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The Evaluation of Vitamin E and TiO_2 Nanoparticles Administration in Parkinson's Rat Model

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Abstract

Objective: Parkinson's disease (PD) is a severely debilitating disease for which no suitable treatment has been found so far. In recent years, nanoparticles (NPs) have shown therapeutic potential in PD. Thus, in the current research, for the first time, we investigated the effects of vitamin E and TiO_2 nanoparticles (TiO_2-NPs) on a rat model of PD.

Materials and Methods: In this experimental study, after preparation and induction of PD, rats were administrated with vitamin E and TiO_2-NPs. One day after receiving the last dose of treatments, rats were killed and substantia nigra was extracted from the brain and its cell suspension was prepared. In each group, female rats were mated, and after confirmation that the female rats were pregnant by vaginal smear test, the fetus was removed. Cell viability was studied by the MTT method and apoptosis, and necrosis were studied by the flow cytometry technique. Also, after RNA extraction and cDNA synthesis, the expression of *Bcl-2* and *circRNA 0001518* genes were studied using the real time polymerase chain reaction (RT-PCR) technique. For analyzing the data, two-way ANOVA was used.

Results: The results showed a sharp decrease in cell viability in female PD rats and fetuses resulting from PD female rats. Vitamin E treatment showed the greatest effect on increasing cell viability. Decreased expression of the *Bcl-2* gene and increased expression of circRNA 0001518 were observed in PD conditions.

Conclusion: Administration of vitamin E may be a good option for reducing PD-induced cell death.

Keywords: Cyclic RNAs, Nanoparticle, Parkinson's Disease, TiO_2, Vitamin E

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Introduction

Parkinson's disease (PD) is one of the severely debilitating diseases, which mainly affects the dopaminergic system of the substantia nigra -striatum pathway and is characterized by four prominent features: muscle stiffness, slowness of movement (bradykinesia), tremor, and gait disturbance (1). Regarding the pathological mechanisms and the cause of neuronal death of dopaminergic cells of the substantia nigra -striatum pathway in this disease, several hypotheses have been proposed, including mitochondrial complex defects (2), iron accumulation (3), and increased free radicals (4). There is ample evidence that oxidative stress eventually leads to neuronal degeneration in PD. Protection against oxidative damage caused by free radicals in the central nervous system is provided by low molecular weight antioxidants including vitamins E and C (5). Because vitamin E is a free radicals scavenger from the brain, its protective role is a new issue in the treatment of degenerative diseases of the nervous system. A study of the animal and clinical model of PD showed that a deficiency of antioxidants such as vitamin E can cause the dopaminergic system to degenerate, leading to PD symptoms (6).

Mitochondrial autophagy is one of the mechanisms of PD. Also, mitochondrial-induced apoptosis plays an important role in which decreased expression of Bcl-2 protein and increased Bax protein expression have been reported (7). In this process, circRNAs can play regulatory functions due to their miRNA sponge action. For example, recently the anti-apoptotic properties of circRNA 0001518 has been demonstrated in testis torsion/detorsion injury by Zarei Moradi et al. (8) and they stated that this circRNA has a positive correlation with the *Bcl-x* gene expression.

In recent years, the use of nanoparticles (NPs) in the medical sciences has increased dramatically due to their lower toxicity, smaller size, and high bioavailability compared to their non-nano counterparts (9). In PD, NPs have been shown to have protective effects and this has

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been attributed to the reduction of α-Synuclein fibrillation (10). Due to their small size, NPs have the ability to cross the blood-brain barrier (BBB). Also, the antioxidant effects of some NPs against oxidative stress in mitochondria under PD conditions have been reported in previous studies (11). The results of a study showed the synergistic effects of NPs in combination with antioxidants and the prevention of nanoparticle toxicity on cells (12). Recently, researchers have drawn attention to the therapeutic properties of NPs in PD treatment. Examples include the therapeutic effects of Iron Chelation NPS (13), poly (DL-lactide-co-glycolide) (PLGA), acidic NP (aNP) (14), and Cerium oxide NPs (CeONPs) (15). Prevention of overexpression of α-synuclein (SNCA), prevention of iron accumulation in substantia nigra cells (13), and antioxidant properties of NPs (15) were stated as their therapeutic mechanisms in PD conditions. TiO_2 nanoparticles (TiO_2-NPs) have many applications in various industries. The neurotoxic effects of this compound have been reported in PD (16). For example, Naserzadeh et al. (17) indicated that TiO_2 NPs induced necrosis in brain cells. However, the impacts of the combination of TiO_2-NPs with antioxidants have not been reported.

The prevalence of PD is increasing in young populations and therefore, these individuals are more likely to become pregnant during the course of the disease (18). Hence, the management of this disease during pregnancy has been considered. Although most studies have reported successful pregnancies, however, because these researches are mostly case or retrospective studies, there is still concern that PD may adversely affect the fetus. Therefore, it is necessary to do more studies in this area.

Therefore, the current study aimed to determine the effects of vitamin E and TiO_2-NPs on the expression of the *Bcl-2* gene and *circRNA 0001518* levels in a rat model of PD and their fetuses. To the best of our knowledge, there were no reports about simultaneous administration of NPs with an antioxidant in the PD condition and evaluation of their therapeutic effects.

Materials and Methods

Materials

TiO_2-NPs and vitamin E were purchased from Sigma Aldrich (German) corporation. 0.02 mg/kg body weight TiO_2-NPs and 24 international unit (I.U.)/kg, intramuscular (i.m.) vitamin E (19) were administrated to the rats. The nanoparticle dose was selected based on a pre-test in which the maximum concentration of TiO_2-NPs did not cause death in rats.

Animals

In this experimental study, Adult female Wistar rats (6 weeks old, n=48) were obtained from the Pasteur Institute of Iran and placed in a room with a temperature of 25 ± 2˚C, 12 hours of light/dark cycle, and relative humidity of 50 ± 10%. All animals had free access to water and food (corn, wheat, barley). After 1 week of adaptation in the laboratory conditions, the animals were divided into 8 groups (n=6):

1. Control group (healthy)

2. Control group received 24 I.U./kg, i.m. vitamin E 3 times/week for one month

3. Control group received 0.02 mg/kg body weight TiO_2-NPs i.m 3 times/week for one month

4. Control group received 0.02 mg/kg body weight TiO2-NPs and 24 I.U./kg i.m. vitamin E 3 times/week for one month

5. PD group

6. PD group received 24 I.U./kg, i.m. vitamin E 1 h before PD induction and 3 times/week for one month.

7. PD group received 0.02 mg/kg body weight TiO_2-NPs i.m. 1 hour before PD induction and 3 times/week for one month

8. PD group 0.02 mg/kg body weight TiO_2-NPs and 24 I.U./kg, i.m vitamin E 1 hour before PD induction and 3 times/week for one month

At the end of the experiment, on day 31 of the experiment, rats were allowed to mate with fertile males. A vaginal smear test was used to confirm pregnancy of female rats and pregnant rats were placed in separate cages. The embryos were then removed on the 21st day of pregnancy from the females' abdomens and their brains were extracted and used for molecular analysis. For each group, 5 embryos were selected.

Ethical statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Teheran Medical Science, Islamic Azad University (IR.IAU.PS.REC.1399.208).

Induction of parkinson's disease

The rats were anaesthetized by intraperitoneal injection of 45 mg/kg 3% sodium pentobarbital. Then, unilateral lesions were done on the left medial forebrain bundle, followed by 6-hydroxydopamine (6-OHDA) stereotaxical injection at a rate of 0.5 µL/minute. at the coordinates described by the atlas of Paxinos and Watson (20).

Apomorphine-induced rotation tests

If injection of 6-OHDA causes extensive neuronal damage in the midbrain, two to four weeks after surgery, animals show successive rotations toward the injection site in response to apomorphine injection. The number of these rotations per time unit is a measure of the severity of neuronal damage in the midbrain. To perform this test, the rats were first placed in a transparent plastic cylinder (28 cm×38 cm) and after 15 minutes, 0.5 mg/kg body weight apomorphine hydrochloride was injected into them. After 60 seconds, the number of rotations towards the injection site or vice versa was recorded at 10-minute intervals

for an hour. Finally, the number of rotations toward the injured side was subtracted from the opposite side, which indicated the number of net rotations to the opposite side. Further rotation indicated the severity of the lesion and the loss of dopaminergic cells (21).

Preparation of substantia nigra cells

After treating PD and control rats with TiO_2-NPs and vitamin E, the rats were transferred to the operating room and anesthetized by intraperitoneal injection of ketamine and xylazine (5 mL ketamine and 3 mL xylazine). The substantia nigra was then rapidly separated from the middle brain tissues according to the atlas of Paxinos and Watson (20). Three steps of tissue dissection, enzymatic digestion, and mechanical dissociation were used to prepare a single-cell suspension. First, the tissues were cut into 1-2 mm sections by a scalpel and then immersed in phosphate-buffered saline (PBS). In the next step, 0.2 µg/mL of collagenase enzyme containing medium (collagen enzyme at 0.1~0.3 µg/mL) was added and placed at 37˚C for 4 hours to digest the tissue. Then filtration was done using 49~74 µm cell strainers and centrifuged at 300 g for 5 minutes. The supernatant was removed and the cells were washed with PBS [or PBS with 0.1% bovine serum albumin (BSA)] and prepared for cell staining. The cells were resuspended in PBS (or PBS with 0.1% BSA) and the concentration was adjusted to 110^7 cells per mL (22).

MTT assay

Tetrazolium (Sigma, Germany) which forms insoluble purple crystals of Formazan was used in the MTT assay. The isolation of cells was described in the previous section. 50 µL of MTT solution was added to the tubes to reach a final concentration of 2 mg/mL. The tubes were then incubated for 3 hours at 37˚C. After that, 500 µL dimethyl sulfoxide (DMSO) was added in 0.01 N HCl and incubated overnight. The solution was poured on 96 wells plate and after 1 hour the absorption was read at 570 nm by spectrophotometry using 690 nm as the reference (23).

Flow cytometry

During apoptosis, phosphatidylserine binds to the surface of the cell membrane and is detected by annexin-V. Therefore, in the present study, the annexin/Propidium iodide (PI) staining method was used to evaluate apoptosis or necrosis (24). The cells were isolated as described above. Five µg/mL of FITC-labeled annexin-V (Sigma, Germany) and 10 µg/mL of PI were mixed in 100 µl of the cell suspension. After 15 minutes, 400 µl of HEPES buffer was added to the suspension to block the binding. Finally, a tube containing stained cells was placed in a FACS caliber flow cytometer (BDbiosciences, USA).

RNA extraction and cDNA synthesis

After killing the animals one day after receiving the last dose, their brain tissue was extracted and after preparing the brain homogenates, RNA was extracted using an RNA extraction kit (Denazist, Iran). Nanodrop was used to quantify extracted RNA and agarose gel electrophoresis was used to determine the quality of the extracted RNA. After that, cDNA Synthesis Kit (Easy cDNA Synthesis Kit, DenaZist, Iran) was used to synthesize cDNA according to the manufacturer's instructions.

The primers for *Bcl-2* and *circRNA 0001518* genes were designed using the Gene runner, Allel ID, and Primer express software V.3.0 (Applied Biosystems, USA). The sequences of primers are given in Table 1.

Table 1: The sequence of designed primers for evaluation of *Bcl-2* and *circRNA 0001518* genes expressions

Genes	Primer sequence (5′-3′)
circRNA 0001518	F: GGCAGAACAGGAAGTTGGTC
	R: GACAGAGAATGGGGCAGAAA
rat-*Bcl-2*	F: ATCGCTCTGTGGATGACTGAGTAC
	R: AGAGACAGCCAGGAGAAATCAAAC
rat-*β-actin*	F: CGGTTCCGATGCCCTGAGGCTCTT
	R: CGTCACACTTCATGATGGAATTGA

Real-time polymerase chain reaction

Real-time polymerase chain reaction (ABI 7300) was done using a master mix and specific gene primers. The PCR timing and temperature program are shown in Table 2.

Table 2: The polymerase chain reaction (PCR) timing and temperature program used in the current study

Steps	Temperature (˚C)	Time	Number of cycles
Initial denaturation	95	2 minutes	1
Denaturation	95	20 seconds	
Annealing	60	30 seconds	32
Extension	72	20 seconds	
Final extension	72	5 minutes	1

Statistical analysis

The results were shown as mean ± SE. Two-way ANOVA was used to analyze the data after ensuring the normal distribution of the data. Tukey test ($P<0.05$) was used to examine significant differences in the means of

the data. The measurements of relative gene expressions were done by the $2^{-\Delta\Delta Ct}$ method. GraphPad Prism V.8 (GraphPad, USA) was used to analyze the data.

Results

Apomorphine-induced rotation test

The results of the apomorphine-induced rotation test in the healthy control group and PD showed that three weeks after injury, in the average number of rotations in the PD group was higher than the healthy controls (P<0.001). While neither TiO_2-NPs nor vitamin E could not reduce the mean number of rotations in PD conditions, their combination reduced the average number of rotations compared to the PD controls (Fig.1).

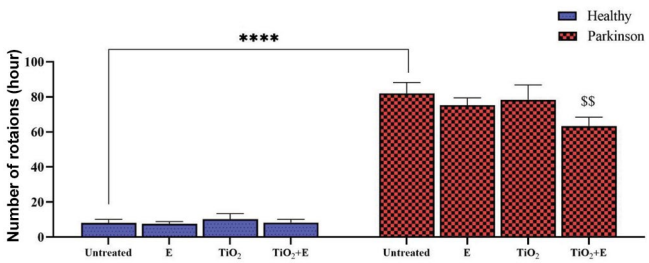

Fig.1: Effect of TiO_2 nanoparticles and vitamin E on the mean number of rotations in apomorphine-induced rotation test (n=6). The animals received Vitamin E (E), TiO_2 nanoparticles (TiO_2), and TiO_2 nanoparticles along with vitamin E (TiO_2+E) three times/week for 1 month. PD; Parkinson's disease, $$; Significant difference at probability levels of P<0.01 compared to untreated PD rats, and ****; Significant difference at probability levels of P<0.0001 compared to untreated healthy rats.

Viability, apoptosis, and necrosis of rat brain

Induction of PD in female rats resulted in decreased viability (Fig.2A), increased apoptosis (Fig.2B), and necrosis (Fig.2C). Neuronal cell death in PD rats was due to increased apoptosis. However, administration of vitamin E or TiO_2-NPs+vitamin E resulted in increased survival, decreased apoptosis, and necrosis. Administration of TiO_2-NPs did not significantly alter the rate of apoptosis and cell necrosis. In healthy rats, administration of vitamin E+TiO_2-NPs and TiO_2-NPs reduced the viability of brain cells and resulted in necrosis-induced cell death. The flow cytometry histograms are illustrated in Figure 2D.

Viability, apoptosis, and necrosis of rat embryonic brain cells

There was a significant decrease in the viability of neuronal brain cells of fetuses from PD female rats compared to fetuses from healthy female rats (P<0.01). However, embryos from PD female rats receiving vitamin E and the combination of TiO_2-NPs with vitamin E showed a significant increase in brain neuronal survival compared with negative control (Fig.3A). However, administration of vitamin E and a combination of TiO_2-NPs+vitamin E in PD rats reduced apoptosis and necrosis of fetal brain cells (Fig.3B, C). The flow cytometry histograms are illustrated in Figure 3D.

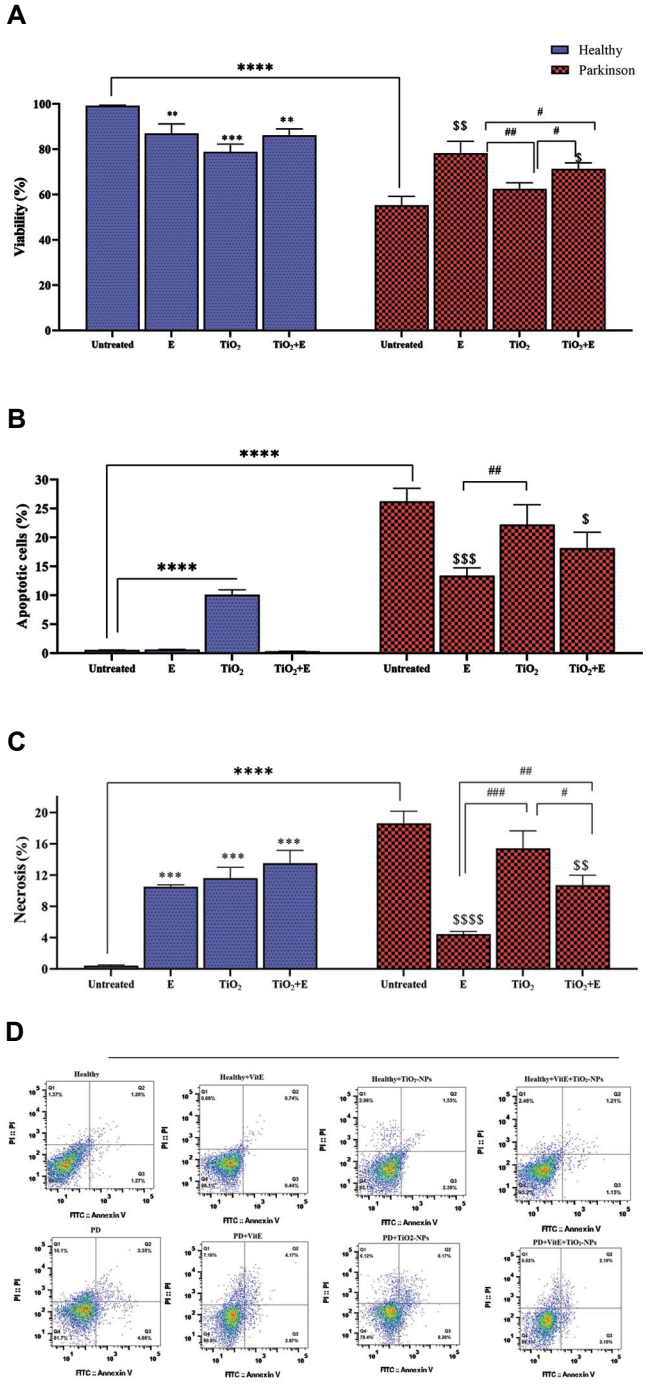

Fig.2: The effect of administration of vitamin E, TiO_2-NPs and their combination on brain cells of female rats. **A.** Viability, **B.** Apoptosis, **C.** Necrosis in brain cells of female rats and **D.** Related histograms (n=3). The animals received Vitamin E (E), TiO_2 NPs (TiO_2), and TiO_2 nanoparticles along with vitamin E (TiO_2+E) three times/week for 1 month. After one month of treatment, the brain was separated and analyzed. PD; Parkinson's disease, NPs; Nanoparticles,**, ***, ****; Show significant differences at probability levels of P<0.01, P<0.001, and P<0.0001, respectively, compared to untreated healthy rats, $, $$, $$$, $$$$; Show significant differences at probability levels of P<0.05, P<0.01, P<0.001 and P<0.0001, respectively, compared to untreated PD rats. The symbol of # shows intragroup mean comparisons.

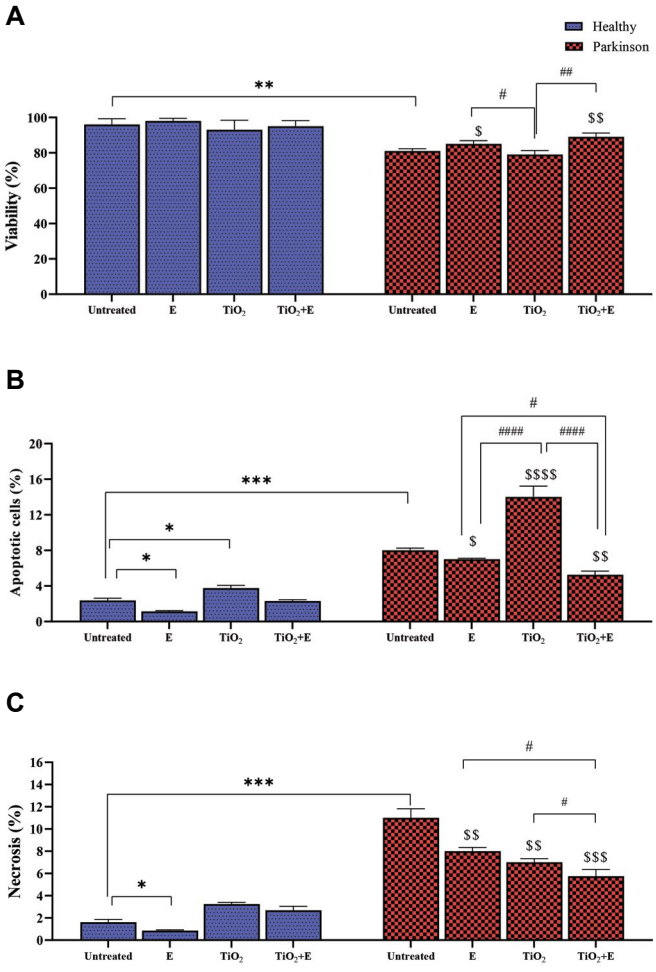

Fig.3: The effect of administration of vitamin E, TiO_2-NPs and their combination on the brain cells of fetus resulted from PD female rats. **A.** Viability, **B.** apoptosis, **C.** and necrosis, and **D.** Related histograms (n=3). The animals received Vitamin E (E), TiO_2 nanoparticles (TiO_2), and TiO_2 nanoparticles along with vitamin E (TiO_2+E) three times/week for 1 month. On the 21st day of pregnancy, the brain was separated and analyzed. *, **, ***; Show significant differences at probability levels of P<0.05, P<0.01, and P<0.001 compared to the healthy group, respectively, $, $$, $$$, $$$$; Show significant differences at probability levels of P<0.05, P<0.01, P<0.001 and P<0.0001, respectively, compared to untreated PD rats. The symbol of # shows intragroup mean comparisons.

Gene expressions analysis

Induction of PD in female rats led to decreased expression of the *Bcl-2* gene in cerebral tissue. However, in the fetuses resulting from PD mothers, an increase in *Bcl-2* gene expression was observed compared to the control group. Decreased expression of the *Bcl-2* gene was observed in the brains of fetuses obtained from PD female rats receiving vitamin E, TiO_2-NPs, and vitamin E+TiO_2-NPs (Fig.4). However, increased expression of the *Bcl-2* gene was observed in the brains of PD rats receiving vitamin E and TiO_2. There was no significant difference in the expression of the *Bcl-2* gene in the female PD brain rats receiving vitamin E+TiO_2-NPs compared to the PD group.

The expression of *circRNA 0001518* in female rats was affected by PD induction and an increase in the expression of this gene under the PD condition was observed in the brains of female rats. However, the treatments used did not affect the expression of *circRNA 0001518*. Increased *circRNA 0001518* expression was observed in the fetal brain of healthy female rats and PD when their mothers received vitamin E, TiO_2-NPs, and vitamin E+TiO_2-NPs.

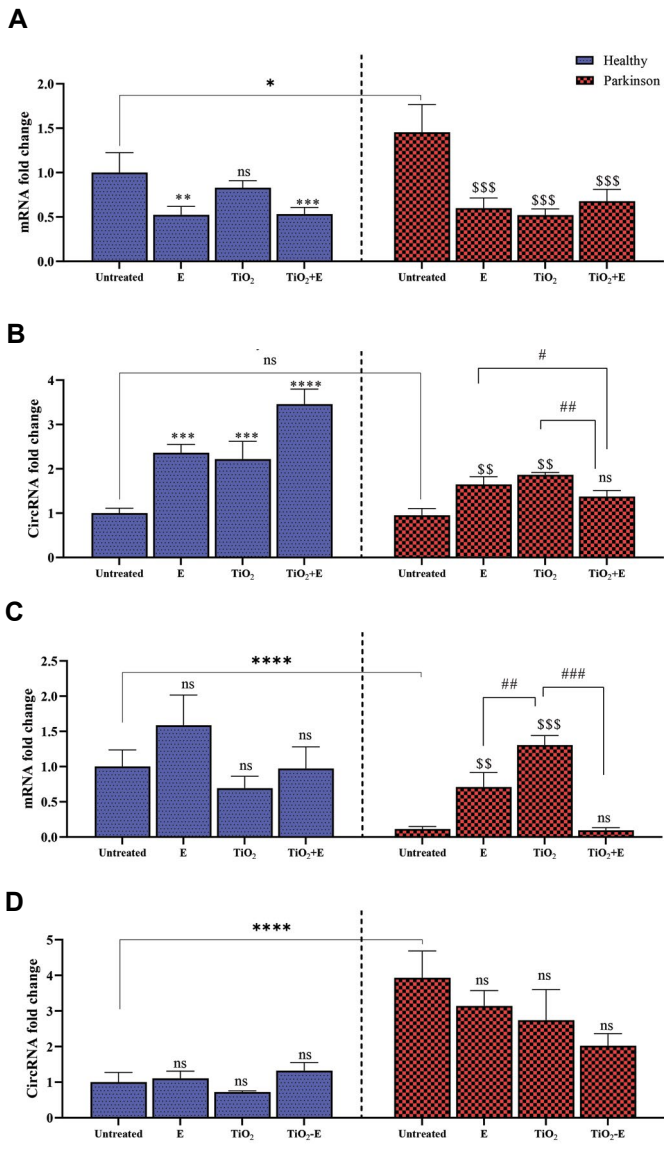

Fig.4: The expression levels of *circRNA 0001518* and *Bcl-2* genes in female rats and fetuses (n=3). **A.** *Bcl-2* gene expression in brain cells of rats, **B.** *circRNA 0001518* expression in brain cells of rats, **C.** *Bcl-2* gene expression in brain cells of the fetus, **D.** *circRNA 0001518* expression in brain cells of the fetus. After one month of treatment, the brain was separated, and the expression levels of genes were analyzed. The animals received Vitamin E (E), TiO_2 nanoparticles (TiO_2), and TiO_2 nanoparticles along with vitamin E (TiO_2+E) three times /week for 1 month. *, **, ***, ****; Show significant differences at probability levels of P<0.05, P<0.01, P<0.001, and P<0.0001 compared to the healthy untreated control group, respectively, $$, $$$; Show significant differences at probability levels of P<0.01 and P<0.001, respectively, compared to untreated PD rats, and ns; Non significant. The symbol of # shows intragroup mean comparisons.

Discussion

The results of the present study showed that the viability of cerebral tissue cells under PD conditions

is greatly reduced in rats. However, the administration of vitamin E with or without TiO_2-NPs increased the survival of brain cells. Therapeutic and protective effects of vitamin E on central nervous system neurons in degenerative diseases of the central nervous system such as PD have been studied by researchers (25, 26). Evidence suggests that the progression of PD is reduced by early administration of vitamins E and C (27). The reduction in PD-induced cell death in vitamin E injection conditions appears to be due to the antioxidant effects of this compound, which prevented oxidative stress and dopaminergic cell death (28).

In the present study, the neuroprotective effect of vitamin E was observed in reducing cerebral tissue cell death. It seems that this compound has strong antioxidant effects against PD-induced oxidative stress, which is in line with the findings of other studies (29). Reducing necrosis and subsequent elimination of inflammation as well as increasing viability are involved in the improvement of neurodegenerative diseases. These results indicated that cerebral cell death due to PD induction in rats is mostly due to necroptosis and vitamin E alone or in combination with TiO_2- NPs was able to prevent this type of cell death. The effects of intravenous vitamin E injection on cerebral ischemia have also been reported (30).

In the current study, the percentages of viable cells, apoptosis, and necrosis after vitamin E administration were different between female rats and embryos. This can be attributed to different vitamin E needs during fetal development and puberty (31). Also, the dose of vitamin E used in the current study could be the reason for this difference in responses. One study showed that the dose of antioxidants such as vitamins E and C determines the beneficial effects on the teratogenic effects of diabetes, not the total amount of antioxidants used. The bioavailability of vitamin E in both mother and fetal brains was reported (32).

Moreover, when vitamin E was administered to the healthy rats, an unexpected reduction in the viability percentage of neurons was observed, which may indicate stress conditions in them. The dose of vitamin E used appears to have toxic effects on brain neurons, leading to reduced survival. Other studies have shown the pro-oxidant effects of high doses of this vitamin (33). Therefore, it can be said that caution should be exercised in taking vitamin E to prevent the high-dose toxic effects of this vitamin on healthy individuals. However, when this dose was administered to PD rats, an increase in neuronal survival was observed compared with the PD group. Therefore, administration of this dose in PD conditions leads to neuroprotective effects. This has been reported in other conditions, such as patients with type 1 diabetes and cisplatin-induced kidney and liver damage (34).

Studies have shown that high concentrations (50 mg/kg) of TiO_2-NPs can exert toxic effects on neurons (16), which are consistent with the effect of TiO_2-NPs on the healthy group in our study. However, when TiO_2-NPs were co-administered with vitamin E in PD rats, there was an increase in the viability of substantia nigra neurons compared to control PD rats. This can be attributed to the neuroprotective effects of vitamin E in PD conditions. In the present study, it was shown that embryos from healthy female rats receiving TiO_2-NPs showed an increase in apoptosis in brain cells compared to healthy controls, which could indicate the toxic effects of this compound on the embryo. Various studies have shown that this compound causes neurotoxicity in offspring mice (17, 35), which is consistent with current research findings. Also, in the study of Naserzadeh et al. (17), it was found that TiO_2-NPs lead to the destruction of the structure of the brain and liver of rat embryos. Therefore, it seems that this compound can cross the placental barrier and has neurotoxic effects on the fetus.

The results of the present study showed a sharp decrease in *Bcl-2* gene expression in the cerebral tissue of PD female rats. However, TiO_2-NPs and vitamin E had a significant effect on *Bcl-2* gene expression in rat cerebral tissue and increased the expression of this gene. In the combined treatment of vitamin E+TiO_2-NPs, no significant difference was observed in the expression of the *Bcl-2* gene in the cerebral tissue compared to the PD group. The role of the *Bcl-2* gene family in the process of mitochondrial apoptosis is well-known. The Bcl-2 protein has an anti-apoptotic role and its overexpression protects neurons (36). Increasing tolerance to oxidative stress by increasing the content of antioxidant compounds such as glutathione is one of the mechanisms of the protective effect of Bcl-2 (37). However, in the present study, an increase in the expression of this gene was also observed in the female rats receiving TiO_2-NPs compared to control PD rats which was contrary to the data obtained from cell viability and apoptosis/necrosis assessment. Therefore, merely increasing the expression of this gene may not indicate the neuroprotective effects of compounds, and other regulatory genes such as *Bax* may be involved.

circRNAs are a class of RNA that show good stability due to their cyclic structure. circRNA acts as a sponge to bind miRNAs and regulate gene expression. These regulatory elements have been used as biomarkers for a variety of diseases and even as a target for the treatment of diseases. circRNAs can accumulate in the brain and cause neurological diseases (38). In the present study, an increase in *circRNA 0001518* expression was observed in PD rats compared to the control rats in cerebral tissue. However, the used treatments did not have a significant effect on changing the expression of *circRNA 0001518*. The increased expression of *circRNA 0001518* in PD rats may indicate its probable role in PD-induced cell death and, therefore, should be considered in future research. The result of the present

research is in contrast to a recent study in which the anti-apoptotic effects of *circRNA 0001518* in Testis Torsion/Detorsion Injury conditions have been reported (8), which can be attributed to the different roles of this circRNA in different diseases.

The patterns of studied gene expressions were different between mothers and fetuses which can be attributed to the different patterns of gene expressions during development.

Conclusion

The current study found that a high dose of vitamin E could lead to neurotoxic effects in healthy rats. However, when this dose was administered to PD rats, the neuroprotective effects were seen. Moreover, although TiO_2-NPs administration had toxicity effects on healthy female rat neurons, the combination of vitamin E and TiO_2-NPs had opposite effects and could increase the survival of brain cells even in the PD group. The mechanism of this effect and its probable attribution to the upregulation of the *Bcl-2* gene decreased apoptosis, and necrosis of brain cells should be further assessed. It was also found that induction of PD in female rats reduced the viability of brain cells in the fetuses, indicating teratogenic effects. Administrations of vitamin E and vitamin E +TiO_2-NPs showed neuroprotective effects in fetuses resulting from PD female rats. It is suggested that the effects of other NPs in combination with antioxidants should be evaluated in future studies. Moreover, identifying the neuroprotective mechanism of these compounds is important and requires further studies.

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Authors' Contributions

B.J., M.H.; Conceptualization, Methodology, Software, Validation, and Formal analysis. M.E.; Supervision and Project administration. N.B.; Review and Editing, Visualization, and Formal analysis. All authors read and approved the final manuscript.

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