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Subacute intoxication with sodium nitrate induces hematological and biochemical alterations and liver injury in male Wistar rats

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ABSTRACT

Nitrate pollution has emerged as a problem of great importance because in recent years, the levels of nitrate in soil and groundwater have increased, mainly through anthropogenic activities, such as the use of fertilizers in agriculture, domestic wastewater and septic tanks, industrial waste and deforestation. In animals, nitrate reduction to nitrite (NO_2) and nitric oxide (NO) promote the formation of methemoglobin in the blood and the generation of highly reactive intermediates that induce oxidative stress in target organs. Exposition to nitrates has been associated with methemoglobinemia, reproductive toxicity, metabolic and endocrine alterations and cancer. This study analyzed acute intoxication with sodium nitrate ($NaNO_3$) in male Wistar rats, aged 12–16 weeks. Four groups with n = 10 rats each were formed: group 1 was the control, and group 2, group 3 and group 4 were treated for 10 days with intragastric doses of 19, 66 and 150 mg/kg/d $NaNO_3$, respectively. Hematological, metabolic and histological biomarkers in the liver were analyzed. The results showed high percentages of methemoglobin, an increase in NO_2 in the plasma and an accumulation in the liver. Moreover, there were high counts of white blood cells and platelets in all treated groups. Additionally, there was an increase in the spleen weight in group 4. High levels of glucose, triglycerides, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were observed and were significantly increased in groups 3 and 4. For oxidative stress biomarkers, there were increases in Thiobarbituric Acid Reactive Substances (TBARS), total GSH and SOD activity, mainly in group 4. Changes in mitochondrial activity were not significant. Histopathological analyses of the liver showed inflammation, infiltration of mononuclear cells, steatosis, ischemia and necrosis, and these findings were more evident at high doses of $NaNO_3$ in which high of S-nitrosylation were found. In conclusion, $NaNO_3$ was reduced to NO_2, thereby inducing methemoglobinemia, whereas other reactive species generated oxidative stress, causing hematological and metabolic alterations and injury to the liver.

1. Introduction

Nitrate pollution has emerged as a problem of great importance because in recent years, the levels of nitrate in water for human consumption have increased. Water quality has deteriorated considerably due to anthropogenic activity, such as the use of fertilizers in agriculture, domestic wastewater and septic tanks, industrial waste and deforestation (Shukla and Saxena, 2018).

High concentrations of nitrates have been reported in the soil and groundwater of developed countries, such as in the USA, China, India, Korea and the European Union, where the European Commission has identified Nitrate Vulnerable Zones in which sustainable agriculture strategies are implemented to reduce nitrates (Ascott et al., 2017). However, in developing countries, up to 70% of the water used in

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human activities is returned to the environment without any treatment, which affects water quality in rivers and coastal areas, affecting the health of human populations and ecosystems (Rojas et al., 2015).

Studies on the spatial distribution of nitrates and the risk to human health were performed, and the main sources of contamination identified were domestic waste and agricultural activities. Additionally, a negative impact on health was observed, mainly in children and women (Rebolledo et al., 2016). The World Health Organization (WHO) established a permissible limit for the nitrate concentration in groundwater of < 50 mg/L as nitrate or < 11 mg/L as nitrate-nitrogen, whereas the Environmental Protection Agency (USA) guideline is < 45 mg/L as nitrate or < 10 mg/L as nitrate-nitrogen. These values are based on epidemiological evidence for methemoglobinemia in infants from short-term exposure.

By contrast, there are reports of the health benefits of nitrate intake. This is because it is a source for the exogenous of production of NO that confers several beneficial cardiovascular effects on blood pressure, platelets, endothelial function and mitochondrial efficiency (Katri et al., 2017). However, these benefits were observed with nitrate ingested from the diet, mainly from green vegetables and during pharmacological administration; therefore, there is controversy about its safety, and the biological/biochemical role of the nitrate – NO_2 – NO pathway has been considered and analyzed.

Nitrate reduction is initiated in the mouth by bacteria with nitrate reductase enzymes, which are also present in the mammalian gut. In the mouth, approximately 25% of the nitrate ingested is reduced to NO_2, and in the stomach, the acidic pH favors the formation of nitrous acid, which can decompose to different nitrogen oxides, such as nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3), depending on the redox environment and gastric content. The enterosalivary circulation of nitrate after absorption in the intestine has been reported, such that it can be reduced again to NO_2 in the mouth and restart the cycle (Pereira et al., 2013).

In the blood, NO_2 can bind to hemoglobin and oxidize ferrous iron in the heme site, forming methemoglobin, which cannot transport oxygen, thereby generating methemoglobinemia that is clinically significant; in 5–12% of methemoglobin cases, cyanosis is observed, and fatal toxicity occurs at levels of 30–50%. During the biotransformation of nitrate to NO_2, other reactive nitrogen species (RNS) are produced, such $NO^{\bullet}$ and peroxynitrite ($ONOO^-$), and these react with proteins to form nitrotyrosine (Belcastro et al., 2017). Additionally, the formation of nitrosamines produced by chemical reactions of NO_2 with certain amines or amides to form N-nitroso compounds, such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), and N-nitrosomorpholine (NMOR), which are likely carcinogenic to humans, were reported. Moreover, NDMA is hepatotoxic, causing fibrosis and tumors (Erkekoglu and Baydar, 2010).

Nitrogen oxides can affect the functionality of mitochondria, increasing reactive oxygen species (ROS) production by mitochondrial complex III, and other reactive molecules, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$) (Akopova et al., 2016). An imbalance between oxidative - nitrosative stress and the antioxidant response can lead to lipid peroxidation, protein carbonylation, modification of amino acids, DNA damage and S-nitrosylation of proteins that alter and compromise cellular function (Kurutas, 2016). Therefore, RNS and ROS induce the antioxidant response as a defense mechanism, and this includes an increase in the glutathione (GSH) level and the overexpression of detoxification enzymes, such as superoxide dismutase (SOD) isoforms, catalase, thioredoxin, glutathione peroxidase and glutathione transferase (He et al., 2017).

Several studies have reported the effects of nitrate intoxication in animals and alterations in several biochemical parameters, such as glucose, cholesterol, creatinine, LDH, AST, ALT, and alkaline phosphatase (AP), and a high percentage of methemoglobin (Azzez et al., 2011). Moreover, biomarkers of oxidative stress were increased (Bouaziz-Ketata et al., 2014). Another study on the rainbow trout *Oncorhynchus mykiss* provided strong evidence that relatively low nitrate-nitrogen levels of 80–100 mg/L were related to chronic effects, such as changes in swimming behavior, as well as slightly decreased survival and reduced total biomass (Davidson et al., 2014); however, in a study of *Danio rerio*, fish were treated with NO_2 in the range of 15–130 mg/L, growth restriction was observed, and there was a negative correlation between the NO_2 concentration and the growth rate (Voslářová et al., 2008).

In humans, the consumption of nitrates or NO_2 in drinking water and food has been associated with diseases, such as methemoglobinemia, some types of cancer, including stomach, liver, colon, lung, thyroid, kidney and non-Hodgkin lymphoma (Espejo-Herrera et al., 2015), cases of hypothyroidism and adverse reproductive effects (Kilfoy et al., 2011). However, other studies have not found this association (Quist et al., 2018); therefore, there is controversy about its adverse effects at an epidemiological level.

In this study, low doses of $NaNO_3$ under the permissible limit (< 50 m/L NO_3) were administered to male Wistar rats. Short-term exposure was evaluated, and the methemoglobin percentage and NO_2 level in plasma and liver were quantified. Additionally, hematological and metabolic parameters were analyzed, and liver injury was assessed.

2. Material and methods

2.1. Animals

Forty male Wistar rats 12–16 weeks of age and weighing between 100 and 150 g were included in this study. The animals were housed in a climatized environment at 25 ± 3 °C and 60% relative humidity with forced ventilation under automatic light cycles of 12 h, with free access to water and food (2014 Teklan Global, 14% protein). Rats were randomized and distributed in groups of 10 individuals. Group 1was treated with 0.9% saline solution as a control, and the three experimental groups were treated with doses of 19 mg/kg (group 2), 66 mg/kg (group 3), and 150 mg/kg (group 4) of $NaNO_3$ (Sigma-Aldrich Darmstadt, Germany). Doses were administered daily for 10 days using an intragastric tube made of stainless steel N. 18 (Pfizer ® New York, NY, USA) to ensure that the exact doses were administered. The experimental protocol was authorized by the Bioethics Committee of the Instituto de Investigaciones Biomédicas of the Universidad Nacional Autonóma de México (CICUAL ID – 210).

2.2. Sample collection

The rats were sacrificed by cervical decapitation. Blood samples were collected in tubes with EDTA to evaluate hematological parameters, methemoglobin percentage and to obtain plasma by centrifugation at 3000 g for 10 min. Blood samples were also collected without anticoagulant to obtain serum in which biochemical parameters were analyzed. Both the plasma and serum samples were stored at − 80 °C until use. The rat livers and spleens were removed, cleaned and weighed. The livers were sectioned, and samples were rinsed and homogenized (10% w/v) in an appropriated buffer. These were centrifuged according to a previous protocol. The supernatants were stored at − 80 °C until use. Other liver samples were fixed in 10% buffered formalin for histological analysis.

2.3. Methemoglobin and hematological analyses

The methemoglobin percentage was measured using the method reported by Sakata et al. (1982). Hematological parameters were evaluated in an automated Hematology Analyzer Cell-Dyn 1800. The hemogram reported the total red blood cells, white blood cells, hemoglobin, hematocrit, mean corpuscular volume and total platelet concentration.