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Sodium nitrite-induced oxidative stress causes membrane damage, protein oxidation, lipid peroxidation and alters major metabolic pathways in human erythrocytes

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ABSTRACT

Nitrite salts are present as contaminants in drinking water and in the food and feed chain. In this work, the effect of sodium nitrite ($NaNO_2$) on human erythrocytes was studied under *in vitro* conditions. Incubation of erythrocytes with 0.1–10.0 mM $NaNO_2$ at 37 °C for 30 min resulted in dose dependent decrease in the levels of reduced glutathione, total sulfhydryl and amino groups. It was accompanied by increase in hemoglobin oxidation and aggregation, lipid peroxidation, protein oxidation and hydrogen peroxide levels suggesting the induction of oxidative stress. Activities of all major erythrocyte antioxidant defense enzymes were decreased in $NaNO_2$-treated erythrocytes. The activities of enzymes of glycolytic and pentose phosphate pathways were also compromised. However, there was a significant increase in acid phosphatase and also AMP deaminase, a marker of erythrocyte oxidative stress. Thus, the major metabolic pathways of cell were altered. Erythrocyte membrane damage was suggested by lowered activities of membrane bound enzymes and confirmed by electron microscopic images. These results show that $NaNO_2$-induced oxidative stress causes hemoglobin denaturation and aggregation, weakens the cellular antioxidant defense mechanism, damages the cell membrane and also perturbs normal energy metabolism in erythrocytes. This nitrite-induced damage can reduce erythrocyte life span in the blood.

1. Introduction

Nitrite is naturally present in humans and in most green vegetables. In humans it acts directly or indirectly (by reducing to nitric oxide) in maintaining vascular homeostasis. It also plays key physiological roles in signalling, cellular respiration and mediation of innate immunity (Lundberg et al., 2008). Inside the cell, nitrite can be reversibly converted to nitrate and nitric oxide.

Sodium nitrite ($NaNO_2$) is widely used as a food preservative and color fixative in fish and meat. It is also used in the manufacture of dyes, nitroso compounds and rubber chemicals. Medicinally, it is used as a vasodilator, bronchodilator and as an antidote for cyanide poisoning. Human exposure to nitrite has increased sharply in recent years because of extensive use of nitrogenous fertilizers in agriculture, improper disposal of human and industrial wastes and atmospheric nitrogen pollution (Galloway et al., 2003). The nitrate–nitrite content in food and water in many areas has increased alarmingly beyond the permissible limits (WHO, 2011). In some areas it has greatly exceeded the levels of 1 ppm (nitrite) and 10 ppm (nitrate) in drinking water set by the U.S. Environmental Protection Agency (EPA).

Although nitrite has important functions in the cell at physiological concentrations, it can be toxic in high amounts to animals and humans. The immediate and major health implication of nitrite intoxication is methemoglobin (MetHb) formation (Chui et al., 2005) and cyanosis, which can be fatal. Infants are particularly susceptible to nitrite intoxication, causing death in most cases (WHO, 2011). Continuous exposure to non-fatal doses of nitrite can

Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; DTNB, 5,5′-dithiobisnitroben zoic acid; G6PD, glucose 6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Hb, hemoglobin; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MetHb, methemoglobin; NAD^+ and NADH, reduced and oxidized nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, reduced and oxidized nicotinamide adenine dinucleotide phosphate; Na,K-ATPase, sodium potassium ATPase; $NaNO_2$, sodium nitrite; NO, nitric oxide; ROS, reactive oxygen species; SEM, scanning electron microscopy; SH, sulfhydryl; SOD, Cu,Zn superoxide dismutase; TR, thioredoxin reductase.

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cause physiological disturbances including permanent growth inhibition, neurological disorders, respiratory failure and paralysis (Mensinga et al., 2003). Pregnant women, anemic and glucose 6-phosphate dehydrogenase (G6PD) deficient individuals are also prone to nitrite toxicity (Huber et al., 2013; WHO, 2011). Nitrite easily transforms into carcinogenic nitrosating compounds in the acidic environment of gut (Brambilla and Martelli, 2007). A relationship between nitrite/nitrate levels and higher incidence of various cancers has been reported in humans (Dellavalle et al., 2013; Coss et al., 2004). $NaNO_2$ administered rats showed increased risk of forestomach neoplasm and squamous papilloma (NTP, 2001). Degenerative changes were observed in various tissues of $NaNO_2$ administered mice (Ozen et al., 2014). Nitrite has thus been classified as cytotoxic, mutagenic, teratogenic and embryotoxic (NTP, 2001).

Human exposure to nitrite results in its uptake and subsequent transfer to blood with rapid binding to erythrocytes (Dejam et al., 2007). Blood acts as a biological nitrite buffer to maintain its concentration throughout the body, and supply it as and when required. About 75% of nitrite enters erythrocytes by diffusion, as nitrous acid or other species, while the remaining 25% uptake occurs by the sodium-dependent phosphate transporter (May et al., 2000).

Erythrocytes have been used as a simple model to study the cellular effects of various compounds (Takebayashi et al., 2010), especially those that generate reactive oxygen species (ROS). Erythrocytes are particularly susceptible to oxidative insult due to their role as oxygen transporters and high content of polyunsaturated fatty acids, transition metals and redox active hemoglobin (Hb) molecules. We have examined the effect of $NaNO_2$ on human erythrocytes under *in vitro* conditions using concentrations to which humans could be potentially exposed, especially in areas with nitrate–nitrite contaminated drinking water (WHO, 2011). Our results show that $NaNO_2$ causes significant oxidative damage to erythrocytes which was also visible in electron microscopic images.

2. Materials and methods

2.1. Materials

$NaNO_2$, 1-chloro-2,4-dinitrobenzene, glutathione reductase (GR), metaphosphoric acid, 2,4,6-trinitrobenzene sulfonate, N-(1-naphthyl)ethylenediamine dihydrochloride and ouabain were purchased from Sigma–Aldrich, USA. Reduced (GSH) and oxidized (GSSG) glutathione, reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH and $NADP^+$), reduced nicotinamide adenine dinucleotide (NADH), N-ethylmaleimide, o-phthalaldehyde, pyrogallol, 2,6-dichlorophenolindophenol, 2,4-dinitrophenylhydrazine, 5,5′-dithiobisnitrobenzoic acid (DTNB), xylenol orange, sulfanilamide, adenosine 5′-monophosphate (AMP), glucose 6-phosphate, sodium nitroprusside, methyl violet and sorbitol were from Sisco Research Laboratory (Mumbai, India). Thiobarbituric acid, tris(hydroxymethyl)aminomethane, trichloroacetic acid and S-acetylthiocholine iodide were purchased from Himedia Laboratories (Mumbai, India). All other chemicals were of analytical grade.

2.2. Isolation of erythrocytes and treatment with $NaNO_2$

This study was approved by the institutional ethics committee which monitors research involving human subjects. Human blood was taken from young (22–30 years), healthy, non-smoking volunteers after getting their informed consent and used immediately. Blood was collected in heparinized tubes, centrifuged at 1500 rpm for 10 min at 4 °C in a clinical centrifuge and the plasma and buffy coat were removed. The erythrocyte pellet was washed three times with phosphate-buffered saline (PBS) (0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4) and resuspended in PBS to give a 10% (v/v) cell suspension (hematocrit).

Stock solutions of $NaNO_2$ were prepared in PBS. Erythrocytes were incubated with different concentrations of $NaNO_2$ (0.1–10 mM, corresponding to 0.0069–0.69 mg/ml) for 30 min at 37 °C. $NaNO_2$-untreated cells were similarly incubated at 37 °C and served as control. The samples were centrifuged at 2500 rpm for 10 min at 4 °C. Cell pellets were washed three times with PBS and erythrocytes were lysed with ten volumes of 5 mM sodium phosphate buffer, pH 7.4, at 4 °C for 2 h. Samples were centrifuged at 3000 rpm for 10 min at 4 °C and the supernatants (hemolysates) were quickly frozen in aliquots to be used later for the analysis of several biochemical parameters.

2.3. Osmotic fragility

Control and $NaNO_2$ treated erythrocytes were incubated at 37 °C and centrifuged as above. The cell pellet was washed and resuspended in PBS to give 10% hematocrit. Then, 0.05 ml of this cell suspension was added to different tubes containing 5 ml of 0.2–0.7% NaCl. After 30 min at 37 °C, the samples were centrifuged at 2500 rpm for 10 min in a clinical centrifuge and the absorbance of supernatants was recorded at 540 nm. The absorbance of untreated erythrocytes, lysed with 5 mM sodium phosphate buffer, pH 7.2, for 2 h at 4 °C, served as a reference and represents 100% lysis.

2.4. Hemoglobin and methemoglobin levels and methemoglobin reductase

Hb concentration in hemolysates was determined by the cyanomethemoglobin method using a commercially available kit (Hemocor-D Kit, Coral Clinical Systems, Goa, India).

The levels of MetHb were determined from the absorbance of diluted hemolysates at 540, 576 and 630 nm (Benesch et al., 1973) and expressed as percent of total Hb. NADH-dependent MetHb reductase activity was determined from the increase in absorbance at 600 nm after incubation of hemolysates with NADH and 2,6-dichlorophenolindophenol (Kuma et al., 1972).

Heinz bodies were detected by staining control and $NaNO_2$ treated erythrocytes with 0.5% methyl violet (in 0.9% NaCl) for 45 min. Cells were then fixed on glass slides and observed under a light microscope at 100× magnification.

2.5. Reduced (GSH) and oxidized (GSSG) glutathione, amino groups and total sulfhydryl levels

GSH and GSSG levels in hemolysates were determined fluorometrically using o-phthalaldehyde and N-ethylmaleimide (Hissin and Hilf, 1976). Total sulfhydryl (SH) groups were determined from the yellow color produced after their reaction with DTNB (Sedlak and Lindsay, 1968). Free amino groups were determined using 2,4,6-trinitrobenzenesulfonate (Snyder and Sobocinski, 1975).

2.6. Carbonyl content, thiobarbituric acid reactive substances, hydrogen peroxide and nitrite levels

Protein carbonyl content in hemolysates was determined after reaction with 2,4-dinitrophenylhydrazine (Levine et al., 1990). Hemolysates, deproteinized using zinc sulfate–NaOH, were used for the analysis of lipid peroxidation, nitrite and H_2O_2 levels.