Iran Red Crescent Med J. 2018 November; 20(11):e83094. doi: 10.5812/ircmj.83094.

Published online 2018 November 13. **Research Article**

Effect of Drinking Water Nitrates and Vitamin C on Rat Liver Enzymes and Oxidative Markers

Mahmoud Moghaddam Dorafshani [1, *], Mohammad Reza Nikravesh [1, **], Mahdi Jalali [1] and Mohammad Soukhtanloo [2]

[1] Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Department of Clinical Biochemistry and Pharmacological Research Center of Medicinal Plants, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[*] *Corresponding author*: Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-5138002491, E-mail: moghaddamdm1@mums.ac.ir
[**] *Corresponding author*: Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-5138002491, E-mail: nikraveshmr@mums.ac.ir

Received 2018 August 08; **Revised** 2018 October 08; **Accepted** 2018 October 11.

Abstract

Background: Liver cells or hepatocytes facilitate different hepatic functions. The liver reportedly accounts for up to 500 separate functions, alongside other systems and organs. The high consumption of the food containing high levels of nitrate in the community and the presence of this harmful substance in water endanger the health of many people. N-nitroso compounds, as potential free radicals, can damage the tissues through oxidative stress.
Objectives: The current study was targeted toward examining the impact of drinking water nitrate and vitamin C on hepatic enzymes and oxidative markers in rats.
Methods: The present experimental study was performed on 49 rats in Mashhad, Iran, during 2017 - 2018. The subjects were assigned into seven groups. Group one received water without nitrates (control) while groups two, three, and four received different concentration of nitrates (10, 45, 200 mg/L). Groups five, six, and seven received the same concentration of nitrates and vitamin C (20 mg/100g body weight). After 91 days, blood samples were obtained to determine hepatic enzymes (namely, ALT/SGPT: alanine aminotransferase, AST/SGOT: aspartate aminotransferase, and ALP: alkaline phosphatase). Furthermore, an autopsy was carried out to examine the liver tissue regarding the markers of oxidation (namely, MDA: Malondialdehyde, SOD: Superoxide-dismutase, CAT: Catalase enzyme, and GSH: Glutathione), according to the protocol.
Results: The results revealed a significant elevation in ALP ($P = 0.034$), AST ($P = 0.018$), and ALT (155.14 ± 25.67, 92 ± 17.72, $P = 0.000$), compared to those in the control group. In addition, the fourth group demonstrated a significant enhancement in MDA level, collated to the group one ($P = 0.44$), while there was a significant drop in CAT ($P = 0.025$), SOD ($P = 0.002$), and GSH levels ($P = 0.000$). Furthermore, use of vitamin C led to a significant drop in the levels of ALP, AST, ALT, and MDA, as well as a significant elevation in GSH, SOD, and CAT in the seventh group in comparison to the fourth group ($P < 0.05$).
Conclusions: As the findings indicated, drinking water nitrate and Vitamin C exerted a non-significant effect on the doses of nitrate (10 and 45 mg/L). Nonetheless, a nitrate dose of 200 mg/L had a significant impact on ALT, ALP, AST, and oxidative stress indicators leading to hepatic diseases.

Keywords: Ascorbic Acid, Drinking Water, Free Radicals, Liver Diseases, Nitrates, Oxidative Stress, Rats

1. Background

One of the main factors responsible for the enhancement of nitrates in the environment is its application as a fertilizer in the agricultural sector. Accordingly, this harmful substance can leak into the surface and groundwater during rain or as a result of the disposal of the human waste, especially sewage (1, 2). The high concentration of nitrates in aquatic environments, especially drinking water, have led to serious concerns, the most important of which is the short- and long-term effect of nitrate in the body that can lead to methemoglobinemia or induce malignant effects on fetuses and cancer (3). Nitrates are naturally produced in the human body at an amount of about 62 mg per day. However, dietary habit is an important source of nitrates to the body (4); accordingly, human exposure to nitrates and nitrites is mainly due to food intake, especially contaminated vegetables, meat, and water (5, 6). The researchers found that nitrates, nitrites, and N-nitroso could pass through the uterus and affect the fetus (7). In the metropolises, the landfill is the pri-

mary method of disposing of the urban, industrial, animal, and plant wastes that contain organic nitrogen. These wastes are converted to ammonium ion (NH) within a process known as ammonification through soil microorganism activity. In spite of the soil potentiality to hold this compound, in another phenomenon referred to as nitrification, ion of ammonium is partly transformed to nitrite (NO_2^-), and afterward to nitrate. The surface layer of the soil is incapable of preserving these two compounds; as a result, nitrite and nitrate can enter the underground waters (8, 9). The solubility of nitrate in water impedes the conventional water treatment techniques to eliminate this ion. Consequently, the reduction or removal of soluble contaminants requires the adoption of advanced purification methods. On the other hand, the nitrate-creating cycle continues in cities where wastewater is improperly disposed through absorbent wells, causing the constant production of nitrate and its release into underground waters (10-12). Upon the exposure of the creatures to contaminants, they initiate certain activities targeted toward the direct purification, metabolization, and minimization of any contaminant-induced cellular damages. These enzymatic mechanisms can be regarded as the biological markers for contaminant exposure (13). Considering the lethality of oxidation injury, the organisms adopts vindicative systems to maintain the cells versus oxidative markers. The most effective agents for the elimination of toxic radicals are antioxidant enzymes (14). Extensive investigations have been conducted on the variations in the operation of enzymes against oxidants with the aim of evaluating the possibility of applying these enzymes as biological indicators of organic pollution. The liver plays an important role in the detoxification of the body. During the prenatal period, any substance passing through the placenta directly enters the liver and, if toxic, it causes impairment in the development and function of the liver (15).

2. Objectives

The current study was conducted to investigate the impact of vitamin C and nitrates in drinking water on hepatic enzymes and biomarkers of oxidation among rats.

3. Methods

3.1. Cure of Animal and Study Plan

The current empirical study was carried out on 55 male Wistar rats relatively weighing 150 - 250 g in Mashhad, Iran, during 2017 - 2018. The rats were obtained from the Standard Animal House of Mashhad University of Medical Sciences, Mashhad, Iran. The animals were housed under a standard condition (i.e., a temperature of 22 $\pm$ 1°C, 12:12 light-dark cycle, and 60% humidity) with free access to water and food. The sample size was calculated as 49 cases based on the Morgan's table and different population sizes with the application the formula of Cochran (Equation 1).

$$n = \frac{NZ^2pq}{Nd^2 + Z^2pq} \quad (1)$$

Wherever, n; Sample sizes, N; Population sizes, Z = 1.96, p = q = 0.5, d; Error = 0.05. The study population was selected through simple random sampling technique, and then assigned into seven groups of seven members as follows: Group one (control): distilled water, Group two: 10 mg/L nitrates, Group three: 45 mg/L nitrates, Group four: 200 mg/L nitrates, Group five: 10 mg/L nitrates+Vitamin C (20 mg/100 g. Body weight), Group six: 45 mg per Liter nitrates+20 mg per 100 g B.wt Vitamin C, and Group seven: 200 mg per Liter nitrates+20 mg per 100 g B.wt. Based on the previous studies, the experiments were carried out after 91 days.

3.2. Sampling Process of Blood Serum

Under deep anesthesia (100 mg/kg ketamine, Sigma Co., USA), 5 mL of blood was collected from the heart of each subject. Serum separation was accomplished by centrifuging the blood samples at 3000 rpm for 12 minutes, followed by the immediate sample examination.

3.3. Hepatic Tissue Homogenization

After liver dissection and removal from the body, the tissue (150 mg) was homogenized with phosphate-buffer saline (PBS, 1.5 mL) using a homogenizer machine (IKA T18, USA), 2 minutes at 5000 rpm and the resultant dilution was centrifuged. In order to preclude enzyme and protein destruction, all stages were implemented at 4°C (refrigerated centrifuge). In the next step, the supernatant was removed from the rest of the solution to be utilized in the appraisal of biochemical indicators.

3.4. Evaluation of Serum Hepatic Enzymes

The evaluation of ALP, ALT, and AST was performed by means of BT 3000 Co., Italy, and AST, ALT, and ALP assay kits (Pars Azmoon Co., Iran). The accuracy of the results was ensured through calibrating the biochemistry analyzer by a serum calibrator, namely Tru Cal U (No. 20003), prior to the measurement of the parameters. Additionally, it was checked both before and during the examinations by means of two controllers of serum (Pars Azmoon Co., Iran) (16-18).

3.5. Superoxide Dismutase Activity Assessment

The measurement of SOD activity was accomplished using the Madesh method (ZellBio Co., Germany), involving the inhibition of the repercussion of tetrazolium (MTT) with O_2^-, generated of Pyrogallol (Sigma Co., USA), via superoxide dismutase enzyme. To this end, based on the

standard enzyme dilution (RX Co., UK), solutions were arranged in different concentrations by PBS (50 mM, pH = 7.4). Subsequently, 10 μL of the homogenized tissue was mixed with PBS (65 μL, pH = 7.4) and MTT (30 μL), in addition to pyrogallol (75 μL). The mixture was then incubated for five minutes at ambient temperature. After the addition of 0.75 μL dimethyl sulfoxide (DMSO) to the mixture, ELISA was used to determine the light absorption of the compound at a wavelength of 570 nm. Furthermore, the percentage of SOD-induced inhibition was estimated by the corresponding formula, according to the instructions of the kit. The inhibition percentage was fitted to the standard curve to determine the enzyme activity that was reported in unit per mg of tissue (19). The activity of SOD enzyme was measured by optical densities (OD) using Equation 2.

$$SOD\ Activity = \frac{OD\ (Control) - OD\ (Sample) \times 100}{OD\ (Control)} \quad (2)$$

3.6. Malondialdehyde Level Measurement

One of the byproducts of lipid peroxidation is malondialdehyde (MDA) with the molecular formula of $CH_2(CHO)_2$, which directly damages the cellular polyunsaturated fatty acids through its oxidative substances. The ultimate vitage of LP (Lipid Peroxidation) was measured by the estimation of malondialdehyde level by means of the Satoh method (ZellBio Co., Germany). The process was done through the mixture of TCA (trichloroacetic acid, 1.5 mL) with homogenized tissue (500 μL). After the centrifugation of the obtained solution for 10 minutes, 1.5 mL of the supernatant was removed and then was added to TBA (thiobarbituric acid, 2 mL, 0.67%).

The resultant solution steam was cooked into Bain-marie weld bowls for 30 minutes and then mixed with 2 mL 1-butanol. The mixture was then centrifuged at 4000 rpm for 15 minutes after a severe vortex. By means of a spectrophotometer, pink supernatant absorption was read at 532 nm (PG Co., U.K). The spectrophotometer was zeroed using 20% trichloroacetic acid containing 0.5% thiobarbituric acid at 532 nm. Subsequently, MDA concentration was estimated based on tetraethoxypropane -1, 1, 3, 3 as standard, and malondialdehyde level was estimated according to μM per mg tissue (20). The standard curve was drawn based on the standard MDA and wavelengths, measured by means of the spectrophotometer.

3.7. Glutathione Level Measurement

Glutathione (GSH) level in the tissue was estimated by means of the Tietze method (ZellBio Co., Germany). To this end, 10 μL of sulfosalicylic acid dehydrate-5 was added to the homogenized sample in a proper density, and centrifuged (4°C, 2000 rpm) during ten minutes. Subsequently, supernatant (100 μL) was mixed with NA_2HPO_4 (0.3 M, 820 μL). Then, mixed DTNB (0.4%) with sodium citrate (1%) and the absorption was read at 412 nm during five minutes. Furthermore, the glutathione level was measured according to μm per mg tissue (21), which was prepared at a density range of 25 - 200 μm.

3.8. Measurement of Catalase Activity

The measurement of catalase (CAT) activity was performed through the Aebi method (ZellBio Co., Germany) after hydrogen peroxide synthesis (240 nm). A certain volume of tissue extract was mixed with 0.01 mL ethanol and then subjected to incubation on ice for 30 minutes. The incubated mixture was then added with Triton (10%). The CAT activity was estimated using the resultant solution. Then, hydrogen peroxide (0.05 mL) was added to tissue extract at a proper volume Na_2HPO_4 (50 mM, pH = 7) led to the onset of reaction. The absorption rate was determined at 240 nm after three minutes (according to Unit/mg tissue) (13).

3.9. Statistical Analysis

The data were analyzed using the Kolmogorov-Smirnov test (K-S test), one-way ANOVA, and Tukey's test in SPSS Statistics for Windows version 16.0 (SPSS Inc., Chicago, ILL., USA). Furthermore, the mean and standard deviation were utilized to express the data. In order to eliminate bias in data, all devices were calibrated prior to the experimentation; moreover, three investigators were employed to examine the samples. In this study, the variables were measured by means of the numerical scale. The Kappa index was > 0.6, and P-value of < 0.05 was considered statistically significant.

4. Results

The results of the one-sample K-S test were indicative of the normal distribution of all data ($P > 0.05$). Consequently, data analysis was performed using the parametric tests of one-way ANOVA and post-hoc Tukey's test.

4.1. Mean of Biochemical Parameters

Table 1 and Figure 1 present the mean values of ALT, ALP, and AST serum levels, density of malondialdehyde, enzymes activity of superoxide dismutase and catalase, and thiol groups of hepatic tissue.

4.2. One-Way ANOVA Test

The one-way ANOVA and post-hoc Tukey's test were run to estimate the mean effect of one variable on several groups for each of the serum liver enzymes and oxidative markers (Table 2).

Table 1. Mean Value of Biochemical Parameters in Each Group

Parameters	Groups						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
ALP, U/L	343.14 ± 122.01	344 ± 141.75	361.28 ± 155.19	531.14 ± 18.64[a]	367.85 ± 88.45	361.85 ± 74.07	350 ± 97.24
AST, U/L	238.14 ± 21.04	270.57 ± 126.41	231 ± 201.14	443.85 ± 100.71[a]	262.57 ± 86.05	266.28 ± 99.94	219.71 ± 17.46
ALT, U/L	92 ± 17.72	95 ± 17.94	98.85 ± 32.66	155.14 ± 25.67[a]	100.42 ± 28.43	97.28 ± 12.06	93.71 ± 20.38
MDA, μM per mg tissue	0.50 ± 0.29	0.62 ± 0.48	0.59 ± 0.3	1.04 ± 0.21[a]	0.48 ± 0.19	0.45 ± 0.41	0.48 ± 0.22
SOD, Unit per mg tissue	1.73 ± 0.44	1.81 ± 0.07	1.83 ± 0.6	0.68 ± 0.16[a]	1.56 ± 0.62	1.87 ± 0.68	1.47 ± 0.27
CAT, Unit per mg tissue	7.25 ± 0.99	7.63 ± 2.69	7.74 ± 2.46	3.91 ± 0.42[a]	7.34 ± 2.03	7.51 ± 1.89	7.01 ± 1.38
GSH, μM per mg tissue	29.94 ± 8.11	21.39 ± 6.73	20.75 ± 13.01	4.81 ± 4.43[a]	28.23 ± 6.36	27.95 ± 7.17	22.11 ± 11.61

Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CAT, catalase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.
[a] Significant difference, compared to other groups (P < 0.05).

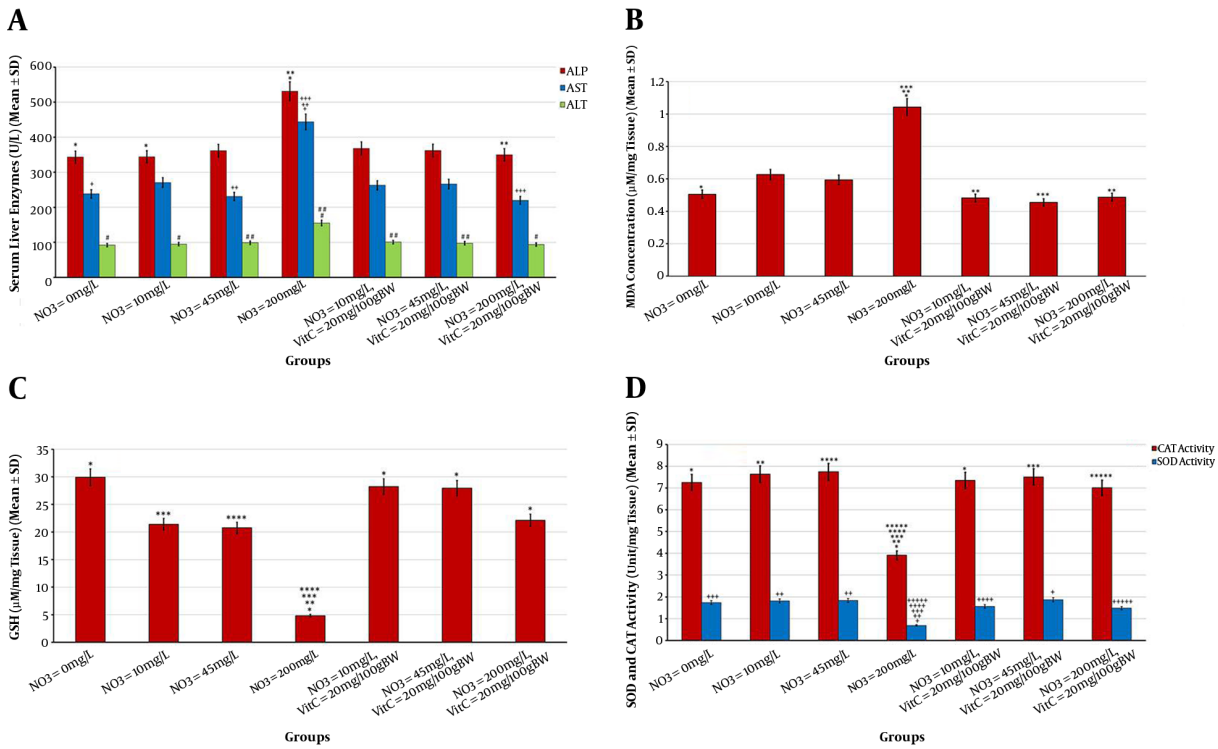

Figure 1. Comparison of nitrates concentration in drinking water and Vitamin C on liver enzymes and oxidative markers (Data are presented as Mean ± SD). A, Serume Levels of ALP, AST and ALT; (*P = 0.03, **P = 0.046; +P = 0.018, ++P = 0.013, +++P = 0.008; #P ≤ 0.001, ##P = 0.001); B, MDA Concentration of Liver Tissue; (*P = 0.44, **P = 0.03, ***P = 0.02); C, GSH Levels of Liver Tissue; (*P ≤ 0.001, **P = 0.009, ***P = 0.014, ****P = 0.021); D, SOD and CAT activities of Liver Tissue; (*P = 0.02, **P = 0.009, ***P = 0.013, ****P = 0.007, *****P = 0.047; +P ≤ 0.001, ++P = 0.001, +++P = 0.002, ++++P = 0.018, +++++P = 0.041).

5. Discussion

The reduction of nitrate to nitrite can result in carcinogenicity through the formation of N-nitroso compounds (22). Nitrites can be converted to highly reactive and biological radical nitric oxide in a large extent (23). Ruth et al. reported that the consumption of drinking water containing 1.3 g/L sodium nitrate during pregnancy and lactation reduces the erythropoietic progress, which slows down growth, resulting in mental retardation and mortality (24). Ozen et al. showed that the cytoplasm of the hepatocytes was reacted in a liver exposed to sodium nitrite using the immunohistochemical method (25). In a study con-

Table 2. Results of One-Way ANOVA Test for Each of Serum Liver Enzymes and Oxidative Markers

Parameters	Sum of Squares	df	Mean Square	F	P Value
ALP					
Between groups	190577.63	6	31762.93	2.7	0.026
Within groups	493102.85	42	11740.54		
Total	683680.49	48			
AST					
Between groups	245744.98	6	40957.49	3.38	0.008
Within groups	508358	42	12103.76		
Total	754102.98	48			
ALT					
Between groups	21195.1	6	3532.51	6.62	0.001
Within groups	22390.28	42	533.1		
Total	43585.38	48			
SOD					
Between groups	7.28	6	1.21	5.54	0.001
Within groups	9.19	42	0.21		
Total	16.48	48			
MDA					
Between groups	1.77	6	0.29	2.90	0.018
Within groups	4.27	42	0.1		
Total	6.04	48			
CAT					
Between groups	76.24	6	12.7	3.68	0.005
Within groups	144.89	42	3.45		
Total	221.13	48			
GSH					
Between groups	3041.63	6	506.94	6.73	0.001
Within groups	3162.95	42	75.3		
Total	6204.59	48			

Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CAT, catalase; df, the degree of freedom; F, statistics; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

ducted by Stokes et al., in 2009, it was reported that the consumption of sodium nitrite in drinking water increases the nitrites and nitrates levels in the plasma, heart, and liver (26). According to Raat et al., the consumption of water containing nitrates increases the concentration of nitrates and nitrites in the stomach and plasma (27). When the liver cells are damaged or destroyed, the cellular enzymes leak into the bloodstream; therefore, they can be measured through blood tests. The ALT and AST are two main liver enzymes. The AST is also present in the heart, muscle, kidneys, and brain. Accordingly, the serum AST levels increase in heart attacks or muscle damage. Regarding this, AST is not a specific indicator of liver damage. However, ALT is found almost exclusively in the liver. The simultaneous elevation of the ALT and AST level in the blood is indicative of a high probability of liver damage. The liver synthesizes the highest amount of ALP enzyme. Consequently, the high level of this enzyme can be caused by liver damage or other reasons. The enhancement of the alkaline phosphatase level can be suggestive of a problem in the bile duct. Bile duct disorders can be due to problems with the liver, gallbladder, or the connected tubes. The AST and ALT are logically sensitive indicators of liver damage or damage caused by various diseases. However, it should be emphasized that the above normal levels of liver enzymes should not be automatically correlated with liver damage. In this regard,

an increase in these enzymes can occur with muscle damage. The interpretation of AST and ALT depends on the overall clinical outcome of the patient. In addition, the exact level of liver enzymes does not correlate with the extent of liver problems and the diagnosis of the disease. As a result, the exact level of AST and ALT cannot be used to determine the severity of liver disease and its prediction and prognosis (28-35). In the present study, the increased levels of liver damage indicators (i.e., AST, ALP, ALT), in the group receiving a nitrate dose of 200 mg/L indicated hepatocellular damage is consistent with the findings of Ramesh et al. (36). Likewise, in the present study, the liver was observed to undergo some biochemical changes, such as the enhancement of the MDA level, and the reduction of SOD, CAT activities, and thiol groups at the nitrate dose of 200 mg/L. Nitrite not only creates a toxic mechanism, but it also activates several other mechanisms. Some of these mechanisms include the reduction of hemoglobin concentration and its resultant anemia, degeneration of the hepatic cells following anemia, damage to the liver lysosomal and microsomal membranes, DNA damage, and alteration of the extracellular homeostasis (e.g., the incidence of hypocalcemia) (37). The SOD facilitates the acceleration of anion superoxide conversion into molecular oxygen and hydrogen peroxide. Consequently, this enzyme plays a key role in the cellular mechanism and antioxidant defense. The CAT is present in the cells of aerobic and anaerobic organism. The function of this enzyme includes the decomposition of hydrogen peroxide into water and oxygen. This enzyme has a relatively high activity level with the highest and lowest concentrations in the liver and connective tissues, respectively. The antioxidant enzymes react to the average of free radicals, strengthen via several contaminants (e.g., nitrate); therefore, these can be regarded as environmental contaminants markers (38). Toxic aldehyde is the result of lipid peroxidation, and MDA is one of the most toxic forms of this compound. In general, MDA is a colorless compound and the final product of fatty oxide decomposition, which is currently regarded as an indicator of lipid peroxidation. Accordingly, the injury severity of cells unto oxidation can be determined by the measurement of this enzyme level (39). As our results indicated, MDA showed a significant elevation with 200 mg/L dose of nitrates ($P < 0.05$), indicating the damage to the liver tissues. Furthermore, the fourth group demonstrated a significant increase in MDA concentration with 200 mg/L dose of nitrates in comparison to the group one (control). The outcome was suggestive peroxidation of lipid leading to the formation of free toxic radicals, and consequently, hepatic tissue injury caused by oxidative stress. Moreover, the first group (i.e., control group) had no significant difference with the second and third groups regarding the MDA concentration. Based on our findings, doses of nitrates (10 - 45 mg per liter) were acceptable and led to nothing stress in the hepatic tissue. Vitamin C can lead to the reduction of oxidative stress damage to the hepatic tissue owing to its antioxidant property. Accordingly, the seventh group treated with 200 mg/L nitrates and Vitamin C showed a significant decrease in this regard ($P = 0.03$).

As our findings revealed, the biochemical indicators were significantly influenced by 200 mg/L nitrate in drinking water. In this respect, this nitrate dose elevated ALT, ALP, and AST in the blood serum, which is suggestive of hepatic malfunction (Figure 1). Daily intake of Vitamin C (20 mg per 100 g B.wt) could result in the prevention of the damage induced by high doses of nitrates. Usunomena et al. investigated the liver enzymes of a rat treated with dimethylamine hydrochloride and sodium nitrite. In the mentioned study, it was shown that the intrahepatic cell damage was caused by N-nitroso amine precursors, necrosis of the cells, and tissue damage (40). The results of the present study revealed that the hepatic tissue oxidative stress indicators and serum biochemical parameters were not adversely affected by doses of nitrates (10 - 45 mg per liter). Accordingly, these doses resulted in no hepatic malfunction. However, 200 mg per liter dose of nitrate significantly influenced the aforementioned markers and resulted in hepatic tissue injury and liver dysfunction by forming free radicals. Nonetheless, even at a nitrate dose of 200 mg/L, Vitamin C as an antioxidant, could prevent the hepatic damage. The current study did not involve the supplementary method (e.g., IHC; immunohistochemistry), which is one of the limitations of the present study. Therefore, it is suggested to perform further studies in this domain using the aforementioned technique.

Acknowledgments

This article was conducted in the Faculty of Medicine in Mashhad University of Medical Sciences, Mashhad, Iran. Hereby, the authors acknowledge their gratitude to the deputy of research and other researchers for their cooperation in this study.

Footnotes

Authors' Contribution: Mahdi Jalali, Mohammad Reza Nikravesh, Mohammad Soukhtanloo and Mahmoud Moghaddam Dorafshani: Study design, managing literature searches, the measurement of oxidative stress indicators and biochemical parameters assistance and data collection.

Ethical Considerations: Ethics Committee of Mashhad University of Medical Sciences with the code of IR.MUMS.fm.REC.1396.202 on 2017/07/12 (www.mums.ac.ir/research).

Funding/Support: The current study was funded by the research council president for Research Affairs of Mashhad University of Medical Sciences (research grant number: 951498).

References

1. Jensen FB, Gerber L, Hansen MN, Madsen SS. Metabolic fates and effects of nitrite in brown trout under normoxic and hypoxic conditions: Blood and tissue nitrite metabolism and interactions with branchial NOS, Na+/K+-ATPase and hsp70 expression. *J Exp Biol*. 2015;**218**(Pt 13):2015–22. doi: 10.1242/jeb.120394. [PubMed: 25908056].
2. Alimohammadi M, Latifi N, Nabizadeh R, Yaghmaeian K, Mahvi AH, Yousefi M, et al. Determination of nitrate concentration and its risk assessment in bottled water in Iran. *Data Brief*. 2018;**19**:2133–8. doi: 10.1016/j.dib.2018.06.110. [PubMed: 30229091]. [PubMed Central: PMC6141436].
3. van Grinsven HJ, Rabl A, de Kok TM. Estimation of incidence and social cost of colon cancer due to nitrate in drinking water in the EU: A tentative cost-benefit assessment. *Environ Health*. 2010;**9**:58. doi: 10.1186/1476-069X-9-58. [PubMed: 20925911]. [PubMed Central: PMC2973935].
4. Brody JG, Aschengrau A, McKelvey W, Swartz CH, Kennedy T, Rudel RA. Breast cancer risk and drinking water contaminated by wastewater: A case control study. *Environ Health*. 2006;**5**:28. doi: 10.1186/1476-069X-5-28. [PubMed: 17026759]. [PubMed Central: PMC1622744].
5. Cockburn A, Brambilla G, Fernandez ML, Arcella D, Bordajandi LR, Cottrill B, et al. Nitrite in feed: From animal health to human health. *Toxicol Appl Pharmacol*. 2013;**270**(3):209–17. doi: 10.1016/j.taap.2010.11.008. [PubMed: 21095201].
6. Halliwell B. Dietary polyphenols: Good, bad, or indifferent for your health? *Cardiovasc Res*. 2007;**73**(2):341–7. doi: 10.1016/j.cardiores.2006.10.004. [PubMed: 17141749].
7. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *Am J Clin Nutr*. 2009;**90**(1):1–10. doi: 10.3945/ajcn.2008.27131. [PubMed: 19439460].
8. Hongo T, Wakasa H, Yamazaki A. Synthesis and adsorption properties of nanosized Mg-Al layered double hydroxides with Cl-, NO3- or SO4 2- as interlayer anion. *Mater Sci Poland*. 2011;**29**(2):86–91. doi: 10.2478/s13536-011-0024-6.
9. Pikul KV, Bobyreva LE, Kotelevska TM, Pryimenko Ncapital O C, Tarasenko KV. Problems of nitrate water pollution in Poltava region. *Wiad Lek*. 2018;**71**(4):839–42. [PubMed: 30099420].
10. Mousavi SR, Balali-Mood M, Riahi-Zanjani B, Sadeghi M. Determination of cyanide and nitrate concentrations in drinking, irrigation, and wastewaters. *J Res Med Sci*. 2013;**18**(1):65–9. [PubMed: 23900450]. [PubMed Central: PMC3719231].
11. Rantanen PL, Mellin I, Keinanen-Toivola MM, Ahonen M, Vahala R. The seasonality of nitrite concentrations in a chloraminated drinking water distribution system. *Int J Environ Res Public Health*. 2018;**15**(8). doi: 10.3390/ijerph15081756. [PubMed: 30111761]. [PubMed Central: PMC6121643].
12. Zhang Z, Wang JJ, Ali A, DeLaune RD. Heavy metal distribution and water quality characterization of water bodies in Louisiana's Lake Pontchartrain Basin, USA. *Environ Monit Assess*. 2016;**188**(11):628. doi: 10.1007/s10661-016-5639-y. [PubMed: 27766564].
13. Aebi H. [13] Catalase in vitro. *Methods Enzymol*. 1984;**105**:121–6. doi: 10.1016/s0076-6879(84)05016-3.
14. Cheung CC, Zheng GJ, Li AM, Richardson BJ, Lam PK. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, Perna viridis. *Aquat Toxicol*. 2001;**52**(3-4):189–203. doi: 10.1016/S0166-445X(00)00145-4. [PubMed: 11239681].
15. Proskocil BJ, Sekhon HS, Clark JA, Lupo SL, Jia Y, Hull WM, et al. Vitamin C prevents the effects of prenatal nicotine on pulmonary function in newborn monkeys. *Am J Respir Crit Care Med*. 2005;**171**(9):1032–9. doi: 10.1164/rccm.200408-1029OC. [PubMed: 15709053].
16. Vozarova B, Stefan N, Lindsay RS, Saremi A, Pratley RE, Bogardus C, et al. High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. *Diabetes*. 2002;**51**(6):1889–95. doi: 10.2337/diabetes.51.6.1889. [PubMed: 12031978].
17. Binder TA, Goodson JM, Socransky SS. Gingival fluid levels of acid and alkaline phosphatase. *J Periodontal Res*. 1987;**22**(1):14–9. doi: 10.1111/j.1600-0765.1987.tb01534.x. [PubMed: 2950223].
18. Huwart L, Sempoux C, Salameh N, Jamart J, Annet L, Sinkus R, et al. Liver fibrosis: Noninvasive assessment with MR elastography versus aspartate aminotransferase-to-platelet ratio index. *Radiology*. 2007;**245**(2):458–66. doi: 10.1148/radiol.2452061673. [PubMed: 17940304].
19. Madesh M, Balasubramanian KA. Microtiter plate assay for superoxide dismutase using MTT reduction by superoxide. *Indian J Biochem Biophys*. 1998;**35**(3):184–8. [PubMed: 9803669].
20. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin Chim Acta*. 1978;**90**(1):37–43. doi: 10.1016/0009-8981(78)90081-5. [PubMed: 719890].
21. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem*. 1969;**27**(3):502–22. doi: 10.1016/0003-2697(69)90064-5. [PubMed: 4388022].
22. Rocha BS, Gago B, Barbosa RM, Cavaleiro C, Laranjinha J. Ethyl nitrite is produced in the human stomach from dietary nitrate and ethanol, releasing nitric oxide at physiological pH: Potential impact on gastric motility. *Free Radic Biol Med*. 2015;**82**:160–6. doi: 10.1016/j.freeradbiomed.2015.01.021. [PubMed: 25645954].
23. Ramezani Noroozani F, Ojinejad D, Ghorbani Ranjbary A. [Effects of sodium nitrite on liver enzymes and histological structure of liver in streptozotocin-induced diabetic rats]. *J Mazandaran Univ Med Sci*. 2017;**26**(144):171–9. Persian.
24. Ward MH, deKok TM, Levallois P, Brender J, Gulis G, Nolan BT, et al. Workgroup report: Drinking-water nitrate and health–recent findings and research needs. *Environ Health Perspect*. 2005;**113**(11):1607–14. doi: 10.1289/ehp.8043. [PubMed: 16263519]. [PubMed Central: PMC1310926].
25. Ozen H, Kamber U, Karaman M, Gul S, Atakisi E, Ozcan K, et al. Histopathologic, biochemical and genotoxic investigations on chronic sodium nitrite toxicity in mice. *Exp Toxicol Pathol*. 2014;**66**(8):367–75. doi: 10.1016/j.etp.2014.05.003. [PubMed: 24947405].
26. Stokes KY, Dugas TR, Tang Y, Garg H, Guidry E, Bryan NS. Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *Am J Physiol Heart Circ Physiol*. 2009;**296**(5):H1281–8. doi: 10.1152/ajpheart.01291.2008. [PubMed: 19252084].
27. Raat NJ, Noguchi AC, Liu VB, Raghavachari N, Liu D, Xu X, et al. Dietary nitrate and nitrite modulate blood and organ nitrite and the cellular ischemic stress response. *Free Radic Biol Med*. 2009;**47**(5):510–7. doi: 10.1016/j.freeradbiomed.2009.05.015. [PubMed: 19464364]. [PubMed Central: PMC2744499].
28. Hyder MA, Hasan M, Mohieldein AH. Comparative levels of ALT, AST, ALP and GGT in liver associated diseases. *Eur J experiment Biol*. 2013;**3**(2):280–4.
29. Lundberg JO, Carlstrom M, Weitzberg E. Metabolic effects of dietary nitrate in health and disease. *Cell Metab*. 2018;**28**(1):9–22. doi: 10.1016/j.cmet.2018.06.007. [PubMed: 29972800].
30. Gonzalez Delgado MF, Gonzalez Zamora A, Gonsebatt ME, Meza Mata E, Garcia Vargas GG, Calleros Rincon EY, et al. Subacute intoxication with sodium nitrate induces hematological and biochemical alterations and liver injury in male Wistar rats. *Ecotoxicol Environ Saf*. 2018;**166**:48–55. doi: 10.1016/j.ecoenv.2018.09.060. [PubMed: 30245293].
31. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hep-*

atology. 2003;**38**(2):518–26. doi: 10.1053/jhep.2003.50346. [PubMed: 12883497].
32. Lum G, Gambino SR. Serum gamma-glutamyl transpeptidase activity as an indicator of disease of liver, pancreas, or bone. *Clin Chem*. 1972;**18**(4):358–62. doi: 10.1016/j.freeradbiomed.2015.01.021. [PubMed: 5012259].
33. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology*. 2006;**44**(4):865–73. doi: 10.1002/hep.21327. [PubMed: 17006923].
34. Nannipieri M, Gonzales C, Baldi S, Posadas R, Williams K, Haffner SM, et al. Liver enzymes, the metabolic syndrome, and incident diabetes: The Mexico City diabetes study. *Diabetes Care*. 2005;**28**(7):1757–62. doi: 10.2337/diacare.28.7.1757. [PubMed: 15983331].
35. Nyblom H, Berggren U, Balldin J, Olsson R. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol Alcohol*. 2004;**39**(4):336–9. doi: 10.1093/alcalc/agh074. [PubMed: 15208167].
36. Ramesh B, Viswanathan P, Pugalendi KV. Protective effect of Umbelliferone on membranous fatty acid composition in streptozotocin-induced diabetic rats. *Eur J Pharmacol*. 2007;**566**(1-3):231–9. doi: 10.1016/j.ejphar.2007.03.045. [PubMed: 17477917].
37. Huertas M, Gisbert E, Rodriguez A, Cardona L, Williot P, Castello-Orvay F. Acute exposure of Siberian sturgeon (Acipenser baeri, Brandt) yearlings to nitrite: Median-lethal concentration (LC(50)) determination, haematological changes and nitrite accumulation in selected tissues. *Aquat Toxicol*. 2002;**57**(4):257–66. doi: 10.1016/S0166-445X(01)00207-7. [PubMed: 11932005].
38. Niyogi S, Biswas S, Sarker S, Datta AG. Antioxidant enzymes in brackishwater oyster, Saccostrea cucullata as potential biomarkers of polyaromatic hydrocarbon pollution in Hooghly Estuary (India): Seasonality and its consequences. *Sci Total Environ*. 2001;**281**(1-3):237–46. doi: 10.1016/S0048-9697(01)00850-6. [PubMed: 11778956].
39. Garcia YJ, Rodriguez-Malaver AJ, Penaloza N. Lipid peroxidation measurement by thiobarbituric acid assay in rat cerebellar slices. *J Neurosci Methods*. 2005;**144**(1):127–35. doi: 10.1016/j.jneumeth.2004.10.018. [PubMed: 15848246].
40. Usunomena U, Sunday JJ, Spencer N, Esosa US, Kingsley O, Emmanuel MN. Toxicity evaluation of the liver and in vitro metabolism in wistar rat on exposure to N-nitrosamine precursors. *Brit J Pharmacol Toxicol*. 2011;**2**(3):138–42.