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Immunohistochemical Evaluation of the Effects of Nitrate in Drinking Water on Laminin Alpha-5 Expression in Rat Renal Glomeruli

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Abstract

Background: Nitrate is a polyatomic ion with the molecular formula NO_3^-. Nitrate poisoning occurs through metabolism in the liver and conversion of nitrate to nitrite, which acts as an intermediary. Laminins are high-molecular weight proteins in extracellular matrix, regarded as one of the major components of the basement membrane.

Objectives: The current study aimed to evaluate the effects of nitrate on laminin α_5 expression in rat renal glomeruli.

Methods: The current interventional study was conducted on 24 male Wistar rats in Iran during 2014 - 2016. Samples were randomly divided into four groups as follows: 1) Normal drinking water, 2) Distilled water containing 45 mg/L of nitrate, 3) Distilled water containing 100 mg/L of nitrate and 4) Distilled water (control). Sample size consisted of 48 kidneys randomly collected from rats. Laminin α_5 content in renal glomeruli was determined using the immunohistochemistry (IHC) protocol. Data analysis was performed using the Kruskal-Wallis test and the Spearman correlation coefficient; P value below 0.05 was considered statistically significant.

Results: In the study, intensity of reaction to anti-laminin was not the same in renal glomeruli. While there was no significant difference between the groups one and two ($P > 0.05$), a significant difference was observed between the groups three and four ($P < 0.05$) in this regard.

Conclusions: According to the results of the study, standard amount of nitrate in drinking water had no significant impact on laminin content. On the other hand, exceeded nitrate concentrations limited the distribution of laminin, leading to potential adverse effects on glomerular basement membrane.

Keywords: Nitrate, Laminin, Kidney, Rat

1. Background

Kidney is the organ responsible to adjust the volume and composition of body fluids, and remove metabolic wastes from the body. Glomerular capillary wall is comprised of two endothelial and podocyte cells separated by an extracellular matrix identified as glomerular basement membrane (GBM). This thick membrane is the most important component of renal filtration barrier, which separates the vasculature from the urinary space. Some of the most important components of basement membrane include fibronectin, type IV collagen, laminin, proteoglycan (agrin), heparan sulfate (perlecan) and nidogen. However, the presence of laminin polymerization in extracellular space is of paramount importance due to its contributions to membrane development (1-8).

Laminins are binding molecules of the glycoprotein family, mainly found in basement membranes. The majority of laminins can self-assemble into a network, which is crucial for most of the physiological activities. Moreover, these proteins have a key role in the initial stages of development and in some of the body parts, such as muscles, skin, kidneys, lungs and arteries (9-14). According to the literature, nitrate (NO_3^-) is one of the inorganic anions, caused by the oxidation of nitrogen. In addition, nitrate is a crucial element of protein synthesis in plants and plays an important role in nitrogen cycle. Due to natural oxidation of nitrate, it can be found throughout the environment (15).

Evidence suggests that nitrate ion cannot be removed by conventional water treatment processes due to its water-soluble nature. Therefore, advanced purification methods with the ability to filtrate soluble contaminants are required. On the other hand, denitrification cycle still continues in cities with improper disposal of sewage through absorption wells, mainly leading to continuous production and dissemination of nitrate to groundwater (16). Previous studies show that high levels of nitrate in drinking water could be observed in areas with traditional wastewater treatment methods and industrial cities (due to the infiltration of sewage and industrial waste to groundwater) (17-19). Nitrite, caused by the reduction of

organic and inorganic nitrate, oxidizes hemoglobin iron (Fe^{2+}) to (Fe^{3+}). This results in the oxidation of hemoglobin to methemoglobin, oxygen-carrying capacity of which is less than hemoglobin and cannot transfer sufficient oxygen to tissues. Inadequate oxygen transfer leads to skin discoloration (around eyes and mouth) over time, which justifies the name of Blue baby syndrome (20-29).

2. Objectives

The current study aimed to evaluate the effects of nitrate in drinking water on laminin α_5 expression in rat renal glomeruli.

3. Methods

The current interventional study was conducted on 60 male Wistar rats (weighing 250 - 300 g) in Iran during 2014 - 2016. Rats were provided by the animal house of Mashhad University of Medical Sciences and kept at standard condition (22 $\pm$ 1°C and under a 12: 12 light-dark cycle). In this research, animals had free access to water and food during the study. In addition, sample size was calculated based on previous studies. Samples were randomly divided into four groups (n = 15) and six rats of each group were randomly selected and allocated to study groups, as follows:

1. Group one: Receiving normal municipal drinking water (n = 6),

2. Group two: Receiving distilled water with the maximum concentration of nitrite (45 mg/L) (n = 6),

3. Group three: Receiving distilled water with exceeded amount of nitrate (100 mg/L) (n = 6),

4. Group four (control): Receiving distilled water (n = 6).

Intervention period was 91 days based on the results obtained by previous studies. At the end of the intervention, rats were anesthetized and their kidneys were carefully dissected and removed from the body. Following that, changes in the expression of laminin α_5 glycoprotein were evaluated using the immunohistochemistry (IHC) protocol. This research project was approved by the research ethics committee of Mashhad University of Medical Sciences (code: IR.MUMS.REC.1393.151) on 2014/09/03.

3.1. Tissue Preparation and IHC Protocol

To perform IHC (30-32), serial sections (5 μm) were obtained from paraffin blocks and tissue sections were washed in Tris-HCl buffers (5.1% sodium chloride, pH 4.7) twice for five minutes after deparaffinization. Afterwards, the sections were placed near 0.3% triton X-100 in Tris buffer and goat serum, followed by setting the androgen peroxidase in a solution of 3% hydrogen peroxide in methanol for one hour to inhibit its activation.

In the next stage, the sections were incubated with laminin antibody with a concentration of 1: 50 (conjugated with horse radish peroxidase) for 24 hours. Following that, they were placed in Tris-buffered saline with 3% triton and 2% saline and washed in Tris buffer for 10 minutes three times. Afterwards, the sections were exposed to diaminobenzidine (with 0.03% hydrogen peroxide) for 15 minutes, followed by the application of hematoxylin to create background staining. Prepared sections were mounted on glass slides (Table 1).

Application of this protocol helped to detect the amount of reaction to laminin antibody, observed in a brown color (from light to dark brown based on its intensity) (Table 2). At the final stage, pictures were provided from the desired areas using a microscope camera, followed by analyzing the data and drawing tables and diagrams after the evaluation of results.

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

Name of Materials	Company	Manufacturing Country
Alcohol 100°	Sina	Iran
Alcohol 96°	Sina	Iran
Xylene	Merck	Germany
Formalin	Merck	Germany
Hematoxylin	Merck	Gemany
Sodium nitrate	Merck	Germany
Sodium chloride	Merck	Germany
Entlan glue	Betagen	Iran
Tris-HCl	Merck	Germany
Laminin alpha 5 antibody	Abcam	USA
Secondary antibody	Abcam	USA
Bovine serum albumin	Sigma	USA
Glycerol gel	Sigma	USA
3,3′-Diaminobenzidine	Sigma	USA
Sodium nitrate	Merck	Germany
Lam and plates	Betagen	Iran
Hydrogen peroxide	Merck	Germany

3.2. Data Analysis

Stained samples were evaluated using a research microscope (Olympus, BX51 model) and the life sciences software. Samples were assessed under microscope and

Table 2. The Grade of Immunoreactivity and Intensity of Reaction for Laminin α_5 Antibody[a]

Reaction	Grade
Negative (-)	0
Weak (+)	1
Moderate (++)	2
Strong (+++)	3
Very strong (++++)	4

[a](-), no reaction; (+), weak reaction: light brown; (++), moderate reaction: brown; (+++), strong reaction: dark brown and (++++), very strong reaction: very dark brown.

ranked by three experts. Optical microscope was calibrated to obtain unbiased data. In the current study, the ordinal scale was used to measure the variables and the Kappa index was greater than 0.6. Based on the ratings of data, samples were assigned specific grades. Data analysis was performed by SPSS version 16 using nonparametric methods (e.g. Kruskal-Wallis test and the Spearman correlation coefficient) due to the non-normality of the data. Moreover, P value below 0.05 was considered statistically significant.

4. Results

In the current study, the results of immunohistochemical evaluation revealed a non-uniform distribution of laminin α_5 protein in GBM. In this regard, severe reactions were observed in some areas around the GBM and in the extracellular matrix, indicating laminin α_5 expression. In addition, assessment of Kruskal-Wallis test results indicated no significant difference among the control, distilled water and water containing 45 mg/L of nitrate groups in terms of laminin α5 expression ($P > 0.05$). Accordingly, a significant difference was found between the group with water containing 100 mg/L of nitrate and other study groups regarding the intensity of reaction to anti-laminin ($P = 0.000$) (Figures 1 and 2; Tables 3 and 4).

The Spearman correlation coefficient was performed to determine the correlation between nitrate intake and severity of reaction to anti-laminin. As observed in Table 5, non-zero test results were obtained, indicating a positive correlation between the groups. In addition, this result was in the direction of the figure, meaning that increased nitrate concentration was associated with alleviated reaction to anti-laminin.

5. Discussion

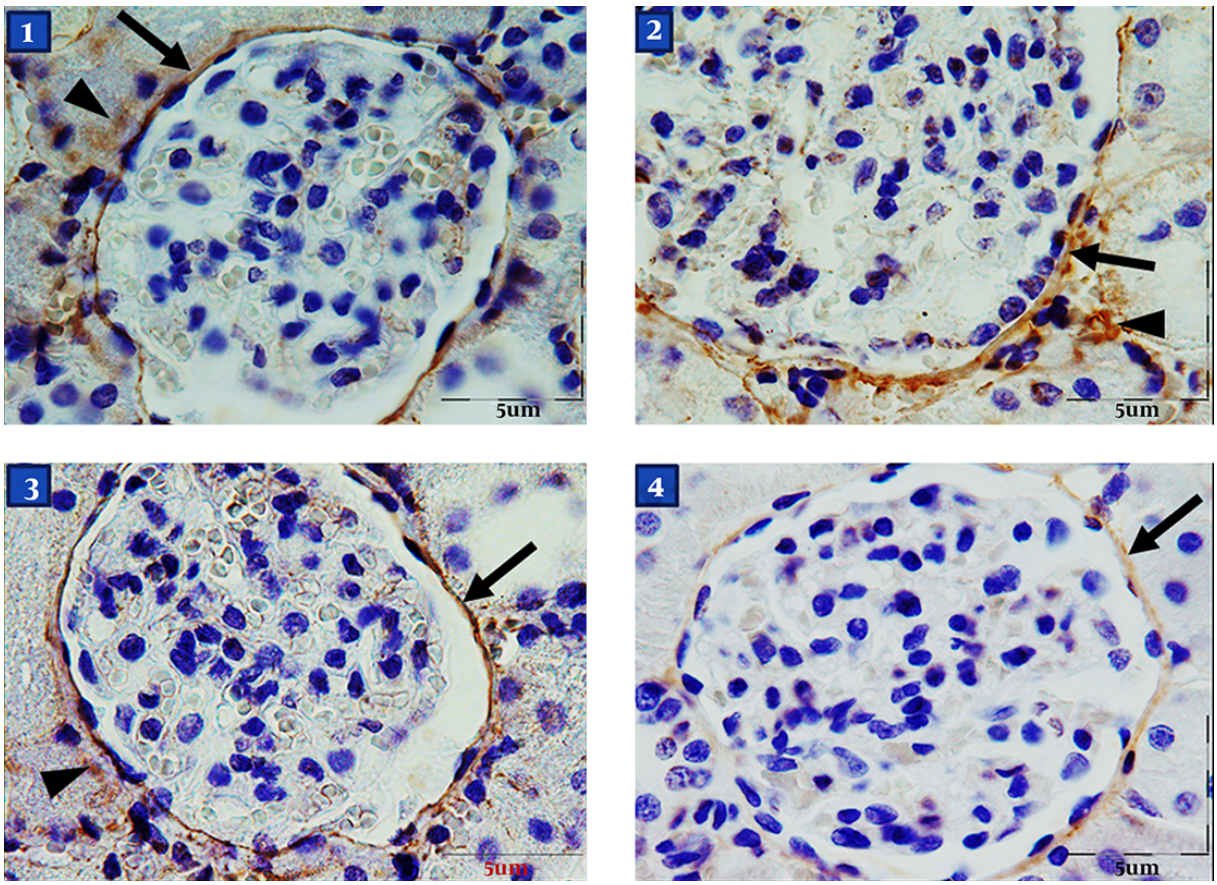
Despite the fact that the majority of renal diseases are of glomerular origin, complications of interstitial fibrosis are more common. Under such circumstances, active fibroblasts turn into a shape similar to those of smooth muscle cells, followed by the initiation of protein and smooth muscle actin isoforms (alpha-smooth muscle actin, α-SMA) synthesis. Therefore, α-SMA containing myofibroblasts are the first influential cells involved in the accumulation of matrix, mostly observed in fibrotic diseases (33). The current research employed immunohistochemistry protocol to determine the pattern of laminin α_5 expression in kidney. According to the obtained results, laminin α_5 had a heterogeneous distribution in various regions of kidney and higher localized expressions in some areas.

No significant difference was observed among the control, and the groups 1 and 2 in terms of laminin reaction intensity in renal glomeruli. Meanwhile, a significant difference was observed between the mentioned groups and the group receiving water with 100 mg/L of nitrite ($P = 0.000$). According to these results, increased concentration of nitrite in water was associated with decreased reaction intensity to anti-laminin, indicating alleviated laminin protein expression under such circumstances. In addition, the results of the Spearman correlation coefficient revealed a significant relationship between nitrate and the intensity of reaction to anti-laminin. However, negative amounts - indicated a reverse association.

Moreover, several reports indicated that laminin α_5 could be expressed by the majority of epithelial, endothelial, myogenic and mature cells from embryonic tissues at different stages of development. Therefore, lung bronchi and evolving kidney nephrons could take advantage of the effects induced by this protein to grow, develop and reveal their highest intensity. Nevertheless, the intensity of this laminin chain was not significant at the early stages of epithelium evolution and was gradually replaced by laminin α_1. Therefore, it seems that each exogenous (e.g. nitrate) and androgen (e.g. hormonal changes) factor could change laminin expression in basement membrane structure and extracellular matrix.

Given the fact that laminin α_5 is distributed in most of the basement membranes of embryonic and mature organs and participates in evolutionary processes, the importance of its role in kidney (known as an organ enriched with basement membrane) should be considered. It is noteworthy that the absence of laminin prevents ureteric bud branching and leads to renal agenesis, which could be associated with incomplete vascularization of renal glomerular (34). One of the most important issues in this regard is the fact that only GBM composition

Figure 1. Photomicrographs of Renal Glomeruli After Immunohistochemical Reaction: 1) Control Group; 2) The First Experimental Group; 3) The Second Experimental Group; 4) The Third Experimental Group



In these figures, the basement membrane turned brown in reaction to anti-laminin (arrow). Heterogeneous laminin α_5 distribution in extracellular matrix and reaction in some areas were high, which was indicative of more laminin α_5 expression in those areas (arrow head) (magnification: X40).

Table 3. Degree of Reaction to Anti-Laminin Cross-Tabulation

Group	Frequency					Mean ± SD	Mean ± SE
	Very Low	Low	Medium	High	Very High		
DW	0	0	0	6	14	4.70 ± 0.47	4.70 ± 0.10
NO3(100 mg/L)	15	5	0	0	0	1.25 ± 0.44	1.25 ± 0.09
NO3(45 mg/L)	0	0	8	12	0	3.60 ± 0.50	3.60 ± 0.11
Water	0	0	0	9	11	4.55 ± 0.51	4.55 ± 0.11
Total	15	5	8	27	25		
Percent	18.8	6.2	10.0	33.8	31.2		

Abbreviations: DW, distilled water; SD, standard deviation; SE, standard error.

changes during the evolution, compared to most of the basement membranes. While hetero-trimmer immature nephrons contain laminin $\alpha_1\beta_1\gamma_1$, mature glomeruli consist of $\alpha_5\beta_2\gamma_1$ hetero-trimmers, which is the only laminin isoform found in mature GBM.

However, $\alpha_5\beta_1\gamma_1$ laminin hetero-trimmers were also

Table 4. Kruskal-Wallis Test Results[a]

	Reaction to Anti-Laminin
The Chi-square statistic (χ^2)	60.967
Degrees of freedom	4
Sig	0.000

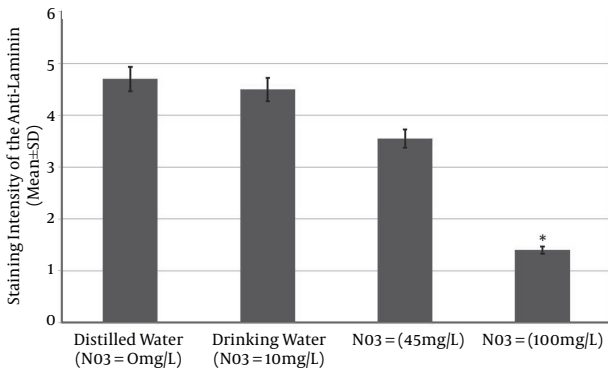
[a] Kruskal-Wallis test is the U Mann-Whitney test extensively and similar non-parametric analysis of one factor variance (Sig: Significance of the results).

Table 5. Evaluation of Spearman Results to Determine the Correlation Between Nitrate Intake and Intensity of Reaction to Anti-Laminina

	Nitrate Levels in Drinking Water	Staining Intensity of the Anti-Laminin
Nitrate levels in drinking water		
Correlation coefficient	1.000	-0.859[a]
Sig.		0
Number of data	80	80
Staining intensity of the anti-laminin		
Correlation coefficient	-0.859[a]	1.000
Sig.	0	
Number of data	80	80

[a] Correlation WAS significant at the 0.01 level (2-tailed); the Spearman test showed correlation coefficient based on the rank of the data.

Figure 2. Comparison of Nitrate Concentration in Drinking Water and Mean Intensity of Reaction to Anti-Laminin Based on the Intensity of Glomerular Basement Membrane Staining



*Significant difference compared to other groups, P = 0.000; data are presented as mean ± SD.

temporarily observed in immature basement membrane. These changes might be due to the synthesis of these chains in collaboration with endothelial cells and podocytes (35). Studies show that transdifferentiation of tubular epithelial cells into myofibroblasts is one of the changing mechanisms of protein expression in renal extracellular matrix. Myofibroblasts are the main extracellular matrix (ECM)-secreting cells in mesenchymal tissues and increased matrix leads to increased fibrosis. Moreover, fibrocytes originate from renal tubular cells during changes in extracellular matrix.

It should be considered that transforming growth factor-β1 (TGFβ1) increases the renal tubular cell proliferation, associated with excessive synthesis of extracellular matrix proteins, including laminin (36). In the present study, a significant reduction was observed in the group with drinking water containing 100 mg/L of nitrate. Therefore, it could be concluded that exceeded amount of nitrate in drinking water was associated with decreased reaction due to lack of laminin expression in GBM. This change in laminin expression resulted in pathologic disorders in GBM and the destruction of the glomerular filtration barrier. In this regard, the presence of laminin in the extracellular matrix is important to form the basement membrane.

It should be noted that toxic products of nitrate metabolism were excreted by glomerular filtration and tubular secretion. Therefore, renal glomeruli were exposed to higher levels of metabolic products (e.g. nitrite), which is considered as the main cause of glomerular and tubular toxicity (37). In the present research, most of the changes were observed in the laminin of glomerular extracellular matrix. According to the results of the current study, exceeded amounts of nitrate led to decreased

laminin α_5 levels. This reduction was associated with increased possibility of defects, such as glomerular sclerosis, interstitial fibrosis and reduced glomerular filtration and might cause glomerulopathy. One of the major drawbacks of the current study was the research methodology, since application of reverse transcription polymerase chain reaction (RT-PCR) might have yielded more accurate results. It is noteworthy that limited research is conducted in this regard and findings of the present study are unique.

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Footnotes

Authors' Contribution: Mahdi Jalali, Mohammad Reza Nikravesh and Mahmoud Moghaddam Dorafshani: study design, managing literature searches, the immunohistochemical protocol assistance and data collection.

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