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Nitrate Metabolism: A Curse or Blessing to Humanity?

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Authors' contributions

This work was carried out in collaboration between both authors. Author DAN conceived, designed the study and wrote the first draft of the manuscript. Both authors managed the literature searches and revised the manuscript. Author DAN is finally responsible for all the information presented. Both authors read and approved the final manuscript.

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ABSTRACT

Nitrate (NO_3^-) is a metabolic waste produced in humans during the detoxification of nitric oxide (NO). Nitric oxide is responsible for the regulation of blood flow, cell signaling and host defense in a number of mammalian tissues. Shortly after synthesis, nitric oxide is oxidized to nitrate in order to terminate its effect. NO can also be synthesized by an alternative mechanism that relies on the sequential reduction of nitrate to nitrite (NO_2^-); thereby making nitrite a storage pool that can be reduced to NO under appropriate condition with a concomitant increase in the methemoglobin (metHb) concentration. The NO and metHb produced can result to adverse health effects if not detoxified. NADH-methemoglobin reductase accounts for most metHb reduction. Nitric oxide dioxygenases (NODs) catalyze the conversion of NO to NO_3^- which help to protect cells from NO poisoning. Nitrate and its reduction products are involved in the modification of the body's physiological functions. We are exposed to nitrate from dietary intake and nitric oxide oxidation. Nitrate metabolism becomes a threat when nitrate is consumed in excess and the body lacks the

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enzymes that catalyze the detoxification of its byproducts. During such condition, the hemoglobin, nucleic acids, dietary amines and glycoproteins acts as the primary molecule that mediates its effects. Nitrate is a potential therapeutic agent in the prevention of cardiovascular diseases and improving cell viability. This review serves to reevaluate nitrate metabolism in human with the intentions of understanding its role in NO homeostasis and its metabolic effect due to excess intake.

Keywords: Nitrite; nitric oxide; nitrogen dioxide; methemoglobin; peroxynitrite; nitric oxide dioxygenase; dietary amines.

1. INTRODUCTION

Nitrate is an important plant nutrient which has found its way into the human system, playing many roles in the general regulation of metabolism in certain tissues and cells; reducing ailment whose etiology involves the insufficiency of NO. The nitrate/nitrite concentration in circulation, cellular nitric oxide synthase (NOS) activity as well as NOD activity has to be maintained by the body due to the varying levels of nitrate and nitrite in tissues. Human nitrate intake and metabolism have received considerable interest because nitrite formation in the saliva is suspected to be involved in infantile methemoglobinemia and mutagenesis [1]. Humans are exposed to nitrate from dietary intake and nitric oxide oxidation [2]. Generation of nitric oxide in the endothelial cells aids in vasodilation, thereby regulating blood flow. Phagocytic cells such as macrophages employ the use of NO as a toxin against microorganisms [3]. Shortly after synthesis, it is oxidized to form nitrate [4,5]. Nitrate is produced in-vivo and humans are exposed to about 1mmol each day from this source [2].

Under normal condition and also without any enzyme dysfunction, nitrate produced in the body will never result to any disease. Dietary nitrate principally derived from vegetables and nitrate contaminated water, increases the concentration of nitrate ion in the human body. Nitrate may have other beneficial physiological roles such as protecting the gastrointestinal tract against a variety of gastrointestinal pathogens, as nitric oxide has an antibacterial property [6,7]. Several studies have been carried out on the formation of mutagenic and carcinogenic *N*-nitroso compounds in relation to nitrate intake in humans [8,9]. High nitrate intake from drinking water has been linked to methemoglobinemia, a condition whereby the hemoglobin is oxidized to methemoglobin making the blood unable to transport oxygen [10,11,2]. Infants under three months are at a greater risk due to high water intake in relation to body weight and underdeveloped enzyme system [12,2,13]. This results in cyanosis, referred to as blue-baby syndrome. Exogenous nitrate uptake may be beneficial, but there is a need to balance the potential benefits with its health risks. The aim of this study is to show how the human body maintains a steady state of nitrate circulation in relation to NO homeostasis and degradation; as a result of which we may be exposed to certain health disorders.

2. ENVIRONMENTAL OCCURENCE

NO_3^- and NO_2^- occur naturally and are part of the nitrogen cycle. The nitrate ion being chemically unreactive due to its stability, however it can be reduced by microbial action to nitrite. NO_2^- is unstable and can undergo oxidation to nitrate or reduced to various nitrogenous compounds by chemical and biological processes. Nitrate is reduced to ammonia (NH_3) by microbes when used as a source of nutrients through the process known as assimilative nitrate reduction. As a terminal electron acceptor in anaerobes, it undergoes denitrification, where it is reduced to NO_2^-, NO, nitrous oxide (N_2O) and finally nitrogen gas (N_2) by a process known as dissimilative nitrate reduction.

Nitrogen from the soil pool enters the biomass principally in the form of NO_3^- taken up by plants and microorganisms [14]. Nitrate taken up during growth is used in the synthesis of organic nitrogenous compounds, making it a key nutrient in plant growth. Nitrates then move further up the food chain when animals ingest plants. The supply of nitrogen in the soil pool is limited and plants must compete with varieties of soil microorganisms for available nitrogen. By the process of denitrification, bacteria reduce nitrate to nitrogen which is then returned to the atmosphere.

Plants depend on the biological nitrogen fixation as a source of nitrogen. The bulk of nitrogen

fixed is accounted for by the reduction of nitrogen to ammonia by living organisms [14]. Nitrogen fixation is limited to prokaryotes because they have the nitrogenase enzyme complex that catalyzes the reduction of nitrogen to ammonia. Nitrogen fixing bacteria may be free living or form symbiotic association with plants i.e. rhizobia. Nitrogen is returned to the soil in the form of ammonia through animal wastes or death and subsequent decomposition of all organisms (see Fig. 1). NH_3 is converted back to NO_3^- by nitrifying bacteria found in the soil. This is achieved by the oxidation of NH_3 to NO_2^- by bacteria of the genus, nitrosomonas; which is further oxidized to nitrate by members of the genus, nitrobacter. When nitrate-nitrogen supply exceeds plant demand, the ground water gets contaminated by leaching which is the downward movement of NO_3^- with water through the soil [15].

3. HUMAN EXPOSURE

Due to the limited supply of nitrate in the soil, nitrate containing fertilizers and organic manures are mostly applied to increase soil fertility. Nitrate can reach both surface and ground water as a result of agricultural activities. The nitrate concentration in surface water is normally low (0-18 mg/L) but can reach high levels as a result of agricultural runoff [2]. The nitrate content of vegetables can be affected by processing of the food, the use of fertilizers and growing conditions [16]. Sodium nitrite is used as food preservatives especially in cured meats which greatly delays the formation of botulinum toxin [17].

Humans are mostly exposed to inorganic nitrates from dietary sources. Vegetables, cured meat and consumption of nitrate contaminated water are the main sources of dietary nitrate. Vegetables constitute the major source of nitrate, providing most of the average daily human dietary intake [18-20]. The level of nitrate in drinking water should not exceed 50mg/L for bottle fed infants [2]. Consumption may be significantly higher in communities where well water contains elevated concentrations of nitrate.

4. NITRATE METABOLISM IN HUMANS

Nitrate is metabolized in an unusual way. Ingested nitrate is absorbed from the stomach or intestine and about 25% is recirculated and secreted by the salivary gland thereby increasing its concentration in the saliva [8,20,21]. As a result, salivary nitrate concentrations are approximately 10 to 20 times those found in plasma [22]. There is therefore a high concentration of nitrate in saliva which increases with oral intake. Facultative anaerobic bacterial flora, mainly from the posterior surface of the tongue reduces salivary nitrate to nitrite which is then swallowed in the saliva [23,24]. Nitrate itself is not toxic to humans. It becomes a problem only when it is converted to nitrite in the human body [15].

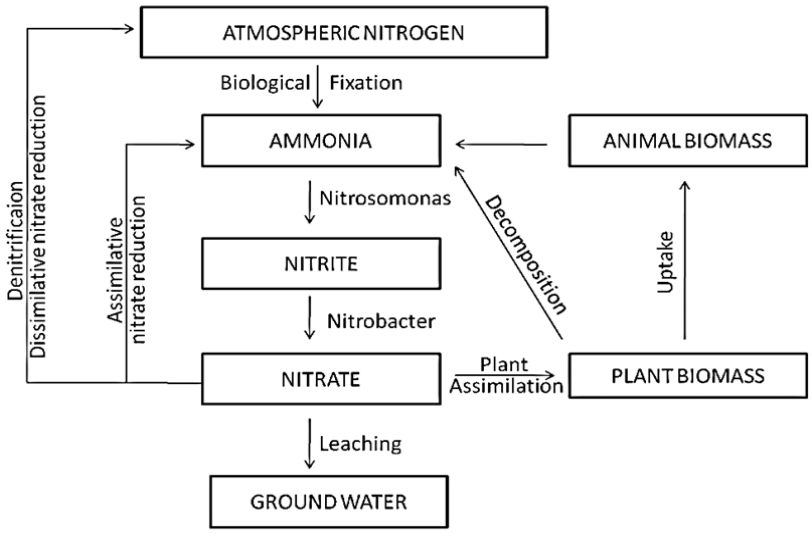

Fig. 1. Nitrogen cycle

Nitrate and nitrite are then swallowed and re-enter the stomach. Nitrate is rapidly absorbed in the small intestines and readily distributed throughout the body [25]. The major part of the ingested nitrate is eventually excreted in urine as nitrate, ammonia or urea, fecal excretion being negligible. Ammonia is utilized for amino acid biosynthesis or converted to urea in the liver by the enzymes of the urea cycle. Urea is then secreted into the blood stream and excreted by the kidney as urine. Little nitrite is excreted [26,27]. This is because of its low stability which makes it highly reactive with endogenous compounds. Urinary excretion of nitrite is as a result of urinary tract infection by facultative anaerobic bacteria which uses nitrate as a source of nutrient i.e. *Escherichia coli* [27].

4.1 Reduction of Nitrite to Nitric Oxide

Nitric oxide is an important biological molecule that is responsible for cell signaling and host defense in a number of mammalian tissues [28]. The gaseous substance acts near its point of release, entering the target cells after diffusion where it activates guanylyl cyclase which catalyzes the formation of cGMP, a second messenger [5]. Many functions of NO are regulated through its interaction with guanylyl cyclase. Macrophages and endothelial cells of the blood vessels synthesize NO from molecular oxygen and the guanidium nitrogen of L-arginine by Ca^{2+} dependent NO synthase (see Fig. 2) [17,5].

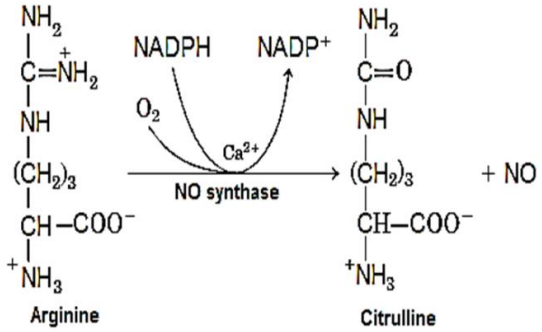

Fig. 2. Biosynthesis of nitric oxide by nitric oxide synthase [5]

NO can be synthesized by an alternative mechanism that relies on the sequential reduction of nitrate to nitrite [29]. Exogenous nitrate contributes to whole body NO production and homeostasis [17]. Nitrate can simply be regarded as a storage pool that can be reduced to NO under appropriate condition. Nitrite is readily protonated under the acidic conditions of the stomach prior to absorption [24].

$$NO_2^- + H^+ \longrightarrow HNO_2$$

Absorption of nitrous acid (HNO_2) by the blood oxidizes the iron moiety of unoxygenated hemoglobin from its ferrous (Fe^{2+}) state to its ferric (Fe^{3+}) state known as methemoglobin (metHb). The metHb is unable to bind oxygen. Also, its presence increases the affinity of the non-oxidized hemoglobin for oxygen, thereby shifting the oxygen dissociation curve to the left [30,11]. The non-oxidized hemoglobin holds on to oxygen more tightly and does not release it, thus blocking oxygen transport [30,11,7]. HNO_2 causes the oxidation of iron in the ferrous state to the ferric ion with the concomitant formation of NO [31].

$$HbFe^{2+} + HNO_2 \longrightarrow HbFe^{3+} + NO^{\cdot} + OH^-$$

Also, the reduction of NO_2^- to NO can occur via proteins possessing NO_2^- reductase activity such as heme globins (the blood deoxyhemoglobin, muscle deoxymyoglobin and the nervous system neuroglobin); molybdenum containing enzymes (xanthine oxidoreductase, aldehyde oxidase, sulfite oxidase) and components of components of the electron transport chain (ETC) [32,33]. The heme globins are regarded to function primarily as the nitrite reductase (Nir) enzyme in humans.

1. Nitrite reductase activity of heme globins

$$HbFe^{2+} + NO_2^- + H^+ \longrightarrow HbFe^{3+} + NO^{\cdot} + OH^-$$
$$MbFe^{2+} + NO_2^- + H^+ \longrightarrow MbFe^{3+} + NO^{\cdot} + OH^-$$

2. Nitrite reductase activity of molybdenum containing enzymes

$$NO_2^- + Mo^{4+} + H^+ \longrightarrow NO^{\cdot} + Mo^{5+} + OH^-$$

Nitrosyl-hemoglobin HbNO or $HbFe^{2+}(NO^{\cdot})$ is generated when nitric oxide reacts with deoxyhemoglobin [31].

$$HbFe^{2+} + NO^{\cdot} \longleftrightarrow HbFe^{2+}(NO^{\cdot})$$

In normoxia, NO is synthesized by nitric oxide synthase (NOS) from L-arginine and O_2. In hypoxia, NO_2^- is reduced to NO by heme globins; xanthine oxidoreductase (XOR), aldehyde oxidase (AO), sulfite oxidase (SO) [32]; complex III [34,32], cytochrome c [35,32] and complex IV [36,32] of the mitochondrial electron transport

chain (see Fig. 3). The nitrite-dependent NO generation at complex III prevents the transport of electrons. Cytochrome c which is an electron donor for complex IV (cytochrome c oxidase) contain heme groups that reversibly alternate between the Fe(II) and Fe(III) oxidation states during electron transport.

Nitrite reduction by components of the ETC inhibits proton generation from coupling of electron transport at complex III and IV which could also inhibit mitochondrial respiration and ATP synthesis due to low proton generation. Complex I can also undergo S-nitrosation by nitrite during ischemia. This can inhibit the flow of electron in the inner mitochondrial matrix. Upon reperfusion (reoxygenation), the generation of reactive oxygen species (ROS) in the mitochondria is decreased due to the inhibition of complex I by nitrite. The inhibition of O_2 consumption at complex IV is as a result of the binding of NO to cytochrome c oxidase. Molybdenum containing enzymes reduces nitrite to NO through the reaction with its molybdenum cofactor [32]. A deficiency in molybdenum could impair this process.

Production of NO by blood vessels endothelial cells regulates the relaxation of the surrounding smooth muscle cells causing vasodilation [37]. The neurotransmission and vasodilation action of NO are largely mediated by the formation of HbNO [38,39]. The persistence of the reddish colour in meat treated with potassium nitrate (KNO_3) or sodium nitrite ($NaNO_2$) is due to the binding of the iron atom to NO; preventing the oxidation of iron to its ferric state in the myoglobin due to the formation of nitrosyl-myoglobin [40,41].

$$MbFe^{2+} + NO^{\cdot} \longleftrightarrow MbFe^{2+}(NO^{\cdot})$$

NO is also synthesized by the anaerobic reduction of nitrite by the bacterial flora of the stomach [42,43] and in the oral cavity [44,45] under low pH. The tongue also contains high numbers of oral *Streptococci species* capable of producing the observed pH change [44]. *Escherichia coli* is an example of a facultative anaerobic bacteria that can synthesize NO in the human gut. Nitrite derived from dietary nitrate is a substrate for NOS-independent production of NO in the acidic condition of the human stomach [29]. The enterosalivary circulation of nitrite and the formation of NO is also important for protection against oral and gastrointestinal diseases [6].

Hemoglobin has a very high affinity for NO. The formation of ferrous nitrosyl-heme ($HbFe^{2+}NO^{\cdot}$) makes NO inert which serves as a means of preserving the bioactivity of NO [46-48]. In order for NO to exert its action, it needs to come out of the HbNO pocket. Processes which oxidize the ferrous nitrosyl-heme can facilitate NO release [49,33]. When exposed to oxidizing agents such as peroxynitrite ($ONOO^-$), NO is released which then initiates signal transduction in target cells by the activation of guanylate cyclase. The release of NO from ferrous-nitrosyl heme upon oxidation with $ONOO^-$ involves the formation of a ferric nitrosyl-heme ($HbFe^{3+}NO^{\cdot}$) intermediate [46]. The NO dissociates from ferric-nitrosyl heme upon hydrolysis to give pure $HbFe^{3+}$ [50,51].

$$HbFe^{2+}NO + ONOO^- \longrightarrow HbFe^{3+}NO + NO^{\cdot}_2$$

$$HbFe^{3+}NO \xleftrightarrow[H_2O]{} HbFe^{3+} + NO^{\cdot}$$

4.2 Nitric Oxide Reaction with Superoxide ($O^{\cdot}_2{}^-$) Forming Peroxynitrite ($ONOO^-$)

$ONOO^-$ is formed by the reaction of NO with superoxide anion ($O^{\cdot}_2{}^-$) [52,53]. This is feasible because, the reaction between NO and O_2^- is faster than that of superoxide dismutase (SOD) catalyzed decomposition of $O^{\cdot}_2{}^-$. The NO/O_2^- reaction predominates over O_2^-/SOD reaction, making NO the only biological molecule with concentration high enough to out-compete superoxide dismutase for superoxide [53]. The formation of both nitric oxide and superoxide does indeed occur simultaneously in macrophages and endothelial cells [54]. The extent of $ONOO^-$ formation is strongly influenced by the relative availability of $O^{\cdot}_2{}^-$ and NO. [55,56].

Besides NO, all isoforms of NOS are capable of producing superoxide under certain conditions such as concentration of substrate or cofactor.

Then the reaction between the two produces toxic $ONOO^-$ leading to a peroxynitrite-mediated cellular injury [57,58,55].

$$NO^{\cdot} + O^{\cdot}_2{}^- \longrightarrow ONOO^-$$

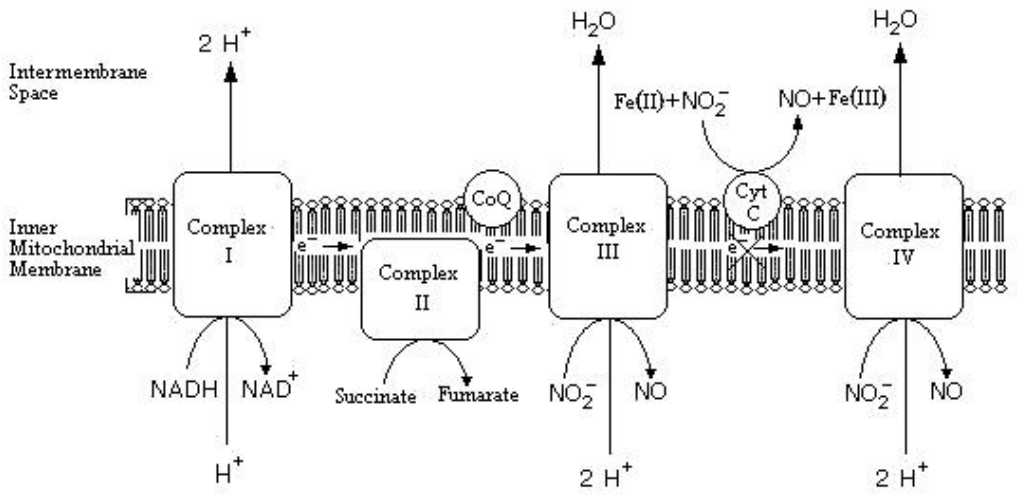

Fig. 3. Nitrite reductase activity by components of the mitochondrial electron transport chain

Peroxynitrite reacts relatively slow with most biological molecules, making it a selective oxidant [52]. $ONOO^-$ undergoes three reaction types namely; oxidation, nitration and isomerization to nitrate. $ONOO^-$ oxidizes protein and non-protein sulfhydryls [59,60,51]. There is also a formation of nitrotyrosine and 8-nitroguanine as a result of nitration in cells exposed to $ONOO^-$ [61,62]. These reactions are cytotoxic making $ONOO^-$ an intermediate which enhances the toxicity of NO. The reactions of $ONOO^-$ with biological molecules will be discussed in more detail in a later section.

4.3 Endogenous Reduction of Methemoglobin

The percentage of metHb in the blood has to be maintained as there is always a conversion of hemoglobin to metHb which is capable of impairing cellular respiration even in the absence of oxidative stress [63]. Several endogenous reduction systems exist to maintain metHb in the reduced state. The NADH-methemoglobin reductase system is the predominant system and accounts for most metHb reduction [63]. Ascorbic acid and glutathione account for small amounts of reduction [64,11].

NADH-metHb reductase is a dual-enzyme system that involves cytochrome b5 and NADH-cytochrome b5 reductase found in both erythrocytes and somatic cells [64]. NADH is a cofactor to cytochrome b5 reductase. Glycolytic intermediates that produce NADH serve as electron donor to cytochrome b5 reductase then to cytochrome-b5 and finally to metHb [11]. The NADH-cytochrome b5 reductase is a flavin adenine dinucleotide (FAD) which catalyzes the transfer of electron from NADH to cytochrome-b5 [65,66,11] (see Fig. 4).

$$\text{NADH} + \text{FAD} \xrightarrow[\text{reductace}]{\text{cytochrome-b5}} \text{FADH}_2 + \text{NAD}^+$$

$$\text{FADH}_2 + \underset{\text{(oxidized)}}{\text{cytochrome-b5}} \longrightarrow \text{FAD} + \underset{\text{(reduced)}}{\text{cytochrome-b5}}$$

$$\underset{\text{(reduced)}}{\text{cytochrome-b5}} + \text{Hb}^{3+} \longrightarrow \underset{\text{(oxidized)}}{\text{cytochrome-b5}} + \text{Hb}^{2+}$$

Fig. 4. NADH-methemoglobin reductase system

4.4 Endogenous Formation of Nitrate from Nitric Oxide

Once nitric oxide is produced, it may directly react with myoglobin or hemoglobin in the extracellular space [67]. This is due to its rapid diffusion through membranes. NO combines strongly with hemoglobin to form nitrosyl-hemoglobin which is rapidly oxidized to NO_3^- [4,46]. NO is unstable and highly reactive and hence a toxic substance. Once it has delivered its message, it is important that it be rapidly eliminated to prevent its interference with subsequent NO signals. Within seconds of its formation, it undergoes oxidation to nitrate, thereby making its action brief.

The excess nitrate excretion that has been observed after low nitrate and nitrite intake originates from endogenous synthesis. In normal healthy individuals, endogenous synthesis results in 1 mmol per day on average, corresponding to 62 mg nitrate per day [2]. Gastrointestinal infections greatly increase nitrate excretion as a result of increased endogenous (non-bacterial) nitrate synthesis, probably induced by activation of the mammalian reticoendothelial system [68,27,26,43]. The mechanism involved in the endogenous formation of nitrate would be discussed further in the next chapter.

4.4.1 Reactions of peroxynitrite

The reactions of peroxynitrite that have been identified till date are; oxidation reaction, isomerization to nitrate and electrophilic nitration [59]. $ONOO^-$ reacts rapidly with CO_2 to form a short-lived nitrosoperoxycarbonate ($ONO_2CO_2^-$) adduct [69,70,59]. $ONO_2CO_2^-$ and its decomposition products are responsible for the oxidation and nitration reactions exhibited by $ONOO^-$ in biological systems [69,70]. The reaction proceeds via the rearrangement of $ONOO^-$ to give nitrocarbonate anion ($O_2N{-}OCO_2^-$) which in turn undergoes hydrolysis to give nitrate and carbonate [59]. The reactions are elaborated below:

1. Formation of nitrosoperoxycarbonate ($ONO_2CO_2^-$) adduct

$$CO_2 + ONOO^- \longrightarrow ONO_2CO_2^-$$

2. Decomposition of $ONO_2CO_2^-$

$$ONO_2CO_2^- \longrightarrow CO^{\cdot}{}_3^- + NO^{\cdot}{}_2$$

3. Rearrangement of $ONO_2CO_2^-$ to give nitrocarbonate anion

$$ONO_2CO_2^- \longleftrightarrow O_2N{-}OCO_2^-$$

4. Hydrolysis of $O_2N{-}OCO_2^-$ to give nitrate and carbonate

$$O_2N{-}OCO_2^- \xrightarrow[H_2O]{} CO_3^{2-} + NO_3^- + 2H^+$$

$ONOO^-$ also isomerizes to NO_3^- in the presence of oxyHb and oxyMb leading to the one electron oxidation of the heme to the ferric form with a concomitant formation of $O^{\cdot}{}_2^-$. [71]. The reaction proceeds through the formation of a metHb-peroxynitrite ($HbFe^{3+}ONOO^-$) intermediate which then undergoes isomerization to NO_3^- [72,71]

1. Formation of a metHb-peroxynitrite ($HbFe^{3+}ONOO^-$) intermediate

$$HbFe^{2}(O_2) + ONOO^- + H^+ \longrightarrow HbFe^{3+}(ONOO^-) + O^{\cdot}{}_2^-$$

2. Isomerization of metHb-peroxynitrite to NO_3^-

$$HbFe^{3+}(ONOO^-) \longrightarrow HbFe^{3+} + NO_3^-$$

3. The $O^{\cdot}{}_2^-$ released spontaneously reacts with SOD, yielding O_2 and H_2O_2 [71].

$$2O^{\cdot}{}_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

4. The H_2O_2 produced, is finally detoxified by catalase.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

4.4.2 Nitric oxide dioxygenases (NODs)

Nitric oxide dioxygenation in NO metabolism help to protect cells from NO poisoning and is induced by NO exposure. The Hb of red blood cell and Mb of myocytes metabolize NO in the vascular lumen, muscles and other mammalian cells by decreasing NO signal and toxicity [73].

The NOD's are group of enzymes that catalyzes the conversion of NO to NO_3^-. NOD is a function of heme globins. Hemoglobin and myoglobin are the primary proteins that catalyze nitric-oxide dioxygenation in humans; acting as enzymatic NOD's when coupled to a reductase [73]. NO produced by the endothelial cells of the blood vessel is detoxified through its reaction with oxygenated hemoglobin (oxyHb) to yield NO_3^- and metHb [74,73]. The metHb is reduced back to hemoglobin through the action of an intracellular methemoglobin reductase discussed earlier.

Net reaction catalyzed by nitric oxide dioxygenase is;

$$2NO + 2O_2 + NAD(P)H \longrightarrow 2NO_3^- + NAD(P)^+ + H^+$$

The reaction mechanism is explained below:

1. Nitrosyl-heme dissociates to unoxygenated hemoglobin and nitric oxide

$$HbFe^{2+}(NO^{\cdot}) \longleftrightarrow HbFe^{2+} + NO^{\cdot}$$

2. NADPH is oxidized to $NADP^+$ by the FAD present in the hemoglobin molecule.

$$NADPH + FAD + H^+ \longrightarrow NADP^+ + FADH_2$$

3. Hemoglobin has a higher affinity for O_2 than NO. This helps in limiting NO inhibition during the dioxygenation reaction [74]. The formation of nitrosyl-heme in the 1st reaction is a mechanism for decreasing NO binding to heme.

$$O_2 + HbFe^{2+} \longleftrightarrow HbFe^{2+}(O_2)$$

4. The ferrous heme forms a stable complex by the transfer of an electron to O_2.

$$HbFe^{2+}(O_2) \longleftrightarrow HbFe^{3+}(O^{\cdot}_2{}^{-})$$

5. The radical complex formed by ferrous heme and O_2 is suitable for association of the radical electron of NO. A peroxynitrite intermediate is formed which then undergoes isomerization to NO_3^- [75,76]. The Fe^{3+} facilitates an oxygen bond rearrangement by participating in an iron mediated oxygen bond shift [75].

$$HbFe^{3+}(O^{\cdot}_2{}^{-}) + NO^{\cdot} \longrightarrow HbFe^{3+}(ONOO^-)$$
$$\longrightarrow HbFe^{3+} + NO_3^-$$

6. FAD is regenerated by the transfer of electron from $FADH_2$ in the 2nd reaction to cytochrome-b5 and then to $HbFe^{3+}$ which is finally reduced to $HbFe^{2+}$. The FAD being a function of cytochrome b5 reductase in red blood cells [11,65,66].

$$FADH_2 + HbFe^{+3} \longrightarrow FAD + HbFe^{2+}$$

Also in the skeletal muscles cells, under normal O_2 concentrations, NO is detoxified through its reaction with oxygenated myoglobin (oxyMb) to yield nitrate ion and metmyoglobin (metMb). The reaction follows the same mechanism as that of hemoglobin above.

The metmyoglobin (metMb) is also reduced back to myoglobin (Mb) through the action of cytochrome b5 reductase [76,77]. The O_2 dependent NO consumption activity provides a feedback mechanism for controlling O_2 delivery to hypoxic tissues via elevated NO concentration, guanylyl cyclase activation, smooth muscle relaxation and increased capillary blood flow [78,79].

5. NITRATE METABOLISM BY ENTERIC BACTERIA IN THE GUT

Nitrate undergoes three major reduction process namely; assimilatory nitrate reduction to ammonia by bacteria, fungi and higher plants when used as a source of nutrient for growth. Nitrate respiration when it is used as a terminal electron acceptor and nitrate dissimilation where it is successively reduced to NO_2^-, NO, N_2O and N_2. It is assumed that nitrate respiration and dissimilatory nitrate reduction are basically the same process [80], as they are both carried out by facultative bacteria mostly found in the human gut. The nitrate respiration is energy conserving and leads to the generation of a proton motive force [80] while the latter involves redox balancing to dissipate excess reducing power under certain metabolic conditions [81,80].

A direct quote from Gibson et al., page 246 suggests that "The relative insensitivity of sulfur reducing bacteria (SRB) to the toxic effects of nitrate or its reduction product, nitrite, compared with methanogenic bacteria, suggest that nitrate could potentially play a role in selecting for SRB in the large gut" [82]. Sulfur reducing bacteria seems to flourish in the presence of excess bioavailability of nitrates in the guts. SRB such as Desulfovibrio can metabolize both sulfate and nitrate, where the reduction of nitrate leads to the formation of ammonium [83,84,82]. The overgrowth of Desulfovibrio in the gut can be associated with inflammatory bowel disease [85] alongside excess nitrate and nitrite in the stool [86].

Enteric bacteria protect themselves against NO generated by their own metabolism or innate immune response [87]. These bacteria are able to reduce NO to a harmless product which is critical for their survival. The periplasmic cytochrome c nitrite reductase (NrfA) of *Escherichia coli* can catalyzes the respiratory reduction of nitrite and also nitric oxide [88]. Under aerobic conditions, the flavohemoglobin (flavoHb) of *E. coli* detoxifies NO through oxidation to nitrate, while under anaerobic conditions the flavoHb is capable of reducing NO to N_2O [89,90]. The flavoHb is a critical protein under aerobic conditions while under anaerobic conditions, the transcription of NrfA is induced which in turn activates nitric oxide reductase (Nor) [87,91]. The NrfA system is particularly well suited to NO removal, because it is located in the periplasm and so can detoxify NO before it enters the cell [88].

6. PHYSIOLOGICAL CHANGES ASSOCIATED WITH NITRATE METABOLISM

Nitrate is involved in the modification of the body's physiological functions. Nitrate does not act directly but depends on its reduction by microbial action, hemoglobin and myoglobin to NO_2^-, NO, N_2O and NH_3. Some of the

physiological changes caused by nitrate will be discussed in this chapter.

6.1 Adverse Effects of Nitrate Metabolism on Cobalamin

The production of N_2O by enteric bacteria in the gut has serious adverse effects on cobalamin. The major source of cobalamin being from animal protein is provided in the coenzyme form, 5'-adenosylcobalamin as a protein-vitamin complex [92]. Cobalamin binds haptocorrin in the saliva and transported to the stomach [93,92]. This complex is acted upon by pepsin which is required for cobalamin release in the stomach [94,92]. Cobalamin binds to the gastric intrinsic factor (IF) and then transported to the ilium.

Cobalamin absorption takes place in the ilium through specific membrane associated receptors [95,92]. Enteric bacteria disrupt the absorption of cobalamin in the gut. The use N_2O anaesthesia and recreational abuse has been reported to cause cobalamin-related disturbances in vivo [96-98]. Prolonged administration can result in megaloblastic anaemia [99,97] and neurological disorder [96,99]. It irreversibly oxidizes the cobalt ion of cobalamin from the +1, cob(I)alamin to a +3 oxidation state cob(II)alamin. The oxidation disrupts the methionine synthase reaction, which blocks the regeneration of the cobalamin coenzyme methylcobalamin [100,96,97]. This inhibits the methylation of the myelin phospholipids, alters incorporation of fatty acids into the myelin sheath leading to the degeneration of the spinal cord [96,97].

6.2 Nitric Oxide and the Human Skin

NO has protective and cytotoxic effect to the skin upon exposure to UV rays of the sun. The UV induced formation of NO can be enzymatic, through the inducible isoform of NO synthase (iNOS) [101] or non-enzymatic through the decomposition of nitric oxide derivates such as nitrate, nitrite and *S*-nitroso compounds i.e. *S*-nitrosoglutathione, *S*-nitrosocysteine [102]. The action of photolabile dermal NO derivates in the skin depends on the redox state of the cell environment [102].

Intracellular NO_3^- in physiological concentrations has protective effects against UV rays of the sun. UV exposure in an antioxidative environment leads to a higher yield of protective NO by UV-induced NO_3^- decomposition with its major effect on cell viability [103]. This justifies the increased use of antioxidants like ascorbic acid and vitamin E against UV rays in skin care products.

In contrast, oxidative stress and low concentrations of antioxidants decrease the NO yield and cause cell damage by the prevailing generation of toxic reactive nitrogen species (RNS), like peroxynitrite [102]. The mechanism of UV-induced nitrite decomposition under hypoxic conditions reveals the generation of NO and a cascade of further reactions. This causes nitrosative stress as a result of the generation of highly toxic nitrogen dioxide ($NO^{\cdot}_2$), which is capable of initiating a chain reaction leading to lipid peroxidation and cell death. The reaction mechanism is explained below.

1. Decomposition of Nitrite to NO

$$NO_2^- + h\nu_{365nm} \longrightarrow NO^{\cdot} + O^{\cdot -}$$

2. Generation of hydroxyl radical thereby initiating a radical chain reaction.

$$O^{\cdot -} + H_2O \longrightarrow OH^{\cdot} + OH^-$$

3. Formation of Nitrogen dioxide

$$NO_2^- + OH^{\cdot} \longrightarrow NO^{\cdot}_2 + OH^-$$

4. Inhibition of NO by $NO^{\cdot}_2$ under hypoxic conditions forming nitrous anhydride (N_2O_3)

$$NO^{\cdot}_2 + NO^{\cdot} \longleftrightarrow N_2O_3$$

5. Regeneration of Nitrite. The reaction is continuous due to the formation of NO_2^-.

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+$$

The scavenging of NO by $NO^{\cdot}_2$ under hypoxic conditions increases lipid peroxidation and cell death. Ascorbic acid protects against UV/nitrite-induced lipid peroxidation by scavenging nitrogen dioxide which simultaneously enhances UV-induced NO formation from nitrite through the oxidative medium. This is important in preserving membrane integrity and effective protection against ROS-induced apoptosis as well as necrotic cell death [104]. The NO can also diffuse into blood vessels, where it is oxidized to nitrate by hemoglobin or in the dermis, where it is oxidized to nitrite by myoglobin. It has been hypothesized that nitrite and other dermal NO derivatives are vasoactive and can evolve changes in blood flow and pressure when exposed to daylight [105].

6.3 Regulation of Heparan Sulphate Activities

Heparan sulfate (HS) is a substituent of cell-surface proteoglycans (PG). Cell-surface proteoglycans are integrated in the plasma membrane via the hydrophobic region of transmembrane proteins like the syndecans or via glycosylphosphatidylinositol (GPI) anchor as seen in glypicans [106,107].

HS chains are continuously cleaved into oligosaccharides by heparanase and terminally degraded by exoglycosidases and sulfatases in lysosomes [108]. In addition to heparanase-catalyzed degradation, the heparan sulfate chains in the intact proteoglycan can be broken down by nitrite-dependent cleavage at internally located N-unsubstituted glucosamine residues [109,110,106]. The nitrite is derived from NO oxidation. NO produced from NO-synthase is unstable and is either converted to nitrite or stored as protein bound S-nitrosothiols which releases NO non-enzymatically [107].

Heparan sulfate is a biological sulfate critical in the lysosomal break down of accumulated cellular debris [110,111] and maintaining blood colloidal suspension [111]. Lysosomes also depend upon internalized sulfate, derived from heparan sulfate proteoglycans (HSPGs), to catalyze its proteolytic activities. Severe neurological dysfunction associated with lysosomal storage diseases involving impaired heparan sulfate homeostasis attest to the importance of sulfate in lysosomal function [112]. Capillary flow is regulated through the water structuring properties of sulfate from heparan sulfate and nitrate from NO oxidation. Sulfate which is an ionic kosmotrope forms a liquid crystalline gel-like structure in the water causing the precipitation of proteins; nitrate a chaotrope on the opposite end of the Hofmeister series promotes disorder in the surrounding water which dissolves proteins [113]. It is critical to control the activities of chaotropes and kosmotropes in order to maintain the proper functioning of active proteins. The role of HSPG in the control of metabolism depends on the availability cholesterol sulphate as it is able to supply both cholesterol and sulfate to tissues [114].

The endothelial nitric oxide (eNOS) is believed to play a dual role, which is essential in regulating blood colloidal suspension and capillary flow. When eNOS is not synthesizing NO, it is rather producing sulfate. It is a homodimer with one monomer containing an N-terminal heme oxygenase domain and the other a C-terminal FAD binding reductase domain [115]. The reductase domain is similar to flavoproteins. Seneff et al. [116] proposed that eNOS synthesize sulfate from sulfane sulfur species (thiosulfate and persulfide) with superoxide as a reactive intermediate; sulfate produced reacts with ATP to form 3'-phosphoadenosine- 5-phosphosulfate (PAPS), a substrate for the sulfation of cholesterol. The union of cholesterol to sulfate acts as a means of transporting the sulfate synthesized by eNOS [116].

$$HS\text{-}SH + O_2 \xrightarrow[eNOS]{} SO_4^{2-} \xrightarrow[ATP]{} PAPS$$

In order to regulate blood flow, there is a constant need for eNOS to determine whether it should synthesize sulfate or nitric oxide which is quickly oxidized to nitrate. A coordinated release of nitric oxide will result in the breakdown of heparan sulfate chains [107], some of which will be released into the blood. These run the risk of increasing the viscosity, leading to a no-flow situation. Nitrate helps to counterbalance this effect by promoting low blood viscosity.

7. TOXIC PRODUCTS OF NITRATE METABOLISM

The products of nitrate metabolism are NO, $ONOO^-$, HNO_2, metHb and metMb. All the toxic effects exhibited by NO_3^- are mediated through the formation of NO. The major cytotoxic fate of NO is;

i. Formation of N_2O_3 leading to the Nitrosation of amines and deamination of DNA base.
ii. Formation of $ONOO^-$ leading to the oxidation and nitration of biological molecules.

7.1 Peroxynitrite ($ONOO^-$)

$ONOO^-$ is a toxic oxidant which is capable of nitrating and oxidizing the DNA molecule [117]; alongside protein and non-protein sulfhydryls [58]. This can lead to DNA damage due to the nitration of the guanine base to 8-nitroguanine upon reacting with $ONOO^-$ [118]. Within a DNA molecule, oxidized bases i.e. 8-oxoguanine and 5-hydroxymethyluracil are also observed when exposed to NO as a result of peroxynitrite formation [117]. The presence of these modified

bases causes depurination leading to abasic sites and potentially strand breaks [118]. A single strand break can also be formed by the withdrawal of a hydrogen atom on the sugar-phosphate backbone by peroxynitrite [119]. The mutagenicity of $ONOO^-$ is observed in base deletions, insertions, multiple mutations as well as G:C ⟶ T:A point mutation [120].

7.2 Nitrous Acid (HNO_2)

HNO_2 is a chemical mutagen that leads to the deamination of nucleic acids [121]. Though nitrate serves as an alternative source for the synthesis of nitric oxide, it also generates a mutagenic by-product, nitrous anhydride (N_2O_3) through HNO_2 and NO. N_2O_3 arises from the condensation of molecular HNO_2 or from the auto oxidation of NO [122,123].

$$2HNO_2 \longrightarrow N_2O_3 + H_2O$$
$$2NO^{\cdot} + O_2 \longrightarrow 2NO^{\cdot}{}_2$$
$$NO + NO^{\cdot}{}_2 \longrightarrow N_2O_3$$

7.3 Nitric Oxide (NO)

Nitric oxide is a free radical which serves as a mediator where macrophages express cytotoxic activities against microorganisms [124]. It can be toxic when the body is overwhelmed with it, resulting to the formation of nitrous anhydride; a nitrosating agent which deaminates exocyclic amino groups of nucleic acids via the formation of a diazonium ion [125,126]. The diazonium ion is stabilized by its conjugation with the aromatic ring. Hydrolysis of the diazonium ion completes the deamination and changes the base pairing ability thereby leading to an AT ⟷ GC transition (see Fig. 5).

The reaction tends to be continuous due to the fact that the NO_2^- liberated goes ahead to form N_2O_3 on reacting with NO. Deamination of cytosine to uracil will give rise to a G:C ⟶ A:T mutation through mispairing. However, most cell types have significant quantities of uracil glycosylase which is known to excise uracil from single and double DNA with preference for cytosine. Therefore, the deamination of cytosine to uracil may not be critical because the resulting uracil will be repaired before causing a mutation [127,125]. The deamination of adenine to hypoxanthine may result in an A:T ⟶ G:C mutation due to the pairing of hypoxanthine with cytosine. The deamination of guanine to xanthine will lead to a G:C ⟶ A:T transition upon pairing; xanthine is unstable in DNA and could depurinate leaving an apurinic site. In addition to mispairing of deaminated bases, the instability of hypoxanthine and xanthine in DNA leads to depurination and subsequent strand breakage [125].

Nitrite can also react with dietary amines in the human stomach to form nitrosamines, which is carcinogenic [128,2,26,9]. This is enhanced by high acidity of the stomach, leading to the formation of N_2O_3 [129]. *N*-nitrosamines are formed as a result of an electrophilic substitution of organic nitrogen with a nitrosating compound, leading to the formation of diazonium ion (see Fig. 6). The diazonium ion is stabilized by its reaction with organic nitrogen derived from dietary amines. The hydrolysis of the diazonium ion leads to the formation of nitrosamines. The reaction is also continuous due to the NO_2^- liberated forms N_2O_3 as enhanced by the high acidity in stomach.

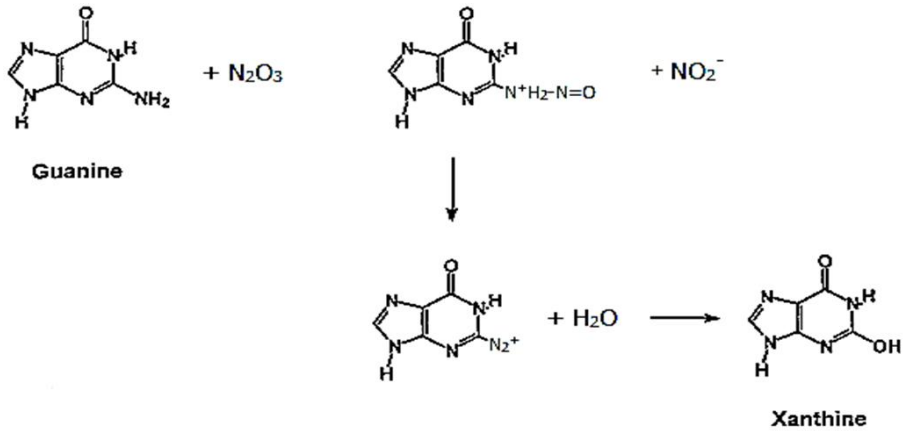

Fig. 5. DNA base deamination-hydrolysis of the diazonium ion leads to deamination of guanine forming xanthine [126]

7.4 Impaired Nitrate Handling and Neurodegenerative Diseases

Lysosomal dysfunction could result from a long-term impairment of eNOS sulfate synthesis. Lysosomes, the "digestive system" of the cell, protect cells from oxidative damage [130]. Nitric oxide interferes with autophagy [131]. A system characterized by nitrate originating from NO is not sustainable owing to continuous breakdown of heparan sulfate chains in the intact proteoglycan and consequent impairment of lysosomal-based autophagy. As a result, cells would eventually be overwhelmed with their own debris. Autophagy inhibition may account for accumulations of aggregated proteins due to nitrosative stress in neurodegenerative diseases.

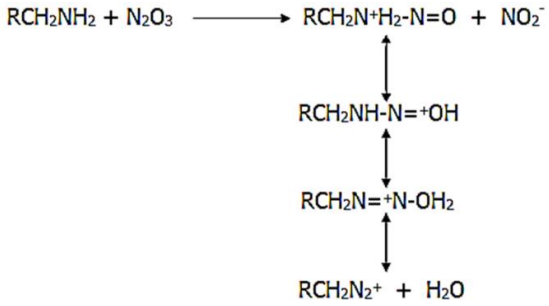

Fig. 6. Nitrosation of a primary amine by N^2O^3 leading to diazonium ion formation. [126]

Alzheimer's disease is characterized by extracellular deposits of amyloid-β peptides (Aβ) forming senile plaques and intracellular neurofibrillary tangles [132,133]. The Aβ protein contains a 40-42 amino acid peptide derived from proteolytic processing of a larger amyloid precursor protein (APP) molecule at the NH_2 and COOH termini of the Aβ domain [134,133]. The APP molecules are proteolytically cleaved by secretases in the Golgi complex and lysosome endosomal system via the α pathway and the β pathway [133].

The beta-site APP-cleaving enzyme 1 (BACE-1), a β secretase, generates soluble fragments which are later degraded by an additional protease, γ-secretase to produce Aβ [135,136]. Heparan sulfate interacts with BACE-1 and regulates its processing of APP [137]. HS specifically inhibits BACE-1 cleavage of APP but not the alternative cleavage by α secretase. The constant breakdown of HS by nitrite derived from NO oxidation will result to increased BACE-1 activity.

The inability of the mitochondria to mediate against ROS and RNS appears to play a prominent role in the early events of Alzheimer's disease progression [138]. Mitochondria are ordinarily constantly broken down and renewed by lysosomal processes [114]. When it becomes impaired, aged mitochondria become a source of reactive oxygen species that contribute significantly to neuronal damage. Since HSPG metabolism takes place in the lysosome, and plays a crucial role in autophagy to recycle cellular debris; excessive nitric oxide synthesis will have direct effect on HSPG which supports proteolytic activities. Lysosomal dysfunction due to NO inhibited autophagy is a major factor in neurodegeneration and the progression of Alzheimer's disease [139,140].

7.5 Methemoglobin and Methmyoglobin

Nitrite oxidizes the ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) within the hemoglobin molecule. This increases the amount of methemoglobin in the blood leading to a blood disorder known as methemoglobinemia. The methemoglobin level of a healthy individual is 1% of the total hemoglobin [10]. This is because the blood has a mechanism of reducing the methemoglobin level when it is higher than normal through the intracellular methemoglobin reductase described previously. Excess methemoglobin generated from nitrate metabolism impairs the ability of the blood to transport oxygen and carbon dioxide, leading to a deficiency in the oxygen concentration of tissues and in severe cases, death [30,11].

On the other hand, myoglobin takes up molecular oxygen from the blood capillaries during relaxation and transports it to the myocytes, where it is supplied to the mitochondria [141,142]. During contraction, the oxygen is made available for cytochrome oxidase for the oxidation of electrons and production of energy in form ATP [143]. The availability of oxygen in the mitochondria as a terminal electron acceptor triggers the transformation of pyruvate into acetyl co-A for further oxidation in the citric acid cycle.

Nitrite inhibits the oxygen transport of myoglobin due to the formation of ferric myoglobin molecule which is unable to transport oxygen [144]. When the myocyte is deprived of oxygen, electron transfer to oxygen slows resulting to an imbalance between the influx of electrons from food oxidation and transfer of electrons to molecular oxygen in the mitochondrial matrix. This leads to the accumulation of lactic acid in

the muscle, increased formation of ROS in the mitochondria and also a decrease in the concentration gradient of protons for ATP synthesis. The accumulation of lactic acid causes muscle fatigue. Also conditions of very high ROS production compromise the mitochondrial function resulting to oxidative stress and ROS related diseases.

8. CONCLUSION AND RECOMENDATIONS

Nitrate metabolism evolved by nature with an adequate means of dealing with its products. It poses a problem to humans due to activities like the use of fertilizer to improve crop yield, curing of meat to improve its appearance and also inhibit botulinum toxin; use of drugs high in nitrates and building wells close to septic pits. The body system is overwhelmed by these activities and the metabolism of the ingested nitrate causes adverse health effects. Man has made it a curse upon himself by increasing the intake of what should be a metabolic waste. The endogenous synthesis of nitrate can't be held accountable for the health effects posed by nitrate metabolism. This is because, the interconversion of NO and NO_3^- is a mechanism adopted by the body system to regulate NO homeostasis. Since the nitrate/nitrite pool for the alternative synthesis of NO comes from either dietary intake or NO detoxification, high levels of nitrate exposure from dietary intake will definitely increase endogenous synthesis.

Despite the fact that nitrate and its reduction products are involved in regulating the physiological functions of the body, there is a great need to reduce its adverse effects due to high levels of exposure. Today, nitrate poisoning has become a major public health issue as a result of human activities; most of which we are ignorant about. Children die of methemoglobinemia and are exposed to the likelihood of a cancer causing mutation due to nitrate contaminated water. Also the accumulation of free radicals in the respiratory system has increased the cases of Alzheimer's disease and other neurodegenerative diseases.

In order to reduce these adverse health effect posed by excess nitrate metabolism, consumption of sodium nitrite treated meat should be discouraged. Placing a ban on the manufacture and sale of infant drugs high in nitrate should be enforced due to the underdeveloped enzyme system of infants below three months of age. A guideline value for nitrate below 50 mg/L for drinking water should be adopted [2]. Also agricultural practices such as fertilizer application should be carefully managed to reduce contamination due to leaching and surface runoff. Finally, careful siting of pit latrines and septic tanks should also be encouraged to prevent groundwater contamination.

Nitrate may be considered as a significant component of certain fruits and vegetables which accounts for the health benefits of certain foods. The consumption of antioxidants will go a long way to inhibit nitrosamine formation in the gastric medium and the onset of carcinogenesis. Food supplements rich in antioxidants should be encouraged especially in people aged 40 years and over. This may go a long way in the prevention of cancers and neurodegenerative diseases. Also, antioxidant rich skin care products have potentials to prevent or ameliorate the cytotoxic effect of UV induced cancer causing mutation and cell death.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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