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Neuroprotection Effect of Quercetin on TNF-α Levels and Gene Expression of Caspase 3 in MPTP-Induced Male NMRI Mice

Neda Nikokalam Nazif [1], Maryam Khosravi [1,*], Ramesh Ahmadi [2], Maryam Bananej [1] and Ahmad Majd [1]

[1]Department of Biology, Faculty of Biological Sciences, North-Tehran Branch, Islamic Azad University, Tehran, Iran
[2]Department of Physiology, Qom Branch, Islamic Azad University, Qom, Iran

[*]*Corresponding author*: Department of Biology, Faculty of Biological Sciences, North-Tehran Branch, Islamic Azad University, Tehran, Iran. Email: maryam-khosravi@iau-tnb.ac.ir

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Abstract

Background: Parkinson's disease is a progressive nervous system disorder caused by a degenerative loss of dopaminergic neurons of midbrain, from the substantia nigra to the corpus striatum pathway. Quercetin has a neuroprotective effect to prevent the greater loss of substantia nigra dopaminergic neurons in Parkinson's disease model.
Objectives: This study aimed to investigate the effect of the flavonoid quercetin on the behavioral test in 1-methyl-4-phenyl,2,3,6-tetrahydropyridine(MPTP)-induced male NMRI mice.
Methods: Animals were divided into eight groups (n = 12). Behavioral tests of bar test and treatment with quercetin began one day after inducing the disease and lasted for 35 days. Then, brains were excluded from craniums for histology, immunohistochemistry tyrosine hydroxylase, measurement of TNF-α levels, and gene expression of caspase 3.
Results: Data showed that orally taking quercetin for 35 days improved the behavioral test of bar tests in Parkinson's disease. Cell density in TH staining was counted and showed considerable decreases in the substantia nigra in Parkinson's disease group (83.67 $\pm$ 12.811) while it was higher in quercetin-treated groups PD + Q1 (103.67 $\pm$ 8.090) and PD + Q2 (145.33 $\pm$ 13.908) than in Parkinson's disease group ($P < 0.05$). Quercetin decreased inflammation due to MPTP in the substantia nigra in PD + Q1 (1395.73 $\pm$ 1.058) and PD + Q2 (1250.66 $\pm$ 1.95), and corpus striatum in PD + Q1 (1207.033 $\pm$ 2.228) and PD + Q2 (1187.44 $\pm$ 1.64) and TNF-α protein levels in the quercetin-treated group ($P < 0.05$). Parkinson's disease decreased gene expression of caspase 3 (0.35 $\pm$ 0.019) and increased it in quercetin-treated groups PD + Q1 (1.26 $\pm$ 0.062) and PD+Q2 (2.27 $\pm$ 0.144) ($P < 0.0001$).
Conclusions: Quercetin is a natural flavonoid with neuroprotection effect and antioxidant, anti-inflammatory, and anti-apoptosis properties preventing the loss of dopaminergic neurons in mice with Parkinson's disease.

Keywords: Parkinson's Disease, MPTP, Quercetin, TNF-α, Caspase 3

1. Background

Parkinson's disease is a neuromuscular disorder caused by brain cell atrophy in people over 60 years of age (1). Losing dopaminergic neurons in the substantia nigra and unusual protein aggregation of Lewy body inside neurons are the main pathologic characteristics of Parkinson's disease (2). Performance can change in ganglia circuits resulting from a lack of dopaminergic neurons, causing motor and cognitive disorders (3). In a previous study, Duty and Jenner (4) considered inflammatory factors as the main players in pathogenesis. In the brain, neurotoxin MPTP is oxidized to produce MPP + and is transferred to dopaminergic neurons by the dopamine transmitter; this destructs dopaminergic neurons in the substantia nigra and decreases dopamine in the nigrostriatal pathway (5).

Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a member of flavonol family with molecule formula $C_{15}H_{10}O_7$ richly found in vegetables, onion, soy, herbs, tea, and grains. It protects the brain and heart. Being full of anti-inflammatory and anti-oxidant materials, quercetin has anti-cancer and anti-apoptosis properties and protects dopaminergic neurons against inflammatory inflammatory injuries evoked by lipopolysaccharide; it also prevents from producing nitric oxide due to its anti-microglia activity (6).

As a pre-inflammatory cytokine, TNF-α is named the tumor necrosis factor. Pathogens of Parkinson's disease have commonly caused inflammation which removes dopaminergic neurons in substantia nigra pars compacta (2). It is suggested that TNF-α and its two receptors (i.e.

TNFR1 and TNFR2) play roles in Parkinson's disease owing to the activation of glial cells.

Accordingly, this happens as a result of producing the inflammatory cascade through releasing the pro-inflammatory cytokine, such as TNF-α and Interleukins (IL-1β, IL-6), inflammatory mediators, nitric oxides (NOs) generated by nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) through activating nuclear factor kappa-light-chain-enhancer (NF-κB) and mitogen-activated protein kinase (MARK).

This cascade has caused the aggregation of nitric oxide and totally removal of dopaminergic neurons.

Moreover, the amount of pro-inflammatory cytokine, including TNF-α and IL-1b has increased in substantia nigra pars compacta of patients with Parkinson's disease (7).

Caspase plays an important role in cell apoptosis and cell cascade activated by caspase. Caspase 3 is a promoter of apoptosis of dopaminergic neurons in the process of caspase cascade in Parkinson's disease (8). In rodents, MPTP-induced disease activates caspases 3, 8, 9, 11, and DNA fragmentation of substantia nigra pars compacta. Concerning the role of caspase in the MPTP-produced toxicant metabolite, it is reported that caspase leads to the death of nerve cells (9).

2. Objectives

This study aimed to investigate the effect of the flavonoid quercetin on the behavioral test, immunohistochemistry of tyrosine hydroxylase (TH), TNF-α levels, and gene expression in 1-methyl-4-phenyl,2,3,6-tetrahydropyridine(MPTP)-induced male NMRI mice.

3. Methods

3.1. Animals

This experimental study examined 96 male NMRI mice in the Physiology Laboratory of Islamic Azad University in Qom, Iran, in 2018. Animals (23 $\pm$ 25 g) were purchased from Karaj Pasteur Institute. They were kept at room temperature (23 $\pm$ 2°C) and humidity (40% - 45%) in plastic cages covered with sawdust in the animal's room. A 12-h light/dark cycle was constantly maintained and animals had free access to water and food. Mice were kept based on the guidelines for animal use approved by the Islamic Azad University and the Helsinki Declaration. This research was carried out after receiving ethical approval from the Ethics Committee of the Islamic Azad University, Science and Research Branch.

3.2. Experimental Groups

Male NMRI mice were randomly divided into eight groups (n = 12). After creating the model and quercetin therapy, they were treated for 35 days. The groups included group 1, control (C); group 2, saline (S); group 3, DMSO + corn oil (DO); group 4, orally treated with 20 mg/kg quercetin (Q1); group 5, orally treated with 30 mg/kg quercetin (Q2); group 6, receiving intraperitoneal administration of MPTP (PD) (25 mg/kg body weight) for four days; group 7, receiving MPTP and 20 mg/kg quercetin (PD + Q1); and group 8, receiving MPTP and 30 mg/kg quercetin (PD + Q2).

3.3. Drug and Treatment

Over the course of four consecutive days, the Parkinson's disease groups received intraperitoneal drug MPTP-HCL (Sigma-Aldrich) dissolved (25 mg) in 5 cc normal saline, at a dose of 25 mg/kg body weight. One gram of quercetin (Sigma-Aldrich) was dissolved in 5% dimethyl sulfoxide in corn oil and each animal was orally gavaged with 20 and 30 mg/kg of the prepared solution for 35 consecutive days. Materials used in the study included ketamine and xylazine (Sigma), formalin 4% (Sigma), Paraformaldehyde (Sigma), Triton X-100 (Abcam), rabbit anti-TH (Abcam), anti-rabbit IgG (Abcam), ELISA kit TNF-alpha (TNF-α; Abeam), DEPC water, RNA extraction kit (Ribospin; GeneAIL), and Primer (Macrogene).

3.4. Bar Test

The bar test was used to evaluate catalepsy in rodents. Animals were placed on a platform and their hands were gently placed on the bar graph. Then, the time animals remained in this situation was recorded. More severe catalepsy was indicated by the longer time they stayed in the position.

3.5. Immunohistochemistry of Tyrosine Hydroxylase (TH) Staining

An anti-tyrosine hydroxylase antibody was used to confirm the destruction of dopamine neurons of the substantia nigra section due to damage by the neurotoxin. To perform this technique, brain tissues were isolated and put in formalin-saline for 24 h, followed by dewatering and processing steps. Tissues were molded in molten paraffin. After 24 h of molding and full cooling, the molds were ready to cut. Cutting was performed by a rotary microtome usually with a thickness of 10 microns. Sections were studied and photographed with a Medacm 107n microscope and Dino-Lite camera using Dino Capture 2.0 software. After immunohistochemical staining, the tissue status and the number of dopaminergic neurons of the substantia nigra were determined and analyzed. Neural neurons were

counted by Image-Pro Plus (V.6) software. In this study, points with a size of greater than 7 μm were considered as nuclei of neural neurons.

3.6. Quantitative Measurement of TNF-α Levels by ELISA

By removing the brain tissue and with respect to the Paxinos and Watson mouse Atlas, the brain regions of the substantia nigra and the corpus striatum were quickly removed from other parts of the brain and placed in liquid nitrogen. After freezing, the tissue was stored in a refrigerator at -80°C. After homogenization and centrifugation, the TNF-α protein level was measured by the Abcam company's ELISA Mouse TNF-α kit (ab108870) with the Elisa reader Stat Fax 2100, produced in France, at a wavelength of 450 nm, compared to a Blank reagent.

3.7. Real-Time PCR

The brains of mice were excised and the RNA was extracted according to the Gene All Extraction Kit (Sinagene-Iran). Then, RNA was prepared. The cDNA was synthesized by the HyperScript TM RT Premix kit (with Random Hexamer). A couple of primers was designed for caspase 3 to determine whether the cDNA was extracted properly or not (Table 1). Synthesized cDNA in different groups, in the presence of specific primers caspase 3, underwent real-time PCR by Mister Mix. In this study, a quantitative real-time PCR technique was used to measure the gene expression by the SYBER GREEN method. To calculate the target gene expression with the reference, we used the relative quantitative method and the $\Delta\Delta$CT method. Also, the *GAPDH* gene was selected as the internal control gene.

3.8. Statistical Analysis of Data

Data were analyzed by one-way Analysis of Variance (ANOVA) and the significant differences between the groups were measured by Tukey post hoc test, bootstrap, and SPSS version 22 software. All data were presented as mean $\pm$ SD and $P < 0.05$ was considered statistically significant. ImageJ software was used for neuronal counting.

4. Results

4.1. Bar Test

The difference found between C and S groups was not significant over time. The mean of catalepsy in DO, Q1, Q2, PD, PD + Q1, and PD + Q2 groups changed during the study; that is, the mean of catalepsy decreased during 35 days. However, the oral administration of quercetin daily in first, 7th, 14th, 21th, 28th, and 35th days has represented a significant reduction in the catalepsy within the period of MPTP induction (Figure 1).

4.2. Immunohistochemistry TH

We observed that MPTP significantly reduced the number of dopaminergic neurons as compared to that of the C group (151.67 $\pm$ 18.187). However, the number of dopaminergic neurons reduced in PD + Q1 group) 103.67 $\pm$ 8.09 (and did not significantly change in PD + Q2 group (145.33 $\pm$ 13.908) compared to that of the control group and increased in these groups compared to that in the Parkinson's disease group (Table 2, Figure 2).

4.3. Effect of Quercetin on Inflammatory (TNF-α)

The TNF-α basal protein levels were measured in the substantia nigra and corpus striatum. The MPTP markedly increased in basal protein levels of TNF-α in the substantia nigra (1354.17 $\pm$ 1.832) and corpus striatum (1272.03 $\pm$ 1.203) in the PD group as compared to the C group (1193.3 $\pm$ 4.636 in the substantia nigra and 1062.03 $\pm$ 2.242 in corpus striatum; $P< 0.05$). There was a significant difference in the mean value of substantia nigra (1354.17 $\pm$ 1.832) and corpus striatum (1272.03 $\pm$ 1.203) in the PD group compared to that in C (1193.3 $\pm$ 4.636 in substantia nigra and 1062.03 $\pm$ 2.242 in corpus striatum), S (1191.6 $\pm$ 4.501 in substantia nigra and 1060.8 $\pm$ 1.998 in corpus striatum), DO (1191.67 $\pm$ 4.068 in substantia nigra and 1067.07 $\pm$ 2.038 in corpus striatum), Q1 (1183.27 $\pm$ 4.443 in substantia nigra and 1056.2 $\pm$ 2.665 in corpus striatum), Q2 (1182.37 $\pm$ 3.67 in substantia nigra and 1006.33 $\pm$ 2.45 in corpus striatum), PD + Q1 (1295.73 $\pm$ 1.06 in substantia nigra and 1207.03 $\pm$ 2.228 in corpus striatum), and PD + Q2 (1250.67 $\pm$ 1.955 in substantia nigra and 1187.74 $\pm$ 1.645 in corpus striatum) groups ($P > 0.05$). In Q1 (1183.27 $\pm$ 4.443) and Q2 (1182.37 $\pm$ 3.67)-treated mice, TNF-α basal protein levels in the substantia nigra remained similar to those of C group (1193.3 $\pm$ 4.636) while decreased in the corpus striatum compared to that of C (1062.03 $\pm$ 2.242), PD + Q1 (1207.03 $\pm$ 2.228), and PD + Q2 (1187.74 $\pm$ 1.645) groups. The basal protein levels of TNF-α increased and decreased in the substantia nigra and corpus striatum compared to those in the control and Parkinson's disease groups, respectively (Tables 3 and 4).

4.4. RT-PCR Analysis

The results showed the highest caspase 3 expression in the Q2 group (4.71 $\pm$ 0.251 times) and the lowest in PD groups (0.35 $\pm$ 0.019 times). There was no significant difference between C, S, DO, and PD + Q1 groups but a significant difference was observed between other groups ($P < 0.0001^{***}$) (Figure 3).

5. Discussion

One of the most common neurogenerative disorders resulting in removing dopaminergic neurons in substan-

Table 1. The Sequence of Designed Primers Used for Real-Time Polymerase Chain Reaction

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Product Length, bp
Caspase 3	GTGTGCGAGATGAGGTGTTG	GCAGCAGCAACAGCAGACTA	107
GAPDH	AAGGTCATCCCAGAGCTGAA	CCCACTGCTCAGGTCACAC	222

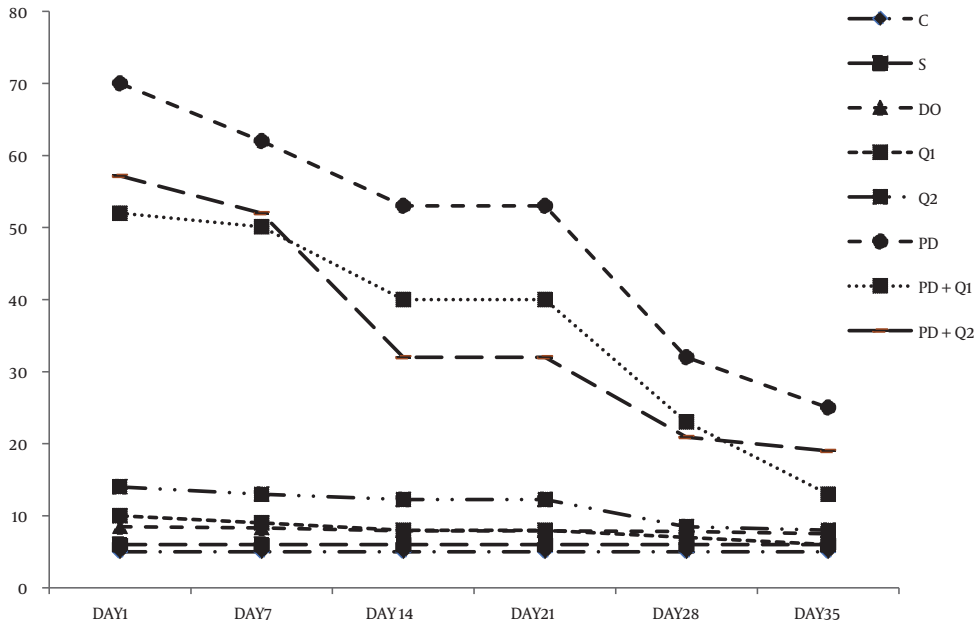

Figure 1. Effect of quercetin on catalepsy reducing MPTP-induced motor dysfunction as determined in the bar test. Catalepsy in days 1, 7, 14, 21, 28, and 35 in groups. The results are shown as mean ± standard deviation (SD), n = 12.

Table 2. Effect of Quercetin on the Number of DA Neurons Stained with Immunohistochemistry of Tyrosine Hydroxylase in the SNpc[a, b, c]

Group	Values	BCa 95% CI
C	151.67 ± 18.187^{B}	(133 - 170.33)
S	106 ± 5.859^{AB}	(99.33 - 112.67)
DO	98.67 ± 11.348^{AB}	(87 - 110.33)
Q1	123.33 ± 9.905^{AB}	(112 - 134.67)
Q2	112.67 ± 9.955^{AB}	(101.33 - 124)
PD	83.67 ± 12.811^{A}	(69 - 98.33)
PD + Q1	103.67 ± 8.09^{AB}	(94.33 - 113)
PD + Q2	145.33 ± 13.908^{B}	(130.67 - 160)

Abbreviations: BCa, bias-corrected accelerated; CI, confidence interval.
[a]Values are expressed as mean ± SD.
[b]F value = 3.892 and P value = 0.012.
[c]Dissimilar letters indicate significant differences between the groups ($P < 0.05$).

Table 3. Effect of Quercetin on Basal Protein Levels of TNF-α in Substantia Nigra in Groups[a, b, c]

Group	Values	BCa 95% CI
C	1193.3 ± 4.636^{A}	(1187.97 - 1198.63)
S	1191.6 ± 4.501^{A}	(1187.03 - 1196.17)
DO	1191.67 ± 4.068^{A}	(1187 - 1196.33)
Q1	1183.27 ± 4.443^{A}	(1178.37 - 1188.17)
Q2	1182.37 ± 3.67^{A}	(1178.3 - 1186.43)
PD	1354.17 ± 1.832^{D}	(1352.07 - 1356.27)
PD+Q1	1295.73 ± 1.06^{C}	(1294.57 - 1296.9)
PD+Q2	1250.67 ± 1.955^{B}	(1248.43 - 1252.9)

[a]Values are expressed as mean ± SD.
[b]F value = 330.986 and P value = 0.000.
[c]Dissimilar letters indicate significant differences between the groups ($P < 0.05$).

tia nigra pars compacta is Parkinson's disease with symptoms like shaking in resting tremor, bradykinesia, flexibility, rigid muscles, and problems in walking (1). In the animal model, MPTP first causes the loss of nerve terminals of dopaminergic in stratum, then leading to the loss of cells in substantia nigra pars compacta. MPTP is an active

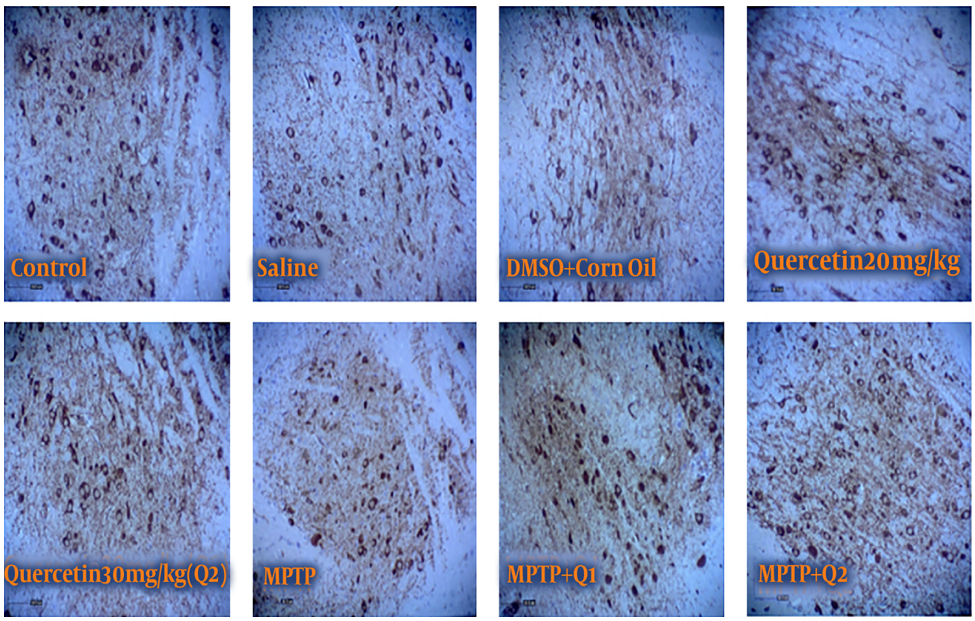

Figure 2. The number of DA neurons in SNpc in groups. Cont, scale bar = 50 μm; 10 ×.

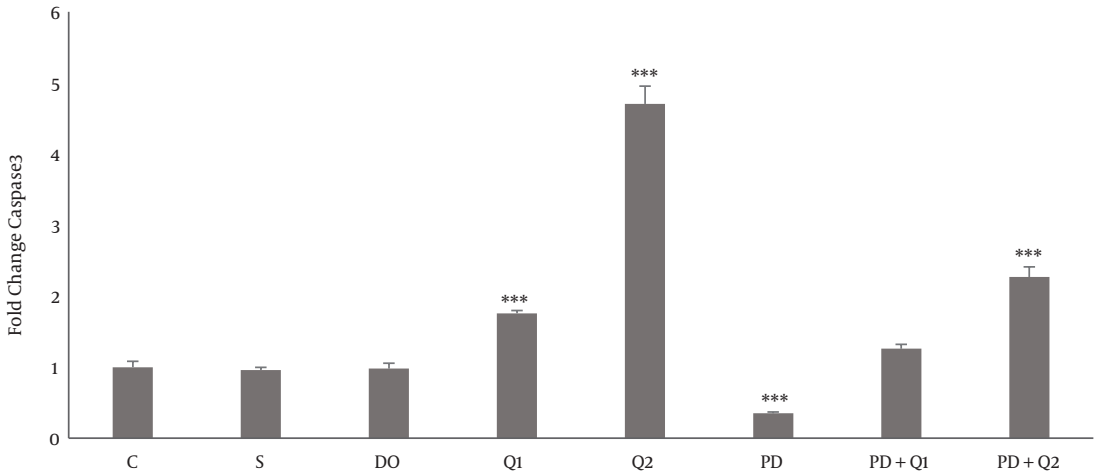

Figure 3. Quercetin caspase 3 gene expression in mice by MPTP. Mice were treated with MPTP (PD) for four days and different concentrations of Q1 and Q2 for 35 days. After treatment, mRNA was extracted from each group; quantitative real-time PCR analysis of caspase 3 gene was performed. The results are expressed as the fold-change calculated by the relative Ct method using GAPDH as the internal reference. Each bar represents mean ± SEM ($P < 0.0001^{***}$).

metabolite; it becomes MPP^+ to enter mitochondria and join mitochondria complex I; then, it inhibits the transmission of electrons from ubiquinone, leads to disorders in producing free radicals and decreases the ATP synthesis, finally leading to apoptosis (5). This study showed that orally taking quercetin for 35 days was effective and improved the motor performance by catalepsy test and prevented cell death due to MPTP injection in immunohistochemistry, decreased protein levels of TNF-α, and increased gene expression of caspase 3 in mice with Parkinson's disease. Quercetin has properties like neuroprotection in striatal neurons, which passes blood-brain barriers (10).

Denny Joseph and Muralidhara (11) believes that quercetin can decrease behavioral changes resulting from rotenone. The behavioral test of catalepsy showed that

Table 4. Effect of Quercetin on Basal Protein Levels of TNF-α in Corpus Striatum in Groups

Group	Values	BCa 95% CI
C	1062.03 ± 2.242^{BC}	(1059.53 - 1064.53)
S	1060.8 ± 1.998^{BC}	(1058.5 - 1063.1)
DO	1067.07 ± 2.038^{C}	(1064.73 - 1069.4)
Q1	1056.2 ± 2.665^{B}	(1053.17 - 1059.23)
Q2	1006.33 ± 2.45^{A}	(1003.6 - 1009.07)
PD	1272.03 ± 1.203^{F}	(1270.67 - 1273.4)
PD + Q1	1207.03 ± 2.228^{E}	(1204.57 - 1209.5)
PD + Q2	1187.74 ± 1.645^{D}	(1185.37 - 1189.37)

[a]Values are expressed as mean $\pm$ SD.
[b]F value = 1991.525 and P value = 0.000.
[c]Dissimilar letters indicate significant differences between the groups ($P < 0.05$).

orally taking quercetin for 35 days decreased catalepsy duration due to MPTP in the PD + Q1 and PD + Q2 groups (Figure 1).

Kappock and Caradonna (12) stated that Tyrosine Hydroxylase (TH) is a dopamine rate-limiting enzyme. After inducing the disease with MPTP, neurons and striatal fibers were investigated to determine the dopamine transmitter and the amount of losing dopaminergic neurons by TH (13). Zbarsky et al. (14) reported that the neuro-protective effect of quercetin on injuries received by the central nervous system, actually as a result of the formation the neurotoxin. MPTP-induced disease and orally taking quercetin for 14 days (50 - 200 mg/kg) decreased the activity of antioxidant enzymes, ATPase, acetylcholine esterase, and levels of 4-dopamine and 2-hydroxy (15). This study showed its neuroprotection effect prevented from dopaminergic neuron depletion due to MPTP. The flavonoid quercetin in treated animals inhibited removing TH + cells in the substantia nigra. In animals of Parkinson's disease model with MPTP, the number of dopaminergic neurons decreased. In animals treated by quercetin, the number of dopaminergic neurons increased in PD + Q1 and PD + Q2 groups compared to the Parkinson's disease group (Figure 2, Table 2).

Parkinson's disease model activated the microglia and increased the production of cytokine and apoptosis before decreasing dopamine neurons of the substantia nigra (2). The inhibition of the activation of microglia with neuroprotection effect has been proven (7). It has been shown that in the MPTP Parkinson's disease model, flavonoids changed the protein levels of TNF-α and IL-1B. TNF-α is a cell-signaling protein (cytokine) involved in inflammatory cascades and its inhibition is the curative purpose in neurogenic disorders. In this study, MPTP increased the TNF-α levels in the substantia nigra (1354.17 ± 1.832) and corpus striatum (1272.03 ± 1.203) of the PD group compared to the C group (1193.3 ± 4.636 in the substantia nigra and 1062.03 ± 2.242 in corpus striatum). Quercetin with anti-inflammatory and anti-oxidant properties decreased the inflammation due to MPTP and protein levels of TNF-α in the substantia nigra and corpus striatum in PD + Q1 (1295.73 ± 1.06 in substantia nigra and 1207.03 ± 2.228 in corpus striatum) and PD + Q2 (1250.67 ± 1.955 in substantia nigra and 1187.74 ± 1.645 in corpus striatum) groups (Tables 3 and 4).

Development of Parkinson's disease typically associates with an increase or decrease in components of proteasome activity and caspase activity, respectively (16). After MPTP injection in the disease model, the activation of caspase 3 and apoptosis occurs in the early stages of disease (17). In other stages, the activation of microglia and Astrocyte occurs in substantia nigra (18). Prema et al. (19) reported that the injection of MPTP increased gene expression of caspase 3, 8, and 9. But, in the current study, gene expression of caspase 3 decreased by injection of MPTP and quercetin increased it. In a study, Chen et al. (20) and Angeloni et al. (21) concluded that quercetin decreased the activity of caspase 3 in Parkinson's disease. In this study, by MPTP injection in the PD group, the lowest gene expression of caspase 3 was 0.35 ± 0.019 times and groups treated with Q2 had the highest gene expression of caspase 3 (4.71 ± 0.251 times) while the PD + Q1 group had a value of 1.26 ± 0.062 times. In the PD + Q2 group, it increased by 2.27 ± 0.144 times more than the C group. Quercetin antiapoptotic properties prevent apoptosis of dopaminergic neurons induced by MPTP (Figure 3).

5.1. Conclusions

Quercetin has anti-apoptosis properties preventing apoptosis of dopaminergic neurons due to MPTP. It has properties like neuroprotective, antioxidant, anti-inflammatory, and anti-apoptosis properties, which can increase the cell neuron survival. MPTP significantly decreases brain dopamine. The results from the study showed that orally taking quercetin improved catalepsy, prevented losing dopaminergic neurons due to MPTP in substantia nigra, decreased TNF-α levels, and increased gene expression of caspase 3. Accordingly, orally taking quercetin for 35 days may be a new approach to protect the life quality of patients with Parkinson's disease.

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Footnotes

Authors' Contribution: Neda Nikokalam Nazif, Maryam Khosravi, and Ahmad Majd designed the experiment. Neda Nikokalam Nazif and Maryam Bananej gathered data. Neda Nikokalam Nazif analyzed and interpreted data. Neda Nikokalam Nazif and Maryam Bananej drafted the manuscript. Neda Nikokalam Nazif and Ahmad Majd critically revised the manuscript for important intellectual content. Neda Nikokalam Nazif statistically analyzed the data. Neda Nikokalam Nazif conducted administrative, technical, and material support of the study. Maryam Khosravi and Ramesh Ahmadi were study supervisors and Maryam Bananej and Ahmad Majd were advisors.

Conflict of Interests: The authors declare no conflicts of interest.

Ethical Approval: The Ethics Committee of the Islamic Azad University, Science and Research Branch, approved the study (ethic code: IR.IAU.SRB.REC.1397.176, 2019-03-12).

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