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NITRATE-INDUCED OXIDATIVE STRESS AND THE EFFECTS OF DIETARY ANTIOXIDANT VITAMINS C, E AND A: INSIGHTS FROM EXPERIMENTAL AND CLINICAL STUDIES

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ABSTRACT
Various studies on nitrates and nitrites including their widespread applications, mechanisms of action and biological effects due to their ability to induce oxidative stress have been reviewed. The current vigorous research efforts to explore the potential uses of antioxidants in various disease conditions have been critically examined and presented. The review is also focused on Vitamins C, E and A, among other antioxidants, as they can be chiefly obtained in natural form from fruits and vegetables in almost all parts of the world.

Keywords: nitrates, oxidative stress, dietary antioxidants, vitamin C, vitamin E, vitamin A

INTRODUCTION

Nitrates are toxic inorganic chemicals commonly found in the environment, foods, and in the bodies of living humans and animals (Antipina *et al.,* 1990; Oladele *et al.,* 1997; IPCS, 1999). Individuals at risk of exposure to nitrates include workers in industries, where fertilizers and explosives are manufactured. They may be chronically exposed to low doses of nitrates through inhalation of dusts containing nitrate salts. The dusts can also dissolve in sweat and expose the skin to concentrated solutions of the salts (IPCS, 1999). This also applies to the farmers who work with fertilizers without using protective gloves, though they are only periodically exposed. Infants can be exposed to nitrates in water used to dilute infant formula (Donovan, 1990; ATSDR, 2001), and while taking mashed carrot used to treat infant diarrhoea, are at special risk of acute poisoning due to immaturity of their enzyme system for regeneration of haemoglobin (IPCS, 1999). Acute poisoning has been reported to occur in animals after feeding with large quantity of food and water contaminated with nitrates and nitrites. The widespread use of nitrate salts in agriculture, food and pharmaceutical industries (Awodi *et al.,* 2005; Manassaram *et al*., 2006), coupled with high rates of air-borne nitrogen compounds emission from industries and automobiles ((USGS, 1996) make the chances of exposure to these chemicals even more inevitable.

Toxicities by nitrates have been well documented. However, with the current widespread use of organic fertilizers on impoverished agricultural lands (Oladele *et al.,* 1997), coupled with increasing water scarcity necessitating the use of water from unregulated sources, the impact of nitrate toxicity is likely to be worse than ever and therefore necessitating further research in this area. Several on-going studies are focused on the modulatory role of many antioxidants during oxidative stress induced by nitrates and other substances.

Sources of exposure to nitrates

The major source of nitrate in the human body is through intake of food and water (IPCS, 1999). Vegetables may account for more than 70 % of the nitrates (ATSDR, 2001), while up to 21% of total intakes of nitrates in a typical human diet is from drinking water (Wogan *et al.,* 1995; ATSDR, 2001; Manassaram *et al*., 2006). Drinking water may contain variable amounts of nitrates and the statutory limits vary from country to country, with maximum being 44.3 mg NO_3/L and 50 mg NO_3/L in the United States of America and European Union, respectively (IPCS, 1999). Other sources of exposure to nitrates include textile, cement and nuclear industry, odour and corrosion control, coagulation of latexes and photography (ATSDR, 2001). Sodium nitrite is used as an antidote in cyanide (IPCS, 1999) and hydrogen sulphide poisoning (Khmelnitsky, 1990). Exposure can also occur through intake of nitrosable drugs (Brenda *et al.,* 2004), and as psychedelics or in suicidal attempts (ATSDR, 2001). There is also endogenous nitrate and nitrite formation through nitric oxide (NO) (Oladele *et al.,* 1997). Thus, the widespread usage of nitrates in the industries, agriculture and medicine has significantly increased the risk of exposure of man to the toxic effects of these compounds.

Pathophysiology of Nitrate Toxic Effects

Biotransformation of nitrates in the body

The principal concern with exposure to nitrates is their biological reduction to reactive and toxic nitrites (Manassaram, 2006). Nitrates themselves are rather harmless. In humans, ingested nitrate is rapidly absorbed from the proximal small intestine into the circulation. Nitrate then reaches the large bowel from the blood, where it is rapidly converted to highly reactive and toxic nitrite. High pH and presence of appropriate intestinal microbial flora favour this conversion reaction (ATSDR, 2001).

The formed nitrite is reabsorbed into the blood, where it reacts with the ferrous (Fe^{2+}) iron of deoxyhaemoglobin to form methaemoglobin, with iron in the ferric (Fe^{3+}) state.

Nitrates are rapidly converted in the liver to denitrated metabolites and inorganic nitrites, which are then excreted in the urine. Approximately 60-70% of ingested nitrate dose is excreted in the urine within the first 24 hours (IPCS, 1999). Half-lives of parent nitrate compounds are usually < 1hr, while that of metabolites ranges from 1-8 hr.

Reactions with Haemoglobin

The major mechanism of acute effects of nitrates and nitrites is methaemoglobinaemia (IPCS, 1999; ATSDR, 2001). Methaemoglobin reduces the oxygen-carrying capacity of the blood and shifts the oxyhaemoglobin dissociation curve to the left, thus interfering with the unloading of oxygen (Dudarev, 1987; Donovan, 1990; IPCS, 1999). Erythrocyte reductases - NADH-dependent and NADPH-dependent, reduce methaemoglobin back to haemoglobin (Prugar and Prugavova, 1990; ATSDR, 2001). A physiological methaemoglobinaemia (1-2% of total haemoglobin) is typical in humans as a result of exposure to oxidising substances and diet (ATSDR, 2001). A congenital methaemoglobinaemia (blue baby syndrome) can be found in persons with either haemoglobin M disease or deficiency of NADH–dependent methaemoglobin reductase. Persons with an NADH-dependant reductase deficiency might be more susceptible to developing acquired symptomatic methaemoglobinaemia after exposure to nitrates and nitrites (ATSDR, 2001). Methaemoglobanaemia may occur concurrently with sulfhaemoglobinaemia, which is usually benign but may confound the diagnosis (ATSDR, 2001).

Nitrites also can oxidise haemoglobin, causing denaturation (Heinz bodies) and erythrocyte haemolysis, resulting in haemolytic anaemia (ATSDR, 2001). Haemolysis is enhanced through the destruction of the phospholipid bilayer of the erythrocyte membrane by reactive NO released by the nitrates (Azhipa *et al.,*1990; Oladele *et al.,*1997). However, decreased erythrocyte osmotic fragility was reported in rats chronically treated with sodium nitrate (Yarube, 2008), indicating that the cells have, apparently, developed capacity for adaptation to the toxic effects (Bensoltane *et al*., 2006).

Free radicals formation

Nitrates and nitrites have been reported to induce free-radical generation *in vivo* (Azhipa *et al.,* 1990; Rubenchik, 1990; Kashko *et al.,* 1993) through the release of nitrite and NO which overwhelm the host antioxidant defence system. Nitric oxide is a potentially reactive free radical gas and a biological messenger in many tissues (Moncada *et al.,* 1991; Hryhorenko *et al.,* 1995; Kerwin, 1995; Peranovich *et al.,* 1995). In low nanomolar concentrations, NO functions as a transmitter in brain and other tissues. Whereas in near-micromolar concentrations, NO which is destroyed partly by cytochrome P450 oxidoreductase (CYPOR) is associated with toxicity and cell death (Hall *et al*., 2009). Nitrates inhibit electron transport in the mitochondrial respiratory chain, leading to blockade of cytochrome oxidase (Antonov *et al.,* 1989; Babsky and Shostakovskaya, 1992). This results in the formation of molecular oxygen (Oladele *et al.,* 1997).

Formation of N–nitroso compounds

N-nitroso compounds are produced in the intestine as a result of interaction of nitrite and nitric oxide with biogenic amines formed from amino acids (such as leucine and valine) under the influence of decarboxylase of *Escherichia coli, Salmonella spp.* and other pathogenic bacteria (Antipina *et al.,* 1990; Antonov *et al.,* 1989). The teratogenic and embryotoxic effects of nitroso compounds such as N-nitroso dimethylamines, N- nitroso diethylamine and N-nitroso methyl-n-butylamine, are based on their ability to pass through the placenta (Rubenchik *et al.,* 1983). The interaction of alkylating products with nitrogen bases of the DNA underlies the mechanism of cancer formation by nitroso compounds. This interaction does not occur chaotically, but rather in a specific pattern at the N-7 position of guanidine and rarely at N-1, N-3 and N-7 positions adenine, N-3 and O^6 positions of guanine, and O^4 position of thiamine. The alkylation of bond C-P which is important in the manifestation of the carcinogenic activity of nitroso amines, produced in the body following nitrate and nitrite poisoning, is apparently related to NO formation (Sorokin, 1991).

Other mechanisms of action

Chronic toxicity of nitrates has been shown to be connected to the formation of carcinogenic N-nitroso compounds (Antipina *et al.,* 1990; Kasyanenko *et al.,* 1992), impairment of acid-base balance, resulting in metabolic acidosis (Sidoryak and Minyaylenko, 1991), stores depletion of vitamins C and E stores (Karpovsky, 1994; Oladele *et al.,* 1997) as well as vitamin A level in the body (Dutsheyko, 1989). Nitrates decrease heat production and cause impairment of energy metabolism in the erythrocyte due to inhibition of redox reaction in the respiratory chain (Dudarev, 1987; Zadorozhnaya, 1991). Nitrates, especially in high concentrations, may impair the activity of digestive enzymes and thyroid gland as well as vitamin A metabolism (Oladele *et al.,* 1997). This may result in cardiac dysfunction and central nervous system lesions. Nitrates may have cumulative effects in humans (ATSDR, 2001). At the same time, evidence of tolerance and adaptation to nitrate toxicity has been reported (Bensoltane *et al*., 2006).

Nitrates as Health Hazards to Humans and Animals

Acute and chronic toxicity of nitrates

Toxic effects of nitrates vary with age, physiological status, diet and other factors (Lewicki *et al.,* 1994). While acute poisoning usually occurs following intake of large doses of nitrates and nitrites, chronic poisoning occurs as a result of intake of small toxic doses over long periods. Toxicity of nitrate is due primarily to its *in vivo* conversion to nitrite after ingestion (NAS, 1977; ATSDR, 2001).

Haematologic effects

Shehata (2005) reported significant decrease in haemoglobin concentration, erythrocyte count, total protein, albumin and globulin concentrations in rabbits exposed to nitrates in drinking water over a short period. However, repeated ingestions of nitrites into the body have been shown to induce a rise in erythrocyte counts, haemoglobin and haematocrit levels (Ogur *et al*., 2000), which is an important compensatory mechanism aimed at improving oxygen supply to the tissues (Manassram *et al*., 2006).

Consequently, body resistance to effects of acute hypoxic hypoxia is increased (Kislyakov and Volzhskaya, 1993).

Circulatory and CNS effects

Nitrates, through a mechanism involving NO, depress the vasomotor centre and dilate blood vessels, thus resulting in a sharp fall in blood pressure and circulatory collapse (Antonov *et al.,* 1989). NO has also been implicated in neuronal damage associated with stroke (Chabrier *et al.,* 1992) and myocardial infarction (Vanin *et al.,*1993). The NO generated in the body during nitrate and nitrite poisoning is involved in regulation of coronary blood vessels (Solodkov *et al.,*1993). Ammonium accumulation in the body associated with nitrate and nitrite poisoning results in excitation and paralysis of the CNS (Ibragimov, 1991).

Carcinogenicity of Nitrates

N-nitroso compounds produced from nitrates *in vivo* have been known to cause malignant tumour in the gastro-intestinal tract, brain, liver, lungs and kidneys (Ariens *et al.,*1976; Gnatyshak, 1988). Zandjani *et al*. (1994) found slightly increased incidence of stomach cancer in one group of workers with occupational exposure to nitrate fertilizer. Further epidemiological studies among high risk groups, such as fertilizer factory workers, populations leaving around irrigation sites, associated with high intake of vegetables and ground water with high nitrate content, patients on long-term treatment with nitrosable drugs, may provide further evidence in this regard.

Reproductive Toxicity

In animal studies, Bruning-Fan and Kaneene (1993) indicated that nitrate, nitrite and N-nitroso compounds may traverse the placenta and affect the foetus *in utero*. L'hirondel and L'hirondel (2000) suggested that the foeto-placental barrier is effective against maternal methaemoglobin from the fourth month of pregnancy in humans. Nitrates and nitrites may induce abortion in experimental animals (Fan and Steinberg, 1996) and decrease in number of litters and live births (Laven *et al*., 2002). Chronic nitrate exposure depresses progesterone levels in cows, which may be responsible for early reproductive toxicity (Manassaram *et al*., 2006).

In human studies, an increased risk associated with intake of drinking water with nitrate level above the MCL by preconception or first trimester pregnant women and neural tube defects, anencephaly (Croen *et al.,* 2001; Brenda *et al.,* 2004); cardiac defects (Cedergren, *et al.,* 2002), and other congenital malformations (Bove *et al.,* 1992) have been reported. Brenda *et al.* (2004) found an association between neural tube defect affected pregnancy and nitrate level ≥ 3.5 mg/L nitrate-N. The risk increased drastically for women who took nitrosable drugs simultaneously.

In a study by Tabacova *et al*. (1997), complications of pregnancy such as anaemia, threatened spontaneous abortion/premature labour and toxaemia were found among women with methaemoglobin levels above the normal physiological (2%) range. Similarly, Bukowski *et al.* (2001) found a significant relationship between intrauterine growth retardation and higher nitrate levels in the diet. Significant decrease in sperm counts and normal serum testosterone (Yarube *et al.,* 2009a), thyroxine and progesterone levels (Hansen *et al*., 2009) were found in rats chronically exposed to nitrate.

Pro-oxidants and Antioxidants

Pro-oxidants

A pro-oxidant (free radical) is a molecule containing unpaired electron in its outer shell (Ayo and Oladele, 1996; Punchard and Kelly, 1997), thereby making it unstable, highly reactive and potentially dangerous to body biomolecules such as proteins, lipids, carbohydrates and DNA (Chan *et al.,*2005). Most free radicals exist only for a fraction of a second before participating in a chemical reaction (Lee *et al.,* 2006). In a reduction-oxidation (redox) reaction, the reductant donates electron and becomes oxidised, while the oxidant accepts electron and becomes reduced. If a free radical reacts with a non-radical, another free radical is generated:

$$A^{\bullet} + B \rightarrow C^{\bullet}$$

This process creates chain reaction that may be thousands of events long till the free radical is deactivated by an antioxidant (Akinwande and Adebule, 2003). An example of such reaction is lipid peroxidation (LPO) of cytomembrane. Free radicals can be generated *in vivo* during physiological or pathological processes (endogenous free radicals) (Ayo and Oladele, 1996; Kharitonov and Barnes, 2003; Lee *et al.,* 2006); or introduced into the body, for example, through diet (exogenous free radicals) (Halliwell, *et al.,* 1992; Aruoma, 1994).

Endogenous free radicals include oxygen free radicals (OFRs) such as superoxide (O_2^-), hydrogen peroxide($H_2O_2^-$), hydroxyl (OH^-), lipid peroxide (LOO^-), alkoxyl (LO^-) and peroxyl (LO_2^-), nitrogen centred free radicals, e. g. phenyldiazine ($C_6H_3N{=}N$); carbon-centred free radicals e. g. trichloromethyl (CCl_3^-); sulphur centred free radicals; and molecular hydrogen (H_2) (Karlson, 1997). A chief source of OFRs is the mitochondrial respiratory chain (Cheesman, 1993; Griendling *et al.,* 1994; Bkaily and d'Orleans-Juste, 1999; Singhal *et al.,* 2001; Johannesson *et al.,* 2003; Valderranma *et al.,* 2006). Exogenous pro-oxidants are environmental factors that increase the production of free radicals in the body, and they include radiation, smoking, aluminum, drugs such as bleomycin, anthracycline, methotrexate, nitrofurantoin (Halliwell *et al.,* 1992); some anaesthetics, certain pesticides, pollutants and hypoxic environment (Yoshikawa, 1993).

Antioxidants

Antioxidants are molecules that serve in biological systems as electron acceptors without subsequent change in their biological activity. Cellular antioxidant enzymes protect against damage caused by exposure to endogenous and exogenous pro-oxidants (Lee *et al.,* 2006). Antioxidants keep the physiological balance between free radical production and its elimination, which is crucial for the survival and functioning of living cells. Endogenous antioxidants are native to the body and include enzymes, for example catalase, superoxide dismutase (Manja *et al.,* 2002), glutathione system, including glutathione peroxidase, glutathione reductase and glutathione transferase (Thomas *et al.,* 1990; Jacob and Jande, 1992; Tomoko *et al.,* 1996; Hultberg and Hultberg, 2006); and ceruloplasmin (Marklund, 1980; Mathys *et al.,* 1995).

Non-enzymatic endogenous antioxidants include copper (Mathys *et al.,* 1995), bilirubin (Maiorino *et al.,* 1991), manganese, metallothionins (Riter and Robinson, 1996), zinc and selenium (Mydlik *et al.,* 2002). Exogenous (nutritional) antioxidants may be naturally occurring (in plant or animal products) or synthetic, taken as normal diet or in form of medicine. They possess free-radical scavenging properties and/or augment endogenous antioxidant status (Seyfulla and Borisoba, 1990).

Examples include ascorbic acid (vitamin C), α-tocopherol (vitamin E), beta-carotene or vitamin A, omega-3 and omega-6 fatty acids (vitamins F_1 and F_2 ,respectively), nicotinic acid, riboflabin (Voskrensky and Bobirev, 1992), aspirin (Halliwell, 1996) and nordihydroguaiaretic acid (Floriano-Sanchez *et al.,* 2006; Meyer *et al.,* 2006).

Oxidative stress

Oxidative stress is defined as a rupture in the pro-oxidant-antioxidant balance in favour of the former, leading to characteristic changes in biomolecules of all types, and to tissue damage (Mircescu, 2008).

During homeostasis, every process is controlled by two antagonising forces to keep internal environment of the body constant. Over-production and/or decreased elimination of free radicals will cause disturbance in pro-oxidant and antioxidant balance, resulting in overproduction of the former (Madesh and Balasubramanian, 1997; Guichard *et al.,* 2006). Oxidative stress occurs if the production of reactive oxygen species (ROS) is abnormally increased or antioxidant concentration decreases (Droge *et al.,* 2006). Free radicals and oxidative stress are involved in the pathogenesis of cardiovascular diseases, artherosclerosis, hypertention, diabetes, cancer and chronic inflammatory diseases (Csovari, *et al.,* 1992; Vaziri *et al.*, 2000; Papov *et al.,* 2003; Galan *et al.,* 2006; Dursun *et al.*, 2008; Sezer *et al.*, 2010), increased susceptibility to infection (Halliwell, 1996), damage and mutation of DNA during human ageing process (Lu *et al.,* 1999; Dykens, 2006). Fundamentally, mechanisms of ageing lead to progressive deficits in functions of cells and organs, which cause diseases that ultimately kill the body such as cancers, cardiovascular and neurodegenerative diseases (Cutler and Mattson, 2006). The pro-inflammatory prostaglandin F_2 is produced *in vitro* and *in vivo* by free radical-catalysed peroxidation of arachidonic acid (Bazan, 1992; Davis *et al.,* 2006). ROS generated by NADPH oxidases are conventionally thought to be cytotoxic and mutagenic, and then induce oxidative stress response (Guichard *et al.,* 2006).

Free radicals and Pathophysiology of Diseases

Oxygen free radicals readily combine with other molecules such as enzymes, receptors and ion pumps, causing oxidation and inhibition or inactivation of normal functions (Punchard and Kelly, 1997; Sung *et al.,* 2000). They can also interact with nucleic acid, thereby causing alteration in base sequence and mutation (Punchard and Kelly, 1997). The primary target of free-radical reaction is the unsaturated bond in lipids found in cellular membrane (Taha *et al.,* 2004). The most destructive effect of free radicals is initiation of LPO as this can result in run-way chain reactions, leading to destruction of cell membrane (Punchard and Kelly, 1997). LPO, which is initiated by ROS, is the process of auto-oxidation of polyunsaturated fatty acid in response to toxic substances (Boots *et al.,* 2003). The change in membrane lipid composition alters membrane permeability, impairs functions of proteins and lipid-dependent membrane-bound enzymes, leading to alterations of cell volume and haemolysis (Halliwell *et al.*, 1992). LPO and lipid-derived oxidised products have been implicated in the pathogenesis of a variety of diseases (Morriel *et al.,* 2000). Lipid hydroperoxide (LOOH) reacts with other lipids, proteins and nucleic acids, propagating the transfer of electrons and subsequent destruction of cells (Papov *et al.,* 2003). Auto oxidation of monosaccharides such as glucose produces peroxide, oxaldehydes and hydrogen peroxide (Sinclair *et al.,* 1990). Hydrogen peroxide induces necrosis and apoptosis of cells (Lee *et al.,* 2006). Free radicals can oxidise sulfhdryl-containing amino acids, resulting in denaturation of proteins and inactivation of enzymes (Manassaram *et al.*, 2006).

Oxidative stress is involved in tissue injury associated with a number of conditions, including rheumatoid arthritis, adult respiratory distress syndrome, immunological disorders, diabetes mellitus, hepatic diseases, psoriasis (Lu *et al.,* 1999) and ischaemia-reperfusion injury of the heart (Ayo *et al.,* 1990; Sax *et al.,* 1992). Other conditions associated with changes in free-radical generation include erythrocytopathy in malaria, sickle-cell anaemia, favism, thalasaemia, G_6PD deficiency (Seyfulla and Borisova, 1990) and trypanosomosis (Igbokwe *et al.,* 1992), lung diseases, such as emphysema, pneumoconiosis, smoking-related lung diseases (Akinwande and Adebule, 2003); infectious diseases such as tuberculosis (Gutkin *et al.,* 1986), influenza (Gorbanov *et al.,* 1992), chlamidial infections (Azenabor *et al.,* 1994); hyperoxygenation syndromes; for example, hyperbaric oxygen and hyperoxygenation respiratory disorders (Del Maestro, 1980; Clark, 1988).

The brain undergoes neurodegeneration, when excess free radicals overwhelm the antioxidative defence system during senescence, head injury or neurotoxic conditions (Chiveh *et al.,* 2000). Free radical-mediated oxidative stress is involved in Alzheimer's disease (Gramov *et al.,* 1993; Opaza *et al.,* 2000), Parkinson's disease (Chiveh *et al.,* 2000) as well as hypoxic and ischaemic brain injuries (Takano *et al.,* 2005). ROS and reactive nitrogen species cause brain damage as a result of high lipid content and low antioxidant defence in the brain (Metodiewa and Koska, 2000). The extent of free radical-induced oxidative stress can be exacerbated by decrease in efficiency of antioxidants in the circulation and is associated with increased risk of cancer (Manja *et al.,* 2002). Oxidative modification of LDL which increases lipid peroxidation and decreases activity of antioxidant systems may contribute to the acceleration of atherosclerosis in renal failure (Mydlik *et al.,* 2002). Oxidative stress is suggested to be central to aging process with reducing endogenous antioxidant defence capacity and repair mechanisms (Halliwell, 1994). The popular use of antioxidant vitamins illustrate the growing awareness of oxidative stress as an important factor in aging process (Droge *et al.,* 2006).

Mechanisms of Disease Prevention by Antioxidants

It is recognised that a major protective goal of antioxidants is to avoid radical-induced damage to polyunsaturated fatty acids and essential fatty acids, in particular the omega-3 and omega-6 fatty acids (Karlson, 1997). Antioxidants can act by scavenging biological ROS, preventing their formation or repairing the damage they induce (Halliwell, 1994). Primary antioxidants prevent the formation of new free radicals species by converting existing free radicals into less harmful molecules before they have chance to induce cellular damage (Karlson, 1997). Superoxide dismutase converts O_2 into H_2O_2, glutathione peroxide converts H_2O_2 into harmless molecules. Secondary antioxidants prevent chain reactions of free radicals and stop tissue damage, for example, vitamin C, α-tocopherol, β-carotene, bilirubin, albumin, uric acid and mannitol. Tertiary antioxidants repair biomolecules damaged by free radicals and they include DNA repair enzymes, methionine and sulphoxide reductase (Heffiner and Repine, 1989).

Some antioxidants act intracellularly; for example, glutathione peroxidase, superoxide dismutase, catalase, AA and metal binding proteins. Also, some antioxidants act extracellularly; for example, transferrin, uric acid and haptoglobin. In addition, some act both intra- and extracellularly (that is, on the cell membrane), for example, reduced glutathionine, urate, ascorbic acid (AA) and α-tocopherol (Halliwell, 1994).

Certain metalloprotein antioxidants act by sequestration of transition metals which are well established pro-oxidants. Transferrin, lactoferrin and ferritin act to reduce iron-induced oxidant stress (Maiorino *et al.,* 1991). Nordihydroguaiaretic acid, a putative anti-cancer, is a potent *in vitro* scavenger of peroxynitrite ($ONOO^-$), singlet oxygen (O_2^-), hydroxyl radical (OH^-), superoxide(O_2^-), hypochlorous acid, and prevents *in vivo* ozone-induced tyrosine nitration in lungs (Floriano-Sanches *et al.,* 2006). Structural antioxidants such as androgens, glucocorticoids and progesterone act by altering their molecular structures (Ayo and Oladele, 1996).

Antioxidant vitamins and their potential uses in nitrate-induced disease processes.

There are associations between oxidative stress, antioxidant nutrients and chronic diseases (Clark, 1988). Nutraceutical therapy with nutrients, vitamins and physical exercise training have been proven to increase antioxidant capacity of the body (Karlsson, 1997). It was shown that alterations of gene expression and protein levels caused by experimentally induced oxidative stress and ROS related diseases can be normalised by dietary antioxidants (Knasmuller *et al*., 2008). Vitamins C, E and A have been well documented to have antioxidant effects among others. The use of these vitamins separately or in combination has been found to significantly affect physiological processes and alter the course of many diseases including, nitrate-induced oxidative stress.

Vitamin C

L-ascorbic acid (AA) is a water soluble vitamin and is widely distributed in fruits and vegetables (Singhal *et al.,* 2001). AA is an effective antioxidant in many biological systems (Ayo *et al*., 2006; Ambali *et al*., 2007). It has an important metabolic role as a result of its reducing properties and function as an electron carrier. It can give up two electrons and in the process is converted to dehydro-AA. It takes part in redox and hydroxylation pathways, acting as a co-factor for cytochrome P_{450}-dependent hydroxylases in some of these reactions (Whitehead and Keller, 2003). Both AA and dehydro-AA possess vitamin C activity. AA can also form an ascorbate radical, giving another route to antioxidant activity by destroying free radicals derived from oxygen, including OH^-, O_2^- and superoxide (Williams, 1997; Whitehead and Keller, 2003). In this role, it may show a synergistic action with other protective enzymes including superoxide dismutase, glutathione peroxidase and catalase. This has been confirmed by Gecha and Fagan (1992), who showed that *in vitro* addition of AA decreased the rate of H_2O_2-induced proteolysis and also destruction of exogenously added superoxide dismutase. AA has been shown to be capable of decreasing haemolysis under *in vitro* conditions, apparently by strengthening the physical integrity of the erythrocytes (Awodi *et al.,* 2005).

Ascorbic acid is also involved in the vitamin E antioxidant system, converting oxidised forms of α-tocopherol back to α-tocopherol (Whitehead and Keller, 2003). AA and vitamin E have been shown to inhibit oxidative stress in cardiomyocytes, resulting in inhibition of all steps of apoptosis including condensation, membrane babbling, shrinkage and cytoplasmic condensation (Guan *et al.,* 2004). Similarly, both vitamins affected nitrate toxicity in a dose-dependent manner (Yarube *et al*., 2009b). AA induces reduction in vascular sensitivity to catecholamines and enhancement of endothelium-dependent relaxation due to increased NO bioavailability (Singhal *et al.,* 2001).

Ascorbic acid is one of several antioxidants shown to play key role in the prevention of many types of cancers. It maintains collagen, a protein necessary for the formation of skin, ligaments and bones. It also enhances the immune systems, helps heal wounds and mends fractures, aids in resisting some types of bacterial and viral infections (Whitehead and Keller, 2003), and participates in control of mood and brain functions (Balz, 2003). AA has been reported to protect sperm DNA from the damage induced by exogenous oxidative stress *in vitro* (Son *et al.,* 2004). It was found to be protective against sodium nitrate-induced toxicity on sperm cells in rats (Yarube *et al*., 2009a).

Although AA is mostly considered to have an antioxidant effect, there are situations when it functions as a pro-oxidant. Podmore *et al.* (1998) reported paradoxical results in their *in vivo* study on DNA base oxidation. Daily supplementation of 500 mg of AA caused decreased oxidation of guanine and increased oxidation of adenine. The oxidation of adenine suggests that AA acted as a pro-oxidant through formation of ascobyl radical. It is also known that this radical is quenched by conversion of glutathione to oxidised glutathione. High doses of AA may increase the concentration of ascorbyl radical, which if not quenched could result in an increased antioxidant burden (Jeffrey, 1998). Yarube *et al*. (2009b) reported decreased erythrocyte count and haemoglobin value by AA, and decreased erythrocyte count, packed cell volume and total serum proteins by vitamin E in sodium nitrate-treated rats in chronic studies. The effects were worse when the two vitamins were administered together, suggesting a negative synergistic effect.

Similarly, AA serves as a pro-oxidant during its interaction with transition metals, especially iron. Ferrous (Fe^{2+}) iron reduces H_2O_2 to generate the OH-radical and in the process becomes ferric (Fe^{3+}) iron. AA can convert Fe^{3+} back to Fe^{2+}, itself being oxidised to dehydro-AA. Continued supply of AA can thus induce a series of cycles of AA-driven free radical generation from iron (Herbert *et al.,* 1996). This system can be used *in vitro* to provide antioxidant stress. Although adverse effects may occur *in vivo* from the combination of elevated dietary concentrations of iron and AA, such effects may be largely restricted to the gastro-intestinal tract since inorganic iron is relatively poorly absorbed from the gut (Whitehead and Keller, 2003). Absorbed iron is usually bound to protein, for example, haemoglobin and ferritin, which may protect the ion from oxidative reactions. In humans, individuals with plasma AA concentrations at the upper limit of the normal range did not show any signs of oxidative damage to DNA when given supplemental iron (Proteggente *et al.,* 2001). Indeed it has been demonstrated that AA can be tolerated at high doses without apparent side-effects (Balz, 2003).

Vitamin E

Vitamin E (α–tocopherol) is a lipid-soluble membrane-localized antioxidant and present in circulating lipoprotein. It is the major lipid soluble chain-breaking antioxidant in plasma, red blood cells and other tissues (Javouhey-Donzel *et al.,* 1993).

It is synthesised only by plants (Basu and Dikerson, 1996), and is therefore found primarily in plant products, the richest source being plant oils. The potent antioxidant properties of vitamin E were first demonstrated by Olcott and Matthil (1931). The effects of lipophilic antioxidant vitamin E, vitamin A and coenzyme Q_{10} are catalysed and expanded in lipid-water interface by vitamin C in the so-called Q-E-C cycles (Karlson, 1997). Vitamin E supplementation significantly lowers lipid peroxidation through increased glutathione levels (Sushil *et al.,* 2000). Boadi (1991) found increased glutathione levels in the brain, liver, lungs and blood of rats treated with vitamin E (120 mg/kg diet) for 30 days. Vitamin E may enhance the body's immune function and inhibit the conversion of nitrites to nitroso-amines in the stomach (Packer, 1991). It is required for normal functions of the immune system and control of aggregation of platelets. Vitamin E is also involved in nucleic acid and protein metabolism, functions of the mitochondria and regulation of hormone production (Guthrie and Picciano, 1995). Other non-antioxidant functions include regulation of protein kinase C, modification of cell growth and proliferation and modification of gene transcription and expression (Azzi and Stocker, 2000).

Vitamin E is involved in an extensive range of protective systems. It reversed the decrease in sperm counts of sodium nitrate-treated rats, apparently by antagonizing the loss of membrane integrity (Yarube *et al*., 2009a), thus serving as an effective antioxidant in antagonizing the free radical effect of nitrates (Hanafy and Soltan, 2004; Ocak, 2007; Suteu *et al*., 2007). It plays a role in prevention of atherosclerosis through inhibition of oxidation of LDL (Reaven, 1993); inhibits platelet adhesion, and is of benefit to diabetics (Jain *et al.,* 2000) and patients with acquired immuno-deficiency syndrome (AIDS) (Liang, 1996; Tang, 1997) as well as Parkinson's disease. Tissue-plasma levels of vitamins E and vitamin C and intracellular glutathione concentration decrease with age (Droge *et al.,* 2006).

Vitamin A

The pro-vitamin A, β-carotene is a lipid soluble vitamin, and like other carotenoids play an important role in the protection of cells from oxidative damage due to their highly conjugated double systems (Mascio *et al.,* 1991). Dietary intake of vitamins A, C and E may influence blood levels of catalase through their antioxidant effect on free radicals (Tabet *et al.,* 2002). Vitamin A plays an important role in the protection of membrane from lipid peroxidation by free radicals (Mydlik *et al.,* 2002). β-carotene reacts with peroxyl radical to form β-carotene radical. It is more efficient in conditions of low O_2 tensions (Olsen and Kobayoski, 1992). It has been proposed that beta-carotene acts as an antioxidant by scavenging lipid peroxyl radicals, not by donating hydrogen atom, like vitamin E and C, but by addition reaction to double bond to give resonance stabilised, carbon–centred conjugated radicals (Burton and Ingold, 1984). Beta-carotene has a protective role during aging as it reduces free radical damage to DNA (Williams, 1997). It has been used with other vitamins to cause down regulation of tumour necrosis factor (Son *et al.,* 2004).

Conclusion and recommendations

Exposure to toxic effects of nitrates is inevitable, considering the increasing applications of nitrates in different sphere of life. It seems there is much more to be discovered about the extent of health hazards induced by nitrates to man and animals. The recent intensive research efforts into antioxidants and their potential uses in various disease processes need to be fully exploited in order to find practical solutions to the nitrate problem. Though such solutions appear to be in sight, some of the positive results must be further evaluated for variations in doses and possible combinations of different types of antioxidants from various sources.

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