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Evaluation of Oxidative Stress Biomarkers in the Renal Tissue of Rats and Serum Biochemical Parameters After the Administration of Nitrate in Drinking Water and Vitamin C: an Experimental Study

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Abstract

Background: Nitrate (NO_3^-) is one of the inorganic anions produced from the oxidation of nitrogen. The organic or inorganic nitrogen may act as a carcinogen depending on the reduction of nitrate to nitrite and the subsequent reactions of nitrite with other molecules, leading to the formation of N-nitroso compounds.
Objectives: This study aimed to determine the effects of nitrate and Vitamin C on oxidative stress in the renal tissue of rats.
Methods: This experimental study was conducted in Iran during the 2017 - 2018. The sample size was estimated to include 55 Wistar male rats using Morgan's table and Cochran's formula. In total, 49 rats were selected and divided into seven groups of: (1) NO_3 = 0 mg/L (control), (2) NO_3 = 10 mg/L, (3) NO_3 = 45 mg/L, (4) NO_3 = 200 mg/L, (5) NO_3 = 10 mg/L + Vitamin C 20 mg/100 g BW (Body Weight), (6) NO_3 = 45 mg/L + Vitamin C 20 mg/100 g BW, and (7) NO_3 = 200 mg/L + Vitamin C 20 mg/100 g BW. Blood samples were obtained to determine blood urea nitrogen (BUN) and creatinine. An autopsy was performed on the renal tissue to evaluate oxidative stress indicators including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH).
Results: In this research, the fourth group showed a significant increase in the level of creatinine (50.14 ± 2.6, 43.14 ± 1.21, P = 0.01) and BUN (0.72 ± 0.04, 0.57 ± 0.11, P = 0.003) compared to the control group. On the other hand, a significant increase was observed in the level of MDA in the fourth group compared to the control group (P = 0.01), whereas a significant reduction was found in the levels of CAT (P = 0.001), SOD (P = 0.02), and GSH (P = 0.02). In addition, the levels of creatinine and BUN significantly reduced in the seventh group compared to the fourth group ($P < 0.05$). Furthermore, the use of Vitamin C resulted in a significant reduction in MDA and an increase in SOD, CAT, and GSH in the seventh group compared to the fourth group ($P < 0.05$).
Conclusions: According to the results of the study, nitrate in drinking water and the prescription of Vitamin C had no significant effect in the presence of nitrate doses of 10 and 45 mg/L. However, a 200 mg/L dose of nitrate significantly affected BUN, serum creatinine, and oxidative stress indicators, causing the kidney disease.

Keywords: Biochemical, Drinking Water, Kidney, Nitrate, Oxidative Stress, Vitamin C, Rat

1. Background

Nitrate (NO_3^-) is one of the most essential elements for protein synthesis in plants and plays an important role in the nitrogen cycle. In general, nitrate can be found everywhere in the environment since it is produced through natural oxidation (1). In the metropolises, the municipal, industrial, animal, and plant wastes entailing organic nitrogen are disposed of in the landfills. In a phenomenon called ammonification, organic nitrogen is converted to ammonium ion (NH_4^+) through the activity of soil microorganisms. Despite the ability of soil to maintain this compound inside, another phenomenon known as nitrification occurs and leads to the conversion of a part of ammonium ion into nitrite (NO_2^-) and then nitrate (2).

The conventional water purification methods fail to remove nitrate due to the solubility of this ion in water. Therefore, advanced purification methods are required to reduce the soluble contaminant. On the other hand, in cities where wastewater is improperly disposed through injection wells, nitrate is constantly produced and released into groundwater (3, 4). Studies show that water has a very high level of nitrate in locations where sewage disposal is carried out traditionally through wet wells, as well as in industrial cities due to wastewater penetration

into groundwater (5). The organic or inorganic nitrogen may act as a carcinogen depending on the reduction of nitrate to nitrite and the subsequent reactions of nitrite with other molecules, especially secondary amines, amides, and carbamates, leading to the formation of N-nitroso compounds (6).

The kidney is responsible for the regulation of the volume and composition of body fluids, as well as the removal of metabolic wastes from the body. The glomerular capillary wall consists of two layers of endothelial cells and podocytes, isolated by an extracellular matrix, known as the glomerular basement membrane. This thick membrane is the most important barrier in kidney filtration, which separates the vasculature from the urine space (7-11). Given the role of the kidneys in the regulation of hemostasis in body fluids, reports have shown that factors, such as malnutrition, disturb the development of the fetus and its subsequent generations (12-14).

Generally, when the creatures are exposed to contaminants, they start certain activities to metabolize, directly purify, and minimize any cellular damage caused by the contaminants. Such enzymatic mechanisms should be considered as the biological markers of exposure to contaminants (15, 16). Given the life-threatening nature of the oxidative damage, the body uses defensive mechanisms to protect against oxidation. The antioxidant enzymes are the most effective routes for the removal of harmful toxic radicals (17). Changes in the activities of antioxidant enzymes (e.g., SOD, CAT) have been extensively evaluated to assess the possibility of their application as a biological indicator of organic pollution (18). With this background in mind, the present study aimed to investigate the possibility of tissue damage caused by nitrate in drinking water using oxidative stress indicators.

2. Objectives

This study aimed to determine the biomarkers of oxidative stress in the renal tissues of rats and evaluate serum biochemical parameters after the administration of nitrate and Vitamin C in drinking water.

3. Methods

3.1. Treatment of Animals and Experimental Design

This experimental study was conducted in Iran from 2017 - 2018. A total of 55 male Wistar rats with the body weight of 150 - 250 g were obtained from the standard animal House of Mashhad University of Medical Sciences, Mashhad, Iran, and kept under a standard condition (22 ± 1°C, under a 12:12 light-dark cycle and 60% humidity). In this research, the animals had free access to water and food during the study. The sample size was estimated according to Morgan's table, which is one of the most common methods in this regard. This table was developed by Robert Krejcie and Daryle Morgan, where the sample size is calculated based on various sizes of populations using the Cochran's formula ($n = Nz^2pq/Nd^2 + z^2pq$; n = Sample size, N = Population size, z = 1.96, p = q = 0.5, d = Error value = 0.05). Therefore, 49 male adult rats were selected through simple random sampling. In the next stage, the rats were divided into seven groups, each of which contained seven cases, as follows (note: rats of each group were kept in separate cages):

1. Group one (control): Receiving distilled water (n = 7),
2. Group two: Receiving distilled water containing 10 mg/L nitrate (n = 7),
3. Group three: Receiving distilled water containing 45 mg/L nitrate (n = 7),
4. Group four: Receiving distilled water containing 200 mg/L nitrate (n = 7),
5. Group five: Receiving distilled water containing 10 mg/L nitrate, along with Vitamin C (20 mg per 100 g BW on a daily basis) (n = 7),
6. Group six: Receiving distilled water containing 45 mg/L nitrate, along with Vitamin C (20 mg per 100 g BW on a daily basis) (n = 7), and
7. Group seven: Receiving distilled water containing 200 mg/L nitrate, along with Vitamin C (20 mg per 100 g BW on a daily basis) (n = 7)

The experiments were carried out 91 days after performing the animal interventions. This duration was determined based on the previous studies. It is noteworthy that the research was confirmed by the Ethics Committee of Mashhad University of Medical Sciences with the code of IR.MUMS.fm.REC.1396.202 on 2017/07/12.

3.2. Blood Sampling

For the purpose of the study, 5 mL of blood sample was obtained from the heart of each rat under deep anesthesia (100 mg/kg ketamine). In order to separate the serum, the blood sample was centrifuged at 3000 rpm (revolutions per minute) for 12 minutes, followed by immediate testing of the samples.

3.3. Homogenization of Tissue

The kidneys were carefully dissected and removed from the body and immediately weighed after being washed with cold and dry saline solution. In the next step, about 150 mg of the tissue, along with 1.5 mL phosphate buffer, was homogenized by a homogenizer (IKA T18, Basic ULTRA TURRAX, USA) for two minutes at 5000 rpm. The obtained solution was then centrifuged. It should be noted that all of the stages were performed at 4°C (refrigerated

centrifuge) to prevent the destruction of enzymes and proteins. After the centrifuging of the samples, the supernatant was separated from the rest of the solution and used for the evaluation of the biochemical indicators.

3.4. Measurement of Serum Biochemical Parameters

The biochemical parameters of BUN and creatinine were evaluated using an autoanalyzer (Biotecnica Instruments, BT 3000 plus, code of UMDNS: 20821-16299, BIOTECNICA Co., Italy) and laboratory kits (Pars Azmoon Co., Iran). In this stage, urea and creatinine levels were measured by the enzymatic method of urease and the modified Jaffe method, respectively. In order to ensure the accuracy of the results, the biochemistry analyzer was calibrated by serum calibrator of Tru Cal U with the number of 20003 before measuring the parameters. In addition, it was tested before and during the examinations using the serum controllers of Tru Lap P and Tru Lab N (made by Pars Azmoon Co., Iran) with the numbers of 301005 and 300005, respectively (19).

3.5. Estimation of MDA Level

MDA with the molecular formula of $CH_2(CHO)_2$ is a product of lipid peroxidation produced by direct damage to cellular polyunsaturated fatty acids (PUFA) due to oxidative agents. In order to determine the final product of lipid peroxidation, the amount of MDA was estimated using the Satoh method. This reaction was performed at the pH of 2 - 3 by adding 1.5 mL 10% trichloroacetic acid (TCA) to 500-μL homogenized tissue. The resultant mixture was centrifuged for 10 minutes. In the next step, after the removal of 1.5 mL of the supernatant, 2 mL of 0.67% thiobarbituric acid (TBA) was added. The final product was boiled in a water bath for 30 minutes. Afterward, 2 mL of 1-butanol was added to the solution and it was centrifuged at 4000 rpm for 15 minutes after a severe vortex. The absorption of the pink supernatant was read at the wavelength of 532 nm using a spectrophotometer (T60 UV-Visible PG). In addition, 20% TCA containing 0.5% TBA was exploited to zero out the spectrophotometer at 532 nm. The concentration of MDA was determined using tetraethoxypropane as the standard agent, and the density of MDA was calculated and expressed as μM/mg (20). For plotting the standard curve, the MDA standard solution was prepared and the wavelengths were determined using the spectrophotometer. Afterward, 0.5 mL of the standard solution was taken at the concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 μM, followed by adding 3 mL of 1% phosphoric acid solution. The rest of the stage was similar to the previous one.

3.6. Evaluation of SOD Enzyme Activity

The activity of SOD enzyme was estimated based on the Madesh's method, in which the reaction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with anion superoxide, produced from Pyrogallol, is inhibited by the SOD enzyme. First, various concentrations of the standard solution of enzyme, which was purchased from Randox Co. of UK, were prepared with 50 mM phosphate buffer at pH of 7.4 and the standard curve was drawn. After that, 65 μL of phosphate-saline buffer with the pH of 7.4 and 30 μL of MTT (1.25 mM), along with 75 μL of Pyrogalum (100 μM), were mixed with 10 μL of the homogenized tissue and incubated for 5 minutes at the room temperature. In the next stage, 0.75 μL of dimethyl sulfoxide (DMSO) was added to the mixture and the light absorption of the compound was read by ELISA at a wavelength of 570 nm. In order to obtain the percent inhibition induced by SOD enzyme, the corresponding formula was used based on the instructions of the kit manufacturer. The enzyme activity was obtained through fitting the percent inhibition to the standard curve and the activity was reported according to the international unit per mg of the tissue (21). In this regard, the activity of SOD enzyme was calculated as the optical density of the control minus the light density of the sample divided by the density of the control multiplied by 100.

3.7. Evaluation of CAT Enzyme Activity

The activity of CAT enzyme was measured using the Aebi's method and following the synthesis of H_2O_2 at a wavelength of 240 nm. In this regard, a proper amount of ethanol (0.01 mL) was added to a certain amount of tissue extract and incubated on ice for 30 minutes. After that, 10% Triton X-100 with the final concentration of 1% was added to the mixture. The obtained solution was used to estimate the activity of the enzyme. The reaction was initiated by adding 0.05 mL H_2O_2 (30 mmol) to an appropriate amount of the tissue extract in 50 mM sodium phosphate buffer with the pH of 7. Subsequently, the absorption rate was read after 3 minutes at the wavelength of 240 nm and special activity was estimated in unit per mg. One unit of the CAT activity was equal to 1 μmol of H_2O_2 decomposed in 1 minute (15).

3.8. Estimation of GSH Level

The estimation of the GSH level in the tissue was carried out using the Tietze's method. To this end, a proper density of the homogenized sample was mixed with 10 μL of Sulfosalicylic acid dehydrate-5 and then centrifuged at 4°C at 2000 rpm for 10 minutes. Afterwards, 100 μL of the

supernatant was taken and added to 820 μL of 0.3 M disodium phosphate (Na_2HPO_4). In the next step, 90 μL of 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) 0.4% started reaction in 1% sodium citrate. Absorption changes at the wavelength of 412 nm were read after 5 minutes. In addition, the standard curve of GSH was plotted in μm/mg using 1 mg/mL GSH solution (22). The standard GSH solution was prepared at the densities of 25 - 200 μm. The name, manufacturing company, and country of the applied materials, as well as the employed tools of the research, are presented in Table 1.

3.9. Data Analysis

Data analysis was performed by SPSS Statistical Software (version 16.0, SPSS Inc. Chicago, ILL, USA) using Kolmogorov-Smirnov test (to determine the normal distribution of the data), one-way ANOVA, and Tukey's test. All devices were calibrated to generate unbiased data. The samples were examined by three investigators. In this study, a numerical scale was used to measure the variables. The Kappa index was greater than 0.6. The data were presented as means and standard deviation (Mean $\pm$ SD). P-value of less than 0.05 was considered statistically significant.

4. Results

First, the one-sample Kolmogorov–Smirnov test was performed on all data, which confirmed the normal distribution of the data ($P > 0.05$). Therefore, the parametric tests of ANOVA and Tukey's test were applied for the analysis of the data.

4.1. Mean Biochemical Parameters

The means and standard deviation of the data obtained from the evaluation of the relative weight of the kidneys, level of the BUN, level of serum creatinine, MDA concentration, SOD activity, CAT enzyme activity, and GSH level in the kidney tissues are tabulated in Table 2 for each research group.

4.2. Results of One-Way ANOVA and Tukey's Post Hoc Test

The mean effect of one variable on several groups was evaluated by ANOVA for each biochemical parameter for all groups (Table 3). Furthermore, the results of the comparison of the groups, including the sum of squares of treatments, the degree of freedom, the mean square of treatments, F statistic, and level of significance are demonstrated in Table 3. The significance level of below 5% is indicative of a significant difference between the mean of the groups. However, since this test fails to independently determine the different means, the Tukey's post hoc test was applied, as well. Given the fact that the source of changes (variances) is divided into two inter-group and intra-group categories in ANOVA, this test evaluates the level of distribution.

4.3. Data Analysis of the Relative Weight of Kidneys

As observed in Table 3, the results of ANOVA indicated no significant difference between the groups in terms of the relative weight of the kidneys ($P = 0.801$). Therefore, it was concluded that nitrate in drinking water had no impact on this value. Therefore, there was no need to implement the Tukey's post hoc test.

4.4. Data Analysis of Serum Creatinine Level

The results of ANOVA demonstrated a significant difference between the groups in terms of serum creatinine level ($P = 0.002$) (Table 3). According to the Tukey's post hoc test, the fourth group (NO_3 = 200 mg/L) showed a significant difference with the first (NO_3 = 0 mg/L), second (NO_3 = 10 mg/L), fifth (NO_3 = 10 mg/L + Vitamin C 20 mg/100 gBW), and sixth (NO_3 = 45 mg/L + Vitamin C 20 mg/100 gBW) groups in this regard ($P = 0.01$). In addition, a significant difference was found between the fourth and seventh groups considering their serum creatinine level (NO_3 = 200 mg/L + Vitamin C 20 mg/100 gBW) ($P = 0.004$) (Figure 1). According to these results, 200 mg/L nitrate significantly damaged the kidney tissue and caused renal malfunction. The consumption of Vitamin C in the seventh group resulted in a significant reduction in the level of creatinine, compared to the fourth group ($P = 0.004$). However, the control group showed no statistically significant difference with the fifth, sixth, and seventh groups in terms of this variable ($P > 0.05$).

4.5. Data Analysis on the Level of the BUN

In the ANOVA test, a significant difference was observed between the groups in terms of the BUN level ($P = 0.002$) (Table 3). In addition, the results of the Tukey's test demonstrated a significant difference among the first, fourth, and fifth groups ($P = 0.003$), among the second, fourth, and seventh groups ($P = 0.006$), between the third and fourth groups ($P = 0.003$), and between the fourth and sixth groups ($P = 0.02$) in this respect (Figure 2). These results clearly demonstrated a damage to the kidney function in a way that the level of BUN significantly increased at a nitrate dose of 200 mg/L in drinking water. On the other hand, the nitrate doses of 10 and 45 mg/L resulted in no significant difference between the control and other groups in this regard. According to the results, the consumption of Vitamin C significantly decreased BUN in the seventh group, compared to the fourth group ($P = 0.006$). Meanwhile, no significant difference was observed between the

Table 1. Name of the Materials, Companies, and Manufacturing Countries

Name of Materials	Company	Manufacturing Country
Sodium nitrate	Merck	Germany
Ascorbic acid (Vitamin C)	Merck	Germany
Hydrogen peroxide	Merck	Germany
Super Oxide dismutase assay kit (SOD)	ZellBio GmbH	Germany
Catalase assay kit	ZellBio GmbH	Germany
Glutathione assay kit	ZellBio GmbH	Germany
Malondialdehyde assay kit	ZellBio GmbH	Germany
Urea (UV) assay kit	Pars Azmoon	Iran
Creatinine assay kit	Pars Azmoon	Iran
Spectrophotometer	PG INSTRUMENT	United Kingdom
Pyrogallol	Merck	Germany
Ketamine	Sigma	USA
Alcohol 100°	Sina	Iran

Table 2. Mean and Standard Deviation of Biochemical Parameters Separated by Groups (Mean ± SD)

Parameters	(Groups, n = 7)						
	Distilled Water NO_3 = 0 mg/L	**Distilled Water NO_3 = 10 mg/L**	**Distilled Water NO_3 = 45 mg/L**	**Distilled Water NO_3 = 200 mg/L**	**NO_3 = 10 mg/L VitC = 20 mg/100 gBW**	**NO_3 = 45 mg/L VitC = 20 mg/100 gBW**	**NO_3 = 200 mg/L VitC = 20 mg/100 gBW**
Relative weight of the kidneys (%)	1.4484 ± 0.35888	1.4636 ± 0.25413	1.3668 ± 0.16653	1.4037 ± 0.23446	1.5736 ± 0.23084	1.4593 ± 0.21612	1.5229 ± 0.29931
BUN (mg/dL)	43.14286 ± 1.214986	43.57143 ± 4.157609	44.57143 ± 4.353433	50.14286 ± 2.609506	43.14286 ± 2.794553	44.42857 ± 3.644957	43.57143 ± 2.225395
Creatinine (mg/dL)	0.57143 ± 0.111270	0.57143 ± 0.048795	0.62857 ± 0.048795	0.72857 ± 0.048795	0.57143 ± 0.075593	0.57143 ± 0.075593	0.55714 ± 0.113389
MDA (μM/mg tissue)	0.34408 ± 0.119252	0.37734 ± 0.195365	0.39974 ± 0.187404	0.76355 ± 0.308301	0.33215 ± 0.170394	0.39859 ± 0.267427	0.38883 ± 0.179610
SOD (U/mg tissue)	1.69118 ± 0.329638	1.69297 ± 0.272461	1.69955 ± 0.504150	0.89341 ± 0.257566	1.71040 ± 0.201624	1.73027 ± 0.173778	1.66432 ± 0.867215
CAT (U/mg tissue)	5.71261 ± 0.503055	5.63435 ± 1.220651	5.55986 ± 1.579212	3.52623 ± 0.717531	5.72424 ± 0.483893	5.30885 ± 0.712150	6.02951 ± 0.308050
GSH (μM/mg tissue)	14.45612 ± 5.284487	11.25420 ± 5.371021	11.08973 ± 9.020065	0.98795 ± 0.507490	11.17906 ± 11.661067	15.04895 ± 7.069243	17.26707 ± 6.955061

Abbreviations: BUN, Blood urea nitrogen; CAT, catalase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

control group and the fifth, sixth, and seventh groups in terms of this variable (P > 0.05).

4.6. Data Analysis on the Level of MDA

According to the results of ANOVA, a significant difference was observed between the groups regarding the level of MDA (P = 0.01) (Table 3). Moreover, the Tukey's test indicated a significant difference between the fourth and fifth groups (P = 0.008), between the first and fourth groups (P = 0.01), between the second and fourth groups (P = 0.02), and among the third, fourth, sixth, and seventh groups (P = 0.03) in terms of the MDA level (Figure 3). These results demonstrated the effect of 200 mg/L nitrate in drinking water on the enhancement of lipid peroxidation in the kidney tissues. In addition, the results revealed that the consumption of Vitamin C in the seventh group significantly decreased the level of lipid peroxidation (P = 0.03), neutralizing the effect of 200 mg/L nitrate through its antioxidant properties. Nonetheless, the consumption of Vitamin C demonstrated no significant difference between the control group and the fifth, sixth, and seventh groups in this regard (P > 0.05).

Table 3. One - Way ANOVA Test Results

Parameters	Sum of Squares	df	Mean Square	F	P Value
Relative weight of the kidneys				0.506	0.801
Between groups	0.202	6	0.034		
Within groups	2.794	42	0.067		
Total	2.996	48			
Creatinine				4.185	0.002
Between groups	0.157	6	0.026		
Within groups	0.263	42	0.006		
Total	0.420	48			
BUN				4.293	0.002
Between groups	259.673	6	43.279		
Within groups	423.429	42	10.082		
Total	683.102	48			
SOD				3.432	0.008
Between groups	3.902	6	0.650		
Within groups	7.958	42	0.189		
Total	11.860	48			
MDA				3.485	0.007
Between groups	0.942	6	0.157		
Within groups	1.892	42	0.045		
Total	2.834	48			
CAT				6.117	0.000
Between groups	29.300	6	4.883		
Within groups	33.528	42	0.798		
Total	62.828	48			
GSH				3.623	0.006
Between groups	1157.381	6	192.897		
Within groups	2236.322	42	53.246		
Total	3393.703	48			

Abbreviations: BUN, Blood urea nitrogen; CAT, catalase; df, Degrees of freedom; F, statistics; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

4.7. Data Analysis on the Activities of SOD and CAT Enzymes

According to the ANOVA test, a significant difference was detected between the groups in terms of the mean level of SOD enzyme activity (P = 0.000) (Table 3). In addition, the Tukey's test demonstrated a significant difference among the first, fourth, and seventh groups (P = 0.01), among the second, fourth, and fifth groups (P = 0.02), between the third and fourth groups (P = 0.04), and between the fourth and sixth groups (P = 0.000) regarding this variable (Figure 4). Regarding the activity of CAT enzyme, the ANOVA test indicated a significant difference among the groups (P = 0.000). Moreover, the Tukey's test revealed a significant difference between the fourth and sixth groups (P = 0.000), between the third and fourth groups (P = 0.001), among the first, second, and fourth groups (P = 0.002), between the fourth and fifth groups (P = 0.003), and between the fourth and seventh groups (P = 0.01) (Figure 4). The results clearly demonstrated a significant decrease in the activities of SOD and CAT enzymes at the nitrate dose of 200 mg/L in drinking water, which was indicative of damages to the kidney tissues caused by oxidative stress (P < 0.05). However, the study groups showed no significant difference with the control group in this respect at the nitrate doses of 10 and 45 mg/L (P > 0.05). Furthermore, the consumption of Vitamin C significantly increased the activities of SOD and CAT enzymes in the seventh group,

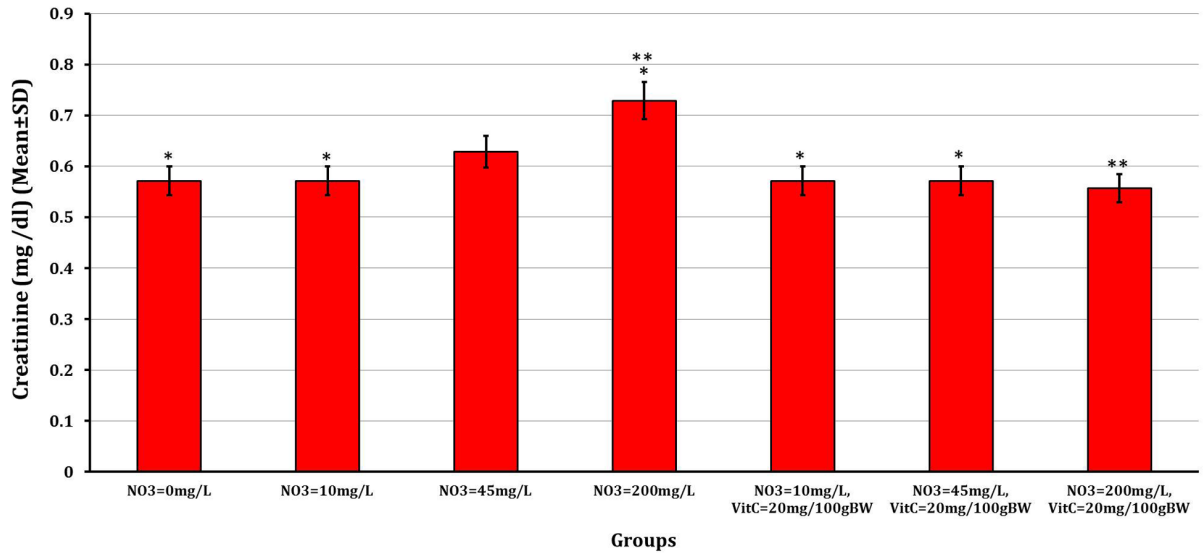

Figure 1. Comparison of nitrate concentration in drinking water and mean of serum creatinine. *A significant difference compared to other groups, *P = 0.01, **P = 0.004; data are presented as Mean ± SD.

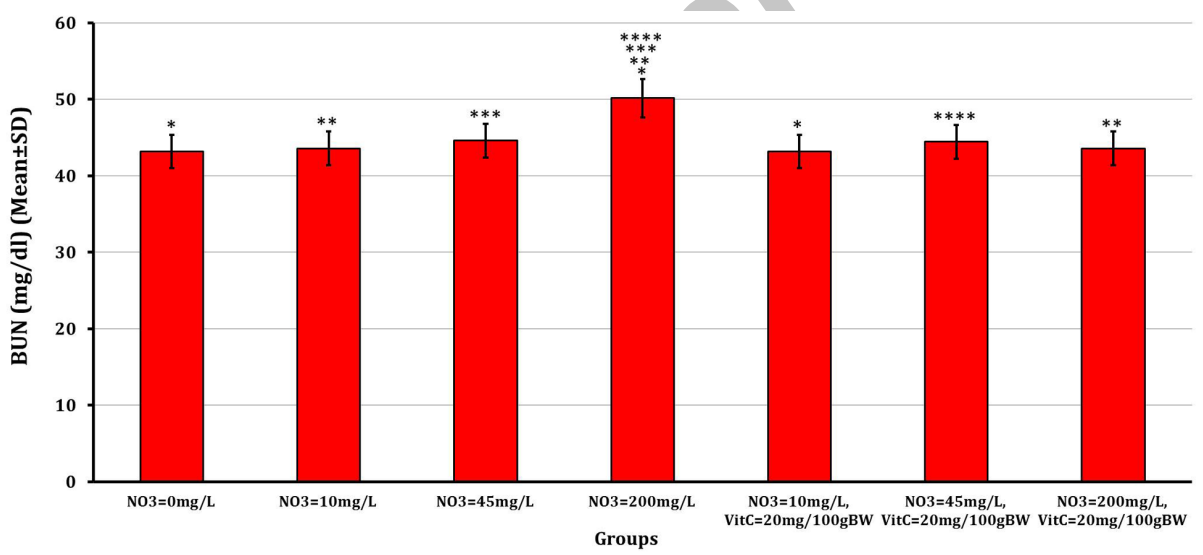

Figure 2. Comparison of nitrate concentration in drinking water and mean of serum BUN. *A significant difference compared to other groups, *P = 0.003, **P = 0.006, ***P = 0.03, ****P = 0.02; data are presented as Mean ± SD.

compared to those in the fourth group (P < 0.05). Nonetheless, the fifth (10 mg/L nitrate), sixth (45 mg/L nitrate), and seventh groups, which received nitrate along with Vitamin C, showed no significant difference with the control group (P > 0.05).

4.8. Data Analysis on the Level of GSH

The results of the ANOVA test showed a significant difference among the study groups in terms of the level of thiol (P = 0.006) (Table 3). According to the Tukey's post hoc test, a significant difference was detected between the first and fourth groups (P = 0.02), between the fourth and sixth groups (P = 0.01), and between the fourth and seventh groups (P = 0.003) in terms of this variable (Figure 5).

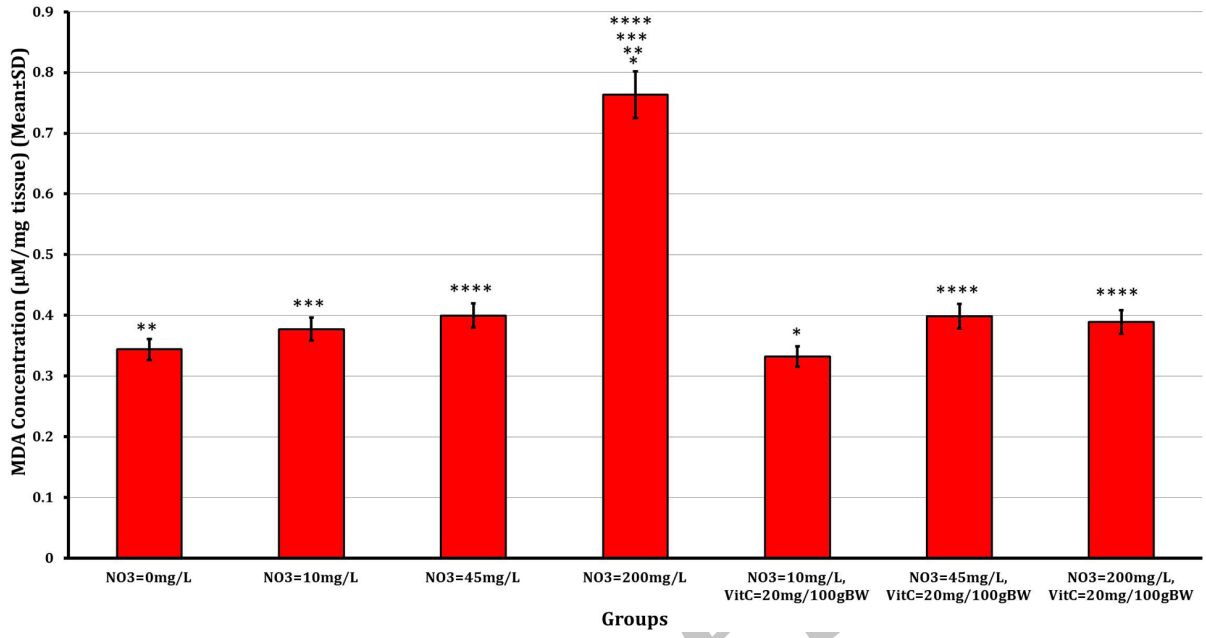

Figure 3. Comparison of nitrate concentration in drinking water and mean of MDA concentration. *A significant difference compared to other groups, *P = 0.008, **P = 0.01, ***P = 0.02, ****P = 0.03; data are presented as Mean ± SD.

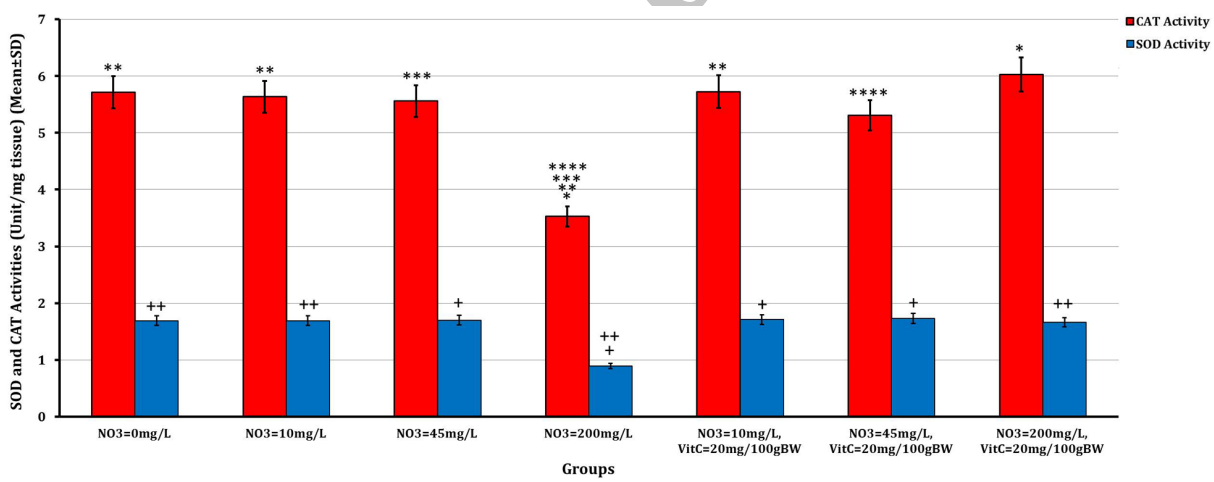

Figure 4. Comparison of nitrate concentration in drinking water and mean of SOD and CAT activities. *A significant difference compared to other groups, *P = 0.000, **P = 0.001, ***P = 0.002, ****P = 0.009; + A significant difference compared to other groups, + P = 0.01, ++ P = 0.02; data are presented as Mean ± SD.

The consumption of Vitamin C significantly increased the level of thiol in the seventh group, compared to the fourth group (P = 0.003). Moreover, the results were indicative of no significant difference in this respect between the control group and the fifth, sixth, and seventh groups using Vitamin C (P > 0.05).

5. Discussion

The organic or inorganic nitrogen may act as a carcinogen depending on the reduction of nitrate to nitrite and the subsequent reaction of nitrite with other molecules, especially the amines of the second type, amides, and carbamates, leading to the formation of N-nitroso compounds. According to several studies performed in Colombia, the

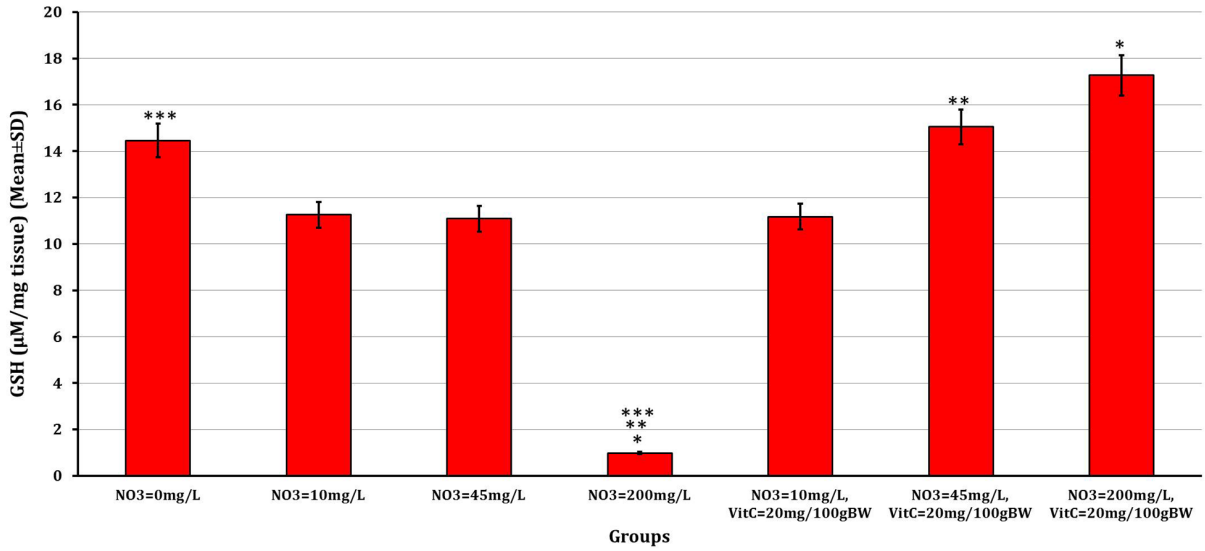

Figure 5. Comparison of nitrate concentration in drinking water and mean of GSH. *A significant difference compared to other groups, *P = 0.003, **P = 0.013, ***P = 0.02; data are presented as Mean ± SD.

incidence of gastric cancer is significantly associated with the concentration of nitrate in the drinking water collected from wells (6, 23). However, the epidemiologic studies conducted in other parts of the world have shown no definitive relationship in this regard. In a study conducted in Germany on a population exposed to high nitrate levels in drinking water, no significant relationship was observed between nitrate concentration and increased brain cancerous tumors (24). On the other hand, in another research performed at the University of Nebraska, Lincoln, USA, a significant relationship was reported between nitrate concentration in water and increased prevalence of Non-Hodgkin's lymphoma (25). The results of the present study demonstrated that 200 mg/L nitrate in drinking water significantly affected the biochemical indicators. Accordingly, this dose of nitrate increased the level of BUN and creatinine in the blood serum. This elevation is indicative of a renal malfunction. Moreover, it leads to the release of free toxic radicals in the kidney tissue through chemical reactions, thereby changing the oxidative stress biomarkers. This nitrate dose also resulted in the significant enhancement of MDA level ($P < 0.05$) in the kidney tissue, followed by the reduction of antioxidant enzymes of SOD and CAT, as well as thiol groups, in the tissues (Figures 1-5).

The SOD is an enzyme that accelerates the conversion of anion superoxide into molecular oxygen and hydrogen peroxide. The CAT is an enzyme that optionally exists in the cells of aerobic and anaerobic creatures. The stimulation of antioxidant enzymes in response to the production of intermediate oxy-radicals is intensified by some pollutants such as nitrates and they are considered as the biomarkers of environmental pollutants (26).

The lipid peroxidation results in the production of toxic aldehyde, one of the most toxic versions of which is MDA. Therefore, the estimation of the MDA level can facilitate the determination of the extent of damage to the cell lymph nodes against oxidation (27). In the present study, the level of MDA significantly increased at the nitrate dose of 200 mg/L ($P < 0.05$), which was indicative of damage to the kidney tissues. As a result, the level of nitrate can be considered as a valid indicator for the evaluation of oxidative stress in samples (28-31). The highly reactive oxygen species are eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. One of the important enzymes in this field is SOD (32). In a study conducted by Carlstrom at the Stockholm University in Sweden, nitrate and nitric oxide inorganic compounds were concluded to protect the body against renal and cardiovascular diseases (33). In addition, Erdogan in a study performed at the Istanbul University in Turkey demonstrated that oxidative stress was not the main threat to the health of individuals with chronic renal failure. An increase in the antioxidant capacity may occur inside and outside the serum to prevent any radical damage in patients with chronic renal failure. In addition, the increased amount of nitric oxide, especially in hemodialysis patients, may also contribute to the antioxidant effect (34).

The mechanism of Vitamin C function in inhibiting ox-

idative stress is determined with three indices, including nitrite level, MDA level, and SOD activity in the tissue. In the current research, a significant increase was observed in the concentration of MDA in the fourth group at 200 mg/L nitrate, compared to the control group. This finding was indicative of the peroxidation of lipids, followed by the production of free toxic radicals and damage by oxidative stress to the kidney tissue (Figure 3). Moreover, no significant difference was observed between the first (i.e., control group) and the second and third groups in terms of MDA concentration ($P > 0.05$). According to these results, 10 and 45 mg/L doses of nitrate are acceptable and cause no stress in the kidney tissue. With regard to the antioxidant property of Vitamin C, the consumption of this compound can reduce damage by oxidative stress to the kidney tissue. Accordingly, a significant decrease was observed in the seventh group in this regard ($P = 0.03$). Based on these findings, the daily consumption of 20 mg Vitamin C per 100 g body weight of rats prevented the stress damages caused by high doses of nitrate.

In the present study, a significant decrease was observed in the oxidant activities of SOD and CAT in the fourth group receiving 200 mg/L nitrate ($P < 0.05$). However, the consumption of Vitamin C significantly increased the activities of CAT and SOD enzymes ($P = 0.001$). At nitrate doses of 10 and 45 mg/L, the activities of these enzymes remained at the same level as that of the control group (Figure 4). Therefore, it could be concluded that 200 mg/L nitrate significantly damages the kidney tissue. The increased concentration of MDA and decreased activity of SOD and CAT enzymes in the fourth group led to a significant reduction in the level of thiol groups ($P = 0.02$), which confirmed stress damage to the kidney tissue. The consumption of the desired dose of Vitamin C increased the level of thiol groups to the same level as that of the control group, preventing more damage to the kidney tissues (Figure 5).

In a research carried out by Sanchez at the University of Mexico City in Mexico, it was concluded that minor hyperuricemia caused oxidative stress in the kidney tissues, leading to systemic hypotension and renal disorders due to increased uric acid. In addition, these researchers marked that the elimination of superoxide anions reduced the side effects of increased uric acid in the blood (35). In another study performed by Arangno at the Turin University in Italy, it was demonstrated that ischemia leads to oxidative stress in the kidney tissue, damaging the proximal organs of the kidney (36), which is in congruence with our findings. One of the major weakness of the current research was the non-unemployment of complementary techniques, such as immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR), which can significantly contribute to this field. Consequently, future studies are recommended to investigate the same issue using these methods.

According to the results of the current study, nitrate doses of 10 and 45 mg/L in drinking water had no adverse effects on the oxidative stress indicators in kidney tissues and the biochemical parameters of the serum. Therefore, no malfunction was observed in the kidney. On the other hand, a nitrate dose of 200 mg/L strongly affected the kidney oxidative stress indicators and serum biochemical parameters; moreover, it caused kidney tissue damage and renal dysfunction by producing free radicals. The consumption of Vitamin C as an antioxidant could prevent the kidney damage even at a nitrate dose of 200 mg/L.

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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.

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