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RESEARCH ARTICLE

# Hydroxytyrosol affects antioxidant Nrf2 expression in the kidneys of diabetic rats

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## ABSTRACT

**Aim:** Diabetic nephropathy is the result of oxidative stress caused by chronic hyperglycemia. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor that responds to oxidative stress. Nrf2 relieves oxidative stress, inflammation, and fibrosis associated with diabetes in the kidneys. In this study, we investigated the effects of hydroxytyrosol, which is a polyphenolic compound with proven antioxidant activity, on Nrf2 expression in diabetic kidneys.

**Methods:** Forty male Wistar rats were used in our study and the rats were divided into four groups as control (sterile water only), hydroxytyrosol (HT) (10mg/kg hydroxytyrosol administered intraperitoneally (ip) for 30 days), streptozotocin (STZ) (diabetes was induced by administering a single dose of 55 mg/kg streptozotocin ip), and streptozotocin + hydroxytyrosol (STZ+HT) (single dose of 55 mg/kg streptozotocin and 10 mg/kg hydroxytyrosol administered ip for 30 days). At the end of the study, Nrf2 expression in kidney tissue was evaluated by immunohistochemistry and Western blot.

**Results:** Immunohistochemistry and Western blot findings of Nrf2 were similar. It was found that while Nrf2 expression increased significantly in the HT group compared to the control group, whereas it decreased significantly in the STZ group ($p<0.001$). In the STZ+HT group, Nrf2 expression was found to be significantly increased compared to the STZ group ($p<0.001$).

**Conclusions:** It was found that hydroxytyrosol with known antioxidant activity increased Nrf2 expression in diabetic rats. These results suggest that hydroxytyrosol may mitigate diabetic nephropathy by Nrf2-induced reduction in oxidative stress, inflammation, and fibrosis.

**Keywords:** diabetes mellitus, hydroxytyrosol, kidney, Nrf2, streptozotocin

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## INTRODUCTION

Diabetes mellitus is a very common disease in society. Diabetes-induced permanent hyperglycemia causes complications such as diabetic nephropathy (DN) (1). DN is a progressive disease that may cause death in developing countries (2). DN is a syndrome that causes death and hypertrophy of glomerular cells, and fibrosis is also observed (3, 4). The most striking phenomenon in DN pathogenesis is the production of reactive oxygen species due to chronic hyperglycemia. Disruption of the balance of reactive oxygen species in the cell causes oxidative stress (5). The balance between the formation and clearance of free radicals in the cells of diabetic patients is disturbed. As a result, a significant increase in lipid peroxidation rate is observed in the cells of diabetic patients (6). This increase may cause damage to many organs of diabetic patients (7). Previous studies show that the development of DN is controlled by oxidative stress, inflammation, and fibrosis. Among these, oxidative stress occurs earliest and causes the activation of pathological pathways in kidney cells (8).

Diabetes-induced oxidative stress causes adverse effects by affecting DNA, proteins, lipids, and cellular pathways (7,9). These adverse effects impair kidney functions by affecting kidney cells. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor that responds to oxidative stress (10). In the oxidative equilibrium state of cells, Nrf2 is found in the cytoplasm bound to Kelch-like ECH-related protein 1 (Keap1). Oxidative stress that occurs due to disruption of oxidative balance causes Nrf2 to detach from Keap1 and translocate to the nucleus. Nrf2 binds to the antioxidant response element (ARE) of antioxidant genes in the nucleus and initiates transcription of antioxidant genes such as superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) (11). A large number of studies have shown Nrf2 activation against DN to be effective in protecting the kidneys (12-19). The triple mechanism including oxidative stress, inflammation, and fibrosis may aggravate DN. However, many studies have shown that Nrf2 activated by different agents can regulate the above-mentioned mechanisms in DN treatment (12). Nrf2 activation has an important role in decreasing oxidative stress, inflammation, and fibrosis (13-15). Nrf2 stimulation reduces renal damage and extracellular vesicular accumulation by decreasing malondialdehyde levels and increasing SOD activity (16). Increased Nrf2 expression reduces renal tissue damage and apoptosis by reducing inflammation and oxidative stress through HO-1 signaling (17, 18). Increased Nrf2 signaling inhibits apoptosis, angiogenesis, inflammation, and oxidative stress by inducing HO-1 and relaxes DN (19). Nrf2 activation is protective against albuminuria and glomerular remodeling observed in DN (15). Herbal remedies used in traditional complementary medicine are widely used to treat diabetes and its complications. The roles of herbal agents should be explored to obtain a DN-effective drug. Mediterranean diet is a source of important antioxidant compounds in the prevention of DN (20). Olive oil is the main component of the Mediterranean diet. Olive oil contains monounsaturated fatty acids and lots of polyphenolic bioactive compounds. The most remarkable of these polyphenolic compounds is hydroxytyrosol with its antioxidant effects (21). Hydroxytyrosol shows its antioxidant effects by reducing inflammation, thrombocyte aggregation, and oxidized low-density lipoprotein (ox-LDL) production. It also shows insulin-like and insulin resistance-reducing effects (21-23). Hydroxytyrosol has also been reported to reduce proteinuria by 67-73% and glomerular volume and glomerulosclerosis by 20-30% (24).

Developing new alternative treatment options is crucial as diabetes is a chronic disease with inadequate treatment options and high treatment costs. Compounds obtained from plants are among the alternatives. Hydroxytyrosol is an anti-oxidative polyphenolic compound with known protective effects on the kidney, and has been shown to have protective and relaxing effects on diabetic nephropathy. We investigated the effects of hydroxytyrosol on Nrf2 expression.

## MATERIALS AND METHODS

### Animals and chemicals

We used 40 Wistar male rats, aged 3-month-old, with a weight of 250-300 grams. Power analysis was used to determine the number of animals in the study. It was determined that there should be 10 animals in each

group. The animals were housed at 24 ± 2 °C, 50 ± 5% humidity, and a cycle of 12 hours light: 12 hours dark. Food and water were provided *ad libitum*. The animals were divided into four groups, each consisting of 10 rats. These groups were as follows: the control group was only injected with sterile water intraperitoneally (ip); the hydroxytyrosol (HT) group was administered ip injection of 10 mg/kg hydroxytyrosol (Cayman, 70604) dissolved in sterile water for 30 days; streptozotocin (STZ) group was administered single dose ip 55 mg/kg streptozotocin injection dissolved in 0.05 M citrate buffer (pH 4.5) to induce diabetes at the beginning of the experiment; streptozotocin+hydroxytyrosol (STZ+HT) group was administered single dose ip 55 mg/kg streptozotocin injection (25) to induce diabetes at the beginning of the experiment and animals with diabetes were administered 10 mg/kg hydroxytyrosol ip injection for 30 days. Hydroxytyrosol dose was determined by considering the doses used in many previous studies (26,27). Blood glucose levels of the animals were measured from tail blood by using an Accu-Chek Nano Performa glucose meter 48 hours after streptozotocin injection. Animals with a blood glucose level ≥250 mg/dl were considered as diabetic and included in the study (25). At the end of the study, the animals were anesthetized with 90 mg/kg ketamine + 10 mg/kg xylazine, their kidney tissues were removed, and they were euthanized by cervical dislocation. Half of each removed kidney was placed in a 4% formaldehyde solution for immunohistochemistry and the other half was placed in cryotubes for Western blotting and kept at -80 ° C. This study was approved by the Düzce University Animal Experiments Local Ethics Committee with decision number 2022/12/05.

### Immunohistochemistry

After the tissues were fixed in 4% formaldehyde for 24 hours, they were dehydrated by passing through increasing degrees of alcohol. Subsequently, the tissues were embedded in paraffin and cleared with xylene. The specificity of the Nrf2 antibody was determined using human breast carcinoma tissue. Negative control immunochemistry was performed by using IgG isotype instead of primary antibody. Sections of 4 µm thickness were taken to positively charged slides from paraffin blocks. The sections were deparaffinized and rehydrated. They were boiled in citrate buffer (pH 6.0) in a 750-watt microwave oven for 7 minutes. They were cooled at room temperature for 20 minutes. The sections were washed with phosphate-buffered saline (PBS). They were incubated in a 3% hydrogen peroxide solution for 15 minutes. The sections were then washed with PBS 3 times for 5 minutes. Their borders were drawn with a hydrophobic pen. They were incubated for 7 minutes in a humidified chamber with UltraV block (Thermo Scientific™ TL-125-UB) and incubated in a humidified chamber overnight at +4 °C with Nrf2 rabbit polyclonal primary antibody (1:100 dilution, Abcam; Cat# ab137550). The next day, the primary antibody was removed and the sections were washed 3 times for 5 minutes with PBS. Biotinylated secondary antibody (Thermo Scientific™ TL-125-PB) was dropped on the sections and they were washed 3 times for 5 minutes with PBS after being incubated at room temperature for 30 minutes. They were incubated for 15 minutes in a humidified chamber at room temperature with peroxidase-labeled streptavidin (Thermo Scientific™ TL-125-PH) and washed. Immunohistochemical reaction was performed with diaminobenzidine. The sections were counterstained with hematoxylin, backtracked, and closed with entellan. Reaction photos were taken with an AxioCam Zeiss digital camera attached to an Olympus Cx41 microscope. Staining intensities in the photos were analyzed using ImageJ Version 1.8.0 software (http://imagej.nih.gov/ij/). The same procedures were applied in our previous study (28).

### Semi-quantitative evaluations

Nrf2 expressions and immunostaining densities in kidney cells were evaluated semi-quantitatively. The scores are shown in Table 1.

### Western blotting

The tissues kept at -80 °C were taken on ice and melted. After the tissues were mechanically lysed, they were incubated for 1 hour by mixing with lysis buffer (Tris–HCl (pH 7.4), Naorthovanadate, sodium dodecyl sulfate (SDS)) and protease inhibitor cocktail (Roche cOmplete™, Mini Protease Inhibitor Cocktail, Cat# 4693124001) and vortexed every 15 minutes. The lysates formed were centrifuged at +4 °C and 15000 g for 10 minutes, the resulting supernatants were taken and protein measurement

**Table 1.** Semi-quantitative distribution of Nrf2 labeling in control, HT (hydroxytrosol), STZ (streptozotocin) and STZ+HT. 0: negative; (+): weak positive; +: positive; ++: dense positive; +++: very dense positive.

| Cells | Nrf2 | | | |
|---|---|---|---|---|
| | Control | HT | STZ | STZ+HT |
| Distal Convoluted Tubule Cells | ++ | +++ | (+) | ++ |
| Proximal Convoluted Tubule Cells | + | +++ | (+) | + |
| Macula Densa Cells | + | + | (+) | + |
| Podocyte Cells | (+) | + | 0 | + |
| Mesangial Cells | 0 | 0 | 0 | 0 |
| Distal Straight Tubule Cells | (+) | +++ | 0 | ++ |
| Proximal Straight Tubule Cells | + | +++ | (+) | +++ |

was performed using Pierce™ BCA Protein Assay Kit (Cat# 23225). The determine the protein content of the samples, they were mixed with 4x lamella buffer (Biorad, Cat# 1610747) and sterile distilled water to equalize the protein content and boiled at 95 °C for 5 minutes. The boiled samples were loaded into SDS polyacrylamide gel wells (50 µg/ml protein in each well). Electrophoresis was performed at 100 V for 2-3 hours. The samples which were then transferred to gel were transferred to polyvinylidene fluoride membranes (PVDF) (EMD Millipore) by performing overnight blotting at 30 V at +4 °C. The membranes were blocked with 5% skimmed milk powder prepared with tris-buffered saline (TBST) containing 0.05% tween-20. The membranes were incubated overnight with Nrf2 rabbit polyclonal primary antibody (1:1000 dilution, Abcam; Cat# ab137550) and internal loading control β-actin rabbit primer antibody (1: 2000 dilutions, Cell Signalling Technology; Cat# 4970). The next day, they were incubated for 2 hours at room temperature (1:2000 dilutions, Cell Signalling Technology, Cat # 7074s) with horseradish peroxidase (HRP) conjugated secondary antibody compatible with primary antibody. Bands were obtained by using a chemiluminescence-based substrate kit (Pierce, Rockford, IL, USA) which reacted with HRP. The bands formed were exposed to X-ray film, scanned, and transferred to the computer. The bands were analyzed by using ImageJ software (http://imagej.nih.gov/ij/). The same procedures were applied in our previous study (29).

### Statistical analysis

The immunochemistry and Western blot results were analyzed with GraphPad Prism 9 (GraphPad Software). After the Shapiro-Wilk test was used to assess the normality of the data obtained with ImageJ, they were expressed as mean ± standard deviation (SD). All data were analyzed with one-way analysis of variance (ANOVA) and Tukey's test was used for the post-hoc test. $P<0.05$ was considered as the level of significance.

## RESULTS

Since we examined the effect of hydroxytyrosol on blood glucose values in detail in another study, we did not repeat it in this study (30).

### Nrf2 immunohistochemistry results;

In the kidney cortex and medulla cells, the Nrf2 immunostaining pattern was both cytoplasmic and nuclear (Figure 1A). In the control group, Nrf2 expression was densely positive in distal convoluted tubule cells, and positive in proximal convoluted tubule, macula densa, and proximal straight tubule cells (Table 1, Figure 1A (1 and 2)). It was weakly positive in podocyte cells and distal straight tubule cells (Table 1, Figure 1A (1 and 2)). Nrf2 expression in the HT group was higher than control and STZ+HT groups ($p<0.001$) (Figure 1A and 1B). Nrf2 expression was very dense positive in distal and proximal

convoluted tubule and distal and proximal straight tubule cells of the HT group (Table 1, Figure 1A (4 and 5)). Nrf2 expression was positive in macula densa and podocyte cells of the HT group (Table 1, Figure 1A (4 and 5)). Nrf2 expression in the STZ group was found to be significantly decreased compared to control and HT groups ($p$<0.001) (Figure 1A and 1B). Nrf2 expression was weakly positive in the distal and proximal convoluted tubule, macula densa, and proximal straight tubule cells of the STZ group (Table 1, Figure 1A (7 and 8)). Nrf2 expressions were negative in podocytes and distal straight tubule cells (Table 1, Figure 1A (7 and 8)). In addition, vacuolization was observed in some distal convoluted tubule cells of the STZ group (Figure 1A (7 and 8)). In the STZ+HT group, Nrf2 expression was significantly increased compared to the STZ group ($p$<0.001) (Figure 1A and 1B). However, no significant difference was found between the STZ+HT group and control group ($p$=0.648) (Figures 1A and 1B). In the STZ+HT group cells, Nrf2 expression was dense positive in distal convoluted and straight tubule cells, positive in proximal convoluted tubule, macula densa, and podocyte cells, and dense positive in proximal straight tubule cells (Table 1, Figure 1A (10 and 11)). In mesangial cells of all groups, Nrf2 expression was negative (Table 1, Figure 1A (1, 2, 4, 5, 7, 8, 10, and 11)).

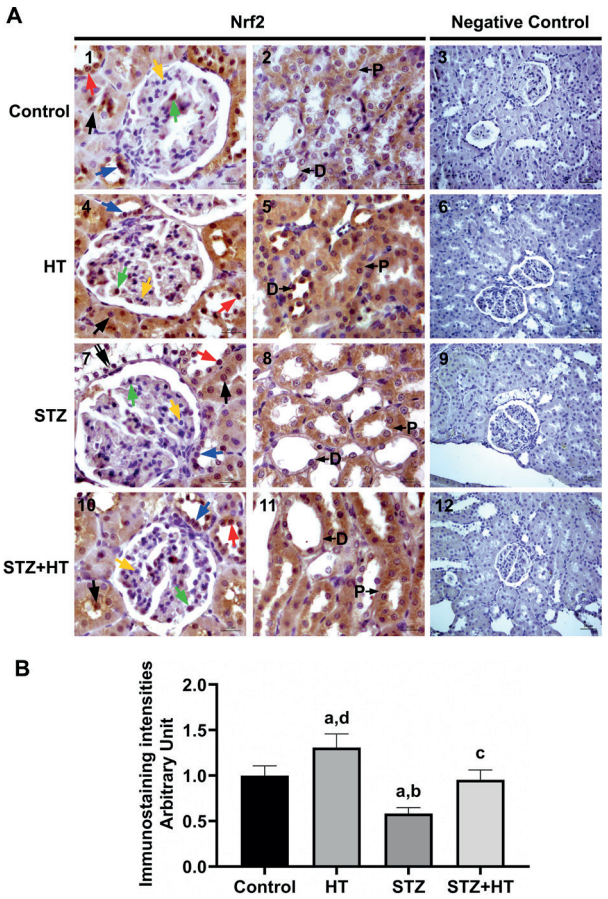


**Figure 1. A.** Representative micrographs of immunohistochemical analysis results of Nrf2. **B.** Quantification of immunohistochemical staining. The data were expressed as mean±SD. Black arrow; proximal convoluted tubule cell, red arrow; distal convoluted tubule cells, blue arrow; macula densa cell, green arrow; podocyte cell, yellow arrow; mesangial cell, double arrow; vacuoilized cells, P; proximal straight tubule cell, D; distal straight tubule cell. Statistical significance ($p$<0.05) compared with [a]control and [b]HT, [c]STZ and [d]STZ+HT. HT; hydroxytyrosol, STZ; streptozotocin. The lens magnification of micrograph 1, 2, 4, 5, 7, 8, 10, and 11 is 100x. The lens magnification of micrograph 3, 6, 9, and 12 is 40x.

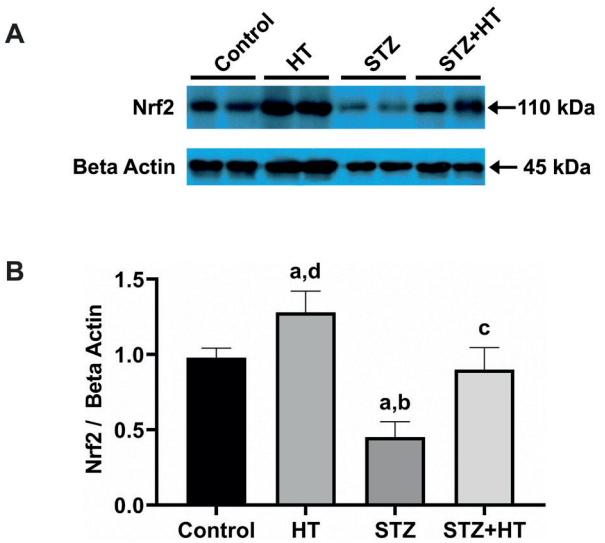


**Figure 2. A.** Representative bands of Western blot analysis results in kidney tissue extracts of Nrf2 protein. **B.** Quantification of Western blot Nrf2 protein level. The data were expressed as mean±SD. Statistical significance ($p$<0.05) compared with [a]control and [b]HT, [c]STZ and [d]STZ+HT. HT; hydroxytyrosol, STZ; streptozotocin.

### Nrf2 Western blot results

In all groups, Nrf2 Western blot results were compatible with immunohistochemistry results. In the HT group, Nrf2 protein was significantly increased compared to the control and STZ+HT groups ($p$<0.001) (Figure 2). STZ group Nrf2 protein level was significantly decreased compared to the control and HT groups ($p$<0.001) (Figure 2). STZ+HT group Nrf2 protein was significantly increased compared to the STZ group. However, the Nrf2 protein level in the STZ+HT group was similar to that in the control group ($p$=0.545) (Figure 2).

## DISCUSSION

In this study, we aimed to investigate the effects of hydroxytyrosol, which is an anti-oxidative polyphenolic compound with known protective effects on the kidney and the basis of the Mediterranean diet, on Nrf2 expression in type 1 diabetic rats. As a result of the study, we determined that Nrf2 expression increased in the HT group treated with hydroxytyrosol compared to the control group, while it decreased in the STZ group treated with streptozotocin compared to the control group. However, we found that in the STZ+HT group, which received hydroxytyrosol together with streptozotocin, Nrf2 expression was at the same level as the control.

In this study, immunohistochemistry and Western blot results of Nrf2 were found to be similar. We found that Nrf2 expression was increased in the HT group that was administered hydroxytyrosol compared to the control group. We found that this increase occurred mostly in proximal and distal convoluted tubules and proximal and distal straight tubules. It has been previously reported that hydroxytyrosol activates Nrf2 in many different tissues and cells (31-33). In the present study, as in previous studies (31-33), hydroxytyrosol may have increased the intensity of Nrf2 expression by activating Nrf2 in many kidney cells, compared to the control group. We found that the streptozotocin, which was given to induce diabetes, reduced Nrf2 expression in the STZ group compared to the control and HT groups, and this decrease was observed in all cells that showed expression. Previous studies have found that streptozotocin decreased renal Nrf2 expression at both the mRNA and protein levels (19,34). Therefore, it can be understood that the reason for decreased Nrf2 expression in the STZ group results directly from the effects of streptozotocin. In the STZ+HT group, which was administered hydroxytyrosol by inducing diabetes with streptozotocin, Nrf2 expression was found to be increased compared to the STZ group. This increase was observed in all cells expressing Nrf2. In diabetic rats, hydroxytyrosol reduces proteinuria by 67-73% and glomerular volume and glomerulosclerosis index by 20-30% (24). Hydroxytyrosol relieves renal hypertrophy index by improving renal dysfunction parameters including creatinine, blood urea nitrogen, serum and urine albumin in serum and urine, and activities of tissue oxidative stress markers and inflammatory cytokines. In addition, hydroxytyrosol increases *Nrf2* mRNA expression and decreases *Keap1* mRNA expression in diabetic rat kidneys (35). In many studies conducted on diabetic kidneys, Nrf2 activated with different agents reduces oxidative stress, inflammation, and fibrosis (13-15). Activated Nrf2 reduces malondialdehyde levels and increases SOD activity, thereby reducing renal damage and extracellular vesicle accumulation (16). Nrf2 activation reduces kidney damage and apoptosis by reducing inflammation and oxidative stress through HO-1 mediation (17,18). In addition, HO-1 induced by Nrf2 relaxes DN by inhibiting apoptosis, angiogenesis, inflammation, and oxidative stress (19). Nrf2 activation is protective for albuminuria and glomerular remodeling observed in DN (15). When these studies are evaluated together, it can be understood that Nrf2 activated by different agents relaxes diabetic nephropathy by reducing oxidative stress, apoptosis, inflammation, fibrosis, and angiogenesis. In addition, hydroxytyrosol has been reported to activate Nrf2 in many different tissues and cells (31-33). It has also been reported that hydroxytyrosol activates *Nrf2* mRNA expression (35). In this study, it is predicted that the Nrf2 protein, which was suppressed in diabetic kidneys with streptozotocin, is activated with hydroxytyrosol and the activated Nrf2 can relax diabetic nephropathy by reducing oxidative stress, apoptosis, inflammation, fibrosis, and angiogenesis.

In conclusion, this study has shown for the first time that hydroxytyrosol, a polyphenolic compound abundant in olive oil and the basis of the Mediterranean diet,

activates the expression of Nrf2 in diabetic kidneys, which protects against kidney damage by reducing oxidative stress, inflammation and fibrosis caused by diabetes. In addition, this study reported in detail the effects of hydroxytyrosol in diabetic kidneys on Nrf2 at the cellular level. Since this study was conducted with an experimental model, the effects of hydroxytyrosol on diabetic nephropathy patients are predictive. Therefore, the course of diabetic nephropathy can be evaluated in future studies in regions where olive and olive oil, which are abundant in hydroxytyrosol, are consumed a lot. In addition, using hydroxytyrosol as an Nrf2 activating agent in diabetes patients can reduce diabetes-related kidney damage. There is certainly a need for new clinical studies to use this on patients.

### Ethical approval

This study has been approved by the Düzce University Animal Experiments Local Ethics Committee (approval date 21/12/2022, number 2022/12/05). Written informed consent was obtained from the participants.

### Author contribution

Concept: HS; Design: HS; Data Collection or Processing: HS, KK; Analysis or Interpretation: HS; Literature Search: KK; Writing: HS. All authors reviewed the results and approved the final version of the article.

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The authors declare the study received no funding.

### Conflict of interest

The authors declare that there is no conflict of interest.

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