



Health effects of exposure to nitric oxide

Report prepared for the British Tunnelling Society

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HEALTH EFFECTS OF EXPOSURE TO NITRIC OXIDE

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HEALTH EFFECTS OF EXPOSURE TO NITRIC OXIDE

Summary

INTRODUCTION AND AIMS

This review of the health effects of exposure to nitric oxide (NO) has been prepared in response to the Health and Safety Executive's (HSE) recent Chemical Hazard Alert Notice that has replaced the long standing occupational exposure limit of 25 ppm with a recommendation that exposures should be controlled to 1 ppm or less. The EU Scientific Committee on Occupational Exposure Limits (SCOEL) has recommended an exposure limit of 0.2 ppm. This review has been based on the toxicological and epidemiological literature reviewed by SCOEL and more recent studies identified through a literature search. In addition, consideration has been given to the role of NO produced naturally in the human body (endogenous NO) in physiological processes and to the use of nitric oxide for the treatment of adults and infants with severe respiratory illness.

The aims of the review were to:

Provide a brief account of the toxicology of NO, building on and updating the reviews published by SCOEL (2003) and the World Health Organization (WHO, 1997);

Review the potential health effects that may arise following high levels of workplace exposure to NO;

Determine the exposure concentrations that would be expected to give rise to adverse effects;

Describe the differences in the properties of NO and nitrogen dioxide (NO₂) and assess the implications in terms of the relative potential of these two gases to cause harm to exposed workers;

Assess the relative levels of risk to health associated with workplace exposure to 0.2, 1, 5, 10 and 25 ppm of NO;

Assess possible interactions between exposure to NO and other substances in the workplace;

Assess what factors could contribute to some individuals being more susceptible to adverse effects than others.

PHYSIOLOGICAL IMPORTANCE OF ENDOGENOUS NO

Endogenous NO plays an important role in intracellular and intercellular signalling, in maintaining the health of blood vessels, in oxygen exchange within the lung, in cellular respiration and in regulating processes of inflammation and the generation or inhibition of reactive oxygen species.

Inhalation of NO would be expected to interfere with the normal balance between endogenous NO synthesis, tissue levels of NO and the NO concentration in exhaled air. A moderate increase in the concentration of NO in inhaled air might cause a reduction in endogenous NO synthesis, but may not necessarily affect tissue levels and the normal operation of physiological processes involving NO. A sudden increase might cause a temporary increase in tissue levels with adverse effects on cellular respiration and the potential formation of peroxynitrite with consequent oxidative tissue damage. A sudden reduction from a high concentration of NO in inhaled air might cause a deficit in tissue levels of NO leading to pulmonary hypertension and reduced oxygenation of circulating blood.

Endogenous NO may play an important role in enhancing the inflammatory response to inhaled mineral dust particles.

Concentrations of NO in some workplace environments are substantial relative to the concentrations normally present in exhaled breath. It seems plausible that exposure to 1 ppm NO in workplace air, a concentration 50 times greater than that normally present in exhaled air, could affect cellular function within the lung.

ANIMAL DATA

The results of animal experiments do not provide consistent evidence for the toxicity of NO at low levels of exposure. Evidence of emphysema like changes in lung structure has been reported in some experiments following continuous exposure to concentrations of ≤ 2 ppm, but similar changes have not been reported in other experiments in which animals have been exposed to concentrations ≥ 2 ppm. In an experiment where emphysema-like changes were observed in rats following long-term exposure to concentrations of less than 2 ppm, twice daily spikes in the concentration of NO may have played a role in the development of lung damage. Emphysema has also been reported following intermittent exposure (2 hours/day, 5 days/week) to 10 ppm. Higher levels of exposure to NO give rise to oxidative damage to lung tissue similar to that observed with NO₂ and ozone and have been associated with effects on epithelial cell turnover that might ultimately lead to lung fibrosis. The lowest concentration associated with effects on lung epithelial cells is 2 ppm.

The inconsistency of the findings of animal experiments may reflect the complexity of the interaction between inhaled and endogenous NO. It is possible that the mechanisms leading to tissue damage at low levels of NO exposure differ from those that are initiated by higher levels of exposure.

Overall, the results of animal experiments suggest that medium term exposure to low concentrations of NO (<1 ppm) is unlikely to cause serious damage to lung tissue whereas long-term exposure to concentrations greater than 10 ppm may cause serious damage. In addition, the small changes in lung tissue that may occur at concentrations of less than 2 ppm, may be associated with the long term development of emphysema. Slightly higher levels of exposure may be associated with an increased risk of developing lung fibrosis.

HUMAN DATA

The results of human volunteer experiments suggest that small effects on lung function may arise in some individuals exposed to a concentration of NO of only 1 ppm. Studies of workers exposed to NO suggest that long term exposure to mean levels of about 1 ppm in coal mines has no important effect on respiratory health in comparison with that arising from exposure to elevated concentrations of respirable dust. Exposure to higher mean concentrations of NO (8.6-26.5 ppm) in the absence of dust, but with co-exposure to NO₂, may have effects on immune function. Repeated exposure to elevated concentrations of NO as a result of explosives use in tunnels may adversely affect lung function and increase the risk of respiratory symptoms. The exact levels of exposure to NO at which respiratory damage may occur in tunnel workers have not been established, but it is likely that, for some workers, short term peak exposures have exceeded 100 ppm. Long term changes in respiratory health reported in tunnel workers may be associated with mean exposure concentrations of about 2.5-5 ppm (as inferred from reported NO₂ concentrations). Long term exposure to mean concentrations of NO of less than 0.1 ppm in ambient air may be associated with an increased risk of mortality, although the effects are difficult to separate from those of other air pollutants. Short term exposure to elevated concentrations of NO (0.5-1 ppm) in ambient air is associated with an exacerbation of symptoms in children with asthma which would be consistent of up regulation of airways inflammation.

Investigations of the therapeutic value of NO suggest that inhaled NO may have physiological effects at concentrations of only 0.2 ppm in some individuals with seriously compromised respiratory health. A concentration of about 5 ppm gives rise to maximum therapeutic benefit whereas concentrations of more than 80 ppm may be damaging in adults.

OVERALL EVALUATION

The effects of exposure to concentrations of NO of about 1 ppm are difficult to predict. The interaction of inhaled NO with processes dependent on endogenous NO may lead to suppression or enhancement of these processes but any changes in cellular function may simply be an adaptive response that is reversed on cessation of exposure.

Repeated exposure to high levels of NO in the workplace may give rise to an increased risk of emphysema. Low level exposure to NO may promote or inhibit the inflammatory response to dust, other pollutants and/or infection. Moderate to high levels of exposure to NO may enhance the inflammatory response to inhaled dust and other pollutants. Exposure to NO may also give rise to an increased risk of pneumoconiosis or silicosis in workers with co-exposure to dust and quartz.

There is little evidence that repeated exposure to concentrations of NO of less than 1 ppm would be likely to lead to irreversible respiratory illness. Both the animal and human data suggest that repeated exposure to concentrations of NO of between 1 to 10 ppm in the workplace is likely to be associated with irreversible effects on the lungs, but only modest effects on respiratory health. The variability of NO concentrations during the working day, before, during and after work and on successive work days may influence on the potential harmfulness of NO exposure.

Short term exposure to concentrations of NO of 80 ppm or more is likely to give rise to acute toxicity arising from the formation of MeHb in blood. The immediate effects are likely to be reversible, but long term exposure to concentrations of 80 ppm in the workplace would be expected to give rise to serious respiratory illness.

The toxicology of NO is distinct from that of NO₂. There may be a greater risk of harm associated with repeated exposure to NO than associated with repeated exposure to NO₂. Repeated exposure to either gas may be associated with an increased risk of respiratory illness.

There may be considerable variability in the response of individuals to a given concentration, but it is not clear what specific characteristics might give rise to an increased risk of adverse effects. Given the impacts of respiratory illness and other diseases on endogenous NO activity, it seems likely that the effects of inhaled NO will be partly determined by an individual's pre-existing health status.

Exposure-response functions have not been established for NO. The table overleaf provides an indication of the relative level of risk associated with different levels of exposure. It is not possible to provide quantified estimates of risk associated with different levels of exposure. As an approximate guide, it is possible that for ten years repeated exposure, a "small risk" might equate to effects developing in <2% of individuals, a "risk" might equate to effects developing in 2-10% of individuals and a "substantial risk" might equate to effects developing in >10% of individuals. These estimates are very uncertain and not scientifically defensible.

Table: Summary of the predicted effects of different levels of exposure to NO

Concentration of NO ppm	Effects of short term exposure	Effects of repeated exposure
0.2	Small reversible effects on cellular function in the lungs in a small proportion of individuals	None expected
1	Small effects on cellular function in the lungs in a large proportion of individuals; small effects on respiratory function in a small proportion of individuals	None expected
2-5	Reversible effects on cellular function in the lungs in a large proportion of individuals	Small risk of irreversible changes in the lung predisposing to emphysema or lung fibrosis, possible long term decline in lung function; increased risk of the development of respiratory illness as a result of co-exposure to other pollutants
10	Reversible effects on cellular function in the lungs in a large proportion of individuals, effects on immune function	Risk of irreversible changes leading to emphysema or lung fibrosis, long term decline in lung function, increased risk of the development of respiratory illness as a result of co-exposure to other pollutants
25	Reversible effects on cellular function in the lungs in most individuals, effects on lung function in a high proportion of individuals	More substantial risk of irreversible changes in the lung leading to emphysema or lung fibrosis, increased risk of the development of respiratory illness as a result of co-exposure to other pollutants

HEALTH EFFECTS OF EXPOSURE TO NITRIC OXIDE

1 Introduction and aims

1.1 INTRODUCTION AND BACKGROUND TO STUDY

This review of the health effects of exposure to nitric oxide (NO) has been prepared in response to the Health and Safety Executive's (HSE) recent Chemical Hazard Alert Notice for NO (HSE, 2004). This notice has replaced the long standing occupational exposure limit of 25 ppm with a recommendation that exposures should be controlled to 1 ppm or less. This HSE recommendation followed a recommendation by the EU Scientific Committee on Occupational Exposure Limits (SCOEL) for an exposure limit of 0.2 ppm based on the findings of lung damage in animals exposed to concentrations of 0.5 to 2 ppm on repeated or continuous exposure. The SCOEL recommendation has not been adopted by the EU.

This review has been based on the toxicological and epidemiological literature reviewed by SCOEL and some additional relevant articles identified through a literature search. In addition, consideration has been given to the role of NO produced naturally in the human body (endogenous NO) in physiological processes and to the use of nitric oxide for the treatment of adults and infants with severe respiratory illness.

1.2 AIMS

The aims of the review were to:

Provide a brief account of the toxicology of NO, building on and updating the reviews published by SCOEL (2003) and the World Health Organization (WHO, 1997);

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Assess the relative levels of risk to health associated with workplace exposure to 0.2, 1, 5, 10 and 25 ppm of NO;

Assess possible interactions between exposure to NO and other substances in the workplace;

Assess what factors could contribute to some individuals being more susceptible to adverse effects than others.

2. Methods

The review built on the criteria documents previously published by SCOEL (2003) and WHO (1997). A search was undertaken of PubMed (an online database of abstracts from the peer reviewed medical literature) to determine whether any more recent relevant information is available. This included searching the "related articles" links for the studies cited by SCOEL and WHO. Copies of the key studies referred to by SCOEL and WHO and some more recent studies identified using PubMed were obtained and critically reviewed in the light of the aims of this study. In addition, key review articles on the production of endogenous NO, the presence of NO in exhaled breath and the therapeutic use of NO were identified using Google with follow-up searches being undertaken using PubMed.

3. Chemical and physical properties of nitric oxide and nitrogen dioxide

3.1 NITRIC OXIDE

NO is a colourless, odourless gas that is only slightly soluble in water. It is a by-product of combustion processes, arising from high temperature oxidation of molecular nitrogen from the combustion air, and from oxidation of nitrogen present in certain fuels such as coal and heavy oil. The ratio of NO to NO₂ in oxides of nitrogen (NO_x) emitted from combustion sources is generally between 9 to 1 and 19 to 1.

NO is a free radical that will react with a wide range of molecules and it is readily oxidised to NO₂. NO may be oxidized to NO₂ by atmospheric oxygen but at low NO concentrations this reaction is slow and is important only when NO > 1 ppm (Boström, 1993). At lower concentrations NO can react with ozone to form NO₂ and molecular oxygen. NO can also react with other radical species present in photochemical smogs. In the absence of ozone arising from photochemical reactions, the conversion of NO to NO₂ in tunnel environments is much slower than in ambient air.

NO may combine with NO₂ and water to form nitrous acid. In sunlight, atmospheric concentrations of HNO₂ are limited by the photolysis of HNO₂ to produce NO and hydroxyl radical, but higher concentrations can develop in indoor air. Nitrous acid is a weak reducing agent and is oxidized to nitrate only by strong chemical oxidants and by nitrifying bacteria.

3.2 COMPARISON OF PROPERTIES WITH NITROGEN DIOXIDE

NO₂ is a reddish-orange-brown gas with a characteristic pungent odour. The boiling point is 21.1 °C, but the low partial pressure of NO₂ in the atmosphere prevents condensation. NO₂ is corrosive and highly oxidizing. Some physical and chemical properties of NO and NO₂ are listed in Table 3.1.

Table 3.1: Physical and chemical properties of NO and NO₂

	Nitric oxide	Nitrogen dioxide
Molecular formula	NO	NO ₂
Molecular mass	30.0 g/mol	46.01 g/mol
Appearance	colourless, odourless gas	red-brown pungent gas
Boiling point	-151°C	21°C
Solubility in water	0.0056g/100 g water	Reacts with water
Relative vapour density (air = 1):	1.04	1.58
Standard enthalpy of formation at 25°C, 100 kPa	90.25 kJ/mol	33.1 kJ/mol
Standard molar entropy at 25°C, 100 kPa	210.8 J/K/mol	240 J/K/mol

NO₂ would be expected to cause cellular damage as a result of its oxidising properties and also its acidity in solution. NO is a much weaker oxidant and, under some conditions, is likely to be a weak reducing agent. The low solubility of NO in water would also lead to a lower potential to cause acid damage than that arising with NO₂, but would also allow NO to penetrate the small airways of the lung to a much greater extent than NO₂, as the latter may be largely absorbed in the proximal airways. Both NO and NO₂ are free radical molecules with NO possibly being more reactive than NO₂ under some circumstances. It is possible that relatively little free NO₂ penetrates the cells of the airways because of its reactivity with water, whereas NO is likely to be reactive with a wide range of intracellular components. One biologically important reaction of NO (but not NO₂) is its reaction with superoxide anion to form the highly reactive species peroxynitrite (see discussion of endogenous NO below).

NO₂ would be expected to cause irritation of the upper airways and the main airways of the lung. NO would be expected to have a lower potential to cause irritation at low to moderate levels of exposure and its effects might be expected to be greatest in the distal parts of the lung.

4. Endogenous nitric oxide

4.1 PHYSIOLOGICAL FUNCTION

NO is generated throughout the body. It has an important role in regulating the muscles of blood vessel walls. It also has a role in cellular respiration, cell signalling in various tissues including the nervous system and modulating inflammation (Wang *et al*, 2003, Moncada & Higgs, 2006). In the lungs NO plays an important role in maintaining normal arterial blood pressure and permeability of blood vessels. NO plays a complex role in the regulation of the inflammatory process (Zeidler & Castranova, 2004). Anti-inflammatory functions include reducing the recruitment and adhesion of inflammatory cells and NO plays an important role in reducing the inflammatory response to bacterial endotoxin. Pro-inflammatory effects of NO include the induction of the production of pro-inflammatory messenger molecules (cytokines).

NO can exhibit antioxidant activity including reactions with alkoxy and peroxy radical intermediates and the inhibition of cellular superoxide anion production through its action on the enzyme NADPH oxidase. In theory, the anti-oxidant activity of NO may prevent damage to the lung surfactant system and the subsequent development of disease (Weinberger *et al*, 2001). Enhanced levels of NO arising from increased endogenous production or administration of exogenous NO, however, can give rise to the formation of cytotoxic reactive nitrogen intermediates and the formation of reactive nitrogen oxides including peroxynitrite. The pro-oxidant activities of NO have been linked with damage to lung surfactant, DNA damage, inactivation of enzymes and proteins, increased cellular proliferation, tumour promotion and inhibition of cellular respiration (Weinberger *et al*, 2001; Liaudet *et al.*, 2000). NO may up regulate the oxidative response to inhaled mineral dust particles causing enhanced levels of tissue damage (Zeidler & Castranova, 2004).

4.2 NO IN EXHALED BREATH

Small amounts of endogenous NO are exhaled. Reported concentrations of NO in exhaled breath are variable and some of the substantial between study differences are likely to be related to differences in measurement protocol. Reported concentrations in exhaled air range from less than 0.01 ppm to more than 0.05 ppm (eg Olin *et al*, 1998, Gomez *et al*, 1998; Schilling *et al*, 1984). At atmospheric concentrations of NO exceeding about 0.035 ppm, concentrations of NO in exhaled breath increase with increasing concentrations of NO in inhaled air (Corradi *et al*, 1998, Therminarias *et al*, 1998). The increase in exhaled NO is less than that in inhaled NO, suggesting that inhalation of NO affects the balance of endogenous NO production within the lungs. Similarly smokers, who inhale substantial quantities of NO in tobacco smoke, show reduced levels of NO in exhaled air (Schilling *et al*, 1994, Kharitonov *et al*, 1995). Emissions of NO in tobacco smoke have been estimated as 2.78 mg/cigarette compared with 0.73 mg/cigarette for NO₂ (WHO, 1997) The implied intake of NO in inhaled air for a smoker smoking 20 cigarettes a day and inhaling a total of 20 m³ of air over 24 hours, would be equivalent to continuous exposure to up to 2.78 mgm⁻³ or 2.2 ppm.

Various disease states have differing impacts on measured concentrations of exhaled NO. Increased levels of NO have been reported in asthmatics (Garnier *et al*, 1996; Gomez *et al*, 1998; Olin *et al*, 2004; de Meer *et al*, 2005), patients with bronchitis (Delen *et al*, 2000), rheumatic heart disease, particularly in those with pulmonary hypertension (Golbasi *et al*, 2001) cirrhosis and liver failure (Sogni *et al*, 1995) or kidney failure (Matsumoto *et al*, 1999). The elevated levels of NO may result from the inflammatory processes associated with these conditions. Other disease states including acute respiratory distress and AIDS may be associated with reduced concentrations of exhaled NO (Brett *et al*, 1998; Loveless *et al*, 1997).

Levels of exhaled NO have been reported to increase on exposure to airborne particles or other pollutants. In a study of Dutch children, Steerenberg *et al* (2001) reported that levels of exhaled NO increased in response to exposure to PM₁₀ (approximately the fraction of airborne particulate that penetrates to the lung), Black Smoke, nitrogen dioxide, and nitric oxide. Increased exhaled NO levels were associated with exposure to PM_{2.5} (approximately the fraction of airborne particulate that penetrates to the gas exchange region of the lung in those with compromised respiratory health) in boilermakers exposed to residual oil fly ash and metal fumes during annual maintenance work, although the findings were not substantiated in a follow up study, the following year.

4.3 EFFECTS OF EXCESS PRODUCTION OF ENDOGENOUS NO

Inflammatory stimuli such as endotoxins lead to the enhanced production of endogenous NO. Over production of endogenous NO is likely to cause dilation of the blood vessels, reduced blood pressure, vascular leakage and disruption of cell metabolism (Moncada & Higgs, 2006). Increased NO production inhibits cellular respiration through increased generation of superoxide and possibly also the formation of peroxynitrite and this may divert oxygen to other areas of the cell and surrounding tissues (Moncada & Higgs, 2006). Both processes may give rise to cellular damage but also contribute to the ability of cells such as macrophages to destroy pathogens through the production of reactive oxygen species. Long-term overproduction of NO may contribute to the development of atherosclerosis (hardening of the arteries) either as a direct consequence of NO on cellular respiration or through the formation of NO adducts such as peroxynitrite (Moncada & Higgs, 2006).

4.4 COMMENTS

Inhalation of NO would be expected to interfere with the normal balance between endogenous NO synthesis, tissue levels of NO and NO concentrations in exhaled air. A modest increase in concentrations of NO in inhaled air (0.1 ppm) might cause a reduction in endogenous NO synthesis, but may not necessarily affect tissue levels and the normal operation of physiological processes involving NO. This would be consistent with the relatively small elevation in exhaled NO concentrations arising from marginally increased exposure to NO (<1 ppm) in inhaled air. A sudden increase in concentration of NO in inhaled air of ≥ 1 ppm might cause a temporary increase in tissue levels with adverse effects on cellular respiration and the potential formation of peroxynitrite with consequent oxidative tissue damage. A sudden reduction in concentration in inhaled air might cause a deficit in tissue levels of NO leading to pulmonary hypertension and reduced oxygenation of circulating blood.

Concentrations of NO in some workplace environments are substantial relative to the concentrations normally present in exhaled breath. It seems plausible that exposure to 1 ppm NO in workplace air, a concentration 50 times greater than that normally present in exhaled air, could affect cellular function within the lung.

Exposure to NO could escalate NO-mediated processes of inflammation and oxidative damage in response to other air pollutants or infection.

5. Experimental investigations of the effects of inhaled NO

5.1 ABSORPTION OF INHALED NO

The results of two studies reviewed by the WHO (1997) suggest that 85 to 92% of inhaled NO was absorbed at concentrations ranging from 0.33 to 5.0 ppm with levels of absorption being marginally increased in exercising individuals. The percentage absorption of NO in rats exposed to 138 ppm, 270 ppm and 880 ppm was 90%, 60% and 20%, respectively (Yoshida *et al*, 1980). The progressive decrease in absorption was ascribed to an exposure-induced decrease in ventilation. In dogs exposed to vehicle exhaust mixtures, 73% of the constituent NO was removed by the nasopharyngeal region. The WHO (1997) concluded that the absorption of NO was similar to that of NO₂, although the lower solubility of NO may result in greater amounts reaching the pulmonary region.

5.2 NON-CANCER EFFECTS OF INHALED NO IN ANIMAL STUDIES

5.2.1 Introduction

The experimental database for NO is very limited, as attention has largely focussed on the potential toxicity of NO₂ rather than NO. Unless indicated otherwise, NO₂ contamination of NO was negligible in the reviewed studies.

5.2.2 Acute effects

Lung injury

Short term (5 hour) exposure of mice to concentrations of NO ranging from 0 to 100 ppm gave rise to a dose-related increase in protein (a marker of cellular injury) in bronchioalveolar lavage (BAL) fluid¹ (Weinberger *et al.*, 1998). The results suggest that NO induces alveolar epithelial injury with a lowest observed effects level of 10 ppm, although a significant increase in BAL protein was only observed at concentrations of 40 ppm or greater. Cells recovered from BAL fluids produced significantly more NO than cells from control animals. Superoxide production and peroxynitrite production were also enhanced. The authors concluded that inhaled NO primes lung macrophages to produce reactive oxygen and nitrogen intermediates. In contrast, in dogs with endotoxin induced acute respiratory distress syndrome, inhalation of 20, 40, or 80 ppm suppressed pro-inflammatory cytokine expression in the lungs leading to a down regulation of inflammatory response (Miao *et al.*, 1998). A much earlier study reported that there was no change in respiratory function in guinea-pigs exposed to NO at 16 ppm or 50 ppm for 4 hours, but no investigation of more sensitive markers of respiratory toxicity was undertaken (Murphy *et al.*, 1964²). Lambs exposed to 80 ppm NO for 3 hours showed no evidence for fluid accumulation or tissue damage in the lungs when compared with control lambs (Frostell *et al.*, 1991).

Airways responsiveness

One study has reported an increase in airway responsiveness following exposure to NO. Guinea-pigs exposed to 5ppm NO for 30 minutes, twice a week for 7 weeks showed an increased response to acetylcholine (WHO, 1997 – reference to source study not provided). Other studies have reported that NO causes dilation of the airways (bronchodilation) that counteracts the constriction of the airways in response to agents such as methacholine. Reversal of methacholine-induced bronchoconstriction by NO has been reported in guinea-pigs at 5 ppm (Dupuy *et al.*, 1992). In rabbits full reversal of methacholine-induced bronchoconstriction was seen at 80 ppm (Högman *et al.*, 1993).

Effects on blood vessels

A large number of studies have demonstrated that NO causes dilation of blood vessels within the lung and can reverse experimentally induced pulmonary hypertension. In sheep, constriction of pulmonary blood vessels in response to an infused thromboxane analogue or oxygen starvation was reversed by acute exposure to 5 ppm NO (Fratacci *et al.*, 1991, Frostell *et al.*, 1991). In dogs with endotoxin induced acute respiratory distress syndrome, inhalation of 20, 40, or 80 ppm improved arterial blood oxygenation and flow (Maio *et al.*, 2002) and concentrations of 5-40 ppm were found to be effective in reducing platelet-activating factor-induced pulmonary hypertension (Yamada *et al.*, 1998). A dose-dependant decrease in pulmonary arterial pressure was reported in rats with monocrotaline-induced pulmonary hypertension exposed to 20-100 ppm NO (Katayama *et al.*, 1994). The reaction to

¹ Procedure in which the lungs of experimental animals are washed out and the recovered fluid analysed to determine the presence of any inflammatory cells, biochemical markers of cell damage or other biochemical markers such as cytokines – messenger molecules – known to be involved in the inflammatory process

² Although the title of this document suggests that only NO₂ and ozone were included in the study, the text includes a brief description of investigations made with NO

NO was almost constant at concentrations of over 60 ppm. The effects of lower concentrations of NO were not investigated. In rats with chronic pulmonary hypertension arising from exposure to a low oxygen atmosphere for periods of up to 29 days, inhalation of NO at concentrations of 0.1-2.0 ppm reduced mean pulmonary artery pressure (Jiang *et al*, 2002). Brady *et al* (1998) showed that in rats exposed to 6 ppm NO, there was an immediate effect on the levels of cytokines (messenger molecules) in lung tissue that are associated with vascular dilation, but, that this response diminished on continued exposure for up to a week. In a study of dogs with induced acute respiratory injury, two hour exposures to concentrations of 10 or 40, ppm NO were effective in restoring arterial blood flow in animals treated with oleic acid and endotoxin but not in animals treated with oleic acid alone (Gust *et al*, 1999). The authors concluded that the effect of inhaled NO on oxygenation in injured lungs depends on the pattern of lung injury prior to NO inhalation.

More recently it has been demonstrated that inhaled NO reduces systemic vascular resistance in normal and septic dogs (Quezado and Eichacker, 2000). In contrast, however, a study in mice established that inhalation of 80 ppm NO had no systemic vascular effects (Hataishi *et al*, 2006).

Blood

Inhaled NO may affect blood oxygenation through its interaction with haemoglobin and may also affect blood clotting tendencies. Oda *et al* (1975) investigated the formation of nitrosyl-haemoglobin (NOHb) in mice and rats exposed to concentrations of NO of 10.6 ppm (with levels of NO₂ contamination of 0.5 ppm). Concentrations of NOHb in blood equilibrated at 0.13% after 20 minutes of exposure. On withdrawal from NO exposure, the NOHb level halved within a few minutes. The extent to which NO interacted with haemoglobin was much smaller than that observed with carbon monoxide at similar levels of exposure, despite the much greater affinity of NO for haemoglobin. It was noted (but not discussed) that NO-Hb was also detected in mice exposed to 12.8 ppm NO₂. In a subsequent experiment (Oda *et al*, 1976), NOHb levels were shown to increase with increasing concentration with levels of 1.58% being achieved following 90 minutes exposure to 66 ppm NO (contaminated with 36 ppm NO₂). Subsequently Oda *et al* (1980) reported that the concentration of NOHb in blood in mice exposed to 40 ppm NO was 0.7% with equilibrium being achieved in 30 minutes. There was an associated 5% increase of methaemoglobin (MetHb). In contrast, exposure to 40 ppm NO₂ produced a concentration of NOHb of 0.2% and no increase in MetHb. In animals exposed to concentrations of NO between 20 and 80 ppm there was an exposure related increase in NOHb accompanied by an exponential increase in MetHb, particularly at high concentrations of NO. In comparison, no effects on haemoglobin were observed in rats exposed continuously to 2 ppm NO over a six week period (Azoulay *et al*, 1977). A subsequent *in vitro* investigation demonstrated that the oxygen carrying capacity of rat blood was reduced on exposure to 10 ppm NO, although similar effects were not observed in human blood at concentrations of less than 100 ppm (Azoulay *et al*, 1978). Lambs exposed to 80 ppm NO for 3 hours showed no increase in MetHb compared with control lambs (Frostell *et al*, 1991). In guinea pigs, a one hour exposure 100 ppm NO did not induce substantial MetHb (less than 2%; Dupuy *et al* 1992). In dogs with endotoxin induced acute respiratory distress syndrome inhalation of 40, or 80 ppm gave rise to MetHb, > 3% (Maio *et al*, 2002).

At very high levels of NO exposure or other conditions leading to lung oedema, the generation of MetHb in the alveoli by NO may severely damage lung surfactant function thus provoking further damage to the lung and respiratory function (Weinberger *et al*, 2001).

Variable effects have been reported on blood clotting that could lead to lung injury. In rats, inhalation of 15 ppm NO for 10 hours reduced the tendency for blood clotting to occur in response to endotoxin-induced pulmonary inflammation as reflected by increased bleeding time, reduced platelet aggregation, and increased platelet cGMP (Kermarrec *et al*, 1998). Nong *et al* (1997) also reported a reduced tendency towards blood clotting in the lungs of rats pretreated with an intravenous thrombotic collagen challenge after exposure to concentrations of 20, 40, and 80 ppm NO. In contrast, however, subchronic exposure to NO may activate blood clotting (Kobayashi *et al*, 2001- see below).

Immune effects and inflammation

Exposure of rats to 10 ppm NO for 24 hours has been reported to lead to improved bacterial clearance in pneumonia induced by alveolar instillation of live *Pseudomonas aeruginosa* (Jean *et al*, 2002). NO exposure was also associated with increased recruitment of alveolar neutrophils (white blood cells associated with inflammation) but had no effects on protein concentration (a marker of cell injury) in the BAL fluids. Exposure to NO had no impact on mortality rates, suggesting that any enhancement in the ability to fight infection was counterbalanced by other factors.

5.2.3 Subchronic effects

Respiratory system

Young rats exposed to 2.0 ppm NO continuously for 6 weeks showed progressive development of mild inflammation and, in some animals, the development of emphysema-like changes in their lungs with small patches of alveolar oedema after 6 weeks of exposure (Azoulay *et al* 1977). Some control animals also showed some signs of a mild inflammatory response, attributed to the experimental procedure, and some changes in lung structure similar to those seen in exposed animals but no evidence of emphysema. The study only employed a relatively small number of animals and only small changes in lung architecture were observed. In a small study involving 4 exposed animals and 4 controls, rabbits exposed continuously to 5 ppm NO for 14 days developed fluid-containing vacuoles inside the endothelial cells of the pulmonary arteries and/or in the intercellular junctions (Hugod, 1979). Fluid accumulation in interstitial space led to a thickening of the alveolar capillary membrane.

Rats exposed for 9 weeks to 0.5 ppm with twice daily 1 hour spikes to 1.5 ppm showed changes in lung structure consistent with the early development of emphysema through the destruction of alveolar tissue (Mercer *et al*, 1995). The number of gaps in lung tissue – fenestrae – was significantly increased in exposed animals compared with unexposed animals in which such fenestrae had not developed. These fenestrae had formed by the degradation of the lung lining, specifically interstitial cells, interstitial matrix and connective tissue. The number of interstitial cells in the lungs of rats exposed to NO was reduced by 29% and the mean thickness of the interstitial space was reduced from 0.32 μm in the control group to 0.24 μm . The authors attributed the morphological changes observed with NO to its effects on the function of interstitial fibroblasts – cells that are critical for the synthesis and maintenance of the interstitial matrix and connective tissue fibres in the lung. Cellular assays have demonstrated that NO can inactivate a key enzyme within this cell type causing inhibition of normal cellular function whereas similar effects do not arise with NO₂. The authors interpreted the absence of damage to epithelial cells as indicating that the levels of NO exposure used in the experiment were too low to give rise to significant formation of peroxynitrite or other reactive radicals.

In a subsequent experiment, Mercer (1999) found little evidence of emphysema-like changes in rats exposed to concentrations of NO of 2 ppm or 6 ppm for six weeks. A slight increase in the numbers of fenestrae was observed in the lungs of animals exposed to 2 ppm in comparison to unexposed controls but the effect was not statistically significant. No increase in fenestrae was observed in the lungs of animals exposed to 6 ppm. The method used to assess such changes was slightly different from that previously employed by Mercer *et al* (1995), but this is unlikely to have caused the substantial difference in the apparent outcome of the two experiments. Morphological analysis of lung tissue did reveal effects on epithelial cells at both 2 ppm and 6 ppm that had not been observed at the lower concentrations employed in the earlier experiment. At 6 ppm, there was also an approximately threefold increase in the number of alveolar macrophages in the airspaces. The increases in the number of types I and II epithelial cells, the percentage of epithelia basement membrane covered by type II cells and numbers of alveolar macrophages were consistent with those observed with the oxidant gases ozone and NO₂. NO exposure also caused sequestration of platelets in capillary blood vessels in the lung, although no changes in platelet morphology or in the numbers of circulating cells were observed. Analysis of BAL fluids revealed no difference in antioxidant activity between exposed and unexposed animals as assessed from

ascorbic acid and glutathione levels but the significance of an increase in uric acid levels observed at 6 ppm was unknown.

In a study undertaken to assess the potential risks associated with long term NO therapy in patients with COPD, Kobayashi *et al* (2001) exposed mice continuously for 3 weeks to concentrations of NO of 0, 2 and 40 ppm with or without additional oxygen. Concentrations of total protein, thrombin and soluble tissue factor in BAL fluids were significantly increased in mice exposed to 40 ppm compared with 0 or 2 ppm and the expression of lung tissue factor mRNA was higher at 40 ppm than at 2 ppm. The authors concluded that long-term inhalation of NO may activate the clotting system by increasing the lung expression of tissue factor, giving rise to an increased risk of lung injury. In a further study undertaken to investigate the effects of inhaled NO on endogenous NO production, Frank *et al* (1998) found no evidence in rats exposed to 20 ppm for 1 to 3 weeks, that vasoconstriction was increased in response to acute oxygen starvation as a result of any progressive down regulation of endogenous NO production. In a two week study in oxygen-starved rats, Kouyoumdjian *et al* (1994) found that continuous exposure to 10 ppm NO induced sustained dilation of pulmonary blood vessels and reduced the extent to which blood vessels were permanently altered in response to chronic oxygen starvation.

Immune function

The results of investigations of the effect of subchronic exposure to NO on immune function have been mixed. In mice exposed continuously to 2 ppm for 4 weeks, no effects in resistance to infection by a bacterial aerosol were observed (Azoulay *et al*, 1981). In mice exposed to 10 ppm for 2 hours/day, 5 days/week, Holt *et al* (1979) reported a slight increase in serum antibody response after 10 weeks of exposure, but no effects on immune function after 30 weeks exposure. In a tumour rejection assay, NO slightly depressed the immune response.

5.2.4 Long term exposure

Oda *et al* (1980) exposed mice to 2.4 ppm NO continuously for up to 23 months and observed no histological changes differences between the lungs of exposed and unexposed animals. Some damage to the lung epithelium and obstruction of the small airways were observed in both exposed and unexposed animals. Very small changes in blood suggested that NO exposure did cause a very slight increase in the rate of turnover of red blood cells. Although Oda *et al* undertook some histological investigation of lung tissue, they did not report such a detailed approach as subsequently employed by Mercer *et al* (1995). It is possible, therefore, that subtle changes in lung architecture may have been missed, but it seems unlikely that important emphysema-like changes could have gone unobserved.

In an earlier experiment, emphysema was observed in mice exposed to 10 ppm for 2 hours/day for 5 days/week for 30 weeks (Holt *et al*, 1979). Many large air spaces were observed in the periphery of their lungs, some of the capillaries in the alveolar septa were congested and some haemorrhaging had occurred. Mice exposed for the same time period to concentrations of NO₂ of 10 ppm also showed congestion of septal capillaries and diffuse interstitial pneumonia, but much less development of emphysema than observed with NO. Holt *et al* (1979) also reported effects on blood cell counts in mice exposed to NO with an increase in the number of white blood cells and the proportion of polymorphonuclear cells. Changes were also observed in red blood cell morphology, spleen weight and bilirubin. The purity of the NO used by Holt *et al* was not stated.

Emphysema-like changes have also been observed in the lungs of dogs exposed to 0 or 1.64 ppm NO (contaminated with 0.14 ppm NO₂) for 16 hours per day for 68 months. Effects included enlargement of alveolar air space, destruction of alveolar septa and an increase in alveolar pores (Hyde *et al*, 1978). The relative importance of NO versus NO₂ was unclear (see section 5.6.2).

In an 18 month study, no differences in lung function were found between dogs exposed to 1.5-2 ppm NO (with 0.2 ppm NO₂) for 16 hours per day and unexposed controls, but as there

was a wide interindividual variation in lung function, this study lacked the sensitivity to detect small effects (Vaughan *et al*, 1969).

5.2.5 Conclusions

The results of animal experiments do not provide consistent evidence for the toxicity of NO at low levels of exposure. Evidence of emphysema like changes in lung structure has been reported in some experiments following continuous exposure to ≤ 2 ppm, but similar changes have not been reported in other experiments in which animals have been exposed to ≥ 2 ppm. It is possible that twice daily spike in concentrations of NO played an important role in the development of lung injury in one of the experiments where effects were observed at concentrations lower than 2 ppm. Emphysema has also been reported following intermittent exposure (2 hours/day, 5 days/week) to 10 ppm. Higher levels of exposure to NO have given rise to similar oxidative damage to lung tissue as observed with NO₂ and ozone and have been associated with effects on epithelial cell turnover that might ultimately lead to lung fibrosis. The lowest concentration associated with effects on lung epithelial cells is 2 ppm.

Some of the inconsistency in the results of animal experiments may result from the complex interaction between inhaled NO and endogenous NO on cellular processes modulated by NO. It is possible that the nature of this interaction is concentration-dependent leading to different types of effects at low levels of exposure from those observed at slightly higher levels of exposure. The results of animal experiments also suggest that pre-existing health status, particularly the nature of any pre-existing lung damage, may have an important influence on the effects arising from NO inhalation.

Overall, the results of animal experiments suggest that medium term exposure to low concentrations of NO (<2 ppm) could cause slight changes in lung tissue that may be associated with the long term development of emphysema. Slightly higher levels of exposure may be associated with an increased risk of developing lung fibrosis. Repeated exposure to concentrations of NO >10 ppm may give rise to a risk of serious respiratory illness.

5.3 GENOTOXICITY AND CARCINOGENICITY

It has been demonstrated in several experimental systems that NO can cause alterations in DNA that could lead to genotoxic effects. Chromosomal aberrations in rat-lung cells *in vivo* and in TK6 human lymphoblastoid cells and mouse macrophage cells *in vitro* have been reported following NO treatment (Arroyo *et al.*, 1992; Isomura *et al.*, 1984; Nguyen *et al.*, 1992; Zhuang *et al.*, 1998; Wink *et al.*, 1991). The experiment in rats reported mutagenic changes in lung cells recovered following 3 hours exposure to 27 ppm but not 9 or 19 ppm (Isomura *et al.*, 1984), but the experiment was not conducted to a standard protocol which limits its value for regulatory toxicology. The mutagenicity of NO has also been demonstrated in bacterial assays (Nguyen *et al.*, 1992).

Peroxynitrite (a potential product of NO exposure) can also initiate DNA base modifications. Incubation of purine nucleotides or of isolated DNA at physiologic pH with peroxynitrite *in vitro* has been demonstrated to cause DNA damage (Yermilov *et al.*, 1995) that could interfere with DNA replication (Szabo & Ohshima, 1997). NO and peroxynitrite have also been shown to cause DNA strand breaks (Salgo *et al.*, 1995) Possible responses include initiation of DNA repair and/or cell death by necrosis or apoptosis.

NO has been shown to increase the activity of the intracellular enzyme, soluble guanylyl cyclase, and several *in vitro* studies have demonstrated how this may give rise to a potential reduction in DNA synthesis and cellular proliferation (Weinberger *et al.*, 2001).

No increased tumour risk has been reported in the limited number of long term experiments that have been undertaken with NO. No evidence of chromosomal damage was seen in white blood cells in human volunteers exposed to 39.5 ppm NO for 2 hours (Luhr *et al.*, 1998).

In conclusion, NO may cause DNA damage leading to mutagenic effects where it comes into direct contact with cells (for example, cells lining the airways), but there is no evidence that

exposure to NO is likely to be associated with an increased cancer risk.

5.4 COMBINED EXPOSURE TO NO AND DUST

Zeidler and Castronova (2004) reviewed several animal studies that had demonstrated enhanced levels of endogenous NO production following exposure to crystalline silica or asbestos and concluded that endogenous NO plays an important role in enhancing the inflammatory response to mineral dust causing oxidative damage leading to lung injury. Blackford *et al* (1997) reported that when normalized to an equivalent exposure in terms of particle number, the production of NO was correlated with the relative potency of silica, coal, carbonyl iron and titanium dioxide as assessed from the protein level and white cell count of lung lavage fluids. In genetically engineered mice that lacked a key enzyme for NO production, the reaction to silica was very much reduced (Zeidler & Castronova, 2004). There is no information about the impacts of combined exposure to mineral dust and NO and it is not possible to predict potential impacts from the studies of endogenous NO production following dust exposure. Although, it has been demonstrated in two experiments in rabbits that inhalation of 10 ppm NO reduced the inflammatory response to lipopolysaccharide (Koh *et al*, 2001; Kang *et al*, 2002), the role of endogenous NO in modulating the response to this type of challenge (endotoxin) has been demonstrated to be different from that modulating the response to mineral dust (Zeidler & Castronova, 2004). The increased production of reactive oxygen and nitrogen species in cells recovered from mice lungs following inhalation of NO reported by Weinberger *et al* (1998) suggests that co-exposure to dust and NO is likely to lead to increased levels of oxidative damage.

5.5 HUMAN VOLUNTEER STUDIES

Most volunteer studies undertaken during the last 15 years have focussed on the potential therapeutic value of NO exposure. In a single much earlier study that was focussed on respiratory effects, Kagawa (1982) exposed 8 healthy volunteers to a concentration of 1 ppm NO for 2 hours. The subjects undertook intermittent light exercise. No symptoms were observed, but a small, statistically significant decrease of specific airway conductance was observed in half of the subjects. The WHO (1997) cite a study by von Nieding *et al* which involved a large number of volunteers (191) who were exposed for 15 minutes to concentrations of NO ranging from 10 to 40 ppm. At concentrations equal to or greater than 20 ppm, they reported an increase in total respiratory resistance and a decrease in the oxygenation of blood in the pulmonary arteries. In a much smaller investigation of the bronchodilatory effects of NO, Hogman *et al* (1993) found that a ten minute exposure to 80 ppm NO, had no effect on airway tone in six healthy volunteers and 4 patients with chronic obstructive pulmonary disease. This level of NO exposure did however reduce the airways response to a methacholine challenge in 3 patients with asthma and 2 patients with hyper-reactive airways. In an experiment in 9 healthy volunteers made hypoxic by breathing an atmosphere with only 12% oxygen, Frostell *et al* (1993) determined that a ten minute exposure to concentrations of 10 to 40 ppm NO selectively induced dilation of the pulmonary arteries, reversing the constriction induced by hypoxia. In the absence of hypoxia, a ten minute exposure to NO had no effect on pulmonary circulation. No adverse symptoms and no effects on blood pressure, heart rate or respiratory rate were reported in a study of 12 volunteers exposed to 39.5 ppm NO for 2 hours (Luhr *et al*, 1998). Mean MetHb levels rose from 0.63 to 1.3% during the course of the exposure.

Several recent studies have examined the effects of inhaled NO on platelet function in healthy humans. Gries *et al* (2000) reported that ADP-and collagen-induced platelet aggregation in healthy male and female volunteers was significantly inhibited following inhalation of 5, 10, and 40 ppm for 20 or 40 minutes, although no clear dose-response relationship was observed. At 40 ppm there was a significant prolongation of bleeding time. Albert *et al* (1996, 1999) reported that there was significant increase in bleeding time in healthy subjects exposed to 30 ppm for 55 minutes and effects on platelet aggregation and secretion were minimal. There is limited evidence arising from the therapeutic use of NO of effects on platelet function giving rise to prolonged bleeding times in both babies and adults treated with NO and an increased risk of intracranial haemorrhages in NO-treated infants than in historical controls (Weinberger *et al*, 2001).

5.6 COMPARISON WITH NITROGEN DIOXIDE

5.6.1 Short term effects in animal studies

The main effects of NO₂ in short term experiments are an increase in airways resistance and bronchoconstriction and evidence of oxidative damage and inflammation in the lung (WHO, 1997). A concentration and time-dependent increase in airways resistance has been reported in guinea pigs exposed to concentrations of 0.6 to 4 ppm for 6 to 12 weeks (Kobayashi & Miure, 1995). Bronchoconstriction has been reported in guinea pigs exposed for 10 minutes to 1 ppm (Halinen *et al*, 2000). Evidence of effects on antioxidant activity in the lungs of rats exposed to NO₂ has been reported at concentrations of 0.4-4 ppm (WHO, 1997) and the lowest concentration at which markers of inflammation have been detected in guinea pigs is 1 ppm (Halinen *et al*, 2000). Effects on immune function have also been reported with the lowest exposure being reported to adversely affect immune response in the mouse being 0.5 ppm after 3 months exposure or 1 ppm after a single 4 hour exposure (WHO, 1997).

The short term effects of NO differ from NO₂ in that NO promotes dilation of the airways. The lowest concentration of NO reported to be associated with markers of airways inflammation is 10 ppm compared with only 1 ppm with NO₂ (although this comparison may partly be an artefact arising from the very limited database available for NO). NO₂ may instigate oxidative damage at concentrations of less than 1 ppm whereas there is no evidence that NO has similar effects at these concentrations. NO may exert antioxidant activity at low concentrations through the scavenging of superoxide and other radical oxidative species, although there are no animal data to confirm this. NO has a range of effects on blood clotting and oxygenation that are not observed with NO₂ and high levels of exposure to NO may give methaemoglobinaemia which is not observed following exposure to NO₂.

5.6.2 Effects of long term exposure in animals

The main effects of NO₂ in long term experiments are changes in lung structure leading to fibrosis and emphysema and a reduction in lung function (WHO, 1997). In short to medium term studies in rats, the lowest level at which lung epithelial cell proliferation has been observed is 0.4 ppm while other studies have found no effects at concentrations of 2 ppm. The results of longer term studies suggest that prolonged exposure of rats to concentrations of 0.5 ppm can give rise to epithelial type 2 cell hyperplasia that could lead to fibrosis, although no effects were seen in monkeys following prolonged exposure to 1 ppm. Mercer *et al* (1995) found that the exposure of rats to 0.5 ppm NO₂ with twice daily spikes to 1.5 ppm was associated with the formation of fenestrae similar to those seen with NO. The number of fenestrae was about two thirds of that observed with the same concentration of NO and, in contrast to the effects of NO, there was no change in the number of interstitial cells or thickness of the interstitial space. In a subsequent 6 week experiment, Mercer (1999) found that the effects of exposure to 2 ppm of NO or NO₂ on epithelial cells appeared to be similar whereas the effect of NO on alveolar macrophages appeared to be greater than that of NO₂. Barth *et al* (1995) found concentration and time-dependent changes in rat lung tissue following exposure to NO₂. Three days exposure to 5 ppm was associated with slight oedema whereas 25 days exposure to 10 ppm was associated with the development of emphysema. Effects on lung function were observed in rats exposed continuously for 78 weeks to 0.5 ppm for 23 hours/day with a one hour 1.5 ppm daily peak (WHO, 1997) and in mice, effects were observed following exposure for 32 weeks to 0.2 ppm with a daily peak of 0.8 ppm. Hyde *et al* (1978) reported the development of emphysema in dogs exposed for 68 months to NO/NO₂ mixtures, and found more extensive damage in animals exposed to 0.6 ppm NO₂/ 0.1 ppm NO than in those exposed to 0.14 ppm NO₂/ 1.64 ppm NO. The mixed nature of the exposure, however, means that it is difficult to be certain of the relative importance of NO versus NO₂. NO₂ interacts with cell membranes by causing lipid peroxidation and prolonged exposure of animals to concentrations of 2 ppm or greater has been observed to cause an influx of inflammatory cells into the lung and to promote changes in cell turnover leading the development of lung fibrosis. It is plausible that co-exposure to NO may enhance the inflammation and oxidant damage associated with NO₂.

In conclusion, the long term effects of high levels of exposure NO and NO₂ may be similar. There is limited evidence that NO₂ may cause oxidative damage leading to epithelial cell proliferation at much lower levels of exposure than NO, but this may simply reflect the paucity of data available for NO. There is limited evidence that the effects of low level exposure to NO may interfere with cell function leading to a risk of emphysema arising by a different pathway from that arising from exposure to NO₂. The damage to lung epithelial cells arising from exposure to NO₂ is focussed at alveoli immediately adjacent to the terminal airways whereas NO may penetrate more deeply into the gas exchange regions of the lung.

5.6.3 Human volunteer studies

Short term exposure to NO₂ causes increased airways responsiveness, constriction of the airways, reduced lung function and/or respiratory symptoms in some individuals. Asthmatics appear to be more susceptible to the effects of NO₂ than healthy volunteers.

There is considerable between study and within study variability in the results of investigations of the effects of NO₂ on airways responsiveness and lung function (WHO, 1997). In a review of 7 studies in healthy subjects, Bylin (1993) found no evidence of effects on airways responsiveness at concentrations of less than 1 ppm. A meta-analysis by Folinsbee (1992) found a statistically significant effect at concentrations greater than 1 ppm. Most studies of lung function have found no effects in healthy volunteers at exposure concentrations of up to 80 ppm, although some have found effects at concentrations as low as 0.15 ppm (WHO, 1997). In a review of 40 studies involving healthy volunteers, Bylin (1993) concluded that there were clear effects on lung function at concentrations of 5 ppm and equivocal evidence for effects at 2.5 ppm. Among 25 studies of asthmatic volunteers, most showed no effects at concentrations of 0.1-0.6 ppm but small effects were seen in some studies at concentrations of 0.3 ppm. There is limited evidence to suggest that the lungs of volunteers adjust to NO₂ exposure so that the lung function effects of NO₂ decline with multiple exposure (Blomberg *et al*, 1999). The limited information available for NO also shows variable effects on lung function.

Exposure to concentrations of NO₂ of more than 0.25 ppm has been shown to increase the asthmatic response to a range of allergens (Strand *et al*, 1998; 1997) with other studies reporting similar effects following exposure to concentrations equal to or greater than 0.4 ppm (Tunnicliffe *et al*, 1994; Wang *et al* 1995). There are no comparable data for NO, although NO has been demonstrated to reduce bronchoconstriction in asthmatics following challenge with methacholine.

The relationship between NO₂ exposure and respiratory symptoms is unclear. Vagaggini *et al* (1996) found that exposure to 0.3 ppm NO₂ for 2 hours was associated with a small increase in symptoms in healthy subjects or patients with COPD but not in asthmatics. No symptoms were observed in healthy subjects exposed to 80 ppm NO (Luhr *et al*, 1998).

Overall, although the data for NO are very limited, the effects of exposure to NO in volunteer experiments appear to be distinct from those arising from NO₂.

5.6.4 Conclusions

The toxicology of NO is distinct from that of NO₂, particularly at low levels of exposure. Lowest observed effects levels for a number of endpoints (inflammation, fibrosis, effects on lung function) appear to be higher for NO than NO₂, but this could largely reflect the paucity of experimental data for lower concentrations. NO has effects on blood vessels, the inflammatory process and cell function at concentrations of less than 1 ppm. Comparable effects are not observed with NO₂. NO penetrates the gas exchange region of the lungs to a greater extent than NO₂.

5.7 CONCLUSIONS

Overall, the results of animal experiments suggest that medium term exposure to low concentrations of NO (<2 ppm) could cause slight changes in lung tissue that may be

associated with the long-term development of emphysema. Slightly higher levels of exposure may be associated with an increased risk of developing lung fibrosis. Repeated exposure to concentrations of NO >10 ppm may give rise to a risk of serious respiratory illness.

Co-exposure to NO and mineral dust may lead to higher levels of lung inflammation and oxidative damage than would arise in the presence of mineral dust alone.

Exposure to NO may lead to DNA damage in the lung although there are no indications of an increased cancer risk in long-term animal experiments.

The results of human volunteer experiments suggest that exposure to concentrations of NO as low as 1 ppm could interfere with the normal cellular processes that occur in the lung. It is unclear whether these changes in cellular function would give rise to an increased risk of adverse health effects on repeated exposure to concentrations of NO of about 1 ppm.

The toxicology of NO is distinct from that of NO₂, particularly at low levels of exposure. There is no clear evidence that exposure to NO is substantially less harmful than exposure to NO₂.

6. Epidemiological studies

6.1 WORKPLACE EXPOSURE TO NO

Workplace exposures to NO usually occur in conjunction with exposure to NO₂ and traditionally NO₂ has been regarded as the more harmful of the two species. Acute exposure to high concentrations of NO may cause toxicity arising from the formation of MetHb leading to abdominal pain, cough, headache, drowsiness, nausea, dizziness, confusion, blue skin, lips or finger nails, shortness of breath, convulsions, and unconsciousness. There is little evidence of adverse effects arising at typical levels of workplace exposure.

In a study of the effects of NO_x on natural killer (NK) cells in 18 glass craftsmen and 12 braziers, Azari *et al* (1996) reported by NK cell activities were significantly lower than in 21 non-exposed controls whereas the percentage of NK cells was significantly greater. The percentage of NK cells was not significantly correlated with age, smoking habit or number of years worked but was significantly related to air concentrations of NO₂ and NO. Mean levels of exposure for NO for braziers and glass craftsman were 8.6 and 26.5 ppm respectively and the levels of NO₂ were 2.9 and 1.2 ppm respectively.

Robertson *et al* (1984) reported a large study in British coalminers exposed to colliery mean concentrations of NO of between 0.11 and 1.23 ppm and mean concentrations of NO₂ of between 0.02 and 0.08 ppm. The most highly exposed workers were shot firers who had long term mean exposures that exceeded 2 ppm in one of the nine collieries investigated. Short-term peak exposures to NO exceeded 100 ppm whereas peak concentrations of NO₂ rarely exceeded 10 ppm (Robertson *et al*, 1981). There was no difference in the long term decline in lung function between men in collieries identified as low NO_x and those with much higher levels NO_x, after exposure to coalmine dust had been taken into account. No association was found between NO_x exposure and persistent cough, sputum production and breathlessness although dust exposure was associated with all three. Cumulative levels of dust exposure were marginally higher in the high NO_x group (174 compared with 148 gh/m³). In a follow up study, Jacobsen *et al* (1988) found no relationship between exposure to NO or NO₂ and susceptibility to respiratory infection. Given the extent of dust exposure and associated respiratory damage, it is possible that dust effects completely masked any impact of NO.

WHO (1997) reviewed several workplace studies in which workers were exposed to NO_x arising from diesel combustion. Although the WHO (1997) considered NO₂ as being the important component of NO_x in these studies, workers would have also been exposed to NO, with the ratio of NO to NO₂ being highest in studies of underground workers (potentially between 5:1 and 10:1). In a study of workers in bus garages, Gamble *et al*. (1987) reported an increased prevalence of acute respiratory symptoms in workers with mean exposures to NO₂ > 0.3 ppm and no effects on lung function. In a study of salt miners with mean exposures

ranging for 0.2 to 2.5 ppm, Gamble *et al.* (1983) found no relationship between exposure to NO₂ and cough, breathlessness or reduced lung function.

SCOEL cited a study that reported a small dust-dose dependent decrement in lung function in salt miners with mixed exposures to salt dust, diesel exhaust, NO, NO₂ and CO (Lotz *et al.*, 1998). Subsequently these authors have reported small effects on the immune system as assessed from white blood cell counts and specific counts of T cells (Backe *et al.*, 2004). The studies were unable to attribute effects to individual pollutants.

6.2 EPIDEMIOLOGICAL STUDIES OF TUNNEL WORKERS

There have been a number of studies of the respiratory health of tunnel workers but these have focused on the potential impacts of exposure to dust and NO₂. Concentrations of NO are typically not reported and the role of NO was not investigated. Although these studies are of very limited value in assessing the potential effects of NO exposure, they do provide some indirect evidence of potential harm arising from NO exposure.

Bakke *et al.* (2004) compared lung function changes over an average period of six years in 651 Norwegian construction workers (drill and blast workers, tunnel concrete workers, shotcreting operators, and tunnel boring machine workers) with outdoor concrete workers, foremen, and engineers. The annual decline in lung function in tunnel construction workers was greater than that of the control group for both non-smokers and ever smokers. After adjustment for age and observation time, reduced lung function was more strongly associated with cumulative exposure to NO₂ than exposure to dust in both non-smokers and ever smokers. NO was not specifically considered. Given that NO₂ and NO concentrations are likely to have been strongly correlated and the probable high ratio of NO to NO₂ concentrations, it is possible that exposure to NO could have contributed to the observed effects. An earlier Norwegian cross sectional study of 212 tunnel workers and a reference group of 205 outdoor construction workers reported that the prevalence of chronic obstructive pulmonary disease (COPD) was 14% compared with 8% in the reference subjects (Ulvestad & Lund, 2003, Ulvestad *et al.*, 2000). The tunnel workers also reported significantly higher occurrence of respiratory symptoms and showed a significantly greater reduction in lung function than the control workers that was related to years of exposure (Ulvestad *et al.*, 2000). In a subgroup of 29 non-smoking concrete workers who had worked in a tunnel environment for one year, markers of nasal inflammation (increased exhaled NO levels and nasal mucosal swelling) were significantly higher than in subjects who had performed similar tasks outdoors. The tunnel workers had a higher geometric mean exposure to total dust and respirable dust than the reference subjects but were also exposed to significantly higher levels of quartz, oil mist, and NO₂. Peak concentrations of NO₂ were 48.5 ppm compared with long term mean concentrations of 0.50 ppm (95% CI 0.4 to 0.7). The highest levels of exposure were measured when the tunnel workers passed through the blast cloud during transportation of the blasted mass. High concentrations of NO₂ were also measured during loading of the blasted rock onto diesel powered trucks (1.86 ppm, 8 h TWA). Exposure to NO was not considered but was likely to have been correlated with that of NO₂. Measurements made in UK collieries during the 1970s suggested that mean ratios of NO to NO₂ after shotfiring were in the range 5 -10.

In a study of 96 Norwegian tunnel workers and a reference group of 249 other heavy construction workers, examined in 1991 and re-examined in 1999, Ulvestad *et al.* (2001) reported a significant decline in lung function associated with exposure to respirable dust and quartz and an increased risk of developing respiratory symptoms associated with respirable dust. The mean exposure to respirable dust and quartz in tunnel workers varied from 1.2-3.6 mgm⁻³ (respirable dust) and 0.019-0.044 mgm⁻³ (quartz) depending on job task performed. Correlations of NO_x were strongly correlated with those of dust and quartz and a separate investigation of their effects was not undertaken. It is not possible to assess the contribution of NO to the observed relationships between exposure to dust and respiratory health. Given the relatively low levels of exposure to dust, it is plausible than concurrent exposure to NO_x contributed to the observed effects.

Previously Bakke *et al.* (2001) specifically investigated the effects of exposure to blasting

fumes in tunnel workers. Two groups of tunnel workers, one using ammonium nitrate fuel oil (ANFO) as the explosive and the other using size-sensitized emulsion (SSE), with 24 workers per group, were compared with a reference group (N=34) with low exposure. The ANFO workers had the highest exposures to respirable dust, quartz, volatile organic compounds, oil mist and vapours, formaldehyde and NO₂ (NO not measured) but their exposure to total dust and carbon monoxide was not consistently greater than that of the SSE workers. The SSE workers were significantly more exposed to total dust and alpha-quartz than the reference group. The ANFO workers had high peak exposures to NO₂ (up to 20 ppm) whereas exposure concentrations for the SSE workers did not exceed 2 ppm. The lung function of the ANFO workers significantly decreased over an 11 day observation period whereas there were no significant changes among the SSE workers and the outdoor workers. It is likely that concentrations of NO were at least five times higher than the recorded exposures to NO₂.

A US study reported that tunnel workers, labourers, and operating engineers in heavy and highway construction are at increased risk for asthma and that tunnel workers also appear to be at increased risk for chronic bronchitis (Oliver *et al* 2001). Inverse relationships were observed between time in the union, and risk for asthma and chronic bronchitis suggesting that symptomatic workers are self-selecting out of their trade.

Several older studies investigated the respiratory health of toll workers exposed to vehicle exhaust in tunnels. The results of these studies are mixed and exposure to NO was not specifically considered. In a study of lung function and respiratory health in 466 bridge and tunnel workers involved in toll collection and traffic direction who were followed up for at least 3 years, the tunnel workers had significantly poorer lung function, more respiratory symptoms, and higher carboxyhaemoglobin levels than the bridge workers (Evans *et al*, 1988). Individuals working over 20 years had the lowest mean lung function values, showed the greatest decline in lung function, and the most respiratory symptoms. The clinical importance of the observed effects was not clear. In contrast, in a study of 175 US tunnel and turnpike workers, Tollerud *et al* (1983) found no relationship between high levels of exposure to automobile exhaust and respiratory symptoms, illness or lung function following a minimum of 3 years exposure. Although these studies of toll workers are not particularly informative, it is possibly of relevance that in one study, greater effects were observed in workers exposed to vehicle exhaust in tunnels than on bridges. The ratio of NO to NO₂ in underground air would be expected to be considerably greater than for a bridge environment.

In conclusion, the results of most studies of tunnel workers suggest that dust exposure is significantly correlated with reduced lung function and respiratory symptoms but do not exclude a role for NO_x. One study found a specific relationship between reduced respiratory health and NO₂ that might be partly or wholly attributable to NO exposure. Levels of NO exposure are likely to have been high. Long term mean levels may have been between 2.5 and 5 ppm or more as an 8 hour TWA and short term peaks of concentration may have exceeded 100 ppm. There is limited evidence that tunnelling work may be associated with a greater adverse impact on respiratory health than other types of construction work. Given that ratios of NO to NO₂ are much higher in underground environments than in other workplaces, this may be partly due to NO exposure and/or the combined effects of NO and dust exposure, although exposure to dust and other pollutants is likely to be higher within a tunnel environment in other construction environments.

6.3 ENVIRONMENTAL EXPOSURE

Most studies of the effects of ambient air pollution have investigated the impacts of NO₂ rather than NO. Typical concentrations of NO in UK air are less than about 0.03 ppm as a long term mean, although peak concentrations may exceed 0.5 ppm (Table 6.1).

Table 6.1: Concentrations of NO measured at UK automatic monitoring sites in 2004 (Netcen, 2005)

Type of site	No of sites	Annual mean (ppm)			Mean maximum 1 hour mean in year (ppm)		
		mean	range		mean	range	
Roadside	14	0.063	0.020	0.119	0.632	0.356	0.872
Rural	5	0.002	0.001	0.003	0.061	0.035	0.088
Suburban	5	0.021	0.010	0.052	0.508	0.405	0.672
Urban background	17	0.011	0.006	0.026	0.574	0.281	1.117
Urban centre	16	0.028	0.014	0.047	0.584	0.371	1.044

In a French study of the long term effects of air pollution on mortality, Filleul *et al* (2005) reported adjusted risk ratios (95% CI) for total suspended particulate (TSP), black smoke, NO₂, and NO for non-accidental mortality of 1.05 (1.02 to 1.08), 1.07 (1.03 to 1.10), 1.14 (1.03 to 1.25), and 1.11 (1.05 to 1.17) for 25 years exposure to 10 $\mu\text{g m}^{-3}$ respectively. On a time averaged basis, community exposure to 10 $\mu\text{g m}^{-3}$ of NO over a 12 month period would be equivalent to workplace exposure to 50 $\mu\text{g m}^{-3}$ (= 0.04 ppm) over the same period. In a study of emergency hospital admissions for children with asthma admissions in Belfast, Thompson *et al* (2001) reported small associations with PM₁₀ (relative risk = 1.10), sulphur dioxide (relative risk = 1.09), NO₂ (relative risk = 1.11), NO (relative risk = 1.07), NO_x (relative risk = 1.10), carbon monoxide (relative risk = 1.07), and benzene (1.14); no associations were noted between meteorological factors (temperature and rainfall) or ozone and asthma emergency-department admissions. Benzene, however, was the only variable that was independently associated with asthma emergency-department admissions in children. In a Danish study that examined the short-term relationship between carbon monoxide, NO, NO₂, NO_x, sulphur dioxide, ozone, and black smoke and respiratory health in children, Keiding *et al* (1995) found that nitric oxide and NO_x, appeared to be associated with slight exacerbation of respiratory illnesses among children. Similarly, in a 3 year study in Helsinki, Ponka (1991) found that hospital admissions for asthma were significant correlated with ambient air concentrations of NO₂, NO, sulphur dioxide, carbon monoxide, ozone and TSP. Regression analysis revealed that NO and ozone were most strongly associated with asthma problems. Long term mean concentrations of NO₂ were 39 $\mu\text{g m}^{-3}$. No data were provided for NO.

6.4 COMPARISON WITH NO₂

6.4.1 Workplace exposure

There are relatively few studies of the effects of workplace exposure to NO₂ as distinct from NO_x. Douglas *et al.* (1989) reported that workers exposed to silo gas (NO₂ levels from 200 to 2000 ppm) experienced hypoxaemia and transient airway obstruction leading to death. Although transient exposures to high concentrations of NO₂ may be followed by a symptom-free period, severe respiratory failure can develop several hours later (Meulenbelt & Sangster, 1990). Overall, there is no evidence that long term exposure to NO₂ (or concurrent co-exposure to NO) in the workplace is associated with severe adverse effects.

6.4.2 Exposure to NO_x in ambient air

The results of the numerous epidemiological investigations of the short-term effects of exposure to NO₂ are inconsistent and there is considerable uncertainty about the relationship between personal exposure concentrations of NO₂ and the measurements used in epidemiological analyses. The health endpoints for which there is the most consistent evidence of a relationship with NO₂ include daily mortality, emergency hospital visits for asthma and use of primary health care facilities (Searl, 2004). There is very limited evidence to suggest that co-exposure to NO₂ may increase the harmfulness of exposure to airborne particles (Katsouyanni *et al*, 2001). The available data do not allow comparison between the effects of exposure to NO and NO₂ in ambient air.

There has been considerable debate about the true importance of NO₂ in the apparent association between NO₂ and adverse health effects that has been reported in many studies of the short term effects of air pollution on health. Concentrations of NO₂ are generally strongly correlated with those of airborne particles and it is difficult to determine the independent effects of each pollutant. Tobacco smoking has a substantial influence on personal exposure to NO and NO₂ and is a much more important source of exposure to these gases for smokers than ambient air. Smoking and exposure to secondary tobacco smoke may dominate exposures to NO and NO₂, thus obscuring the role of ambient NO_x in the relationship between air quality and health. NO₂ is strongly associated with traffic pollution and a number of authors have suggested that NO₂ is merely a marker of traffic pollution and the actual causal agent is some other compound of traffic pollution such as fine particles (Seaton & Dennekamp, 2003). The apparent health effects of NO₂ often disappear in multipollutant models that include particles and/or ozone. Other studies, however, have reported significant associations between NO₂ and various health endpoints even in the absence of a strong correlation between concentrations of NO₂ and fine particles. Overall, the evidence points towards a small independent role for NO₂ in the association between air quality and health that is separate from that associated with fine particles (Searl, 2004). Given that concentrations of NO and NO₂ are strongly correlated and the lack of investigation of any potential independent role for NO, it is possible, perhaps likely, that effects attributed to NO₂ in epidemiological studies are partly or wholly due to exposure to NO. The inconsistency of the results of epidemiological studies of the impacts of NO₂ would be consistent with the role of NO/NO₂ being much less important than either particles or ozone, in the association between air quality and health.

6.5 CONCLUSIONS

Most studies of the potential health effects of workplace or environmental exposure to NO_x have focussed on the potential role of NO₂ and it is difficult to determine the potential influence of NO on health. Studies in miners suggest that long term exposure to a mean concentration of NO of about 1 ppm does not have an important influence on health in comparison with that arising from concurrent dust exposure. There is limited data from studies of tunnelling workers, that long term exposure to a mean concentration of NO₂ of about 0.5 ppm may adversely affect lung function. The inferred level of concurrent exposure to NO might be of the order of 2.5-5 ppm.

Long-term exposure to relatively low concentrations of NO_x in ambient air (mean levels of NO <0.05 ppm) may be associated with an increased risk of respiratory illness. Exposure to NO_x in ambient air may contribute to the overall burden of ill health due to air pollution. The separate role of NO and NO₂ have not been extensively investigated and, although effects have been attributed to NO₂, there are no data to exclude a role for NO.

7. Therapeutic use of NO in humans

7.1 CLINICAL USE OF NO

NO is administered to patients with severe hypoxia due to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) and neonatal ARDS in order to improve the uptake of oxygen by the lungs. Administered by inhalation, NO diffuses to the vascular smooth muscle cells and activates enzymes leading to relaxation of the smooth muscle in the vessel walls and vasodilation (McDonald & Murad, 1995). As NO is rapidly bound to and inactivated by haemoglobin, its effects remain limited to the pulmonary circulation, without causing systemic vasodilation (Rimar & Gillis, 1993). The dilation of the blood vessels in well ventilated areas of the lung in response to NO inhalation, increases blood flow and the circulation of oxygenated blood. NO may also reduce blood flow in poorly ventilated areas of the lung by inhibiting the endogenous production of NO (Wang *et al*, 2003). In transplant operations, NO may protect the lung against damage by inhibiting the release of chemical signals that would trigger an influx of white blood cells (specifically polymorphonuclear neutrophils) into the lung and the adhesion of these inflammatory cells to the alveolar surface (Kubes *et al*, 1991). Inhaled NO therapy is delivered by blending dilute NO gas into the ventilator inlet gas and via an endotracheal tube / tracheostomy to the lower airway.

NO therapy is still controversial. Although inhaled NO has been demonstrated to improve oxygenation, it has not been proven to significantly affect survival (Gaston, 2006). The use of NO is most widely accepted for the treatment of newborn babies and the RxList Monograph indicates that the recommended maximum dose is 20 ppm. Treatment should be maintained for up to 14 days or until arterial oxygen levels are sufficient and the neonate is ready to be weaned from NO therapy. In clinical trials, some patients who showed no improvement in blood oxygenation at 20 ppm showed improvement on an increase in dose to 80 ppm. The risk of methaemoglobinaemia and elevated NO₂ levels is significantly increased in neonates at doses >20 ppm. In one study in neonates, NO₂ levels were <0.5ppm at concentrations of up to 20 ppm NO compared with 2.6 ppm at NO concentrations of 80 ppm. Abrupt discontinuation of NO therapy may cause worsening oxygenation and pulmonary hypertension. Severe contraction of pulmonary blood vessels may result as a result of the down regulation of endogenous NO production in the presence of exogenous NO (Wang *et al*, 2003).

Most current dosing recommendations for the treatment of adults do not exceed 40 ppm. The timing of NO administration has a critical impact on its therapeutic benefit for patients with acute respiratory illness. Administration before the onset of inflammation or very early in the inflammatory process may attenuate the inflammatory response in the lung and elsewhere in the body. Inhaled NO does not reduce the inflammatory response, once inflammation is already in process, and may even increase peroxynitrite production causing increased oxidative damage (Wang *et al*, 2003; Gaston, 2006).

Methemeoglobinaemia is generally uncommon in adults at NO concentrations less than 80 ppm. In adults with pulmonary hypertension, peak MetHb levels of 9.6% and 14% were reported in two patients after 108 and 18 hours of 80 ppm NO inhalation (Wessel *et al*, 1994). Maximal MetHb levels in adults are reached 3–5 hours after initiation of NO inhalation (Young *et al*, 1994). At concentrations of greater than 100 ppm combined with oxygen therapy, extensive formation of NO₂ is likely.

Patients vary widely in their response to NO and most studies have failed to demonstrate clear dose-response relationships. In many studies, fewer than half of the patients showed improvement on NO therapy, although others within the same study have shown substantial benefits. The effective dose level for ARDS has reported as 20 ppm (Benzing *et al*, 1998), 5 ppm (Iotti *et al*, 1998) or as low as 2 ppm (Yoshida *et al* (1997; Puybasset *et al*, 1994). Puybasset *et al* (1994) reported that in two patients, 91% and 74% of the pulmonary vasodilation achieved was obtained for inhaled NO concentrations of 0.1 ppm and Benzing *et al* (1998) also reported that dilation of blood capillaries occurred at 1 ppm. Radovancevic *et al* (2005) found that concentrations of 40, 60 or 80 ppm were effective in treating pulmonary hypertension in some heart transplant patients.

Studies of NO therapy in neonates and children with acute respiratory distress syndrome have produced variable results. Demirakca *et al* (1996) reported maximum benefits at 20 ppm in neonates compared with 10 ppm in older children. Okamoto *et al* (1998) reported benefits in children at concentrations of 1 ppm and an optimal concentration of 4 ppm. In a study of the newborn, Cornfield *et al* (1999) found that a concentration of 2 ppm did not improve oxygenation but did slow the rate of clinical deterioration, whereas treatment at 20 ppm improved oxygenation in initially untreated infants but not in infants initially treated at 2 ppm. A US study that examined the outcomes of NO therapy in infants at 36 centres found no evidence that higher doses improve outcome (Ream *et al* 1999). Patients receiving prolonged inhaled NO at doses of ≤20 ppm maintained MetHb levels of <3.0% and circuit concentrations of NO₂ of <1 ppm.

There is limited evidence that prolonged administration of NO to treat pulmonary arterial hypertension leading to severe breathlessness over a 2 years period led to an improvement of symptoms (Perez-Penate *et al*, 2005). There is some evidence that chronic administration may damage the cells lining the terminal airways, specifically causing ciliary depletion and epithelial hyperplasia (abnormal cell proliferation, potentially leading to fibrosis).

7.2 RELEVANCE OF CLINICAL EXPERIENCE WITH NO TO RISK ASSESSMENT

The value of NO in the treatment of respiratory illness, particularly pulmonary hypertension and ALI/ARDS is still not fully established. While some patients have clearly experienced short term benefits as a result of NO therapy, others have failed to respond to treatment. The concentrations used by different groups are widely variable but beneficial effects have been reported at inhaled concentrations of <1 ppm. The therapeutic use of NO is usually restricted to a few hours or days and there is little experience of long-term effects. There is some evidence that prolonged administration could cause damage to the terminal airways.

It is difficult to extrapolate from impacts in adults or children with severely compromised respiratory health to potential impacts in healthy individuals but it would seem prudent to control workplace exposures to levels below those observed to have important clinical effects.

8 Summary of findings and assessment of risks associated with nitric oxide exposure

8.1 PHYSIOLOGICAL IMPORTANCE OF ENDOGENOUS NO

Endogenous NO plays an important role in intracellular and intercellular signalling, in maintaining the health of blood vessel walls, in oxygen exchange within the lung, in cellular respiration and in regulating processes of inflammation and the generation or inhibition of reactive oxygen species.

Inhalation of NO would be expected to interfere with the normal balance between endogenous NO synthesis, tissue levels of NO and NO concentration in exhaled air. A moderate increase in the concentration of NO in inhaled air might cause a reduction in endogenous NO synthesis, but may not necessarily affect tissue levels and the normal operation of physiological processes involving NO. A sudden increase might cause a temporary increase in tissue levels with adverse effects on cellular respiration and the potential formation of peroxynitrite with consequent oxidative tissue damage. A sudden reduction from a high concentration of NO in inhaled air might cause a deficit in tissue levels of NO leading to pulmonary hypertension and reduced oxygenation of circulating blood.

Endogenous NO may play an important role in enhancing the inflammatory response to inhaled mineral dust particles.

Concentrations of NO in some workplace environments are substantial relative to the concentrations normally present in exhaled breath. It seems plausible that exposure to 1 ppm NO in workplace air, a concentration 50 times greater than that normally present in exhaled air, could affect cellular function within the lung.

8.2 ANIMAL DATA

The results of animal experiments do not provide consistent evidence for the toxicity of NO at low levels of exposure. Evidence of emphysema like changes in lung structure has been reported in some experiments following continuous exposure to ≤ 2 ppm, but similar changes have not been reported in other experiments in which animals have been exposed to concentrations ≥ 2 ppm. In an experiment where emphysema-like changes were observed in rats following long-term exposure to concentrations of less than 2 ppm, twice daily spikes in the concentration of NO may have played a role in the development of lung damage. Emphysema has also been reported following intermittent exposure (2 hours/day, 5 days/week) to 10 ppm. Higher levels of exposure to NO give rise to oxidative damage to lung tissue similar to that observed with NO₂ and ozone and have been associated with effects on epithelial cell turnover that might ultimately lead to lung fibrosis. The lowest concentration associated with effects on lung epithelial cells is 2 ppm.

The inconsistency of the findings of animal experiments may reflect the complexity of the interaction between inhaled and endogenous NO. It is possible that the mechanisms leading

to tissue damage at low levels of NO exposure differ from those that are initiated by higher levels of exposure.

Overall, the results of animal experiments suggest that medium term exposure to concentrations of NO of less than 1 ppm is unlikely to cause serious damage to lung tissue whereas long term exposure to concentrations greater than 10 ppm may cause serious damage. In addition, the small changes in lung tissue that may occur at concentrations of less than 2 ppm, may be associated with the long term development of emphysema. Slightly higher levels of exposure may be associated with an increased risk of developing lung fibrosis.

8.3 HUMAN DATA

The results of human volunteer experiments suggest that small effects on lung function may arise at concentrations of NO of only 1 ppm. Studies of workers exposed to NO suggest that long term exposure to mean levels of about 1 ppm in coal mines has no important effect on respiratory health in comparison with that arising from exposure to elevated concentrations of respirable dust. Exposure to higher mean concentrations of NO (8.6-26.5 ppm) in the absence of dust, but with co-exposure to NO₂, may have effects on immune function. Repeated exposure to elevated concentrations of NO as a result of explosives use in tunnels may adversely affect lung function and increase the risk of respiratory symptoms. The exact levels of exposure to NO at which respiratory damage may occur in tunnel workers have not been established, but it is likely that, for some workers, short term peak exposures have exceeded 100 ppm. Long term changes in respiratory health reported in tunnel workers may be associated with mean exposure concentrations of about 2.5-5 ppm (as inferred from reported NO₂ concentrations). Long term exposure to mean concentrations of NO of less than 0.1 ppm in ambient air may be associated with an increased risk of mortality, although the effects are difficult to separate from those of other air pollutions. Short term exposure to elevated concentrations of NO (0.5-1 ppm) in ambient air is associated with an exacerbation of symptoms in children with asthma which would be consistent of up regulation of airways inflammation.

Investigations of the therapeutic value of NO suggest that inhaled NO may have physiological effects at concentrations of only 0.2 ppm in some individuals with seriously compromised respiratory health. A concentration of about 5 ppm gives rise to maximum therapeutic benefit whereas concentrations of more than 80 ppm may be damaging.

8.4 OVERALL EVALUATION OF THE AVAILABLE DATA

The effects of low level exposure to NO are difficult to predict. The interaction of inhaled NO with processes dependent on endogenous NO may lead to suppression or enhancement of these processes. Exposure to concentrations of between 0.2 and 1 ppm NO in inhaled air is likely to affect cellular function within the airways and lungs. The health significance of these effects in most individuals is uncertain as any changes in cellular function may simply be an adaptive response that is reversed on cessation of exposure.

Long term exposure to nitric oxide may be associated with the development of respiratory illness. Repeated exposure to high levels of NO in the workplace may give rise to an increased risk of emphysema. Low level exposure to NO may promote or inhibit the inflammatory response to dust, other pollutants and/or infection. Moderate to high levels of exposure to NO may enhance the inflammatory response to inhaled dust and other pollutants. Exposure to NO may also give rise to an increased risk of pneumoconiosis or silicosis in workers with co-exposure to dust and quartz.

There is little evidence that repeated exposure to concentrations of NO of less than 1 ppm would be likely to lead to irreversible respiratory illness. Both the animal and human data suggest that repeated exposure to concentrations of NO of between 1 to 10 ppm in the workplace is likely to be associated with irreversible effects on the lungs, but only modest effects on respiratory health. The variability of NO concentrations during the working day, before, during and after work and on successive work days may influence on the potential

harmfulness of NO exposure.

Short term exposure to concentrations of NO of 80 ppm or more is likely to give rise to acute toxicity arising from the formation of MeHb in blood. The immediate effects are likely to be reversible, but long term exposure to concentrations of 80 ppm in the workplace would be expected to give rise to serious respiratory illness.

The toxicology of NO is distinct from that of NO₂. There may be a greater risk of harm associated with repeated exposure to NO than associated with repeated exposure to NO₂. Repeated exposure to either gas may be associated with an increased risk of respiratory illness.

There may be considerable variability in the response of individuals to a given concentration, but it is not clear what specific characteristics might give rise to an increased risk of adverse effects. Given the impacts of respiratory illness and other diseases on endogenous NO activity, it seems likely that the effects of inhaled NO will be partly determined by an individual's pre-existing health status.

Exposure-response functions have not been established for NO. Table 8.1 gives an indication of the relative level of risk associated with different levels of exposure. It is not possible to provide quantified estimates of risk associated with different levels of exposure. As an approximate guide, it is possible that for ten years repeated exposure, a "small risk" might equate to effects developing in <2% of individuals, a "risk" might equate to effects developing in 2-10% of individuals and a "more substantial risk" might equate to effects developing in >10% of individuals. These estimates are very uncertain and not scientifically defensible.

Table 8.1: Inferred exposure-response information for NO based on the reviewed studies in animals and humans

Concentration of NO ppm	Effects of short term exposure	Effects of repeated exposure	Comments
0.2	Small reversible effects on cellular function in the lungs in a small proportion of individuals	None expected	Inhalation of less than 0.1 ppm NO affects exhaled NO concentrations suggesting interference with endogenous NO; Concentrations of NO of 0.1 ppm have been shown to be of therapeutic benefit to some individuals with severe respiratory illness; some evidence that NO in ambient air (concentrations typically less than 0.1 ppm) may be associated with a small loss of life expectancy and increased risk of respiratory symptoms
1	Small effects on cellular function in the lungs in many individuals; small effects on respiratory function in a small proportion of individuals	None expected	Inhalation of low concentrations of NO may interfere with endogenous NO processes in the lung leading to an adaptive response. In a human volunteer experiment, effects on airways conductance were observed in some individuals at 1 ppm. No association was found between NO and respiratory ill health in miners with long term exposure to about 1 ppm, although effects may have been masked by concurrent exposure to dust.
2-5	Effects on cellular function in the lungs in a large proportion of individuals	Small risk of irreversible changes in the lung predisposing to emphysema or lung fibrosis, possible long term decline in lung function; increased risk of the development of respiratory illness as a result of co-exposure to other substances	Emphysema-like changes in the lung have been reported in rats exposed continuously to 0.5 ppm with twice daily peaks of 1.5 ppm although similar effects were not observed in rats exposed continuously to 6 ppm. Emphysema like changes were also observed in dogs exposed continuously to 1.6 ppm with concurrent exposure to 0.3 ppm NO ₂ . Damage to epithelial cells has been observed following long term exposure of rats to 2 ppm. Effects on lung function reported in tunnel workers with long term mean exposures to NO ₂ of about 0.5 ppm that are likely to have been associated with concurrent exposures to NO of about 2.5-5 ppm. There is no specific information about the effects of co-exposure to NO and other pollutants but endogenous NO has been shown to be important in mediating the inflammatory response to mineral dust.
10	Reversible effects on cellular function in the lungs in a large proportion of individuals,	Risk of irreversible changes leading to emphysema or lung fibrosis, long term decline in lung function, increased risk of the development of respiratory illness as a result of co-exposure to other substances	Mice exposed to 10 ppm NO for 2 hours/day, 5 days/week for 30 weeks developed emphysema. Effects on natural killer cell activity were observed in workers exposed to concentrations of about 9 ppm with concurrent exposure to about 3 ppm NO ₂ .
25	Reversible effects on cellular function in the lungs in most individuals, effects on lung function in a high proportion of individuals	More substantial risk of irreversible changes in the lung leading to emphysema or lung fibrosis, increased risk of the development of respiratory illness as a result of co-exposure to other substances	Tunnel workers with short term exposures to NO that are likely to have exceeded 25 ppm showed a measurable decline in lung function over an 11 day period

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