Turkish Journal of Agriculture - Food Science and Technology, 12(8): 1359-1365, 2024
DOI: https://doi.org/10.24925/turjaf.v12i8.1359-1365.6573

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

Determination of the Effect of Thymoquinone on DNA Damage in Kidney Cells Treated to High Glucose Depending on Time and Dosage by Comet Assay

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ARTICLE INFO

Research Article

Received : 04.12.2023
Accepted : 04.07.2024

Keywords:
Comet assay
Genotoxicity
NRK-52E
Thymoquinone
High glucose

ABSTRACT

The purpose of this study was to assess the anti-genotoxic potential of thymoquinone (TQ) against DNA damage in NRK-52E cells treated with high glucose using the comet assay technique single cell gel electrophoresis method. Cells were propagated by regular passages in *in vitro* conditions. TQ proliferative concentration (10µM) and IC25 (3rd-hour: 550 mM, 12th-hour: 240 mM, 24th-hour: 200 mM) and IC50 (3rd-hour: 760 mM, 12th-hour: 400 mM, 24th-hour: 280 mM) values for each hour of high glucose and were determined separately with MTT method. At these concentrations, the cells were divided into control(C), Thymoquinone (TQ), high glucose(G) and high glucose plus thymoquinone (GT) groups; It was incubated with the indicated substances for 3, 12, 24 hours. DNA damage was evaluated by applying the comet assay protocol and the results were calculated as DNA damage index (DDI). While DDI levels were observed to be significantly higher ($p<0.05$) in all groups administered high glucose compared to the control, a significant decrease was determined in all groups in which TQ was added along with high glucose. It was determined that high concentrations of glucose had genotoxic effects on kidney cells, and TQ administration together with high glucose, depending on concentration and time, had a significant effect on reducing DNA damage. However, it was concluded that the application of only thymoquinone significantly increased the DDI value compared to the control, and this was a data worth investigating in future studies. Additionally, TQ inhibited DNA damage. These results demonstrated the importance of TQ against nephrotic syndrome with its high antioxidant properties.

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Introduction

Hyperglycemia in patients with diabetes increases free radical formation, and when endogenous antioxidants are not sufficient to balance toxic reactive oxygen species, diabetes-related oxidative stress increases. Oxidative stress is caused by several processes, including glucose auto-oxidation, protein glycation, and the production of advanced glycation end products. This condition leads to many significant diabetic complications, including nephropathy (Habib et al., 2016; Lopez-Sanz et al., 2018). In cells exposed to high glucose, cytotoxicity, reactive oxygen species (ROS) formation, DNA damage, increased lipid peroxidation, and decreased enzymatic and non-enzymatic antioxidants were observed (Subramaniyan & Natarajan 2016). Reactive oxygen species, which increase with oxidative stress, damage cell components, especially lipids, proteins, and DNA. It is known that abasic regions, single and double-strand breaks, DNA-Protein cross-linking, base and sugar modifications caused by oxidative stress cause DNA damage. (Maynard et al., 2009).

TQ, which constitutes the main bioactive component of Nigella sativa (Black Cumin) oil, is a phenolic compound with a 2-methyl-5-isopropyl-1,4-benzoquinone structure with high antioxidant properties (Figure 1). It is the most bioactive compound responsible for the biological effects of black cumin and the most abundant among black cumin essential oils (Pari & Sankaranarayanan, 2009).

In addition to acting as a free radical scavenger, TQ is known to protect the activity of antioxidant enzymes glutathione peroxidase, glutathione-S-transferase, catalase and to have an anti-diabetic effect (Abdelmeguid et al, 2010; Woo et al., 2012; Kurt et al., 2015; Usta & Dede, 2017).

Single-cell gel electrophoresis analysis, a fluorescence microscopic method, sensitively and quickly detects DNA damage at the cellular level. The reason why it is called the comet test is that the damaged DNA moves according to the size of the damage and when stained under the microscope, the damaged part appears as a tail. The method's basic principle is that DNA fragments exiting the

cell move in the direction of the electric current, forming a tail. (Kumaravel, 2009; Boutet-Robinet et al., 2013; Afanasieva & Sivolob, 2018).

Using a single-cell gel electrophoresis comet assay, this study aimed to assess TQ's potential anti-genotoxic effects against potential DNA damage in NRK-52E cells treated with high glucose, taking into account concentration and time.

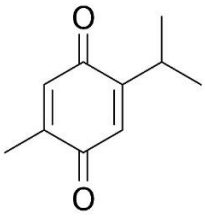


Figure 1. Chemical structure of thymoquinone

Materıal and Methods

Culture Conditions of Cell Line

NRK-52E cells obtained from the American Type Culture Collection (ATCC) were used as live material (Wesel, Germany). Regular passage in DMEM (Capricorn RPMI 1640, Germany) medium supplemented with 10% FBS (Capricorn FBS-11A, Germany), 1% L-glutamine (Capricorn, GLN B, Germany), and 1% penicillin/streptomycin (Capricorn PS-B, Germany) was used to propagate NRK-52E cells in vitro. All cell incubations were carried out throughout the study in a CO2 incubator (Esco Celculture CO2, Singapore) containing 5% CO2 at 37°C.

Preparation of Solutions

Glucose was used to induce high glucose toxicity. Stock 1000 mM glucose (D-glucose, Sigma Aldrich; Saint Louis, USA) solution was resolved in a complete cell culture medium. Thymoquinone was used as a therapeutic agent. TQ (Sigma-Aldrich; Saint Louis, ABD) master stock solution was prepared as 76.8 mM by dissolving it in DMSO (DMSO ≤0.05). From the master stock thymoquinone solution, 1000 µM of TQ was diluted with complete cell culture medium to create an intermediate stock solution. Then, solutions at various concentrations determined from Thymoquinone and glucose stock solutions were prepared by diluting them with the cell medium as necessary.

MTT Assay

Cytotoxic levels of glucose and proliferative levels of thymoquinone were determined in NRK-52E cells by the MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazoliumbromide) method. NRK-52E kidney cells were seeded in 96-well plates with 8000 cells per well. It was left incubated for 24 hours for the cells to adhere to the bottom of the plate. After incubation, the cell medium was replaced with the medium in which glucose and thymoquinone were dissolved in sufficient concentrations. Glucose-containing mediums were prepared in 11 different concentrations between 50 and 800mM. Mediums containing thymoquinone were prepared at 9 different concentrations between 1 and 40 µM. Each dose was administered at least 3 times. Normal medium without glucose and thymoquinone was added to the control wells. Cells were then incubated for 3, 12, 24 hours.

Each well received a 10% MTT solution after the incubation period. Cells were incubated at 37°C for two to four hours. At the end of this period, formosan crystals form in living cells depending on MTT. The well contents were removed. Lysing solution was added to each well to dissolve the formosan crystals. The optical density of the samples was determined at 570 nm with an ELISA microplate reader (Biochrom, Anthos Zenyth-200, UK). In the control group where no substance was treated, cell viability was accepted as 100% and the presented formula was used to calculate the cell viability of other concentrations (Hazman et al., 2021).

$$\text{Cell viability (\%)} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Cytotoxic glucose doses (IC25, IC50) effective in NRK-52E cells were determined by viability rates for each dose. Microsoft Office Excel program was used to calculate of cytotoxic doses. IC50 doses that kill half of the kidney epithelial cells and IC25 doses that are half of this dose have been found. After the cytotoxic concentrations were determined, experimental groups were created and administrations began.

6 different doses of glucose were applied to NRK-52E cells. To determine the possible toxic effects of glucose on NRK-52E cells, the 3rd, 12th, and 24th hour IC25 and IC50 doses used in the study were preferred. In addition to these groups, both glucose and thymoquinone were administered together. In total, a total of 14 groups were studied, including the control group and the thymoquinone-only group (Table 1).

Genotoxicity analysis with comet analysis

NRK-52E cells were seeded in flasks and incubated for 24 hours. Cells were treated with IC25 and IC50 concentrations of glucose alone and its combinations with thymoquinone for 3, 12, and 24 h and only medium (control) and only thymoquinone. The single-cell neutral comet stage was initiated by trypsinization to the cells whose incubation period had expired. For this purpose, the cell suspension was applied to slides treated with agarose. After lysis and electrophoresis steps, it was left to dry. Painted preparations were scored by counting 1000 cells each under a fluorescence microscope using fluorescent dye. DNA damage levels were calculated according to the DNA damage index (DDI) formula. The DNA damage index reflects the number of Arbitrary Units (AU).

$$\text{DDI} = \frac{(0 \times \text{N0}) + (1 \times \text{N1}) + (2 \times \text{N2}) + (3 \times \text{N3}) + (4 \times \text{N4})}{(\text{N0} + \text{N1} + \text{N2} + \text{N3} + \text{N4})} \times 100$$

The number of cells that belong to each damage level is indicated by the letter "N" in the formula (Yüksek et al., 2020a; Yüksek et al., 2020b; Çetin et al., 2021).

Statistical Analysis

The Z-ratio test comparison was used to determine the P values for the study groups (Minitab Statistical Software, 18).

Table 1. Treatment conditions with glucose or thymoquinone applied to the groups

	3 h	12 h	24 h
control			Group1
Thymoquinone			Group2:10 µM
glucose IC25-mM	Group 3: 550	Group 4: 240	Group 5: 200
glucose IC50-mM	Group 6: 760	Group 7: 400	Group 8: 280
glucose IC25-mM+T	Group 9: 550+ 10 µM	Group 10: 240 +10 µM	Group 11: 200 + 10 µM
glucose IC50-mM+T	Group 12: 760 +10 µM	Group 13: 400 +10 µM	Group 14: 280 +10 µM

Table 2. Cytotoxic (IC25, IC50) doses of glucose in NRK-52E cells

Incubation times	glucose IC25 (mM)	glucose IC50 (mM)
3 h	550	760
12 h	240	400
24 h	200	280

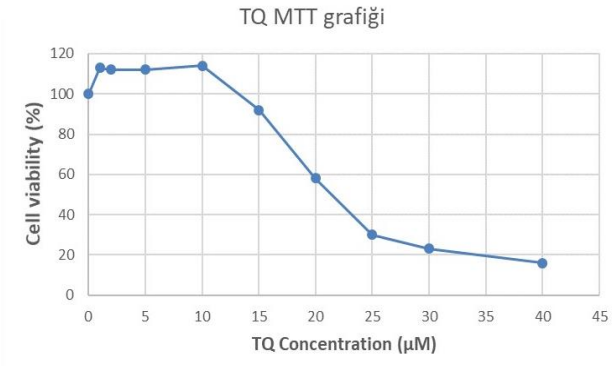


Figure 2. MTT result of TQ treated to NRK-52E cells at 24 hours

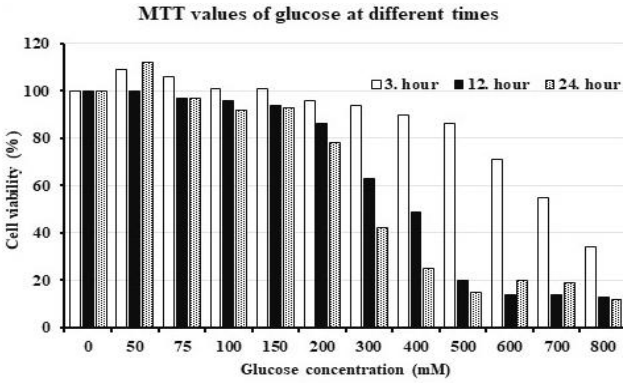
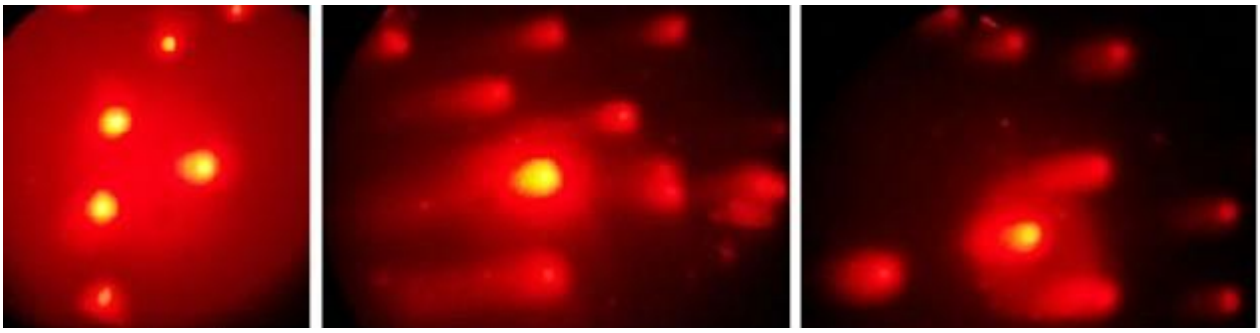


Figure 3. MTT results (%) at various times in glucose-treated NRK-52E cell



Control High glucose High glucose + TQ

Figure 4. Comet images of groups

Results

MTT Results

The proliferative concentration of TQ in NRK-52E cell

Cell viability rates for each dose were calculated by comparing the cells treated with different concentrations of TQ at the 24th hour and the data of the control group are presented in Figure 2.

According to the MTT results at the 24th hour in NRK-52E cells, the proliferative concentration of non-cytotoxic doses of TQ was determined to be 10µM.

Cytotoxic effects of high glucose on NRK-52E cell

Compared to the control group, there is cellular proliferation in cells administered glucose at concentrations up to 100 mM at 3, 12, and 24 hours. It was found that the 200 mM glucose was the starting point for cytotoxicity during all incubation times. It was found that as Figure 3 indicates, cell viability declined with time and concentration.

The IC25 and IC50 values of cytotoxic glucose doses were determined separately for the 3rd, 12th, and 24th hours in NRK52E cells based on the above-mentioned data as follows (Table 2).

As a result of the MTT test, glucose IC25 and IC50 concentrations were obtained at different times presented in Table 2.

Effect of high glucose on genotoxicity in NRK-52E cells

The cells were formed as control (C), Thymoquinone (TQ), high glucose (G), and high glucose+thymoquinone (GT) groups and were incubated at the determined concentrations for 3, 12, and 24 hours. By performing the DNA damage Neutral Comet protocol, the DNA damage index (DDI) parameter was calculated and damage levels were evaluated (Garcia et al., 2011).

Genomic DNA damage results obtained from the groups are presented in Table 3.

Table 3. DNA Damage Index (DDI) levels obtained from groups treated with different concentrations of substances

	Group name	DDI × 100 (AU)
24th hour	Control	16
	TQ	74
3th hour	Glucose IC25	138
	Glucose IC25+TQ	104
	Glucose IC50	171
	Glucose IC50+TQ	123
12th hour	Glucose IC25	192
	Glucose IC25+TQ	106
	Glucose IC50	241
	Glucose IC50+TQ	198
24th hour	Glucose IC25	163
	Glucose IC25+TQ	107
	Glucose IC50	258
	Glucose IC50+TQ	128

Table 4. p values for DNA damage rates based on z ratio comparison

		1		2				3				4			
				G	G	G	G	G	G	G	G	G	G	G	G
		C	T	IC25	IC25+T	IC50	IC50+T	IC25	IC25+T	IC50	IC50+T	IC25	IC25+T	IC50	IC50+T
1	C		0.121	0.207	0.173	0.228	0.194	0.238	0.176	0.254	0.24	0.224	0.177	0.258	0.199
	T			0.001	0.001	0.012	0.001	0.024	0.001	0.059	0.028	0.008	0.001	0.07	0.001
2	G IC25				0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	G IC25+T					0.001	0.001	0.001	0.001	0.012	0.001	0.001	0.001	0.019	0.001
	G IC50						0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	G IC50+T							0.001	0.001	0.002	0.001	0.001	0.001	0.005	0.001
3	G IC25								0.001	0.001	0.001	0.001	0.001	0.001	0.001
	G IC25+T									0.01	0.001	0.001	0.001	0.017	0.001
	G IC50										0.001	0.001	0.009	0.001	0.001
	G IC50+T											0.001	0.001	0.001	0.001
4	G IC25												0.001	0.001	0.001
	G IC25+T													0.016	0.001
	G IC50														0.003
	G IC50+T														

C: Control; 1: 24th hour; 2: 3th hour; 3: 12th hour; d: 24th hour

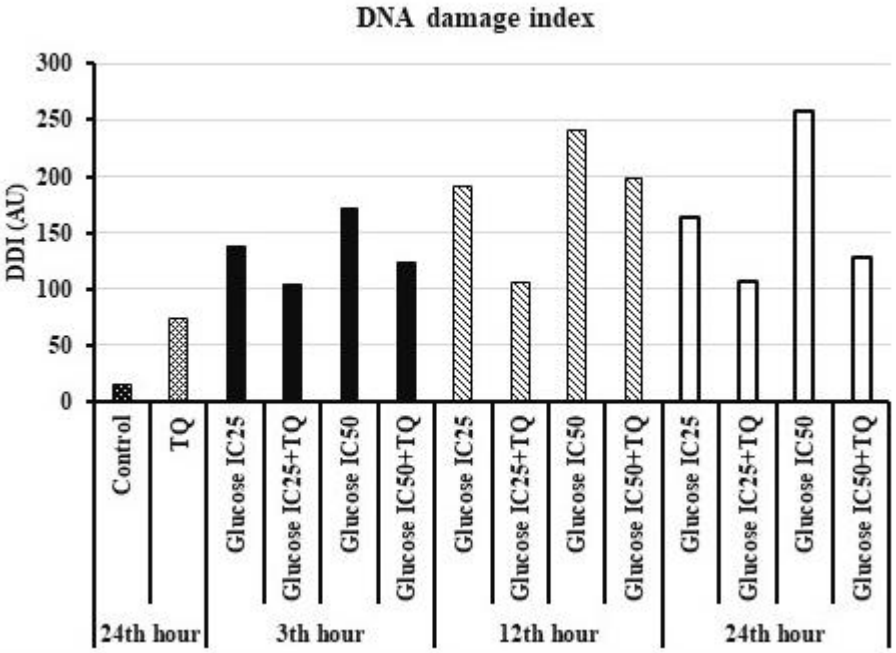


Figure 5. DNA Damage Index (DDI) levels of groups

The data obtained revealed that comet scores by genotoxicity analysis in the groups given glucose at IC25 and IC50 doses in NRK52E cells were higher than the control group (Table 4).

All groups treated with high glucose showed significantly greater ($p<0.05$) DDI levels than the control group; however, all groups treated with thymoquinone also showed significantly lower DDI levels. A significant increase was also detected only in the thymoquinone group compared to the control ($p<0.05$). The difference from the control group in all groups given high glucose at 3, 12, and 24 hours was found to be statistically significant ($p<0.05$). The difference between the groups from thymoquinone was also found to be statistically significant ($p<0.05$).

Discussion

Although it is known that high glucose causes DNA damage in various *in vivo* studies, *in vitro* studies showing the effect of TQ on this damage are limited. In this study, a high glucose-induced early-stage diabetic nephropathy model in the kidney cell line was used *in vitro*. NRK52E cells are rat kidney proximal tubular epithelial cell lines.

Approximately one-third of patients with diabetes suffer from diabetic nephropathy (DN), the main cause of kidney disease. Nephropathy is associated with hyperglycemia and impaired glucose metabolism. Excessive kidney damage occurs during diabetes by causing microalbuminuria, an increase in glomerular filtration rate, and kidney damage index. Hyperglycemia, which causes dysfunction and abnormal growth of glomerular and tubular cells, causes functional and structural changes in the progression of diabetic nephropathy (Samikkannu et al., 2006). Reactive oxygen species, chronic hyperglycemia, advanced glycation end products, and inflammatory cytokines are the main factors that cause the development and emergence of DN. By triggering a series of signaling pathways such as Akt, AMPK, and mTOR, it causes impaired glomerular filtration function, glomerular basement membrane thickening, mesangial expansion, accumulation of proteins such as extracellular matrix and fibronectin (Wu et al., 2023). The most harmful microvascular consequence of diabetes and the primary cause of chronic kidney disease globally is still diabetic nephropathy. Because it arises from unclear mechanisms, effective treatments are limited. Therefore, more studies on the mechanisms of diabetic nephropathy should be conducted.

It was observed that high-density lipoprotein decreased and fasting blood sugar, urine sugar, total cholesterol, triglyceride, urine volume and low-density lipoprotein parameters increased in diabetic rats (Gondi et al., 2015). Free radicals formed in diabetes increase the oxidation of lipids, proteins and nucleic acids by disrupting the function and structure of the mitochondrial electron transport chain (Kim et al., 2012). DNA modifications that can become permanent are one of the most important consequences of Oxidative stress, which can cause some genomic mutations and cellular dysfunction (Devi et al., 2013; Sun et al., 2016; Poetsch, 2020). High glucose leads to the formation of harmful products such as ROS, oxidative stress, lipid peroxidation and therefore MDA. High glucose causes an increase in DNA strand breaks as a result of the intracellular methylation reaction with the production of nitric oxide and free radicals. Hydrogen peroxide causes the DNA chain to break by producing radicals with hydroxyl content near the DNA molecule through the Fenton reaction (Orsolic et al., 2011; Pessôa et al., 2012; Hou et al., 2014). It has been claimed that increased micronucleus frequency in type 2 diabetes mellitus patients can be considered an important biomarker of genotoxic damage (Smail, 2023). The increase in the amount of 8-OHdG (8-hydroxy-2-deoxy guanosine), which indicates oxidative DNA damage, occurs due to the destruction of nuclear and mitochondrial DNA by free radicals. It is one of the markers used to determine DNA damage levels (Othman et al., 2013; Karahan et al., 2018; Bazyel et al., 2019; Yaycı et al., 2021). In this study, the degree of DNA damage was examined using comet analysis. For more reliable and sensitive results, the alkaline Comet Assay technique is mostly used in studies on genotoxicity. The aim is to detect damage at the cellular level by examining cell DNAs one by one. This indicates that the amount of genetic material damaged determines how big the comet's tail is (Alam et al., 2015; Yu et al., 2016; Cui & Yu, 2018; Usta et al., 2024).

It can be hypothesized that defects following DNA damage from high glucose may cause premature aging in different kidney cells and further promote the progression of kidney diseases (Xiong & Zhou, 2019). DNA damage can cause various post-translational modifications that can activate the p53 gene and increase its ability to promote apoptosis (Verma et al., 2010). For diabetic renal tubular epithelial cells, hyperglycemia inhibition helps control DN

For diabetic renal tubular epithelial cells, hyperglycemia inhibition helps control DN (Nakamichi et al., 2021). Glycemic control is the main determinant to prevent progression to DN. Diabetic nephropathy lesions are reversible and these effects can be reduced by the ability of the kidney to remodel upon prolonged normoglycemia. The role of DNA damage and repair is important in the development of diabetic complications (Varun et al., 2023). In patients with type 1 diabetes, a genetically superior DNA damage repair protects against the progression of complications (Bhatt et al., 2015).

Since DN is reversible in its early stages, phytotherapy is an option for prevention and early treatment (Ping et al., 2013). The safety profile of the phytochemical to be used must be evaluated, which necessitates toxicological studies such as cell cytotoxicity and DNA damage analyses. Thymoquinone shows pharmacological activities in many disorders, including diabetes, nervous and gastrointestinal disorders, kidney and liver disorders, cancer and related inflammatory diseases, rheumatism, and allergies (Talebi et al., 2021; Yılmaz et al., 2021). For this purpose, this study attempted to find the scientific basis for the importance of thymoquinone use in diabetic nephropathy.

There have been some studies showing the beneficial and curative effects of TQ administration in the treatment of nephrotoxicosis experimentally produced by different factors (Usta & Dede, 2017; Aybastıer et al., 2018; El-Shemi et al., 2018; Gümüş et al., 2018). In various *in vivo* studies, a significant increase in the level of 8-OHdG, which indicates DNA damage, was noted in diabetic groups compared to the control group (Simone et al., 2008; Al-Aubaidy et al., 2011). It has been shown that the use of low doses of thymoquinone,

which is not cytotoxic in cancer cells, can reduce the cytotoxic and genotoxic effects caused by etoposide (Çelebioğlu et al., 2022). In this study, high glucose (HG) caused abnormal DNA methylation in cell lines, consistent with *in vivo* studies. TQ inhibited DNA damage. These results demonstrated the importance of TQ against nephrotic syndrome with its high antioxidant properties.

Conclusion

As a result, it was determined that high concentrations of glucose had genotoxic effects on kidney cells, and TQ administration together with high glucose, depending on concentration and time, had a significant effect on reducing DNA damage. However, it was concluded that the application of only thymoquinone significantly increased the DDI value compared to the control, and this was data worth investigating in subsequent studies. It was predicted that thymoquinone could reduce DNA damage by blocking hyperglycemia, oxidative stress, apoptosis, and inflammatory processes. It may be an important strategy to prevent and protect against complications caused by high glucose in the early stage of kidney damage.

Declarations

Funding Statement: The author received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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