in oxygen.

Results:

- No evidence of a lessening of drug effect during the 120-min inhalation period.
- Dinitrogen oxide impaired cognitive and psychomotor performance.
- Statistically significant changes were not noted in any of the physiological parameters.
- Little evidence suggestive of acute tolerance to either the subjective or the cognitive/psychomotor impairing effects of dinitrogen oxide.
- Subjective effects and psychomotor impairment remained relatively invariant throughout the inhalation part of the session.
- Lack of acute tolerance to the psychomotor impairing effects of dinitrogen oxide
- Generally, the magnitude of cognitive and psychomotor impairment was concentration dependent. The recovery of psychomotor/cognitive function was rapid, with performance returning to near baseline levels 5 min into the recovery period for most of the measures.

2.2.1.3 Fagan et al., 1994

Study reference:

Fagan, D., D. L. Paul, B. Tiplady, et D. B. Scott. « A Dose-Response Study of the Effects of Inhaled Nitrous Oxide on Psychological Performance and Mood ». *Psychopharmacology* 116, n° 3 (1994): 333-38.

Detailed study summary and results:

Study design and population

- 20 healthy volunteers (8 males and 4 females), age = 20-35 years.
- The study used a five-period crossover design, comparing placebo; 50,000; 100,000; 200,000; and 400,000 ppm dinitrogen oxide in oxygen. Treatment order was randomised. Each period took place on a separate day. Test mixtures were given by inhalation over a period of 1 h. Effects on performance were determined using objective and subjective measures over this period.
- The following measures of performance and mood were used: critical flicker fusion threshold, choice reaction time, body way, Digit symbol substitution, tapping, Gibson spiral maze, continuous attention task,visual vigilance, visual analogue scale,
- 5 session in separate days.
- Each subject completed a set of visual analogue scales (Vas) after which the mask were attached and the inhalation began. After 15 minutes inhalation, the subject completed a second set of VASs, and then completed the full battery of tests, which took about 45 rain. Finally, a third set of VASs was completed, and the inhalation of dinitrogen oxide ended

Results

- All values for inspired and expired concentrations of dinitrogen oxide were within 0.5% of the intended concentration of dinitrogen oxide, and that the coefficient of variation never exceeded 6%.
- All test measures in the battery of test with the exception of critical flicker fusion showed significant dose related effects of dinitrogen oxide.
- Clear dose-related effects were seen for the Dizzy- Steady and Clearheaded-Muzzy scales (visual analogue scales). For these scales, there was little difference to be seen between the results obtained at 15 and 60 min post-drug.

- The commonest symptoms reported during experiment were dizziness, paraesthesia and euphoria, all of which occurred most commonly at the highest dose of dinitrogen oxide. One subject vomited near the end of the testing session with 400,000 ppm dinitrogen oxide. Another subject became unconscious shortly after the 400,000 ppm session started. In both cases the session was terminated, the subjects were given 100% oxygen for a few minutes, and recovery was rapid.

2.2.1.4 Mahoney et al., 1988

Study reference:

Mahoney, F. C., P. A. Moore, E. L. Baker, et R. Letz. « Experimental Nitrous Oxide Exposure as a Model System for Evaluating Neurobehavioural Tests ». *Toxicology* 49, n° 2-3 (1988): 449-57.

Detailed study summary and results:

Study design and population

- 15 male volunteers, aged 24-34
- Testing on 4 separate days over 2 days
- Nasal inhalation mask
- Neurobehavioral evaluation system battery (continuous performance test, hand-eye coordination test, serial digit learning, symbol-digit substitution test, pattern recognition, pattern memory, switching attention, finger tapping, mood scale

Results:

- Dinitrogen oxide at 200,000 ppm significantly impaired performance on symbol-digit and finger tapping and approached statistical significance for continuous performance test response latency (P = 0.055).
- A significant drug effect on the confusion items contained in the NES Mood Scales was seen at the low dose.
- The dinitrogen oxide at 400,000 ppm impaired performance on virtually all NES measures. The serial digit learning task was the only NES test not significantly affected although statistical significance was approached (P -0.055). Also, as seen in the lower dinitrogen oxide dose, only 1 of the 5 selfrating scales within NES Mood Scales (Confusion) was significantly affected.
- A significant improvement in performance was observed between training and control sessions when no dinitrogen oxide was administered. Significant "practice" or "learning" effects were observed for continuous performance test, symboldigit, hand-eye coordination, switching attention and finger tapping. Internally consistent performance and effects of dinitrogen oxide were observed on the Switching Attention task. No impairment was noted with 200,000 ppm dinitrogen oxide in the first 2 'simple' conditions, but 2 separate variables associated with the 'complex' condition were significantly affected (switchingside and switching-direction).
- Significant improvement in performance was observed between training and control sessions for both switching conditions while response times for the simple conditions, side and direction, remained relatively stable across all sessions. The lowest order of stimulus complexity in the Switching Attention task (which requires subjects simply to respond to the location of a large rectangle) produced the lowest mean response time of 4 variable measures. Adding to the perceptual complexity of the stimulus in the second condition, i.e. determining the direction of an inset arrow, raised the response time. The same relationship was also observed when comparing the stimuli used in both switching conditions, that is, the switching-side respose latency was substantially lower than switching- direction latency. In addition, the added cognitive load of switching repeatedly from one response criterion to the other increased response latencies above those for the corresponding simple response conditions.

2.2.1.5 Estrin et al., 1988

Study reference:

Estrin, W. J., P. Moore, R. Letz, et H. H. Wasch. « The P-300 Event-Related Potential in Experimental Nitrous Oxide Exposure ». *Clinical Pharmacology and Therapeutics* 43, n° 1 (1988): 86-90.

Detailed study summary and results:

Study design and population

- 6 healthy volunteers (sex not specified), 27-35 years
- Dinitrogen oxide administered at 4 different concentration during a 2-hour testing period. The subject were kept at dose for 10 minutes initially and remained at that dose for an additional 20 minutes during which the subtests of the psychometric test battery were given and the P-300 determination was made.
- Flow rate: 10l/min
- Dinitrogen oxide concentration: 100,000, 200,000, 400,000 ppm mixed in oxygen.

Results

- Each of the six subjects tolerated the initial administration of 100% 02 by nasal inhalation and the subsequent administration of 100,000, 200,000, and 400,000 ppm dinitrogen oxide.
- Performance on all three behavioral outcomes was impaired by the 400,000 ppm dinitrogen oxide dose.
- With the exception of SDT, all variables showed trends in the expected direction by the 10% dose.
- Difference scores relative to performance during the 0% dinitrogen oxide condition were calculated, and 18 data point for each outcome variable were correlated. The highest correlation was between CPT latency and P-300 amplitude ($r = 0.61$; $P < 0.05$).
  Change in CPT latency and FTT were well correlated ($r = 0.59$; $P < 0.05$). Change in P-300 latency and amplitude were negatively correlated ($r = 0.33$). Although only two of these correlations were statistically significant, all exhibited trends in the expected direction.
  A dose-dependent reduction in the amplitude of the P-300 wave, as well as a dose-dependent increase in P-300 latency, was present, correlating with increased concentration of dinitrogen oxide.

2.2.1.6 Venables et al., 1983

Study reference:

Venables, H., N. Cherry, H. A. Waldron, L. Buck, C. Edling, et H. K. Wilson. « Effects of Trace Levels of Nitrous Oxide on Psychomotor Performance ». *Scandinavian Journal of Work, Environment & Health* 9, n° 5 (1983): 391-96.

Detailed study summary and results:

Study design and population

- 24 healthy volunteers students, 25.2 mean age
- Each Each subject received a placebo or 50 ppm of dinitrogen oxide, in a design balanced for order effects, over two experimental sessions in an exposure chamber. The sessions were 4 h in duration, and performance testing took place during the final 40 min in the chamber.
- 2.5 m^3 inhalation chamber
- Performance tests: audiovisual tasks, simple reaction time, four choice reaction time, stressanalyser, visual analogue scale.

Results

- One subject did not attend the session in which he would have received the placebo treatment. His results for the other session have been included in the analysis however.
- There was no difference in performance scores in the performance test between the two conditions. It is particularly worth noting that, in the case of the audiovisual task, the mean reaction time is exactly the same for both treatments.
- Visual analogue scores. It can be seen that there was a greater deterioration in mood on all four dimensions with exposure to 50 ppm of dinitrogen oxide; the differences were not, however, statistically significant.

2.2.1.7 Bruce and Bach et al., 1976

Study reference:

Bruce, D.L., et M.J. Bach. « Effects of trace anesthetic gases on behavioural performance of volunteers ». *British Journal of Anesthesia* 48, n° 9 (1976): 871-76.

Detailed study summary and results:

Study design and population

- 100 healthy male volunteers (20-30 year-old), tested twice, 1 week separating the two exposure.
- Ten were exposed first to air while the other received anaesthetic first.
- 5 exposure unit (20 subjects per unit), 4 hours of exposure
  - Dinitrogen oxide 500 ppm + halothane 10 ppm
  - Dinitrogen oxide 500 ppm
  - Dinitrogen oxide 50 ppm + halothane 10 ppm
  - Dinitrogen oxide 50 ppm
  - Dinitrogen oxide 25 ppm + halothane 0.5 ppm
- Administration: 8l/min, mask worn by the subjects
- Testing began two hours after start of exposure
- Performance tests: tachistoscope, raven matrices, O,connor dexterity, 3-min audiovisual, digit span,

Results

- Considerable variation in the anaesthetic concentrations of endexpired air samples was observed. This was particularly true of the first group of subjects, exposed to dinitrogen oxide 500 p.p.m. plus halothane 10 p.p.m., and the authors noted that this may explain why their test scores were actually better than those exposed to 500 p.p.m. dinitrogen oxide only in a subsequent set. At first, the tightness of mask fit was not checked closely, and a few subjects had very low end-expired dinitrogen oxide concentrations. It would appear from these data that the measured expired dinitrogen oxide concentrations correlate closely with decrements in test scores.
- The test data indicate that measurable and statistically significant decrements in performance may result from exposure to anaesthetic agents in concentrations as small as 50 p.p.m. dinitrogen oxide, a figure well below those measured in studies of anaesthetic content of operating room air. The tests were not equally sensitive to the effects of the anaesthetic gases, varying from the extreme sensitivity of the audiovisual task to no change in the O'Connor Dexterity test.
- Visual perception was impaired by anaesthetic agents, as shown by the effects on both the tachistoscopic and audiovisual tasks. This was not confirmed by the vigilance task, however, which was primarily a test of visual perception. Perhaps the infrequent changes in that task, compared with the rapid reactions required in the tachistoscopic and audiovisual tasks, allowed the subject to focus his attention more effectively on the changes requiring a response. The improvement in vigilance response in the group exposed to dinitrogen oxide 50 p.p.m. and halothane 1 p.p.m. cannot be explained.

- The ability to reason logically was relatively resistant to the effect of anaesthetic gases, as judged by the Raven Matrices test. This function may be ingrained so deeply that it is relatively insensitive to stressors. Alternatively, the authors noted that there might exist a different test which would have been sensitive.

2.2.1.8 William et al., 1984

Study reference:

Williams, D. J., R. J. Morgan, P. S. Sebel, and D. E. Maynard. 'The Effect of Nitrous Oxide on Cerebral Electrical Activity'. *Anesthesia* 39, no. 5 (May 1984): 422–25.

Detailed study summary and results:

Study design and population

- 15 subjects (21-35 y – staff of the London hospital), males and females
- Health outcome: cerebral activity (cerebral function analyzing monitor (CFAM))
- Exposure assessment:
  - Mask :
  - exposed to room air, 100% oxygen and 100,000, 300,000 and 500,000 ppm dinitrogen oxide
  - During 10 min for air and oxygen
  - During 15 min for dinitrogen oxide mixtures (each concentration)

Results

- Data only on 9 subjects (6 subjects unable to cooperate or finding the study unpleasant at high concentration).
- Statically significant reductions in amplitude of the proccesse electroencephalogram (EEG) at 300,000 and 500,000 ppm dinitrogen oxide
- Subjectif effects: hyeracusis, emotina states (fear and panic to elation and euprhoria).
- At 300,000 and 500, 000 ppm, dinitrogen oxide caused a significant reduction in the amplitude of CFAM

2.3 Specific target organ toxicity – repeated exposure

2.3.1 Animal data

2.3.1.1 Misra et al., 2020

Study reference: Misra, Usha Kant, Sandeep Kumar Singh, Jayantee Kalita, et Alok Kumar. « Astrocyte Activation Following Nitrous Oxide Exposure Is Related to Oxidative Stress and Glutamate Excitotoxicity ». Brain Research 1730 (2020): 146645.

Test type

- Rats, non-guideline repeated-dose neurotoxicity study

Test substance

- Dinitrogen oxide (no information on purity)

Test animals

- Male Wistar rats
- N= 5-10 per group

Administration/exposure

- Inhalation
- Whole body, 60-day exposure, 2h/d
- Concentration levels: control, dinitrogen oxide (500 000ppm) and O_2 (1:1 ratio)

Description of test design:

- Behavioral, Biochemical, Histopathological study.
- For behavioral changes experiments, activity scores were recorded for 3 sessions of 5 min each.

Results and discussion

- No animal died during the experiment
- Statistically significant decrease in bw gain at the end of the experiment (200.6 mg vs 245.6 mg in controls)
- Significant reduction in total distance travelled, time moving and number of rearing whereas time resting increased compared to the control rats. Grip strength was significantly decreased in exposed group compare to control animals.
- Homocystein levels, glutamate and malanodialdehyde (MDA) levels were significantly increased, however GSH and total antioxidant capacity (TAC) level decreased in N_2O exposed group compared to the controls.
- Astrocyte phenotype and its activation was significantly altered more so in spinal cord compared to cerebral cortex and was associated with neurobehavioral changes, oxidative stress and glutamate level.

2.3.1.2 Singh et al., 2015

Study reference: Singh, Sandeep Kumar, Usha Kant Misra, Jayantee Kalita, Himangsu K. Bora, et Ramesh C. Murthy. « Nitrous Oxide Related Behavioral and Histopathological Changes May Be Related to Oxidative Stress ». *NeuroToxicology* 48 (2015): 44-49.

Test type

- Rats, non-guideline repeated-dose neurotoxicity study

Test substance

- Dinitrogen oxide (no information on purty)

Test animals

- Male Wistar rats
- N= 6 per group

Administration/exposure

- Inhalation
- Whole body, 1-month exposure, 90min/day
- Concentration levels: control (air and oxygen), dinitrogen oxide (500,000 ppm) and O_2 (1:1 ratio)

Description of test design:

- Behavioral, Biochemical, Histopathological study.

Results and discussion

- There was progressive weight loss in exposed group compared to controls (303.8±14.11 vs 244.6±8.75 g; p = 0.0073).
- After exposure the rats appeared sluggish, lethargic and developed limb weakness which was more marked in the hind limbs. These symptoms recovered in 1–1.5 h.
- In the exposed group, the total distance traveled (2001.66± 118.27 cm; p = 0.037), time moving (80.16± 5.7 s; p = 0.028), number of rearing (10.33 ±1.45; p = 0.014) and grip strength (1042.40 ±51.3 N; p = 0.041) were significantly decreased whereas, resting time significantly increased (219.83 ±5.7 s; p = 0.030) compared to controls.
- Serum vitamin B12 level was below 150 ng/dl in 5 of 6 rats in both exposed and control groups. HCY level was significantly increased in the exposed group compared to control (20.56±1.30:10.40±1.42 mm/ml; p = 0.0007)
- Glutathione level (2.21 0.60 mg/dl; p = 0.018) and total antioxydatn capacity (TAC) level was significantly decreased (0.76 0.16 Trolox_Eq_mmol/l; p = 0.036) following N2O exposure. Both GSH and TAC had negative correlation with homocysteine (HCY). With serum B12 GSH and TAC were not significantly correlated. GSH and TAC correlated with different behavioral parameters
- Histopathological examination of brain tissues did not reveal any abnormality in the control group. However, there were variable changes in the meninges and brain parenchyma in the exposed group.
    - The meninges showed localized capillary congestion with vascular dilatation. Vascular congestion and focal hemorrhage was observed in cerebral cortex. Neuronal degenerations characterized by shrinkage and vacuolation were invariably observed in cerebrum. There was thickening of vascular endothelial wall with infiltration of mononuclear and polymorphonuclear cells which were more marked in subarachnoid space. Outer molecular layer of cerebral cortex showed focal demyelination characterized by depletion of myelin and vacuolation. Degenerative changes such as neuronophagia and satellitosis were invariably observed in external pyramidal layer.
    - No histological abnormalities were observed in cerebellum except focal capillary congestion
    - There were multiple demyelinating areas characterized by vacuolation and depletion of myelin throughout the white matter

2.3.1.3 Dyck et al., 1980

Study reference: Dyck, P. J., L. A. Grina, E. H. Lambert, C. S. Calder, K. Oviatt, K. Rehder, B. A. Lund, et K. A. Skau. « Nitrous Oxide Neurotoxicity Studies in Man and Rat ». *Anesthesiology* 53, n° 3 (1980): 205-9.

Abstract: To assess the effects of chronic exposure to low levels of nitrous oxide on neural function of man, the authors evaluated the neurologic condition, motor and sensory nerve conduction, and computerized tests of sensation of approximately half of the dentists in Rochester, Minnesota. Results of scored tests of neural function were not significantly different for dentists who used nitrous oxide extensively in their practices and dentists who did not. To assess the effects of chronic exposure to high levels of nitrous oxide on neural function and structure of experimental animals, groups of rats were exposed to 70 per cent N_2O in 30 per cent oxygen for four hours, five days a week, for six months. Rats exposed to N_2O and control rats showed no difference in well-being, in caudal nerve conduction, in axonal content and transport of acetylcholinesterase and dopamine-beta-hydroxylase, or in number and size distribution and pathologic abnormality of teased myelinated fibers. Although these results indicate a lack of peripheral nerve neurotoxicity of N_2O in the rat, one cannot assume a similar lack of neurotoxicity in man with heavy exposures.

2.3.1.4 Hayden et al., 1974

Study reference: Hayden, Jess, G.D. Allen, L.A. Butler, G.B. Lewis, and R.L. Schultz. 'An Evaluation of Prolonged Nitrous Oxide-Oxygen Sedation in Rats'. *The Journal of the American Dental Association* 89, no. 6 (December 1974): 1374–80.

Abstract: The effect of long-term exposure to an analgesic concentration of nitrous oxide-oxygen on the cerebral cortex of rats was determined in this experimental study.
Observable effects on the cerebral cortex of rats after prolonged exposure to nitrous oxide-oxygen in an analgesic concentration were determined.
Sixteen rats in two experimental groups were housed in airtight cages through which 40% nitrous oxide in oxygen was passed for eight hours a day. Four rats composed the control group. The animals in the first experimental group were killed after seven days' exposure and rats in the second group after 14 days' exposure. Rats in the control group were killed at the same intervals. Blocks of tissue from the frontal, parietal, and occipital portions of the brain were removed from the animals, and a cortical cell count was completed.
Observable signs of damage to cortical cells, especially those in the occipital region, were found. Differences in some aspects of cellular response seem to be sex related.

2.3.1.5 Fung et al., 1993

Study reference: Fung, Y. K., M. R. Brown, et R. E. Sullivan. « Effects of Nitrous Oxide Exposure on Behavioral Changes in Mice ». *Pediatric Dentistry* 15, n° 2 (1993): 93-98.

Test type

- Mice, non-guideline repeated-dose neurotoxicity study

Test substance

- Dinitrogen oxide (no information on purity)

Test animals

- Male adult mice
- N= 8 per exposed group and 7 in controls

Administration/exposure

- Inhalation
- Whole body, 8h per day for 8 consecutive days
- Concentration levels: control (air), dinitrogen oxide at 1000 and 2000 ppm

Description of test design:

- Endpoints assessed: motor coordination, locomotor activity, stereotypic behavior and anxiety level, cellular examination of the occipital cortex.
- Locomotor activity: measured one-day after exposure (horizontal activity was measured) at 10 min. for 2h (10, 30, 60, 90, 120 min time-points).
- Anxiety assessment was investigated during 30 min. Side changes, dark-like area time spent measured
- Stereotypy: total number of stereotypies at 10 rainintervals for 2hr.
- Coronal sections of the occipital lobe of control and animals exposed to 2000 ppm dinitrogen oxide were examined under light microscopy. The large cells (neural cells) and small cells (neuroglial cells were counted in a 0.03 mm2 area.
- Motor coordination: rolling-roller performance step (rotaring rod)

Results and discussion

- No effect was found on motor coordination or anxiety level.

- Mice exposed to N_2O showed reduced locomotor activity compared to control animals; however, with the exception of the longest time period (120 minutes), this decrease was not statistically significant and not dose-related.
- Mice exposed to N_2O showed a dose-dependent reduction in stereotypic behaviour. Necropsy was performed in the 2,000 ppm group.
- Although the number of neural cell counts was less in the N_2O exposed group compared to control mice, this was not statistically significant (206±8 cells in control vs 186±7.2 in 0.2% N_2O group).
- No significant differences were seen in the number of neuroglial cells or total cells counted in the control tissues, as compared to the neural tissues from mice exposed to N_2O.

2.3.1.6 Rice et al., 1985

Study reference: Rice, S. A., R. I. Mazze, et J. M. Baden. « Effects of Subchronic Intermittent Exposure to Nitrous Oxide in Swiss Webster Mice ». *Journal of Environmental Pathology, Toxicology and Oncology: Official Organ of the International Society for Environmental Toxicology and Cancer* 6, n° 2 (1983): 271-81.

Test type

- Mice, repeated-dose toxicity study

Test substance

- Dinitrogen oxide (purity not stated)

Test animals

- Swiss Webster mice
- N= 15/sex/group

Administration/exposure

- Inhalation
- 14-week whole body exposure, 4h/d, 5d/w
- Concentration levels: control (air), dinitrogen oxide at 5000, 50,000 and 500,000 ppm.

Description of test design:

- Endpoints assessed: liver, kidney, spleen and testis were weighed and examined histologically along with brain, stomach, seminal vesicle and ovary. Blood smears were performed (blood count, white blood cell count, reticulocyte and platelet counts), liver P450 content, rate of defluorination of enflurane and methoxyflurane).

Results and discussion

- All animals survived to necropsy.
- Statistically significant treatment-related decrease in body weight at the top dose (decreased in body weight gain in males and females by 77 and 63% vs control, respectively).
- No effect on brain weight, no histopathologic findings in brain.

2.3.2 Human data

2.3.2.1 Thayabaran et al., 2021

Study reference:

Thayabaran D., Burrage D. Nitrous oxide-induced neurotoxicity: a case report and literature review. Brit J Clinical Pharma. 2021;87:3622–3626.

Detailed study summary and results:

A 29-year-old man presented with a 2-week history of ascending lower limb numbness, pins and needles, and difficulty walking. He reported symptoms developing 3 days after the abrupt cessation of Dinitrogen oxide use. He described ascending symptoms from feet to thigh before developing right foot pain and unsteadiness, prompting his attendance to the emergency department. At peak, he was using 60 dinitrogen oxide canisters (whippets) per day for the first 5 days followed by use every 2-3 days thereafter. His past medical history was unremarkable and he did not use any regular medication. Dinitrogen oxide was obtained using multiple catering canisters to fill up a balloon from which he would directly inhale. Prior to neurological symptoms, he experienced profuse sweating which began the day after stopping use of Dinitrogen oxide. He denied any unwell contacts, recent illness or travel, and disclosed intermittent low-level alcohol and cannabis use. On examination he had an unsteady gait, reduced sensation to light touch and pinprick anteriorly from ankle to mid-thigh, absent deep tendon reflexes and down-going plantars. His neurological examination was otherwise unremarkable. Vitamin B12 levels were low at 164 ng L^{-1} (normal range 197–771) and his homocysteine levels were high at 51.6 μmol L^{-1} (normal range 0–15). Magnetic resonance imaging (MRI) spine was unremarkable. He was subsequently treated with intravenous vitamin B substances and ascorbic acid account for suspected nutritional deficiencies, and reported an immediate improvement in symptoms, most notably in his gait and paraesthesia. He was discharged with a loading course of 1000 μg of hydroxocobalamine intramuscularly twice weekly (for 10 doses) and 5 mg daily folic acid.

2.3.2.2 Zheng et al., 2020

Study reference:

Zheng, D., Ba, F., Bi, G. et al. The sharp rise of neurological disorders associated with recreational nitrous oxide use in China: a single-center experience and a brief review of Chinese literature. J Neurol 267, 422–429 (2020).

Detailed study summary and results:

a. Retrospective single-centre study report

Study method:

The authors retrospectively collected data of patients diagnosed with neurological disorders associated with recreational use of N2O in Shengjing Hospital of China Medical University from January 2018 to June 2019.

Results:

There were 43 patients who met the inclusion criteria (average age: 21.9 ± 3.3 years, range 15–30 years, 21 males and 22 females). On average, the patients' usage rate of N2O was 10.8 ± 6.6 times/month (range 1–28). The duration of recreational N2O use was 10.7 ± 6.5 months (range 0.7–48). The most common clinical manifestations were limb numbness and weakness (98%) followed by unsteady gait (70%). Other neurologic manifestations included urine and bowel dysfunction, memory impairment, headache and dizziness, seizures, and involuntary movement. The most common psychological symptom was anxiety (14%) followed by insomnia, hallucination, and lethargy. The patients also showed chest tightness (12%), eating disorders (9%), skin hyperpigmentation (7%), and sexual dysfunction (5%).

The main abnormal findings in the blood tests were vitamin B12 deficiency in 63% of the patients (169.4 ± 79.1 pg/mL, normal range: 180–910 pg/mL), hyper-homocysteine in 93% of the patients (78.1 ± 32.2 μmol/L; normal range: 0–20 μmol/L), and anaemia in 19% of the patients. The other blood tests performed were unremarkable. Three patients underwent lumbar puncture with only one showing slightly increased protein levels in the cerebrospinal fluid. In 77% of patients, MRI of the spinal cord revealed longitudinal high T2 signal lesions in the dorsal spinal cord. Except in one patient who showed lesions in the cervical and thoracic spinal cord, the rest only showed lesions in the cervical spinal cord. Majority of the patients with abnormal MRI findings showed lesions extending to 4–6 spinal segments. In the axial MRI image, the lesions were in an inverted "V", "Triangle", or "oval" shape and mainly presented in the posterior column of the spinal cord. Patients with mental symptoms underwent brain MRI scans. Among these patients, only one with prominent memory impairment and seizures showed diffuse brain atrophy on brain MRI, which showed significant improvement after treatment for 1 month. Electromyography showed sensory and/or motor nerve impairment in all the patients. Mixed axonal and demyelinating neuropathy was the typical electromyography finding (93%) and was more prominent in the lower limbs.

Based on the history of N2O use, neurological examination, laboratory results, and electromyography and MRI findings, the following diagnoses were made: peripheral neuropathy (10/43), myeloneuropathy (30/43), and combined myeloneuropathy and toxic encephalopathy (3/43). All the patients were required to stop recreational N2O use and received vitamin B12 supplementation (daily injections for 1–2 weeks followed by weekly injections for 4–8 weeks; monthly injections were administered if needed). The follow-up time varied from 4 to 32 weeks (mean 20.5 ± 11.8 weeks). At the end of follow-up, most of the patients showed satisfactory clinical recovery with only two patients having the same difficulty walking without assistance as before treatment. The most common residual symptom was numbness of the lower limbs.

b. Review

Methods:

Literature search using the "nitrous oxide" and "neurological disorder" as keywords in the Chinese literature databases of WANFANG and CNKI and the English literature databases of Pubmed and Web of Science.

The results of the literature search showed that from 2016, when the first case report from mainland China was published, to June 2019, a total of 40 papers containing 83 patients with neurological disorders associated with recreational N2O use had been published. There was a gradual annual increase in both the number of articles and patients from 1 article and 1 patient in 2016 to 12 articles and 48 patients in the first half of 2019. The neurological disorders reported in these articles were similar to the ones in the current study.

2.3.2.3 Oussalah et al., 2019

Study reference:

Oussalah A, Julien M, Levy J, et al. Global Burden Related to Nitrous Oxide Exposure in Medical and Recreational Settings: A Systematic Review and Individual Patient Data Meta-Analysis. J Clin Med. 2019;8(4):551.

Detailed study summary and results: review and meta-analysis

Material and method:

- Literature search was performed in Medline.
- Regular N_2O exposure was defined as repeated exposure to N_2O, especially in a recreational setting, pain management or occupational exposure, with minimum consumption of one cartridge per month. For each patient, the authors estimated the average number of cartridges consumed per day and the duration of exposure in years. In the recreational setting, N_2O was commonly available in the form

of small pressurized cartridges, which can deliver the equivalent of 8 L of N_2O gas at standard temperature and pressure (8 g) (Randhawa et al., 2016). The authors quantified the exposure to N_2O using the following formula: Amount of N_2O exposure = (average number of cartridges consumed per day × duration of exposure expressed in years).

Summary of results: literature review

- All the case reports included in the systematic review reported individual-level data on 100 patients. Among the 100 reports, most originated from North America (n = 51), Western Europe (n = 22), China/Taiwan (n = 12) and Australia/New Zealand (n = 10).
- The male:female gender distribution was 60:40 and the median age was 27 years (Interquartile range, 22–36; range). Most patients included in the meta-analysis were exposed to N_2O in the setting of recreational use (57%) or surgery (25%)

Table 30: Individual included in the meta-analysis (Oussalah et al., 2019)

Setting of dinitrogen oxide exposure	**N**	**n**	**Percentage (95% CI)**
Recreational	100	57	57.0 (47.1–66.9)
Surgery	100	25	25.0 (16.4–33.6)
Occupational exposure	100	9	9.0 (3.3–14.7)
Pain management	100	6	6.0 (1.3–10.7)
Manipulation under general anaesthesia	100	1	1.0 (0–3.0)
Munchausen syndrome	100	1	1.0 (0–3.0)
Management of sleep disturbance	100	1	1.0 (0–3.0)
Frequency of dinitrogen oxide exposure	**N**	**n**	**Percentage (95% CI)**
Regular	100	76	76.0 (67.5–84.5)
Once	100	24	24.0 (15.5–32.5)
Quantitative estimates of dinitrogen oxide exposure*	**n**	**Median**	**IQR (25^{th}–75^{th})**
Number of dinitrogen oxide cartridge per day	30	25	8–85
Duration of dinitrogen oxide duration (year)	52	0.7	0.3–1.5
Quantification of dinitrogen oxide exposure (cartridge-years)	28	18.5	1.4–99.9

The three most frequently reported diagnoses were: subacute combined degeneration (28%), myelopathy (26%), and generalized demyelinating polyneuropathy (23%). A T2 signal hyperintensity in the spinal cord was reported in 68% of patients who underwent magnetic resonance imaging of the spinal cord.

Table 31: Magnetic Resonance Findings and Diagnoses among the Patients Included in the Individual Patient Data Meta-Analysis.

Magnetic resonance imaging findings	**N**	**n**	**Percentage (95% CI)**
Presence of T2 signal hyperintensity in the spinal cord	75	51	68.0 (57.2–78.8)
Reported diagnoses*	**N**	**n**	**Percentage (95% CI)**
Subacute combined degeneration	100	28	28.0 (19.0–37.0)
Myelopathy	100	26	26.0 (17.3–34.7)
Generalized demyelinating polyneuropathy	100	23	23.0 (14.6–31.4)
Vitamin B12 deficiency	100	14	14.0 (7.1–20.9)
Axonal polyneuropathy	100	11	11.0 (4.8–17.2)
Encephalopathy	100	2	2.0 (0–4.8)

Recurrent paraparesis	100	1	1.0 (0–3)
MTHFR deficiency	100	1	1.0 (0–3)
Toxicity due to N_2O with no specific diagnosis applied	100	19	19.0 (11.2–26.8)

N: total number of studied patients; n: number of observations; N_2O: dinitrogen oxide. * Any patient could have more than one diagnosis applied.

- At least one neurological symptom was reported in 96% (92.1–99.9%) of patients and included the following clinical manifestations by decreasing order of frequency: paresthesia in the extremities (80%), unsteady gait or walking difficulties (58%), weakness (43%), fallings or equilibrium disorders (24%), Lhermitte's sign (15%), and ataxia (12%).
- Patients had a high risk of macrocytic anaemia with a median MCV of 100 fL (IQR: 94–103) and median values of hemoglobin of 12.8 g/dL (IQR: 10.8–14.2) and 10.7 g/dL (IQR: 8.3–12.4) in males and females, respectively. At least one hematological abnormality was retrieved in 71.7% of cases (59.9–83.4%). The proportions of patients with low hemoglobin level (<13.0 g/dL in men; <12.0 g/dL in women), low hematocrit level (<39% in men; <36% in women), and MCV >100 fL were 55.8%, 52.4%, and 41.8%, respectively .
- The median plasma vitamin B12 concentration was low (101 pmol/L, IQR: 74–161) with 70.7% of patients exhibiting a vitamin B12 level <150 pmol/L, considered as the threshold of vitamin B12 deficiency. The median plasma concentrations of homocysteine and methylmalonic acid were 55 µmol/L (IQR, 29–111) and 5.0 µmol/L (IQR: 1.1–6.6), respectively, with a vast majority of patients exhibiting high homocysteine (>15 µmol/L) and methylmalonic acid (>0.4 µmol/L) levels (90.3% and 93.8%, respectively). According to the cB12 scoring system, 90.9% of patients exhibited at least a decreased vitamin B12 status and 84.8% of patients exhibited a possibly or probably deficient vitamin B12 status. The serum folate concentration was in the normal reference range with a median of 12.8 µg/L (IQR, 7.3–14.6)
- Data regarding the amount of N_2O exposure was available in 28 patients. In exploratory analyses, the amount of N_2O exposure was not significantly correlated with any biological variable. Furthermore, the amount of N_2O exposure was not significantly associated with the most frequently reported diagnoses (subacute combined degeneration, generalized demyelinating polyneuropathy, and myelopathy), the most commonly reported clinical findings (paresthesia in extremities, numbness, tingling; unsteady gait, walking difficulty; weakness; and fallings or equilibrium disorders), or the presence of T2 signal hyperintensity in the spinal cord.

2.3.2.4 Lundin et al., 2019

Study reference:

Lundin MS, Cherian J, Andrew MN, Tikaria R. One month of nitrous oxide abuse causing acute vitamin B_{12} deficiency with severe neuropsychiatric symptoms. *BMJ Case Rep*. 2019;12(2):e228001.

Detailed study summary and results: case report

A 21-year-old university student studying abroad in the USA presented to the emergency department with double vision, lower extremity weakness with difficulty ambulating and other neuropsychiatric symptoms. Review of systems was positive for double vision, falls, dizziness, weakness in lower extremities and anxiety. There was no upper extremity paraesthesia or weakness, respiratory difficulty or shortness of breath. The patient described three episodes characterised by then momentary syncope preceded by a prodrome of loss of vision followed by tinnitus. On physical examination, the patient's extra-ocular muscles were intact, pupils were normal in size, and reactive to light and accommodation. The patient had 4/5 strength in the

lower extremities. The Romberg test was positive. No orthostasis was present and respiratory effort was normal. MRI of the brain and spinal cord were normal. Vitamin B12 was 78 pg/mL (58 pmol/L, reference 211–911 pg/mL). The patient had been using dinitrogen oxide capsules used for whipped cream recharging, which she obtained from other students, a few times daily for a month for the purpose of anxiety relief. The patient denied use of illicit substances or alcohol use. She smoked half a pack per day. She related that she had last used the N2O 3 days prior to presentation. She had stopped using it because it was no longer helping the anxiety. Also, the patient had been trying to aggressively diet in order to lose weight she had gained after returning from vacation, although she had not been a vegan or vegetarian.

Differential diagnosis: There was no significant medical history or family history of neurological diagnoses, genetic or metabolic disorders. Multiple Sclerosis, Vitamin B12 deficiency and other vitamin and nutrient deficiencies, Guillain–Barré syndrome, Miller Fisher variant, Neuromyelitis optica (Devic's disease), neurosyphilis, subacute sclerosing panencephalitis, Lyme disease, cerebellar ataxia (toxin mediated-alcohol, drugs vs infectious (prion, viral, toxoplasmosis), neoplasm (Lymphoma, meningioma etc), neurodegenerative disorders, psychogenic ataxia, conversion syndrome, Wilson's disease.

Treatment and outcome. The patient was treated with 1000 mcg intramuscular injections of B12 daily. the patient was treated with intramuscular vitamin B12 repletion. The patient was instructed to abstain from N2O. After parenteral treatment, the patient had improvement in her gait, muscle strength and anxiousness. Serum vitamin B12 prior to discharge was 1130 pg/mL (211–911 pg/mL).

2.3.2.5 Lan et al., 2019

Study reference:

Lan SY, Kuo CY, Chou CC, Kong SS, Hung PC, Tsai HY, Chen YC, Lin JJ, Chou IJ, Lin KL; PCHAN Study Group. Recreational nitrous oxide abuse related subacute combined degeneration of the spinal cord in adolescents - A case series and literature review. Brain Dev. 2019 May;41(5):428-435.

***Detailed study summary and results*: retrospective clinical study**

Methods. The study is a retrospective hospital-based case series conducted at Chang Gung Children's Hospital,Taoyuan, and Saint Paul's Hospital in Taiwan from March 2012 to January 2018. Patients under 20 years of age who were diagnosed with N2O-induced subacute combined degeneration of the spinal cord were enrolled. Clinical symptoms, onset and duration, laboratory data, nerve conductive velocities, somatosensory evoked potential and spinal magnetic resonance images of these patients were analyzed. The treatments and outcomes were compared. Degeneration of the spinal cord was defined as increased signal intensity on T2-weighted magnetic resonance imaging (MRI) within the white matter of the spinal cord without contrast enhancement. It was considered to be related to dinitrogen oxide if the patient had a history of dinitrogen oxide abuse during the period of 6 months before the onset of symptoms, and the degeneration was predominantly restricted to posterior and lateral columns. Detailed clinical history, diet behavior, physical and laboratory examinations were carefully obtained to exclude the other possible causative agents and etiologies.

A literature review was performed through searches of MEDLINE and PubMed databases, with the key-words ''spinal cord degeneration dinitrogen oxide" and/or ''myelopathy dinitrogen oxide" and/or ''neurotoxicity dinitrogen oxide" with the studies limited to human studies and case reports. Reference lists of the selected studies were viewed as a secondary source. Cases below 20 years of age who abused or used N_2O for recreational purposes were included. We excluded cases older than 20 years of age, those caused by dinitrogen oxide anesthesia, with underlying vitamin B12 deficiency or following a vegetarian diet. The search yielded seven case reports.

Results. Nine patients (eight girls and one boy) aged 14–19 years were included. Eight of these patients presented with numbness as the initial symptom, and three experienced hyperesthesia. All suffered from muscle weakness, including four with lower limb weakness alone and five with both upper and lower extremity involvement. Ascending weakness was noted in two cases and descending weakness in three. Eight patients eventually developed varying degrees of ataxia. When seeking medical help, the initial chief complaints included numbness or weakness (7/9), abdominal pain with constipation (1/9), and lower urinary tract symptoms (1/9). All patients had a history of dinitrogen oxide abuse during the period of 6 months before onset of the symptoms, and seven of the patients had used dinitrogen oxide over a long period of time (more than 6 months). None of the patients were veganists. A decreased or absence of deep tendon reflex was found in all of the patients, and eight had proprioception defects. All of the patients had decreased muscle power. Four of the patients had low levels of serum vita-min B12, two of whom were within lower limits. Eight patients had an elevated serum homocysteine level, and only two patients had anemia. Six patients received neurophysiology studies. All had demyelinating features, four had evidence of spinal cord involvement and one had axonal degeneration. Cervicothoracic MRI revealed degeneration of cervical areas in 6 patients, thoracic regions in 1, and simultaneous cervicothoracic involvement in the other. Cobamamide at a dose of 500–1500㎎ per day was prescribed after the diagnosis in all patients, and all had full recovery of muscle power within 2 months. Five patients had persistent sensory deficits, and sensory ataxia persisted in one patient. Constipation was noted in one patient, while none of the patients had urinary tract symptoms. A search of the literature found seven articles describing adolescent cases of subacute combined degeneration attributed to recreational dinitrogen oxide abuse, including two males and five females. All of the patients had numbness/paresthesia and five had weakness, including two with an ascending pattern.

Table 32: Published case of spinal cord degeneration/myelopathy in adolescents caused by recreational use/abuse of dinitrogen oxide (Lan et al., 2019)

Date	Reference	Age/sex	Symptoms	Neurologic exam	Lab	MRI changes	Treatment	Outcome
2012	Hsu CK, et al.	19/M	4-limb numbness Gait imbalance for 1 month	Muscular weakness Areflexia Ataxic Positive Romberg sign Sensory level	Low Vitamin B12 (156 pg/nL)	Posterior& anterior column of C-spine	1000 μg/day vitamin B12 × 5 days then 1000 μg/ week × 2 months	Full recovery after 2 months
2016	Chen HJ, et al.	20/F	Unsteady gait Involuntary movement Distal tingling sensation for 1 month	Dystonia Muscle power normal Hyperreflexia Extensor plantar-response Vibration/proprioception impairment	Vitamin B12 normal Normocytic anemia	Posterior column of C1-C6	2000 μg/day × 3 days then oral form, dose not mentioned	Involuntary movements disappeared, Walk independent slowly 3 weeks later
2011	Lin RJ, et al.	18/F	Lower limb numbness for 2 months, ascending Leg weakness Unsteady gait	Hyporeflexia Pinprick, joint position, vibration impairment Weakness Steppage gait Constipation	Vitamin B12 normal High homocysteine	Posterior column of C2-C6	Intramuscular vitamin B12, dose and duration unknown	Loss follow-up
2012	Ghobrial GM, et al.	19/M	Progressive numbness and weakness	Decrease muscle power Hyperreflexia Absence of vibration, proprioception and sensory light touch	Vitamin B12 normal	Posterior column of C2-C7	Intravenous methylprednisolone	Symptom resolution in 36 h
2015	Matthew Jones, et al.	18/F	Hand numbness Leg weakness Drop foot for 2–3 weeks	Decreased muscle power of lower limbs and tendon reflex Ataxic gait	Vitamin B12 normal MMA normal	Dorsal column of cervical and thoracic spine	Vitamin B12, dose not mentioned	Ambulate 100 ft when discharge
2015	Duque MA, et al.	20/F	Lower leg paresthesia for 3 weeks Difficulty walking	Hip flexor weakness Thoracic sensory level Reduced proprioception in distal lower limbs	Low Vitamin B12 (126 pg/ mL) High MMA No anemia High folate	Dorsal column & around central canal	Vitamin B12, dose not mentioned	Full recovery in 1 month
2017	Yuan JL, et al.	20/F	4 limb progressive paresthesia Unsteady gait for 15d	Mild cognition impairment, Decreased vibration & proprioception, bilateral hyporeflexia, sensory ataxia, positive Babinski sign and Romberg sign	Low folic acid No anemia Normal vitamin B12 and homocysteine	Posterior column of C1-T12	Intramuscular Vitamin B12 1 mg/day	Resolved markedly in 3 months

MRI: magnetic resonance imaging; C: cervical; T: thoracic.
MMA: methylmalonic acid.
Data collected from references [8,34–39].

Table 33: Clinical features of the 9 patients with dinitrogen oxide abuse (Lan et al., 2019)

Patient No.	Gender	Age	Symptoms			Neurological examination	Laboratory data		NCV Study* /SSEP study	Spinal MRI changes	N_2O abuse
			Numbness	Limbs weakness (Direction)	Others		Vit. B_{12} (pg/ml) (Ref. 197–771)	Homocysteine (μmol/L) (Ref. < 12)			
1	F	17	+	U + L	Hyperesthesia Ataxia	Decreased DTR Loss of P Decreased MP	221	12.1	Moderate motor neuropathy	C Spine	Heavily use, dose and duration not sure
2	F	16	+	U + L (Descending)	Ataxia	Absence of DTR Loss of P Decreased MP	219	10	Mild sensorimotor polyneuropathy/Normal	C1-C6 T7-T8	4L/day for 3 months
3	F	15	+	U + L (Descending)	Hyperesthesia Ataxia	Decreased DTR Loss of P Decreased MP	114	43	Mild polyneuropathy/ Spinal cord involvement	C2-C6	4-10*L* weekly for 6 months
4	F	18	+	U + L	None	Decreased DTR Loss of P Decreased MP	309	31	Moderate axonal sensorimotor polyneuropathy/Spinal cord involvement	NP	1 year, dose and frequency not sure
5	F	16	+	L (Descending)	Dizziness Hyperesthesia Ataxia	Decreased DTR Decreased MP	380	81.9	NP	C3-C6	Heavily use around 1 year, dose not sure
6	F	16	+	L	Ataxia LUTS	Absence of DTR Loss of P Decreased MP	112	119.6	NP	T8-T11	1 cylinder (6 kg)/month for 1 year
7	F	18	+	U + L (Ascending)	Ataxia	Decreased DTR Loss of P	195	18	Extreme sensorimotor polyneuropathy/Spinal cord involvement	C Spine	1 cylinder (6 kg) share with others, 3–4 times/ week for 1 year
8	M	19	–	L	Ataxia Constipation	Decreased DTR Loss of P Decreased MP	422	29	Moderate axonal sensorimotor polyneuropathy/Spinal cord involvement	C2-C7	More than 1 year; dose and frequency not sure
9	F	14	+	L (Ascending)	Ataxia Constipation Cramping	Absence of DTR Loss of P Decreased MP	<83	82.1	NP	C2-C8	4L/week for more than 1 year

C: cervical; T: thoracic; DTR: deep tendon reflex; F: female; M: male; L: lower; U: upper; LUTS: lower urinary tract symptoms; MP: muscle power; MRI: magnetic resonance imaging; NCV: nerve conduction velocity; NP: not performed; P: Proprioception; Ref.: reference of normal limits; SSEP: somatosensory evoked potential.

* The severity of axonal neuropathy is based on Electrophysiological Severity Scale, modified from Mondelli et al. [33]. "Mild" meant decreased amplitude of sensory nerve action potential (SNAP), 20–50% decrease of compound muscle action potential (CMAP) amplitude, decreased by <25% with respect to lower limits of motor conduction velocity(MCV) or reduced interferential pattern at full effort with or without denervation activity at rest in electromyography (EMG); "moderate" meant absolutely decreased amplitude of SNAP with normal sensory conduction velocity (SCV), 50–99% decrease of CMAP amplitude, decreased by >25% with respect to lower limits of MCV or discrete interferential pattern at full effort in EMG; "severe" meant decreased SCV and amplitude of SNAP, absence of CMAP or single interferential pattern at full effort in EMG; "extreme" meant absence of SNAP or no motor unit action potential.

2.3.2.6 Shah et al., 2019

Study reference:

Shah K, Murphy C. Nitrous Oxide Toxicity: Case Files of the Carolinas Medical Center Medical Toxicology Fellowship. *J Med Toxicol*. 2019;15(4):299-303.

Detailed study summary and results: human case report

A 45-year-old man presented to the emergency department with paresthesias and a history of inhaling 100 canisters of whipped cream chargers per week for approximately 4 weeks. During this time period, he noted progressively worsening numbness and tingling in his distal extremities. These symptoms were worse in his lower extremities compared to his upper extremities. He presented to the emergency department after he was nearly involved in a motor vehicle collision. While driving that day, he could sense neither where his feet were in relation to the car pedals nor the vibration of the pedals. He ate an omnivorous diet, had no history of bariatric surgeries, and did not have any gastrointestinal symptoms.

A detailed neurologic examination was performed, given concern for neurotoxic effects. The patient was alert and oriented to person, place, time, and situation. Cranial nerve testing revealed pupils that were equally round and reactive to light. Extra ocular motions were intact, with smooth pursuits, quick saccades, and conjugate eye movements present. There was no ptosis of the eyelids nor nystagmus observed. Sensation was intact to light touch in the V1, V2, and V3 distributions. Symmetric smile, frown, and eyebrow elevation were noted along with good buccinator tone and muscle mass. Soft sounds of rubbing fingers together near the ears were heard bilaterally. The soft palate elevated symmetrically, and the uvula was midline. Sternocleidomastoid strength was intact and symmetric bilaterally. His tongue protruded in the midline with normal lateral movement. Strength testing revealed 4/5 strength bilaterally in shoulder abductors, elbow flexors, elbow extensors, wrist extensors, finger interosseous, hip flexors, knee extensors, knee flexors, ankle dorsiflexors, and ankle plantar flexors. There was no pronator drift. Sensory exam revealed decreased sensation to light touch in upper extremities from fingertips to mid-forearm and in lower extremities from toes to mid-thigh, neither distribution in a dermatomal pattern. Muscle tone in upper and lower extremities was relaxed, symmetric, and neither diminished nor enhanced. Biceps, triceps, brachioradialis, patellar, and Achilles reflexes were symmetric and 2+. Two beats of clonus were present bilaterally on ankle jerk. Rapid alternating movements were slowed bilaterally on index finger-thumb tapping and pronation/supination of hand on the supinated palm of the other hand. Finger-to-nose testing showed dysmetria. Vibration sense was absent in bilateral distal lower extremities. Patient had an ataxic gait, with inability to safely ambulate independently due to instability, and he was unable to perform tandem gait. Romberg test was positive. He was also unable to tie his shoes on his first two attempts.

On a complete blood count, the white blood cell count was 4.6 × 103/μL; hemoglobin, 12.3 g/dL; hematocrit, 37%; and platelet count, 177 × 103/μL. Mean corpuscular volume was 98 fL; mean corpuscular hemoglobin, 33 pg; mean corpuscular hemoglobin concentration, 34 g/dL; and red cell distribution width, 19.7%. The patient's comprehensive metabolic panel was within normal limits. HIV panel and syphilis testing were nonreactive. The vitamin B12 level was 154 pg/mL (normal 180–914 pg/mL). Folic acid level was > 24.8 ng/mL (normal > 5.8 ng/mL). Serum homocysteine level was 178.2 μmol/L (normal 6–15 μmol), and serum methylmalonic acid level was 1145 nmol/L (normal 0–378 nmol/L).

Non-contrast MRI of the brain and cervical, thoracic, and lumbar spine revealed a normal MRI of the brain with abnormal T2 hyper intense signal involving the dorsal columns of the cervical and thoracic cord. The radiologist commented that the appearance was nonspecific with a causative differential diagnosis including sequelae of HIV, copper deficiency, underlying hereditary condition such as Friedreich's ataxia or leukoencephalopathy, or potential vitamin deficiencies including vitamin B12 deficiency. There was no noted acute fracture, significant central spinal stenosis, or cord compression.

The patient was started intramuscular vitamin B12 daily for 14 days. On day 11, his homocysteine level had normalized to 9.5 μmol/L (normal 6–15 μmol/L), and his vitamin B12 level was > 1500 pg/mL (normal 180–914 pg/mL). His ataxic gait, diminished position and vibration sense, and dysdiadochokinesia had significantly improved, and he was able to ambulate independently, but he still had not returned to baseline. On day 14, he was discharged home with home physical therapy arranged, and he was scheduled for weekly 1000 μg vitamin B12 intramuscular injections for the next month. He was also advised to buy over the counter methionine supplements and take 1000 mg once a day until symptom resolution.

2.3.2.7 Johnson et al., 2018

Study reference:

Johnson K, Mikhail P, Kim MG, Bosco A, Huynh W. Recreational nitrous oxide-associated neurotoxicity. J Neurol Neurosurg Psychiatry. 2018 Aug;89(8):897-898. doi: 10.1136/jnnp-2017-317768.

Abstract :

A previously healthy 21-year-old woman presented with subacute onset of confusion and gait ataxia. She reported habitual inhalation of dinitrogen oxide (N_2O) purchased online over the past year, with increased consumption over the preceding weeks of up to 300 canisters/week. Examination revealed pale skin with diffuse hyper pigmented macular patches over the trunk and atrophic glossitis. Cognitive impairment was evident with impaired insight, orientation, short-term memory and attention. She had impaired limb proprioception with sensory ataxia and positive Romberg's test. Distal limb power was significantly reduced with milder weakness proximally and globally depressed reflexes.

2.3.2.8 Keddie et al., 2018

Study reference:

Keddie S, Adams A, Kelso ARC, et al. No laughing matter: subacute degeneration of the spinal cord due to nitrous oxide inhalation. *J Neurol*. 2018;265(5):1089-1095.

Detailed study summary and results: case reports

Methods. All adult patients presenting between 1st of November 2016 and 1st May 2017 to the Emergency Department of the Royal London Hospital, with a history of N_2O use and symptoms suggestive of subacute degeneration of the spinal cord were included. Blood tests were performed to rule out alternative causes of myelopathy. All patients were seen and examined by an attending consultant neurologist and imaging reported by a consultant neuroradiologist.

Results. There were approximately 150,000 attendances to the Emergency Department over the 6-month study period. The median age of patients was 22 years (range 17–26), three were women and seven were men. Eight were current smokers, six drank alcohol more than twice per week, and three used other drugs recreationally (two used cocaine and one marijuana). On average, patients used N_2O around two-three times per week, and the number of N_2O canisters consumed ranged between 75 and 2000 per week. Altered sensation in the limbs was the predominant presenting feature (seven had symptoms in the upper limbs and all ten had symptoms in the lower limbs). Strength was well preserved in most patients. Additional clinical features were gait ataxia (n = 8), falls (n = 3), Romberg's sign (n = 6), pseudoathetosis (n = 5), Lhermitte's phenomenon (n = 1), Uhtoff's phenomenon (n = 1), and segmental myoclonus (n = 1).

All patients had normal mean cell volume and haemoglobin level. Four patients had low vitamin B12 levels (median 191; range 109–2000 ng/L). Methylmalonic acid (MMA) was measured in eight patients (median 2.9; range 0.16–110 μmol/L) and was not taken in patients whose B12 level was below normal or elevated as a result of replacement. MMA was elevated in seven of the eight patients. Four of these could be deemed clinically relevant/revealing, with an associated normal B12 level. Three were 'complimentary', with an associated low B12 level. The MMA level was normal in one patient whose vitamin B12 level was low (case

7), but it transpired that they had been aware of the risks of myelopathy and had been concurrently taking oral B12 once a day as prophylaxis. Despite this they had nonetheless developed subacute degeneration of the spinal cord.
A cerebrospinal fluid examination was performed in four patients. White cell counts were < 1 in all cases. Protein was raised in two patients at 0.5 and 0.7 g/L (normal ≤ 0.4 g/L). Glucose was within normal limits in all. Unmatched oligoclonal bands were demonstrated in one patient, the significance of which is unclear but would suggest a degree of intrathecal immunological response. MRI of the spinal cord was performed in nine patients and showed T2 signal change affecting the dorsal columns of the cervical spine, consistent with subacute degeneration of the spinal cord. In one patient, pathological enhancement was detected initially. Follow-up MRI was performed in four patients after an average of 14 months (range 5–27 months) from presentation. In two patients the signal change persisted and in the other two it had resolved. In the cases where MRI signal change had resolved (cases 7 and 10), both received treatment for a minimum of 4 months. Case 7 had abstained from N_20, had been taking oral B12 prior to presenting, and was asymptomatic following treatment. Case 10 continued to use N_20 once a fortnight and experienced persistent paraesthesia in the feet. The patient also had poor diabetic control, and nerve conduction studies demonstrated mixed axonal and demyelinating features consistent with diabetic neuropathy.

Four patients were lost to follow-up. Of the remaining six patients, two recovered without residual symptoms, three continued to have paraesthesia in the feet and one continued to have paraesthesia, gait ataxia, and proprioceptive sensory loss to the ankles.

2.3.2.9 Kaski et al., 2017

Study reference:

Kaski D, Kumar P, Murphy E, Warner TT. Iatrogenic B12-deficient peripheral neuropathy following nitrous oxide administration for functional tonic leg spasm: A case report. Clin Neurol Neurosurg. 2017 Sep;160:108-110.

Detailed study summary and results: case report

A 27-year-old man had suffered recurrent patellar dislocations resulting in multiple bilateral knee operations from the age of 15 years. He was admitted for investigation of a 5-year history of severely painful abrupt onset right paroxysmal leg spasms. He required manipulation under anesthesia (MUA) to abort the spasm and straighten the leg. These events were preceded by a prodrome of anxiety followed by a severe ascending pain from the toes to the hip. This pattern recurred on >50 occasions over a period of 5 years. During these exacerbations he would typically be administered Entonox, a 50:50 mixture of dinitrogen oxide and Oxygen in the emergency department. Spasms would resolve over 5-45 min, but not necessarily related to N2O administration. He was prescribed baclofen, clonazepam, and pregabalin, all with variable results.

Incidentally, he complained of bilateral paraesthesia of the soles, and numbness of the feet. The neurological examination was normal apart from absent ankle jerks bilaterally and a distal sensory loss over his feet bilaterally to both pain and vibration, extending to the ankles. The patient did not drink alcohol, was not vegan, and had no gastrointestinal conditions. He was not taking any regular or over-the-counter medications.

MRI of the brain and spine performed as a work-up for the leg spasms were normal. Nerve conduction studies showed a mild large fibre, length-dependent axonal sensorimotor polyneuropathy. Haemoglobin and mean cell volume were normal, as was a full autoimmune, vasculitis, infectious, metabolic and paraneoplastic blood test screen. Vitamin B12 level was normal. Homocysteine and mehtylmalonate levels were elevated.

He was diagnosed with a functional (psychogenic) left leg spasm, and a sensorimotor neuropathy secondary to a functional (organic) B12 deficiency, related to controlled dinitrogen oxide administration. He was advised complete cessation of dinitrogen oxide and commenced B12 replacement. Two months after intramuscular hydroxycobalamin injections, his sensory symptoms had improved, laboratory tests of B12 were normal but the reflexes remained absent and the neurophysiological findings unchanged. He continued to experience frequent leg spasms and significant psychological distress, and was referred for psychological support and in-patient treatment of the functional disorder as part of a multidisciplinary team setting.

2.3.2.10 Egan et al., 2018

Study reference:

Egan W, Steinberg E, Rose J. Vitamin B_{12} deficiency-induced neuropathy secondary to prolonged recreational use of nitrous oxide. Am J Emerg Med. 2018 Sep;36(9):1717.e1-1717.e2.

Abstract: case report

A 24-year-old female, otherwise healthy, presented to the Emergency Department (ED) with difficulty walking and bilateral leg pain. The patient was a recreational dinitrogen oxide (NO2) user, also known as “whippets” or simply nitrous. Neurologic examination demonstrated an unsteady gait and positive Romberg sign along with normal deep tendon reflexes and normal muscle strength in upper and lower extremities. Laboratory results demonstrated macrocytic erythropoiesis, reduced B12, elevated homocysteine, and elevated methylmalonic acid. Outpatient MRI later demonstrated degeneration of the posterior spinal column. The patient was empirically treated in the ED with intramuscular B12 and admitted to the evaluation unit for pain control and Physical Therapy (PT) evaluation.

2.3.2.11 Middleton et al., 2018

Study reference:

Middleton JA, Roffers JA. Peripheral Neuropathy Due to Recreational Use of Nitrous Oxide Presenting After an Ankle Sprain With Foot Drop. Orthopedics. 2018 May 1;41(3):e432-e433.

Abstract: case report

A 22-year-old man was referred for orthopedic follow-up after an ankle injury. Initial evaluation in urgent care included radiographs with negative findings. After a delayed presentation, a course of functional treatment was recommended. Subsequently, he developed a deep venous thrombosis and pulmonary emboli. He was found to be factor V Leiden deficient and was fully anticoagulated on warfarin. Later reevaluation revealed a steppage gait and foot drop. Electrodiagnostic studies (ie, electromyography and nerve conduction studies) revealed a severe peripheral polyneuropathy. The patient admitted to engaging in high-volume recreational use of dinitrogen oxide. Neurological evaluation confirmed vitamin B12 deficiency consistent with the toxic effects of dinitrogen oxide. The patient's condition improved with vitamin B supplementation, bracing, and avoidance of dinitrogen oxide and similar neurotoxins. He participated in a 3-month physical rehabilitation program, and he displayed partial recovery at most recent follow-up

2.3.2.12 Buizer et al., 2017

Study reference:

Buizert A, Sharma R, Koppen H. When the Laughing Stops: Subacute Combined Spinal Cord Degeneration Caused by Laughing Gas Use. J Addict Med. 2017 May/Jun;11(3):235-236

Abstract: case report

The authors describe a case of subacute combined spinal cord degeneration caused by dinitrogen oxide (N_2O, laughing gas) use. Because of its euphoric effects, the use of N_2O has become increasingly popular in recent years. Unfortunately, the use of N_2O leads to inactivation of vitamin B12. Vitamin B12 plays an essential role in the synthesis and maintenance of myelin, a fatty substance that surrounds nerve cells and is crucial for their functioning. Deficiency of vitamin B12 could typically result in degeneration of posterior and lateral columns of the spinal cord. Treatment with intramuscular vitamin B12 injections and abstinence of N_2O generally leads to gradual improvement of symptoms. The author's case demonstrates the importance of the methyl malonic acid test to detect early or mild vitamin B12 deficiency as a cause of myelopathy while serum vitamin B12 level may be normal.

2.3.2.13 Glijn et al., 2017

Study reference:

Glijn NHP, van der Linde D, Ertekin E, van Burg PLM, Grimbergen YAM, Libourel EJ. Is nitrous oxide really that joyful? Neth J Med. 2017 Sep;75(7):304-306.

Detailed study summary and results: case report

A 23 year-old women with a previous history of iron deficiency and recurrent venous thromboembolism presented acute paresis of her legs and tingling of her limbs. She was not taking any medication, used alcohol socially and mentioned the recreational use of N_2O multiple times daily. On neurological examination, she had symmetrical weakness of the iliopsoas muscle and quadriceps MRC grade 4, paralysis of dorsal flexors of the feet MRC 0, plantar flexors MRC 3, areflexia of the legs and feet, indifferent plantar reflex response, loss of vibration sense from knees to toes and paraesthesia in both legs.

No abnormalities of the cranial nerves and the arms were observed. Vital signs and general medical examination were not affected.

Laboratory analysis revealed a direct antiglobulin test (DAT)-negative haemolytic anaemia: haemoglobin 5.5 mmol/l (7.5-10), leukocytes 1.4 x 109 (4.3-10.0), platelets 266 x 109 (150-400), MCV 98 fl (80-100), vitamin B12 85 pmol/l (130-700), folic acid 36.6 nmol/l (> 5), homocysteine 120.4 μmol/l (3.6-13.0), and methylmalonic acid 1.10 μmol/l (< 0.45).

Electromyography showed axonal polyneuropathy with demyelination. Additional magnetic resonance imaging showed a normal cerebrum and spine. Lumbar puncture revealed no abnormalities in the cerebrospinal fluid, and testing for tuberculosis and polymerase chain reaction for viral infections was negative.

The authors reported that the patient was diagnosed with a non-immune haemolytic anaemia, leukopenia and severe neurological signs as a result of a severe vitamin B12 deficiency due to recreational use of N_2O.

Treatment consisted of vitamin B12 supplements, folic acid and intensive physiotherapy. After starting the supplements all the laboratory abnormalities gradually normalised but the paraparesis persisted. After 6 months of intensive physiotherapy and rehabilitation, a slight improvement of the paraparesis has occurred; however, the patient is still only capable of walking within her own home with a walking frame.

2.3.2.14 Sleeman et al., 2016

Study reference:

Sleeman I, Wiblin L, Burn D. An unusual cause of falls in a young woman. J R Coll Physicians Edinb. 2016 Sep;46(3):160-162.

Detailed study summary and results: case report

A 29-year-old woman attended the emergency neurology clinic in a wheelchair with a two month history of increasing falls and urinary frequency. She described bilateral leg numbness below the knee, pins and

needles from the level of the hips distally and altered sensation over the palms of both hands. Her past medical history included 20 years of intermittent abdominal pain following a ruptured appendix. She had undergone multiple laparotomies, which revealed adhesions and a right-sided ovarian cyst. She was still in severe pain and took regular long and short-acting morphine preparations, hyoscine butylbromide, paracetamol and ondansetron. As her GP decreased her opioid dose, she made increasingly frequent ambulance trips to A&E for pain relief and at the time of referral was attending twice a day. She had recently given up her job as a shop assistant due to falls. She smoked ten cigarettes a day and was teetotal. Her abdominal pain led to erratic eating habits.

On examination, upper limbs, cranial nerves, speech, and cognition were all normal. Tone was difficult to assess but there was no clonus. Lower limb strength was grade 4 out of 5 bilaterally. Ankle reflexes were normal, knee reflexes were brisk and both plantar responses were extensor. Joint position was impaired at the toes and pain perception was impaired below the level of the T6 dermatome. Full blood count, thyroid stimulating hormone, copper and caeruloplasmin were all normal. Vitamin B12 was low at 162 pg/ml; (normal range = 145–569 pg/ml). She underwent magnetic resonance imaging (MRI) scan of her spinal cord, which revealed hyper-intensity of the posterior cord from C2–T5. The combination of myelopathy, peripheral neuropathy and typical MRI changes suggested subacute combined degeneration of the spinal cord. The most common cause of this presentation is vitamin B12 deficiency; however, her levels were not low enough to account for her symptoms. She had been receiving inhaled dinitrogen oxide in A&E as an opioid-sparing agent and, coupled with poor nutritional status, this had precipitated subacute combined degeneration of the spinal cord (‘dinitrogen oxide myelopathy’). Dinitrogen oxide analgesia was withdrawn and she received vitamin B12 injections and intensive physiotherapy. Two years later, she was able to walk with the aid of crutches and was continuing to improve, though she was still liable to falls when walking outside and unable to work.

2.3.2.15 Chen et al., 2016

Study reference:

Chen HJ, Huang CS. Nitrous Oxide-induced Subacute Combined Degeneration Presenting with Dystonia and Pseudoathetosis: A Case Report. Acta Neurol Taiwan. 2016 Jun 15;25(2):50-55.

Detailed study summary and results: case report

A 20-year-old woman presented with one month of progressive unsteady gait, involuntary movement in the four limbs and mild tingling sensation in a stocking glove distribution. She was previous healthy without a contributory family history. Further questioning with regard to her habits and social history revealed that she had used dinitrogen oxide and ketamine intermittently for recreational purposes for about two years; however, she did not give the detailed information about the dosage or the frequency. She had difficulty in walking independently, feeling that she was unable to control her lower limbs voluntarily rather than weakness. She also had problem in writing or using chopsticks. She had to hold a spoon effort fully while having meals. No symptom implying autonomic dysfunction was noted. Neurological examination demonstrated normal mental status and cranial nerve function except for mild stuttering in speech and dystonia in the facial muscle and tongue, especially while she was trying to protrude her tongue or make more facial expression. Manual test revealed full muscle strength. Hyperreflexia and extensor plantar response were present bilaterally. She exhibited dystonia-like posture in four limbs and athetoid movements in fingers and toes, worsened by eye closure. Sensory examination showed impaired vibration and proprioception distal to wrists and ankles. There was no significant cerebellar signs though she did not perform those tests smoothly, interfered by the involuntary movements. She needed support to keep in a broad-based stance and merely made few steps unsteadily with assistance.

Initial laboratory tests revealed anemia (Hb: 9.9 g/dl), normal mean corpuscular volume (MCV) (85.7 fL; normal range 81-99 fL), increased red cell distribution width (RDW) (29.8%; normal range 11-16%),

decreased iron level (22 μg/dl; normal range 50-175μg/dl) and normal serum vitamin B12 level (626 pg/ml; normal range 180- 914 pg/ml). Iron deficiency anemia (IDA) with suspected megaloblastic anemia was diagnosed. Since the patient stated she had taken some pills containing vitamin B complex before admission, the vitamin B12 level was presumably corrected. Serum copper, zinc, ceruloplasmin and thyroid function were within normal ranges. She had negative results in human immunodeficiency virus (HIV) antibody, nonreactive serologic tests for syphilis (STS) and the autoimmune profile (ANA, anti-ENA, RA factor). Analysis of the cerebrospinal fluid (CSF) was normal. Nerve conduction study (NCS) showed sensorimotor polyneuropathy. Visual evoked potentials (VEP) study suggested impaired bilateral visual conduction pathway. The results of somatosensory evoked potentials (SSEP) were suggestive of impaired both central sensory conduction and peripheral sensory conduction over bilateral extremities. Magnetic resonance imaging (MRI) of spinal cord showed long segmental hyper intense lesion from C1 to C6 level without obvious enhancement with gadolinium in the posterior column. MRI of brain showed unremarkable findings. Based on the history, clinical presentation, image findings and laboratory tests, she was diagnosed as spinal cord degeneration (SCD) caused by dinitrogen oxide abuse, presenting with generalized dystonia and pseudoathetosis. The patient was treated with high-dose intravenous vitamin B12 supplementation (2000 μg per day) for the first three days followed by oral supplementation. Her balance and gait improved gradually as well as the involuntary movement after treatment. With rehabilitation, she could walk independently slowly three weeks later.

2.3.2.16 Li et al., 2016

Study reference:

Li HT, Chu CC, Chang KH, Liao MF, Chang HS, Kuo HC, Lyu RK. Clinical and electrodiagnostic characteristics of nitrous oxide-induced neuropathy in Taiwan. Clin Neurophysiol. 2016 Oct;127(10):3288-93.

Detailed study summary and results: retrospective case-control study

Method: 33 patients with dinitrogen oxide-induced neuropathy over a 10-year period were reviewed and the authors reported their demographic data, spinal cord MRI, laboratory examinations and nerve conduction studies. 56 healthy controls' nerve conduction studies were collected for comparison analysis.

Motor and sensory nerve conduction study (NCS)

Thirty-two patients (97%) exhibited abnormal results in at least one motor or sensory nerve. The mean CMAP amplitudes were significantly reduced to 81% in the peroneal nerve and 76% in the tibial nerve. We also observed a significant prolonged distal latency and F-wave latency, and conduction velocity slowing, especially in the lower limbs. The peroneal/tibial CMAP ratio was calculated to evaluate the possible injury pattern between these two nerves, although there was no significant difference between the control and N_2O neuropathy groups (0.36 ± 0.02 vs. 0.47 ± 0.15, respectively; p = 0.15). This result indicated relatively non-selective lower-limb motor nerve involvement in N_2O-induced neuropathy. The authors observed a significant decrease in the sural nerve SNAP amplitude (67%). These data demonstrated a trend towards a sensori-motor neuropathy, with predominant lower-limb involvement. Furthermore, electromyograms were performed for 18 patients and revealed that 11 patients had active denervation changes, including positive sharp wave in one patient and simultaneous fibrillations plus positive sharp waves in 10. The The demyelinating features in the motor nerves, include distal latency prolongation (>120% of the ULN), conduction block, conduction slowing (<80% of the LLN), and F-wave latency prolongation (>125% of the ULN). Among them, conduction slowing in peroneal (36%) and tibial (30%) nerves were the most frequently encountered features.

Overall, mixed axonal and demyelinating neuropathy was the most common type of neuropathy (12 patients, 36%), followed by axonal neuropathy (10 patients, 30%) and demyelinating neuropathy (2 patients, 6%).

The other 8 patients (24%) only exhibited mildly abnormal findings and were not classified as axonal or demyelinating neuropathy. One patient had completely normal finding.

Patients with low vitamin B12 and/or high homocysteine levels demonstrated similar NCS characteristics, nutritional condition, and hematological state compared with those with normal vitamin B12 levels.

To examine possible factors that were associated with severe axonal impairment, we compared the total neuropathy score, dinitrogen oxide exposure duration, and blood levels of vitamin B12, folate, and homocysteine between patients with and without severe CMAP or SNAP impairment in the lower limbs. Among 13 patients with CMAP <20% LLN in either peroneal or tibial nerve, significant higher homocysteine levels were noted compared with CMAP >20% LLN group (37.0 ± 6.8 vs. 21.4 ± 3.6 lmol/L, p = 0.047). In addition, there was a trend that severely reduced sural SNAP amplitude associated with a higher total neuropathy score (18.0 ± 1.3 vs. 14.4 ± 1.2, p = 0.06) and homocysteine levels (37.3 ± 7.1 vs. 22.3 ± 3.8 lmol/L, p = 0.06). However, there were no other significant differences between the groups for the other factors (vitamin B12 levels, folate levels, and dinitrogen oxide exposure duration).

2.3.2.17 Massey et al., 2016

Study reference:

Massey TH, Pickersgill TT, J Peall K. Nitrous oxide misuse and vitamin B12 deficiency. *BMJ Case Rep*. 2016;2016:bcr2016215728.

Detailed study summary and results: case report

A 36-year-old man was hospitalised after becoming unable to stand after 5 weeks of ascending limb paraesthesiae and progressive balance difficulties. His medical history was unremarkable. He denied recent foreign travel or alcohol ingestion but admitted to habitual N_2O inhalation from 'whippits'. Neurological examination revealed pseudoathetosis in the upper limbs, brisk but symmetrical reflexes throughout, flexor plantars, reduced vibration sensation to the hips bilaterally and an inability to stand unaided due to sensory ataxia. The remainder of the general and neurological examination was normal.

The patient was diagnosed with a myeloneuropathy. Differential diagnoses included copper deficiency, vitamin E deficiency and vitamin B12 deficiency causing a subacute combined degeneration of the cord. Causes of vitamin B12 deficiency would include poor dietary intake, malabsorption, pernicious anaemia due to lack of intrinsic factor or, as in this case, heavy N_2O use (300 'whippits' per day).

He was treated with intramuscular B12 replacement and intensive physiotherapy, and advised to stop inhaling N_2O. On discharge at 4 weeks, he was mobilising short distances using a zimmer frame; at 12 weeks, he was mobilising independently. Repeat spinal cord MRI at this time demonstrated significant improvement.

2.3.2.18 Duque et al., 2015

Study reference:

Duque MA, Kresak JL, Falchook A, Harris NS. Nitrous Oxide Abuse and Vitamin B12 Action in a 20-Year-Old Woman: A Case Report. Lab Med. 2015 Fall;46(4):312-5.

Detailed study summary and results:

Patient: A 20-year-old woman complaining of bilateral paresthesia of the lower extremities and lower limb weakness. The patient had experienced bilateral paresthesia of the lower extremities for approximately 3 weeks before admission to the hospital. Her numbness progressively ascended to the trunk and she then developed difficulty walking, followed by numbness in the hands and associated back pain without bladder

or bowel incontinence. The patient reported having received an influenza vaccination approximately 1 week before the onset of symptoms. The patient was diagnosed with neuroblastoma at age 5 years and was successfully treated with chemotherapy.

At examination, the following were noted: bilateral hip flexor weakness, a thoracic sensory-level deficit (sensitivity to sharp touch was reduced below T10), and reduced proprioception distally in the bilateral lower extremities.

The clinical picture suggested an autoimmune etiology based on the patient's previous history of vaccine exposure (influenza vaccine); thus, treatment was started with intravenous immunoglobulin (IVIG) and intravenous methylprednisolone for 5 days.
Spinal magnetic resonance imaging (MRI) findings were consistent with demyelination of the dorsal regions of the spinal column and demyelination around the central canal (Image 1). Brain MRI results were negative. Results of a nerve conduction study showed changes consistent with demyelinating polyneuropathy.
During the course of her hospitalization, further investigation identified that the patient abused dinitrogen oxide (specifically, she had ingested the contents of small canisters of N2O, known colloquially as whippets) before admission. Serum homocysteine was not measured because the possibility of B12 deficiency was not considered due to the relatively normal hematologic findings.
The patient was discharged with vitamin B12 supplementation and counselling prior to discharge. At her 1-month follow-up visit, the patient showed remarkable improvement of her neurologic symptoms with intact neurological sensitivity and no symptoms of muscular weakness.

2.3.2.19 Bäckström et al., 2015

Study reference:

Bäckström B, Johansson B, Eriksson A. Death from Nitrous Oxide. J Forensic Sci. 2015. Nov;60(6):1662-5.

Detailed study summary and results: case reports

Case Report 1

The first case involves a 25-year-old, previously healthy industrial worker who was found dead in his home. He was lying on the floor in front of the TV set, which was switched on. The sweater was pulled up, the trousers and underwear were pulled down, and the face was covered by a modified gas mask. The mask was connected to a rubber tube and a plastic bag, forming a closed system. A whipped cream pump was loaded with a dinitrogen oxide cartridge and connected to the plastic bag. Everything in the apartment was in good order, and there was no suicide note. The deceased had no history of any somatic or psychiatric disease and did not abuse alcohol or illicit drugs. His girlfriend found the deceased when she returned home after the evening shift. She denied that he had expressed any suicidal thoughts or behaved in a depressed manner. Furthermore, she reported to the police that he earlier had bought dinitrogen oxide cartridges instead of carbon dioxide "by mistake" and soon thereafter he started to abuse the gas which was inhaled from the whipped cream nozzle or released into a plastic bag and subsequently inhaled. Often when he inhaled, he became warm and sweated profusely. She had also inhaled a few times but did not like the (side) effects. He had never made any attempt to hide his activities, and there were never, according to her, any sexual activities related to the breathing sessions. To her knowledge, this was the first time the mask was used. The mask was probably taken from the industry where he worked. At autopsy, the areas covered by the gas mask were pale, sharply demarcated from cyanotic areas. Besides signs of congestion of the inner organs, there were no pathological findings Extensive microscopical analyses, analysis of alcohol, as well as screening for licit and illicit drugs, were all negative.

Case Report 2

The second case was that of a 35-year-old, previously healthy man, who was found dead on his living room floor. He was lying in front of the TV set and a VCR loaded with a pornographic videotape. His face was covered by a gas mask connected to a gas cylinder marked “N2O”.

2.3.2.20 Pugliese et al., 2015

Study reference:

Pugliese RS, Slagle EJ, Oettinger GR, Neuburger KJ, Ambrose TM. Subacute combined degeneration of the spinal cord in a patient abusing nitrous oxide and self-medicating with cyanocobalamin. Am J Health Syst Pharm. 2015 Jun 1;72(11):952-7.

Abstract: case study

A 27-year-old woman was treated in the emergency department for complaints of abdominal pain and inability to urinate for about 12 hours. The patient also complained of worsening lower-extremity weakness for 10 days and a "pins and needles" sensation in the lower extremities for approximately 1 year. She reported dinitrogen oxide abuse over 3 years (an average of 100-200 "whippit" cartridges daily on 3 or 4 days per week), as well as long-term self-medication with oral and i.m. cyanocobalamin for the purpose of preventing dinitrogen oxide-induced neurologic symptoms. Results of magnetic resonance imaging (MRI) were highly suggestive of SCD, which is typically seen in primary vitamin B12 deficiency but has been reported in the context of chronic dinitrogen oxide exposure. Treatment was initiated with cyanocobalamin 1000 μg i.m. daily, to be continued for 5 days and followed by a four-week regimen of 1000 μg i.m. weekly. The patient was discharged after 3 days, despite continued symptoms, with instructions to obtain ongoing care but was lost to follow-up. Conclusion: A patient who abused dinitrogen oxide chronically developed ataxia, paresthesia, and urinary retention while self-medicating with cyanocobalamin. A diagnosis of SCD was supported by MRI findings, symptoms, and the known relationship between dinitrogen oxide exposure and vitamin B12 deficiency.

2.3.2.21 Rhainbolt et al., 2015

Study reference:

Rheinboldt M, Harper D, Parrish D, Francis K, Blase J. Nitrous oxide induced myeloneuropathy: a case report. Emerg Radiol. 2014 Feb;21(1):85-8.

Detailed study summary and results: case reports

A 35-year-old male without significant past medical history, presented to the ER with complaints of disorientation, rapidly worsening subjective weakness and developing flexion contractures of the fingers, urinary urgency, and loss of balance. Physical exam was notable for a weakly positive Babinski sign, abnormally brisk reflexes in all four limbs, diminished sensation in a stocking and glove distribution, and ataxia. Relevant social history included the daily consumption of over 100 small canisters of dinitrogen oxide (so-called “whippets”), used commercially as a propellant for whipped cream, as well as 6 mg of Xanax and three to six beers daily.

The use of other recreational substances was denied. Laboratory findings, including serum vitamin B12 levels, were normal. Notably, methylmalonic acid and homocysteine levels, however, were not assayed. There was no evidence of megaloblastic anemia. Because of concerns for potential proximal cord compromise, contrast-enhanced MRI of the cervical spine was ordered, demonstrating an increased T2 signal within the dorsal columns bilaterally from C2–C3 through C6–C7 without evidence of cord expansion or extrinsic compression. Post-contrasted images demonstrated mild intramedullary enhancement in a linear distribution constrained to the dorsal column region of involvement seen on T2 images. Cord expansion as would be typified more by tumoral involvement was not seen. Demyelinating disease, though also

commonly manifested with multisegment involvement, was deemed unlikely, as well, due to the constrained geographic pattern of involvement. The appearance and distribution, together with the relevant clinical history, were deemed most compatible with dinitrogen oxide-related myeloneuropathy. High-dose vitamin B12 therapy was instituted in the ER, and the patient was transferred to the neurology service for supportive care and continued metabolic supplementation. Recovery has been slow to date.

2.3.2.22 Ghobrial et al., 2012

Study reference:

Ghobrial GM, Dalyai R, Flanders AE, Harrop J. Nitrous oxide myelopathy posing as spinal cord injury. J Neurosurg Spine. 2012 May;16(5):489-91.

Detailed study summary and results: case report

A 19 year-old male presented with complaints of progressive numbness and weakness in all extremities after a fall over a six foot fence onto his back, one day prior. He denied back pain, bowel or bladder incontinence. Additionally, he denied any relevant past medical or surgical history. Over the course of twenty-four hours, his symptoms progressed to an inability to ambulate and numbness in the distal extremities. The patient stated he occasionally abused oxycodone, alcohol, and "huffed" dinitrogen oxide canisters several times per week. Moreover, he stated he would "huff" as much as twenty pound canisters of dinitrogen oxide at one setting. On initial assessment, the patient had minimal neck pain, was hemodynamically stable, and had a Glascow Coma Scale of 15. He was alert and oriented, without apparent cognitive or language deficits. On motor exam however, proximal weakness of 3/5 in the deltoids bilaterally and distally of 2/5 was found. In the lower extremities, the iliopsoas muscle groups were weak (4/5) and distal groups were 3/5. Hyperreflexia was noted in the bilateral lower extremities. Sensation to light touch and pin prick was absent in the distal extremities as well as vibration and propioception. The sensation loss was not dermatomal. The remainder of the neurological exam was insignificant.

Investigations

Due to the history of dinitrogen oxide abuse a vitamin B-12 and folate level were sent and returned within the normal reference ranges. A MRI scan of the cervical spine did not demonstrated any acute traumatic signs such as bony fracture, ligamentous injury, or subluxation. However, the spinal cord parenchymae had significant T-2 signal abnormalities in the posterior columns from C2-7.

Clinical Course

Intravenous methylprednisolone was started at an outside institution according to the NASCIS II criteria and since the patient was making progressive improvements was maintained. Thirty-six hours after injury, the patient noted complete resolution of his symptoms. The following day, the patient was discharged home.

2.3.2.23 Hsu et al., 2012

Study reference:

Hsu CK, Chen YQ, Lung VZ, His SC, Lo HC, Shyu HY. Myelopathy and polyneuropathy caused by nitrous oxide toxicity: a case report. Am J Emerg Med. 2012 Jul;30(6):1016.e3-6.

Detailed study summary and results: case report

A 19-year-old man had a 1-month history of progressive 4-limb numbness and gait imbalance. He reported a kicking-related injury to the left foot while climbing stairs about 1 month previously, after which he developed left foot pain, bilateral lower limb numbness, and an unstable gait. Conservative treatments with nonsteroidal anti-inflammatory drugs proved to be ineffective.

The regions of numbness expanded to include the upper limbs and eventually his entire body (complete numbness from shoulders to his toes). When the condition progressed to include general weakness, the

individual visited the outpatient department (OPD) of our hospital for help. The individual had no known medical history, was on a normal diet, had a 3-year cigarette smoking history, and indicated no use of alcohol or illicit drugs. He reported no constipation or urinary incontinence. He was fully conscious, with normal mentality and speech; eyeballs
were conjugated and freely movable without nystagmus. Cranial nerve function was intact, except for mild bilateral facial paresis. Muscle strength was generally reduced to grade 4 on the Medical Research Council scale, and muscle tone was reduced; anal tone, however, was normal. There was no muscle fasciculation, but the brachioradialis and knee jerk tendon reflexes were absent bilaterally. Plantar responses were negative on both sides. The finger-nose-finger test was normal. No tremor was observed, but pseudoathetosis of the fingers was found when the upper limbs were outstretched with the eyes closed. The patient exhibited an equivocal Lhermitte sign and a positive Romberg sign, and he walked with a wide-based, ataxic steppage gait. The sensory tests showed diminished superficial sensation below a segmental level around his cervical-thoracic junction. Also, symmetric decreased pinprick and temperature sensations over all his distal extremities (glove-and-stocking pattern) was noted; the sensory decrease was greater distally than proximally.

Most of the laboratory tests were within the reference range, except for the presence of megaloblastic red blood cells. The complete blood cell count indicated hemoglobin, 15.7 g/dL (reference range, 14-18 g/dL); hematocrit, 47.1% (reference range, 42%-52%); and mean corpuscular volume, 96.5 fL (reference range, 80-94 fL). A peripheral blood smear showed anisocytosis and 2% metamyelocyte. The serum vitamin B12 concentration was 156 pg/nL (reference range, 246-900 pg/nL), and red cell folate concentration was 9.5 nmol/L (reference range, 5-30 nmol/L). Serologic tests for syphilis, Epstein-Barr virus, cytomegalovirus, and human immunodeficiency virus antibodies were negative. Thyroid function and autoimmune screen were within normal limits; intrinsic factor antibodies were absent. Results of the Cerebrospinal Fluid studies were normal, with no evidence of albuminocytological dissociation (protein: 23 mg/dL, glucose: 60 mg/dL).

Initial cervical spine magnetic resonance examination was done under the suspicion of spinal cord lesion. It revealed increased signals in posterior and anterior columns bilaterally on T2-weighted images in cervical cord. The results of motor nerve conduction velocity (NCV) revealed prolonged distal latency in bilateral median nerves, reduced NCV in bilateral ulnar and left tibial nerves, poor response in bilateral peroneal and right tibial nerves, and prolonged F latency in all of the excitable nerves. The H reflexes of tibial and median nerves were markedly prolonged on both sides. Sensory NCV revealed no response in the right sural nerve and markedly reduced sensory nerve action potential in the left sural nerve. Needle electromyography showed no spontaneous activity during rest in the left anterior tibialis, gastrocnemius, vastus medialis, right abductor pollicis brevis, flexor digiti minimi, brachioradialis, left lower cervical paraspinal, and left lower lumbar paraspinal muscles. We did find, however, a decrease in the number of motor unit potentials during voluntary contractions in these 6 muscles. After reviewing the NCV and electromyography findings, we concluded that the patient had a demyelination-predominated polyneuropathy. All evoked potentials (EP), brainstem auditory EPs (BAEPs), and median nerve somatosensory EPs (SEPs) were in the reference range, with a trend toward the upper limit. However, the markedly prolonged cortical potential (P37) in the bilateral tibial nerve SEP implied a delayed, prolonged conduction in posterior column of spinal cord.

After further inquiry, the patient was admitted to the recreational use of N_2O inhalant over a period of 2 months up until the time that his ascending paresthesia became worse. During this period, he inhaled approximately 500 to 600 cartridges from N_2O-filled balloons during 5- to 6-hour sessions, 4 to 5 times per week. The final diagnosis was N_2O induced polyneuropathy and myelopathy.

One week after hospital admission, the patient began a course of vitamin B12 injections for 2 months. The numbness decreased within 1 week, but a mild sensory ataxic gait remained. After 2 months of N2O abstinence, the patient recovered fully without any neurologic sequela.

This case presented a subacute course of symmetrical numbness, which had the mixed characters of sensory level below cervical-thoracic junction and glove-stocking pattern. The initial anatomical diagnosis included cervical cord lesion and polyneuropathy. On the other hand, the pseudoathetosis, Romberg sign, and the ataxic gait were derived from a proprioceptive disturbance, which could result from a dorsal cord lesion. Therefore, a cervical magnetic resonance imaging (MRI) was performed first. The signs of areflexia and absent plantar response indicated that the lesion of lower motor neuron caused his motor deficit. The MRI revealed pathological changes in posterior and anterior columns, but the lateral column was spared.

2.3.2.24 Richardson et al., 2010

Study reference:

Richardson PG. Peripheral neuropathy following nitrous oxide abuse. Emerg Med Australas. 2010 Feb;22(1):88-90.

Detailed study summary and results: case report

A 28-year-old man presented with bilateral numbness and weakness in both lower limbs. He first noticed the symptoms 1 week previously but they had worsened over the 2 days preceding presentation. He saw his general practitioner who referred him to the emergency department with difficulty walking and maintaining balance with either possible dinitrogen oxide drug abuse, or evolving Gillian–Barré syndrome as the suspected cause.

The patient admitted to dinitrogen oxide abuse, the full extent only being declared after rigorous history taking. He had a 2 year history of recreational dinitrogen oxide bulb use. These are readily available at most supermarkets for use in a dispenser for making whipped cream. The bulbs come in a pack of 10 and contain 8 g of dinitrogen oxide under pressure. The gas is inhaled either from a secondary reservoir like a thick-walled balloon or can be inhaled directly from a whipped cream dispenser, effectively acting as nitrous bong. The latter was the method used by the patient. In the last week prior to presentation he admitted to using up to at least 80 bulbs a day. He was a manual labourer and had been unable to work for the last 5 days. Clinically he was mildly obese, afebrile. He had normal vital signs. His cranial nerve examination was completely normal. He had a broad based gait but was Romberg's negative, Lhermitte's sign was absent (is elicited by neck extension or flexion resulting in an 'electrical like sensation shooting down the back and limbs – suggests dorsal column lesion), and Unterberger's test was negative (patient walks on the spot with the eyes closed, if they rotate to one side it suggests labyrinthine lesion on that side). He had 5/5 power in the upper and lower limbs and had symmetrical knee and ankle jerk on reinforcement. He had normal position sense but did have a subjective sensory deficit to soft touch in both lower limbs from the thighs down. He had decreased proprioception in both feet and decreased vibration sense to the level of both knees. Previous case reports of dinitrogen oxide neuropathy have also reported a negative Romberg's test in the presence of abnormal proprioception.

Investigations

Electrolytes and liver functions tests were normal. Full blood examination showed an Hb of 142 (135–180 g/L), platelet count of 339 (150–450), white cell count of 9.1 (4.0–11.0) and an MCV 97 (80–98). CRP was 1.7 (0–6) and ESR was 10 (1–15). Vitamin B12 was 184 pmol/L (166–811). A methylmalonic acid (MMA) level and homocysteine (HC) level were ordered with only the latter performed coming back at 55 mmol (<5); serum folate was normal.

A diagnosis of polyneuropathy secondary to dinitrogen oxide abuse was made and the patient was referred for medical admission to commence parenteral vitamin B12. The patient refused to be admitted for further tests to exclude other causes of B12 deficiency and nerve conduction studies. He did attend outpatient follow up twice and continues to see his general practitioner on irregular intervals and has received in total

approximately 7 B12 injections (1000 mg IMI) over a 3 month period. Although he denies ongoing dinitrogen oxide use his neurological symptoms have persisted, he is unable to work, and he has refused further blood tests to date to confirm normalization of his HC or MMA levels, which are markers of ongoing vitamin B12 inactivity.

2.3.2.25 Scapellato et al., 2008

Study reference:

Scapellato, Maria Luisa, Giuseppe Mastrangelo, Ugo Fedeli, Mariella Carrieri, Isabella Maccà, Luca Scoizzato, et Giovanni Battista Bartolucci. « A Longitudinal Study for Investigating the Exposure Level of Anesthetics That Impairs Neurobehavioural Performance ». *Neurotoxicology* 29, n° 1 (2008): 116-23.

Detailed study summary and results:

In an Italian hospital, operating-theatre workers exposed to anesthetic gases (N=38) and 23 unexposed nurses participated in a longitudinal study (Scapellato et al., 2008) during one year to investigate effects on neurobehavioral functions. Neurobehavioral functions were assessed using a battery of tests:

- Euroquest self-administered questionnaire, exploring symptoms,

- Block design subtest (WAIS) measuring visuospatial and motor skills,

- Mood scale measuring stress and arousal state,

- Colour word vigilance test, which is a complex reaction time test.

The study was designed to consider potential pre-existing abilities and potential changes over time (repeated cross-sectional study). Three measures were taken for each subject at each of four time points: before and after work shift on Monday and Friday of a working week, twice a year. To attenuate learning effects, the subjects were allowed to practice the tests before the experimental session. The colour word vigilance (CWV) test was the endpoint used to appraise short-term effects induced by N_2O and isoflurane. Exposure was assessed *via* biological concentration in urine (urinary N_2O and isoflurane) at the end of work shift (Monday and Friday), twice a year (not stated if tests are performed on the same week). Contamination of urine was avoided and urine was analysed using gas chromatography with electron capture detection. The authors gathered information on the subjects to identify potential confounding factors. The subjects were classified into 4 groups (A: unexposed, B: <13; C: ≥ 13 to <27 and d: ≥ 27 µg/L). The urinary concentrations of 13 and 27 µg/L correspond respectively to air concentrations of 25 and 50 ppm (Imbriani et al 1995). The correlation between urinary N_2O excretion and end shift CWV was investigated by using the analysis of simple linear regression. For the analysis of repeated measures of CWV, a model of two-stage regression was used, which was built as follows. In the first-stage, reaction times (or CWV test results) were plotted against time in each subject, obtaining through the simple regression analysis a slope (or coefficient of regression), which expressed the individual change of CWV test results over a working week. At the second-stage, the slope was the dependent variable in a multiple regression analysis in order to select factors which affected longitudinal changes in reaction times among the following variables: general characteristics of subjects (age, gender, years of schooling, alcohol and coffee consumption, smoking habit, length of work); subjective symptoms (EQscores); basic cognitive abilities (BD test results); Monday morning CWV test result (the baseline value, conveying the pre-existing ability of the subjects); and occupational exposure. This approach consists therefore of a regression model for the average response over time and the effects of covariates on this average response. Stress and arousal were taken concurrently with CWV and, since the contingent mood state could affect CWV, they were analysed simultaneously using a multiple analysis of variance for repeated measures

Although the overall means were below the reference values of 27 µg/l for N_2O and 3.32 µg/l for isoflurane, urinary concentrations of N_2O exceeded the biological exposure limit in 12 out of 38 exposed subjects (32%), and that of isoflurane in 4 out of 38 (11%). No significant difference was found for all variables except sex (effect of sex distribution on reaction time was of borderline significance). There was no significant correlation between urinary levels of N_2O and end-shift CWV values, separately on Monday and Friday. With respect to the unexposed group, CWV test results over a working week were significantly ($p < 0.020$) higher in the Group D, but not in Group B nor C. There was a rough dose–effect relationship between increasing N_2O level of exposure and impairment of neurobehavioral performance. Since they were not significantly different, Groups B and C were considered equal to Group A and the two groups were collapsed into a single unit. Therefore, subjects were then categorized in two classes, according to the level of N_2O urinary concentrations being below or above 27 µg/L. Figure 2 shows that the weekly profiles of reaction times for the two groups were not parallel.

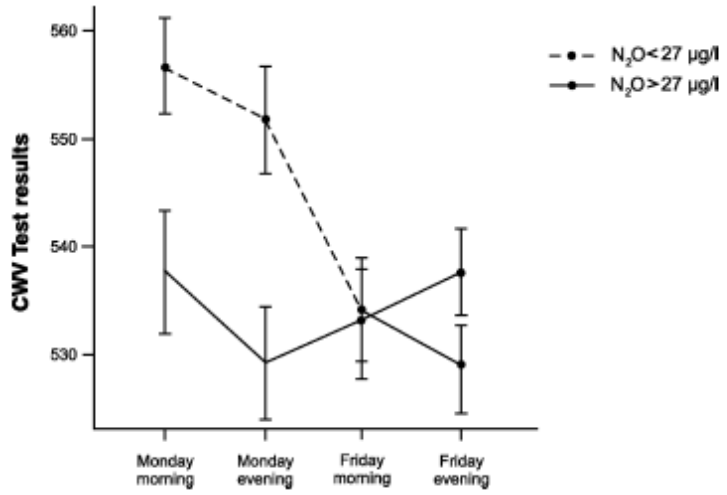

Figure 1: Means of colour word vigilance test as reported in Scapellato et al., 2008

In subjects with urinary concentrations of N_2O below 27 µg/l, there was a linear decrease in reaction times from Monday morning to Friday evening, indicating a learning effect. In subjects with N_2O urinary concentrations above 27 µg/l, the means of the CWV were essentially steady across a work week, indicating that performances may have been impaired. The highest difference is located between Monday end-shift and Friday before shift. For arousal, the tests of within-subjects contrasts were significant at ‘‘trial 1 vs. 2’’ ($F = 9.845$; $p < 0.003$) and at ‘‘trial 3 vs. 4’’ ($F = 5.719$; $p < 0.020$). In subjects with N_2O urinary concentrations above 27 µg/L, arousal was low on Monday morning, increased at end of the workshift, and remained high until Friday evening. In subjects with urinary N_2O below 27 µg/l, arousal was high before workshift, and low after workshift, on both Monday and Friday. It seems, therefore, that significant changes in reaction times and arousal occur from Monday to Friday, thus suggesting a cumulative effects of anesthetic gases over a week of exposure. No contrast was significant for the stress.

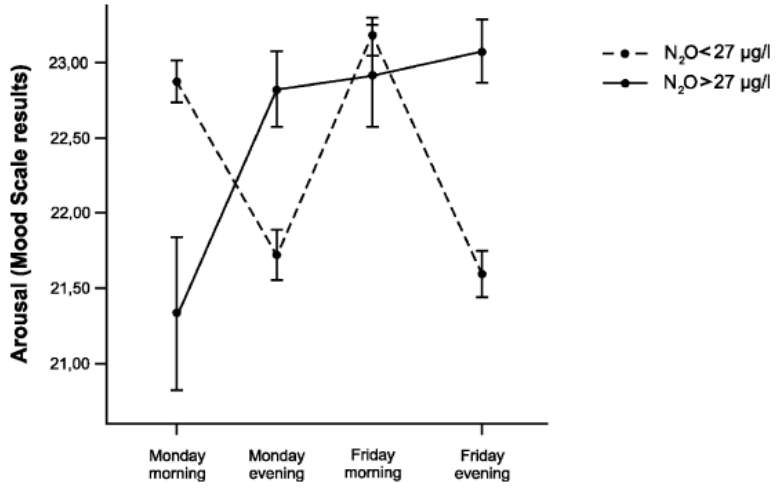

Figure 2: Means (and standard error of the mean) of arousal across a working week in subjects with urinary N_2O above or below 27 µg/l;

According to the authors, for N_2O urine concentration at the end of shift, 27 µg/L is a threshold under which vigilance alteration is not expected in exposed workers; it corresponds to 50 ppm for TWA air concentration. However, their results should be considered with caution, due to the small number of workers concerned in their study and also because occupational co-exposures were not taken into account.

2.3.2.26 Lin et al., 2007

Study reference:

Lin CY, Guo WY, Chen SP, Chen JT, Kao KP, Wu ZA, Liao KK. Neurotoxicity of nitrous oxide: multimodal evoked potentials in an abuser. Clin Toxicol (Phila). 2007;45(1):67-71

Abstract: case report

A 41-year-old male dinitrogen oxide abuser had used dinitrogen oxide (4-5 cans/per day, about 2000 ml/can) for more than 10 years. He complained of progressive motor clumsiness and distal paresthesia in the four limbs. Abnormal laboratory tests were megaloblastic red blood cells (102.3 fL, normal 80-94 fL) and serum vitamin B12 concentration of 143 pg/nL (normal 160-970 pg/nL). A magnetic resonance image did not show significant findings in the brain but demonstrated conspicuous changes in the posterior and lateral columns at the C2-C7 level, in accordance with the anatomical lesions of the subacute combined degeneration of the spinal cord. In addition to sensori-motor axonal polyneuropathy, multimodal evoked potentials (Eps) showed abnormal visual EPs with prolonged peak latencies of P100, abnormal brainstem auditory EPs characterized by delayed wave V and difficulty in the recognition of waves I and III, abnormal somatosensory EPs with significant decreased peak amplitudes of cortical potentials bilaterally, and abnormal motor EPs to transcranial magnetic stimulation with prolonged central motor conduction time.

2.3.2.27 Shulman et al., 2007

Study reference:

Shulman RM, Geraghty TJ, Tadros M. A case of unusual substance abuse causing myeloneuropathy. Spinal Cord. 2007 Apr;45(4):314-7.

Detailed study summary and results:

A 23-year-old female presented with a history of profound tetraparesis. She had an extensive history of intravenous drug abuse. Approximately eight months prior to presentation, the patient began inhaling dinitrogen oxide; a practice known as ‘nanging’. Initial use was limited to 1 box of 10 whipped cream (‘whippet’) bulbs per day, but had escalated to 13 boxes per day for 6 weeks before presentation. The patient described progressive limb weakness for 3 weeks, evolving such that she remained paralysed for the 3 days prior to admission. Her partner found her and he was unable to rouse her. On presentation, examination revealed evidence of a flaccid tetraparesis. Patchy paraesthesia (pain and light touch) was noted in upper limbs and trunk. Absent pelvic reflexes and GlasgowCom a Scale of 14 were noted. Routine investigations showed a raised creatinine and urea consistent with acute renal failure. Enzymology revealed raised creatinine kinase (9160 U/l (o160 U/l)) and the presence of high urinary myoglobins reflecting rhabdomyolysis. Apart from a normocytic anaemia, routine haematology was unremarkable. The patient required admission to an intensive care unit, which was complicated by bilateral femoral vein DVTs and a PE confirmed by duplex ultrasound and Computer Tomography Pulmonary Angiogram. Magnetic resonance imaging (MRI) revealed increased symmetrical signal intensity on T2 weighted images in the cervico-thoracic spinal cord. Clinical diagnosis Further investigation included a toxicological screen, thyroid function tests, as well as cobalamin and folate levels. Nerve conduction studies showed peripheral axonal sensorimotor neuropathy. An electroencephalogram demonstrated a severe diffuse encephalopathy. Cobalamin levels were found to be significantly depressed at 125 pmol/l (4210). Red cell folate was within normal limits. A clinical diagnosis of toxic myeloneuropathy due to dinitrogen oxide was made.

2.3.2.28 Sethi et al., 2006

Study reference:

Sethi NK, Mullin P, Torgovnick J, Capasso G. Nitrous oxide "whippit" abuse presenting with cobalamin responsive psychosis. J Med Toxicol. 2006 Jun;2(2):71-4.

Detailed study summary and results: case report

A woman brought her husband, a 33-year-old unemployed Indian-American male with no past psychiatric history, to the hospital because of his bizarre behavior and delusions. The patient believed that he was part of an experiment for NASA in which he serves as an “interface” between humans and machines. The patient noted commands, and he stated that he felt compelled and controlled when he destroyed furniture and broke a window at his home. He also reportedly rode his bike into a moving vehicle because his mind commanded him to do so. The patient stated that the actions were part of his “training” but that he was unable to identify the purpose of his training. He also stated that he had been told that he had a second wife and must therefore kill his current wife. The patient and his wife reported that the patient never had similar thoughts or delusions in the past. He worked as a medical technologist in the past, and he denied occupational exposure to any toxic substance. He was in a monogamous relationship with his wife and had no children. He initially denied use of any recreational drugs and alcohol but on further questioning reported excessive use of inhalants, namely, dinitrogen oxide. He reportedly bought several cases of dinitrogen oxide containers, also known as “whippits,” from a cookware store. Using a plastic container fitted with an airtight facemask, he inhaled the dinitrogen oxide on a daily basis for nearly 4 weeks prior to his current hospitalization. He denied any auditory or visual hallucinations, or any suicidal ideations. Sleep and appetite were reported as normal and no stressors were identified. Neurological consultation was requested to rule out any organic cause of his delusions. An examination revealed a young man in no apparent respiratory distress. He was oriented to person, place, and time with fluent speech and intact comprehension and repetition. The patient exhibited fixed delusions as a participant in a NASA experiment. His episodic and remote memory was intact and there was no ophthamoplegia or nystagmus. All cranial nerves were intact. No motor or sensory deficits were identified, and position and vibration sensations were intact. He had no gait difficulties; a Romberg test was

bilaterally normal with flexor plantar reflexes. A negative urine toxicology screen did not indicate any other commonly abused drugs such as cocaine and amphetamine. The thyroid profile was normal and he tested non-reactive for serological tests of syphilis and HIV. Taking the history of dinitrogen oxide abuse into consideration, we decided to send out a serum B12 level even though his CBC indices and MCV were normal [Hb/Hct=14mg/dl and 44% and MCV=92 fl]. A peripheral smear showed no hypersegmented neutrophils or macro-ovalocytes. The patient's vitamin B12 level was 202 pg/ml (normal 180–900). Because the level was in the low normal range, we decided to send out blood for methymalonyl CoA and homocysteine levels, which are known to be elevated even when the B12 level registers in the low normal range. Methylmalonyl CoA was 1078 nmol/L (normal 90–279), and homocysteine was 48.4 mcmol/ L (normal 5–15). We decided to treat him intramuscularly with 1000 micrograms of Vitamin B12 every day for one week and with low dose Quetiapine fumarate (Seroquel 25 mg PO every day). He was discharged with instructions to take 1000 micrograms, intramuscularly, of vitamin B12 every week for one month and advised to follow-up in the psychiatry out-patient clinic. At the time of discharge, 2 weeks after his presentation to our hospital, his fixed delusions started resolving and he felt better. He was lost to follow-up.

2.3.2.29 Wu et al., 2006

Study reference:

Wu MS, Hsu YD, Lin JC, Chen SC, Lee JT. Spinal myoclonus in subacute combined degeneration caused by nitrous oxide intoxication. Acta Neurol Taiwan. 2007 Jun;16(2):102-5.

Detailed study summary and results:

A 26-year-old woman suffered from weakness and numbness of both lower limbs for 2 days. She was previously healthy and denied history of abuse of toxic substances or illegal drugs. There was no history of gastrointestinal disease or deficient intake of B12. On neurological examination, her mental status, speech, and cranial nerves were normal. Strength was 3/5 in the lower limbs and 4/5 in the upper limbs. The tendon reflexes were absent and the plantar responses were bilaterally flexor. Sensory examination showed paresthesias in the hands and feet with markedly decreased vibratory sensation in her feet and legs following a distal-accentuating pattern. On the 2nd hospital day, she further developed respiratory difficulty. She was initially diagnosed as Guillain-Barré syndrome (GBS). Plasma exchange was done but her symptoms did not improve. Results of the CSF studies were normal without evidence of albuminocytological dissociation (protein: 32 mg/dl, glucose: 58 mg/dl, chloride: 128 mmol/L). The anti-HCV, anti-HIV, Venereal Disease Research Laboratory test (VDRL) and thyroid function test were unremarkable. The nervous conduction velocity (NCV) and electromyography (EMG) studies disclosed sensory-motor demyelinating polyneuropathy. Blood biochemistry tests and blood cell counts revealed no abnormalities except higher MCV (101.8 fL). The B12 level (normal value ≥ 211 pg/ml) was low (187 pg/ml). She was prescribed intramuscular B12 injections with a 1000 μg daily for 7 days then 1000 μg every week for 2 months under the impression of B12 deficiency. The next day after B12 injection, she developed multifocal, jerky movements over four extremities and in the trunk asynchronously. The brief and rapid twitching of muscle groups persisted during sleep with a lesser severity. The severity of myoclonic movements increased in the first four days, then they ceased spontaneously after the 7th day of B12 therapy when the dose was reduced to 1000 μg per week. Electroencephalogram did not reveal any abnormality. Further electrophysiological examination could not be performed, because the patient refused investigation. When reviewing her history again, she disclosed a history of weekly dinitrogen oxide inhalation for 2-3 months due to recreational purpose. The MRI of cervical spine revealed abnormal demyelinating lesion affecting the posterior column between C2 and C7 spine on T2- weighted images. Dinitrogen oxide intoxication was finally diagnosed. Her symptoms completely resolved within 2 months after B12 supplementation.

2.3.2.30 Waclawik et al., 2003

Study reference: case report

Waclawik AJ, Luzzio CC, Juhasz-Pocsine K, Hamilton V. Myeloneuropathy from nitrous oxide abuse: unusually high methylmalonic acid and homocysteine levels. WMJ. 2003;102(4):43-5. Erratum in: WMJ. 2003;102(6):5.

Abstract: case report

A 23-year-old patient developed diffuse paresthesias and sensory loss. He had mildly reduced serum vitamin B12 (B12) concentration with unusually high levels of methylmalonic acid (MMA) and homocysteine and no evidence of B12 malabsorption. Following parenteral B12 administration, his neurological deficit promptly resolved and B12 and MMA levels normalized, but elevated levels of homocysteine persisted. One year later, he admitted to inhaling dinitrogen oxide. After halting dinitrogen oxide abuse his homocysteine level normalized. This case demonstrates the importance of serum homocysteine level measurements in cases of suspected dinitrogen oxide toxicity [corrected].

2.3.2.31 Iwata et al., 2001

Study reference:

Iwata K, O'Keefe GB, Karanas A. Neurologic problems associated with chronic nitrous oxide abuse in a non-healthcare worker. Am J Med Sci. 2001 Sep;322(3):173-4.

Abstract: case report

Chronic exposure to dinitrogen oxide is known to be associated with hematologic and neurologic abnormalities. When this syndrome occurs, it is generally seen in health care workers, especially dentists and anesthesiologists, who have access to dinitrogen oxide. Here, however, we report a case of a 55-year-old non-healthcare worker who presented with multiple neurological abnormalities. His serum vitamin B12 level was low but his Shilling test was normal. His neurologic symptoms improved after cessation of inhaling dinitrogen oxide and starting vitamin B12 therapy. Physicians should consider dinitrogen oxide abuse in non-healthcare workers presenting with neurologic symptom of unclear cause.

2.3.2.32 Lucchini et al., 1997

Study reference

Lucchini, R., L. Belotti, M. G. Cassitto, A. Faillace, M. Margonari, G. Micheloni, M. L. Scapellato, et al. « Neurobehavioural Functions in Operating Theatre Personnel: A Multicenter Study ». *La Medicina Del Lavoro* 88, n° 5 (1997): 396-405.

Detailed study summary and results

In order to define a safe exposure level, Lucchini et al., 1997, conducted a multi-centre study in Italy evaluating neuropsychological symptoms, subjective stress and response speed functions in subjects occupationally exposed to low levels of anesthetic gases. A group of 112 operating theatre workers from 10 Italian hospitals was exposed to anesthetic gases (Dinitrogen oxide and isoflurane), and 135 non-exposed workers were used as control group. People with daily alcohol intake exceeding 80 g were excluded as well as those with daily coffee consumption exceeding 5 cups, and/or with CNS medications, and/or neurological or psychiatric disorders and/or occupational or non-occupational exposure to neurotoxic metals or solvents and/or aged of more than 59 years. The workers were examined before and after the shift on the first and the last day of the working week. The testing comprised a complex reaction time test (the Stroop Colour Word) and a subjective mood scale. The week preceding the first testing a training session was organized in order to limit the learning effect of neurobehavioral testing. During this session, a questionnaire for neuropsychological symptoms (EURO-QUEST) was administered together with the block design subtest from the WAIS battery. The aims of these supplementary tests were to examine basic intellectual abilities of the participants. Biological and atmospheric indicators of exposure were measured at the beginning and the end of the working week for N2O and isoflurane. The results of these measurements indicated moderate exposures: for atmospheric N2O geometric mean and 95th percentile were 23.2 ppm and 127 ppm on the 1st

day and 20.6 ppm and 114 ppm on the last one; the corresponding values for isoflurane were 0.4 ppm and 3.8 ppm, and 0.3 ppm and 2.7 ppm. For end-of-shift urine dinitrogen oxide concentrations geometric means and 95th percentiles were 7.1 µg/L and 12.4 µg/L on the 1st day, 7.8 µg/L and 21.5 µg/L on the last one.
No statistical difference was observed between exposed and control subjects for neurobehavioral effects, stress and arousal levels.
The authors concluded that the biological exposure limits of 13 µg/L for urine N2O concentration (corresponding to 25 ppm for TWA air concentration) is adequately protective for the integrity of workers neurobehavioral functions, as measured with the tests used.

2.3.2.33 Lucchini et al., 1996

Study reference

Lucchini, R., D. Placidi, F. Toffoletto, et L. Alessio. « Neurotoxicity in Operating Room Personnel Working with Gaseous and Nongaseous Anesthesia ». *International Archives of Occupational and Environmental Health* 68, n° 3 (1996): 188-92.

Detailed study summary and results:

Lucchini et al. (1996) examined 30 operating room workers in an Italian hospital. The group of volunteers represented 80% of the entire personnel of the department and was composed of surgeons, anesthetists, operating room nurses and technicians. A control group consisted of 20 subjects randomly selected among medical and paramedical personal in other departments in the same hospital. Dinitrogen oxide atmospheric concentration was measured using personal sampling during a 3 hours period of time. Urinary Dinitrogen oxide was also measured in urine at the end of the shift. Simple reaction time (SRT) test was selected as psychomotor test and was performed at two different times: first, during a week with constant use of non-gaseous anesthesia and secondly, during a week with constant use of gaseous anesthesia, with a two-week interval between these weeks. In addition, biological measurements were performed: serum cortisol as a biological stress indicator and serum prolactin to investigate interference with the dopaminergic system. The authors used a –so-called "double-blind testing condition": as a matter of fact, only the 4 (exposed) anesthetists knew during which week gaseous anesthesia was used. Potential confounding factors such as age of alcohol consumption were checked. No information on potential co-exposures was provided in this study.
On the last day of the gaseous anesthesia week, mean dinitrogen oxide air concentration was 54.2 (SD= 22.8) ppm and mean urine concentration was 25.6 (SD= 22.1) µg/L. A good correlation between Dinitrogen oxide in air and in urine (r=0.89; p=0.0001) was found. The study shows a prolonged reaction time and increased serum prolactin levels in exposed workers only when they worked with gaseous anesthesia. No effect of Dinitrogen oxide exposure was observed for serum cortisol levels.
The authors concluded that their results indicate neurobehavioral effects of Dinitrogen oxide exposure below 100 ppm. However these results should be considered with caution, as co-exposures were not taken into account and the number of workers included in the study was small.

3 ENVIRONMENTAL HAZARDS

Hazards not addressed in the CLH report.

4 ADDITIONAL HAZARDS

4.1 Hazardous to the ozone layer

4.1.1 Ravishankara et al., 2009

Study reference

Ravishankara AR, Daniel JS, Portmann RW. Nitrous oxide (N2O): the dominant ozone-depleting substance emitted in the 21st century. Science. 2009 Oct 2;326(5949):123-5

Detailed study summary and results:

Dinitrogen oxide shares many similarities with the chlorofluorocarbons (CFCs). The CFCs and N2O are very stable in the troposphere, where they are emitted, and are transported to the stratosphere where they release active chem- icals that destroy stratospheric ozone through chlorine- or nitrogen oxide–catalyzed processes. They both have substantial anthropogenic sources. Unlike CFCs, dinitrogen oxide also has natural sources, akin to methyl bromide, which is another important ODS. Assigning an ODP for dinitrogen oxide and separating out the natural and anthropogenic emissions are therefore no more conceptually difficult than they are for methyl bromide.

The authors shows that the ODP of dinitrogen oxide is positive and nonzero showing that dinitrogen oxide is an ozone- depleting substance on the basis of the extent of ozone depletion it causes.

The authors calculated the Ozone Depleting Potential (ODP) of N_2O by using the Garcia and Solomon two-dimensional (2D) model. The ODP of N_2O under current atmospheric conditions is computed to be 0.017. This value was considered comparable to the ODPs of many hydrochlorofluorocarbons (HCFCs) such as HCFC-123 (0.02), -124 (0.022), -225ca (0.025), and -225cb (0.033) that are currently being phased out under the MP. The authors conclude that the value of the ODP of N_2O is robust because (i) similarly calculated ODPs for CFC-12 (1.03) and HCFC-22 (0.06) agree with the accepted values ; (ii) ozone depletion by NO_x from N_2O dominates the chemical control of ozone in the mid-stratosphere, a region well represented with 2D models; and (iii) ozone reductions by enhanced N_2O have been reported in other studies, although no published study, to the best of our knowledge, has previously presented an ODP for N_2O.

The authors examined a few important factors that influence the ODP of N_2O. At mid-latitudes, chlorine-catalyzed ozone destruction contributes most to depletion in the lowest and upper stratospheres, that is, below and above the ozone maximum. Nitrogen oxides contribute most to ozone depletion just above where ozone concentrations are the largest. This leads to efficient ozone destruction from NO_x. The ODP of N_2O is lower than that of CFCs primarily because only ~10% of N_2O is converted to NO_x, whereas the CFCs potentially contribute all their chlorine.

The auhors quantified the dependence of the ODP of dinitrogen oxide on atmospheric concentrations of chorine by calculating it for 1959 concentrations of stratospheric Cly (essentially preindustrial). They find the ODP for 1959 to be 0.026, showing that Cly concentrations have a moderate effect on the efficiency of N2O-caused ozone destruction. These results for the 1959 and 2000 Cly concentrations bracket the range expected for the rest of the 21st century; it shows that the dinitrogen oxide's ozone destructiveness per emitted unit mass should increase by about 50% when the stratospheric chlorine loading returns to preindustrial concentrations.
Nitrogen oxide chemistry is also dependent on odd hydrogen, bromine, and methane levels, but the dependence of dinitrogen oxide's ODP on these factors is expected to be much smaller than the effect of chlorine.

Whereas enhanced stratospheric sulfate aerosols after volcanic injections increase the effectiveness of chlorine to destroy ozone, they will decrease the effectiveness of NO_x emissions by sequestering the catalytically active NO_x in HNO_3. Such an influence has been observed after the Mount Pinatubo eruption. Therefore, we anticipate that the ODP of N_2O will be reduced when the sulfate loading is enhanced. However, high volcanic sulfate loadings are unpredictable and sporadic, and their effects are short-lived, lasting only a few years. We assess the extent of their influence by calculating ODPs at peak sulfate loadings observed after the eruption of Mount Pinatubo.