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Associate Editor: SUSMITA CHATTERJEE

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Pathophysiology of Nitrate Toxicity in Human and its Mitigation Measures

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ABSTRACT

Nitrate and Nitrite are natural ions of the nitrogen cycle and are commonly found in groundwater and surface waters. The nitrate ion (NO_3^-) is the most stable form of nitrogen in oxygenated environments, thus all nitrogen-containing molecules can act as sources of nitrates. Under acidic conditions, nitrites (NO_2^-) are formed naturally from nitrates, and nitrites in turn may combine with amines or amides to form N-Nitroso compounds (nitrosamines).

For human consumption, WHO report, 2004 permits up to 50 mg/L but for short term exposure only, whereas IS-10500 permits 45 mg/L (as NO_3) as desirable limit and 100 mg of NO_3 per liter as maximum permissible limit in the absence of alternate source.

The problem of nitrates is endemic–both internationally and nationally. The epidemiological data are scarce but some surveys indicate that in certain areas people are per force consuming water with 1000 mg/l nitrate due to absence of any alternate source. It is very difficult to remove nitrates from water because it is chemically non reactive in dilute aqueous solutions.

In human the sources of nitrate are drinking water, vegetables, preservatives of food and cooking in aluminum utensils. Main sources of nitrate contamination in water are improper disposal of sewage and industrial effluents; and, indiscriminate and excessive use of manure/ nitrogenous fertilizers in agriculture.

After ingestion the ingested nitrate gets converted to nitrite by micro flora in the oral cavity and in the gastrointestinal tract. This results in increased oxidation of hemoglobin to methemoglobin, leading to methaemoglobinemia. Simultaneously, increased production of free oxide radical and free radical nitric oxide occurs. These radicals, predispose cells for irreversible damage. Further this metabolic outcome predisposes person for carcinogenic and other effects. The other effects observed were increased infant mortality,

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abortions, birth defects, recurrent diarrhea, recurrent stomatitis, early onset of hypertension, histopathological changes in cardiac muscles, alveoli of lungs and adrenal glands, recurrent respiratory tract infection in children, hypothyroidism and diabetes. Recent ongoing studies indicated that high nitrate ingestion adversely affects the immune system of the body.

Recently some studies have indicated that an adaptation process (Cytochrome b_5 reductase adaptation) in the human body becomes active with increasing nitrate consumption to compensate for undesired effects of nitrate toxicity.

Ascorbic acid, methionine, alpha-tocopherol and methylene blue have been found to be effective in treating the nitrate toxicity. Role of antioxidants to counter act the nitrate toxicity is controversial.

For prevention denitrification of drinking water can be done, but these processes are difficult to implement and maintain. The other way of prevention is to protect most vulnerable age group from nitrate toxicity through simple interventions, e.g., breast-feed infants preferably up to an age of 4 months, avoid high nitrate containing food for weaning, and avoid high nitrate water to pregnant women and avoiding WHO ORS preparation with locally available high nitrate water during diarrhoea. Recent studies are in favor that long term uses of anti acid secretary agents e.g. H_2 receptor inhibitors/proton pump inhibitors/ antacids etc. especially in pregnant mothers and children may be hazardous. In case if it is necessary to use these drugs, they should be used cautiously and preferably with the antioxidants.

1. INTRODUCTION

Naturally occurring nitrate levels in surface and ground water are generally a few milligrams per liter. In many ground waters higher levels are found due to water percolating through nitrate rich rocks and also due to an excessive use of chemical fertilizers. World Health Organisation (WHO) report, 2004 maintains that extensive epidemiological data support limiting the value of nitrate-nitrogen to 10 mg/L or as nitrate to 50 mg/L (WHO, 2004) for human consumption whereas IS-10500 prescribes maximum permissible limits of nitrate in drinking water as 45 mg of NO_3 per liter (IS 10500, 1995).

The problem of nitrates is endemic internationally, as well as nationally but so far no compiled data at national level are available. The data available are either of small areas or of scattered zones/areas. Many workers tried to compile status of nitrate in drinking water in Rajasthan state, India (Kumar *et al*., 2002; PHED Habitat Survey, 1991–93).

2. EPIDEMIOLOGY

The problem of nitrate toxicity is global and has been reported from a number of countries (Prakasa Rao and Puttanna, 2006).

2.1. International Perspective

Nitrate is a widespread contaminant of ground and surface waters worldwide (Prakasa Rao and Puttanna, 2006; Singh *et al*., 1994). In the United States, the problem appears to be concentrated in the Mid-West and the Far-West, with large areas of Iowa, Illinois, Kansas, Michigan, Wisconsin, Washington and California being heavily affected (Revenga and Mock, 2001; Smith *et al*., 1994). The USGS

(US Geological Survey-1994) reported that nitrate concentration in the nation's groundwater supply was increasing steadily. In South America, nitrate concentrations in the monitored watersheds are relatively low and follow human land use. The highest nitrate concentrations are found in the Uruguay watershed, where intensive agriculture is practiced. Likewise, nitrate concentrations in water are also greater in the Magdalena watershed of Colombia than in the less densely populated watersheds of the Amazon basin. The nitrate concentrations in South America correspond to lower fertilizer application rates in comparison to Europe.

Nitrate concentrations are higher in watersheds that have been intensively used and modified by human activity, such as the Weser, Seine, Rhine, Elbe, and Senegal. High levels are also found in such watersheds in China, South Africa, and the Nile and Mississippi basins. South Africa has some of the highest natural nitrate levels in the world (more than 550 mg/L NO_3^--N) especially in the Kalahari region (Colvin *et al*., 2008).

More detailed data are available in Europe and show distinct regional trends in the concentrations of nitrates in rivers (Scheidleder *et al*., 1999). Nitrate loadings are highest in areas with intensive livestock and crop production, especially in the northern parts of Western Europe. Nitrate concentrations are lowest in Finland, Norway, and Sweden.

2.2. National Perspective

Nitrate from sources like mineralized organic matter, fertilizers, organic manures, industrial effluents or urban wastes, results in groundwater pollution which has received attention in various parts of India. The available data from various studies point to the fact that nitrate pollution from any of the above sources can pose a serious health hazard to humans, animals, birds and aquatic life. The data available on concentrations of nitrate in groundwater samples from Punjab, Haryana, U.P., Delhi, Orissa, Tamil Nadu, Bihar, A.P., M.P., Maharashtra, Karnataka and other states point to the fact that in many of these samples, the nitrate content in groundwater has been much more than permissible limits. In many parts of Punjab and Haryana nitrate levels in groundwater is continuously increasing beyond the critical standard. Nitrate levels in groundwater over vast agricultural areas can be correlated with intensive irrigated agriculture, use of nitrogenous fertilizers, and groundwater exploitation. Workers tried to evaluate the nitrate pollution and its strategies, for reducing it (Prakasa Rao and Puttanna, 2006). Nitrate pollution of groundwater by fertilizer use in India has also been reviewed (Nitrate Pollution in ground water, 2007).

The details of International and National status of nitrate pollution is given in Annexure I

3. SOURCES

There are two sources of nitrogen nitrate for human beings. (1) Exogenous sources and (2) Endogenous sources.

3.1. Exogenous Sources

Wastes containing organic nitrogen are decomposed in soil or water by microbial action forming ammonia, which is then oxidized to nitrite and nitrate. Because

nitrite is easily oxidized to nitrate, it is primarily nitrate that is found in groundwater and surface waters. Nitrate-containing compounds in the soil are generally soluble and readily migrate with groundwater. Nitrosamines have a short lifespan in ambient air. Vegetables (cauliflower, spinach, collard greens, broccoli, and root vegetables) account for more than 70 percent of the nitrates ingested in the human diet. The remainder of nitrate in a typical diet comes from drinking water (21 percent) and meat and meat products (6 percent). Nitrates are naturally occurring inorganic ions, which are part of the nitrogen cycle, and are commonly found in groundwater and surface waters. The nitrate ion (NO_3^-) is the most stable form of nitrogen in oxygenated environments, thus all nitrogen-containing molecules can act as sources of nitrates.

3.1.1. Water and Food

Main sources contributing to nitrate content of natural waters are atmosphere, geological features, anthropogenic sources, atmospheric nitrogen fixation and soil nitrogen. Oxides of nitrogen are generated through lightening and reach to ground with rain water. Recent reports indicated that atmospheric contributions amount to 25% of total load of nitrate (WHO, 2003). Direct discharge to septic tanks, sewage and industrial effluent are other contributors. Excessive use of chemical fertilizers is one of the main sources of nitrate in water.

Nitrogen is an essential major element for plant growth (Allan Wild, 1988). Nitrogen nutrition of crops is very often the limiting factor for food production and nutritional quality of food as indicated by its protein content. Thus, nitrogen has a key role to play in feeding the world population. Since a large response to nitrogenous fertilizers is a universal phenomenon, farmers usually apply large doses of fertilizer. But the recovery of applied nitrogen by the crop rarely exceeds 50% and a large fraction of the surplus nitrogen gets transformed into nitrate form and pollutes the environment including surface and ground waters. The interaction between the various forms of nitrogen in soil, plants, animals and atmosphere constitute the nitrogen cycle (Fig.1).

Although there has been a concurrent rise in fertilizer usage with nitrate concentration in water, particularly where irrigation is practiced, there exist several other sources of nitrate-N, namely sewage effluent, animal excreta, natural soil nitrate and decomposition of soil organic matter. In many regions, natural deposits of nitrate may constitute a major source for nitrate pollution of groundwater. For example, in selected locations in Punjab, Haryana and western Uttar Pradesh, nitrate-N concentration in well water samples was several folds higher than the upper safe limit.

3.1.2. Leafy Vegetables

Being a rich source of nutrients and antioxidants, leafy vegetables occupy an important place in human diet. Some vegetables are known to accumulate high concentration of nitrate under the current practice of heavy fertilization. Vegetables are the major source of the daily intake of nitrate by human beings, supplying about 72 to 94 % of the total intake. On consumption by humans, part of this nitrate is converted to nitrite and N-nitroso compounds that have detrimental effects on human health.

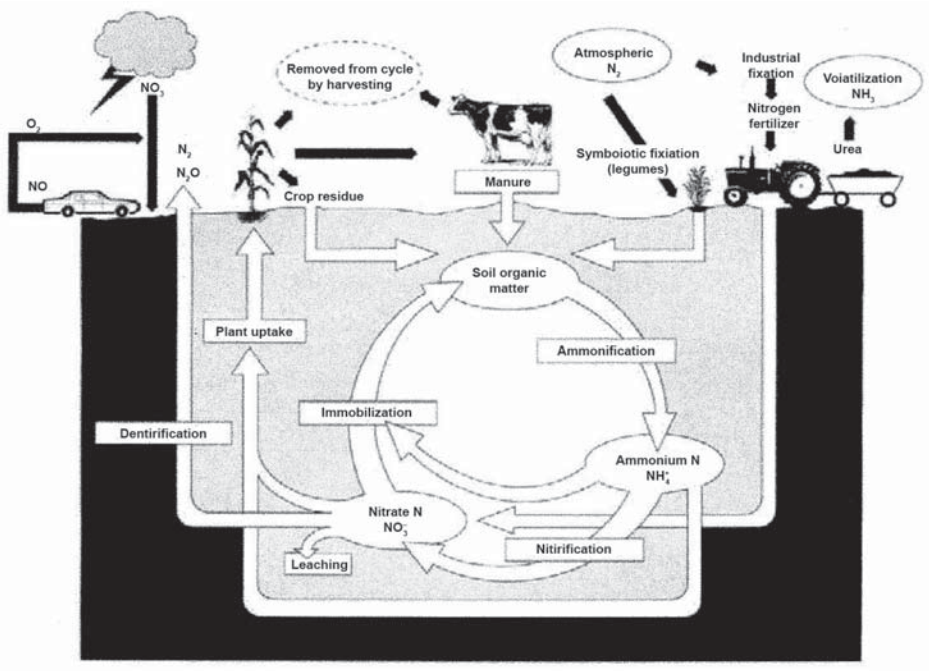


Fig. 1: Nitrogen in an agricultural system (Wolkowski *et al*., Internet pub)

In 1995, the European Commission's (EC's) Scientific Committee for Food (SCF) established the Acceptable Daily Intake (ADI) of nitrate ion as 3.65 mg kg^{-1} body weight (equivalent to 219 mg day^{-1} for a person weighing 60 kg) (Scientific Committee on Food, 1995) and the Joint Expert Committee on Food and Agriculture (JECFA) in the United Nations/WHO established the Acceptable Daily Intake of nitrate as 0−3.7 mg kg^{-1} body weight (Speijers, 1996). Therefore, assuming a 60 kg body weight, the ingestion of 100 g of fresh vegetables with a nitrate concentration of 2500 mg kg^{-1} fresh weight exceeds the Acceptable Daily Intake for nitrate by approximately 13%. For a real assessment, however, nitrate content in all other sources as well as their average daily consumption amount needs to be considered. On the other hand, the USA Environmental Protection Agency (EPA) Reference Dose (RfD) for nitrate is 1.6 mg nitrate N kg^{-1} body weight per day (equivalent to about 7.0 mg NO_3 kg^{-1} body weight per day) (Mensinga *et al*., 2003).

The nitrate content in vegetables available in the markets is reaching as high as 4451 mg kg^{-1} fresh weight in chenopodium and 4293 mg kg^{-1} fresh weight in spinach (Anjana *et al*., 2007a, 2007b). Studies on genotypes of spinach have shown enormous intraspecific variation of nitrate content. One genotype at the three-week stage of plant growth, and six genotypes at the six-week stage, exceeded the ADI limit. Petioles possessed several times higher level of nitrate than leaf lamina. All of the genotypes studied showed diurnal variation in nitrate accumulation with a minimum at noon (Anjana *et al*., 2007a; Anjana *et al*., 2007b).

Studies suggest that nitrate content possesses a significant reverse relationship

with nitrate reductase (NR) activity, the first enzyme in nitrate assimilatory pathway. It is held that NR expression level is largely responsible for different nitrate accumulation patterns in different cultivars of a given species. Therefore, nitrate accumulation in plants can be significantly reduced by over expression of NR genes in high nitrate-accumulating genotypes. In addition, it is also important to enhance expression of NR so that nitrate does not accumulate in the green tissue after converting into nitrite. Overexpression of these genes will reduce the nitrate concentration in vegetables to safe limits for human consumption. Moreover, a careful selection of vegetable genotypes based on their relative levels of nitrate content and NR activity, harvesting young plants at noontime, and removal of petioles from leaves could minimize the dietary intake of nitrate through leafy vegetables. Also, at least 50 % of accumulated nitrate can be removed by cooking vegetables in water (with low nitrate concentration).

The nitrates and nitrites values in vegetables (mg/kg) and lettuce (mg/kg) are depicted in Annexure II.

3.1.3. Other Sources of Nitrate Pollution

Malik (2000) suggested that the presence of nitrate bearing rocks in geological formations as in some parts of Haryana contributed to high nitrate levels in groundwater (Malik RPS, 2000) (Table 1).

3.1.4. Contamination from Agro-Based Industries

The methods of disposal of industrial effluents include diversion to surface water sources (rivers, lakes, canals, etc.), stored in lagoons or ponds for percolation, or spreading or spraying onto the ground surface. The effluents may be treated, partly treated or untreated. The polluted water percolates either directly from the surface water bodies or from agricultural fields (when such water is used for irrigation), and pollutes the ground water. The extent of pollution depends upon the volume of effluents discharged per day and their characteristics (Wakida *et al*., 2004). Under certain circumstances forests also contribute to nitrate pollution.

Food including meat and meat products and vegetables account for 76%, and drinking water accounts for 21% of total human nitrate intake (WHO, 1985 b).

Vegetables and cured meat are the main sources of nitrate and nitrite in diet, but small amounts can be present in fish and dairy products. Nitrate is added to meat as a preservative and to ensure better color and flavor. In general, vegetables

Table 1: Other sources of nitrate pollution (Malik RPS, 2000)

	Agriculture	Municipal	Industrial
Diffuse sources	Use of synthetic nitrogen fertilizers	Combustion engines in vehicles	Atmospheric emission (oxides of nitrogen) from combustion engines and energy production
	Use of organic manures	Disposal of municipal effluents	Disposal of effluents
Point sources	Accidental spills	Badly designed land fills	
	Improper, shelter less composting pits, heaps	Septic tanks	Disposal of wastes into wells, ponds, injection into tube wells, etc.
		Leaking sewerage systems	Badly designed landfills
Linear sources	Contamination of rivers with major groundwater connections		

will be the main source of nitrate intake when nitrate levels in drinking water are below 10 mg per liter (WHO, 1985a). Several vegetables and fruits contain 200 to 2500 mg of nitrate per kg (Chaudhary *et al*., 2010). The nitrate content in vegetables can be affected by processing of the food, the use of fertilizers, and growing conditions, especially the soil temperature and day light intensity. Vegetables such as lettuce, spinach, beetroot, celery, brinjal (egg plant), beets, banana, strawberry, tomatoes, and peas (Paliwal, 1989) often contain nitrate concentration above 2500 mg/kg, especially when they are cultivated in greenhouses. Nitrate levels in food are generally well below 10 mg/kg and rarely exceed 100 mg/kg. Exceptions to this are vegetables that have been damaged, poorly stored, or stored for extended periods as well as pickled or fermented vegetables. In such circumstances, nitrate levels of up to 400 mg/kg have been found (Sinha *et al*., 2008). Nitrate in vegetables have been reviewed (Surindra *et al*., 2009).

Preservatives used in food industry e.g. for preservation of meat and fish, nitrite for meat curing, are significant nitrate sources. Cooking in aluminum utensils enhances reduction of nitrates to nitrite (WHO, 1977e) hence increasing the risk.

Continuous application of nitrogen fertilizers can cause an increase in the concentration of nitrates in fruits, vegetables and food. Maximum admissible level of nitrites in vegetables is 1 mg/kg (Mondal NC *et al*., 2008). In India, Usha *et al. (*Usha, G *et al*., 1993) have studied dietary intake of nitrates. Nitrate contents of cereals ranged from 20 to 76 mg/kg, pulses 39 to 114 mg/kg, leafy vegetables 30 to 270 mg/kg, roots and tubers 31 to 2043 mg/kg and condiments and spices 145 to 4680 mg/kg. The calculated percentage contributions of nitrates from diet in Andhra Pradesh, India reported are: cereals and tubers 41.2; nuts and oil seeds 3.5 and condiments and spices 3.1. Nitrate content in tea has been studied (Regina *et al*., 1998). 50% of the nitrate in dried tea was found to be released into infusion. Variation of nitrate concentrations in spinach genotypes has been studied (Anjana *et al*., 2006). In Brazil, the legally permissible limit of nitrate in milk is 1 mg per litre (Seraphim, 1998). However, measurements have shown 50 to 180 µg per liter nitrate and 20 to 2100 µg per liter nitrate in pasteurized milk (Seraphim, 1998) Groundwater is most frequently used for cultivation of vegetables.

The concentration of nitrate in groundwater has to be taken into consideration for calculating nitrogen requirement of vegetables (Prakasa Rao and Puttanna, 2006). Use of nitrification inhibitors helps to reduce nitrates in food.

Recently, organic methods of cultivation, which exclude the use of chemical fertilizers and pesticides, are gaining importance, especially for horticultural crops. Accumulation of nitrate in soil is not expected in properly implemented organic farming. Therefore, a reduction in nitrate content of fruits and vegetables is expected due to organic method of cultivation. Prakasa Rao *et al.* (2000) reported that nitrate content in *Averrhora carambola* fruit, tomato, guava, papaya, shaddock and *Chrysanthamum coronarium* were not significantly affected by organic fertilization (Prakasa Rao *et al.*, 2000). However, in actual practice, nitrate content in the produce will depend on proper management of the crop. Excessive use of animal manures can lead to high nitrate content in vegetables. It is necessary to analyze nitrate content in organically grown fruits, vegetables and other food articles.

Hydro geological investigations show that nitrate levels are high in sandy soil because of low water holding capacity and high permeability of pollutants like chloride and nitrate than in clayey soil.

Top feed prepared with water containing high nitrate also exposes the infants to high nitrate. As nitrate levels in breast milk are low, the probability of exposure to high nitrate in breast fed infants is very low.

Cooking in aluminum utensils increases the reduction of nitrates to nitrite (WHO, 1985a) hence increases the intake.

3.2. Endogenous Sources

Nitrates known as endogenous nitrate are also produced in body. A major pathway for endogenous nitrate production is conversion of arginine by macrophages to nitric oxide and citrulline, followed by oxidation of the nitric oxide to nitrous anhydride and then reaction of nitrous anhydride with water to yield nitrite. Gastrointestinal infections and non-specific diarrhea increase endogenous (non-bacterial) nitrate synthesis, probably induced by activation of the mammalian reticuloendothelial system (WHO, 1985 b; WHO, 1996).

3.3. Total Daily Intake of Nitrate (WHO, 1985c)

In general, diet is the major source of human intake of both nitrate and nitrite. Certain vegetables, e.g. lettuce, spinach, beetroot and celery, contain relatively high levels of nitrate (often over 226 mg/kg), but the nitrite levels are usually very low. Nitrates and nitrites are also added as preservatives in some foods, such as cured meats and certain types of cheese.

Daily dietary intakes of nitrate and nitrite have been estimated in different countries (Ellen G and Schuller PL, 1983). The variation in the quantity of nitrates and nitrites ingested *via* the diet is extremely high. For example, individuals who seldom eat vegetables and cured meats have a low intake, whereas vegetarians have a relatively high intake.

In most European countries, the mean nitrate intake is about 10-30 mg/day. Vegetarians usually have a two- to four-fold higher intake of nitrates than non vegetarians.

For most people in Europe, drinking-water does not contribute more than 30% of their total dietary intake of nitrate. However, when the nitrate level in drinking-water exceeds 10 mg/l, this contribution may be considerably higher.

For bottle-fed infants, drinking-water is usually the major source of dietary nitrate. Nitrate levels in breast milk are low, even when the lactating mother consumes nitrate-rich drinking-water. Breastfeeding should thus be encouraged, even in areas with high nitrate levels in drinking-water.

4. KINETICS AND METABOLISMS

About 20 % of ingested nitrate is reduced to nitrite by nitrate-reducing micro flora present in saliva (Eisenbrand *et al*., 1980) at the base of the tongue (Walker, 1995). The factors which influences the oral micro flora and hence the reduction of nitrate are nutritional status, infection, environmental temperature and age (more in elderly) (Eisenbrand *et al*., 1980).

Ingested nitrate reduced to nitrite by nitrate reducing micro flora in stomach (in favorable conditions *viz*. pH≥4) and upper part of intestine and may be at other parts of the human gastrointestinal tract. In normal conditions the reduction of

nitrate to nitrite do not occurs in stomach but in situations where the stomach pH is high, such as achlorhydria (Ruddell *et al*., 1978), atrophic gastritis (Walker, 1995, Mirvish, 1975), artificially fed infants, or patients using antacid or similar drugs e.g. Omeprazole (Farinati *et al*., 1996; Colbers *et al*., 1996; Vermeer *et al*., 2001), the conversion of nitrate to nitrite occurs even in stomach. The high pH of stomach favors the growth of nitrate reducing organisms.

This nitrite, readily and completely absorbed from both the stomach and the upper small intestine. Approximately 25% of ingested nitrate is actively secreted into saliva, where it is partly (20%) reduced to nitrite by the oral microflora; nitrate and nitrite are then swallowed and reenter the stomach.

Absorbed nitrite is then rapidly distributed throughout the tissues. Absorbed nitrite is rapidly oxidized to nitrate in the blood, with the formation of methemoglobin.

4.1. Fate of Nitrates Ingested

The following is flow chart of the fate of ingested nitrate (Li *et al*., 1997; WHO, 1977a) (Fig. 2).

4.1.1. Metabolism in Mouth

Nitrate is converted to nitrite by microorganisms in the saliva. About 4–7% of ingested nitrate was detected as nitrite in the saliva (Eisenbrand *et al*., 1980; Speijers et al., 1987; Bruning-Fann and Kaneene, 1993). The major site for this reduction appears to be at the base of the tongue where a stable, nitrate-reducing microflora is established (Walker, 1995). Once nitrite is formed, it has a short biological half-life, being rapidly oxidized to nitrate in the blood. Nitrate undergoes active secretion in humans not only in the salivary duct cells but also in the gastric parietal cells and, is in passive equilibration with other intestinal secretions. This active secretion also occurs at a number of other sites leading to enterosystemic cycling of nitrate and nitrite.

Factors that may influence the oral microbial flora are nutritional status, infection, environmental temperature and age (Eisenbrand *et al.,* 1980). Salivary nitrite levels were generally higher in older age groups, although considerable variation between

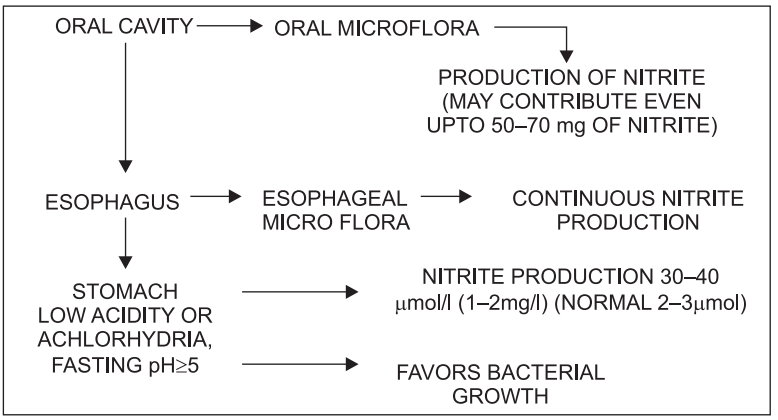


Fig. 2: Fate of ingested nitrate (Li *et al*., 1997; WHO, 1977a)

individuals was noted (Eisenbrand *et al*., 1980; Forman *et al*., 1985).

4.1.2. Metabolism in Stomach

A low pH (1–2) in the fasting stomach is considered normal for adults, and under these conditions bacterial nitrate reduction does not take place because of poor bacterial growth. High gastric pH values and sometimes correspondingly high nitrite levels were observed in patient with achlorhydria, stomach cancer, gastric ulcer, atrophic gastritis (Mirvish, 1975; Walker, 1995) and in patients treated with cimetidine, Omeprazole, which are used to treat hyperacidity and antacids (Farinati *et al*., 1996 and 1989; Jaskiewicz *et al*., 1990; Vermeer *et al*., 2001).

4.1.3. Metabolism in Intestine

Small intestine and lower part of the gut are rife with microorganisms, but nitrate and nitrite have not been found generally in the lower gut or in faeces. Studies on ileostomy patients given a conventional or high nitrate/nitrite meal indicated that the type of foodstuff ingested can significantly alter levels of nitrite and nitrate in the distal ileum and is a factor in determining nitrite/nitrate input into the proximal colon (Radcliffe *et al*., 1989).

Wagner (1983a) observed that ascorbic acid did not affect nitrate plasma levels nor the amount of nitrate excreted in urine, faeces or saliva, indicating that ascorbic acid does not interfere with nitrate metabolism (Wagner *et al*., 1983b).

In general, body sites containing both micro flora and nitrate will generate nitrite.

4.2. Formation of N-Nitroso Compounds

Formation of N-nitroso compound is a multiple step process (Choi, 1985). First, nitrate is converted into nitrite after consumption. Second, the nitrite reacts with natural or synthetic organic compounds (known as secondary amines or amides) in food or water to form new combinations; called N-Nitroso compounds (either nitrosamines or nitrosamides). Many of these N-nitroso compounds have been found to be carcinogenic in all the animal species tested, although some of the most readily formed compounds, such as N-nitrosoproline, are not carcinogenic in humans. At least 75% of the 120 N-nitroso compounds have been found to be carcinogenic to animals (Gilli *et al*., 1984, Terblanche, 1991). The most common N-nitroso compounds are dimethylnitrosamine (DMN), N-methylmethanamine (DMA), trimethylamine (TMA) and trimethylamine oxide (TMAO). The N-nitrosocompounds are carcinogenic in animal species and are probably also carcinogenic to humans. The data from number of epidemiological studies are at most, only suggestive, relating to carcinogenicity in human, but it had been reported that a link between cancer risk and endogenous nitrosation as a result of high intake of nitrate and, or nitrite and nitrosatable compounds is possible (RIVM, 1989; WHO, 1996).

4.3. Endogenous Synthesis of Nitrate and Nitrite

The excess nitrate excretion that has often been observed after low nitrate and nitrite intake, originates from endogenous synthesis, which amounts, in normal healthy humans, to 1 mmol/day on average, corresponding to 62 mg of nitrate per day or 14 mg of nitrate-nitrogen per day. Gastrointestinal infections greatly increase nitrate excretion, as a result, at least in part, of increased endogenous

(non-bacterial) nitrate synthesis, probably induced by activation of the mammalian reticuloendothelial system (WHO, 1985b, 1996; Speijers *et al*., 1987; Wishnok *et al*., 1995; Shephard, 1995). This endogenous synthesis of nitrate complicates the risk assessment of nitrate. Increased endogenous synthesis of nitrate, as reported in animals with induced infections and inflammatory reactions, was also observed in humans. Infections and non-specific diarrhea played a role in the increased endogenous synthesis of nitrate (Tannenbaum *et al*., 1978; Green *et al*., 1981; Bartholomew and Hill, 1984; Lee *et al*., 1970; Gangolli *et al*., 1994). These observations are all consistent with the induction of nitric oxide synthase by inflammatory agents, analogous to the experiments described in animals and macrophages.

A major pathway for endogenous nitrate production is conversion of arginine by macrophages to nitric oxide and citrulline, followed by oxidation of the nitric oxide to nitrous anhydride and then reaction of nitrous anhydride with water to yield nitrite. Nitrite is rapidly oxidized to nitrate through reaction with hemoglobin. In addition to macrophages, many other cell types can also form nitric oxide, generally from arginine.

4.4. Predisposing Factors

A direct correlation between gastric pH, bacterial colonization and gastric nitrite concentration has been observed in healthy people (Mueller *et al*., 1986). In individuals with gastrointestinal disorders and achlorhydria, high levels of nitrite have been reported (Ruddell *et al*., 1978; Dolby *et al*., 1984). Infections and non-specific diarrhea played a role in the increased endogenous synthesis of nitrate (Tannenbaum *et al*., 1978; Green *et al*., 1981; Hegesh and Shiloah, 1982; Bartholomew and Hill, 1984; Lee *et al*., 1970; Gangolli *et al*., 1994). These observations are all consistent with the induction of nitric oxide synthase by inflammatory agents. This induction in humans has been difficult to demonstrate directly, but administration of [^{15}N] arginine to two volunteers resulted in the incorporation of ^{15}N into urinary nitrate in both individuals, confirming the arginine-nitric oxide pathway in humans (Leaf *et al*., 1989).

4.5. Trans Placental Cross

Nitrite has been shown to cross the placenta and cause the formation of fetal methaemoglobinemia in rats (El Nahas *et al*., 1984).

4.6. Half Life

The half-life of nitrate in the body after ingestion is approximately 5 h (Wagner *et al*., 1983a). Nitrite was not detected in any of the body fluids studied except saliva where it appeared to increase as nitrate levels decreased (Cortas and Wakid, 1991).

5. METABOLISM OF INGESTED NITRATE IN HUMAN BODY AT CELLULAR LEVEL

Inorganic or organic nitrates were ingested. This will result in increased oxidation of hemoglobin to methemoglobin and increased production of nitric oxide (Murray *et al*., 1993; Waldman *et al*., 1987; Craven *et al*. 1978) (Figure 1). The conversion of nitrite to nitric oxide is non-enzymatic (Robertson, 1996; Smith *et al*., 1997; Vane *et al*., 1994; Lowestein *et al*., 1994). The oxidation of hemoglobin

to methemoglobin results in the formation of the superoxide radical, by the transfer of single electron. The enzyme superoxide dismutase, present in the erythrocytes, catalyses the conversion of superoxide radical ($O^{\bullet -}$) to H_2O_2 and O_2. The H_2O_2, is decomposed, by glutathion peroxidase or catalase, both are present in erythrocytes (Winterbourn *et al*., 1976; Sutton *et al*., 1976). Once the rate of oxidation of hemoglobin increases sufficiently in erythrocytes and overwhelms the protective and reductive capacities (e.g. cytochrome b_5 reductase system etc.) of the cells (Bodansky, 1951; Jaffe, 1981) there is increased production of reactive free radicals of nitric oxide ($NO^{\cdot}$) and oxygen (Winterbourn *et al*., 1976).

5.1. Fate of Free Radical Nitric Oxide ($NO^{\cdot}$)

Hemoglobin scavenges nitric oxide through the high affinity ferrous sites on heme to form S-nitrosothiol which has an affinity to nitric oxide 8000 times higher than the affinity for oxygen (Hsia, 1998) by binding at b 93 cysteine residue on the globin chain. As hemoglobin binds oxygen in the lungs, its binding affinity to S-nitrosothiol is increased. As hemoglobin releases oxygen at the periphery its affinity for S-nitrosothiol is reduced and nitric oxide is released in the tissues (Hsia, 1998). The thiol group of S-Nitrosothiol essentially protects nitric oxide from being scavenged by the binding site on heme. Thus, in addition to carrying oxygen, hemoglobin acts as a carrier of nitric oxide. The enhanced release of nitric oxide from S-nitrosohaemoglobin in hypoxic tissue in turn reduces regional vascular resistance.

Nitric oxide is a biogenic messenger, an endothelial derived relaxing factor (EDRF) (Jaffe,1981; Hsia, 1998)) and activates guanylyl cyclase system (Berger *et al*., 1997) [converts guanosine triphosphate (GTP) to 3'5' cyclic guanosine monophosphate (cGMP)], raising the cGMP pool and therefore inducing *inter alia* vasodilatation (Berger *et al*., 1997) by lowering intracellular calcium ion (Smith *et al*., 1997)**.**

5.2. Fate of Free Oxide Radical ($O^{\cdot}$)

In a normal cell, $O^{\bullet -}$ will be scavenged by the enzyme superoxide dismutase, and H_2O_2, which is a product of reaction, and removed by glutathion peroxidase and catalase (Roediger *et al*., 1986; Comly, 1945). Any $O^{\bullet -}$ that escapes this mechanism leads to production of much more reactive substances such as hydroxyl radical ($OH^{\bullet}$) and peroxynitrit radical. $O^{\bullet -}$ and H_2O_2 are highly selective in their reaction with biological molecules, whereas $OH^{\bullet}$ attaches everything around it and react with other cell constituents, causing irreversible cell damage. This mechanism is likely to become more significant if $O^{\bullet -}$ is produced in abnormally high amount (e.g. excessive nitrate ingestion), or if any of the protective mechanisms are defective (Sutton *et al*., 1976; Hsia, 1998). Thus, increased consumption of nitrate will leads to (a) increased production of nitrite (Allison *et al*., 1984; Cole *et al*., 1980); (b) enhanced absorption of sodium from the intestinal lumen (Roediger *et al*., 1986); (c) excess $NO^{\cdot}$ (free radical nitric oxide) generation, having vasodilatory effect (Winterbourn *et al*., 1976; Hsia,1998; Berger *et al*., 1997; Nitric Oxide,1997), and (d) increased production of $O^{\bullet -}$, which will react with other cell constituents, possibly causing irreversible cell damage (Berger *et al*.,

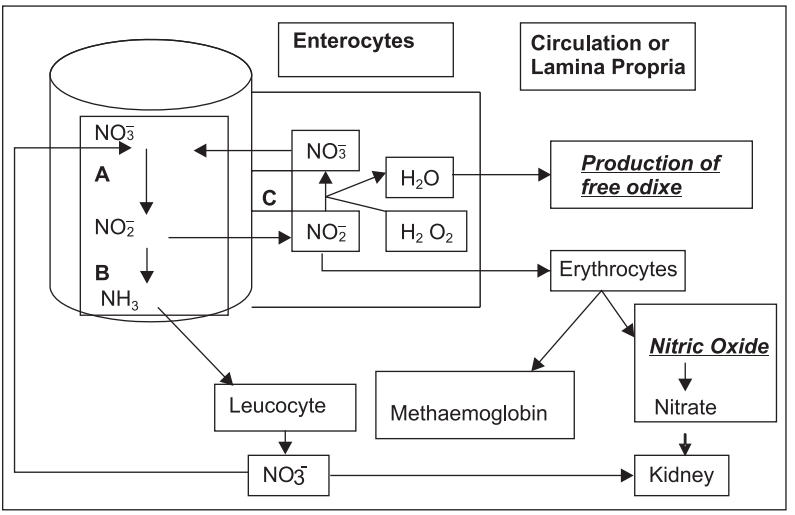


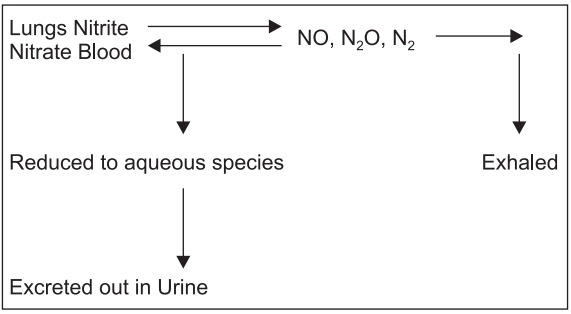
Fig. 3: Metabolism of ingested nitrate in human body at cellular level
A – Bacterial nitrate reductase; *B* - Bacterial nitrite reductase; *C* – Catalase

1997; Gupta *et al*., 1998) (Fig. 3).

6. EXCRETION

Nitrate excretion in urine generally reflects nitrate intake. The major part of the ingested nitrate is eventually excreted in urine as nitrate, ammonia, or urea, faecal excretion being negligible. Little nitrite is excreted (WHO, 1985b; RIVM, 1989) in urine. About 70–75% of ingested nitrate is excreted within 24 hours of ingestion irrespective of amount of intake. The excretion was noticed high in first 5 hours (Bartholomew and Hill, 1984; Wagner *et al.,* 1983b).The mean nitrate clearance after an oral dose of $NaNO_3$ of 470 µmol/kg body weight was 25.8 ml/minute corrected for a body area of 1.73 m^2. The urinary nitrate/creatinine ratio increased 25 to 70 times after dosing. These results indicated a predominantly tubular excretion of nitrate (Ellen *et al*., 1982). However, various authors have reported that urinary nitrate excretion may exceed nitrate intake even in infants if the latter is low, as a consequence of endogenous nitrate formation (Hegesh and Shiloas, 1982). Urinary nitrate excretion in infants was reported to be 80–100 % of the average intake, but no specific data were given for exposure levels (Turek *et al.,* 1980). Low levels of nitrate and nitrite were detected in the feces of humans (Saul *et al.,* 1981).

A small amount of ingested nitrate converting to nitrite, changes to oxides of nitrogen (nitric oxide, nitrous oxide, nitrogen etc.) in blood and gets excreted by exhalation through lungs (as shown in flow chart given below).



7. ACUTE TOXIC EFFECTS

Exposure to high nitrate may cause acute toxicity due to acute exposure.

7.1. In Human

Human lethal doses of 4–50 g NO_3^- (equivalent to 67–833 mgNO_3^-/kg BW) have been reported. Toxic doses with methemoglobin formation as a criterion for toxicity – ranged from 2 to 5 g (Corré and Breimer, 1979) of NO_3. These values are equivalent to 33–83 and 100–150 mg NO_3^-/kg body weight respectively.

Signs and symptoms of Acute Nitrate Toxicity: Fassett (1973) reported a rapidly occurring severe gastroenteritis with abdominal pain, blood in the urine and faeces as symptoms of acute nitrate intoxication. Repeated doses gave rise to dyspepsia, mental depression, headache and weakness. Cyanosis (bluish discoloration) will be present.

7.2. In Animal Studies

The studies relating to acute exposure of nitrate were conducted in animals. The acute oral toxicity of nitrate to laboratory animals is low to moderate. LD50 values of 1600–9000 mg of sodium nitrate per kg of body weight have been reported in mice, rats, and rabbits (Til *et al*., 1988). Ruminants are more sensitive to the effects of nitrate as a result of high nitrate reduction in the rumen; the LD50 for cows was 450 mg of sodium nitrate per kg of body weight. Nitrite is more toxic than nitrate: LD50 values of 85–220 mg of sodium nitrite per kg of body weight have been reported for mice and rats (RIVM, 1989; WHO, 1996).

8. CHRONIC TOXIC EFFECTS

It is caused due to long term exposure of non lethal doses. The following effects have been reported:

8.1. Methaemoglobinemia

Nitrates in drinking water have been reported to cause Methaemoglobinemia in infants up to 6 months of age.

8.1.1. Mechanism of Methemoglobin Formation

The essential action in the formation of methemoglobin is an oxidation of the ferrous to the ferric ion. This oxidation may be brought about in one of the following ways (Bodansky, 1951): by the direct action of the oxidants, or by the action of hydrogen donors in the presence of oxygen, or by auto oxidation. In

the presence of nitrites, the ferrous ion of hemoglobin gets directly oxidized to ferric state. Normally, the methemoglobin that is formed is reduced to hemoglobin by cytochrome b_5/cytochrome b_5 reductase system, as the following reaction (Benz, 2001):

$$Hb^{+3} + \text{Red cyt } b_5 \rightarrow Hb^{+2} + \text{oxy cyt } b_5$$

Oxidised cytochrome b_5 (Red Cyt b_5) is regenerated by the enzyme cytochrome b_5 reductase:

$$\text{oxy cyt } b_5 + \text{NADH} \xrightarrow[\text{Reductase}]{\text{cyt } b_5} \text{Red cyt } b_5 + \text{NAD}$$

Cytochrome b_5 reductase is a NADH dependent enzyme. NADH is supplied from glycolysis (Fig. 4).

Thus, the enzyme cytochrome b_5 reductase plays a vital role in counteracting the oxidative effects of nitrate ingestion. However, permissible concentration (50 mg/L as nitrate as per (WHO, 2004) normally present in water or food do not cause any health risk to adults but the infants constitute a vulnerable group for the following reasons (WHO, 1977b):

- Relatively higher stomach pH (2.0–5.0) which permits growth of nitrate reducing organisms such as *Coliforms, E. coli, Pseudomonas fluorescence, B. subtilis, S. albus*
- Relative higher consumption of water per unit weight of body
- The presence of fetal Hb which readily gets oxidized to methHb.
- Poorly developed cytochrome b_5 reductase system
- Nitrate gets concentrated by repeated boiling of water for feeding
- Bacterial contamination of the water itself or dried milk powder
- Early weaning on to nitrate rich vegetables e.g. Spinach
- Diarrhea causes increase in stomach pH

In India where the breast-feeding is a common practice especially up to the age of 6 months, protects the infants from nitrate toxicity. The probability of toxicity to fetus in pregnant women can not be ruled out as transplacental passages of nitrate metabolite (Nitrite) have been documented in animal experiment.

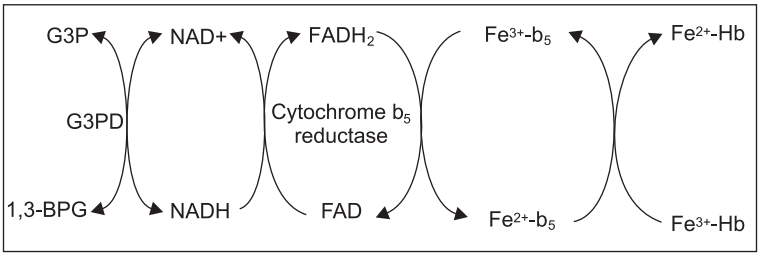


Fig. 4: Reduction of methemoglobin (Nagel, 2007)

While a few cases of methaemoglobinemia in infants have been reported to be associated with water nitrate levels of less than 50 mg nitrate ion/L, most cases occurs with nitrate level of 90 mg nitrate ion/L or more (Comly, 1945; Cornblath *et al*., 1948; Jaffe, 1981; Marshall *et al*., 1945; Knotek *et al*., 1964; WHO, 1977a). Recently methaemoglobinemia have been reported in all age groups (Gupta *et al*., 2000). It was observed that methaemoglobinemia was prevalent among all age groups, but was more severe in infants and older age groups (> 45 years).

Once methemoglobin levels in blood exceeds 10% of total Hemoglobin, it manifests as clinical cyanosis and causes cellular anoxia. Effect of methemoglobin (in relation to %) and clinical presentation are given below (WHO, 1977c).

Meth-Hb	**Clinical Presentation**
<10%	No signs & symptoms
10–25%	No symptoms
	Cyanosis Present
25–50%	Cyanosis, Dyspnoea, Headache
50–60%	Dyspnoea even on lying,
	Cyanosis, Disorientation
60%	Lethal levels

8.2. Cytochrome b_5 Reductase Adaptation

In several Indian villages, people have been consuming water containing high nitrate concentrations, at times up to 1200 mg nitrate ion/L. High nitrate concentrations are causing methaemoglobinemia in all ages (Gupta *et al*., 2000). It was observed that this methaemoglobinemia was more in infants and elderly people (Gupta *et al*., 2000). Children and adolescents have lower levels of methemoglobin. Adaptation to the reserve of cytochrome b_5 reductase activity with increasing water nitrate concentration to compensate methaemoglobinemia was reported to be a possible mechanism. The study (Gupta *et al*., 1999b) indicated that high nitrate concentrations are hazardous not only to infants but also to groups over 18 years of age. It was reported that adaptation of cytochrome b_5 reductase activity peaks at about 95 mg nitrate ion/L nitrate concentration and gets exhausted by nitrate level 200 mg nitrate ion/L, hence making people more prone for toxicity.

8.3. Infant Mortality Rate (IMR)

A study (Super, *et al*., 1981) on African mothers and other studies also (Schwiede, 2005; Fewtrell, 2004; Spalding and Exner, 1993; CDC, 1996; Dorsch *et al*., 1984) reported an increase in infant deaths with increasing exposure of pregnant mothers and infants to nitrate. This may either be due to undetected toxic methaemoglobinemia or to malformations and weaknesses in the infant caused by fetal nitrate exposure. It was further suggested that because of high IMR there is need to revise the nitrates standards for drinking water (Kumar S *et al*., 2002).

8.4. Nitrate, Nitrite, Nitrosamines and Cancer

Nitrate itself is not carcinogenic, but instead acts as a “procarcinogen”, meaning that it reacts with other chemicals (amines and amides) to form carcinogenic

compounds (N-nitroso compound). Under acidic conditions, nitrites (NO^{2-}) are formed naturally from nitrates, and nitrites in turn may combine with amines or amides to form N-Nitroso compounds (nitrosamines). The endogenous formation of carcinogenic N-nitroso compounds can occur following ingestion of nitrate from drinking water. Nitrate is first reduced in the saliva to nitrite, which can react in the stomach with secondary amines and amides. The nitrosamines most commonly reported in foods are dimethylnitrosamine, diethylnitrosamine, nitrosoproline and nitrosopyrrolidine. Nitrosamines can also be found in some alcoholic beverages and in tobacco products.

The amount of N-nitroso compounds which can be formed *in vivo* depends in part on the availability of nitrite, which is itself dependent on the availability of nitrate, the presence of a microbial population with nitrate reductase activity, and conditions favorable to chemical nitrosation (Tannenbaum, 1983). The physiological studies (Weisenberg *et al*., 1982; NAS, 1977; Moller, 1989) provide strong support indicating the association between nitrate contamination of drinking water and increased cancer rates. It has been reported that endogenously formed N-nitroso compounds are important in human cancer (Michaud, 2004; Mirvish, 1995). Szaleczky *et al*. (2000) reported that endogenously formed nitrogen and oxygen free radicals are believed to be involved in human cancer etiology. They reported that plasma nitrate/nitrite originates from endogenous nitric oxide production in fasting humans, decrease in superoxide scavenger activity (SSA), and free sulfhydryl groups (SH) reflects the amount of superoxide anion generated, and nitrotyrosine is believed to be formed by the interaction of tyrosine and peroxynitrite.

Population ingesting larger amounts of nitrate might be expected to have a higher incidence of cancer of the relevant target organ (NAS, 1981). In addition, products of nitric oxide, generated by macrophages during inflammation, can react with water at neutral pH to form nitrite and nitrate and with amines to form nitrosamines (Mirvish, 1995). Hence the International Agency for Research on Cancer (IARC, 1978; Fraser *et al*., 1980) has classified nitrate/nitrite as possibly carcinogenic to humans.

In animal or human studies, N–Nitroso compounds has been associated with 15 different types of cancers, including tumors in the bladder, stomach, brain, esophagus, bone and skin, kidney, liver, lung, oral and nasal cavities, pancreas, peripheral nervous system, thyroid, trachea, acute myelocytic leukemia, and T and B cell lymphoma. This group found to be carcinogenic in a wider range of tumors than any other group of carcinogens (NAS 1977, 1978; IARC 1978; Mirvish, 1991 and 1983). More than one hundred of these N-Nitroso compounds have been tested for carcinogenicity in animals, and 75–80% of them have been found to be carcinogens (NAS, 1977). IARC (1978) concluded that 11 N-Nitroso compounds were carcinogenic in man. In humans, the organs thought to be most at risk from cancers are the stomach, esophagus, nasopharyngeal cancer, and cancer of the bladder.

To summarize, the carcinogenesis due to high nitrate ingestion is multifactorial. The possible mechanism may be:

1. Increased formation of nitrosamines (known carcinogen), a metabolic intermediate product of nitrate metabolism.

2. Increased formation of free oxide radicals
3. Interference in NO metabolism

Still there are studies (RCEP, 1979; Fraser *et al*., 1980; Zaldivar *et al*., 1977; Armijo *et al*., 1975; Cuello, 1976; Hill *et al*., 1973) which have drawn no firm conclusion to prove nitrate as carcinogen.

8.4.1. Nitrate, Nitrite, Nitrosamines and Cancer in Infants and Children

A High Cancer Risk: A series of human and animal studies have indicated that infants, children, and even the foetus may face elevated risk later in life due to the effects of nitrate or nitrite exposure. Human epidemiology studies found an increase in stomach cancer rates with consumption of well water high in nitrate and that individuals exposed during the first ten years of life formed a high risk group (Gray *et al*., 1991; Cuello *et al*., 1976). Studies with N-Nitroso compounds on both fetal and infant equivalent animals support this finding (Gray *et al*., 1991; Cuello *et al*., 1976). Animal studies have documented transplacental passage of nitrite and reported that a high dose of nitrate to the pregnant ham can cause subacute methaemoglobinemia in fetal rats (Shuval and Gruner, 1972). Further, animal studies with nitrosamine compounds showed that exposure during infancy increases the cancer risk from N-nitroso compounds by a factor of six (Gray *et al*., 1991).

8.4.2. Nitrate and Gastric Cancer

Since 1976, a relationship between increasing rates of stomach cancer and increasing nitrate intake have been documented (Mirvish, 1983; Armijo, 1975 and 1981; Cuello, 1976; Xu *et al*., 1992; Reed *et al*., 1981 and 1983). An increased risk of gastric cancer in conditions associated with low gastric acidity is well recognized and lends support to the hypothesis that N-nitroso compounds may be involved in its development. High levels of gastric juice nitrites and elevated urine levels of nitrosamines have been reported in patients with chronic atrophic gastritis or high intragastric pH levels (Farinati *et al*., 1996; Jaskiewicz *et al*., 1990; Farinati *et al*., 1989; Vermeer *et al*., 2001; Dallinga, 1998). High nitrate intake with low vitamin C intake, high meat intake, or chronic bowel inflammation has been shown to be associated with increased cancer incidence. A study suggests that the *Cyp2E1 RsaI* polymorphism is associated with a reduced risk of gastric cancer (Nishimoto *et al*., 2000).

Reed *et al* (1983) reported first time in humans a significant lowering of gastric juice and N-nitroso compounds by ascorbate treatment in 51 achlorhydric subjects. This may be an important observation for preventing gastric cancer in high-risk subjects (NAS 1981; WHO 1985b, 1996; ECETOC 1988; RIVM 1989).

8.4.3. Nitrate and non-Hodgkins Lymphoma (NHL)

Epidemiological study of nitrate in well water in Nebraska showed an association between nitrate contamination and a different kind of cancer, non-Hodgkins lymphoma (Weisenburger, 1990). The authors concluded that “these findings suggest that NHL in eastern Nebraska may be related to the use of pesticides and nitrogen fertilizers”.

8.4.4. Nitrate and Colorectal Cancer

De Roos *et al*. (2003) observed negligible overall associations of either cancer with average nitrate level and with the number of years with average nitrate–nitrogen level greater than 5 or 10 mg/L. They reported an increased risk of colon cancer associated with drinking water nitrate among certain subgroups expected to have high rates of nitrosation, but they did not observe the same patterns for rectum cancer. The colon and rectum have similar epithelial tissues, but these two cancer types have somewhat different risk factors. Although N-nitroso compounds formed in the digestive tract would be expected to pass through the rectum, contractile activities in the rectum cause fecal matter to pass through quickly, resulting in less contact time with the rectum than with the colon (Vander *et al*., 1994).

8.4.5. Nitrate and Urinary Bladder Cancer

Recently, studies have pointed out risk of bladder cancer and high nitrate ingestion. Nitrate ingestion contributes to stomach nitrite levels, which can react with amines (nitrosation) to form endogenous nitrosamines (Michaud, 2001; Mirvish, 1995). In a recent epidemiological study, drinking water nitrate levels were associated with a significant elevation in bladder cancer risk (Weyer *et al*., 2001). In addition, products of nitric oxide, generated by macrophages during inflammation, can react with water at neutral pH to form nitrite and nitrate and with amines to form nitrosamines (Mirvish, 1995). High levels of gastric juice nitrites and elevated urine levels of nitrosamines have been reported in patients with chronic atrophic gastritis or high intragastric pH levels (Farinati *et al*., 1996 and 1989; Jaskiewicz *et al*., 1990; Vermeer *et al*., 2001). Significant correlations between mean intragastric pH values and mean *N*-nitrosodi-*n*-butylamine level (P=0.005) and total volatile *N*-nitrosamine contents (P=0.009) were also observed (Dallinga 1998).

8.4.6. Pathophysiology of Carcinogenesis due to NO and Free Oxide Radicals

Cancer is the endpoint of a multistep process that includes three fundamental components: initiation, promotion, and progression (Fig. 5). Arginine is involved in a number of biosynthetic pathways that significantly influence carcinogenesis and tumor biology. Since the discovery that arginine metabolism generates a ubiquitous signal transduction molecule, nitric oxide (NO), 3 arginine-derived NO has been found to play a significant role in many of the specific events that lead to cancer.

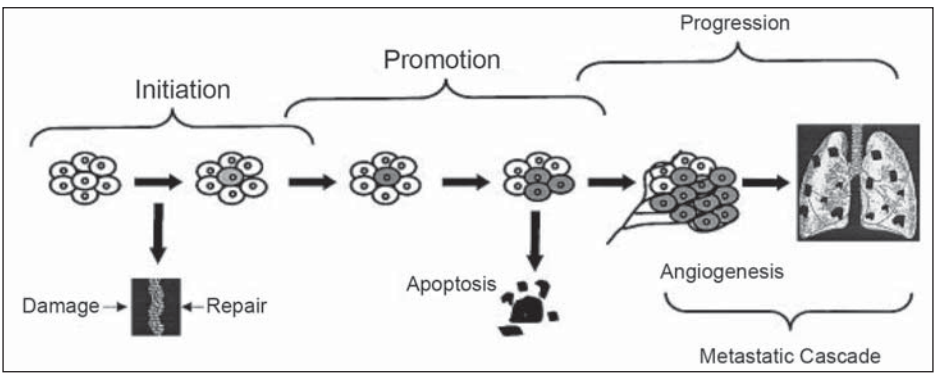


Fig. 5: Simplified schematic of the multi-step process resulting in cancer

Long-term exposure to NO induced by chronic inflammation may promote carcinogenesis in a number of organs (Lala and Chakraborty, 2001).The association between malignancy and chronic inflammation has long been known. Chronic tissue inflammation is associated with head and neck, lung, esophageal, stomach, colon, liver, and skin cancer.

The increased incidence of cancer in ulcerative colitis (UC) patients may be related to excess NO generation as a result of chronic colonic inflammation. A role for NO in sporadic colorectal cancer has also been suggested by the findings of increased iNOS activity in colon adenomas, colon cancer, and metastases (Lagares-Garcia *et al*., 2001). NO may contribute to tumor progression from a colorectal adenoma to a colorectal carcinoma.

Experimental evidence also exists implicating NO in the development of cholangiocarcinoma (Jaiswal *et al*., 2001) and hepatocellular carcinoma (Majano and Garcia-Monzon, 2003). NO's carcinogenic effects may involve several mechanisms including direct DNA and protein injury or the inhibition of programmed cell death, thus promoting abnormal cell growth. NO may also regulate key proteins involved in carcinogenesis through several posttranslational modifications.

In some cholangiocarcinoma cell lines, NO-mediated modification of pro-apoptotic caspases can contribute to the carcinogenic process (Torok *et al*., 2002). NO may also promote tumor growth through the stimulation of tumor angiogenesis (Morbidelli *et al*., 2004). NO promotes several steps required for tumor angiogenesis including endothelial cell proliferation, vascular permeability and stimulation of angiogenic growth factors. These NO mediated mechanisms of carcinogenesis may also be relevant to the development of a variety of malignancies.

NO is involved in the initiation of numerous cancers because it is involved in:

8.4.6.a. Initiation

1. High levels of NO may modify DNA directly or indirectly by inhibiting DNA repair activities (Roy *et al*., 2004) and hence can cause irreversible injury to several fundamental cancer control genes.
2. NO and superoxide rapidly react to form peroxynitrite which can cause oxidative damage to DNA.
3. NO can also block DNA synthesis through the inhibition of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (Ghafourifar and Colton, 2003).
4. In addition, NO can directly inhibit enzymes in mitochondrial electron transport chain or act indirectly by interfering with DNA repair mechanisms leaving the cell susceptible to other DNA damaging agents (Xu *et al*., 2002).

8.4.6.b. Progression

NO also can influence the progression of established neoplasms. Solid tumor consists of number of cell types including tumor cells, fibroblasts, lymphocytes, macrophage, neutrophils and endothelial cells.

During tumor growth, NOS activity may be increased in these cell types by pro-inflammatory cytokines and hypoxia present in the tumor microenvironment. The highly lipophilic nature of NO makes transcellular passage easy and facilitates NO-mediated effects between tumor cells and other cell types.

All three NOS isoforms (iNOS, eNOS, nNOS) have been detected in tumor cells (Lala and Orucevic, 1998). NOS isoforms may be involved in tumor cell proliferation, survival, migration, and invasiveness. Exactly the roles of 3 NOS isoforms have not been understood so far.

NO also promotes tumor invasiveness by affecting matrix metalloproteinase expression (Jurasz *et al*., 2001). Some tumor cells exposed to NO exhibit increased expression of the p53 tumor suppressor gene. In laboratory experiments it was observed that arginine-enhanced diets (NO precursor) increases tumor growth and increases tumor spread via increased polyamine synthesis.

8.4.6.c. Apoptosis

Apoptosis is a programmed cell death is a highly regulated active process characterized by cell shrinkage, membrane blebbing, chromatin condensation, cell fragmentation, and the formation of apoptotic cell bodies.

NO-mediated apoptosis or programmed cell death has been demonstrated in a variety of cell types including tumor cells. Several reports demonstrate that NO and peroxynitrite can directly cause either necrotic cell death or apoptotic or programmed cell death depending upon the NO level and cell type (Xie and Huang, 2003).

Some cells are intrinsically resistant to NO mediated cell death due to genetic determinants of the cell expression of p53 and other tumor suppressor or apoptosis-related proteins.

NO may inhibit apoptosis through pathways that are dependent upon the cyclic nucleotides cyclic AMP or GMP. In hepatocytes, NO has been shown to inhibit apoptosis through suppression of caspase activity (Torok *et al*., 2002). NO can function in an anti-apoptotic role through the posttranslational modification of a number of proteins that regulate apoptosis (Salvucci *et al*., 2001).

8.4.6.d. Angiogenesis

Neovascularization or angiogenesis is an absolute requirement for tumor growth. NO being a endogenous vasodilator, it have been shown that NO promotes new vessel growth through NO mediated up regulation of angiogenic factors such as vascular endothelial growth factor and basic fibroblast-derived growth factor (Cianchi *et al*., 2004). It also affects the proliferation of vascular cells such as endothelial and smooth muscle cells through the cGMP signaling pathway (Schlossmann *et al*., 2003). Modulation of NO and its effect on tumor microcirculation and new vessel formation represents a novel anti-tumor strategy. Still there are conflicting data suggesting that NO can inhibit or stimulate angiogenesis depending upon the NO level and angiogenic model (Xie and Fidler, 1998).

8.4.6.e. Immune Response

Consistent with the aforementioned roles of arginine-derived NO in cancer, NO also has a disparate role in tumor immunity. Hibbs *et al*. (1987) first described the role of NO in activated macrophage cytotoxicity in 1987. Subsequent to this landmark observation, NO-mediated tumor cell cytotoxicity has been demonstrated in a variety of immune cells including natural killer cells, T-cells and endothelial cells (Albina, 1998). Recent data have shown that the metastatic capability of human colorectal cancer cells correlates with their sensitivity to NO mediated liver sinusoidal endothelial cell cytotoxicity (Ozawa, 2003).

NO also modulates the expression of cell adhesion molecules critical to the inflammatory response, such as vascular cell adhesion molecule, and P-selectin (Kevil *et al*., 2004). In addition, NO influences chemokine signal transduction pathways (Macphail, 2003).

NO overproduction has been implicated in tumor-induced immunosuppression through the inhibition of immune cell activation pathways.

8.4.6.f. Metastasis

Increasing evidence suggests that NO influences the metastatic process. This review has previously summarized the role of NO in apoptosis, adhesion, invasion, and the host immune response to cancer. While the vast majority of circulating tumor cells dies rapidly in the circulation, NO mediates several events that promote tumor cell dissemination and survivability.

NO affects cell deformability and the ability to form tumor cell: platelet aggregates. Aggregation with platelets can protect tumor cells from immune cell attack. As a result of the aforementioned findings, researchers have speculated that NO may represent a potential target for chemoprevention (Hofseth, 2003). A few studies examining the chemopreventative effects of NOS inhibition have been performed in rat colon (Schleiffer 2000) and esophageal (Chen, 2004) cancer models and a mouse mammary adenocarcinoma model (Jadeski, 2003). Rao *et al*. (2002) demonstrated a reduction in the development of aberrant crypts, a precursor to colon cancer, by inhibiting iNOS in a chemically-induced rat colon cancer model. On the other hand, Schleiffer (2000) recently demonstrated that the NOS inhibitor L-NAME promoted carcinogen-induced preneoplastic changes in a rat colon carcinogenesis model by inhibiting NOS activity and stimulating polyamine synthesis. These conflicting reports illustrate the need for further studies in this area.

Hence there is possibility of cancer development in conditions where NO production is enhanced e.g.

1. High nitrate ingestion – as have been observed increases rate of colorectal cancer
2. Use of sildnafil citrate
3. Drugs which have good amount of L Arginine
4. Inhibition of cancer growth by NOS inhibitor L-NAME.

8.5. Respiratory System

A correlation among drinking water nitrate concentration, high methemoglobin levels and pathological changes in bronchi and lung parenchyma have been reported in animal studies (Shuval *et al*., 1972; Gruener *et al*., 1970). Changes in lungs reported were frequent dilation of bronchi with lymphocytic infiltration of mucosa and muscles, frequent purulent bronchial exudates, interstitial round cell infiltration and fibrosis at certain areas. WHO (1977d) reported an association of increased asthmatic attacks and high air borne nitrate concentrations.

A high percentage (40–82%) of cases of acute respiratory tract infection with history of recurrence has been reported in children drinking high nitrate in water (Gupta *et al*., 2000). These findings were further substantiated (Gupta *et al*., 1999c) in an animal experiment on rabbits. Significant changes in lungs were

observed with congestion, presence of inflammatory cells and breakdown of alveoli. These changes were absent when animals were fed water containing 45 mg/L of nitrate, as the concentration of nitrate increased the changes were more pronounced and severe.

8.5.1. NOx, Tobacco and Malignancy

Tobacco use causes 20% of cancer deaths worldwide. The International Agency for Research on Cancer predicts 10 million tobacco-related deaths annually by 2020, of which 70% will occur in the developing world (IARC, 2004)

This study conducted by Sleiman *et al*. (2010) reported that residual nicotine from tobacco smoke sorbed to indoor surfaces reacts with ambient nitrous acid (HONO) to form carcinogenic tobacco-specific nitrosamines (TSNAs). Substantial levels of TSNAs were measured on surfaces inside a smoker's vehicle.

Laboratory experiments using cellulose as a model indoor material yielded a >10-fold increase of surface-bound TSNAs when sorbed secondhand smoke was exposed to 60 ppbv HONO for 3 hours. In both cases we identified 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal, a TSNA absent in freshly emitted tobacco smoke, as the major product. The potent carcinogens 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone and N-nitroso nornicotine were also detected (Sleiman *et al*., 2010).

Time-course measurements revealed fast TSNA formation, with up to 0.4% conversion of nicotine. Given the rapid sorption and persistence of high levels of nicotine on indoor surfaces—including clothing and human skin—this recently identified process represents an unappreciated health hazard through dermal exposure, dust inhalation, and ingestion.

These findings raise concerns about exposures to the tobacco smoke residue that has been recently dubbed "thirdhand smoke." (SHS, smoke inhaled unintentionally) The work of Sleiman *et al*. (2010) highlighted the importance of reactions at indoor interfaces, particularly those involving amines and NOx/HONO cycling, with potential health impacts. Whereas direct inhalation of SHS is an exposure pathway of concern, nonsmokers, especially infants, are at risk through contact with surfaces and dust contaminated with residual smoke gases and particles (Matt *et al*., 2004). Reactions of atmospheric species [O_3, nitrous acid (HONO), NOx] with residual smoke on surfaces (furniture, walls, skin and clothing) have been overlooked as a source of long-term exposure to harmful pollutants. Nicotine, their precursor, is the most abundant organic compound emitted during smoking (up to 8 mg per cigarette). It is deposited almost entirely on indoor surfaces and persists for weeks to months (Singer *et al*., 2003 and 2004). HONO is often present in indoor environments at higher levels than outdoors.

Typical indoor levels are 5–15 ppbv, with [HONO]/[NO_2] ratios ~0.15 to 0.4 (vs. ~0.03 outdoors). Indoor levels up to 100 ppbv have been reported (Finlayson-Pitts and Pitts, 2000; Wainman *et al*., 2001; Lee, *et al*., 2002). The main indoor sources of HONO are direct emissions from unvented combustion appliances (Spengler, 1993; Pitts *et al*., 1989), smoking (Eatough *et al*., 1989), and surface conversion of NO_2 and NO (Goodman *et al*., 1999; Finlayson-Pitts *et al*., 2003; Mertes and Wahner,1995; Hirokawa *et al*., 2008; Yabushita *et al*., 2009; Enami *et al*., 2009). Heterogeneous formation of HONO also occurs inside automobiles, leading to [HONO] up to 30 ppbv and [HONO]/[NO_2] ~ 0.4 in polluted urban areas (Febo

and Perrino, 1995). Pitts *et al*. (1978) first described the atmospheric production of N-nitrosamines by reactions of nitrogen oxides and HONO with amines.

N-nitrosamines were found to be unstable in sunlight, rendering the reaction unimportant in outdoor daytime conditions. However, this process can be relevant in indoors where N-nitrosamines and HONO are less vulnerable to photochemical decomposition.

8.5.2. Nitrate and Animal Experiments

Rabbit studies in lungs (Gupta *et al*., 1999) indicated no damage at 100 ppm nitrate in drinking water. As the nitrate concentration in water increases to 400 ppm (Fig. 6), the changes in lungs indicated in form of congestion, presence of inflammatory cells and breakdown of alveoli.

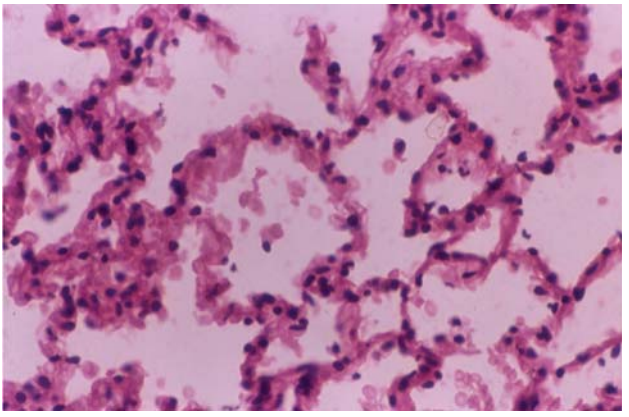


Fig. 6: Histopathological changes in lung at 400 ppm
(Haematoxylin and eosin, x 400, Gupta *et al*., 1999).

8.6. Cardiovascular System 3

The cardiac toxicity of nitrate is mediated through its metabolic effects and metabolic products e.g. metheoglobin formation, increased NO formation, increased in free oxide radicles (ROS) and high levels of compensatory enzymes (Cytochrome b_5 reductase etc.). The following effects have been reported:

1. Early onset of hypertension
2. Inflammation and degeneration of cardiac musculature
3. Causing endothelial dysfunction
4. Making the person prone for ischaemia
5. Enhances arteriosclerotic and atherosclerotic process

8.6.1. Early Onset of Hypertension

Earlier onset of hypertension has been reported with high nitrate ingestion (Malberg 1978). Some reports are also there which reported an inverse relationship between cardiovascular mortality and nitrate concentration in water supplies (Morton 1971). Pomeranz *et al*. (2000) reported that elevated salt and nitrate levels in drinking water cause an increase of blood pressure in school children.

8.6.2. Inflammation and Degeneration of Cardiac Musculature

The nitrate toxicity on cardiovascular system have been reported in both animals and human beings. Animal studies in early seventies (Shuval *et al*., 1972; Gruener

et al., 1970) reported correlation among drinking water nitrate concentration, high methemoglobin levels and cardiac muscles (Shuval *et al*., 1972; Gruener *et al*., 1970). The changes reported in cardiac muscles were small foci of inflammatory cells and fibrosis. Diffuse interstitial cellularity with pronounced degenerative foci was frequent in the highest nitrate groups only. The intramural coronary arteries provided surprising results with some degree of thinning and dilation in comparison to the control group who showed some degree of thickening, a marked hypertrophy and narrowing. Further, Gupta *et al*. (1999) in an animal experiment reported that high nitrate ingestion was associated with changes in cardiac muscles in form of branching of myosites, presence of inflammatory cells and focal degenerative changes in cardiac muscles. These changes were absent when animals were fed with water containing 45 mg/L of nitrate, but as the concentration of nitrate increased the changes were more pronounced and severe.

The changes in the cardiac muscles with different concentration of nitrate in water are given below (Table 2).

Figure depicts the changes in the Cardiac muscle in the rabbits fed with water containing 400 mg of NO_3 ion per liter (Fig. 7). The figure shows branching of myosites with focal degenerative changes.

The changes in cardiac tissue even in animal study may be of importance, in view of the side/adverse effects related to the use of nitrate containing drugs for the management of cardiac disorders and increasing drug tolerance of these nitrate containing drugs (USP DI, 1990).

Table 2: The effect of nitrate in drinking water on cardiac muscles

Water nitrate (mg/L)	Branching of Myosites	Inflammatory cells	Focal degenerative changes
45	NIL	NIL	NIL
100	+	NIL	NIL
200	+	NIL	NIL
400	+	+	+
500	+	+	+

(+) Mild, (++) Moderate, (+++) Severe, NIL: Normal histopathology (No change observed).

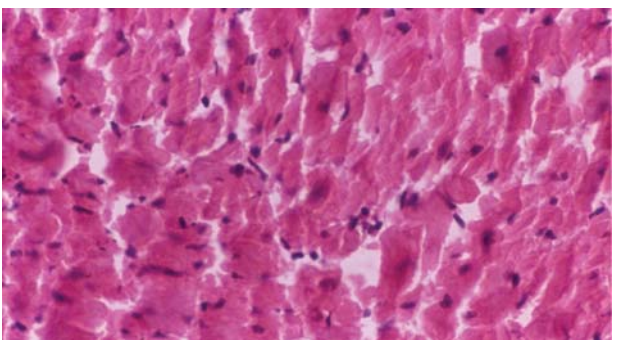


Fig. 7: Histopathological changes in the cardiac muscle at 400 ppm
(Haematoxylin and eosin, x 400, Gupta *et al*., 1999)

8.6.3. Endothelial Dysfunction

Endothelial dysfunction, or the loss of proper endothelial function, is a hallmark for vascular diseases, and is often regarded as a key early event in the development of atherosclerosis. Impaired endothelial function is often seen in patients with coronary artery disease, diabetes mellitus, hypertension, hypercholesterolemia, as well as in smokers. Endothelial dysfunction has also been shown to be predictive of future adverse cardiovascular events. One of the main mechanisms of endothelial dysfunction is the diminishing of nitric oxide, often due to high levels of asymmetric dimethylarginine, which interfere with the normal L-arginine-stimulated nitric oxide synthesis. The most prevailing mechanism of endothelial dysfunction is an increase in reactive oxygen species (ROS), which can impair nitric oxide production and activity via several mechanisms. (Toledo *et al*., 2004; Deanfield *et al*., 2005) The signaling protein ERK5 is essential for maintaining normal endothelial cell function (Roberts *et al*., 2009)

An association has been observed between increase in plasma nitrite/nitrate levels and an excessive production and/or inactivation of NO leading to impaired vascular smooth-muscle reactivity leading to impairment in endothelium dependent, shear stress-induced vasodilatation.

8.6.4. Making the Person Prone for Ischemia

Nitrate and Nitrite has emerged as a viable alternative source of NO under ischemic conditions. NO has been shown to be one of the most important molecules for the prevention of injury from Ischemia. Paradoxically, in conditions of inadequate oxygen, the NO is dangerous and may be the cause of Ischemia. (Garg and Bryan, 2009)

Nitrate inhalation exposure for long term, of individuals working in munitions or dynamite manufacturing leads to an apparent biocompensation of the cardiovascular effects of organic nitrates. These workers, when removed from the source of the nitrates, experienced symptoms of angina and were subject to sudden and sometimes fatal heart attacks (Carmichael and Lieben, 1963; Stokinger, 1982).

8.6.5. Atherosclerosis

Nitrate is a source of ROS in human body. ROS is known to cause enhanced atherosclerosis process.

Atherosclerosis develops from low-density lipoprotein molecules (LDL) becoming oxidized (ox-LDL) by free radicals, particularly reactive oxygen species. When oxidized LDL comes in contact with an artery wall, a series of reactions occur to repair the damage to the artery wall caused by oxidized LDL. The LDL molecule is globular shaped with a hollow core to carry cholesterol throughout the body. Cholesterol can move in the bloodstream only by being transported by lipoproteins (Anthea *et al*., 1993). The body's immune system responds to the damage of artery wall caused by oxidized LDL via sending specialized white blood cells (macrophages and T-lymphocytes) to absorb the ox-LDL and forming specialized foam cells. Unfortunately, these white blood cells are not able to process the ox-LDL, and ultimately grow then rupture, depositing a greater amount of oxidized cholesterol into the artery wall. This triggers more white blood cells to make a vicious cycle (Sloop *et al*., 1997). Eventually, the artery becomes inflamed. The

cholesterol plaque causes the muscle cells to enlarge and form a hard cover over the affected area. This hard cover is what causes a narrowing of the artery, reduces the blood flow and increases blood pressure (Duguid, 1960).

It is important to discuss the preventive role of thioredoxin proteins to counteract the toxic effects of nitrate. Zhang *et al*. (2007) demonstrate regulation of such events by a mitochondria-specific thioredoxin, which reduces oxidative stress and increases NO bioavailability, thus preserving vascular endothelial cell function and preventing atherosclerosis development. Thioredoxin proteins are classically defined by their ability to reduce disulfides (a SOS bond) to dithiols (two OSH groups), and in the process the thioredoxins are oxidized from a dithiol to a disulfide. The thioredoxin disulfide is then cycled back to its active form by the enzyme thioredoxin reductase, using NADPH as the electron donor (Mustacich and Powis, 2000; Arnér and Holmgren, 2000). The role of thioredoxin proteins as an antioxidant that reduces protein thiols and various forms of ROS is widely appreciated.

It has long been described that superoxide rapidly reacts with NO to inactivate it. In fact, this was a major clue to the original identification of endothelial-derived relaxaing factor as NO. The Superoxide reacts directly with NO with high affinity to form the reactive nitrogen species peroxynitrate. In addition to inactivating NO, ROS have a host of potentially deleterious effects and antioxidants are widely touted as having some potential for heart disease prevention. However, initial results from clinical trials of antioxidants have been disappointing, suggesting that we have insufficient understanding of the specific roles of ROS and antioxidants to design effective therapies.

8.7. Gastro Intestinal System

In Gastrointestinal system 4 major findings have been reported

1. Recurrent diarrhea in children
2. Recurrent stomatitis
3. GI Malignancy
4. Histopathological changes in animal study

8.7.1. Recurrent Diarrhoea in Children

Gupta *et al*. (2001) reported a problem of recurrent diarrhea in children upto 8 years of age. They suggested that increased consumption of nitrate leads to (a) increased production of nitrite (Allison *et al*., 1984, Cole *et al*., 1980); (b) enhanced absorption of sodium from the intestinal lumen (Roediger *et al*.,1986); (c) excess $NO^{\cdot}$ (free radical nitric oxide) generation, having vasodilatory effect (Winterbourn *et al*., 1976; Hsia 1998; Berger *et al*., 1997; Nitric oxide; Smith *et al*., 1997); and (d) increased production of O_2^-, which will react with other cell constituents, possibly causing irreversible cell damage (Berger *et al*., 1997; Gupta, 1998). These changes in enteric mucosa cause hyperemia and edema in the enteric mucosa and later on possibly cause irreversible mucosal damage, and therefore provide high-risk conditions suitable for recurrent diarrhoea.

These findings were correlated well with histopathological study conducted on rabbits (Gupta *et al*., 2001). It was observed that the histopathological changes observed in the intestinal mucosa on rabbit study revealed that the degree of damage in colon was progressive as the nitrate content of the ingested water increased.

These findings are of interest since infants and children consuming nitrate rich water, specially during diarrhea which is an aggravating factor for nitrate toxicity (Murray *et al*., 1993), where the use of WHO oral rehydration solution (ORS) is a normal routine. The use of WHO ORS could be of grave concern, if prepared with water containing high nitrate.

8.7.2. Recurrent Stomatitis

Recurrent stomatitis was another problem reported in people using high nitrate containing drinking water. This finding was observed in all groups and was well correlated with increased Cytochrome b_5 reductase activity following high nitrate ingestion. The increased activity of enzyme Cytochrome b_5 reductase is associated with stomatitis (Gupta *et al*., 1999a). Another possible cause may be the production of NO which is a metabolic product of nitrates and is know inflammatory agent.

8.7.3. GI Malignancy

The formation of N-nitroso compounds in the stomach has been connected with drinking water nitrate, and excretion of N-nitroso compounds by humans has been associated with nitrate intake at the acceptable daily intake level through drinking water (Vermeer *et al*., 1998). The metabolism of nitrate and nitrite, the formation of N-nitroso compounds, and the development of cancers in the digestive system are complex processes mediated by several factors. Individuals with increased rates of endogenous formation of carcinogenic N-nitroso compounds are likely to be susceptible. Known factors altering susceptibility to the development of cancers in the digestive system are inflammatory bowel diseases, high red meat consumption, amine-rich diets, smoking, and dietary intake of inhibitors of endogenous nitrosation (e.g., polyphenols and vitamin C) (Vermeer *et al*., 1998, De Kok *et al*., 2005, De Roos *et al*., 2003). In 1995, when the Subcommittee on Nitrate and Nitrite in Drinking Water reported that the evidence to link nitrate to gastric cancer was rather weak (NRC, 1995), the stomach was still thought to be the most relevant site for endogenous nitrosation. Previous studies, such as those reviewed in the NRC (1995) report, which found no link between nitrate and stomach cancer, concentrated on the formation of nitrosamines in the stomach. Recent work indicated that larger amounts of N-nitroso compounds can be formed in the large intestine (De Kok *et al*., 2005, Cross *et al*., 2003).

Since 20–90% of flatus is formed by Nitrogen (*Encyclopedia Britannica, 2007–08*). Further ingestion of high nitrate water or constipation enhances the nitrate toxicity. The major components of the flatus, which are odorless, by percentage are (*Encyclopedia Britannica, 2007–08*):

- Nitrogen: 20–90%
- Hydrogen: 0–50%
- Carbon dioxide: 10–30%
- Oxygen: 0–10%
- Methane: 0–10%

8.7.4. Histopathological Changes in Animal Study

Animal studies conducted by Gupta *et al*. (2001) indicated changes in structure of liver and intestine with high nitrate ingestion. Further it was observed that the pathological changes were more with increasing nitrate concentrations. The changes observed were:

8.7.4.a. Liver

Hepatocytes are more eosinophilic and grannular. Hepatic cords are distorted. Hepatocytes show marked degeneration. Marked congestion was noted. Inflammatory cells were observed. Degenerative changes of nucleus were founded. Portal tract shows slight congestion and presence of inflammatory cells. These changes were more pronounced as the nitrate concentration in water increases (Table 3, Fig 8).

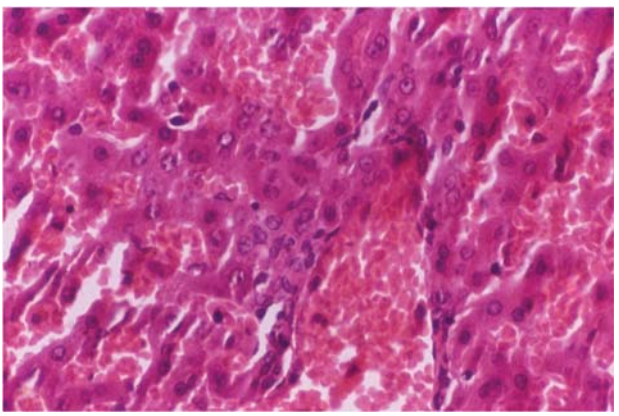


Fig. 8: Histopathological changes in the liver at 500 ppm
(Haematoxylin and eosin, x 400, Gupta *et al*., 2001)

8.7.4.b. Intestine

The histopathological changes in Intestine were not observed at 100 ppm nitrate but as the nitrate concentration in drinking water increases from 100 ppm, focal collection of inflammatory cells starts at sub mucosa with slight lymphoid hyperplasia, which was more pronounced as the water nitrate concentration reaches to 400 ppm and at this level degenerative changes also starts (Fig 9).

8.8. Abortions

Health effects associated with ingestion of nitrate-contaminated water have included stillbirth, low birth weight, and slow weight gain and even death of the animals

Table 3: Different nitrate dosage and histopathological changes in the liver

Nitrate (ppm)	Histopathological changes
100	HP
200	HP, Granular degeneration, mild congestion and inflammation
400	HP, Congestion and inflammation of portal tract
500	HP, Congestion, degeneration and inflammation of portal tract

HP: High power magnification (400X) on microscopic examination

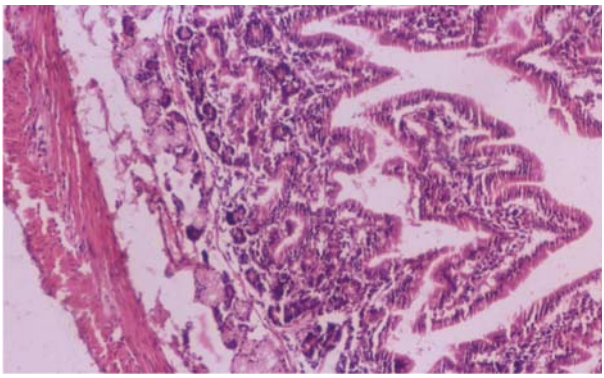


Fig. 9: Histopathological changes in the intestine at 400 ppm (LP)
(Haematoxylin and eosin, x100, Gupta *et al*., 2001).

affected (Committee on nitrate accumulation, 1972). Spontaneous abortions were also observed in laboratory animals and livestock (FDA, 1972; Sund *et al*., 1957). In 1959, for the first time in humans, spontaneous abortions were reported to have an association with increased methemoglobin levels due to high nitrate ingestion (Muhrer, 1959). In subsequent years some studies (Schmitz, 1861; CDC, 1996; Fewtrell, 2004) found an increased risk of spontaneous abortion or certain birth defects if the mother drank water high in nitrate. Therefore it has been suggested that women who are pregnant or who are trying to become pregnant should not consume water containing high nitrate.

8.9. Birth Defects–Malformations

The risk of birth defects due to nitrate exposure is a particular concern because of the fact that risk could be due to a single high dose of nitrate early in the pregnancy that later has profound effects on long-term fetal development. Animal studies have indicated that there is transplacental transfer of N-Nitroso compounds to the fetus (Shuval and Gruener, 1972) and this fetal exposure can cause cancer later in life (Druckrey, 1966). It was also reported that a single dose of a nitrosamide given to pregnant rats on day 15 of the pregnancy was reported to cause birth defects in the offspring. In rats and hamsters studies multiple birth defects, including malformations of the eye, central nervous system, and musculoskeletal system, were observed when a single dose of N-Ethyl-N-Nitrosourea, a nitrosamine, was given to the mother (Druckery, 1966; Givelber, 1969).

Extending these finding on human being, a number of human epidemiology studies were conducted (Dorsch, 1984; Knox, 1972; Super, 1981). A link was found between anencephaly rates and intake of cured meat containing high levels of nitrite (Knox, 1972). This study provided the first suggestive evidence in humans that nitrite consumption in food could have adverse impacts on the foetus. A 1984 study (Dorsch *et al*., 1984) found statistically significant dose response relationships between birth defects of the central nervous system and musculoskeletal system and increasing nitrate concentration of drinking water. Arbuckle *et al*. (1988) found the evidence for an association between nitrate and birth defects to be weaker. Fan (1987) reported that nitrate contamination of 45

ppm nitrate ion, or 10 ppm nitrate-nitrogen, adequately protects the very young from nitrate-induced toxicity, both pre- and post-natally. The adverse reproductive effects reported occurred at doses that were about one thousand times and higher than the estimated human intake. Croen *et al*. (2001) found that exposure to nitrate in groundwater at concentrations above the 45 mg/L maximum contaminant level was associated with increased risk for anencephaly (Odds ratio 4.0; confidence interval 1.0–15.4). Risk for anencephaly increased for mothers with the highest nitrate exposure 36–67 mg/L in groundwater compared with nitrate exposure less than 5 mg/L (Odds ratio 6.9; confidence interval 1.9–24.9).

8.10. Diabetes

A positive correlation between high nitrate levels in drinking water and increased incidence of type 1 diabetes was observed independently from length of mother's education, child's or mother's age, place of residence or mother's smoking status (Kostraba, 1992; Virtanen *et al*., 1994; Parslow *et al*., 1997; Van Maanen *et al*., 2000). Further studies indicated that consumption of high levels of N-nitroso compounds (NOCs) by human mothers may result in an increased incidence of type 1 diabetes in male offspring (Helgason *et al*., 1981). In animal studies NOCs have been reported to be toxic to pancreatic beta cells, providing the rationale for these observations (Wilson *et al*., 1983). Kostraba (1992) postulated that exposure to nitrate in drinking water causes increased production of free radicals, which may play a role in the etiopathogenesis of insulin-dependent diabetes mellitus (IDDM). The production of free oxide radicals following high nitrate ingestion have been further supported by studies conducted by Gupta *et al*. (1999a and 2000)

Further it is to be noted that there are studies, which indicated no relationship between nitrites and nitrates in drinking water and increased incidence of type 1 diabetes (Dahlquist *et al*., 1990; Virtanen *et al*., 1994; Verge *et al*., 1994). Thus, while some studies suggest an association between intake of nitrate/nitrites and risk of type 1 diabetes, the data remain limited and inconsistent (Longnecker *et al*., 2001). Hence the association of dietary nitrate/nitrites with diabetes remains tenuous and further research needs to be supported.

IDDM is an autoimmune disease characterized by the selective destruction of insulin secreting pancreatic β-cells found in the islets of Langerhans (Gepts, 1965). Since nitrates are source of nitric oxide, It has been shown that nitric oxide, produced in micromolar levels by the β-cell in response to cytokines, mediates the damaging effects of cytokines through the inhibition of mitochondrial enzymes, inhibition of glucose stimulated insulin secretion and the induction of DNA strand breaks and base modifications (Hughes *et al*., 2009; Corbett *et al*., 1992; Southern, 1990).

Cytokines, released from invading leukocytes during insulitis, are believed to participate in the initial destruction of β-cells and precipitating in the autoimmune response (Mandrup-Poulsen, 1996; Rabinovitch *et al*., 1998).

Nitric oxide, produced in micromolar levels following enhanced expression of the inducible nitric oxide synthase (iNOS) in β-cells, mediates the damaging actions of cytokines on β-cell function (Corbett *et al*., 1992a and 1992b; Southern, 1990). Nitric oxide inhibits insulin secretion by attenuating the oxidation of glucose to CO_2, reducing cellular levels of ATP and, thereby, attenuating ATP-inhibited

K^+-channel activity (Misler *et al*., 1992; Hughes *et al*., 1990). The net effect is the inhibition of β-cell depolarization, calcium entry, and calcium-dependent exocytosis. In addition to the inhibition of β-cell function, nitric oxide induces DNA damage in β-cells (Eizirik *et al*., 1996; Steer *et al*., 2006). Nitric oxide, or the oxidation products N_2O_3 and $ONOO^-$, induce DNA damage through direct strand breaks and base modification (Burney *et al*., 1999; Tamir *et al*., 1996; Niles *et al*., 2006), and also by inhibition of DNA repair enzymes, thereby enhancing the damaging actions of nitric oxide (Jaiswal *et al*., 2001; Graziewicz *et al*., 1996). Recent studies have shown that β-cells maintain a limited ability to recover from cytokine-mediated damage (Scarim *et al*., 1996; Corbett *et al*., 1994).

8.11. Thyroid

Nitrate competitively inhibits iodine uptake. If dietary iodine is available at an adequate range (corresponding to a daily iodine excretion of 150–300 μg/day), the effect of nitrate is weak, with a tendency to zero. The nitrate effect on thyroid function is strong if a nutritional iodine deficiency exists simultaneously (Höring *et al*., 1991; Höring, 1992). An association between high nitrate concentrations in drinking-water and goiter has been (Höring *et al*., 1991; Höring, 1992; Van Maanen *et al*., 1994) described by various workers. A dose–response relationship has been demonstrated by Höring *et al*. (1991) (nitrate in drinking-water v/s incidence of goiter) as well as by Van Maanen *et al*. (1994) (nitrate in drinking-water v/s thyroid volume). Both the experimental and epidemiological studies give the impression that nitrate in drinking-water has a stronger effect on thyroid function than nitrate in food.

8.11.1. Effect of Nitrate on Thyroid Function and Morphology

The thyroid gland contains a transport mechanism that provides sufficient iodine substrate for hormone formation. This process, called iodine trapping, is accomplished by a membrane protein, the sodium-iodine symporter. The transport of iodine is an active process. Depending on the presence of sodium gradient across the basal membrane of the thyroid cell, the downhill transport of two Na^+ ions results in the entry of one iodine atom against an electrochemical gradient. This trapping mechanism for iodine is shared by other monovalent anions, including pertechnetate, perchlorate, thiocyanate and nitrate. The relative potency of perclorate for inhibiting iodine uptake by the sodium-iodine symporter was 15 and 240 times greater than that of thiocyanate and nitrate, respectively, on a molar concentration basis in serum (Tonacchera *et al*., 2004). Although nitrate is not a powerful goitrogenic substance as thiocyanate and perchlorate, chronic nitrate exposure may cause an inhibition in the accumulation of iodine in the thyroid gland and consequently it may result in thyroid malfunction (Fig. 10).

The nitrate is found in a high concentration in soil, agricultural products and drinking water, because of the use of fertilizer. The people living in high nitrate polluted area have a chronic nitrate exposure.

It has been shown that the children exposed to water pollution by nitrates have a higher relative risk of goiter (Vladeva *et al*., 1998). It has been reported that the thyroid gland volume of the schoolchildren and adults living in high nitrate

polluted area is higher than those living in low nitrate polluted area (Van Maanen *et al*., 1994; Tajtakova *et al*., 2006). It has also been reported that the frequency of hypoechogenicity in schoolchildren living in high nitrate polluted area was higher than those living in low nitrate polluted area (Tajtakova *et al*., 2006). Various experimental studies showed that especially long-term nitrate exposure via drinking water may result in an increase in thyroid gland weight (Eskiocak, 1995; Eskiocak *et al*., 2005; Zaki *et al*., 2004) and histomorphological changes, including retention of lobular architecture, prominent vascular congestion, follicular hyperplasia, a vacuolisation and an increase in the colloidal volume of the follicles thyroid (Eskiocak *et al*., 2005; Zaki *et al*., 2004) (Fig 11).

Several investigators showed that low dose or short-term nitrate intake causes a decrease in thyroid radioiodine uptake (Katti and Sathyanesan 1987; Shrivastava *et al*., 1987; Szokeova *et al*., 2001). Whereas Eskiocak *et al*. (Eskiocak, 1995;

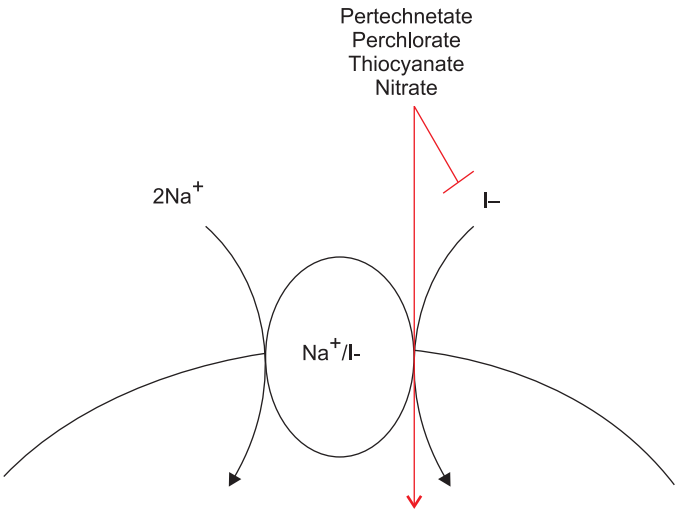


Fig. 10: Competition with iodine for sodium-iodine symporter resulting in decreased iodide uptake by thyrocytes

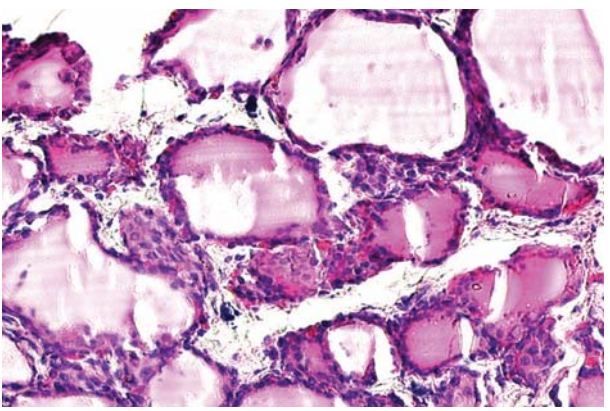


Fig. 11. Histopathological changes in the thyroid glanse at 250 mg/L
(Haematoxylin and eosin, x100, Eskiocak *et al*., 2005).

Eskiocak *et al*., 2005) reported that high dose and long-term nitrate exposure results in an increase in the thyroid radioiodine uptake. These findings suggest that the effect of nitrate on thyroid iodine uptake is dose dependent and the inhibition of thyroid iodine uptake may be stronger at higher amounts of nitrate.

Several research groups reported that nitrate cause thyroid hormone dysfunction. It found that the thyroid secretion rate is significantly reduced in the goat which intake 50 mg/L nitrate via water in comparison with the control group (Simon *et al*., 2000). Van Maanen *et al*. (1994) reported that there is a decrease in TSH levels in humans exposed to high nitrate pollution, whereas Tajtakova *et al*. (2006) reported that an increase in TSH levels in schoolchildren living in high nitrate pollution area. There are also some experimental studies which support that thyroid hormone dysfunctions may be caused by nitrate intake via drinking water. It has been reported that the total and free T_3 and T_4 levels are decreased at high dose and long-term nitrate exposure (Eskiocak, 1995; Eskiocak *et al*., 2005; Jahreis *et al*., 1987). However, Szokeova *et al*. (2001) demonstrated that short-term nitrate administration may result in a significantly higher serum level of total T_3. These findings indicate that short or long-term nitrate exposure may be strongly responsible for the prominent change in thyroid hormone production.

8.12. NOx (nitrite/nitrate) and Nephrotic Syndrome

NOx levels in serum obtained from patients with nephritic syndrome showed significantly higher levels than those of healthy controls. Balat *et al*. (2000) measured plasma and urinary, total NOx in children with minimal change nephritic syndrome (MCNS). In comparison with healthy controls, children with MCNS had increased urinary nitrite excretion. Plasma nitrite levels were high in relapse compared with controls (Balat *et al*., 2000). Trachtman *et al*. (1996) also reported that patients with MCNS had increased urinary nitrite excretion regardless of whether the disease was in relapse or remission.

Zachwieja *et al*. (2002) found that the number of apoptotic T cells is greater in patients with nephrotic syndrome than in children in remission from nephrotic syndrome and in controls. High serum NOx may be related to an increased apoptotic rate of circulating lymphocytes.

There are three isoforms of NOS—iNOS, eNOS, and nNOS—in the renal tissue of patients with IgA nephropathy, lupus nephritis, and MCNS. The eNOS was present in glomerular endothelial cells and endothelium of cortical vessels in control and diseased kidneys. The iNOS were localized in mesangial cells, glomerular epithelial cells, and infiltrating cells in IgA nephropathy and lupus nephritis, whereas immunostaining for iNOS was hardly detected in control kidneys and nephrotic syndrome (Kawashima *et al*., 2007). It seems that there is no direct injury of glomerulus caused by oxidation products of NO in pediatric nephritic syndrome. iNOS showed partially positive in the tubular cells of nephrotic patients. These findings may suggest that NO has a role in electrolyte regulation. Excess NO production and following oxidation products and electrolytic loads in tubules and interstitial cells might influence kidney damage in patients with nephrotic syndrome over a long duration. As eNOS is immunoexpressed in most cases studied, NO derived from eNOS may be involved in the progression of glomerulonephritis. However, the numbers of immunostaining studies are limited,

and therefore, further studies are needed to understand the role of NO in human glomerulonephritis (Kawashima *et al*., 2007).

Further the role of NO was evaluated and reported that NO has both direct effects, mediated by the NO molecule itself, and indirect effects, mediated by reactive nitrogen species produced by interaction of NO with oxygen (O_2) or superoxide radicals. Under a high NO concentration, the indirect effects mediated by reactive nitrogen species prevail, and then reactive nitrogen species induce cell toxicity by nitrosating DNA and tyrosine residues and inducing lipid peroxidation (Davis *et al*., 2001). NO is needed particularly to maintain the blood flow in kidneys. On the other hand, oxidative stress is believed to affect the development of diabetic-associated vasculopathy, endothelial dysfunction, and nephropathy (Hiragushi 2001). However, the role of NO is obscure.

An experiment on rabbit indicated increasing damage to kidney with increasing nitrate concentration in drinking water of the animal (Gupta *et al*. – unpublished data). Histopathological changes at different nitrate concentration have been depicted in Table 4 and Fig. 12 given below.

Table 4: Different nitrate dosage and histopathological changes in the kidney

Nitrate (ppm)	Histopathological changes
100	HP, Tubular changes, Necrosis (+++)
200	HP, Tubular changes, Eosiniphilic changes
400	HP, Tubular changes, Eosiniphilic changes,granular changes, increased cellularity

HP: High power magnification (400X) on microscopic examination.

8.13. Adrenal Gland

High nitrate ingestion affects the adrenal gland. In addition to the effect of nitrite on the adrenal zona glomerulosa in rats, a study in humans indicated that sodium

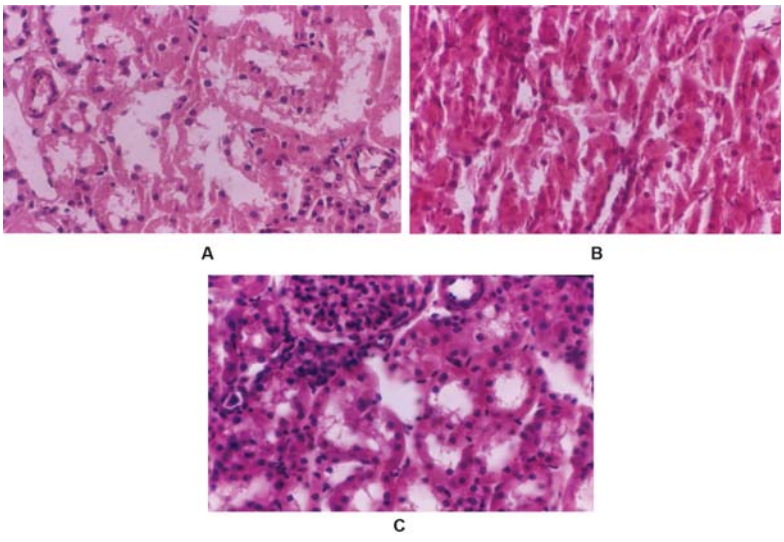


Fig. 12. Histopathological changes in kidney at different nitrate concentration.
100, 200 and 400 ppm nitrate intake group at A, B and C, respectively.
(Haematoxylin and eosin, x 400, Gupta *et al*.– unpublished data).

nitrite (0.5 mg of sodium nitrite per kg of body weight per day, during 9 days) causes a decreased production of adrenal steroids, as reflected by the decreased concentration of 17-hydroxysteroid and 17-ketosteroids in urine (Til *et al*., 1988; Kuper and Til, 1995). Similar results were also found in rabbits (Violante *et al*., 1973). Although the mechanism is not clear, the effects of nitrite seen in rats seem relevant for the hazard assessment in humans.

8.14. Immunity

Few studies (Ustyugova IV, 2002; Kozliuk *et al*., 1989; CDFA,1989) had reported the effect of nitrate/nitrite ingestion on immune system. The effect of nitrate ingestion (Ustyugova, 2002) on human immune system indicated that nitrate had no effect on lymphocyte growth, but nitrite decreases proliferation of lymphocytes. Fibroblast growth remains unaffected. A decreased production of Th1 cytokines (interleukin-2, interferon-gamma, and tumor necrosis factor-beta), which is responsible for resistance to a variety of infectious diseases was noted. No effect on the production of the Th2 cytokine interleukin-10, which is responsible for disease susceptibility, was noted. Because nitrate/nitrite shifted the balance from a Th1 to a Th2 response in some individuals, exposure to these compounds may decrease these people's responsiveness to infectious diseases. The levels of nitrate used in this study are relevant to human health because they are present in the liquid portion (non breastfed) of some 2-month-old infant diets in rural Romania. Animal studies also reported an immune suppression due to high nitrate ingestion (Porter *et al*., 1999).

8.15. Air Pollution and Nitrate Toxicity

Nitrogen oxides or NOx is the generic term for a group of seven compounds, highly reactive gases, all of which contain nitrogen and oxygen in varying amounts. The most important of these are nitric oxide (NO), nitrogen dioxide (NO_2) and nitrous oxide (N_2O). Nitric oxide and nitrogen dioxide are air pollutants. Nitrous oxide is not generally considered to be an air pollutant, but it is an ozone depleting gas, and also contributes significantly to visibility impairment in the form of plumes and hazes. Most of the nitrogen oxides in our environment come from natural rather than anthropogenic sources. Natural sources of NOx are lightning, and biological and non-biological processes in soil; they also descend from the stratosphere.

NOx cause a wide variety of health and environmental impacts because of various compounds and derivatives in the family of nitrogen oxides, including nitrogen dioxide, nitric acid, nitrous oxide, nitrates, and nitric oxide. They react to form nitrate particles, acid aerosols and contribute to formation of acid rain. NOx constitute one of the main ingredients involved in the formation of ground level ozone, which can cause serious respiratory problems because of its potent oxidizing properties. This occurs when NOx and volatile organic compounds (VOCs) react in the presence of heat and sunlight.

Nitrogen oxides form when fuel is burned at high temperatures, as in combustion in power plants and automobiles and during processes used in chemical plants. The general population is primarily exposed to nitrogen oxides by breathing in

air polluted with these gases. In addition, people residing in areas close to coal burning power plants or areas with heavy vehicular traffic are mainly exposed to higher levels of nitrogen oxides. Households that burn biomass fuels or coal or gas stoves have higher levels of nitrogen oxides in them when compared to houses using electrical appliances. Another source of indoor NOx is tobacco smoke. Active or passive inhalation of second-hand smoke exposes one to nitrogen oxides. Occupationally, workers employed in facilities that produce nitric acid or certain explosives like dynamite and trinitrotoluene (TNT), as well as workers involved in the welding of metals may breathe in nitrogen oxides during their work.

Health effects are related to levels of NOx as well as the duration of exposure (Health effects of outdoor air pollution, 1996; Bernstein *et al*., 2004). Low levels of nitrogen oxides in the air irritate eyes, nose, throat, and lungs leading to cough and shortness of breath, tiredness, and nausea. Breathing high levels of nitrogen oxides can cause rapid burning, spasms, and inflammatory swelling of tissues in the throat and upper respiratory tract. High exposures may lead to pulmonary edema leading to hypoxemia and even death. Industrial exposure to nitrogen dioxide may cause genetic mutations, damage to developing fetus, and decrease fertility in women. Industrial exposure to nitric oxide can cause unconsciousness, vomiting, mental confusion, and damage to the teeth. So far, there is no evidence that nitrogen oxides are potential carcinogens.

A review of studies published in the last decade has shown urban pollution to be an environmental cardiovascular risk factor (Maitre, 2006). This link was significant for NOx and PM10. A study of short-term effects of nitrogen dioxide on total, cardiovascular and respiratory mortality in 30 European cities found significant association between the two (Samoli, 2006). Significant associations of daily changes in particle concentrations, nitrogen dioxide and carbon monoxide were found with hospitalizations for respiratory diseases (CODP, pneumonia, asthma) and cardiovascular diseases (Hinwood, 2006). There are only a few reports of methaemoglobinemia caused by the inhalation of automobile exhaust fumes. Laney and Hoffman (1992) reported a patient who had methaemoglobinemia induced by automobile exhaust fumes while driving a sports car.

Exposure to indoor NO_2 at levels well below the Environmental Protection Agency outdoor standard (53 ppb) was associated with respiratory symptoms among children with asthma. Each 20-ppb increase in NO_2, increases both likelihood of any wheeze or chest tightness and days of wheeze or chest tightness (Belanger *et al*., 2006).

When we are exposed to gases like CO, NO, NO_2 etc. they may have many undesired effect on the blood haemoglobin (Borland and Higenbottam, 1989). These gases tend to reduce the oxygen carrying capacity of haemoglobin by oxidising the ferrous ions present in it, forming methemoglobin (due to NO*x* exposure) and carboxyhaemoglobin (due to CO exposure). This retards the oxygen carrying capacity of the blood haemoglobin and thus resulting in lesser oxygen being transported to various body parts. This may cause problems including suffocation, anxiety and hypertension and in extreme cases it may even lead to death. Although, the effect of such gases on the blood haemoglobin is reversible (Gibson and Roughton, 1957; Wark and Warner, 1981), but it may really pose a

threat in case of an excessive and prolonged exposure to such gases. Biyani *et al*. (2009) investigated the effect of such gases through mathematical modeling and the model validation was carried out by a simple non invasive parameter like percent saturation of peripheral oxygen (SpO_2) on exposure to a controlled environment. Percent oxygen saturation in the blood of human beings is reduced during exposure to environment where appreciable amount of NO*x* and CO are present in the ambient air (Kamat, 2002; Wennmalm *et al*., 1992). They formulated a model to quantify the effect of CO and NO*x* on oxygen carrying capacity of blood and the amount of carboxyhaemoglobin and methemoglobin formed due to such exposure. The model estimated using the data for individuals' working environment (kitchens using LPG), the SpO_2 values as they are exposed to such gases during cooking for a considerable period and was validated using their observed values. A similar form of model has been proposed earlier for production of methemoglobin during inhalation of nitric oxide for a fixed inspired concentration (Young *et al*., 1994 and 1996) but the values of the model parameters were not estimated. Another study has also been made on the effect of NO*x* on the somatic chromosomes of Goldsmiths earlier (Yadav and Seth, 1998).

It has been reported that nitric oxide-induced methaemoglobinemia can be reduced effectively by a medium to high concentration of methylene blue, or by a high dose of riboflavin in vitro, but not by N-acetylcysteine. Methylene blue acts as a cofactor for nicotinamide adenine dinucleotide phosphate, reduced (NADPH) methemoglobin reductase, an alternative hemoglobin reduction pathway, and greatly increases the enzymatic reduction of methemoglobin through this pathway (Khan and Kruse, 1999) (Fig 13). Methylene blue is commonly administered intravenously at a dose of 1 to 2 mg/kg, as a 1% solution, over 5 to 10 min, and sometimes via the oral route; further, an intraosseous infusion has been reported for infant methaemoglobinemia (Coleman and Coleman, 1996; Chongtham *et al*., 1997; Herman *et al*., 1999).

The EPA has established that the average concentration of nitrogen dioxide in ambient air in a calendar year should not exceed 0.053 parts of nitrogen dioxide per million parts of air (0.053 ppm). The Occupational Safety and Health Administration (OSHA) have set a limit of 25 ppm of nitric oxide in workplace air during an 8-hour workday, 40-hour work week. OSHA has also set a 15-minute exposure limit of 5 ppm for nitrogen dioxide in workplace air.

8.16. Animal Experiments

Clinical manifestations on different systems, of nitrate experiments are as follows:

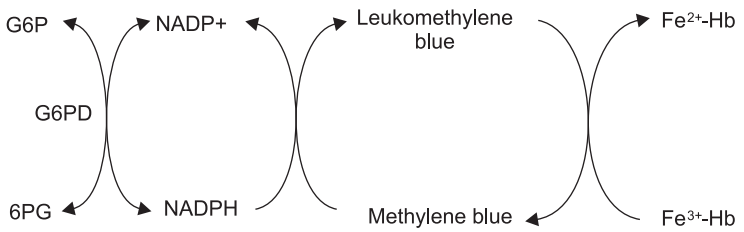


Fig. 13: Reduction of methemoglobin via methylene blue

8.16.1. Central Nervous System

Experiments on mice indicated that nitrites had some form of sedative effects. The development of sensoro-motor functions and adult learning behavior was studied in rats exposed to nitrate. The results indicated a nitrate-induced deviation in behavioral development, and impairment in learning behavior, particularly of the discriminative type (Markel *et al*., 1989).

8.16.2. Gastro Intestinal System

In a 19-month study effects of nitrate on gastric epithelium were studied (Ptashekas 1990). Ultrastructural examination showed that sodium nitrate alone or in combination with saphrol caused atypical changes in the gastric epithelium.

8.16.3. Cardiovascular System

Animal studies (Shuval *et al*., 1972; Gruener *et al*., 1970) reported increased cardiac muscle contraction which correlated directly to methHb levels. At 10–15% methHb, ECG shows shortening of Q-T interval, reduction in T wave.

8.16.4. Embryo Toxicity

Experiments on rats showed anemia, increased meth Hb, and increased mortality in off springs. Nitrite appeared to cause fetotoxicity in rats at drinking-water concentrations equivalent to 200 and 300 mg of sodium nitrite per kg of body weight per day, causing increased maternal metHb levels. However, after similar doses in feed in other studies, no embryo toxic effects were observed in rats. In a reproductive toxicity study in guinea-pigs at dose levels of 0, 50, or 60 mg of sodium nitrite per kg of body weight per day given by subcutaneous injection, fetal death followed by abortion occurred at the highest dose level. Teratogenic effects were not reported in studies on mice and rats (RIVM 1989; WHO 1996).

8.16.5. Genotoxicity

In an animal experiment on mice an increase in chromosome aberrations was found in a group of animals, who were consuming nitrate more than 707 mg/kg BW. It was observed that the number of micronuclei was enhanced at 79 and 236 mg/kg BW. The cyto toxicity occurred in the bone marrow as shown by a concomitant depression of the bone marrow. According to the authors, it cannot be excluded that formation of N-nitroso compounds was responsible for the bone marrow damage (Luca *et al*., 1985).

8.16.6. Reproductive Behavior

Reproduction in female guinea pig (RIVM 1989; WHO 1996) was grossly impaired by high nitrate ingestion (30,000 mg of potassium nitrate per litre). In rabbits, dose levels of 250 or 500 mg of nitrate per litre administered during 22 weeks revealed no detrimental effects on reproductive performance after successive gestations. In sheep and cattle, no abortions were observed at dose levels causing severe methaemoglobinemia (RIVM 1989; WHO 1996).

8.16.7. Mutagenicity and Related End-points

Nitrite is mutagenic. It causes morphological transformations in *in vitro* systems; mutagenic activity was also found in a combined *in vivo-in vitro* experiment with

Syrian hamsters. The results of *in vivo* experiments were controversial (RIVM 1989; WHO 1996).

8.16.8. Serum Somatomedin Activity

A decrease in serum somatomedin activity due to nitrate administration was also observed (Jahreis *et al*., 1987).

9. TREATMENT AND PREVENTION

9.1 Treatment

Supplementations of ascorbic acid, methionine, alpha-tocopherol, methylene blue have been found to be effective in overcoming the problem of nitrate toxicity. These substances are effective in inhibiting nitrosation if present in gastric juices at all time in significant concentrations.

9.1.1. Role of Antioxidants

As ROS are derived from a number of sources including nitrates, it was deemed feasible to readjust the balance of ROS production and detoxification by supplementing antioxidants. How to prevent or treat this oxidative stress? (Armitage *et al*., 2009)

However, clinical trials testing this hypothesis showed little benefits in reducing respiratory, GIT, cardiovascular etc. events or mortality; some of the treatments even caused harm (Dotan *et al*., 2009).

Indeed, *in vivo* detoxification of increased ROS by antioxidant supplementation may be impossible due to the poor bioavailability of the antioxidants at the correct subcellular localisation and at the optimal time point. Antioxidants may also promote new radical chain reactions initiated by their oxidised forms.

Importantly, the global removal of ROS may result in unwanted effects since ROS also regulate important physiological functions. Therefore, the current strategy is to identify and target the major sources of ROS, including NADPH oxidases (NO_X). A major constraint in NADPH oxidase research and translation is the lack of specific inhibitors. All commonly used compounds, including apocynin and diphenyleneiodonium, are unspecific (Heumuller *et al*., 2008; Wind *et al*., unpublished observations), and there are no specific NO_X inhibitors commercially available. The novel NADPH oxidase inhibitor, VAS2870, inhibits PDGF-stimulated cell migration and NADPH activity in vascular smooth muscle cells (Ten Freyhaus *et al*., 2006) as well as ox-LDL-stimulated ROS formation in endothelial cells (Stielow *et al*., 2006). However, its mechanism of action and selectivity for different NO_X isoforms is unknown.

In vivo experiments with a small peptide inhibitor, gp91ds-tat, which interferes with the interaction between NO_X^2 and the regulator p47phox, had promising results, e.g. it reduced angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery (Jacobson *et al*., 2003). As individual NO_X isoforms are regulated by different subunits, targeting these protein–protein interactions is one strategy for the development of isoform-specific NO_X inhibitors.

For more details on NADPH oxidase inhibitors and their limitations, the published reviews (Cave, 2009; Williams *et al*., 2007; Lambeth *et al*., 2008; Selemidis *et al*., 2008) can be looked. Once specific inhibitors become available,

their potential to manage CVD by preventing ROS formation can be fully explored. This concept may also be applicable to other chronic diseases associated with oxidative stress.

Hence the dissection of the oxidative stress hypothesis into a detailed identification of clinically relevant enzymes targeted by ROS offers for the first time a more mechanistic approach to treatment of oxidative stress including CVD. eNOS recouplers/enhancers as well as sGC stimulators/activators target specific oxidative damage, which currently can only be detected at a functional level. Nevertheless, clinical efficacy and proof-of-concept animal models promise a far more positive outcome than for previous antioxidant approaches. Thinking forward, the NADPH oxidase field represents an uncharted territory in the management of CVD. We believe that the development of NOX isoform selective inhibitors in the next decade will reveal the clinical relevance of this enzyme family. Twenty years after its discovery (Palmer *et al*., 1987), NO has come of age. It may be not so much NO synthesis itself but the signalling and oxidative events surrounding this unusual molecule that may produce the therapeutic outcomes that have been postulated for this highly protective vascular pathway and for management of oxidative stress including CVD.

9.2. Prevention

9.2.1. At Environmental Level

9.2.1.a. Avoid Water Pollution:

- Minimize the contamination of water supplies by nitrates originating from agricultural practice : Avoid inadvertent and excessive use of nitrogenous fertilizers
- Avoid water pollution
- Avoid the habit of open air defecation
- Avoid the stagnation of waste water around the source of water
- Avoid the sewage disposal directly to ground water table

9.2.1.b. Removal of Nitrate from Drinking water– Denitrification

The removal of nitrate from water is costly and difficult to implement and maintain both at domestic as well as community level. The following are the known processes of denitrification

a. *Physiochemical Process*: The commonly used processes under this category are (a) Reverse osmosis (b) Ion exchange (c) Electrodialysis
b. *Biological Treatments*: The details of only biological denitrification have been given in Appendix 1. This process is important since it bears similarity with metabolism of nitrates in living organisms.

Details of these processes have been depicted in Annexure III

9.2.2. At Human Level

- Breast feeding only, at least upto the age of 4 months.
- If top feed is necessary then preferably use cow milk (of course unadulterated) or dry milk preparations reconstituted with water containing low level of nitrate.
- Avoid the use of high nitrate containing food for weaning.

- Avoid use of nitrate and nitrite as preservative to the minimum extent possible especially to cured and canned meats and fish.
- Use of drinking water with low level of nitrates especially to pregnant mothers.
- Avoiding WHO ORS preparation with locally available high nitrate water during diarrhea.
- Avoid long term use of anti acid secretary agents especially in pregnant mothers and children. In case if it is necessary to use these drugs, they should be used cautiously and preferably with the use of antioxidants.

9.2.3. Reducing Nitrate Levels in Vegetables

The following hints will reduce nitrates in vegetables. The problem is that some of them will reduce essential nutrients such as vitamin C as well.

- Washing and cooking in water (nitrates are soluble in water)
- Peeling e.g. in potatoes because the nitrates are concentrated in the skin and just below (again, this is part of failsafe eating)
- Discarding: in leafy vegetables such as lettuce, discarding the stem and mid-rib can decrease up to 40 per cent of the nitrates
- A study of French fries showed that peeling reduced levels by 30 per cent; preheating and cutting reduced the nitrate content by a further 20 per cent and blanching by 30 per cent. After final frying only about five per cent of the original nitrate content remained (Reheating cold cooked vegetables has been shown not to increase nitrate levels as previously thought)
- For homegrown vegetables, choose low nitrate varieties (iceberg lettuce is one of the lowest and is failsafe); use slower releasing nitrogen sources such as manure and compost; avoid fertilising just before harvest; grow vegetables outside if possible (lower light due to plastic or glass covering contributes to higher nitrates); harvest in full sun
- A number of studies have suggested a protective effect of Vitamin C and other nutrients against either nitrates or particular cancers. Some experts think that the naturally occurring nutrients in vegetables are enough to protect against effects of nitrates. The WCRF warns: don't use high-dose supplements to protect against cancer. Research shows that high-dose nutrient supplements can affect our risk of cancer, so it's best to opt for a balanced diet without supplements

10. POLICY ISSUES AND RECOMMENDATIONS

For human consumption, WHO report, 2004 permits up to 50 mg/L but for short term exposure only, whereas IS-10500 permits 45 mg/L (as NO_3) as desirable limit and 100 mg of NO_3 per liter as maximum permissible limit in the absence of alternate source.

Since, the literature available on nitrate toxicity in human beings is limited and does indicate grave effects of nitrate ingestion e.g. methaemoglobinemia (effecting all systems of body due to lack of oxygen carrying capacity), Cancer, Diabetes (especially in children), damage to lungs and heart muscles, increased infant mortality, abortions, birth defects, recurrent diarrhea etc., the reports are by an large non conclusive.

Considering these toxicities and limited role of the protective system (adaptation of enzyme cytochrome b_5 reductase) available in human body against nitrate toxicity, probably the present WHO guide lines for short exposure may be adhered to. Looking to the ground reality that available technologies options are not affordable for public supplies, it is recommended that:

- Simple policy changes, to minimize the toxicity of nitrate may be urgently introduced in government campaign at least in high nitrate belt
- It is highly desirable that more detailed epidemiological health related studies covering a large sample are carried out to arrive at new acceptable standards
- A simple and cost effective method for nitrate removal should be developed.
- Health education system should be developed to make people aware about the toxic effects of nitrate ingestion and the ways of its prevention
- All measures to avoid the exposures to the tobacco smoke residue i.e. "thirdhand smoke." (SHS, smoke inhaled unintentionally) should be strictly implemented. Since this is a source of nitrosamine compounds, which are carcinogen
- Certain recommendations made by the Council on Scientific Affairs, were adopted by the AMA House of Delegates as AMA directives at the 2004 AMA Annual Meeting can be adopted in other countries facing the problem of nitrate toxicity; (1) The AMA supports the current Food and Drug Administration and United States Department of Agriculture regulations, including current labeling requirements, for nitrites in food. (Directive) (2) The AMA encourages continued research and surveillance of the safety of nitrite use in foods, with particular attention to its possible effects on type 1 diabetes (Directive)
- Guidelines should be laid down to reduce the nitrate in vegetables.

11. CONCLUSIONS

Nitrate and Nitrite are natural ions that are a part of the nitrogen cycle. Naturally occurring nitrate levels in surface and ground water are generally a few milligrams per liter. In many ground waters an increase of nitrate levels has been observed due to water percolating through nitrate rich rocks and owing to the farming practices of using chemical fertilizers recommends.

For human consumption, WHO report, 2004 permits up to 50 mg/L but for short term exposure only, whereas BIS-10500 permits 45 mg/L (as NO_3) as desirable limit and 100 mg of NO_3 per liter as maximum permissible limit in the absence of alternate source.

Not much literature on human studies is available on nitrate toxicity except reports documenting Methaemoglobinemia in infants due to high nitrate ingestion. Apart from methaemoglobinemia, few studies indicated nitrate as a cause of cancer, but it is still controversial and no firm conclusions have been drawn so far. The other effects observed were increased infant mortality, abortions, birth defects, recurrent diarrhea, recurrent stomatitis, early onset of hypertension, ischaemia, CVD, histopathological changes in cardiac muscles, alveoli of lungs and adrenal glands, recurrent respiratory tract infection in children, Neprhotic syndrome in children, hypothyroidism and diabetes. Recent studies have indicated that high

nitrate ingestion adversely affects the immune system of the body. Recently an adaptation system to nitrate ingestion has also been reported. This adaptation to an enzyme cytochrome b_5 reductase has been shown to be protective to human being, but to a limited extent only.

More detailed epidemiological health related studies covering a large sample are required to provide insight to the nitrate toxicity on human being. It is possible that a detailed study on pathophysiology of nitrate metabolism and its effect on human being would yield a better understanding of the various diseases caused by nitrates, and, their prevention and treatment.

Since it is very difficult and costly to remove nitrates from water because it is chemically non reactive in dilute aqueous solutions, it is recommended that a simple change in habits and adoption of simple preventive measures may be urgently introduced in government campaign at least in high nitrate belts. Indiscriminate use of nitrogenous fertilizers should be avoided. The most important strategy is to promote breast feeding up to the age of at least 6 months.

12. FUTURE RESEARCH AREAS FOR NITRATE TOXICITY

12.1. Research in the Field of Human Health

Nitrate is perhaps the most extensively studied anion in water for its chronic toxic manifestations, as it is one of the most common pollutants of drinking water all across the world. Despite the availability of vast literature on nitrate toxicity, the studies on humans are limited because of ethical and other considerations, and, the most toxic manifestations are available in animal studies only. Many epidemiological studies have been conducted to establish certain cause-effect relationships for many clinical manifestations of nitrate toxicity and the results of such studies are normally inconclusive or the correlations are statistically non significant. This has led to a very insufficient knowledge generation among researchers and practicing doctors about the preventive and curative aspects of different manifestations. There is wide field of research in this area; still the few suggestions are as follows

- Researches relating to nitrate, nitrite, NO, NOx, ROs and various diseases e.g. malignancy, diabetes, thyroid, kidney, heart etc. should be explored and a possibility should be sarched out for the treatment of these disorders at cellular level.
- The interaction of tobacco smoke especially third hand smoke should be established, so that mitigation strategies may be formulated to over come this problem especially to protect our infants and new born.
- Further researches may be initiated to find out the specified antioxidants, so that treatment guideline may be initiated for the treatment of certain untreatable disorders e.g. malignancy, CVD, asthma, Kidney disease etc.
- The effect of long term use of antacids, H_2 receptor inhibitor, proton pump inhibitor with high nitrate ingestion should be studied among human volunteers with adequate control. Microbiological analysis of salivary samples as well as the swab samples can help monitor the progress of biological conversion of ingested nitrate in these localized areas. Further it should be seen that is there any improvement with addition of antioxidant?

- Role of ingested and endogenous nitrate is also important to delineate in human.
- Understanding the nitrate metabolism and its relation to free oxide radical production with their role in carcinogenesis may give rise to an insight to the pathophysiology of cancer development in human beings. This may provide a guide line in treatment and prevention of cancer.
- A study is required to suggest changes in ORS where nitrate is high in drinking water, since ORS supplementation in diarrhea patient is inevitable but if prepared in high nitrate water, it may be hazardous to these children.
- Since high nitrate effects the immune response, a study should be taken to evaluated the sero conversion of vaccines in these children consuming high nitrate in drinking water.

12.2. Research in the Field of Environmental Protection, Agricultural Modification and Mitigation Measures

Sufficient data on nitrate content in groundwater must be collected systematically and periodically; and analysed to understand the sources contributing to it.
Relative contributions of different sources of nitrate in different regions must be investigated so that appropriate recommendations can be made for control of pollution.

Practices that result in high fertilizer use nitrogen efficiency provide environmental protection, high crop yields and profits. Therefore, increasing fertilizer use efficiency should be the highest research priority. Holistic approach to farming systems will have to be adopted and it should be based on soil characteristics, climatic constraints, moisture availability and management skills of the individual farmers. One of the major challenges is to understand the timing and amount of N supplied by mineralization while adjusting application rates of additional inorganic or organic N fertilizer to ensure that plant demand for N is met. Other important research areas that can improve fertilizer use efficiency are integrated management of organic, biological and mineral nutrient sources, identification of best management practices and fertilizers for specific conditions (precision farming). Crops which act as scavengers of nitrate from deep soil layers need to be identified and included in cropping sequences.

Suitable methods of estimating nitrate leaching have to be evaluated for different situations. Long term studies are required to evaluate management practices. Mathematical models should be developed, evaluated and used in conjunction with geographical information system. The contributions from various sources such as agriculture, industry, urban activities, deforestation, dairy industry etc. to the nitrate contamination of drinking water must be evaluated.

Methods of controlling leaching losses of nitrates from agricultural fields through better cropping practices, better fertilizer management and better irrigation management have to be developed. Various cropping management practices include use of crop rotations, cover crops, optimum water management and soil conservation. Different fertilizer management practices include need based application of nitrogen fertilizers (based on colour chart, sap test total N or chlorophyll determination), split applications, slow release fertilizers and nitrification inhibitors, integrated

nutrient management etc.

Calculation of residence time of nitrate-N in the soil profile after it escapes the root zone will enable prediction of time trends for nitrate-N levels in groundwater bodies.

Biogeochemical processes controlling nitrate attenuation in aquifers have been critically reviewed (Srikanth, 2009). Further research is needed to improve current understanding on the influence of organic carbon, sulphur and iron electron donors, physical restrictions on microbial activity in dual porosity aquifers, influences of environmental condition (e.g. pH in poorly buffered environments and salinity in coastal or salinized soil settings), co-contaminant influences (particularly the contrasting inhibitory and electron donor influences of pesticides) and improved quantification of denitrification rates in the laboratory and field.

Global estimates of gaseous emissions of NH_3, N_2O and NO from fertilizer are 11.2, 0.9. 0.6 Mt N/Year, respectively (Rivett *et al*., 2008). N discharged into the sea in Asia is likely to be 9.6 Mt in 2030 (one third of global). These estimates should be refined and ways of limiting pollution should be looked into.

ANNEXURE 1

NITRATE LEVEL SCENARIO

Naturally occurring nitrate levels in surface and ground water are generally a few milligrams per liter. Nitrate, either from the mineralized organic matter, fertilizers, organic manures, industrial effluents or urban wastes are sources of groundwater pollution. In many ground waters higher levels are found due to water percolating through nitrate rich rocks and also due to an excessive use of chemical fertilizers, sewage and animal manures. The available data from various studies point to the fact that nitrate pollution from any of the above sources can pose a serious health hazard to humans, animals, birds and aquatic life. WHO maintains that extensive epidemiological data support limiting the value of nitrate-nitrogen to 10 mg/l or as nitrate to 50 mg/L (WHO, 2004) for human consumption whereas IS-10500 prescribes maximum permissible limits in drinking water as 45 mg of NO_3 per liter (IS 10500, 1995).

The problem of nitrate toxicity is global and has been reported from a number of countries (Prakasa Rao, 2006).

International Perspective

Nitrate is a widespread contaminant of ground and surface waters worldwide (Prakasa Rao, 2006; Singh *et al*., 1994). The accumulation of nitrate in the environment results mainly from:

- non-point source runoff from the over-application of nitrogen fertilizers
- point-sources such as Concentrated Animal Feeding Operations (CAFOs)
- point-sources from poorly or untreated human sewage

In addition, nitrate-containing wastes are produced by many industrial processes including paper and ammunitions manufacturing. Combustion of fossil fuels in power plants and cars, SUVs and other internal combustion engines results in the production of nitric acid and ammonia and causes air pollution.

In the United States, the problem appears to be concentrated in the Mid-West and the Far-West, with large areas of Iowa, Illinois, Kansas, Michigan, Wisconsin, Washington and California being heavily affected (Revenga and Mock, 2001; Smith *et al*., 1990). The US Geological Survey (USGS) reported that nitrate concentration in the nation's groundwater supply was increasing steadily.

The level of nitrates in freshwater ecosystems is a problem worldwide (Prakasa Rao and Puttanna, 2006; Singh *et al*., 1994; Revenga and Mock, 2001; Smith *et al*., 1994; Hallberg, 1989; Puckett, 1995; Davies and Bradley, 1995; Haung, 1995; Kviteck, 1996; Burt, 1993; Hirata, 1996; Kutrz, 1980; Cameron and Haynes, 1986; David *et al*., 1997; Shiklomanov, 1997; Camargo and Alonso, 2006; Zhang *et al*., 1996; Scheidleder *et al*., 1999; Colvin *et al*., 2008).

Data on nutrient trends in global waters are scarce. Nitrate concentrations are higher in watersheds that have been intensively used and modified by human activity, such as the Weser, Seine, Rhine, Elbe, and Senegal. High levels are also found in such watersheds in China, South Africa, and the Nile and Mississippi basins.

South Africa has some of the highest natural nitrate levels in the world (more than 550 mg/L NO_3^--N) especially in the Kalahari region (Colvin *et al*., 2008).

In South America, nitrate concentrations in the monitored watersheds are relatively low and follow human land use. The highest nitrate concentrations are found in

the Uruguay watershed, where intensive agriculture is practiced. Likewise, nitrate concentrations in water are also greater in the Magdalena watershed of Colombia than in the less densely populated watersheds of the Amazon basin. The nitrate concentrations in South America correspond to lower fertilizer application rates, compared to Europe.

More detailed data are available in Europe and show distinct regional trends in the concentrations of nitrates in rivers (Scheidleder *et al*., 1999). Nitrate loadings are highest in areas with intensive livestock and crop production, especially in the northern parts of Western Europe. Nitrate concentrations are lowest in Finland, Norway, and Sweden.

Nitrate pollution of groundwater is also of serious concern. In general, the risk of nitrate pollution for groundwater supplies is directly related to the amount of fertilizers or other nitrogen inputs to the land, as well as the permeability of the soil. For example, half the groundwater samples in a heavily fertilized region of northern China contain nitrate levels above the safe limit for drinking water. In the United States, high nitrate concentrations are widespread in shallow groundwater aquifers in agricultural areas. Groundwater pollution in Europe is similarly widespread (Scheidleder *et al*., 1999).

National Perspective

Nitrate either from the mineralized organic matter; fertilizers, organic manures, industrial effluents or urban wastes are sources of groundwater pollution which has received attention in various parts of India. The available data from various studies point to the fact that nitrate pollution from any of the above sources can pose a serious health hazard to humans, animals, birds and aquatic life. The data available on concentrations of nitrate in groundwater samples from Punjab, Haryana, U.P., Delhi, Orissa, Tamil Nadu, Bihar, A.P., M.P., Maharashtra, Karnataka and other states point to the fact that in many of these samples, the nitrate content in groundwater has been more than permissible limits. In many parts of Punjab and Haryana nitrate levels in groundwater is continuously increasing beyond the critical standard. Nitrate levels in groundwater over vast agricultural areas can be correlated with intensive irrigated agriculture, use of nitrogenous fertilizers, and groundwater exploitation.

Nitrate pollution and strategies for reducing it has been reviewed (Prakasa Rao, 2006). Nitrate pollution of groundwater by fertilizer use in India has also been reviewed (Singh *et al*., 2007).

In the riparian states of Punjab, Haryana, Uttar Pradesh and Tamil Nadu the problem of nitrate pollution of groundwater is acute. A report on State of India's Environment discussed nitrate contamination of water in India (Reidhead, 1996). Excessive use of fertilizers has led to an increase in the levels of nitrate in the shallow groundwater sources. The nitrate content in the well water of U.P., Haryana and Punjab are far beyond the standard prescribed safe limit of 45 mg/L (Reidhead *et al*., 1996; Agrawal *et al*., 1999). In agriculturally intensive areas of Punjab (Kansal *et al*., 1992), Delhi (Pathak *et al*., 1999; Kansal *et al*., 1994; Gupta *et al*., 1999), Maharastra (Deshpande *et al*., 1999) Andhra Pradesh (Srinivasa Rao, 1998), where fertilizer applications are high, there is ample evidence of pollution of groundwaters by nitrates. Even in semi-arid regions of Deccan plateau (Power

and Sheikh, 1995; Patra and Rego, 1997) and arid regions of Rajasthan (Ozha *et al*., 1993; Nagaraj and Chandrashekar, 2005) nitrate leaching was found prevalent. A study of groundwater quality in Karnataka during 2000 – 2001 indicated 37% of habitations (numbering 20929) are facing water quality problems and nitrate was a problem in 4077 habitations (Nagaraj and Chandrashekar 2005).

Numerous reports indicate sporadic groundwater pollution with nitrate in different parts of the country (Malik, 2000; Singh and Sekhon, 1977; Sankararamakrishnan *et al*., 2008; Kundu, *et al*., 2008; Kundu and Mandal, 2009; Reddy *et al*., 2009; Datta *et al*., 1997; Sehgal *et al*., 1989; Rangarajan *et al*., 1996; Rajmohan and Elango, 2005; Narula *et al*., 2003; Mishra *et al*., 2000; Thirumathal and Shivakumar, 2003; Nemade and Shrivastava, 1997; Nagaraju and Sastri, 1999; NEERI, 1991; IFA, 2002; Handa, 1986; Laksmanan, 1986; Gupta, 1981; Kulashreshta, 2003; Gonnade, 2004; Raju, 2009; Suthar *et al*., 2009; Chaudhary V, 2010; Sinha, 2008; Dar, 2009; Mondal, 2008; Sardessai and Sundar, (2007); Sankararamakrishnan *et al*.,2008; Reddy, 2009; WHO, 2003; Wild, 1988)

National Environmental Engineering Research Institute (NEERI) has published Information on the occurrence of excessive nitrate in groundwater in India (NEERI, 1991). About 56% of the total nitrogen fertilizers manufactured in India is used for producing rice, maize and wheat (IFA, 2002). Using the data generated by reconnaissance of nitrate content in shallow groundwaters by Central Groundwater Board (Handa, 1986) (Table 5). The states of Punjab and Haryana have been placed in high risk zone. North eastern states have been placed in low risk zone. The other states lie in between.

NO_3^- enrichment in groundwater has been appearing as a major threat in few intensively cultivable states: Punjab, Haryana, Maharastra, Andhra Pradesh, and Uttar Pradesh; West Bengal, Rajasthan and Delhi (Table 6).

Northwestern India

Nitrate N content in groundwater in Punjab has been continuously increasing since 1975. In 1999, several samples of water drawn from shallow hand pumps in Punjab contained nitrate N levels much above the WHO limit of 10 mg N l^{-1}. In 1997 predicted that mean concentration of nitrate in groundwaters in Punjab will increase from 0.42 mg nitrate N l^{-1} in 1975 to around 8 mg nitrate N l^{-1} in years to come (Singh and Sekhon, 1977). Probability plots indicated that 90% of the well water samples contained nitrate N less than the safe limit. The geometric mean of nitrate N content registered an increase from 0.42 to 2.29 mg l^{-1} during 1975 to 1988. In a study undertaken by National Environmental Engineering

Table 5: Risk of groundwater nitrate pollution in different parts of India (Handa, 1986)

Risk zone	Average fertilizer N consumption (kg/ha)	Average NO_3 in groundwater (mg/L)	Region (states)
Little or no risk	2	6–8	Jammu and Kashmir, north eastern states
Low risk	4–11	8–45	HP, MP, Orissa, Maharashtra
Moderate risk	14–53	13–50	UP, Uttarakhand, Bihar, Jharkhand, WB, AP, Gujarat
High risk	118–163	55–100	Punjab, Haryana

Table 6. Reports of nitrate nitrogen contamination of ground waters in various parts of the country

Sampling/study site	State	Range (mg/L)	Referans
Ludhiana district (n =?)	Punjab	12–30	Shirahatti, 2000
Kanpur district (n = 297)	Uttar Pradesh	1.0–166	Sankararamakrishnan et al., 2008
Hooghly district (n = 412)	West Bengal	0.01–4.56	Kundu et al., 2008
Nadia district (*n* = 342	West Bengal	0.01–5.97	Kundu and Mandal 2009
Anantapur district (*n* = 48)	Andhra Pradesh	3.0–684	Reddy et al., 2009
New Delhi (n = 95)	Delhi	0.04–98.3	Datta et al., 1977
Jaipur district (n = 5)	Rajasthan	26–459	Gupta et al., 1981

Research Institute, Nagpur, 1290 out of 4696 (27%) groundwater samples from selected districts in 17 states in India (excluding northeastern states) contained more than 45 mg nitrate N (NEERI, 1991). High nitrate content (45 to > 600 mg l^{-1}) in shallow and deep tube wells due to seepage from industrial effluents and urban sewage has been reported from around Jodhpur city in Rajasthan and Lucknow in Uttar Pradesh (Sankararamakrishnan *et al*., 2008).

Malik (2000) analysed data on nitrate content of groundwater samples collected by Punjab and Haryana state Groundwater Boards. More than 33% of the water samples in Punjab and Haryana had nitrate levels above the desired limit of drinking water standards. In about 17% of the samples, the nitrate N level exceeded 22 mg/L. A close scrutiny of the available data revealed that there is no discernible pattern of distribution of nitrates in groundwaters of Punjab and Haryana and pollution is localized (Malik, 2000).

In ground water of northwest Rajasthan, nitrate concentration was in the 0.0–278.68 mg/L, respectively (Kundu *et al*., 2008). Most of the water samples were in the categories of nitrate < 45 mg/L. High nitrate in groundwater of Churu Block, Rajasthan indicates possibility of seepage from sewage (Kundu and Mandal, 2009).

The level of nitrate in some agro-economy based rural habitations of northern Rajasthan, India has been evaluated (Reddy *et al*., 2009). NO_3^- level in groundwater was 7.10–82.0 L with an average NO_3^- level of 60.6±33.6 (SD) mg/L.

Uttar Pradesh

Groundwater quality in the Lower Varuna River Basin, Varanasi District and Uttar Pradesh has been reported (Raju *et al*., 2009). Nitrate–N concentrations were analyzed in shallow and unconfined ground water aquifers of Kanpur district along the Ganges Alluvial Plain of Northern Bithore zone, 19% of the samples exceeded the limit and as high as 166 mg/ L as nitrate–N was observed. 10% and 7% samples in Kanpur city and beyond Jajmau zone respectively, exceeded the BIS limit (Sankararamakrishnan *et al*., 2008). Especially in Bithore zone, the point sources could be attributed to the animal wastes derived from cows

and buffaloes and non point sources could be due to the extensive agricultural activity prevalent in that area. No significant seasonal variation in water quality parameters was observed.

Bihar

Ranchi city is coming under the threat of nitrate poisoning (Kundu *et al*., 2008). The accumulation of high nitrate in cracks, fissures in the hard rocks of the Jharkhand State, is obvious due to the relatively thin soil cover and occurrence of groundwater at shallower depth; pollutants find their way to the groundwater body. Other than anthropogenic sources metamorphic rocks present in Jharkhand Plateau can be the other source of nitrate to ground water because nitrogen is found in the metamorphic rocks.

In Bihar State other than Rafiganj and Bhagalpur area concentration of nitrate in groundwater is below toxic level. In Rafiganj the concentration of nitrate varies from 100 ppm to 300 ppm. In Bhagalpur it varies from 90 to 160 ppm. In some parts of Motihari district the concentration of nitrate has reached up to the level of 155 ppm. In Valmiki Nagar few places have been identified where the concentration of nitrate has crossed the toxic level.

West Bengal

Reports of nitrate contamination in Hooghly district (412 samples, 0.01–4.56 mg NO_3 /L) (Kundu *et al*., 2008) and Nadia (342 samples 0.01–5.97 mg NO_3 / L) (Kundu and Mandal, 2009) have appeared in literature.

Kashmir

An attempt has been made to evaluate the concentration of nitrate in groundwater and its management in Sopore and its environs (Dar *et al*., 2009). Linear Trend Analysis on seasonal and annual basis clearly depicted that nitrate pollution in the study area is increasing significantly. About 85% of samples during summer season and 67% of the samples during winter season were showing a high concentration of nitrate, exceeding permissible limit of 50 mg/ L, which is due to the use of nitrogenous fertilizers in the study area.

Karnataka

In a study entitled ground water quality scenario in Karnataka, conducted under the aegis of the World Bank for the rural water supply and sanitation an extensive survey was conducted in 2000–01 (Nagaraj and Chandrashekar, 2005). Water quality mapping done by GIS based on 154,491 ground water samples showed that nitrate content in drinking water ranged from 101 to 6064 mg nitrate/L. A large number of villages are affected by high nitrate content in drinking water and pose a serious health problem. The relative numbers of villages affected are given in parenthesis for some of the worst affected districts: Tumkur (35%), Mysore (20%), Kolar (21%), Raichur (10%), Mandya (5%), and Davanagere (15%).

According to a mines and geology department study on excess chemicals in the state's groundwater, most districts where drinking water is supplied through bore wells have a high concentration of nitrates and fluorides.

The main cause for increase in nitrates in groundwater is open sewage disposal and use of nitrogen fertilizers. Some districts like Gulbarga, Bijapur, Raichur and

Tumkur have sanitation coverage below 20%.

NCR Delhi

Except Gautham Budh Nagar and Bulandshahar districts, in many parts of all the NCR sub-regions, groundwater is severely affected by nitrate pollution (Singh *et al*., 2007). Most of these parts are under NCR Plan land use of agricultural and regulated area. As low as 20 mg/ L to abnormally high levels (100–130 mg/ L) of nitrate in groundwater have been found at different places in the districts of Rohtak, Sonepat, Faridabad and Gurgoan of Haryana sub-region.

Groundwater in Delhi shows wide range in nitrate (10–1600 mg/L) content (Singh *et al*., 2007).

The relationship between NO_3 and ^{18}O also suggests that the groundwater originates from two or more isotopically distinct, non-point sources which vary spatially as well as temporally due to different degrees of evaporation/recharge (Datta *et al*., 1997) and different amounts of fertilizers applied.

Maharashtra

The studies carried out by Central Ground Board in the state of Maharastra (Deshpande *et al*., 1999) revealed that out of 688 samples, 75% of the samples had nitrate levels below the critical limit and 14% have nitrate levels above 100 mg/L. High nitrate levels in 73% samples in Nagpur metropolitan area should be due to urban sewage and industrial sources. Nitrate contamination is prominent in Yavatmal, Nagpur and Chandrapur and is only of isolated importance in other districts.

Rajasthan

In Rajasthan, nitrate rich groundwaters have been observed in many parts of Churu, Alwar, Bharatpur, Jalore, Jaipur, Sikkar, Tonk and Jhunjhunu districts posing a serious threat to human health (Suthar *et al*., 2009).

Gujarat

In Mehsana district 18.5 % of habitations were affected by excess nitrate in water but in Amreli district there was no affected habitations in 1991 (NEERI, 1991). There are 773 nitrate-affected villages in Gujarat (Nagaraju and Sastri, 1999).

Andhra Pradesh

Nitrate concentrations in the groundwater samples of Krishna delta, showed large variations of nitrate from 10–135 mg/L. In 79 groundwater samples, about 39% shows high nitrate contents (>50 mg/L), which is more than the permissible limit in drinking water. In north Krishna delta 49% and in south Krishna delta 26% water samples were found to exceed the permissible limit. This study indicates that groundwater of north Krishna delta is more polluted than south. Nitrate pollution level is found more in dug wells compared to hand pumps/bore wells. In this region 49% dug wells and 31% hand pumps have exceeded the desirable limits. The possible sources for the high nitrate level in groundwater were identified as excessive utilization of nitrogenous fertilizers for agricultural purposes. The studies carried out on the nitrate contents have shown that concentration varies from 10–135 mg/L.

Nitrate in groundwater in Vamsadhara basin of Srikakulam district, AP, was found to range from below detection limit to 450 mg NO_3 /L and it was traced to point sources (poultry farms, cattle sheds and leakages from septic tanks) and non-point sources (agriculture) (Srinivasa Rao, 1998). High nitrate concentrations were derived from animal sources.

In the north eastern part of Anantapur district, 65% of the samples were found to be unsuitable for drinking purposes in the pre monsoon season and 45% in the post monsoon due to excess nitrate content in the groundwater (Reddy *et al*., 2009). Among the different seasons and environs, nitrate was in highest concentration in the granitic terrain and canal command areas during pre monsoon season. The nitrate was found to decrease with depth in all the hydrogeological set-ups in both the seasons. Intense agricultural practices, improper sewerage and organic waste disposal methods were observed to contribute nitrate to the shallow and moderately deep aquifers.

Goa

Observations carried out during the dry season April-May 2002, and March 2003 and wet season September 2002, showed temporal and spatial variability of nitrate in the Mandovi and Zuari estuaries, Goa, India (Sardessai S & Sundar D, 2007). During the month of September in the wet season nitrate concentration in the mid estuarine region of the Mandovi estuary increased from 4.4 μM (2.7ppm nitrate) in the upstream region to 6.0 μM (3.7ppm nitrate). Nitrates are found to decrease during the dry season possibly due to horizontal mixing of the shelf water, which contains less than 1 μM of nitrate or its utilisation in biogeochemical processes. Significant inter-annual variability in nitrate concentration has been reported. The Zuari estuary also showed similar variability as that of Mandovi.

North East

North Eastern provinces with high nitrate levels: Assam 0.02–49.0 mg/L Arunachal Pradesh 0–40mg/L (non monsoon) 0–45.3 (monsoon) Manipur 0–24.6 mg/L (monsoon) (Handa, 1986)

Kerala

Nitrate has been found in drinking water (Laksmanan,' 1986). Nitrate concentration ranged between 0.35 and 35.44 mg/L. Nitrate was relatively higher in the well water samples from Nedumkandam, Kalkoonthal, and Kallar, due to contamination from a septic system, sewage, and agricultural runoff.

Tamil Nadu

Nitrate contamination of groundwater in Chennai, Bhavani basin (Kulashreshta *et al*., 2003) and Palar and Cheyyar river basin (Rajmohan and Elango, 2005), Chennai (Rangarajan *et al*., 1996) (3–226 mg NO_3 –N/L) and Swaminathapuram (Thirumathal and Shivakumar, 2003), Dindigul district in Tamil Nadu has been reported.

An Overview

Punjab and Haryana have been placed in high risk zone. Irrigation without

artificial drainage in the poorly drained flat plains of Punjab and Haryana (with a thick pile of unconsolidated and permeable Late Quaternary–Holocene alluvial sediments, increases the nitrate pollution hazard compared to that in the freely drained regions of northern and northeastern states and peninsular plateau in the southern part of the country. Agriculture is not intensive in some northern hilly states and northeastern states as reflected in the meager consumption of nitrogenous fertilizers and negligible groundwater use. The situation in peninsular states is in between the two. But nitrate levels are showing an upward trend throughout the country (Table 7).

Table 7: Information on the occurrence of excessive nitrate in ground waters of India (NEERI, 1991)

Name of the State	No of samples analysed for nitrate (NO_3)	Number of samples in which nitrate is more than 45mg/L
Andhra Pradesh	151	8
Assam	94	–
Gujarat	48	32
Haryana	365	142
Arunachal Pradesh	41	5
Jammu and Kashmir	125	17
Karnataka	336	116
Kerala	45	1
Madhya Pradesh	225	50
Maharashtra	–	–
Orissa	201	28
Punjab	351	93
Rajasthan	581	314
Tamil Nadu	–	–
Uttar Pradesh	397	85
West Bengal	257	33
Delhi	390	212

ANNEXURE II

Table 8: Nitrates and Nitrites in Vegetables (mg/kg)

Nitrate Food	Low	Average	High	Year	Source*
Beans	6	392	810	2008	EFSA
Beetroot	110	1370	3670	2008	EFSA
Brussels sprouts	1	24	100	2008	EFSA
Cabbage	47	311	833	2008	EFSA
Carrots	21	296	1574	2008	EFSA
Cauliflower	7	148	148	2008	EFSA
Celery	18	1103	3319	2008	EFSA
Garlic	8	69	161	2008	EFSA
Green Beans	9	323	735	2008	EFSA
Leek	5	345	975	2008	EFSA
Lettuce iceberg	210	875	1537	2008	EFSA
Parsnip	2	16	83	2008	EFSA
Peas	1	30	100	2008	EFSA
Potatoes	10	168	340	2008	EFSA
Pumpkin	8	894	4617	2008	EFSA
Rhubarb	28	2943	6550	2008	EFSA
Spinach	64	1066	3048	2008	EFSA
Spinach organic		2138		2005	FSA
Lettuce glasshouse		5700		2004	FSA
Lettuce rucola		4800		2008	EFSA
Lettuce curly		3263		2005	FSA
Lettuce iceberg u/cover		2500		2004	FSA
Lettuce organic		1115		2005	FSA
Lettuce		875		2008	EFSA
Lettuce organic		596		1982	SA

http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/contam_ej_689_nitrate_en.pdf
The EFSA maximum permitted level for nitrates in lettuce is 4,500 mg/kg

ANNEXURE III

Biological Denitrification

The biological reduction of nitrate to nitrite and subsequently to dinitrogen gas requires a suitable electron donor. The electron donor is usually an organic molecule and methanol is the most commonly used carbon source. Equations 1 and 2 represent the reduction of nitrate to nitrite and nitrite to nitrogen gas respectively and the equation 3 represents the overall reaction using methanol as the electron donor (McCarty *et al*., 1969).

$$NO_3^- + 0.33\ CH_3OH \rightarrow NO_2^- + 0.67\ H_2O + 0.33\ CO_2 \quad (1)$$

$$NO_2^- + 0.5\ CH_3OH \rightarrow 0.5\ N_2 + 0.5\ H_2O + 0.5\ CO_2 + OH^- \quad (2)$$

and overall:

$$NO_3^- + 0.833\ CH_3OH \rightarrow 0.5\ N_2 + 1.16\ H_2O + 0.833\ CO_2 + OH^- \quad (3)$$

Other organic carbon source such as ethyl alcohol sucrose, acetone, brewery waste, chemical process waste, corn starch waste, molasses, wharf, sulfide liquor and winery residue etc. are taken as inexpensive carbon substrates.

Autotrophic denitrification has been studied using hydrogen or various sulfur compounds. Autotrophic denitrifying bacteria use molecular hydrogen or other inorganic compounds as the reductants and carbon-dioxide as the source of carbon as shown in equation below (Smith *et al*.,1994; Garcia de Lomas *et al*., 2006).

$$5\ H_2 + 2H^+ + 2\ NO_3 \rightarrow N_2 + 6\ H_2O \quad (4)$$

Some researchers have evaluated reduced sulfur compounds such as sulfide and thiosulfate for the denitrification of water and domestic and industrial wastewater (Sutton *et al*., 1979; Amminudin and Nicholas, 1973). Sulfate is a by product of denitrification using sulfur compounds as given in following equations.

Thiosulfate (Claus and Kutzner, 1985)

$$5\ S_2\ O_3^{2-} + 8\ NO_3^- + H_2O \rightarrow 4\ N_2 + 10\ SO_4^{2-} + 2H^+ \quad (5)$$

Sulfide (Barrenstein *et al*., 1986)

$$5\ S^{2-} + 8\ NO_3^- + 8\ H^+ \rightarrow 5\ SO_4^{2-} + 4\ N_2 + 4\ H_2O \quad (6)$$

Such reactions have been utilized gainfully by researchers for developing simultaneous anaerobic sulfide and nitrate removal systems (Gommers and Kuenen, 1988; Cai *et al*., 2010). While the heterotrophic denitrification produces alkalinity, the autotrophic denitrification consumes alkalinity (Park *et al*., 2010). A sequencing batch reactor is the most frequently applied system for biological nitrogen removal as anoxic and aerobic phases can be separately handled with high efficiency in a single reactor (Terada *et al*., 2006). An interesting modification of conventional denitrification has been reported by Modin *et al*. (2010) in the form of aerobic methane oxidation coupled to denitrification. Here aerobic methanotrophs oxidize methane and release organic compounds that are used by co-existing denitrifiers as electron donors for denitrification.

Though oxygen inhibits denitrification, there have been periodic reports of aerobic denitrification (Marshall *et al*., 1953; Krul, 1976). Recently under

controlled conditions in homogeneously suspended bacterial cultures at D.O. concentrations ranging from 10% to twice air saturation, persistent denitrification has been reported by many workers (Krul and Veeningen, 1977; Meiberg *et al*., 1980, Gupta and Kumar, 1999).

Robertson and Kuenen (1988) not only detected the presence of appropriate enzyme but also demonstrated the production of nitrogen containing gases from nitrate by *Thiosphaera pantotropha* (isolated from desulphurizing, denitrifying waste water treatment systems) at dissolved oxygen concentrations up to 90% of air saturation.

In situ Denitrification

In situ groundwater treatment has been reported to be used for removing various constituents from groundwater. Several investigators have evaluated the injection of various substrates and nutrients into aquifers in an effort to simulate *in situ* denitrification. Liquid substrates such as acetic acid, ethanol and treated wastewater have been used and gaseous substances have been evaluated.

Application of *in situ* ground water denitrification depends on the prevention of well clogging (Kruithof *et al*., 1988) and biomass accumulation is thought to be the main cause of *in situ* clogging. In shallow aquifers accumulation of gas may represent a major contributor to the clogging of wells (Soares *et al*., 1988).

Following considerations should be made while designing an *in situ* denitrification scheme:

1. Complete utilization of carbon sources is required since no organic carbon is allowed to present in drinking water.
2. NO_3 removal efficiency is limited by geometrical arrangement of wells.
3. Clogging of aquifer must be anticipated particularly in soil that provides small pore size. Release of N_2 gas must be ensured however clogging of aquifer may be alleviated by smart feed regime & large possible dispersion of carbon source releases gases (N_2 etc.) (Soares *et al*., 1988).

The movement of bacteria in porous media depends on following factors (Janda, 1988):

1. Ratio of cell size & pore size.
2. Shape of microorganisms.
3. Flow velocity
4. Injection concentration of bacteria
5. Way of adding carbon source

A proper balance of above factors should be made to ensure uniform dispersion of bacteria in sub-soil. Some studies evaluated underground treatment of nitrate contaminated ground water in Germany by infiltration with treated wastewater or methane gas. In both the cases treated wastewater was found to stimulate faster rates of denitrification than methane. It was suggested that methane utilization by bacteria could occur only in the presence of oxygen and that a symbiotic relationship may be required to achieve denitrification with methane as a substrate (Gayle *et al*., 1989).

Kruithof *et al*. (1988) carried out *in situ* denitrification experiments at the J.H. van Hoek water treatment plant in the Netherlands. Nitrate polluted water (80

mg/L NO_3) was mixed with 49 mg/Lmethanol, injected to the phreatic aquifer and pumped back 25 meters downstream mixed with original groundwater. After 18 days of operation nitrate reduction was about 30% but aquifer clogging problem and rise in nitrite concentration were encountered.

Mercado *et al*. (1988) reported the performance of a pilot plant for *in situ* nitrate removal in Czechoslovakia. Ethanol was injected to the aquifer through a shallow injection well located 15 m upstream of a pumping well. NO_3-N concentration of the aquifer was 14 mg/L. Discharge of the pumping well was 6.5 L/sec. and ethanol dose was 24 mg/l (related to discharge flow). An average nitrate removal of 97.4% was achieved and no clogging problems were encountered.

Some Recent Developments

Robertson and Kuenen (1983) isolated a bacterium *Thiosphaera pantotropha* from a sulfur oxidizing wastewater which was capable of simultaneous heterotrophic nitrification and aerobic denitrification. Gupta (1997) gave a comprehensive review of the literature pertaining its enzyme system which is responsible for the nitrification and denitrification properties and its potential applications for wastewater treatment.

The feasibility of simultaneous organics and nitrogen removal from a synthetic sewage using a mixed culture biofilm containing *T. pantotropha* in a three-stage RBC was assessed for wastewater having a COD of 250–1000 mg/L and NH_4 -N concentration of 27.5–110 mg/L. The organic removal rates in the first stage varied between 5.8–14.1 g COD/m^2.d for the corresponding loading rates of 6.9–20.7 g COD/m^2.d showing a first order relationship. The nitrification rates in this stage varied between 0.47–1.10 g N/m^2.d for nitrogen loading rates of 0.69–2.09 g N/m^2.d despite high simultaneous organic load. A plot of NH_4^+-N loading versus removal rates for first stage indicated a probable change in the order of reaction from first to zero above a nitrogen loading rate of about 1.5 g N/m^2.d. The overall NH_4^+-N loading versus removal rates yielded a straight line relation (Gupta and Gupta, 1999).

A two-stage simultaneous feeding system with feed rates of 72 L/d in first and 12–72 L/d in the second stage was started. Effluent COD remained very low and was below detectable limits. NH_4^+-N varied between 1.60 and 3.35 mg/L. The most important point was the low concentrations of NO_2^--N (0.82–2.70 mg/L) and NO_3^--N (3.57–6.04 mg/L). The overall nitrification rate increased from 0.596 to1.086 g N/m^2.d. NH_4^+-N removal ranged from 97–94% while the removal of total nitrogen varied between 55–78%. The minimum total effluent nitrogen level achieved was 6.21 mg/L at an overall nitrogen loading rate of 1.044 g N/m^2.d (Gupta and Gupta, 1998). This process is yet to tried out on actual sewage where the effect on organic forms of nitrogen can be studied.

The same system was used for treating high strength wastewaters having two and four times the concentration of the above synthetic sewage. The first stage having *T. pantotropha* dominated biofilm showed high carbon and NH_4^+-N removal rates of 8.7–25.9 g COD/m^2.d and 0.81–1.85 g N/m^2.d for the corresponding loading rates of 10.0–32.0 g COD/m^2.d and 1.0–3.35 g N/m^2.d. The ratio of carbon removed to nitrogen removed was close to 12.0. The nitrification rate increased from 0.81 to 1.8 g N/m^2.d on increasing nitrogen loading rates despite a large simultaneous organic

loading rate but fell to 1.53 g N/m^2.d at a high load of 3.35 g N/m^2.d and 32 g COD/m^2.d showing a possible inhibition of the process. A simultaneous 44–63% removal of nitrogen was also achieved without any significant NO_2^--N or NO_3^--N build-up. The second and third stages, almost devoid of any organic carbon, acted only as autotrophic nitrification units, converting the NH_4^+-N from stage 1 to nitrite and nitrate (Gupta and Gupta, 2001).

Another study by Gupta *et al* (1994) involved the growth of a mixed biofilm in a rotating biological contactor (RBC) containing *T. pantotropha*, autotrophic nitrifiers and other heterotrophs for treating a synthetic fertilizer industry wastewater. The influent had a high TKN up to 1386 mg/L and nitrate-nitrogen of 400 mg/l. TKN removal of 44–95% and nitrate removal of 97–98% were achieved simultaneously at different hydraulic retention times and nitrogen loadings.

The anaerobic ammonium oxidation (ANAMOX) process is a novel biological nitrogen removal process, in which the ammonium is oxidized to nitrogen gas using nitrite as the electron acceptor (Mulder *et al*., 1995, van de Graaf *et al*., 1995). This reaction is very gainful from energy and material balance view points and holds lot of promise for future nitrogen removal strategies (Imajo *et al*., 2004). Since this process is autotrophic, there is no requirement for a carbon source and the biomass yield is also low (Strous *et al*., 1998).

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