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Protective Effect of Vitamin C on Protein Expression of Type-IV Collagen Following the Consumption of Nitrate-Containing Drinking Water in Rat Kidney

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Abstract

Background: Evidence shows that chemical fertilizers used for agricultural purposes have high levels of nitrate. These agricultural products consumed by livestock are the most important sources of nitrate. Type-IV collagen, found primarily in the base membrane, is significantly vital for the performance of glomerular base membrane in the kidney.
Objectives: The present study aimed to investigate the effect of nitrate and vitamin C on the expression of type-IV collagen in rat kidney.
Methods: This empirical research was conducted on 49 Wistar rats in Iran from 2017 to 2018. The sample size was determined using Morgan Table Samples were randomly divided into seven groups: (1) no nitrate (control), (2) nitrate at 10 mg/L, (3) nitrate at 45 mg/L, (4) nitrate at 200 mg/L, (5) nitrate (10 mg/L) + vitamin C (20 mg per 100 g of body weight), (6) nitrate (45 mg/L) + vitamin C (similar amount), and (7) nitrate (200 mg/L) + vitamin C (similar amount). After 91 days, the content of type-IV collagen in the kidney tissue was determined using the immunohistochemistry (IHC) protocol. The expression of type-IV collagen gene was detected by real-time polymerase chain reaction (RT-PCR) in all groups.
Results: There was no significant difference among the groups of 1-3 (4.55 ± 0.51, 4.7 ± 0.47, 3.6 ± 0.5, $P > 0.05$) in terms of type-IV collagen. However, the obtained results of group 4 indicated a significant reduction in the content of type-IV collagen (1.25 ± 0.44), compared to the control group (4.55 ± 0.51, $P = 0.000$). In terms of vitamin C consumption, the groups of 5 (3.45 ± 0.51) and 6 (3.4 ± 0.5) did not differ significantly from the control group (4.55 ± 0.51, $P > 0.05$). Nonetheless, the severity of response to anti-type-IV collagen antibody significantly increased in group 7 (3.55 ± 0.6) compared to group 4 (1.25 ± 0.44, $P < 0.05$).
Conclusions: The investigated doses of nitrate in drinking water (up to 45 mg/L) had no significant effect on the content of type-IV collagen. On the other hand, the excessive concentrations of nitrate limited the distribution of type-IV collagen and led to potential side effects on the glomerular base membrane. Moreover, vitamin C had no significant effect on 10 and 45 mg/L doses of nitrate. Nevertheless, 200 mg/L dose of nitrate improved the destructive effects of type-IV collagen on the kidney.

Keywords: Ascorbic Acid, Drinking Water, Fertilizers, Immunohistochemistry, Kidney, Livestock, Nitrates, Real-Time Polymerase Chain Reaction, Rats

1. Background

The kidneys are responsible for adjusting and combining the volume of body fluids and excreting the metabolic wastes. The glomerular capillary wall in the kidney encompasses two types of cells, namely endothelial and podocyte, separated by an extracellular matrix called the glomerular basement membrane (GBM). This thick membrane is the main component of the filtration process of the kidney, separating the vessels from the urinary space. In this regard, type-IV collagen is one of the most important components of the base membrane that contributes to the development of the membrane (1-7).

Type-IV collagen is mainly found in the base membrane. The C-terminal region of type-IV collagen is not removed during processing, and the fibers lie alongside each other not parallel. In addition, type-IV collagen contains

no regular glycine, which increases the strength of the collagen triple helix structure. These two properties form the collagen into a sheet and create the base blade. Each molecule of type-IV collagen is a heteroatom consisting of three α-chains. The formation of these heterotrimers is initiated by the interaction of the C-terminal NC1 domain with the pulling of collagenic domains to triple fibers.

In most cases, type-IV collagen can form a network, which is extremely important for several physiological activities. In addition, this protein plays a significant role in the early stages of physical development after birth, especially in the muscles, skin, kidneys, lungs, and arteries (8, 9).

According to the literature, nitrate (NO3-) is one of the inorganic anions formed by nitrogen oxidation. In fact, nitrite is a vital element of protein synthesis in plants and plays an important role in the nitrogen cycle. Considering the natural oxidation of nitrate, nitrite can be found throughout the environment (10-15). Evidence shows that nitrate cannot be removed via conventional water purification due to its high degree of solubility in water. Therefore, it is essential to have advanced filtration methods to filter soluble contaminants. On the other hand, the inappropriate waste disposal through wells in some cities leads to the continuous production and release of nitrate to the underground water (16). Previous studies have demonstrated that high levels of nitrate in drinking water are observed in areas with traditional wastewater treatment and industrial cities (due to wastewater infiltration to underground water) (17, 18). Nitrite produced by the reduction of organic and inorganic nitrates reduces iron oxide hemoglobin (Fe^{2+}) to (Fe^{3+}). As a result of this oxidation, hemoglobin is changed to methemoglobin, which has a lower oxygen-carrying capacity compared to hemoglobin. Furthermore, it is unable to deliver sufficient oxygen to the tissues. As a result, inappropriate oxygen transfer leads to a change in the skin color (around the eyes and mouth) of newborns over time, which is known as the blue baby syndrome (19-24).

2. Objectives

The current study aimed to evaluate the effect of nitrate-containing drinking water and vitamin C on the expression of type-IV collagen in the kidneys of rats.

3. Methods

3.1. Animals Treatment and Test Organization

This empirical test was conducted in Mashhad, Iran, from 2017 to 2018. In total, 49 male Wistar rats (mean weight: 150 - 250 g) were selected from Standard Animal Home, Mashhad University of Medical Sciences, Iran, using simple random sampling technique. The samples were kept at standard condition (22 $\pm$ 1°C, 12:12 light-dark cycle, and 60% humidity). Animals had free access to water and food during the research. At first, the sample population was estimated as 55 male Wistar rats using Cochran formula ($n = Nz^2pq/Nd^2 + z^2pq$, n = sample size, N = population size, z = 1.96, p = q = 0.5, d-error level = 0.05). In the next stage, rats were divided into seven groups (n = 7), as follows: 1: (control): Distilled water (Dw), 2: Dw containing 10 mg/L nitrate, 3: Dw containing 45 mg/L nitrate, 4: Dw containing 200 mg/L nitrate, 5: Dw containing 10 mg/L nitrate + vitamin C (20 mg/100 g (body weight [Bw])/day), 6: Dw containing 45 mg/L nitrate + vitamin C (20 mg/100 g Bw/day), and 7: Dw containing 200 mg/L nitrate + vitamin C (20 mg/100 g Bw/day). After 91 days (according to previous studies), relevant tests were carried out on the samples.

3.2. Preparation of Kidney Tissues and Performance of Immunohistochemistry Protocol

Serial sections (5 μm) were prepared from paraffin blocks. The separated sections were washed in phosphate buffer (PBS) (Sina Co., Iran) at pH 7.4 for 5 min twice a day after paraffin dehydration. The samples were exposed to Triton X-100 (0.3%; Merck Co., Germany) in phosphate buffer and goat serum (Sigma Co., USA). In the next phase, these sections were incubated for 24 h with anti-type-IV collagen antibody (Abcam Co., USA). In the following, they were exposed to Diaminobenzidine (DAB) (Sigma Co., USA) for 15 min, and then background stain was created for samples using hematoxylin. This protocol facilitated the detection of the rate response to anti-type-IV collagen antibody shown in a brown spectrum. In this regard, (0) indicated no reaction, (1) weak reaction in light brown, (2) moderate reaction in brown, (3) strong reaction in dark brown, and (4) very strong reaction in very dark brown (25).

3.3. RNA Extraction and Complementary DNA (cDNA) Synthesis

Renal tissue RNA was extracted using the manufacturer's protocol (Favorgen Co., Taiwan). The RNA was precipitated using ethanol and stored at -80°C. The concentration of RNA was measured by a spectrophotometer. Using the kit (Takara Co., Japan), cDNA was synthesized from the desired RNA.

3.4. Quantitative Real-time PCR Protocol

The levels of type-IV collagen gene expression were determined by qPCR reaction. Afterward, the qPCR reaction was performed using SYBR Green Master Mix (Ampliqon

Co., Denmark) in a final volume of 25 μL. The cDNA was synthesized using the primers (Table 1), and the PCR run protocol in RT-PCR machine (Roche Co., Germany). After the standard curve was drawn for the type-IV collagen gene with 92% efficiency, the expression of type-IV collagen gene was measured in the experimental groups.

Table 1. Primer Sequences for qRT-PCR Analyses

Gene Name	Primer Name	Primer Sequence
Type-IV collagen	Forward	5' - GCG AGA TGT TCA AGA AGC CC - 3'
	Reverse	5' - AGG AGG GAG TAG CAC CAT GT - 3'
GAPDH	Forward	5' - AAT GCA TCC TGC ACC ACC AA - 3'
	Reverse	5' - GTA GCC ATA TTC ATT GTC ATA - 3'

3.5. Statistical Data Analysis

The stained samples were evaluated using a research microscope (Olympus, BX51 model) and Life Science software. Three experts examined the samples by the microscope. In addition, the optical microscope was calibrated to obtain sequential data. According to the Kolmogorov-Smirnov test, it was found that the data had a normal distribution. Ordinal variables in the current study were analyzed using a non-parametric test. The results indicated that the Kappa index was above 0.6, meaning that the obtained data had certain scores. Data analysis was performed with the SPSS Statistics for Windows, version 16.0 (SPSS Corp., Chicago, Ill., USA) using non-parametric methods for immunohistochemistry results (Kruskal-Wallis test and Spearman's coefficient) and parametric tests for RT-PCR results (One-way ANOVA and Tukey). A P value of less than 0.05 was considered statistically significant. Moreover, the data were presented as mean $\pm$ SD.

4. Results

In the current study, the immunohistochemical evaluation revealed a non-uniform distribution of type-IV collagen in GBM. In this regard, severe reactions were observed in some points around GBM and in the extracellular matrix, demonstrating the expression of type-IV collagen. Kruskal-Wallis test is a type of Mann-Whitney U Test that broadly assesses non-parametric data. The obtained results of this test were indicative of no significant difference between groups 1 - 3 in terms of type-IV collagen expression ($P > 0.05$). There was a significant reduction between the fourth group and other groups ($\chi^2 = 63.15$, df = 4, P = 0.000) (Figures 1-5 and Table 2). Spearman correlation coefficient was used to determine the relationship between nitrate administration and the severity of response to anti-type-IV collagen antibody. As observed in Table 3, non-zero results were obtained, indicating a positive correlation among the groups. In addition, this correlation was one-directional meaning that the increase in nitrate concentration was associated with a reaction to anti-type-IV collagen antibody.

5. Discussion

Despite the fact that most kidney diseases are caused by the glomeruli, the complications of interstitial fibrosis are more common. In such a situation, active fibroblasts change into the shape of smooth muscle cells and begin to produce protein isoforms and alpha-smooth muscle actin (α-SMA). Therefore, α-SMA-positive myofibroblasts are the first effective cells involved in the accumulation in the matrix, which are most commonly observed in fibrotic diseases. In the present study, immunohistochemical protocols were employed to determine the expression of type-IV collagen in the kidney. According to the obtained results, type-IV collagen had a heterogeneous distribution in different parts of the kidney, and it was higher in some regions (26-32).

Moreover, the groups of two and three did not differ significantly from the control group in terms of the severity of the reaction to anti-type-IV collagen antibody in the renal glomeruli. There was also a significant difference between the mentioned groups and the group receiving 200 mg/L nitrite nitrate water (P = 0.00). According to these results, an increase in the concentration of nitrate was associated with a decrease in the severity of the reaction to anti-type-IV collagen antibody, which showed the decreased expression of type-IV collagen in such conditions. Moreover, the obtained results of Spearman's correlation coefficient demonstrated a significant correlation between nitrate and the severity of the reaction to anti-type-IV collagen antibody. Nevertheless, the negative value indicated a reverse relationship in this regard.

Furthermore, several reports indicated that type-IV collagen can be expressed by a majority of epithelial, endothelial, and myogenic cells, as well as adult cells from embryonic tissues at different stages of growth. Therefore, the evolution of lung bronchi and kidney nephrons can be indicative of the highest severity of the reaction and developmental growth of this protein. It seems that any element (e.g., nitrate) and androgen (e.g., hormonal changes) can be a factor that is able to express collagen in the structure of the base membrane and the extracellular matrix. Type-IV collagen is the component of the main proteins of the membrane. This collagen builds the network structure under epithelial and endothelial cells and acts as a barrier between different tissue sections. Type-IV collagen has nu-

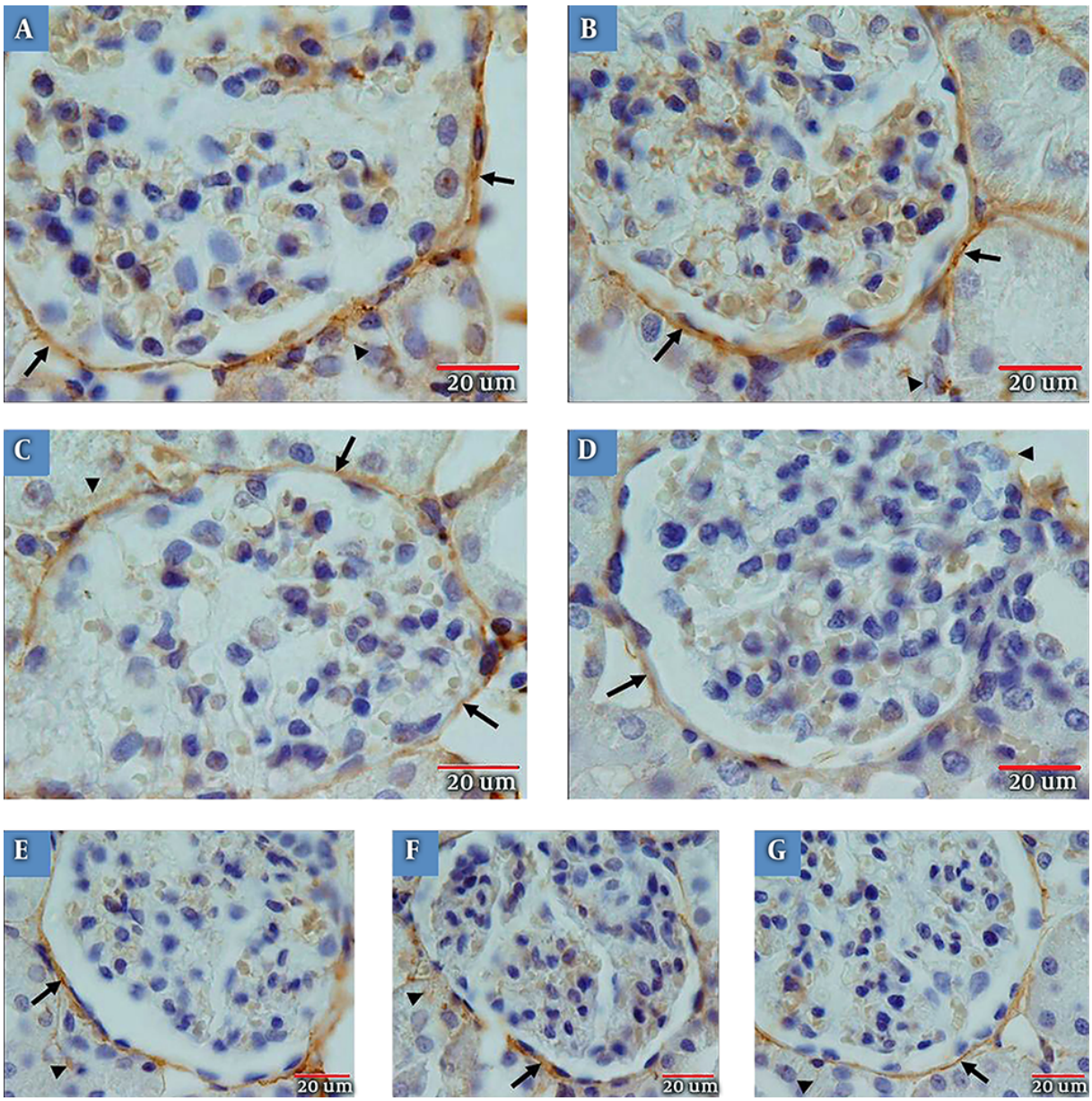

Figure 1. Micrograph of renal tissue after immunohistochemical reaction; (A) group one (control): NO3 = 0 mg/L, (B) group two: No3 = 10 mg/L, (C) group three: NO3 = 45 mg/L, (D) group four: NO3 = 200 mg/L, (E) group five: NO3 = 10 mg/L + VitC = 20 mg/100 gB.wt), (F) group six: NO3 = 45 mg/L, + VitC = 20 mg/100 gB.wt, and (G) group seven: NO3 = 200 mg/L, VitC = 20 mg/100 gB.wt. The base membrane becomes brown in reaction to the anti-type-IV collagen antibody (arrow). The distribution of type-IV collagen was asymmetric in the extracellular matrix and the reaction was severe in some regions, indicating a higher expression of type-IV collagen in these regions (arrowhead). It is noteworthy that reducing the expression of type-IV collagen causes a malfunction of the base membrane.

merous connective components and forms the core of the membrane core. This collagen has a significant signaling potential since certain proteins are released from proteins as subtypes, including tumstatin. As a result, type-IV collagen is the most important structural membrane collagen and also includes the key signaling potential that is vital for various physiological and pathological performances. The mutations in type-IV collagen are responsible for the Alport syndrome, which is a chronic kidney disease. Accordingly, it is of utmost importance to consider the role of type-IV collagen in the kidney (i.e., known as a constructive gland with base membrane) due to the fact that type-

Table 2. The Severity of the Reaction to Anti-Type-IV Collagen Antibody

Group	Frequency					Mean ± SD	Mean ± SE
	Very Low	Low	Medium	High	Very High		
NO3 (0 mg/L)	0	0	0	9	11	4.55 ± 0.51	4.55 ± 0.11
NO3 (10 mg/L)	0	0	0	6	14	4.70 ± 0.47	4.70 ± 0.10
NO3 (45 mg/L)	0	0	8	12	0	3.60 ± 0.50	3.60 ± 0.11
NO3 (200 mg/L)	15	5	0	0	0	1.25 ± 0.44	1.25 ± 0.09
NO3 (10 mg/L) + Vit C (20 mg/100 gBw)	0	0	11	9	0	3.45 ± 0.51	3.45 ± 0.11
NO3 (45 mg/L) + Vit C (20 mg/100 gBw)	0	0	12	8	0	3.40 ± 0.50	3.40 ± 0.11
NO3 (200 mg/L) + Vit C (20 mg/100 gBw)	0	0	10	9	1	3.55 ± 0.60	3.55 ± 0.13

Abbreviations: SD, standard deviation; SE, standard error; Vit, vitamin.

Table 3. Results of Spearman's Test to Determine the Relationship Between Nitrate and Vitamin C Use and the Severity of the Reaction to Anti-Type-IV Collagen Antibody

	Levels of Vit C and Nitrate	The Intensity of the Reaction to Anti-Type-IV Collagen Antibody
Levels of Vit C and nitrate		
Correlation coefficient	1.000	-0.644[a]
P value		0.000
Number of data	140	140
Intensity of reaction to anti-type-IV collagen antibody		
Correlation coefficient	-0.644[a]	1.000
P value	0.000	
Number of data	140	140

Abbreviation: Vit, vitamin.
[a]Correlation at 0.01 level is significant. Spearman's test shows the correlation coefficient based on ordinal data

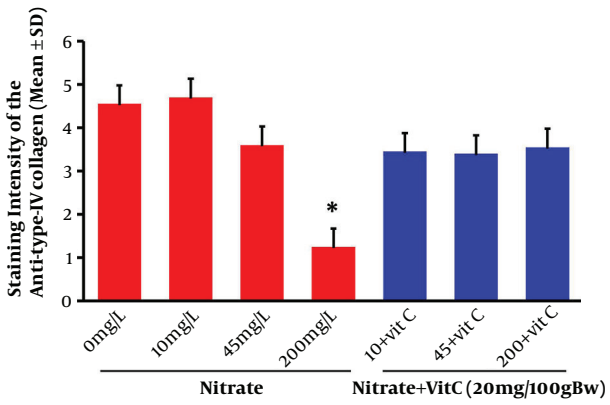

Figure 2. Comparison of nitrate and vitamin C concentrations in drinking water and the mean response rate to anti-type-IV collagen antibody based on glomerular base membrane (GBM) staining are shown (*Significant difference compared to other groups).

IV collagen is distributed in most membranes of the embryonic and adult organs, and participates in evolutionary processes (33, 34).

These changes may be due to the synthesis of these chains in collaboration with endothelial cells and podocytes (35). Studies indicated that the transdifferentiation of tubular epithelial cells into myofibroblasts was one of the changing mechanisms of protein expression in the renal extracellular matrix. Myofibroblasts are the main extracellular matrix (ECM)-secreting cells in mesenchymal tissues, and increased level of matrix elevates fibrosis. Moreover, fibrocytes originate from renal tubular cells during changes in the extracellular matrix. It should be noted that transforming β1-growth factor (TGFβ1) increases the renal tubular cell proliferation, which is associated with the excessive synthesis of extracellular matrix proteins, including collagen (36). In the present study, there was a significant decrease in the group 200 mg/L nitrate water. Therefore, it could be concluded that excessive nitrate in drinking water led to a reduction in the reaction due to the lack of expression of type-IV collagen in GBM. This change in collagen expression resulted in pathological disorders in GBM and degradation of the

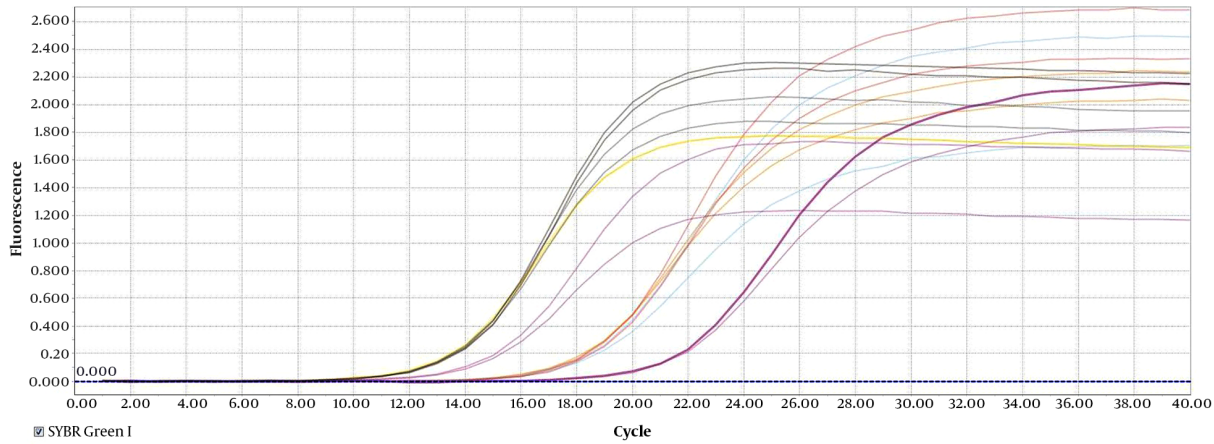

Figure 3. Multiplication cycle curves of RT-PCR of GAPDH (yellow) and type-IV collagen (red) genes in different treatments are shown.

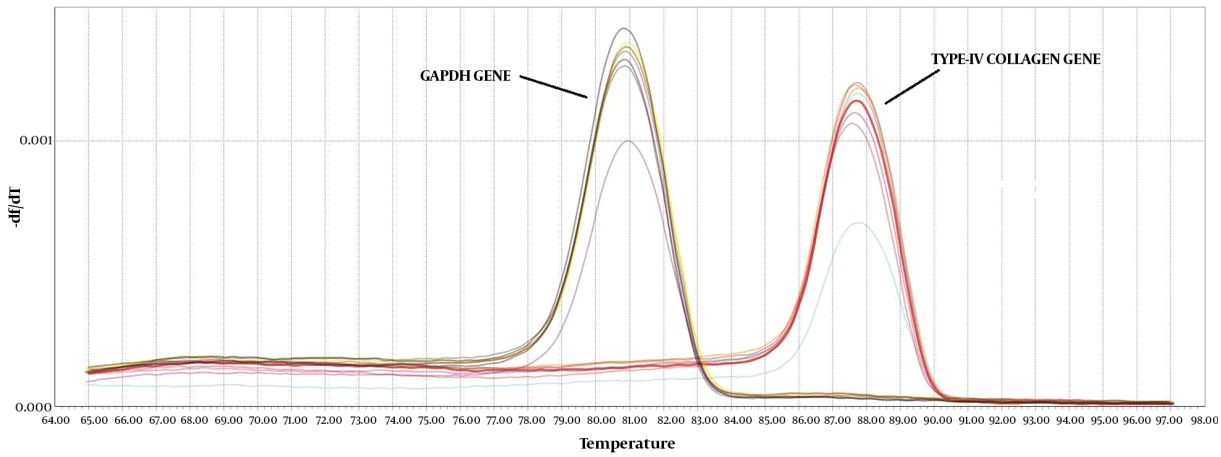

Figure 4. Melting curve analysis of RT-PCR of GAPDH and type-IV collagen genes in different Groups is shown.

glomerular filtration dam. In this regard, the presence of collagen in the extracellular matrix is important for the formation of the base membrane. In addition, the groups of five and six did not significantly differ from the control group in terms of vitamin C consumption ($P > 0.05$) (11). Meanwhile, the severity of the reaction to anti-type-IV collagen antibody significantly increased in group seven, compared to group four ($P < 0.05$). It should be mentioned that the toxic products of nitrate metabolism are eliminated by glomerular filtration and tubular secretion. Therefore, renal glomeruli were exposed to higher levels of metabolic products (e.g., nitrites), which are considered the main causes of glomerular and tubular toxicity (37-39). It is worth mentioning that the implementation of RT-PCR method was one of the main strengths of the current study. Given quantitative studies have been conducted in this field, the results of this study are unique.

5.1. Conclusions

In the present study, a majority of changes in type-IV collagen were observed in the glomerular extracellular matrix. According to our findings, the excessive consumption of nitrate causes a decrease in the expression of type-IV collagen. This could be associated with the increased probability of defects, such as glomerular sclerosis, interstitial fibrosis, and glomerular filtration loss, and may cause glomerulopathy. It is noteworthy that the findings of the present study are of utmost importance due to the limited number of studies in this area.

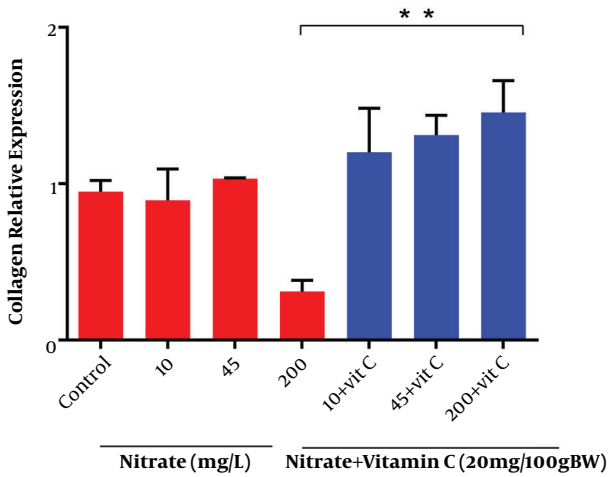

Figure 5. Changes in the relative expression of type-IV collagen gene in different groups is shown. Data were presented as mean ± SD (** P < 0.001).

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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 RT-PCR, Immunohistochemistry Indicators, and data collection.

Conflict of Interests: None.

Ethical Approval: Ethics Committee of Mashhad University of Medical Sciences approved this study with the code of IR.MUMS.fm.REC.1396.202 on 2017/07/12.

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