MEDICAL GASES

REVIEW OF TOXICOLOGICAL DATA ON NITROUS OXIDE

MGC 153/08/E

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1 Introduction

Nitrous oxide has traditionally been used as an anaesthetic gas for over 150 years and is now also used as an analgesic, as a pre-mixed gas mixture with medical oxygen. Nitrous oxide is a very safe gas from the aspect of administering to the patient for anaesthetic purposes, but care is needed to ensure that the healthcare professionals administrating the gas are not continually exposed to high levels of nitrous oxide within their working environment.

Over the years, scavenging systems have been developed for areas where nitrous oxide or nitrous oxide gas mixtures are being administered to maintain the levels of the gas in the working environment below the nationally set levels. These Occupational Exposure Levels (OEL) specified within each country vary, but are set between 25 ppm and 100 ppm, measured over an eight hour period.

There have been a lot of clinical papers published over the last thirty years providing information about the risks to healthcare professionals and patients of being exposed to different levels of nitrous oxide for different periods. The information is not consistent in its recommendations, and in extremes advises that nitrous oxide should not be used for medicinal purposes. This document was commissioned by the Medical Gas Council of EIGA to provide an unbiased view of the appropriate safety requirements for the safe use of nitrous oxide. The information relates to both the exposure levels for the patient and the workplace requirements for where the healthcare professionals are using the gas.

The information in this document, prepared by Dr Paul Brantom, is based on published literature and no new studies have been carried out. Prior to preparing the document, a literature search was undertaken to identify all of the papers that have been published on the subject. It does not attempt to contradict the Occupation Exposure Levels (OEL's) that are specified within the various countries within the EU but does indicate the implications of working at these levels. Neither does the document specify the type of equipment to be used to maintain the levels in the working environment to the appropriate levels. This information is specified within the appropriate international and national standards, such as Part 2 of the Medical Gas Pipeline standard (ISO 7396 – Medical gas Pipeline Systems, Part 2: Anaesthetic Gas Scavenging Disposal Systems).

2 Scope

The scope of this document covers the review of the use of nitrous oxide in healthcare premises where it is administered as an anaesthetic or analgesic gas. It a summary of the information published in medical journals of the effects of being exposed to levels of nitrous oxide to indicate the safety of the product for both the patient and the healthcare professional.

The document does not cover the methods of scavenging the gas when used for medicinal purposes nor recommend specific occupational exposure levels for those administering it in the healthcare environment.

3 Purpose

The document provides information for the medical gas industry to provide to its customers to identify the risks associated with being exposed to nitrous oxide in the working environment and indicates the need to take the appropriate precautions to maintain levels below those set nationally.

4 Nitrous Oxide Review

In view of the concerns relating to possible risks associated with nitrous oxide exposure and the lack of a comprehensive review of available data the European Industrial Gases Association (EIGA) agreed to support the preparation of such a review.

It was recognised that there were a number of specific situations of concern that the review should address. These were identified as:

- Effects of normal occupational exposure, particularly with reference to the potential for reproductive effects.
- Effects of exposure of pregnant women due to surgery during pregnancy.
- Effects of use as both analgesic and anaesthetic.

The scope of the review is given in full in Appendix 1, but the key details are reproduced below:

4.1 Scope of Review

A database of published toxicological information has been accumulated by BOC after comprehensive review of the Medline and Embase databases and has been reviewed and supplemented by a trawl by Dr Paul Brantom, a toxicology consultant (CV appended), using internet search tools relevant to toxicology (primarily Toxnet, supplemented by direct search of specific sites such as WHO, and US government agency data). From this comprehensive database of published information papers have been provisionally selected to provide a complete summary of the known toxicological effects of nitrous oxide. Where papers were not selected for review the reason for their rejection will be documented using standardised criteria.

The review will be categorised by effect, separating the information derived from in vitro studies, animal studies and human studies. For each effect a summary and conclusions will be generated, in addition to the in-depth review. The review will particularly seek to describe any evidence for occupational exposures which may be associated with a significant risk:

From a preliminary review of the database the following areas of study of effects of nitrous oxide have been identified:

4.1.1 Reproductive effects – teratogenicity & infertility

In 1995 the Committee on Toxicity of Dept of Health (COT) reviewed the reproductive effects of nitrous oxide at the request of the UK Health & Safety Executive (HSE) and derived a no adverse effect level (NOAEL) for the rat effects. This NOAEL was extrapolated to a safe working level of 100ppm. Further studies have been published since that review and these will be examined for any impact on the original conclusion. The COT opinion will also be examined to ensure that the conclusions are robust, based on the data cited. If it is considered that it would assist clarity the teratogenic effects will be reviewed separately from those related to infertility. The relevance of Vitamin B_{12} (Vit B_{12}) and methionine synthase MS inactivation will be given particular attention as possible markers of reproductive effects.

4.1.2 Carcinogenicity / Genotoxicity

Investigations of endpoints relevant to genotoxicity have been reported in humans with mixed exposures to anaesthetic gases. The results appear to be mixed and this information will be evaluated for specific relevance to human exposure to nitrous oxide.

4.1.3 Haematological and immunological effects

Studies of effects on the blood and immune response in animals have been identified and will be evaluated to assess the evidence for such effects and, if any, the relevance to human exposures.

4.1.4 Neurological effects

There is evidence from both clinical and non-clinical studies for some adverse effect of chronic exposure to nitrous oxide on the Central Nervous System (CNS), over and above the anaesthetic effects. The evidence for these effects will be evaluated. Slight effects of this nature are used by the American Conference of Industrial Hygienists (ACGIH) to derive the safe working level of 50ppm, described in the background document to their Occupational Exposure Limits (OELs), however this document is of limited value since it does not appear, from the references cited, to have been updated in any significant way since the late 1980s.

4.1.5 Exposure assessment

Many of the recent publications have been concerned with providing more accurate data on exposure levels. Since knowledge of actual exposures is critical to performing a valid risk assessment this body of information will be assimilated to provide an overview of current knowledge of exposure levels in various contexts.

During the course of preparation of the review various additional publications were identified and were provided to the author. For clarity, papers that did not contribute to the overall risk assessment have not been cited but have been included in the reference lists with a brief comment identifying why they were excluded from consideration.

The following document describes the data reviewed and provides summaries of that information in a form that allows different levels of access. For each type of effect the papers for clinical and non-clinical data are reviewed in detail in separate sections, providing critical comment on the data and conduct. At the head of each of those sections the detail is condensed into a referenced overview providing the key facts deriving from the detailed review. Under each type of effect a summary section brings together both clinical and non-clinical information, without specific references but identifying the key facts and any conclusions that might be drawn. Two additional sections, on exposure and mechanism are included, at the start of the review, before considering the specific effects to provide a broad context for those detailed reviews.

The summaries of all the sections are brought together in the executive summary that precedes the contents section.

5 Occupational Exposure

5.1 Summary

From the available data occupational exposure to N2O has significantly reduced over the last 25 years. This has been mainly due to the introduction of scavenging and ventilation systems in all situations of regular use. Despite these improvements most recent papers reporting monitoring of staff exposed to N2O find that exposures can reach well above 100ppm for short periods, and values > 1000ppm are still being reported. As a consequence there are still frequent situations where the 100ppm 8hr Time-Weighted Average (TWA) exposure limit is exceeded.

In reviewing the data for potential effects of exposure all of these possibilities are considered, especially when there appears to be some potential for effects at or close to the likely exposure levels.

Due to the apparent lack of short-term effects below an exposure of 500ppm and the slow recovery from effects when they do occur, it is the frequency and duration of periods in excess of this level which is probably more important to risk assessment than the overall TWA. While there is no reason to question the current Occupational Exposure Limit (OEL) of 100ppm TWA, applied in several European countries, there may be grounds for considering a Short-Term Exposure Limit (STEL) to supplement that limit.

5.2 Detailed review

Country	8hr TWA		STEL
	ppm	mg/m^3	mg/m^3
UK	100	180	
USA (American Conference of Industrial Hygienists - ACGIH),	50		
USA (National Institute for Occupational Safety and Health - NIOSH)	25		

Australia	25		
Sweden	100	180	900
France	25		
Denmark	25		

Table 1 Published Occupational Exposure Limits for N2O

OELs below 100ppm are all based on the effects on performance observed and reported by Bruce *et al.* in the 1970s. These studies have been severely criticised and despite several attempts the results have not been reproduced. It may help the arguments for standardisation of OELs to conduct new studies of such endpoints using current methodologies.

5.3 Papers published up to 1980

The papers summarised in Table 2 demonstrate that, without the use of scavenging, exposure levels could quite frequently reach more than 2000ppm for periods during the working day. The benefits of scavenging were demonstrated by the comparison of different conditions in some of the studies, although the levels of exposure observed were very variable between studies.

Facility	No.	Sample type 1	Sample type 2	Sample type 3	Mean (range) Sample 1 N2O (ppm)	Mean (range) Sample 2 N2O (ppm)	Mean (range) Sample 3 N2O (ppm)	Ref
Operating theatres - scavenged	**146**	**Inspired - Breathing zone (30secs)**	**Expired - End-tidal (Mean of 4-6 expirations)**	**-**	**67 (5-580)**	**77 (8–305)**		**Beynen *et al.*, 1978**
Operating theatres - unscavenged	**39**	**Inspired - Breathing zone (30secs)**	**Expired - End-tidal (Mean of 4-6 expirations)**	**-**	**85 (10-300)**	**93 (9-475)**		**Beynen *et al.*, 1978**
Unscavenged operating theatre	**19**	**Anaesthetist's area (10 min)**	**Anaesthetist's Mask (2hr)**	**Theatre periphery**	**189 (60-650)**	**381 (75-1100)**	**171 (50-670)**	**Davenport *et al.*, 1980**
Scavenged (passive) operating theatre	**15**	**Anaesthetist's breathing zone §**	**Floor by anaesthetic machine**	**Periphery of theatre**	**246 (22-680) 274 (54-535) †**	**666 (22-1200)**	**257 (30-870)**	**Mehta *et al.*, 1978**
Unscavenged operating theatre	**15**	**Anaesthetist's breathing zone §**	**Floor by anaesthetic machine**	**Periphery of theatre**	**872 (35-1870) 1201 (583-1932) †**	**1582 (55-3440)**	**586 (210-1020)**	**Mehta *et al.*, 1978**
Unscavenged operating theatre	**7**	**Background**			**660 (309-1253)**			**Halliday *et al.* ,1979**
Dental clinics	**16**	**Background with air conditioning**	**Background with scavenging**	**Background no control**	**327 (140-550) (n=9)**	**25 (15-40) (n=5)**	**719 (320-1118) (n=2)**	**Halliday *et al.* ,1979**

§ spot sample also obtained in induction room showing higher exposure levels than operating theatre
† personal samples, other values are for spot samples

Table 2 Published analyses of exposure to N2O pre 1980

Further data on exposures were provided in the following publications, which are not suited to inclusion in the above table:

Piziali et al., (1976) demonstrated that air conditioned operating and delivery facilities could achieve background N_2O concentrations of less than 150ppm

Korttila et al., (1978) reported the results of measurements on the blood and expired air of ten nurses exposed to levels of 380ppm (233-436) N_2O over a period of 4 hours working in an operating room. Expired air contained 75µg/l N_2O immediately after the end of working and this had declined to 8µg/l N_2O 21hr later. Blood levels at the same time-points were 153µg/l N_2O and 18µg/l N_2O. Expired air samples measured on mornings during the working week and at the weekend, showed similar levels of 7-22µg/l N_2O, and demonstrated no accumulation of N_2O during the course of the working week

Krapez et al. (1980) report the results of blood N_2O measurements for anaesthetists, surgeons and nurses both with no precautions and with passive scavenging compared with the breathing zone concentrations obtained by 60 sec spot sampling. The exposure of anaesthetists in 3 different theatres with no precautions ranged from 52 to 2385ppm with a mean value of 530ppm. Scavenging reduced the level to 53 (6-203) ppm. Blood levels were 11.9µmol/l (3.1-50.5) without precautions and 1.5µmol/l with scavenging (limit of detection 0.9µmol/l)

5.4 Papers published between 1981 and 1990

Additional data published in this period is summarised in Table 3.2. and provides further background on actual exposure levels particularly a little more on the effect of scavenging on exposures. Generally however the levels of exposure reported for this period, significantly exceed all published OELs.

Facility	No.	Sample type 1	Sample type 2	Sample type 3	Mean (range) Sample 1 N_2O (ppm)	Mean (range) Sample 2 N_2O (ppm)	Mean (range) Sample 3 N_2O (ppm)	Ref
UK Delivery rooms unscavenged	**14 5 21 23**	**Personal samplers of midwives in 4 hospitals**	**-**	**-**	**98 (5-364) 59 (10-121) 41 (1-330) 363 (42-953)**	**-**	**-**	**Munley *et al.*, 1986**
UK Delivery rooms scavenged	**13**	**Personal samplers of midwives in 1 hospital**	**-**	**-**	**150 (42-441)**	**-**	**-**	**Munley *et al.*, 1986**
Scavenged operating rooms in 6 UK hospitals[§]	**89,75, 89 69,56,69 20,19,20 19,18,19 15,15,15 16,9,9**	**Anaesthetist personal sampler**	**OD Assistant, personal sampler**	**Background, sampler on wall**	**169 (16-1580) 168 (17-1580) 172 (16-696) 622 (38-5120) 310 (72-1350) 97 (26-187)**	**59 (4-343) 61 (12-213) 52 (4-343) 155 (22-408) 126 (23-236) 63 (19-100)**	**49 (4-250) 42 (5-248) 73 (4-250) 63 (8-173) 88 (18-254) 37 (17-58)**	**Gray, 1988**
US Dental treatment rooms - unscavenged	**16**	**Dental breathing zone**	**Dental chair foot**	**-**	**409 (69-2000[†])**	**258 (40-750)**	**-**	**Kugel *et al.*, 1989**
US Dental treatment rooms - scavenged	**18**	**Dental breathing zone**	**Dental chair foot**	**-**	**98 (3-1000)**	**86 (0-320)**	**-**	**Kugel *et al.*, 1989**
Netherlands Operating theatres - scavenged	**6,4,8**	**Anaesthetists and assistants**	**Circulating Nurse**	**Surgeon and instrument nurses**	**99.6 (57-120)**	**41.2 (33-54)**	**32.6 (9-57)**	**Borm *et al.*, 1990**
Italian Operating theatres - unscavenged		**Anaesthetist (5)**	**Surgeon (5)**	**Nurse (10)**	**444 ± 144 (Gynaecology)**	**422 ± 87 (Otorhinolaryngology)**	**387 ± 124 (Gynaecology)**	**Trevisan & Gori, 1990**

[§] The scavenging systems used in each hospital were of different types and ages but the data support no conclusion about the benefits of particular aspects of those systems
[†] Levels greater than 2000ppm could not be measured by the methods employed.

Table 3. Published analyses of exposure to N2O between 1981 and 1990

Trevisan & Gori (1990) measured exposure during different types of operation and found a significant influence of the procedure on the exposure of different categories of operating theatre staff. Urinary N_2O was also measured in the same subjects and a strong correlation with the exposure levels was found.

5.5 Papers published after 1991

A diversity of papers has been published during this period and while many show some benefit of the introduction of scavenging systems other factors still seem to create circumstances of high exposure for staff working with N2O in all contexts. Firstly in a number of studies of midwives there is some reassurance that achieving levels of exposure below the 100ppm TWA is possible, although much higher exposures still occur:

Newton (1992) reported the results of monitoring the exposure of 9 midwives over one working day and 4 midwives over a whole working week, using samplers worn in a breast pocket. The exposures recorded were 482ppm (range 5-1558) for the 1 day group and 402ppm (range 12-1151) for those carrying the sampler for a week.

Newton et al. (1999) compared the exposure of UK midwives to N_2O in a newly constructed force-ventilated labour suite compared with an old unventilated facility. For 15 midwives monitored in the new facility the TWA did not exceed 100ppm, but the range of exposure was not recorded. This compare with TWA values ranging from 8 to 1558ppm in the older facility.

Mills et al. (1996) reported results of monitoring 242 midwife shifts on the labour wards of two UK hospitals. While 7 shifts recorded exposures greater than 500ppm (max 1638) 53 had levels in excess of 100ppm. In total the exposure exceeded 100ppm in 26% of shifts. Analysing data from some shifts on which N_2O was not used exposure was still recorded at 22ppm (range 0-233), presumably from general background air contamination.

Henderson & Matthews (2002) examined midwives during 50 shifts in a UK hospital delivery suite by analysing exhaled N_2O and monitoring N_2O exposure by a personal sampler. While the sampler measurements gave a mean exposure of 313ppm (range 2.4-1300) the expired air had a mean content of 64ppm (range 0-727). The correlation between individual exposure and expired air values was poor. Although the 8hr TWA may not be exceeded in all cases the exposure levels measured in this study exceeded the 100ppm TWA for the 4 hour measurement period in 35 of the 50 shifts.

Henderson et al. (2003) monitored the occupational exposure of 46 midwives over a 4-hour period and also examined urine samples for N_2O both before and after the monitoring period. The occupational exposure levels were stated to be 313ppm (range 2.4-1300). Of the urine samples taken before the start of exposure 22/46 had non-zero levels of N_2O and in five cases the later sample had a lower content then the first one. The mean value for urinary concentrations of N2O in the second sample was 114µg/l (range 0-1102). For those midwives with a zero urinary value at the start of the shift there was a good correlation between the exposure level and the urinary concentration after 4 hours. Imbriani & Maestri (2004) note in a letter responding to this paper that apparent N_2O contamination of urine samples can arise due to microbiological activity or sampling in a contaminated environment. These factors can be controlled to improve the value of urinary N_2O as a monitoring tool.

In other areas of N_2O use the recent picture is rather mixed, with evidence from Germany that very low exposures are achievable and data from UK showing that there is still some improvement to be made.

Henderson & Matthews monitored N2O exposure in 8 hospitals in Wales for compliance with the 100ppm TWA and found that there were no instances of the TWA being exceeded. Despite this compliance the maximum concentrations in several instances exceeded 1,000ppm in the anaesthetic room. The same authors (Henderson & Matthews, 2000a) monitored exposure to N2O in dental hospitals and practices. Although for the duration of the procedures monitored several reached a mean concentration of more than 100ppm over more than 2 hours the 8hr TWA was below 100ppm in all cases. Reporting further data from the monitoring of labour suites and other facilities (Henderson &

Matthews, 2000b) the conclusions were similar but instance of mean exposures in excess of 200ppm for more than 1 hour were more frequent than might be expected.

Raj et al. (2003) studied the exposure of UK paediatric anaesthetists to N_2O, monitoring the exposure by personal sampler and blood, breath and urine concentrations by samples taken at the start of anaesthesia of each patient on the list during a whole day of surgery. Excluding one unexplained very high value the exposure data give a mean of 23.8ppm (range 10-172). Urinary, blood and breath samples gave values which correlated poorly with the exposure data but 18% of blood samples and 43% of breath samples exceeded 100ppm. Differences in scavenging were strongly correlated with exposure levels, demonstrating a positive benefit of scavenging systems.

Comparing N_2O exposure levels in 9 different operating theatres in 1996 and 1997 Wiesner et al. (2000) found that introduction of scavenging systems between the two sets of measurements did reduce exposure from a median of 168 (56-7490) to 104 (10-2140) however the ranges shown in parentheses do indicate peak levels of exposure which are well in excess of what might be expected from the TWA values. Many of the TWA values exceed the 100ppm limit established in many European countries. Further analyses of the same data are included in a second paper (Wiesner et al., 2001) but add no additional data.

A clinical trial was conducted in Germany by ***Byhahn et al. (2000)*** to allow detailed measurement of the exposure to N_2O and desflurane during Ear, Nose and Throat surgery. Mean exposure levels were obtained for anaesthesiologist and surgeon during surgery on both adults and children. The results respectively, reported as mean ± SD, were 0.41 ± 0.23 and 2.24 ± 1.93 for adults and 1.20 ± 0.32 and 5.30 ± 0.6 for children. These values are significantly lower than any previously reported and this is attributed to special care in the design of the facility and conduct of the trials.

Mierdl et al., (2003) monitored the $_{N2O}$ exposure of surgeons during cardiac surgery on cardiopulmonary bypass on 10 adults. The monitoring collected samples via tubes in the region of the surgeon's mouth, collecting samples at 90-second intervals, which were estimated on-line. Two stages of the operation were monitored, prior to bypass and while on bypass. During each stage respectively the N_2O concentrations were measured as 9.32 ± 1.93 and 3.00 ± 1.41ppm.

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6 Mechanism of N2O toxicity and Methionine synthase

6.1 Summary

This brief review of mechanism is included to provide a background to the following sections on specific effects of N_2O and is restricted to the review of those aspects which are relevant to the risk assessment.

The only known metabolic interaction of N_2O is the oxidation of the Co (I) form of Vitamin B_{12} (Vit B_{12}) to the Co (III) form. The oxidised form cannot function as a co-enzyme for methionine synthase (MS) thus the effect is to inhibit the activity of this enzyme. Since all of the major effects of N_2O appear to be mediated by this primary interaction, the markers of an effect on this pathway may be useful indicators of thresholds of an overall effect of exposure, particularly in respect of chronic low-level occupational exposure. Markers such as deoxyuridine suppression (dU-suppression), red cell folate, serum folate, Vit B_{12} and homocysteine levels have all been used to monitor for effects of N_2O exposure as well as direct measurements of MS activity. Serum Vit B_{12} seems to be unaffected by N_2O exposure. Studies have shown a good link between depletion of MS activity and both serum homocysteine levels and increased rates of dU-suppression and in the recovery of both to normal levels. These are generally the preferred markers of Vit B_{12} deficiency.

Since all of the known adverse effects of N_2O seem to be mediated via the MS pathway it would be reasonable to conclude that exposures which have no effects on that pathway represent an overall no adverse effect level (NOAEL). This would certainly be appropriate for haematological and neuropathy effects and most probably for reproductive effects, however in that context some further data needs to be considered, relating to possible alternative mechanisms of reproductive effects. A small body of work relating to a possible role of direct N_2O effects on Luteinising hormone (LH) and gonadotrophin release needs further investigation to evaluate the relevance to human risk assessment.

In addition to the MS interaction there is consistent evidence that administration of N_2O is associated with elevated prolactin levels. This is interpreted by some as an indicator of dopaminergic interactions. A reduction in LH levels has also been reported and this has been associated with the same interaction. These effects have only been demonstrated after prolonged exposure to analgesic levels of N_2O and may be more related to those properties. They would appear to be more transient than those associated with the MS pathway, but as endocrine effects they may also play some part in the reproductive effects of N_2O seen in animal studies. Overall it seems unlikely that these additional factors are relevant to any human exposures to N_2O, thus the NOAEL derived from lack of effect on the MS pathway could still be considered relevant. From the available data on rats it would appear that inactivation of MS is no greater in the foetus than in the mother at the end stage of pregnancy (19 days), thus an exposure without effect on the mother at this stage is unlikely to affect the foetus. There are no available data giving an insight into relative foetal sensitivity to MS inactivation at earlier stages of pregnancy.

From the above considerations a NOAEL in rats, on the basis of the mechanism of action, could be concluded to be in the region of 500ppm, as continuous exposure. There appears to be some slight effect on dU-suppression, a marker of MS inhibition, at exposures of 50ppm. While this is not, alone, an indicator of any adverse effect, the relationship would benefit from more detailed investigation.

Comparison of MS inactivation in rats and humans indicates that for a given exposure the rate of inactivation is slower in humans. Although no specific data have been identified on recovery rates

limited evidence from haematological studies, in which dU-suppression was measured, is that recovery may also be slower than in rats. These factors need to be considered when assessing the relevance of animal studies to human exposure at analgesic and anaesthetic concentrations.

For those exposures which result from anaesthetic use or analgesic use there is a predictable effect on Vit B_{12} and MS activities and there are populations who may be vulnerable to those effects, such as those with marginal Vit B_{12} deficiency.

A second population of concern is those subject to N_2O anaesthesia during pregnancy. There is no doubt that some inactivation of MS and Vit B_{12} will occur in that population since the half-time for depletion of activity has been shown to be less than 1 hr, however it is important to recognise that all of the main effects seen in animals at low doses derive from exposures of at least 24hr duration. Marginal Vit B_{12} deficiency in the pregnant women would also tend to increase the possibility of adverse consequences.

6.2 Detailed Review

The metabolic pathways related to the effects of N_2O are summarised below in Figure 1 copied from the review of ***Weimann (2003)***. (Permission applied for)

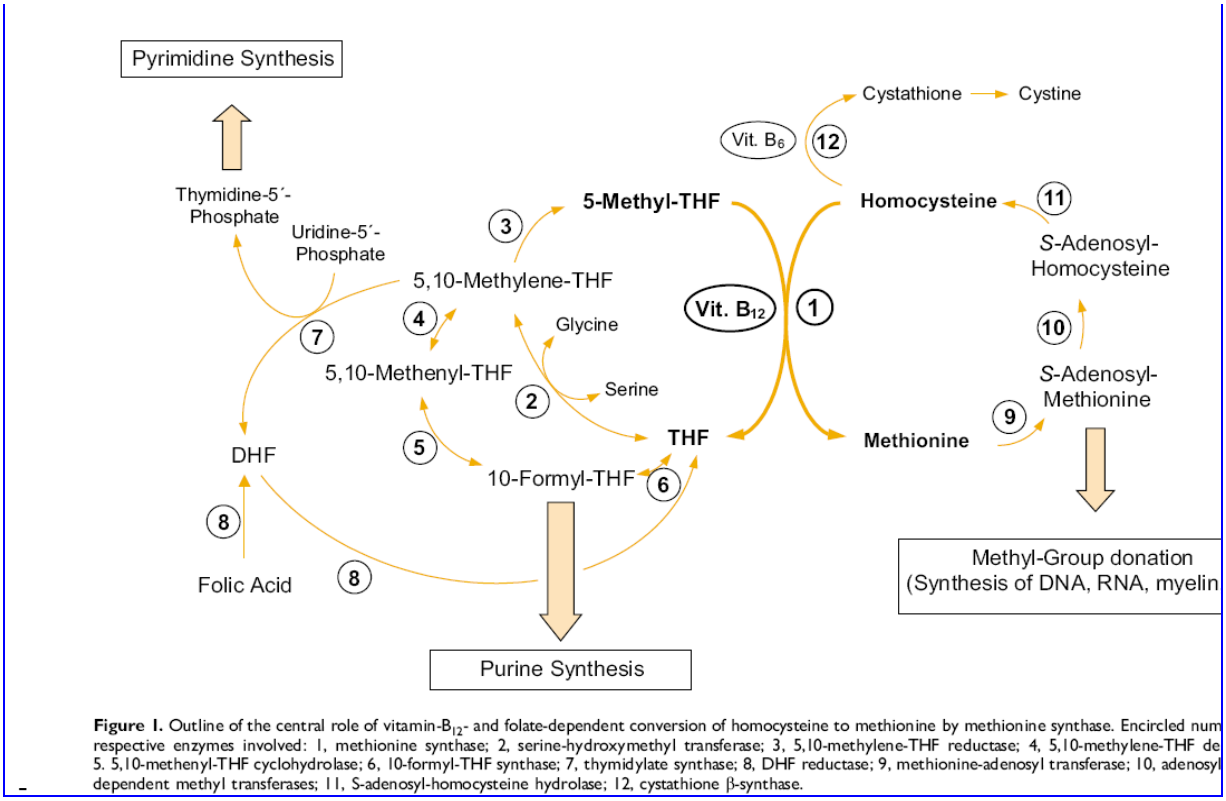

Figure 1. Outline of the central role of vitamin-B_{12}- and folate-dependent conversion of homocysteine to methionine by methionine synthase. Encircled num respective enzymes involved: 1, methionine synthase; 2, serine-hydroxymethyl transferase; 3, 5,10-methylene-THF reductase; 4, 5,10-methylene-THF de 5. 5,10-methenyl-THF cyclohydrolase; 6, 10-formyl-THF synthase; 7, thymidylate synthase; 8, DHF reductase; 9, methionine-adenosyl transferase; 10, adenosyl dependent methyl transferases; 11, S-adenosyl-homocysteine hydrolase; 12, cystathione β-synthase.

Figure 1 Methionine synthase and related pathways (taken from Weimann, 2003)

The only known metabolic interaction of N_2O is with Vit B_{12}. N_2O by irreversibly oxidising Vit B_{12} from the Co(I) state to the Co (III) state blocks its activity as co-enzyme to MS both depleting methionine stocks and blocking the folate cycle. The consequence of these effects is directly comparable to the effects of Vit B_{12} deficiency. This highlights the potential for special vulnerability of those already marginally Vit B_{12} deficient. In this population the effects of even brief anaesthetic or analgesic exposures may have significant consequences ***(Deleu et al., 2000)***.

The most comprehensive available data on dose-relationship and kinetics of the effect of N_2O on MS in animals comes from the paper of ***Sharer et al. (1983)***:

To characterise the effects of N_2O on MS groups of 4-15 male Sprague-Dawley rats were exposed to 500, 1,000, 2,500, 5,000, 10,000, 20,000 or 50,000ppm N_2O for 1, 2, 4 or 8 days (Other groups were exposed to 1,000 or 5,000ppm for up to 28 days. All exposures were 24 hr/day 7 days/week. After

exposure the animals were anaesthetised and exsanguinated. Slices of liver were preserved frozen at -20ºC and subsequently assayed for MS activity. The results obtained have been plotted and a best fit curve obtained. From the curves obtained it is concluded that there was no detectable reduction of MS activity below a 24-hour exposure of 860ppm or a longer exposure of 450ppm.

Inhibition of MS in mice was also studied by ***Koblin et al. (1981)***. Groups of 8 mice were exposed for various durations to 0.6atm N_2O in the presence of 1 atm Oxygen and immediately afterwards liver samples were taken for assay of MS. After 4 hr activity was 5% of the control value of around 300 $nmol.h^{-1}.\ g^{-1}$ liver. A study of recovery showed that 24hr after a 4hr exposure to 0.8atm the MS activity was 70% of control and was nearly complete after 2 days.

Additional studies were conducted to investigate the effects of different concentrations of N_2O and it was concluded that exposures to <0.05atm N_2O for 4 hr had no significant effect whereas 0.1atm for the same duration resulted in a 50% reduction of MS activity. 1100ppm was shown to significantly inhibit MS by 27% after 8 days but by 15 or 22 days exposure to the same concentration the difference was slightly less and was not significant.

Deacon et al. (1980) investigated the rate of MS inhibition by exposure of rats to 50% N_2O for up to 6hr, monitored at intervals over this period and over the subsequent 84hr. The activity was already significantly reduced after 30 minutes and almost undetectable after 6hr. 60hr after exposure activity was less than 50% of normal and had not returned to control levels in some animals after 84hr. dU-suppression in the bone-marrow was increased after 60 minutes, and showed an increasing level of difference over the 6 hr exposure period. The rate had returned to control levels 60hr after exposure but was already recovering markedly by 24hr.

Information regarding the relative effects of similar exposures in man and animals is an important part of extrapolating the results of animal studies to human exposures. The following studies shed some light on possible differences in human and animal responses to N_2O exposure:

The rate of inactivation of MS in human and rat liver was reported by Nunn *et al.* (1988) who studied liver biopsy samples from two groups of patients, one anaesthetised with N_2O and the other who received other anaesthetics. For those anaesthetised with N_2O there was a gradual reduction in MS activity with considerable inter-individual variation but a calculated half-time of 46 minutes. Patients anaesthetised with other anaesthetics showed no correlation of MS levels with duration of anaesthesia. To provide a comparison of the effects of N_2O in rats groups of 6 animals were exposed to 50% N_2O for 5, 10, 20, 40 or 80 minutes and then liver samples assayed for MS activity. The onset of inhibition was much more rapid than in the patients and the half-time was only 5.4 minutes. The starting levels of MS in rat and human samples were 2.23 $nmol.h^{-1}.mg^{-1}$ protein and 1.18 $nmol.h^{-1}.mg^{-1}$ protein respectively.

Koblin et al. (1982) studied the MS activity in biopsy samples from 7 patients who had received N_2O and 7 who had other anaesthetics. The range of activity was 110-320 $nmol.h^{-1}.\ g^{-1}$ liver for those anesthetised with N_2O and 285-523 $nmol.h^{-1}.\ g^{-1}$ liver for those given other anaesthetics. If the activities of MS are plotted against total N_2O received (Concentration times duration) the relationship was quite linear and showed a 50% reduction in starting levels after approximately 1.5hr of exposure to 70% N_2O.

Nunn et al. (1982) compared the results of serum analyses from 10 staff exposed to N_2O at a mean concentration of 150-400ppm in the preceding 24hr with 10 staff without any exposure. Serum was analysed for methionine, valine, isoleucine, leucine, Gamma glutamyl transferase (γ-GT) and Aspartate aminotransferase. There was no difference between the two groups in the results of any of these analyses.

Since the reproductive effects of N_2O are of specific interest to this risk assessment the available data relevant to this aspect are reviewed below:

Baden et al. (1987) demonstrated that N_2O could affect both foetal and maternal methionine synthase. Enzyme solutions were prepared from livers of dams and foetuses, collected on day 19 of gestation and incubated for up to 24hr with 50% nitrous oxide/oxygen or 50% nitrogen/oxygen. MS activity was reduced to 14 and 17% of foetal and maternal starting activity respectively.

Further studies provide some background to the effects of 24hr exposures to N_2O on methionine synthase and other markers at critical stages of gestation and the effect of different durations of N_2O exposure on both maternal and foetal MS activity in pregnant rats:

Groups of 16 pregnant rats were exposed to 0%, 0.75%, 7.5% or 75% N_2O for 24hr on day 9 of gestation ***(Baden et al., 1983)***. Half of each group was killed immediately after exposure and the remainder 72hr later. Liver was assayed for MS activity and bone marrow was assayed for suppression of deoxyuridine-thymidine conversion (dU-suppression). The level of dU-suppression was significantly greater than controls for rats exposed to the highest two concentrations of N_2O but had returned to control levels by 72hr after the exposure. MS activity was not measurable immediately after exposure, in any of the groups exposed to N_2O. 72hr later the activity was 75%, 21% or 11% of control values for the group exposed to 0.75%, 7.5% and 75% N_2O respectively.

Groups of 3-8 pregnant rats were exposed to 10% N_2O on day 19 of gestation for 30, 60, 120 or 240 minutes ***(Baden et al., 1984)***. MS activity was measured in both maternal and foetal livers immediately after the end of exposure. Activity was decreased by all exposures in an exposure-related manner, reaching 32% or 56% of control after 240 minutes for maternal and foetal samples respectively. A second study investigated the effects of exposure to 50% N_2O for up to 60 minutes by which time activity of MS was decreased to 11% and 18% of control for maternal and foetal samples respectively. Rats killed 72hr after exposure to 50% N_2O had MS activity 49% and 85% of control for maternal and foetal samples respectively.

Additional factors to be considered as possibly contributing to the mechanism of reproductive effects of N_2O in rats have been identified by ***Kugel et al. (1991)***:

A group of 8 female rats was exposed for 8hr/day to 30% N_2O for 1 complete oestrous cycle (4days), a control group was exposed to air alone. At the end of exposure animals were killed and brain and pituitary tissue isolated for analysis of LHRH, met-enkephalin, β-endorphin, and substance P. Seven of the eight rats exposed to N2O were arrested in the prooestrous stage of the cycle and Luteinising hormone releasing hormone (LHRH) levels were significantly increased in the preoptic area and marginally decreased in the basal hypothalamus. Other differences observed were increased Met–enkephalin in the pons and medulla regions of the brain, increased β-endorphin levels in the anterior lobes of the pituitary and substance P levels decreased in the preoptic area and increased in the median basal hypothalamus. The possibility that many of these changes are secondary to the disrupted oestrous cycle cannot be ruled out, thus the role of these factors in the mechanism of effect of N_2O remains unclear.

Investigation of these neuroendocrine effects in an *in vitro* model suggests that this line of research needs more exploration in order to clarify the relevance of this effect to the exposure scenarios of interest to a risk assessment:

Zhang et al. (2003) have studied various endpoints after exposure of Gonadotrophin-releasing hormone (GnRH) neuronal (GT1-7) cells to N_2O . The cells showed an 80% reduction in GnRH mRNA levels and significant inhibition of GnRH release after 24hr exposure to 60% N_2O.

One of the most sensitive and specific indicators of functional change in the MS pathway is the dU-suppression test, which uses the rate of the conversion of deoxyuridine to thymidine as an indicator of tetrahydrofolate deficiency, a secondary impact of MS inactivation. Since this is an indirect measurement of the effects of N_2O it can be subject to variation due to factors unrelated to N_2O exposure but is frequently found to be a useful marker of the Vit B_{12} status of an individual and is the preferred clinical method for investigating possible clinical Vit B_{12} deficiency with better specificity than serum Vit B_{12} or red cell folate levels ***(Wickramasinghe & Matthews, 1988)***

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7 Reproductive Effects

7.1 Summary

7.1.1 Teratogenicity

Regular exposure to N2O during pregnancy in the normal course of occupations such as anaesthetist or assistant, dentist or dental assistant and midwife has been of concern since the first studies of N2O in pregnant animals demonstrated the capacity to cause teratogenic effects. Even in animals the teratogenic effects are only caused by the most extreme conditions of exposure, such as anaesthetic concentrations for 24hr at critical stages of foetal development. When the exposure is to a lower concentration or the duration is shorter then such severe effects are not seen. Although two studies have shown higher levels of congenital abnormalities in the offspring of women exposed to anaesthetics these studies have serious flaws which would tend to lead to an overestimate of such risks. Review of all other available human data has not identified any excess of congenital abnormalities in the offspring of mothers exposed to N2O under various circumstances, thus under the circumstances of occupational exposure it is not considered that there is evidence for a teratogenic risk. Such a risk would not be expected from the available animal data and the likely occupational exposure levels.

7.1.2 Foetal loss and spontaneous abortion

Since exposure to N_2O has been associated with foetal loss in animal studies, the evidence for a similar effect following occupational N_2O exposure has been explored in a range of clinical studies. At the exposure levels or schedules which are relevant to occupational exposure the animal studies do not show such losses, thus occupational effects would not be expected.

Although numerous clinical investigations have shown that staff working in operating theatres have an excess risk of spontaneous abortion an equal number of other studies, generally those with more robust designs, have shown no such effects. Those studies which do show some effects provide no formal link of these differences to anaesthetic exposure in general or to N_2O specifically. The nature of some of the studies means that they may be more likely to confirm the hypothesis that they are investigating than to establish any association between cause and effect. This problem arises from the self-selection to participate in questionnaire trials. Those with preconceptions may be more likely to respond than others with no specific reason to participate. Many of the studies have additional severe deficiencies in both conduct and design, such as selection of inappropriate comparator groups; thus the true cause of any of the differences seen cannot be ascertained. In those studies where N_2O exposure has been specifically investigated no effect has been shown.

7.1.3 Use of N_20 anaesthesia during pregnancy

The use of anaesthetics during surgery in pregnancy and the subsequent effect on the developing foetus has been studied by several groups and although surgery is itself associated with some risk to the survival of the foetus there is no evidence that the exposure to anaesthetics, including N_2O has any effect on any aspect of development including abortion and development of congenital abnormalities. This is confirmed by the lack of such effects in animal studies using exposures of less than 24hr, even on the most critical days of foetal development.

There is very limited evidence, from animal studies, of a small effect on post-natal development and behaviour of a brief exposure to 75% N_2O, during the latter stages of gestation. There is no evidence from clinical studies that such effects occur in humans, but there is no report of a specific investigation for such effects. At exposures below 10,000ppm, throughout gestation, there is no evidence in animals for any effect of *in utero* exposure to N_2O on behaviour of the offspring. After exposure of mice to 50,000ppm over 9 days of gestation some slight effects on startle reflex were identified.

7.1.4 Fertility

While exposures in excess of 10% N_2O for various durations have shown some evidence of effects on fertility this seems to be associated with MS inactivation in that longer daily durations of exposure seem to have the greatest effect. No study has shown any effect on fertility in animals at daily exposures of 1,000ppm or below. This would indicate that occupational exposure is unlikely to have any effect on fertility and the limited clinical data available indicate no male fertility effects.

The clinical data which claim to show an effect on female fertility are based on very small numbers of participants and a questionnaire design which almost certainly attracted participation by women who believed that occupational exposure may have been the reason for their own misfortune.. In the context of the available data on animal effects and mechanism there is little likelihood that any effects seen in those studies are truly the result of occupational exposure to N_2O.

Much of the uncertainty regarding the reproductive risks of N_2O has come from poorly constructed retrospective clinical studies plus animal studies that were not designed to provide answers to the current questions. The evidence from mechanistic studies and some clinical studies provides reassurance that the risks are negligible but good quality prospective epidemiological data would be of significant value in providing information on the various putative reproductive effects of anaesthetics in general and N_2O specifically. Additional data on comparative kinetics and metabolism in animal and human tissues might also allow a better extrapolation from the existing animal studies.

A small body of data indicating direct effects of N_2O exposure on gonadotrophin release and Luteinising Hormone (LH) levels needs some additional data in order to assess the relevance to human risk assessment.

7.1.5 General comments

Review of all available published animal data confirms that exposure to atmospheres containing 500ppm N_2O shows no evidence of any reproductive effects in animals, even when exposure is continuous throughout pregnancy. Male fertility is also unaffected at similar exposures. Foetal loss has been seen in rats following constant daily exposure to 1,000ppm N_2O but not at 500ppm. With intermittent exposure (such as 6hr/day 5days/week) such effects are not seen even at 1,000ppm. Since there is strong evidence that methionine synthase (MS) inhibition is the underlying mechanism of the reproductive effects in animals this lack of effect at 500ppm would be consistent with the observations that there is no effect on activity of this enzyme at this exposure level, even after chronic exposure.

Based on the animal data the current Occupational exposure Limit (OEL) for some European countries for N_2O of 100ppm 8hr time-weighted average (TWA) is sufficient to protect against any potential adverse reproductive effect of exposure to N_2O in an occupational setting.

7.2 Teratogenicity and foetal toxicity

7.2.1 Teratogenicity & foetal toxicity - Non-clinical data

7.2.1.1 Overview

Exposure of pregnant rats to high doses of N_2O (>50% or 500,000ppm), for a full 24 hr on a single day of gestation, has been shown to result in both foetotoxic and teratogenic effects (***Fink et al, 1967; Fujinaga et al., 1989; Fujinaga et al, 1990; Lane et al., 1980; Mazze et al., 1984)***. The severity of the effect is influenced by both the timing and duration of exposure ***(Fujinaga et al., 1989; Fujinaga et al, 1990)*** with exposure at the time of organogenesis having much greater effect than at subsequent times ***(Ramazzotto et al., 1979)***.

Only one study in rats has been identified which investigated high exposures for a shorter period ***(Tassinari et al., 1986)*** and this demonstrated no adverse effects after an 8hr exposure to 75% N_2O on 4 different days during gestation, however it should be noted that in the same study the results from animals exposed for 24hr failed to reproduce the effects reported by other authors in animals exposed over a 24hr period. A study in mice using 4hr exposure periods to N_2O at concentrations up to 50%, from day 6 to 15 of pregnancy ***(Mazze et al., 1982)*** has shown no adverse effects on the foetus. A second mouse study ***(Mazze et al., 1986)*** showed that 75% N_2O exposure 6hr/day for 3 day periods during pregnancy increased the incidence of resorptions when given between days 14 and 16 but showed no adverse effects in groups exposed between days 11-13 or 8-10.

Exposure levels as low as 1,000ppm (0.1%) throughout pregnancy in the rat, have also been associated with reproductive effects such as an increased incidence in minor anomalies in the foetus, an increased rate of foetal loss and impaired post-natal development ***(Vieira et al., 1980; Vieira et al., 1983; Corbett et al., 1973)***. Again, both timing and duration of exposure affect the severity of the effect seen ***(Vieira et al., 1978)***.

Since the potential for adverse effects on the foetus in rats has been well characterised by numerous studies it is reassuring to find that there is no evidence of these effects at exposures of 500ppm (0.05%) or lower, even when administered throughout gestation ***(Vieira et al., 1980; Vieira et al., 1983)***. Since exposures between 500 and 1,000pppm have not been specifically included in test programmes the NOAEL for reproductive effects must be concluded to be 500ppm, but probably lies between 500 and 1,000ppm. This conclusion is consistent with the view of UK COT (COT review, 1995)

Limited studies in mice and hamster ***(Mazze et al., 1982; Mazze et al., 1986; Shah et al., 1979)*** add little to the information available from the rat studies, apart from confirming that the effects seen are not rat-specific. The relationship of the effects in other species with exposure provides some evidence for similar sensitivity in all species tested although the exposure regimes are not easily compared.

Investigations of foetal effects *in vitro* ***(Baden & Fujinaga, 1991; Fujinaga et al, 1988)*** have demonstrated that such models can predict a similar outcome as *in vivo* studies at high exposures but these have not been extended to explore low exposure effects.

The mechanism of the teratogenic and foetotoxic effects of N_2O has been the subject of investigation but not systematically, thus the picture is incomplete. The impact of the inactivation of MS activity on DNA synthesis and on cell division has been recorded in various contexts. While some authors consider the mechanism of effect of N_2O on the developing foetus to be predominantly a result of this inhibition of the methionine synthase pathway, other factors may also play a part at higher exposures ***(Mazze et al., 1988; Fujinaga et al., 1991; Fujinaga et al, 1987; Hansen et al., 1993)***. At these higher exposures it is possible that other physiological changes may supplement the metabolic effects however the involvement of MS is demonstrated by the benefits of folate and methionine supplementation in N_2O-exposed rats ***(Keeling et al., 1986; Mazze et al., 1988; Fujinaga & Baden, 1994)***. The possibility that fertility may be affected by the effect of N_2O on LH levels was raised by the studies of ***Kugel et al., (1990)*** but has not been explored further, either in animals or in clinical studies.

7.2.2 Teratogenicity & foetal toxicity - Detailed review

7.2.2.1 Rat

7.2.2.1.1 Low-dose exposure - in the region of 0.1% (1,000ppm)

Corbett et al (1973) exposed small groups of pregnant rats to N_2O at 0, 100, 1,000 or 15,000ppm for 8 or 24 hr/day for 3-9 days between days 8 and 19 of gestation:

Rats exposed to N_2O at 1,000ppm or 15,000ppm for 24 hr/day and two groups exposed to 1,000ppm for 8hr/day had an increased rate of foetal death compared with all controls (Table 6.1). These results show consistently higher levels of foetal loss at exposure levels of 1,000ppm. The variation present between groups exposed on the same days of gestation to the same level of N_2O suggests that the differences seen may not all be due to the exposures, thus this study has limited value in establishing no-effect-levels.

Group	No. rats	No. implant-ations	N_2O (ppm)	Duration (hr/day)	Gestation days	Implant-ations /rat	Foetal death-rate
1	12	72	15,000	24	8-13	6.0*	11.1*
2	10	112	0	24	8-13	11.2	1.8
3	6	53	1,000	24	12-19	8.8*	18.9*
4	9	100	0	24	12-19	11.1	4.0
5	10	109	1,000	8	10-13	10.9	18.4**
6	9	97	100	8	10-13	10.8	15.5*‡
7	7	76	1,000	8	14-19	10.9	14.5*
8	10	99	100	8	14-19	9.9	6.1
9	7	82	1,000	8	10-19	11.6	7.3
10	7	80	100	8	10-19	11.4	5.0
11	11	112	0			10.2	5.4

Results marked with an asterisk are significantly different from control:

** P<0.05; **P<0.01*

‡ Significantly different only from Control group 2

Table 6.1

The reservations from the above study are reinforced by the results of a study reported by Pope et al (1978) which included small groups (7-10) of pregnant rats exposed to 0%, 1.0%, 10.0% and 50% N_2O for 8 hours per day for the whole gestation period.

Dams were killed at day 21 and uterine contents examined. Exposure resulted in decreased foetal weight at the highest two exposures and decreased placental weight at all exposures. Foetal loss was reported to be slightly greater than one set of controls at the two lowest exposures of N_2O but not at the highest level; however there is also comment that the levels do not differ from the general background. Since full data and analysis are not presented further conclusions are not possible, but a slight effect on foetal survival at the lowest exposure would be consistent with other studies.

However the study of groups of 12 pregnant Wistar rats exposed continually from the day after mating until day 19 of gestation to N_2O in air at concentrations of 0, 250, 500 and 1,000ppm (Vieira *et al.*, 1980) did reveal some effects at the 1,000ppm level:

The rats were killed at 19 days and the uterine contents examined. The mean litter size and crown-rump length of the 1000ppm group were significantly reduced compared with controls; other exposure levels showed no difference from control in these respects. The 1000ppm group also had 4 resorptions compared with zero in the control and lower dose groups. Soft part abnormalities were not reported for any group and skeletal abnormalities were only seen in the group exposed to 1000ppm N_2O

N_2O (ppm)	**Litter Size**			**Crown-rump measurements (mm)**			**Resorptions**	
	n	range	Mean ± SD	n	range	Mean ± SD	n	No.
0 (Control)	12	9-13	11.3 ± 1.4	120	32-50	44 ± 1.4	120	0
1,000	12	3-9	6.3 ± 4.0***	66	30-45	35 ± 1.6*	66	4***
500	12	9-13	11.0 ± 1.4	118	32-50	43 ± 1.3	118	0
250	12	8-13	11.3 ± 1.3	120	32-50	43 ± 1.4	120	0

- **Results marked with an asterisk are significantly different from control: * P<0.05; **P<0.01; ***P<0.001**

Table 6.2. Measurements on foetuses exposed to low concentration of N_2O (Vieira et al., 1980)

At higher exposures to N_2O the same group identified foetal abnormalities (Vieira, 1979):

12 pregnant wistar rats were exposed to a constant level of 0.5% N_2O (5,000ppm) throughout pregnancy, compared with a similar group exposed to air alone. Dams were killed on day 19 and uterine contents examined. Increased foetal loss and resorption was seen in the N_2O treated group. Foetal weight and crown-rump length were also decreased in N_2O exposed foetuses. Skeletal abnormalities were not seen in the controls but were observed in 9% of the foetuses exposed to N_2O. These were not described in detail although it is commented that the incidence was greater in females than males. No abnormalities of the internal organs were recorded.

A comparison of the effects of intermittent exposure with these earlier results, which followed continuous exposure, is reported by Vieira *et al.* (1983) and demonstrates significantly less effects than seen after continuous exposure to similar levels:

Groups of 12 pregnant wistar rats were exposed for 6 hours per day 5 days per week to 0, 250, 500, 1,000, and 5,000ppm N_2O in air throughout gestation Dams were killed on day 19 of gestation and the uterine contents examined. Litter size and mean crown-rump length of the foetus was reduced in the group exposed to 0.5% N_2O. There were no malformations or resorptions in any of the groups.

N_2O (ppm)	Litter Size			Crown-rump measurements (mm)		Body weight	
	No. foetuses	range	Mean ± SD	range	Mean ± SD	range	Mean ± SD.
0 (Control)	120	9-13	11.3 ± 1.4	32-50	44 ± 1.4	2.0-3.5	2.6 ± 1.4
5,000	98	6-10	7.0 ± 2.3***	29-50	38 ± 1.2***	2.0-3.1	2.2 ± 1.2
1,000	117	8-13	10.0 ± 1.2	30-50	40 ± 1.3	2.0-3.2	2.4 ± 1.2
500	117	8-13	11.0 ± 1.3	31-50	42 ± 1.2	2.0-3.2	2.5 ± 1.3
250	119	9-13	11.2 ± 1.3	32-50	42 ± 1.4	2.0-3.5	2.6 ± 1.4

*Results marked with an asterisk are significantly different from control: ***P<0.001*

The effects of N_2O exposure during different periods of gestation on pup development was explored by Vieira *et al.* (1978):

Groups of 8 pregnant rats were exposed to air containing N_2O at 1% (10,000ppm) for 6 hours per day 5 days per week for week 1 of gestation, the first two weeks of gestation or for the whole 3 weeks of gestation. All rats were allowed to give birth to their litters and litters were monitored for 8 weeks post-partum. The litter size of all the groups exposed to N_2O was significantly lower than control and body weight of those pups was lower than that of controls throughout the 8-week post-partum monitoring. Pups from mothers exposed during the first week of pregnancy only were lighter than all others on all occasions. Tail length and body length were reduced for all N_2O treated groups throughout the 8-week post-partum period, compared with controls, although the differences were not always statistically significant.

7.2.2.1.2 Single 24-hour exposure – high exposure (>5%; 50,000ppm)

The potential teratogenicity of N_2O was demonstrated in one of the earliest studies of reproductive effects of N_2O (Fink et al, 1967):

Rats at day 8 of pregnancy were exposed to a concentration of between 45 and 50% N_2O for 24 hours per day for 2 4 or 6 days Although the number of rats in each group was very small there was evidence of an increased resorption rate after 2 days which was increased by the longer exposures. A similar pattern was seen with skeletal malformations. The authors speculate about possible sex differences in the effects since the litters from treated animals had a significantly lower number of males compared with controls. Overall the study is too limited to conclude any detailed results but the effect of N_2O on resorptions and abnormalities is clearly demonstrated.

Exploring the importance of timing of exposure during gestation groups of 20 pregnant rats were exposed for 24 hours to 60% N_2O on day 6, 7, 8, 9, 10, 11, or 12. A group of 30 unexposed rats acted as controls (Fujinaga *et al.*, 1989):

All rats were killed at day 20 and the uterine contents examined in detail. Each live foetus was examined for abnormalities and equal numbers assigned for skeletal and soft-part examinations. 15 of the 140 rats from the treated group died during exposure. The study demonstrates a wide range of abnormalities resulting from N_2O exposure but with a peak incidence of rib and vertebral abnormalities following day 9 exposure. Foetal wastage results showed two peaks, one at day 8 which is taken to represent the occurrence of fatal teratological effects but a second at day 11 is more difficult to assign to a cause. The authors suggest that it may be linked to a failure of the switch to placental nutrition which normally occurs at that time.

Based on the assumption that day 9 was the critical time for teratogenic effects, Mazze et al (1984) report the results of four experiments involving the exposure of pregnant rats for 24 hours on day 9 of pregnancy to 0%, 0.75%. 7.5%, 25% or 75% N_2O.

In total the number of animals exposed was 160, 27, 25, 49 and 62 respectively. Results from different studies were pooled for analysis. Factors such as shipping while pregnant, stress of confinement in exposure systems and lack of food intake were studied, with no effect demonstrated on the outcome of exposure. The effects seen in rats after exposure to N_2O at 75% for 24hr on day 9 of gestation were increases in resorptions and major soft part and skeletal malformations (e.g. runts, cleft palate, exencephaly, occular malformations and limb deformities). Such effects did not occur at lower doses; however the incidence of minor skeletal variants, such as extra lumbar ribs, showed a dose-related increase from 7.5% N_2O upwards. Total minor variants (e.g. extra lumbar rib, delayed ossification, asymmetric sternum) were statistically significantly increased compared with controls even at the lowest exposure level. dU-suppression was measured in the bone marrow and foetuses of animals exposed to 25% N_2O, both immediately after exposure and 24 hours later; the results demonstrated significantly greater dU-suppression than in controls at both time-points although values were returning to normal at the latter point. The authors conclude a threshold for teratological effect of this exposure regime at between 26 and 75% N_2O, based on the absence of major effects in the 25% group. The presence of significant increases in the incidence of minor anomalies, even at the 0.75% exposure level, leaves this conclusion open to challenge.

Fujinaga et al (1990) studied rats exposed on day 8 of gestation:

Following up a suggestion that N_2O exposure affected the laterality of foetuses 36 pregnant rats were exposed to 75% N_2O for 24hr on day 8 of gestation and a further 34 rats were exposed to air alone at the same time 4 to 6 rats from each group were killed on days 11, 12, 13, 14, 15, 16, 18 and 20 and the uterine contents examined in detail Laterality of the foetuses regarding tail flexion, umbilical artery emergence and aortic arch curvature was recorded for all live foetuses. Mortality and resorptions increased dramatically in the N_2O exposed group at day 14. Altered laterality was detected in a high proportion of foetuses from N_2O treated dams on all occasions.

To test the hypothesis that the teratogenicity of N_2O was due solely to physiological effects of anaesthetics the effects of Xenon and N_2O were compared by Lane *et al.* (1980):

Four groups of 8 pregnant rats were exposed to 25% O_2, 70-75% N_2, 70-75% N_2O or 70-75% Xenon for 24 hours on day 9 of pregnancy. The animals were killed on day 20 and the uterine contents examined. Resorptions and number of abnormalities were increased only in the N_2O group. The authors concluded that the foetal effects of N_2O must therefore be due to specific chemical effects rather than general physiological effects which would be common to both anaesthetics.

Exploring the possibility that the effects of N_2O might be enhanced by another anaesthetic groups of at least 54 pregnant rats were implanted with osmotic mini-pumps delivering either saline or Fentanyl on day 7 of pregnancy (Mazze *et al.*, 1987):

On day 9 the rats were exposed to atmospheres containing 0, 35 or 50% nitrous oxide for 24 hours. On day 21 of pregnancy dams were killed and the uterine contents examined, including full skeletal or soft part examination of all live foetuses. There were significant numbers of deaths during nitrous oxide exposure in the groups given both anaesthetics. An increased rate of resorption and consequent reduced foetal numbers were seen in rats exposed to 50% N_2O but not at 35%. Minor skeletal abnormalities were more frequent than in controls in foetuses from the 50% N_2O group. Developmental skeletal variants were significantly increased in foetuses from the combined treatment groups at both levels of N_2O. Increases in these variants in groups given N_2O alone were not statistically significant.

7.2.2.1.3 High exposure (>5%) for more than 1 day

The effects of short periods of exposure to N_2O during pregnancy were studied by Ramazzotto *et al.* (1979).

Groups of 10 pregnant rats were exposed to 50% N_2O for 25 minutes daily for three consecutive days during gestation (9-11; 12-14; 15-17) and sacrificed either immediately after the last exposure(days 14 or 17) or at 20 days. A control group of 10 rats was sacrificed at the same time as the treated groups. The percentage of resorptions was increased compared with controls in all rats exposed between 12 and 14 days and slightly in those exposed between 15 and 17 days. No other differences were reported in this study although the foetuses were subject to only limited examination.

The effect of N_2O exposure during gestation was investigated by Tassinari *et al.* (1986):

Small groups of pregnant rats (5-7 animals) were exposed to 75% N_2O for either 8 hours per day between days 9-13 or 11-15 of pregnancy, or for 24 hours per day between days 11-15 or 16-20 of pregnancy. All dams were killed on day 21. Litter size and resorptions were not affected by any of the treatment regimes but 24 hr exposure did result in lower maternal and foetal body weights. No significant effects were identified on foetuses in respect of malformations, skeletal abnormalities or total brain and liver protein or DNA content.

The authors also report details of examinations carried out on pups which were reared from dams exposed to N_2O for 8 hr per day on days 14-15 of pregnancy. There appeared to be a very slight retardation of development in the N_2O exposed pups in the first 14 days of life otherwise no differences were seen.

7.2.2.2 Mice

Mazze *et al.* (1982, 1986) studied the effects of Nitrous oxide in mice:

Groups of between 34 and 24 pregnant mice were exposed to atmospheres containing 0%, 0.5%, 5.0% or 50% N_2O for 4 hours per day from day 6 to 15 of gestation. The dams were killed on day 18 and uterine contents examined. There was no difference between control and any of the treated groups in the incidence or occurrence of resorptions or foetal abnormalities.

Mazze et al (1986) report the results of exposure of groups of 18-29 pregnant mice to 75% N_2O or one of three other anaesthetics for 6 hours per day for a period of 3 days (either 14-16, 11-13 or 8-10). Dams were killed on day 21 and the uterine contents examined. There was some reduction of foetus weight with all anaesthetics following exposure at 14-16 days and with all apart from N_2O after the other exposure periods. Otherwise, the only effect observed was an increase in resorptions after exposure to N_2O over days 14-16. There was no increase in foetal abnormalities with any exposure to N_2O.

7.2.2.3 Hamster

As a confirmation that the effects seen in the rat were relevant to other species groups of 5 or 6 hamsters were exposed to 0%, 70%, 80%, 90% or 95% N_2O for 24 hours on day 7, 8, 9, 10 or 11 of pregnancy (Shah *et al.*, 1979):

All animals were killed at day 15 of gestation and the uterine contents examined. An increased incidence of resorptions was seen with 95% N_2O at day 7 and with 90% N_2O at days 10 and 11. In total 44 of 1109 foetuses from treated groups showed malformations while none were seen in controls. The incidence of malformations was highest in the higher exposure groups and in the animals exposed on days 9, 10 and 11 of pregnancy. Malformations included cleft palate, limb defects, gut herniation and generalised oedema. An additional group of hamsters exposed to 50% N_2O for 4 or 5 days from days 7 or 8 of gestation showed no malformations or increase in resorption rates.

7.2.2.4 In vitro

Some effort has been made to model the effects of N_2O using in vitro models but so far these have not added significantly to understanding of the effects:

Baden & Fujinaga (1991) studied the effects on the *in vitro* development of day 9 rat embryos of 24 hour exposure to 0%, 5%, 25% or 75% N_2O. Cultures were maintained for a further 24 hours without N_2O, after treatment. Embryos treated with 25% or 75% N_2O had smaller crown-rump length, somite number and protein content compared with controls. An increased incidence of abnormalities was seen in the 75% group and some effects at 25%. The group treated at 5% was not distinguishable from the control.

Fujinaga et al (1988) exposed 10-day rat embryos for approximately 22 hours to either a control atmosphere (75% N_2, 20% O_2 and 5% CO_2) or an atmosphere in which the N_2 was replaced by N_2O. There were no differences between the treatments in crown-rump length, number of somites and limb bud index. Mean DNA content was 20.5% lower in the N_2O-treated embryos than the controls while mean protein content was 9.3% lower. Seven embryos were abnormal in the N_2O treated group compared with none in the controls. Abnormalities included kinked tails, severe head malformations, and left-sided tails. While these results are indicative of a direct effect on the foetus, interfering with DNA synthesis the authors are reluctant to accept this as the only mechanism for N_2O teratogenicity.

In a brief note Fujinaga & Baden (1989) indicate that they have established a more robust in vitro teratology model using foetuses at 9 days. This model shows significant effects of 24–hour exposure to 50-75% N_2O on the incidence of malformations and on protein content of embryos.

7.2.2.5 Mechanism of teratogenicity and foetotoxicity

It is assumed that if the methionine synthase pathway is involved in the foetotoxicity of N_2O then supplementation with Folate might reduce the effects, thus supplementation studies have been conducted, demonstrating some beneficial effect:

> Groups of 6-10 pregnant Sprague-Dawley rats were given water or folinic acid by i.p. injection in the afternoon of day 8 of pregnancy and again 12 hours later (Keeling *et al.*, 1986). These rats were exposed either to air or 70-75% N_2O for 24hr immediately after the second injection. One group given water by injection was deprived of food for the 24hr exposure period. An additional group received no injection before exposure to air. On day 21 the dams were killed and the uterine contents examined. Levels of folinic acid were measured after exposure to air or N_2O and methyl folates and non-methyl folates were both slightly increased compared with

the unsupplemented group in both air and N_2O exposed rats. There were no significant differences between treated and control groups in the numbers of implants, live foetuses, or resorptions. Foetal weight was significantly reduced in both N_2O-exposed groups. In the absence of folinic acid the N_2O exposure resulted in a significantly increased incidence of major skeletal malformations. The incidence of these malformations in rats treated with folinic acid and exposed to N_2O was higher than that in controls but not statistically significantly different.

In a further study based on the same assumptions Mazze *et al.* (1988) investigated the effect of folinic acid supplementation or co-administration of 0.27% halothane on the teratogenic effects induced by exposure of pregnant rats to 75% N_2O for 24hr on day 8 of pregnancy. While folinic acid supplementation reduced only slightly the severity of some of the effects of N_2O the co-administration of halothane eliminated those effects completely. The authors conclude that this indicates that the teratogenic effects of N_2O may be due more to physiological effects on uterine blood supply rather than the inactivation of methionine synthase. The relevance of this result to lower levels of exposure of N_2O is uncertain.

Fujinaga et al (1987) have questioned whether methionine synthase inhibition was the sole causative factor in N_2O teratogenicity and compared the effects of another anaesthetic:

On day 8 of pregnancy groups of 30 rats were administered 0.35% isoflurane, 50%N_2O or a combination of 50%N_2O and 0.35% isoflurane for 24 hr. A control group of 40 rats were administered air at the same time. While the effect of the 50% N_2O treatment showed the expected increases in resorptions and foetal losses these effects were significantly reduced by the simultaneous administration of isoflurane. The same pattern was evident for soft-part abnormalities, however skeletal variants were increased compared with controls by both N_2O alone and by the combined exposure. The protective effects of co-administration of isoflurane leads the authors to question the inhibition of methionine synthase as the mechanism of N_2O teratogenicity and an inhibitory effect on uterine blood flow is cited as a possible alternative

Pursuing the idea that the effects of N_2O may be due to factors other than inactivation of methionine synthase groups of rats were exposed to air or 60% N_2O on day 8 of gestation after pre-treatment with Phenoxybenzamine at 0, 2.5, 5, 25, 50, 100, 250, 300, 500 or 3,000µg/kg (Fujinaga *et al.*, 1991). The pre-treatment with phenoxybenzamine at 5 or 50 µg/kg reduced the rate of some minor abnormalities and the overall foetal wastage including resorptions. The results are interpreted to indicate the involvement of α-1 adrenergic receptor stimulation as part of the mechanism for N_2O teratogenicity. Overall the results do indicate some benefit of the pre-treatment but not sufficient to regard this as a significant part of the overall mechanism.

Hansen *et al.* 1993 studied the effect of N_2O on maternal and foetal adenosylmethionine (AdoMet) and adenosylhomocysteine (AdoHcy)..

Groups of approximately 10 pregnant rats were exposed on day 10 of gestation to 0% or 50% N_2O for 24hr or for 22hr followed by an intraperitoneal injection of methionine and a further 2hr of N_2O exposure. Rats were sacrificed immediately after exposure and samples of maternal liver and foetuses were frozen on dry ice. Tissue samples were analysed for content of folates, methylated folates, ornithine decarboxylase (ODC), AdoMet and AdoHcy. Although the post-implantation losses were greater in the N_2O–exposed groups the difference was not statistically significant and methionine treatment had no effect on the frequency. N_2O exposure decreased total folate levels but not the percentage present as methylated folates and methionine injection did not alter this situation. There was no effect of N_2O exposure on maternal or foetal AdoMet or maternal AdoHcy but methionine injection did increase the maternal levels of both while maintaining the same ratio between them. ODC was not affected by N_2O exposure or methionine injection. Overall this study did not provide any mechanistic explanation for the foetotoxic effects of N_2O, due mainly to the failure of the protocol to reproduce previous observations.

Using 9-day rat embryos Fujinaga & Baden (1994) explored the preventive effects of an α_1 adrenergic antagonist (Prazosin), methionine and folinic acid on N_2O-induced teratogenic changes:

Foetuses were exposed to N_2O at 75% for 24 hours. The most marked effect of the different treatments was of methionine which almost totally eliminated all of the effects of N_2O on malformations, crown-rump-length and somite number. Other supplements made little difference to the pattern of changes induced by N_2O.

7.2.3 Teratogenicity & foetal toxicity - Clinical data

7.2.3.1 Overview

7.2.3.1.1 Exposure to anaesthetic concentrations during pregnancy

The use of anaesthetics during surgery in pregnancy and the subsequent effect on the developing foetus has been studied by several groups (Mazze & Kallan, 1989; Smith 1963; Shnider *et al.*, 1965; Brodsky *et al.*, 1980; Duncan *et al.*, 1986; Crawford & Lewis, 1986; Konieczko *et al.*, 1987) and although surgery is itself associated with some risk to the survival of the foetus there is no evidence that the exposure to anaesthetics, including N_2O has any effect on any aspect of development including abortion and development of congenital abnormalities.

7.2.3.1.2 Evidence for teratogenicity

Although two studies (Cohen *et al.*, 1980; Corbett *et al.*, 1974) indicated an increased rate of congenital abnormalities in the offspring of anaesthetists and dental assistants exposed to anaesthetics the studies suffered from major deficiencies of approach, being both based on retrospective questionnaire data, which almost certainly will have led to bias in the results. Other studies have not shown any indication of such an effect (Crawford & Lewis, 1986; Konieczko *et al.*, 1987; Mazze & Kallen, 1989; Cohen *et al.*, 1971)

7.2.3.1.3 Effects of occupational exposure

Occupational exposure to anaesthetics during pregnancy has been the subject of a large number of studies, summarised in Table 5.4. Some of these studies have provided an indication of a possible increased incidence of spontaneous abortion in populations which are exposed to anaesthetics as part of their occupation (Cohen *et al.*, 1971, 1980; Knill-Jones *et al.*, 1972; Rosenberg & Kirves, 1973; Knill-Jones *et al.*, 1975; Mirakhur *et al.*, 1975; Cohen *et al.*, 1980; Guirgis *et al.*, 1990; Saurel-Cubizolles *et al.*, 1994). Other studies have shown no such effects (Cohen, 1974; Pharoah *et al.*, 1977; Lauwerys *et al.*, 1981; Axelsson *et al.*, 1982; Heidam, 1984; Ericson & Kallen, 1985; McDonald *et al.*, 1986; McDonald *et al.*, 1988; Rowland *et al.*, 1995; Hemminki *et al.*, 1985; Johnson *et al.*, 1987). It should be noted that some of the latter studies may have claimed to have found differences but the confidence intervals on the data provide no reassurance that the difference is real. A meta-analysis of many of these studies claimed to show that overall there was a positive effect, but of necessity the inclusion of data and scoring of the value of each study is a subjective assessment which has some significant limitations. Unfortunately much of this data comes from retrospective questionnaire-based studies with no independent verification of data. Such studies are recognised widely as being subject to bias due to the self-selection of respondents and the inaccuracies of recall of the circumstances of traumatic events such as stillbirth, miscarriage or abortion. Over all the studies there is an indication of a trend towards increased foetal loss in women exposed to anaesthetics during pregnancy and this may be due to the bias described above, but may equally be a real difference but due to stress or other occupationally–related factors. The highest quality studies which have been identified (Hemminki *et al.*, 1985; Ericson & Kallen, 1985), which include some check on the accuracy of the data, do not support the association of foetal loss and anaesthetic exposure. In two studies where exposure was known to be limited to N_2O (Axelsson *et al.*, 1996; Heidam, 1984) no such association was found. A study by Bodin *et al.*, (1999) indicated a possible link between N_2O exposure of pregnant midwives and low birth weight however the data-set used was not adequate for firm conclusions to be drawn and the difference seen could easily have been the result of other factors known to affect birth-weight.

7.2.3.2 Teratogenicity & foetal toxicity - Detailed review

A review of data on the reproductive effects of N_2O prepared for the UK Committee on Toxicity (COT) in 1995 identified a number of investigations of the effects of surgery during pregnancy, none of which provide any evidence for an adverse effect of such procedures beyond the consequences of the surgery itself:

Smith (1963) reported on 67 women who had surgery during pregnancy out of 18,503 pregnancies recorded by a US naval hospital. The foetus was lost in 10 of these cases but no conclusions could be drawn. No foetal abnormalities were identified in the offspring of women who had surgery during pregnancy.

Shnider *et al.* (1965) report two sets of data. The first relates to 9073 pregnancies between 1959 and 1964 1.6% of whom had surgery during the pregnancy (47 in trimester 1, 58 in the second trimester and 42 in the third.). Birth defects occurred in 5.4% of the surgical group, comparable to 6% in the control group. Premature delivery occurred after surgery with a frequency of 6.3% following surgery in the first trimester, 8.62% if surgery occurred in the second trimester and 11.4% when it was performed in the last trimester. Perinatal mortality was higher than control at 7.5% compared with 2% and birth-weight < 250g was also more frequent (15.6% compared with 9.9%) but exclusion of those cases undergoing the Shirodkar procedure for suturing the cervix reduced the incidence of both of these findings to control levels.

The second set of data was based on all obstetric cases from 17 hospitals for one year (1961). The total number of pregnancies was 10,912 of whom 2.4% underwent surgery during pregnancy. Those undergoing the Shirodkar procedure had a much higher rate of premature mortality and low birth weight than controls. Those undergoing other surgical procedures, including appendectomy were not different from controls in the incidence of birth anomalies, premature mortality and low birth weight.

Brodsky *et al.* (1980) report the results of a postal questionnaire study on male dentists and dental assistants conducted between 1962 and 1978. 287 women were identified who had surgery during pregnancy. The analysis indicated significantly increased rates of spontaneous abortion, particularly in those undergoing surgery during the first trimester 8% vs 5.1% in controls. There is also a suggestion that there was a greater incidence of spontaneous abortion amongst those who had previous occupational exposure to anaesthetics and underwent surgery in the first trimester. No account seems to have been taken in these analyses, of the nature of the surgical procedure involved or any other factors which might also affect spontaneous abortion rates.

Duncan *et al.* (1986) used health insurance data to identify 2565 women undergoing incidental surgery during pregnancy in Manitoba Canada. Each of these cases was linked to a specifically matched control pregnancy not undergoing surgery. There was no difference between the two groups in the incidence of anomalies. There appeared to be an increased risk of spontaneous abortion following surgery using general anaesthesia, however this is clearly affected by the type of surgery (Obstetric/Gynaecological - RR 2.0 95% CI 1.10 -3.64) (Other sites – RR 1.54 95% CI 1.03 – 2.30) with some sites of surgery showing no difference from control (Abdominal - RR 1.38 95% CI 0.66 – 2.83).

Crawford & Lewis (1986) specifically investigated the effect of N_2O anaesthesia in pregnancy. Case reports for 433 surgical procedures carried out under general anaesthesia (involving at least 33% N_2O), in a UK maternity hospital during the first 2 trimesters of pregnancy over a 12-month period of 1968/9 were analysed. 375 of the procedures were for cervical cerclage, involving an average of 22 minutes exposure to anaesthetic. The incidence of congenital abnormalities, spontaneous abortion and low birth weight was the same as when this procedure was conducted using local anaesthetic. The 58 cases involving other surgical procedures required an average anaesthesia of 38 minutes and did not show any evidence for an adverse effect of the procedure on pregnancy outcome. Based on this limited data there is no evidence for an adverse effect of a brief exposure to anaesthetic during pregnancy on foetal fate.

Konieczko *et al.* (1987) report a review of the incidence of anaesthesia in women delivering and miscarrying abnormal babies at one UK hospital over the period 1982-1984. An additional group studied was all pregnancies during 1977 1984 resulting in a child with a neural tube defect. The data-set consisted of 53 children with neural-tube defects and 97 with other major congenital abnormalities. In only one case was the mother exposed to anaesthetic and this was administered after the critical period of development.

In a second investigation the same group reviewed the cases of 471 mothers, 72 of whom had received anaesthetic during the year before pregnancy. There was no evidence for any adverse effect on pregnancy outcome and 6 of these mothers who had anaesthesia during the pregnancy showed no unusual incidence of abnormality.

The consequence of anaesthetic exposure during pregnancy for pregnancy outcome was explored in a registry-based study of 5405 mothers who were subject to a non-obstetric operation with anaesthesia during pregnancy (Mazze & Kallen, 1989)

Within the cohort three subgroups were identified based on trimester of pregnancy. The data were examined for congenital abnormalities, still-born infants, infants dying within 7 days of birth and low birth-weight infants (2 categories: <1500g; <2500g). The incidence of abnormalities observed in infants in total was not different from that in the general population and was not affected by the trimester of operation. It is particularly important to note that the 2252 operations performed in the first trimester resulted in an almost identical number of abnormalities to that expected in a random sample of the population (44:42.6).

The number of still-born infants was not affected by operations. Birth-weight and 7-day survival were both significantly adversely affected by operations. Although there is no information about the duration of anaesthetic exposure the specific anaesthetics used in more than half the operations were recorded on the registry data. More than 98% of operations involved use of N_2O, but generally in conjunction with another agent.

The possibility of adverse effects on the child may be reduced by the slow transfer of N_2O via the placenta, at least in the later stages of pregnancy:

Karasawa et al. (2003) measured the concentration of N2O in maternal blood and in placental vessels immediately after Caesarean delivery involving N2O anaesthesia. Dividing the exposure into 3 categories, Short (2-9mins), medium (9- 14 mins) and long (14-50 mins) it was possible to show a significant difference in the maternal blood N2O concentrations as the levels increased with time. The umbilical artery and vein levels increased much more slowly.

The possibility of effects of occupational exposure to N_2O on foetal development has been the subject of several investigations. A study of birth-weight of children born to midwives is described by Bodin *et al.* (1999):

In further analysis of data from a questionnaire–based survey of Swedish midwives, reported elsewhere (Axelsson *et al.*, 1996), the effects of work pattern and N_2O exposure during pregnancy on birth weight and gestational age were explored:

The data-set used included supplementary data on the births from the Swedish birth registry. After exclusion of unsuitable information the data-set consisted of 1685 pregnancies from 1244 women and was analysed mainly for effects during the second trimester of pregnancy. Exposure to N_2O was only analysed on a yes/no basis. A supplementary analysis was conducted on 1125 pregnancies where the mother was still working in the second trimester of pregnancy.

Almost 50% of the mothers reported N_2O use and although this is not specified in the report this proportion was presumably higher in the supplementary analysis of mothers who continued to work as midwives into the 2nd trimester. In the midwife mothers who continued practising into the 2nd trimester use of N_2O was associated with a higher odds ratio for low birth-weight (OR 3.4 and 95% CI (0.9-13.4) and babies that were small for gestational age (OR 3.0 and 95% CI (1.2-7.2). There were only 43 cases of low birth-weight in the whole data set. It should be noted that the whole data set showed slightly higher birth weight than the Swedish national average.

Although there was an association between two variables studied (shift pattern and N_2O use) this was recognised and is considered in some detail, but still may confound the analysis.

Since the analysis of women who continued to work as midwives showed the most marked effects and this probably included more than 70% of women exposed to N_2O it is conceivable that all the variables might not have been taken into account, particularly the difference between different types of work at this stage of pregnancy. The study provides an indication of a small effect on foetal development in this group of midwives but the confounding factors, lack of quantification of exposure to N_2O and wide confidence intervals on the results provide no solid reason to consider N_2O exposure as being a causative factor in the differences seen.

A second endpoint suspected of association with N_2O exposure in the workplace is foetal loss or abortion. This specific endpoint has been examined in many studies. A meta-analysis of 19 studies was made by Boivin (1997):

Studies included in this meta-analysis are not generally cited separately in this review, although the original publications have been reviewed where it appeared that some additional information might be obtained. The details of the meta-analysis are shown in Table 6.4.

Author	Study type	Number		Exposed population	Unexposed population	Meta score	RR	95% CI
		Exp	Control					
- Cohen *et al.*, 1971	- Int	- 36	- 34	- Op. room nurses	- Other nurses	- 0	- 3.15	- 0.95 - 10.5
-	- Int	- 37	- 58	- Anaesthetists	- Other physician	- 1	- 3.66	- 1.54 - 8.67
- Knill-Jones *et al.*, 1972	- Q	- 737	- 2150	- Anaesthetists	- Other physician	- 3	- 1.24	- 1.03 -1.49
- Rosenberg & Kirves, 1973	- Q	- 257	- 150	- Anaesth. nurses	- Other nurses	- 3	- 1.72	- 1.03 – 2.86
- Cohen, 1974	- Q	- 468	- 138	Anaesthetists	- Non-exposed	- 4	- 1.07	- 0.67 – 1.72
-	- Q	- 1826	- 676	- Anaesth. nurses	- Non-exposed	- 3	- 1.18	- 0.95 – 1.47
-	- Q	- 2781	- 1533	- Op. room nurses	- Non-exposed	- 3	- 1.29	- 1.08 – 1.55
Knill-Jones *et al.*, 1975	Q	435	435	Op. room worker	Unspecified	3	2.71	1.73 – 4.24
Mirakhur *et al.*, 1975	Q	114	118	Anaesthetists	Unspecified	1	3.11	1.37 – 7.02
Pharoah *et al.*, 1977	Q	670	6337	Anaesthetists	Other physician	2	1.01	0.83 – 1.23
Cohen *et al.* 1980	Q	400	8184	Dental assist.	Dental assist.	5	2.35	2.07 – 2.65
Lauwerys *et al.*, 1981	Q	259	1651	Op. room Physic. & nurses	Other Physic. & nurses	1	1.35	0.87 – 2.1
Axelsson *et al.*, 1982	Q R	139	573	Hospital worker	Any other	3	1.19	0.67 – 2.12
Heidam, 1984	Q R	179	80	Dental assist.	Dental assist.	6	0.9	0.4 – 2.1
	Q R	76	17	Dental assist.	Dental assist.	6	0.3	0.0 – 1.8
Ericson & Kallen, 1985	R	1436	1495	Op. room nurses	Other nurses	2	1.04	0.78 – 1.39
McDonald *et al.*, 1986	Int	45	30919	Op. room nurses	Any other	4	0.29	0.01 – 16
McDonald *et al.*, 1988	Int	70	22543	Health workers	Any other	4	1.07	0.67 – 1.72

Guirgis *et al.*, 1990	Q	4659	2113	Hospital workers	Hospital workers	5	1.98	1.53 – 2.56
Saurel-Cubizolles *et al.*, 1994	Int	284	480	Op. room nurses	Other nurses	5	1.9	1.1 – 3.5
Rowland *et al.*, 1995	Q	356	684	Dental assist.	Dental assist.	5	1.0	0.8 – 1.1
Hemminki *et al.*, 1985	CC	169	469	Nurses	Nurses	4	1.2	0.7 – 2.4
Johnson *et al.*, 1987	CC	?	?	Veterinarians	Veterinarians	4	2.86	0.86 – 9.53

Q = Questionnaire study; Int = Interview study; R = Registry study; CC = Case-control study

Table 6.4. Meta-analysis of studies investigating relationship between occupation and spontaneous abortion. (Boivin, 1997)

Differences have been seen frequently between the populations studied; however the true cause of those differences is uncertain, since the populations are not sufficiently matched to exclude many causative factors including those known to influence abortion rates, such as the physical demands of the work. None of the studies are constructed so as to allow any assessment of the role of anaesthetics in the results seen; in fact the exposure to anaesthetics is poorly reported for most of these studies. Unfortunately most of the studies are questionnaire studies which are suspected to generate reporting bias (Ericson & Kallen, 1979) as the respondents are self-selecting to some extent and the data they provide is generally not independently checked for accuracy.

The meta-analysis of the data-sets shown above provides an overall relative risk estimate of 1.48 (95% CI 1.4–1.58). Exclusion of studies with a global ranking < 3 increased the relative risk estimate to 1.57. When studies with scores of 5 or greater were considered separately the estimate was increased to 1.9. The consideration of confounding factors is taken into account in the global scoring assigned to each study; however in most cases this is quite limited, and the final allocation of the scoring may not be universally agreed. Although this meta-analysis provides an overall indication that there may be some causative factor(s) in the occupational experience of staff working in anaesthesia units it does not provide any further indicators of what such factor(s) might be.

All of the studies included in the meta-analysis, apart from the most recent were also considered by the UK COT, when reviewing the reproductive effects of N_2O in 1995. The conclusion reached by COT on the basis of this information was that there did appear to be an association between working in operating rooms and a slightly increased risk of spontaneous abortion, however they also indicated that such an increased risk "may reflect stress associated with the job rather than exposure to waste anaesthetic gases"

There is no basis, in any of the studies, for considering anaesthetic exposure to be the causative factor in this difference. Two studies, described in more detail below, have looked specifically at the question of N_2O exposure and have concluded that there is no effect of N_2O exposure on abortion rate:

Axelsson *et al.* (1996) reported an analysis of data collected from a survey by questionnaire of Swedish midwives. The analysis for was conducted on a data set of 1717 pregnancies which occurred during the time when the women worked as a midwife. The effects of work pattern and N_2O exposure on spontaneous abortion rate were considered and a full range of confounding variables was also taken into account as mentioned previously. While various aspects of working such as shifts and night work increased the rate of abortions the abortion rate was unaffected by N_2O exposure.

Heidam (1984), in the most highly rated study in the meta-analysis, describes a historical prospective study of the female population of a Danish county, investigating the spontaneous

> abortion rate in 10 different occupations, covering the entire reproductive life of participants up to 1980. One category studied was dental assistants of which 772 were identified and details sought by questionnaire (further details of procedure were described in a 2nd paper). The response rate was 94%. The odds ratio of spontaneous abortion for this category was 1.0, showing no difference from the controls, even when specifically analysed from questionnaire data for sub-groups exposed to mercury, solvents or N_2O.

One of the more recent studies in the meta-analysis was not previously reviewed by UK COT and is deserving of more detailed assessment:

> Rowland *et al.* (1995) reviewed 4,855 responses collected from a questionnaire survey of 7,000 dental assistants aged 18-39 yr. From these responses the incidence of abortion was analysed in a total of 1,465 respondents who had worked during the period of conception and pregnancy. The duration of exposure to anaesthetics for each was classified *post hoc* into one of 4 categories (0.5-2, 3-4, 5-9 or >10 hr/wk) and whether the facility used scavenging or not. An analysis excluding the *post hoc* categorisation of exposure showed no difference in risk levels for spontaneous abortion. Further analysis included adjustment for gestational week, age, smoking, and number of amalgams prepared each week.
>
> By breaking down the exposure into subgroups a higher risk was identified in women with between 3 and 9 hours of unscavenged anaesthetic exposure per week. Rates above and below this level were indistinguishable from control. The total number of assistants falling into the unscavenged category was 113 and of these only 64 had >3hr/wk exposure to anaesthetics. A group of 28 exposed to >10hr/wk had a risk level indistinguishable from control, although the spread was greater. The excess risk thus arose from a group of 36 women, thus is likely to be the difference between a small number of actual abortions for that group compared with an expected value of about 2.5. The report does not provide the actual numbers for any of the variables on which the final analysis is performed, simply quoting relative risk levels.
>
> In pursuit of an effect and ignoring the original lack of a dose-response relationship the authors decide to break exposure at the 3 hr point which still leaves a significant difference in the abortion rate for those with exposures >3hr. Various subsets of analysis have been included, all based upon this arbitrary cut-off point. The numbers and group sizes involved in the specific point of study are too small for this study to be useful despite the original database of 7,000 on which it is claimed to be based. The meta-analysis, described above, utilised the overall odds ratio from this study, which showed no difference between the groups. This would appear to be the most appropriate choice, given the limitations of the other analyses.

A further concern regarding occupational exposure to N_2O has been the possibility that exposure, particularly in early pregnancy, might lead to congenital abnormalities. Foetal loss and abnormality were analysed in a registry-based investigation of births, abortions and abnormalities among nurses working in Swedish operating rooms between 1973 and 1978 (Ericson & Kallen, 1985):

> Since all data analysed in this study are obtained from official registry information the opportunity for reporting bias is eliminated in this study. Other confounding factors are identified and taken into account where possible. Although anaesthetic gas exposure is assumed there is no quantitation of that exposure. A total of 1323 births, 87 spontaneous abortions and 6 legal abortions occurred in the operating room group compared with 1382 births, 85 spontaneous abortions and 28 legal abortions in a group working in medical wards. There were no differences between the groups, apart from a slightly lower rate of malformations in the operating room group, considered to be a chance variation. The 25 infants who died perinatally or had severe malformations were compared with 2 controls each born the same year to a similar mother by gathering more detailed questionnaire information from the mothers. This analysis revealed no differences of relevance, but mothers who stopped work

before week 28 had a higher level of neonatal malformations than those who did not; a difference which cannot be attributed to any occupational factors.

The occurrence of congenital abnormalities in children born to mothers occupationally exposed to anaesthetics has also been investigated in the studies described below:

Corbett *et al.* (1974) sent a questionnaire to 625 registered female anaesthetists in Michigan, USA. Of 525 replies 268 had delivered at least 1 child and there were a total of 695 births. The mothers were categorised according to practice of anaesthesia during pregnancy or not. There is no mention of considering other risk factors in the questionnaire. On a simple analysis 62.4% of the mothers practiced anaesthesia at some time during pregnancy and 16.4% of the children born to this group had birth defects compared with only 5.7% for those who did not practice anaesthesia.

The results are analysed in more detail for each type of birth defect showing the major differences between the study populations to be on the incidence of skin and musculoskeletal abnormalities.

Cohen *et al.* (1980) summarised a range of conclusions from a survey by questionnaire of 30,650 dentist and 30,547 dental assistants.

The survey was based on responses to a postcard sent to all US dentists enquiring about use of anaesthetics. Based on the response to this dentists were selected for further survey with approximately equal numbers of anaesthetic users and non-users. Each dentist was also asked to supply data for their dental assistants. Responses were received from 22,555 dentists and 21,390 assistants with the majority of dentists being male and assistants being female. Rates of congenital abnormalities and spontaneous abortion were higher in the anaesthetic users than in the non-users. Such a result would not be unexpected when the objective of the survey would have been obvious to all participants and those who had experienced problems with miscarriage or congenital abnormalities would naturally be more inclined to respond. The results are reported as means ± SE whereas the relative risk and confidence intervals on that risk would have been more informative. Generally despite the large numbers of individuals questioned this study cannot be regarded as providing even indicative information regarding the risk rates of reproductive effects due to the unreliable methodology employed. The most likely outcome of such a study would be reinforcement of preconceptions and this is almost exactly what it has delivered.

An earlier study was conducted by Cohen *et al.* (1971) by personal interview of 67 operating room and 92 general duty nurses and in a second stage by questionnaire to a group of 131 women including 50 female anaesthesia specialists. The age range of both groups was 25-50.

In the first part of the study the coincidence of pregnancy and work in anaesthesia was not well controlled but the operating room group averaged 78% full-time employment in that activity over the 5 years of the study (1966-1970). The miscarriage rate in the operating room nurses was 29.7% of pregnancies compared with 8.8% in other nurses.

For the second phase of the study, the births during the period 1965-1970 were considered and there was a 37.8% miscarriage rate in the anaesthetists group compared with 10.3% in the controls. The rate of congenital abnormalities was slightly lower in the anaesthetists group than in the controls (2% vs 4.2%).

The numbers involved in both stages of this study and the control of additional variables is very limited (e.g. the mean age of the operating theatre nurse was 3.4 yr greater than their control group at 34.3yr; the mean age of anaesthetists in the second phase was 39.6yr compared with 36.8yr for the controls).While there appears to be a consistent difference between the groups

in this study the selection and characterisation are inadequate to permit any conclusions concerning the cause of those differences.

The investigations of Crawford & Lewis (1986), Konieczko *et al.* (1987) and Mazze & Kallen (1989), described earlier, found no association between the incidence of congenital abnormalities and limited N_2O exposure due to surgery during pregnancy.

7.3 Behavioural effects

7.3.1 Behavioural effects - Non-clinical data

7.3.1.1 Overview

Exposure of pregnant rats to N_2O at up to 10,000ppm throughout gestation does not affect the subsequent behavioural development of the offspring (Holson *et al.*, 1995) whereas 50,000ppm over a more limited period (Rice, 1990) may have a very slight effect on subsequent behaviour, as measured by the startle reflex.

Limited exposure of mice and rats to 75% N_2O on days 14 and/or 15 of gestation was associated with small differences from control in reaching developmental milestones and in behaviour Mullenix *et al.*, 1986; Koeter & Rodier, 1986).

7.3.1.2 Behavioural effects - Detailed non-clinical review

Potential of N2O exposure to affect the behaviour of offspring of dams exposed during pregnancy has been investigated in 4 separate studies:

Holson *et al.* (1995) describe the effects of exposure of groups of 12 pregnant rats to 0%, 0.1%, 0.5% or 1.0% N_2O in air for 6hr/day for 20 consecutive days from Day 1 of gestation. Dams were allowed to deliver their litters and the behaviour of the litters was assessed at specific points up to 100 days after birth, using eight different parameters (Negative geotaxis, developmental activity, auditory startle, amphetamine challenge, 23-h activity, barbiturate anaesthesia duration, complex maze and passive avoidance response). Although some differences were seen between the responses of the groups the results are interpreted by the authors as likely to have arisen by chance. Paternally mediated effects were studied in offspring of groups of 12 males exposed to the same concentration of N_2O for 6hr/day 5 days/week for 9 weeks prior to mating. Each male was mated with 2 females. Again no effects of treatment were identified.

To investigate post-natal effects litters from pregnant mice exposed to 0%, 5%, 15% or 35 N_2O *in utero* from days 6-15 of gestation were subject to examinations for growth and performance up to 95 days after birth (Rice, 1990). Examinations were conducted on 10 litters from each treatment (Litters were culled at day 1 to eight animals and roughly equal sex distribution). No differences were found between the groups in any reproductive indices or offspring survival. Body weight was claimed by the author to show some differences both at pre- and post-weaning although the graphs provided do not indicate any consistent treatment-related effects. Using pinna detachment, incisor eruption and eye-opening as indicators there was no evidence of any effect of treatment on development. Rotating rod trials at 49 days after birth showed no treatment–related effects. The startle reflex was tested, using both acoustic and tactile stimuli on days 60 and 95 after birth, in two mice of each sex from each litter. On day 60 the N_2O groups all appeared to be less responsive than controls with the difference most marked in the lowest exposure group; however the differences were not statistically significant. On day 95 the amplitude of response of N_2O-exposed groups was again lower than that of controls but on this occasion the differences were statistically significant although there was no dose-relationship in the N_2O-exposed groups. It is noteworthy that the base-line amplitude for control response

showed a bigger difference between the two test days than any difference between the experimental groups.

Seeking to identify any behavioural effects of N_2O in the offspring 15 pregnant rats were exposed to 75% N_2O for 8hr on day 15 of gestation. A further 5 pregnant rats were exposed to a similar atmosphere for 8hr on 2 consecutive days (days 14 and 15 of gestation), while a control group were exposed only to air over this same 2 day period (Mullenix *et al.*, 1986). All rats were allowed to deliver their litters which were culled to 4 pups of each sex at birth. Pups were monitored for growth and tested in a residential maze at 1 and 5 months of age. Body weight of pups was not adversely affected by N_2O treatment. It is concluded that N_2O exposure particularly for 2 days resulted in hyperactivity in the offspring detectable more clearly in females and at 5 months after birth. Hyperactivity describes only a greater frequency of normal activities such as turning, smelling and rearing, rather than any abnormal behaviour.

Koeter & Rodier, (1986) exposed pregnant mice to Nitrogen/oxygen (75/25), N_2O/Oxygen (75/25) or halothane for 6hr on gestation day 14. Additional litters from control animals were exposed to the same atmospheres on post natal day 2 for 4 hr. Litters were examined for developmental and behavioural changes for 24 days after birth. Ear-opening was delayed in all treated groups at day 5 after birth and eye-opening was delayed on day 15 for litters exposed to N_2O after birth. There was evidence for delayed locomotory coordination in all N_2O–exposed litters as well as a general decrease in activity over all periods of measurement.

7.3.2 Behavioural effects - Clinical data

7.3.2.1 Overview

A number of clinical reports (Ou *et al.,* 2001; Hay ,1978; Su *et al.,* 2002; Wang *et al.,* 1994) describe observations on mothers and new-born when N_2O is used as an analgesic during labour. Overall these do not indicate any adverse effect of N_2O use but there is an indication from one study (Palahniuk et al., 1977) comparing 3 anaesthetics that there may be some slight effects during the 24hr postnatal period.

7.3.2.2 Behavioural effects - Detailed clinical review

Several studies have reviewed the effects of N_2O during delivery including the APGAR score for the new-born child The APGAR score assesses Activity, Pulse, Grimace, Appearance and Respiration thus is a very basic measure of well-being.

Ou *et al.* (2001) working in a Chinese hospital, compared 100 normal births with N_2O with 100 normal births in which analgesia was not used. The APGAR scores for infants were similar in both groups and no other adverse effects were reported.

Hay (1978) reported a positive beneficial relationship between APGAR score and the achieved N_2O concentration in the umbilical vein compared with maternal blood. This observation was however based on only 14 births

Palahniuk et al. (1977) compared the effects of methoxyflurane, N_2O and epidural injection as anaesthetic in 37 caesarean deliveries in a Canadian hospital. No difference was seen in APGAR scores between the groups. However, more extensive neurophysiological testing of the newborn at 6 and 24 hr. showed a reduced level of muscle tone and alertness and a tendency towards reduced general performance in the N_2O-exposed group, compared with other anaesthetics used.

Su *et al.* (2002) compared 658 births in which N_2O analgesia was used with 642 in which no analgesia was utilised during an 8-month period in a Chinese obstetric unit. The total labour time was similar in both groups but the conversion to a caesarean delivery was greater in the control group. Staining of amniotic fluid and foetal distress were assessed to be similar in both groups. Neonatal weight was also similar. Blood analyses performed on similar numbers from both groups showed no differences. Side effects reported in the N_2O group such as dizziness, drowsiness, numbness around the lips and tickling in the throat were not reported in the control group. Nausea levels were similar in both groups.

Wang *et al.* (1994) compared 34 N_2O assisted births in a Chinese hospital with the remainder of 305 births over a 3-month period. No differences were seen for caesarean rate, duration of labour, neonatal suffocation or post-partum haemorrhage. Only one case of vomiting was observed in the experimental group otherwise no adverse effects of N_2O use were seen.

7.4 Fertility effects

7.4.1 Fertility effects - Non-clinical data

7.4.1.1 Overview

Histological changes typified by depletion of spermatogenic cells, have been identified in the testes of rats following continuous exposure to 20% N_2O for up to 35 days (Kripke *et al.*, 1976). Similar effects were not seen in mice exposed for 14 weeks for 4hr/day to levels of N_2O up to 50% (Mazze *et al.*, 1983). This is in contrast to effects seen in rats exposed to 0.5% N_2O for 6hr/day for 30 days which showed some evidence of reduced fertility in one study (Vieira *et al.*, 1983) but only a very slight and non-significant difference in another (Holson *et al.*, 1995). 5days exposure to 80% N_2O for 4hr/day did not cause any morphological changes in sperm (Land *et al.*, 1981)

Inactivation of Methionine Synthase (MS) has been demonstrated in the testes of rats following exposure to 10% N_2O for one hour with recovery being complete within 24 hours of cessation of exposure (Brodsky *et al.*, 1984). The observed effects of exposure for longer durations and/or to higher levels of N_2O are all likely to be due to this effect on methionine synthase.

The lowest exposure level tested for fertility effects on the testes has been at 1,000ppm N_2O for 6 hr/day for 9 weeks and this may be considered to be a no-effect–level for these effects in the rat. The lack of effects at 1,000ppm would be more reassuring if this study had reproduced the positive result seen previously with 5,000ppm, however the differences seen may represent strain differences in response (Buckley & Brodsky, 1987).

Studies of female fertility have given mixed results. Webman 1980 studying rats exposed daily for 2hr/day for 30 days to 70% N_2O showed no effect on fertility of either sex when mated with unexposed animals or when exposed animals of both sexes were mated. No effect was seen in the histology of ovaries or testes after this exposure regime. In contrast Kugel (1990) found a significant reduction in fertility following only 4days of exposure to 30% N_2O for 8hr/day. These differences seem too great to allocate to strain difference although these may play some part. Taking account of all of the studies it does seem that where fertility effects arise from exposures greater than 10% N_2O the daily duration of exposure is more important than the total duration or exposure level. Intermittent exposure of up to 8hr per day 5 days per week, simulating the occupational situation, is associated with a much lower level of effects than constant exposure to the same N_2O concentrations. The assignment of the female fertility effects by Kugel *et al.* (1990) to effects of N_2O on Luteinising hormone releasing hormone (LHRH) needs further exploration since this mechanism has not been previously identified. The lack of any adverse effect of N_2O when used as an anaesthetic during a clinical procedure of assisted fertilisation provides considerable reassurance regarding fertility effects.

7.4.1.2 Fertility effects - Detailed non-clinical review

Effects of N_2O exposure on male fertility has been investigated in several studies:

Vieira *et al.*, (1983a) exposed groups of 12 male wistar rats to air or 0.5% N_2O for 6hr/day 5days/week for a total of 30 days. Immediately after the end of exposure and again 6 weeks later each rat was mated with 3 females. The females were allowed to produce their litters and pups were monitored for abnormalities and for size and weight for 8 weeks. The mean number of pups per litter was reduced from 12 (9-15) in the controls to 7 (2-14) in the litters derived from males exposed to N_2O immediately after the end of exposure. This effect was not present 6 weeks later. Body weight, body length and tail length were all reduced in the pups from N_2O treated males compared with controls and with the same males after 6 weeks of recovery.

Described as a Dominant Lethal study (Holson *et al.*, 1995) groups of approximately 15 male Crl : COBS CD (SD) BR outbred rats were exposed to 0%, 0.1%, 0.5% or 1.0% N_2O in air for 6 hr per day over a whole spermatogenic cycle (5 days per week for 9 weeks). At the end of the period the males were each housed with 2 females for 5 days. All females were killed 14 days later and the uterine contents examined. There was a dose-related trend towards increased resorption rate with increasing dose of N_2O, and a slight dose-related reduction in the number of live foetuses. The differences were not statistically significant.

N_2O (ppm)	No of implants	Live foetuses	Resorptions	Dead
0 (control)	15.6 ± 0.4	14.4 ± 0.4	1.1 ± 0.2	0.07 ± 0.05
1,000	15.6 ± 0.5	13.8 ± 0.6	1.2 ± 0.3	0
5,000	14.6 ± 0.5	13.3 ± 0.6	1.3 ± 0.3	0.04 ± 0.04
10,000	14.8 ± 0.5	13.0 ± 0.6	1.8 ± 0.5	0.03 ± 0.03

Table 6.5. Results of dominant lethal study in rats (Holson et al., 1995)

In a detailed study of testicular effects groups of approximately 45 male rats were exposed to 20% N2O, 20% O2 or 60% N2 for either 8hr/day or 24hr/day for 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 32 or 35 days (Kripke et al., 1976). Groups of 4-6 rats were killed immediately after the end of exposure and examined for macroscopic abnormalities. Tissues were preserved for histological examination and blood samples were assayed for testosterone. Results are not systematically analysed particularly in relation to the time course of changes, but the histology of the final effects on the testis is described in detail. N2O exposure affected only those cells which were dividing. Continuous exposure over 35 days resulted in tubules almost empty of spermatids and spermatozoa while intermittent exposure had milder effects typified by a reduction in the number of spermatogenic cells. Following 6 days without exposure there was clear evidence of recovery of spermatogenesis but in some animals this took longer. There was no effect on testosterone level which was consistent with the lack of any histological changes in interstitial cells.

Land *et al.* (1981) exposed groups of 5 male mice to one of 9 anaesthetics, including N_2O at 80%, for 4hr/day for 5 days. 28 days later sperm samples were examined for morphological abnormalities. The assay identified adverse effects of chloroform, trichloroethylene and enflurane but showed no effect of N_2O exposures.

Two studies have looked for histological effects of N_2O exposure relevant to fertility in both sexes:

To identify the mechanism of N_2O effects on the testis groups of 24 male Sprague-Dawley rats were exposed to 0%, 10% or 50% N_2O for 1 hour (Brodsky *et al.*, 1984):

> Six rats from each group were killed immediately after exposure and a further 6 from each group at 24, 48 and 72 hr following exposure. The right testis of each animal and a sample of liver were removed and assayed for methionine synthase (MS) activity. MS activity was reduced to 37 and 71% of control immediately after exposure to N_2O for the 50% and 10% groups respectively. Recovery was complete at 24-48hr for the 10% exposure and at 72hr for the 50% exposure. Methionine synthase activity in the liver, although higher than in the testis, followed a similar pattern of decrease and recovery.

Effects of N_2O on fertility of female rats has been less extensively investigated but was included in a study by Webman (1980):

> Groups of twenty-two 39-day old rats of each sex were exposed to 70% N_2O / 30% O_2 for 2 hours/day for 30 consecutive days. A control group of 12 rats of each sex was exposed to O_2 only over the same schedule. There was a further unexposed control group of 5 rats of each sex. Two rats of each sex from the N_2O group were killed at 5, 10, 20 or 30 days of treatment and the gonads removed and fixed in formalin. One rat of each sex from the same group was killed before the start of exposure to provide a histological baseline. After 30 days of exposure the rats were mated as follows:
>
> 7 pairs from N_2O exposed males and N_2O exposed females
> 6 pairs from N_2O exposed males and O_2 exposed females
> 6 pairs from O_2 exposed males and N_2O exposed females
> 6 pairs from O_2 exposed males O_2 exposed females
> 5 pairs from unexposed males and females
>
> Dams were allowed to give birth to their litters and pups were weighed, measured and examined for abnormalities. No effects on fertility, litter size or pups were found in this study. Histological examination of testes and ovaries revealed no evidence of adverse effects.

> To identify any adverse effects on the gonads of intermittent exposure to N_2O groups of 15 mature (13-14-week old) mice of each sex were exposed to room air, 0.5%, 5% or 50% N_2O for 4hr/day 5days/week for 14 weeks (Mazze *et al.*, 1983). After the last exposure mice were killed. Testes and ovaries were subject to detailed examination including, sperm morphology, oocyte counts, testis and ovary weight and histological examination of ovary and testis. No significant differences were found between the treatments in any of the endpoints examined.

In contrast to the results of the above studies Kugel *et al.* (1990) found significant effects of N_2O on fertility in female rats. It was also proposed that this involved a mechanism for N_2O induced infertility arising from disruption of the release of LHRH from the hypothalamus:

> 64 female Sprague-Dawley rats were checked daily for stage of oestrous and 32 were exposed to 30% N_2O for 8hr/day for 4 days. At the end of exposure 8 N_2O-exposed rats and 8 air-exposed controls were terminally anaesthetised and the brains perfuse-fixed *in situ*. Sections of brain were stained using an immunocytochemical technique to show LHRH within cells of the hypothalamus. Counts of cells with a positive staining result showed differences from control only if the exposure had begun on the morning of prooestrous. The difference was very marked, approximately 4 times control levels. A group of 12 rats exposed to N_2O in a similar regime and a similar group of controls were mated immediately after exposure with untreated males. Only 50% of the N_2O- exposed group produced litters compared with 100% of controls. The results indicate a possible mechanism whereby N_2O disrupts the normal oestrous cycle, leading to reduced fertility. Oestrous was disturbed in exposed females for approximately 3 weeks after the end of N2O exposure.

There has been remarkably little follow-up of this mechanism although a study of *in vitro* effects of N_2O exposure on gonadotrophin-releasing-hormone suggests that more work is essential (Zhang *et al.*, 2003). Additional work is needed to establish relevance of these findings to risk assessment and would need the development of data on dose response or possible thresholds of effect.

7.4.2 Fertility effects - Clinical data

7.4.2.1 Overview

The primary concern regarding fertility is the frequent low-level occupational exposure to N_2O.

For males only one study was found, but this provides some clinical reassurance that there is no effect on sperm morphology of mixed anaesthetic exposures, including N_2O at levels up to 300ppm, (Wyrobek, 1981).

Rowland (1993) reviewed questionnaire data from dental nurses and concluded that there was evidence for a longer time to pregnancy in those who had the highest levels of N_2O exposure. A similar conclusion was reached by Ahlborg et al (1996) in a questionnaire study of midwives. Both studies were based on questionnaire data and ultimately on very small numbers, despite large starting populations. They have potential for serious bias in the way that data were obtained. The final group size indicating a positive effect is very small in both studies (19 and 41, respectively) and thus very susceptible to bias by a self-selecting sub-group with preformed views on the cause of a traumatic experience for them.. The possibility that these results represent a real effect must be considered in relation to the available non-clinical data.

7.4.2.2 Fertility effects - Detailed clinical review

Male fertility has been investigated in a study of sperm morphology, with no evidence of any adverse effect in individuals regularly exposed to anaesthetics including N_2O.

Wyrobek *et al.* (1981) compared sperm morphology in samples from 46 anaesthesiologists and 26 controls. The controls were staff who were about to begin residency in anaesthesiology. Sperm samples were taken from each on one occasion and from the controls on two occasions; before the start of residency and after one year of working in operating theatres (13 only). The operating theatre atmospheres are characterised only in respect to N_2O values obtained on monitoring programmes (once weekly to once monthly) although other anaesthetics were in use. The exposures to N_2O are estimated to average 50ppm with a range of 5-300ppm. The mean age of the control group was slightly lower than that of the anaesthesiologists group (30.0±0.4 vs 27.9±0.5). No differences were found between the two groups and of those controls measured before and after exposure all but 3 had similar levels of abnormal sperm, 2 had lower levels after 12 months and 1 had higher levels.

Two questionnaire-based retrospective studies have explored the possible effects of anaesthetic exposure on female fertility with conclusions that some association does exist. The studies are both subject to many of the common problems of such data, in that a small group with concerns abut the effects of exposure can bias the outcome by overestimating their exposure levels and there is no independent verification of exposure data.

Rowland *et al.* (1992) report the results of a survey by questionnaire and telephone interview of a sample of 418 dental nurses who had been pregnant in the last 4 years. This sample was the result of an initial approach to 7,000 dental assistants. Since the individuals were likely to be aware of the purpose of the research when they responded it is impossible to rule out bias when the final population represented only 9% of the original target. The primary objective was

to obtain detailed information on the number of menstrual cycles which elapsed between first unprotected intercourse and pregnancy. The information obtained on time to pregnancy and N_2O exposure was based entirely on the account of the subject without independent verification. Covariates were used to adjust the fecundability ratios. These were oral contraceptive use, cigarettes smoked, age, history of pelvic inflammatory disease, frequency of intercourse and non-white race.

In the analysis of covariates it is given that 203 of the women were unexposed, 121 were exposed to scavenged N_2O and 60 were exposed to unscavenged N_2O. For 34 of the final sample some covariate information was missing, thus they were excluded from further analysis. The group exposed to unscavenged N_2O showed evidence of a difference in "fecundability" from the unexposed control as given in Table 4.5 below:

- N_2O exposure	- n	- Adjusted fecundability ratio (AFR)†	- Combined	- P
- **0-1hr/wk**	- **21**	- **0.94**	- **1.01 (0.73-1.39)**	- **0.53**
- **2-4 hr/wk**	- **20**	- **1.03**		
- **5-9 hr/wk**	- **9**	- **0.45**	- **0.41(0.23-0.74)**	- **0.003**
- **.>10 hr/wk**	- **10**	- **0.37**		
-				

Table 4.5 Effect of unscavenged N2O exposure on fecundability ratio of dental nurses (Rowland et al., 1992)

Assistants exposed to scavenged N_2O were not distinguishable from controls. Although the authors claim to have removed any bias due to self-selection by individuals who believe there was an association between fertility and exposure to N_2O five of the 19 in the high unscavenged group had experienced a spontaneous abortion and had associated that event with N_2O exposure. The categorisation of exposures was based on post hoc data analysis and is thus somewhat suspect in maximising any potential differences, particularly when the reporting of both exposure and consequences is from the same source, without independent verification. While the analysis provided does indicate a statistic worthy of further follow-up it cannot be regarded as a true indicator of effect due to the nature of the study and the opportunity for bias due to many factors including self-selection, particularly given the small numbers actually involved.

In a letter commenting on this study Gray (1993) points out that the incidence of foetal loss in the group exposed to unscavenged N_2O is statistically significant. The response of Rowland *et al.* (1993) indicates that they have found an overall statistically significant increased risk of spontaneous abortion in their study population with a relative risk of 3.1 (C.I. 1.5-6.2). They also comment that their data do not allow distinction between early foetal loss and spontaneous abortion which suggests that they are not directly measuring abortion rates in this analysis.

Ahlborg *et al.* (1996) report the results of a questionnaire-based survey of 3358 Swedish midwives. After excluding data for various reasons data for 972 women were analysed. The specific variable of study was the time to conception for the most recent pregnancy with an upper limit of 13 menstrual cycles for any respondent. Confounding variables taken into account included shift-work pattern, use of N_2O (gauged by number of N_2O-assisted deliveries per month), exercise, medication, smoking, alcohol coffee and tea intake and prior use of oral contraceptives. Actual exposure levels to N_2O were not measured but peak exposures at the time relevant to the study were estimated to be in excess of 500ppm. A total of 41 women within the study reported exposure to N_2O for more than 30 deliveries per month

The data showed a possible adverse effect on time to pregnancy due to age (Adjusted Fecundability ratio (AFR) and (95% CI) – 0.77 (0.64-0.94), full-time working (AFR and (95% CI) – 0.86 (0.74-0.99), shift–working (AFR and (95% CI) - 0.77 (0.51-0.98). and exposure to N_2O for more than 30 deliveries per month (AFR and (95% CI) - 0.63 (0.43-0.94). Effects of N_2O were said to be unaffected by reported use of scavenging equipment. Although there is an apparent difference in the ratios, they are not sufficiently different from controls to be convincing. In addition the reliability of questionnaire-based data may be seriously criticised due to selection and recall bias, thus the results obtained in this study can only provide some indication of potential adverse effects on fertility after exposure of pregnant women to unknown levels of N_2O. The cases involved would ideally require to be explored in much more detail to identify whether there are any additional contributory factors which have not so far been included in the analysis or whether there is a sub-group within the group involved in >30 deliveries per month, which is accounting for the apparent effects.

A study on use of anaesthetics during a procedure described as Gamete Intrafallopian Transfer Technique (GIFT) (Beilin *et al.*, 1999) provides a sensitive indicator of the effect of N_2O on fertility.

The procedure involves combining oocytes and motile sperm in the fallopian tube to encourage fertilisation and pregnancy. The procedure is generally conducted under anaesthesia. 455 such procedures were studied with an overall pregnancy rate of 35%. A range of anaesthetics (Propofol, N_2O, Isoflurane, Enflurane/desflurane, midizolam) were compared and there was no difference between any of the anaesthetics in the outcome.

7.4.3 Reproductive Toxicity References

7.4.3.1 Teratogenicity & Foetal toxicity

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8 Haematological and Immunological effects

8.1 Summary

Exposure of animals to N_2O at concentrations of up to 10,000ppm has not been associated with any effects on the haematological or immunological system. Clinical studies have not been sufficiently controlled to provide any insight into the possible haematological effects of occupational exposure. It would therefore appear that haematological and immunological effects are not a limiting toxicity for N_2O.

The studies conducted to investigate haematological effects in animals have identified that a marker of N_2O interaction with the methionine synthase pathway, deoxyuridine (dU) suppression, is altered compared with controls after 13 weeks continuous exposure at a level of 50ppm in mice. Although this difference is not statistically significant it is consistent with a trend seen at higher exposures but is inconsistent with the known kinetics of methionine synthase (MS) inhibition, which has been shown to be unaffected at exposures below 500ppm. Data from a limited study in dentists, where exposures were less directly quantifiable, indicate an effect on this marker at 8hr Time-Weighted Average (TWA)

exposures in excess of 1800ppm. Although the marker is affected this does not in itself represent a functionally adverse effect on the bone-marrow. However, the precise relationship between the marker and any adverse effects of N_2O exposure is not clearly defined.

There is evidence for an effect on the immune system in animals after 13wks exposure to 5,000ppm N_2O. This effect is characterised by reduced immunoglobulin levels and a reduced response to challenge with sheep red blood cells.

Severe haematological effects, such as megaloblastic erythropoiesis with bone marrow depletion, follow prolonged exposure to analgesic or anaesthetic levels of N_2O, often with reduced White Blood Cell (WBC) results. Since these effects are a direct result of the known interaction between N_2O and the methionine synthase pathway they tend to be more severe in individuals compromised by pre-existing conditions including Vitamin B_{12} (Vit B_{12}) and folate deficiency. Exposure for only 1 hour shows some evidence of these effects

8.2 Non-clinical data

8.2.1 Overview

While two earlier studies, at higher exposures, failed to show any effect of N_2O on white blood cells (Rice *et al.*, 1985; Cleaton-Jones *et al.*, 1977) mice exposed to 50, 500 or 5,000ppm N_2O for 13 weeks (Healy *et al.*, 1990) showed a dose-related increase in dU-suppression in the bone-marrow and a non dose-related reduction in White blood cells (WBC) in all treated groups. The difference in dU-suppression at the lowest exposure was not statistically different from the controls and although representing a continuation of the trend seen at higher doses may not be due to exposure to N_2O. dU-suppression is a marker of the effects of N_2O on MS, but as an indirect indicator does not constitute evidence of an adverse effect. Neither the exposure duration nor regimes of this latter study were significantly different from those showing no effects, thus the WBC result must be regarded with caution, especially as there was no relationship between the severity of effect and exposure. Since in most circumstances a toxic effect would be expected to be more severe with increasing dose it is possible that the observed differences represent an aberrant control result rather than an effect of treatment.

The dose-related dU-suppression results indicate an effect of N_2O on this marker even at 50ppm, although in isolation the difference from control at this low exposure was not statistically significant from controls.

With exposures of 80% N_2O there are significant effects on the WBC counts and on bone-marrow. Rats exposed to this level of N_2O for 6 days, have WBC depleted to 10% of control and with much of the mitotic activity eliminated from the bone marrow (Green & Eastwood, 1963).

8.2.2 Detailed review

Healy *et al.*, (1990) investigated the effects on mice of daily exposure to low concentrations of N_2O.

> Groups of 6 male mice were exposed to 0, 50, 500 or 5,000ppm N_2O for 6hr/day 5 days/week for 2 or 13 weeks. 24hr after the final exposure the animals were killed, blood and bone-marrow sampled and spleen, liver, kidney, thymus and adrenal gland were weighed. Among other measurements the function of spleen cells and lymphocyte cultures was tested using response to mitogens and sheep red blood cells; a dU suppression test was performed on the bone-marrow.
>
> Liver weights of all N_2O-exposed groups were lower than controls after 13 weeks. WBC counts were lower than those of controls for all N_2O-exposed groups after 13 weeks. Neither of these differences showed any dose-relationship. Tritiated thymidine uptake by splenic cells was higher than control values, without any mitogenic stimulus, in animals exposed to 500 and 5000ppm N_2O for 13-weeks, although the difference was only significant at the highest exposure. Response to sheep red blood cells was reduced in the highest exposure group after

13 weeks and this was confirmed by immunoglobulin measurements. dU-suppression was greater than controls in a dose-related manner in all groups exposed to N_2O for 13 weeks although the difference was only significant for the highest two exposure groups. A similar effect was not seen after 2 weeks.

N2O (ppm)	Normalised Deoxyuridine suppression (% of control)	
	2-week study	13-week study
0	100.0 ± 3.3	100.0 ± 18.1
50	111.3± 4.5	133.8± 15.8
500	110.4 ± 5.9	169.3 ± 17.0*
5000	111.3 ± 8.8	204.4 ± 5.9**

Results marked with an asterisk are significantly different from control: *P<0.05; **P<0.01

Table 7.1. Bone Marrow dU - suppression values for rats exposed to low levels of N_2O (Healy et al., 1990)

Effects on white blood cell counts were not seen in rats exposed to a slightly higher level of N_2O daily in a similar regime (Cleaton-Jones *et al.* ,1977):

> In an attempt to clarify the pattern of haematological changes in response to N_2O exposure a group of 30 rats was exposed to 1% N_2O for 6hr/day 5 days/week while a group of 13 rats were exposed to room air. 2 or 3 rats were taken at weekly intervals up to 6 weeks or monthly intervals from 1 to 6 months). Blood and bone-marrow samples were taken from each rat for examination. Over the first 6 weeks there was a higher Packed Cell Volume (PCV) and a lower reticulocyte count in N_2O-exposed rats. Overall there was a significant increase in PCV and Haemoglobin. There was no effect on white cell counts.

The differences between the two studies may owe something to the strain differences in sensitivity to the haematological effects of N_2O which were reported by Green (1968)

Rice *et al.* (1985) investigated the haematological effects of N_2O in mice at concentrations up to 50% for a slightly shorter daily exposure period and found no effects:

> Groups of 15 mice of both sexes were exposed to air, 0.5%, 5% or 50% N_2O in air for 4hr/day 5 days/week for 14 weeks. All animals were monitored throughout the study and blood taken at necropsy for haematology and serum chemistry examinations. Samples of liver were assayed for P-450 levels. There were no significant differences in body weight or liver and kidney weight at necropsy. Limited histological examination revealed no treatment-related differences. Haematological and biochemical investigations revealed no treatment-related differences.

Modelling the known human effects of chronic high exposure in rats Green & Eastwood (1963) found a typical pattern of bone-marrow depletion:

> The blood and bone-marrow of 25 rats exposed to 80% N_2O continuously for 2, 4 or 6 days was compared with that of 32 control animals exposed only to air. After 2 days the white blood cell count (WBC) of N_2O-exposed rats was lower than that of controls but not statistically significantly so. By 4 days the difference was significant and there was some depletion of granulocytes. After 6 days of exposure granulocytes were absent from the blood of N_2O treated rats and the WBC was less than 10% of the control value. The bone marrow of rats after 6 days exposure to 80% N_2O was hypoplastic with no reproduction of cells and mitoses absent. Megakaryocytes appear to be the only cell population unaffected.

Some evidence of a beneficial effect of N_2O exposure on immunosuppression is provided by a study of skin grafts:

> Kripke *et al.* (1988) exposed rats to air, 20% or 40% N_2O either for 48hr before or continuously after skin graft surgery. Exposure to N_2O significantly extended the time to

rejection of the transplant in a dose-related manner whether given before or after surgery. The effect of pre-treatment at 40% N_2O was slightly less than the effect of post-operative exposure.

A further study provided limited evidence for a lack of effect of N_2O exposure on normal immune function

Investigating the possible effect of N_2O on the immune system Cullen *et al.*, (1976) used peritoneal exudate cells (PEC) effective against a mouse ascites tumour with ^{51}Cr-labelled tumour cells. The PEC normally kill the tumour cells, releasing the ^{51}Cr, which can then be measured. PEC and tumour cells were incubated together in the presence of 80% N_2O for 4 hr. Although the cytotoxicity of PEC was lower after N_2O incubation than with air the difference was not statistically significant. Results with halothane in the same model system demonstrated significant reductions in cytotoxicity of PEC.

8.2.3 Clinical data

8.2.3.1 Overview

Exposure to levels of > 60% N_2O for even brief periods (approx 1 hr) may show effects on Red Cell folate levels even 1 month later, particularly those individuals who are marginally deficient at the time of exposure (Deleu *et al.*, 2000). Longer–term (up to 24hr) exposure to similar concentrations is associated with severe disruption of bone marrow with consequent changes in haematological values (Skacel *et al.*, 1983). These effects are all typically mediated through the known interaction of N_2O with Vit B_{12} and folate metabolism. dU suppression is increased by these exposures and only gradually returns to normal after 7 days. Even short periods of N_2O exposure in seriously ill patients can cause megaloblastic bone-marrow change and associated increased rate of dU-suppression (Amos *et al.*, 1982).

Due to the observation of these effects on bone-marrow and WBC and similar findings in animal studies, a study was conducted to investigate the effects of N_2O exposure during surgery on post operative infection and recovery. This study showed no adverse effect of N_2O exposure.

Levels of N_2O exposure relevant to assessing the risks of occupational exposure have been investigated in several studies (Bargellini *et al.*, 2001; Peric *et al.*, 1991; Karakaya *et al.*, 1992; Salo & Vapaavuori,, 1976) and particular attention has been paid to identifying any effects on lymphocyte sub-populations. The studies do not give a consistent picture but the exposures in all of the studies are to mixed anaesthetics, which may be a source of variation. Bargellini *et al.* (2001 found an effect on lymphocyte sub-populations in anaesthetists but when analysed specifically for correlations with N_2O exposure no effect was found. Salo & Vapaavuori (1976) found no effects of N_2O exposure up to 860ppm on haematological values of anaesthesiology staff. Karakaya *et al.* (1992) studied similar endpoints in anaesthetists but found only an effect on lymphocytes which was already known for halothane exposure. Peric *et al.* (1991, 1994) found a range of differences in haematological values of anaesthesiology staff working in a temporarily unscavenged operating theatre. TWA exposure to N_2O and halothane was said to be 1600ppm and 350ppm respectively with maximum levels of 10000ppm and 2244ppm. The effects seen cannot be specifically associated with any anaesthetic exposure but provide an indication of the maximum haematological consequences of exposures well in excess of current standards. Chronic exposure to levels of 1800ppm and above may be associated with Vit B_{12} inactivation, as indicated by increased rates of dU suppression, according to the results of a study of dentists (Sweeney *et al.*, 1985) however, the possibility that these effects, which occurred in only 3 individuals, were a result of abuse of N_2O at a much higher level cannot be ruled out.

8.2.3.2 Detailed Review

Analgesic or anaesthetic exposures to N_2O over prolonged periods have been shown to affect haematological parameters and individuals marginally deficient in Vit B_{12} or folate may have some additional vulnerability to such effects:

Skacel *et al.* (1983) report on 12 patients receiving 70% N_2O for between 4 and 36 hr during operation and post-operatively. All patients showed a rise in hypersegmented neutrophils on day 5 post-operation peaking at day 7-9 then declining to control levels. Marrow examination on 6 of those with prolonged exposure to N_2O showed grossly megaloblastic erythropoiesis after 24hr. The marrow went through a series of changes over the next few days and was still abnormal after 7 days. dU-suppression tests were abnormal in all who were exposed to N_2O for 24hr but had returned to normal by 7days after the exposure apart from one individual who was folate deficient at the time of operation and had an abnormal dU-suppression result before N_2O exposure.

51 elderly patients (mean age 59 yr) who received either 66% N_2O or Propofol anaesthesia for approximately 1 hour while undergoing ophthalmic surgery were screened haematologically before operation and again one month later (Deleu *et al.*, 2000). The N_2O-exposed group showed decreased Folate levels. While both groups showed decreased Haemoglobin, Haematocrit, and RBC the differences were greater for the N_2O-exposed group. Three individuals in the N_2O - exposed group had neurological symptoms, two of these had abnormal folate levels pre-operatively and all had abnormal levels after N_2O anaesthesia. Symptoms improved following folate therapy. Even in these individuals there was no evidence for neutrophil hypersegmentation on blood slides although the one month delay in post operative monitoring may have been too long to pick up such effects.

Seriously ill patients who had been administered N_2O prior to admission to an intensive care unit showed increased rates of dU-suppression and in the case of the most severely ill megaloblastic bone-marrow change also occurred:

Amos *et al.* (1982) report data on 70 consecutive patients admitted to the intensive care unit of one UK hospital, with serial observations made on 34 of these patients for up to 28 days. 22 patients (31%) had megaloblastic bone-marrow change on admission and of these 18 had been anesthetised with N_2O for at least 2 hr within the 24hr period prior to admission. 22 other patients had also been anaesthetised with N_2O for a mean of 3.1hr prior to admission but showed no megaloblastic change. The patients showing no bone-marrow change were generally less seriously ill on admission than those with such change. All patients who had been exposed to N_2O had increased rates of dU-suppression which was proportional to the duration of anaesthesia. Only 2 patients were clinically deficient in Vit B_{12} as indicated by serum levels. The follow-up of patients was severely complicated by the poor rate of survival (44%) but in survivors both the bone-marrow change and dU-suppression slowly returned to normal values, in some cases with the assistance of supplementary Vit B_{12} or folate.

Amess *et al.* (1978) provide an account of 22 patients having cardiac surgery 8 of whom received 50% N_2O for 24hr, 9 who received 50% N_2O for 5-12hr and 5 who did not receive N_2O at all. Haematological and bone-marrow dU-suppression data were obtained from each patient. After 24hr N_2O all of the bone marrow showed megaloblastic erythropoiesis. Most of those exposed for a shorter period showed the same effect. dU-suppression was increased most in those exposed for 24hr but also to a lesser extent in those exposed for a shorter period. Only some patients were followed up 6 days after exposure and by this time all results had returned to normal; in the group exposed or less than 24hr the dU-suppression results were already normal 24hr afterwards. Serum folate was also increased.

There is no indication of effects of N_2O on the immune system from studies on wound healing:

A randomised trial of 418 patients scheduled for colon resection in 3 hospitals in Austria and Hungary was conducted by Fleischmannn *et al.* (2005). A number of healing-related endpoints were compared between a group anaesthetised with nitrous oxide and another group in which nitrogen, remifentanil and isoflurane were used. There was no difference in infection rate, ASEPSIS wound healing score, wound collagen deposition, number of patients admitted to critical care unit, time to first food ingestion, duration of hospital stay or mortality.

Studies of lymphocyte sub-populations in blood samples taken from anaesthetists and anaesthesiology staff are reported by Bargellini *et al.* (2001), Peric *et al.*, (1991) and Karakaya *et al.*

(1992). The studies demonstrate some possible effects on anaesthetists of some factor(s) involved in working in an operating theatre but fail to associate those effects convincingly with any specific cause and certainly provide no evidence for an effect of N_2O:

The blood samples taken from 51 anaesthetists by Bargellini *et al.* (2001) were compared with 20 controls matched for age, BMI, sex , seniority in employment, marital status, number of children, physical activity, coffee and alcohol intake. The anaesthetists group differed from controls in including more smokers or ex-smokers and having slightly shorter declared sleep duration. All but 6 of the anaesthetists were also exposed to X-rays. N_2O exposure levels in the operating theatres over a 3-month period were used to rank the exposure of each anaesthetist over that period, however exposure to other anaesthetics was not specifically included in the analysis. Overall differences in lymphocyte sub-populations were identified in the study. CD3 (T-cells) and $CD16^+CD3^-$ (NK cells) were the only cell types to show statistical differences overall between the two groups. The former were slightly decreased in anaesthetists while the latter were increased. More detailed analysis of the data showed a significant reduction, compared with controls, in both CD3 and CD4 (helper) cells for those anaesthetists exposed within the last 2 weeks (41/51). The increased NK cell numbers were statistically significantly associated with X-ray exposure (45/51). This study demonstrates some effect of working in an operating theatre on lymphocyte sub-populations but shows no association with extent of N_2O exposure, despite specifically analysing for that relationship. The differentiation between the effects of X-rays and anaesthetic gases is suspect given that the majority of anaesthetists were exposed to both factors. Although there is evidence in the data that anaesthetists unexposed for the last 14 days or not exposed to X-rays are similar to controls, in levels of the 3 cell types, the group sizes are too small to give confidence in the differences seen.

Karakaya *et al.* (1992) studied the blood of 32 anaesthetists and 20 health volunteer controls, matched for age. The sex distribution was equal in controls but was 10M/21F for the anaesthetists group. The anaesthetists were said to be exposed mainly to halothane and rarely to N_2O. Serum was analysed for Immunoglobulin levels (IgG, IgM and IgA) while blood was subject to a total leukocyte count and specific determination of levels of lymphocyte sub-populations. No differences were found between the two groups apart from an increased T-helper/ T-Suppressor cell ratio which was identified by the authors to be a known effect of halothane anaesthesia.

Peric *et al.*, (1991) describe the results of analysis of blood samples taken from 21 staff employed in an anaesthesiology department, compared with 35 healthy volunteer controls. The precise function of the members of the anaesthetic group is not defined. Blood samples were taken from the subjects before and after summer holidays of an unspecified duration. The operating theatres of the department were temporarily unscavenged due to equipment failure and measurements of maximum exposure levels for Halothane and N_2O respectively were 2244ppm and 10000ppm with a TWA of up to 350ppm and 1600ppm. Prior to holidays a range of differences were seen including reduced WBC, non-segmented neutrophils, basophils and lymphocytes. Lymphocyte sub-populations were altered with CD2 and CD3 numbers unaffected, but increased as a percentage of the total. CD20 and NK cells were significantly increased both in numbers and in proportion of the total. The lack of background data on the exposure of the specific population and the mixed exposures only allow a conclusion that the unscavenged atmospheres and possibly other practices in the operating theatres and working life of the population studied are associated with effects on the blood including differences in the lymphocyte sub-populations. It is reassuring to note that the majority of these differences were not present in the same group after the summer holiday. Only the reduced CD20 population persisted. Immunoglobin measurements (IgM, IgG, IGD and IgA) showed no differences.

In a follow-up paper on the same 21 individuals Peric *et al.* (1994) report age-related differences in the rates of recovery from the changes seen in erythrocyte count and WBC count. The group sizes are 9 in the younger group (<40) and 12 (8 for post-vacation measurements) in the older group (>40) thus have very limited value. The older group showed a generally lesser effect and higher rate of recovery following vacation.

A limited study of a normal range of haematological parameters, including lymphocyte sub-populations, in a group of operating theatre staff was reported by Salo & Vapaavuori, (1976):

> Blood samples from 10 healthy operating theatre staff were compared with samples from 12 hospital staff working outside the operating theatre environment. The anaesthetic most frequently used was N_2O and levels of up to 860ppm were found by analysis of theatre atmospheres. Blood samples were analysed to identify T- and B- lymphocytes as well as a limited range of normal haematological and clinical chemistry endpoints including ESR, Hb, Haematocrit, RBC, SGOT, SGPT and creatinine. None of the endpoints measured showed any differences between the two groups. The N_2O content of expired air from operating theatre staff, made at the end of a 3hr working period was 31 ± 16 ppm.

Sweeney *et al.*, (1985) report investigations of 21 dentists exposed occupationally to nitrous oxide:

> The time-weighted mean exposures range from 159ppm to 4,600ppm. Three individuals on the trial showed evidence of vitamin Vit B_{12} inactivation (dU suppression test) and two of these had morphological abnormalities of both bone-marrow and peripheral blood cells. None of these individuals had exposures below 1800ppm thus the results are in accord with known effects of N_2O, as a consequence of vitamin Vit B_{12} inactivation.

8.2.4 Haematology & Immunology References

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