Original Article

Protective Effect of Whey Protein Supplement Against Rotenone Induced Motor Dysfunction in a Rat Model of Parkinson Disease

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Abstract

Background: We aimed to investigate the effects of whey protein (WP) supplements in a rat model of rotenone-induced locomotor and biochemical features of Parkinson's disease (PD).

Materials and Methods: Male Wistar rats were used. Daily injections of rotenone (2 mg/kg; i.p.) for 16 days were used to induce PD. WP or soy protein (SP) at 1, 2, and 4 g/rats were administrated daily by gavage. Motor skills were measured in rats 24 h after the last injection using the bar test, grid test, rearing, and open field tests. In the following, striatum tissue was isolated for biochemical measurements. ELISA kits were used for biochemical assessments.

Results: While rotenone caused a significant increase in the delay time in both the bar and grid tests and a significant decrease in the motor activities were observed in both rearing and spontaneous movement tests in the rotenone group, supplementation with 2 and 4 g of WP, but not SP, significantly decreased the delay time in the bar and grid tests and also significantly increased both rearing and spontaneous movements. Additionally, rotenone caused a significant decrease in striatal levels of dopamine and glutathione and significantly increased apoptotic caspases 8, 9, and Cytochrome C, while 2 and 4 g of WP, but not SP, significantly reversed these effects.

Conclusions: WP appears to have neuroprotective effects against rotenone-induced neurotoxicity and motor dysfunction, so it may be effective in the control of PD.

Keywords: Neurologic manifestations, Parkinson's disease, rotenone, whey proteins

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Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder associated with age. Statistically, over 10 million people across the world are diagnosed with PD. The condition is marked by the degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) and the presence of Lewy bodies as pathological features within the brain.[1,2]

Alterations in cellular factors related to the development of PD encompass genetic mutations, excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), dysfunction in the mitochondrial electron transport chain, and ultimately, disturbances in catecholamine metabolism.[3-5] Current therapies can neither reverse nor prevent PD from progressing. Levodopa is a medication of choice for the treatment of PD, which is quickly converted to dopamine in the brain by DOPA decarboxylase.[6,7]

Rotenone, an organic insecticide and herbicide commonly used in agriculture, has the ability to easily cross the blood-brain barrier due to its high lipophilicity. Once inside the brain, it

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interacts with and inhibits the activity of mitochondrial complex I. As a result of this inhibition, mitochondrial dysfunction occurs, leading to the generation of ROS. Furthermore, rotenone also disrupts lipid and glutathione metabolism, further contributing to cellular damage. Ultimately, these effects induce apoptosis or programmed cell death.[8-11]

Whey protein (WP) is a soluble by-product obtained from the separation of casein during cheese production. It is primarily used as an energy drink for athletes, with many therapeutic applications. WP is a source of bioactive peptides and has strong antioxidant properties.[12,13]

In this study, we aimed to investigate the beneficial effects of WP oral administration, in comparison with a normal protein diet, on behavioral and biochemical features of a rotenone-induced rat model of PD.

Materials and Methods

The experiment was started with 72 male Wistar rats (11–12 weeks old; 210–220 g). Rats were subjected to a 12-hour light/dark cycle at a controlled temperature (22 ± 3°C). Water and food were freely accessible to the animals. In accordance with the Guide for the Care and Use of Laboratory Animals (8th edition; National Academies Press; 2011), all animal care research and procedures adhered to the approved protocols by the Review Board and Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC.1397.178). Throughout this study, meticulous attention was given to ensure that the animals experienced minimal discomfort.

Sterile sunflower oil was used to suspend rotenone (Cayman Chemical, US) at a concentration of 2 mg/ml.[10,11,14] To ensure a uniform suspension, the preparation was vortexed before the injection. WP and soy protein (SP) (Kalleh, Isfahan, Iran) dissolved in distilled water (1, 2, 4 g/ml)[15,16] and administrated by gavage. Rats were randomly assigned to nine groups of eight rats; each received daily i.p. injections for 16 days, as follows:

1) control (saline 0.9%); 2) vehicle (sunflower oil); 3) R (2.5 mg/kg rotenone); 4) R + WP 1 g (2.5 mg/kg rotenone + 1 g/rat of WP); 5) R + WP 2 g (2.5 mg/kg rotenone + 2 g/rat of WP); 6) R + WP 4g (2.5 mg/kg rotenone + 4 g/rat of WP); 7) R + SP 1 g (2.5 mg/kg rotenone + 1 g/rat of SP); 8) R + SP 2 g (2.5 mg/kg rotenone + 2 g/rat of SP); and 9) R + SP 4 g (2.5 mg/kg rotenone + 4 g/rat of SP).

For blinding the experiment, all injections to induce PD and gavages of WP or SP were performed by one experimenter, and the behavioral assessments were performed by another who was blind to the groups.

Catalepsy test, bar, and grid tests were used to quantify bradykinesia and rigidity.[17] During the bar test, the rats were positioned with both forepaws on a flat horizontal bar, approximately 9 cm above the base, and kept parallel to it, while being in a half-rearing stance. Subsequently, a timer was initiated, and the latency was measured when the rat lifted one paw off the bar. If the rat failed to lift its paw within 120 seconds, the test was discontinued. Each rat underwent the test three times, with a 30-minute break between each trial, and the average latency was calculated.

The test involved placing each rat in the center of a vertical grid, with a height of 34 cm and bars spaced 1 cm apart. The rats were trained to grab onto the grid with all four paws. Once the rats had a secure grip on the grid, it was then overturned horizontally, causing the rats to hang upside down. A timer was then started to measure the amount of time it took for each rat to release their grip and fall off the grid. The test was performed three times for each rat, with a 30-minute break between each trial. After completing the three trials, the mean time taken by each rat to release their grip was calculated.

Diminish in motor skills and hypokinesia were assessed by rearing behavior and spontaneous locomotor activity. To assess rearing, each rat was placed in a plexiglass cylinder (height = 35 cm, diameter = 25 cm) for 5 minutes and the number of rears was counted. During rearing behavior, rats raise forepaws above the shoulder and contact the cylinder wall. To score next rear, the rat had to remove both forelimbs from the wall and touch the cylinder surface.

In order to measure the spontaneous locomotor activity of the animals, we conducted an experiment where each rat was introduced into a black open field. The dimensions of this field were 60 cm × 60 cm × 40 cm, and it was circular in shape. A camera positioned above the field recorded a video for a duration of 5 minutes. The total distance traveled was calculated using a video-tracking system and analyzed using MATLAB software (MathWorks, Massachusetts, USA). After completing the behavioral tests, all rats were given diethyl ether anesthesia. Subsequently, their brains were removed and placed in chilled phosphate buffer at pH 7.4. The right hemisphere's striatum tissue was immediately dissected and stored at −80°C for future evaluations. The striatum tissue was then homogenized in 1000 µl of phosphate buffer at pH 7.4, along with a protease inhibitor (Abcam, USA: ab271306). The homogenate was centrifuged at 14,000 rpm for 40 minutes at 4°C, after which the supernatant was separated from the pellet. The protein concentration of the tissue homogenate was determined using the Bradford method, with bovine serum albumin utilized as a standard.

The glutathione (GSH/GSSG) assay kit (Abnova, Taiwan) was used to measure reduced and oxidized glutathione in samples. Dopamine the enzyme-linked immunosorbent assay (ELISA) kit (Abcam, USA) was used to measure the concentration of dopamine in the striatum tissue. Also, ELISA kits for apoptosis indicators caspase-8 (LSBio, US), caspase-9 (Cusabio, China), and cytochrome C (Cusabio, China) were used. All assessments were done according to the manufacturer's protocols; standard curves were plotted using standard solutions in the kit. The dopamine, GSH, GSSG, caspase-8, caspse-9, and cytochrome C levels were calculated based on the optical density of the

samples using the standard curve. The values were expressed as units per milligram protein, the estimation was performed in duplicate, and the mean was used for calculation.

Statistical analysis

The power analysis was performed using the Gpower software 3.1. For the effect size >0.5 (based on a pilot study for bar test) and the sample size of 72 with 8 animals/groups, the power was >0.9. Statistical analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software, CA, USA). The normality of data distribution was assessed using the Kolmogorov-Smirnov test. Group differences were analyzed using one-way or two-way analysis of variance (ANOVA), followed by the Bonferroni posttest. To compare mortality rates, the Chi-square test was used. All data are presented as mean ± SD, and also, the statistical significance of the *P* value was carefully considered.

Results

The mean body weight for each experimental group was measured on the beginning (day 1) and on the last day (day 16). As shown in Figure 1a, two-way ANOVA revealed a significant difference in the body weight between day 1 and day 16 of the experimental groups ($F1, 8 = 11$, $P < 0.001$; n = 8). Bonferroni posttest showed a significant increase in body weight in both control (cnt) and vehicle (veh) groups after 16 days ($P < 0.01$), while in the Rotenone (R) group, the body weight significantly decreased after 16 days ($P < 0.001$). Administration of 4 g of WP to the R-treated animals not only prevents weight loss but also caused a significant increase in the mean of body weight of these groups on day 16 as compared to day 1 ($P < 0.01$). In addition, 1 and 2 g of WP and all doses of SP prevent weight loss due to rotenone administrations as there were no significant differences in days 1 and 16 of these groups. This finding suggests that oral WP administration can significantly reverse weight loss induced by rotenone [Figure 1a].

The incidence of death in each experimental group is represented in Figure 1b. Chi-square analysis did not show significant differences in death between experimental groups. However, the increased mortality rate due to rotenone injections (three in eight rats) was remarkably reduced by administration of 2 and 4 g of WP, while SP did not show such an effect.

The bar and grid tests were performed on day 16 to examine catalepsy. The results of one-way ANOVA have shown significant differences between experimental groups ($F8, 63 = 166.7$, $P < 0.001$ for bar test; $F8, 63 = 340.8$, $P < 0.001$ for grid test; n = 8). Bonferroni posttest showed a significant increase in the delay of both bar [Figure 2a] and grid [Figure 2b] tests in the R group as compared with the cnt group ($P < 0.001$). However, administration of 2 and 4 g of WP to the rotenone induced PD-like motor dysfunction significantly decreased the delay time in both bar and grid tests when compared with the R group ($P < 0.001$). However, they had significant differences with the cnt group. In addition, soy diet in the SP groups had no significant effect in comparison with the R group.

The locomotor activities were evaluated using the open field and rearing behavioral tests on day 16 of the study [Figure 2c and d]. One-way ANOVA has shown significant differences between experimental groups ($F8, 63 = 63.2$, $P < 0.001$ for rear test; $F8, 63 = 56$, $P < 0.001$ for spontaneous motor activity; n = 8). Bonferroni posttest showed a significant decrease in the motor activity of the R group compared to the cnt group in both tests ($P < 0.001$). Administration of 1 g of WP and doses of SP in rats treated with rotenone did not significantly change the effects of

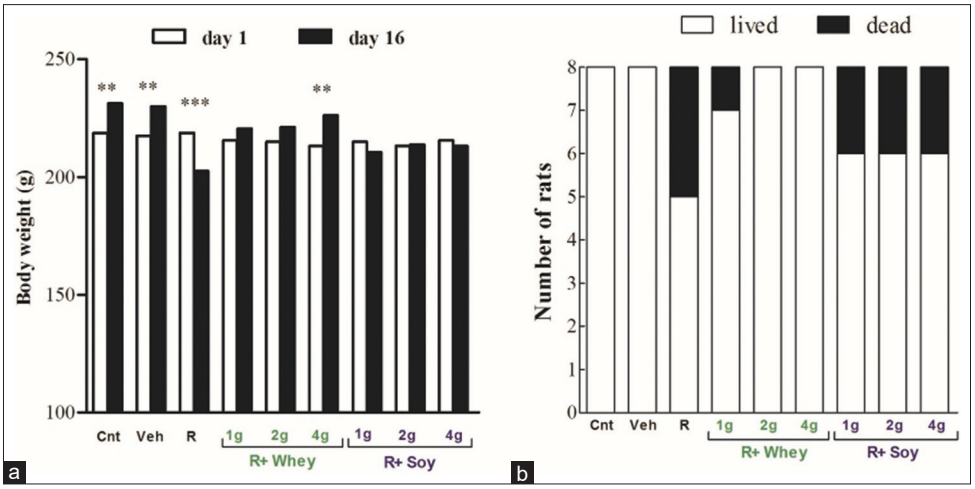

Figure 1: (a) Oral administration of WP prevents weight loss induced by rotenone. Comparison of body weight on days 1 and 16 revealed a significant increase in control (cnt) and vehicle (veh) groups. In the Rotenone (r) group, chronic injections of rotenone significantly reduced body weight on day 16 in comparison with day 1. Whey (1 and 2 g) and soy (1, 2, and 4 g) supplements prevent weight loss, while whey (4 g) reversed this effect of rotenone. (b) Mortality associated with rotenone treatment resulted in a decrease in the administration of whey supplements at doses of 2 g and 4 g. In contrast, soy supplementation did not exhibit a similar effect. Data are Mean ± SD; $n = 8$ for all groups; $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparison between day 1 and 16

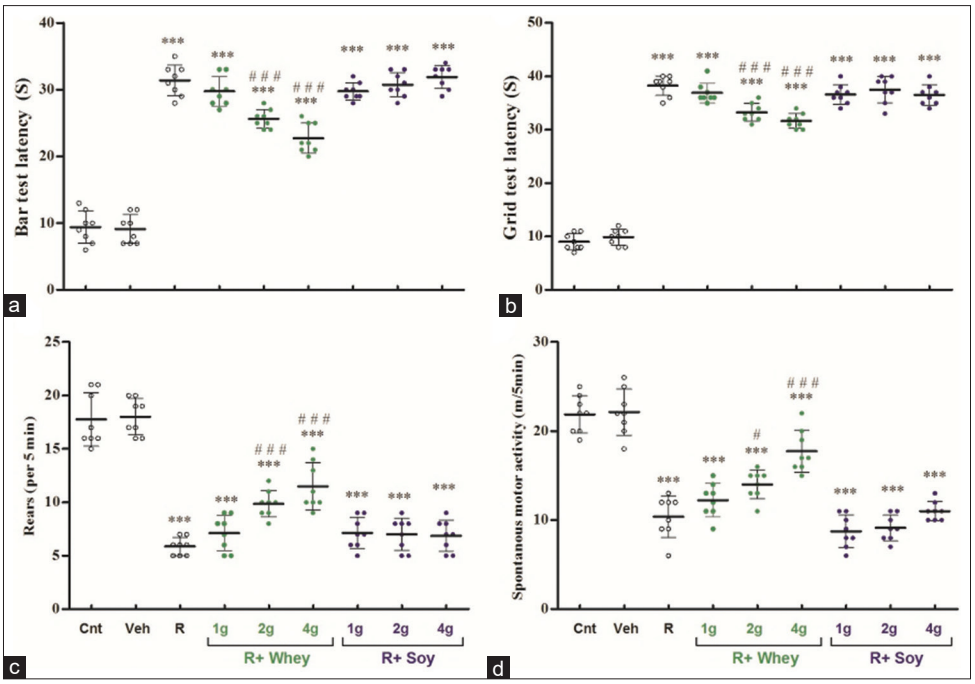

Figure 2: WP supplement improved rotenone-induced signs of catalepsy and motor dysfunction. Rotenone injections significantly enhanced the delay of both bar (a) and grid (b) tests and also, significantly decreased both rears (c) and spontaneous motor activities (d) in the R group compared to the control (cnt). Whey (2 and 4 g) in the rotenone treated rats significantly reversed these effects in comparison with the R group while, whey (1 g) and soy (1, 2, and 4 g) supplements were not significantly effective. Data are Mean ± SD; n = 8 for all groups; ***$P < 0.001$ vs. cnt; #$P < 0.05$ and ###$P < 0.001$ vs. R group

rotenone on the motor activities, and these groups showed no significant differences with the R group in both rear test and spontaneous motor activities. However, 2 and 4 g of WP significantly influenced the rotenone effects. As tTe WP 2g + R and WP 4g + R groups showed a significant increase in rearing ($P < 0.001$) [Figure 2c] and spontaneous motor movement ($P < 0.05$ for WP 2g + R; $P < 0.001$ for WP 4g + R compared with R group) [Figure 2d].

As demonstrated in Figure 3, dopamine assay in the striatum of the experimental groups has shown a significant difference ($F_{8, 63} = 70.7$, $P < 0.001$; n = 8). Dopamine levels were significantly reduced in the striatum of the R group in comparison with the cnt group ($P < 0.001$). While 2 and 4 g of WP significantly increased the dopamine level in comparison with the R group ($P < 0.01$ for 2 g of WP and $P < 0.001$ for 4 g of WP). However, 1 g of WP and all doses of SP had no significant effect on dopamine levels as these groups showed significant differences within the cnt group ($P < 0.001$) and showed no significant differences in the R group.

One-way ANOVA has shown significant difference between experimental groups ($F_{8, 63} = 49.3$, $P < 0.001$ for GSH; $F_{8, 63} = 52.5$, $P = 0.0011$ for GSSG; n = 8). As demonstrated in Figure 4a, the GSH level was significantly reduced in the striatum of the R group in comparison with the cnt group ($P < 0.001$), while the GSSG level [Figure 4b] was significantly increased ($P < 0.001$). In addition, no significant differences were found between the R group and WP 1g + R group in both GSH and GSSG levels. Also, no significant

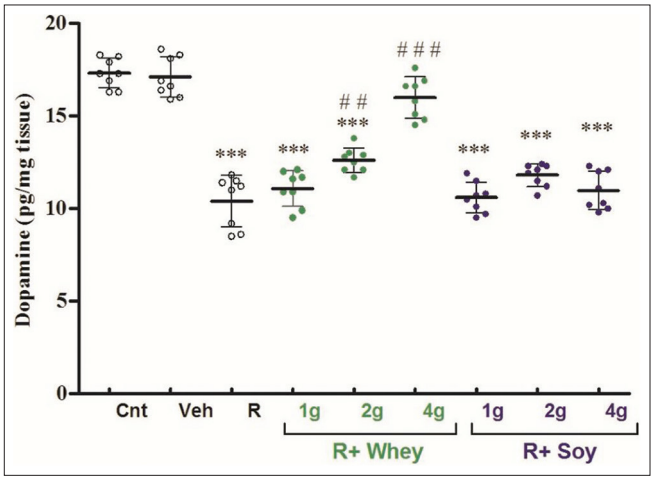

Figure 3: WP supplement improved dopamine levels in the striatum tissue of rotenone treated rats. As rotenone injections caused a significant depletion in the striatum dopamine level, whey (2 and 4 g) supplement in the rotenone treated rats significantly increased the dopamine level in comparison with the R group. However, whey (1 g) and soy (1, 2, and 4 g) show no significant differences with the R group. Data are Mean ± SD; n = 8 for all groups; ***$P < 0.001$ vs. cnt; #$P < 0.05$ and ###$P < 0.001$ vs. R group

differences were found between the R group and all SP groups, which mean neither administration of 1 g WP nor SP prevents rotenone effects on GSH and GSSG levels in the striatum. However, administration of 2 and 4 g WP significantly reversed these effects of rotenone. The mean

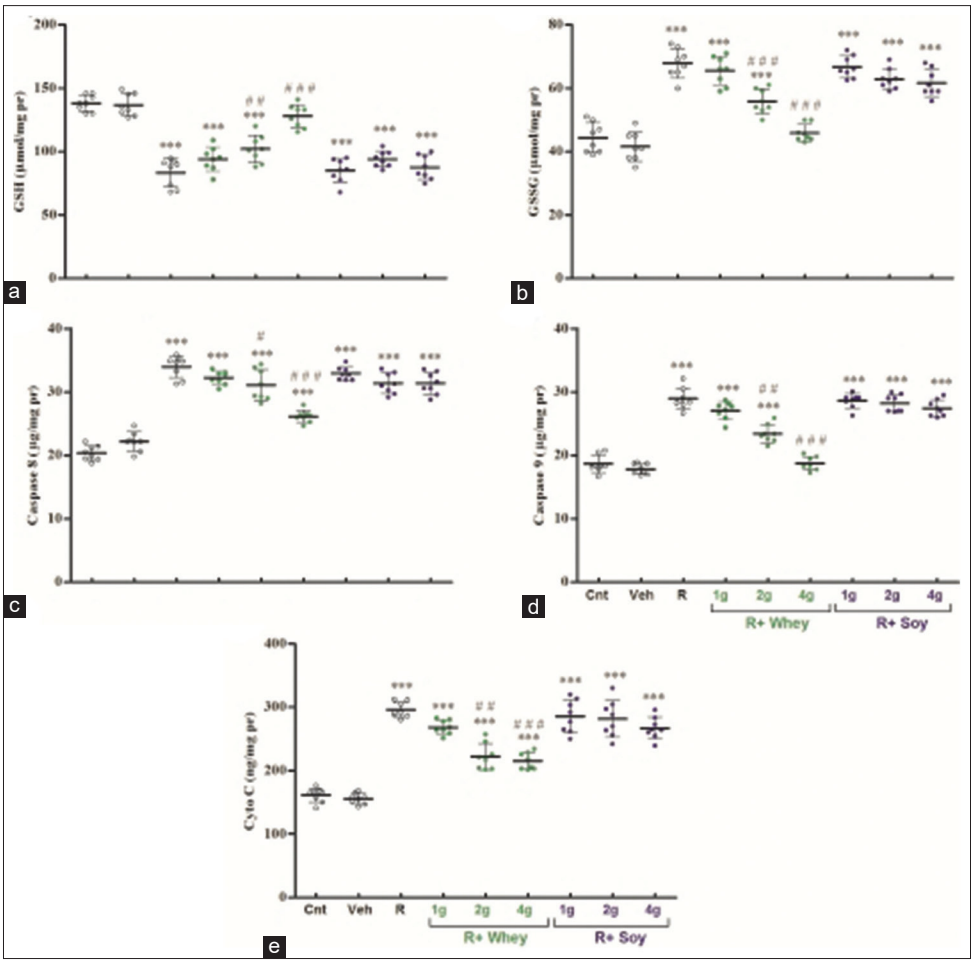

Figure 4: WP improved striatum level of GSH and decreased apoptotic caspases and cytochrome C levels. Striatum level of GSH (a) significantly decreased while GSSG (b), caspases 8 (c), caspases 9, (d) and cyto C (e) significantly increased in the Rotenone (R) group in comparison with control (cnt). Whey 2 and 4 g administration in the rotenone treated rats significantly reversed these effects in comparison with the R group. Whey (1 g) and soy (1, 2, and 4 g) show no significant differences with the R group. Data are mean ± SD; n = 8 for all groups; ***$P < 0.001$ vs. cnt; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ vs. R group

values of GSH levels were significantly increased in the WP 2g + R ($P < 0.01$) and WP 4g + R ($P < 0.001$) groups compared with the R group, while GSSG mean values were significantly decreased ($P < 0.001$).

Caspases 8, 9, and Cyto C levels were assessed in the striatum of the experimental groups and as shown in Figure 4(c-e), One-way ANOVA has shown significant differences between experimental groups (F8, 63 = 81.7, $P < 0.001$ for Caspase 8; F8, 63 = 106.8, $P < 0.001$ for Caspase 9; F8, 63 = 71.5, $P < 0.001$ for Cyto C; n = 8). Bonferroni posttest showed a significant increase of all three factors in the R group compared to the cnt group [$P < 0.001$, Figure 4c-e]. Administration of 1 g of WP and all three doses of SP had no significant effect on these factors when compared with the R group. However, 2 and 4 g of WP significantly decreased the Caspase 8 ($P < 0.05$ for 2 g of WP and $P < 0.001$ for 4 g of WP), Caspase 9 ($P < 0.01$ for 2 g of WP and $P < 0.001$ for 4 g of WP), and Cyto C ($P < 0.01$ for 2 g of WP and $P < 0.001$ for 4 g of WP) striatum levels compared to the R group.

Discussion

In this study, rotenone-induced catalepsy and locomotor dysfunction were associated with decreased striatum levels of dopamine, and GSH was associated with increased levels of apoptotic caspases 8, 9, and cyto C. In addition, oral administration of WP (2 and 4 g) in the rotenone-treated rats significantly improved motor functions and reversed striatum concentrations of dopamine, GSH, caspases 8, caspases 9, and cyto C. However, SP supplement as another protein diet had no such effects.

A pilot study by Tosukhowong *et al*.[16] which investigated the biochemical and clinical effects of 6 months of supplementation with WP in PD patients reported significantly increased plasma glutathione, branched-chain amino acids, and essential amino acids in patients with PD, associated with reduced plasma homocysteine. However, they reported no significant changes in clinical outcomes which were assessed by unified Parkinson's disease rating scale (UPDRS) and striatal L-3,4-dihydroxy-6-[16] F-fluorophenylalanine (FDOPA) uptake. Their biochemical

data were beneficial changes for improving oxidative stress and stimulating muscle and neuronal protein synthesis to improve muscle strength and neuronal performance. However, a small number of PD patients were included in the whey supplement group with no equal distribution of males and females (15 PD patients including 10 males and 5 females). Also, different anti-Parkinson medications that they were received during the study are among the study design problems which may affect the clinical outcomes. In addition, at the time of the study, PD patients had already had the disease for years (7 ± 4). According to the progressive nature of the disease, the clinical outcome of the whey supplement might be different, if it is administrated at the very beginning of the disease. Another important issue of the mentioned study is the dose of whey supplement (20 g/day). A higher dose may show different clinical outcomes. In a previous study,[18] the effects of lactoferrin, an important protein in milk and whey, were examined in a mouse model of PD induced by 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). As iron accumulation is common in patients with PD, they were designed to use lactoferrin as an iron-binding protein to regulate iron accumulation. Their results revealed improvement in motor functions, reduction in cell death of DA neurons, and increased brain-derived neurotrophic factor (BDNF) and antioxidant activity. In another study, intravenous injections of lactoferrin nano-particles in a rotenone-induced PD in rats improved motor activities, reduced DA neuronal loss, and increased the monoamine neurotransmitter levels in rats.[19] Regarding the lactoferrin presence in the WP,[20] the results of these studies support our data, which means lactoferrin might be responsible for at least some of our results by affecting the GSH levels as an important antioxidant to decelerate rotenone-induced neurotoxicity and DA neuronal damage. Accordingly, it has been reported that lactoferrin prevents MPTP-induced neurotoxicity due to its antioxidant properties by increasing the expression of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), and decreasing ROS levels. Also, its neuroprotective effects were associated with the activation of BDNF signaling.[21]

Recent interesting studies have found that a dipeptide Trp-Tyr (WY) from WP can directly inhibit monoamine oxidase-B activity and increase dopamine levels in the hippocampus and frontal cortex which prevent age-related cognitive decline.[22,23]

Although their studies were about the age-related decline in cognitive performance and related areas of the brain such as the hippocampus and frontal cortex, it is very likely that such a WP-containing dipeptide had a similar effect on the striatum area and by increasing the dopamine level in this area, improve the rotenone-induced PD-like motor dysfunctions.

In addition, PD is characterized by decreased GSH levels in the DA neurons of the SN, and as we know GSH and GSH-dependent detoxification enzymes play an essential role in the scavenging of ROS and reducing oxidative stress levels. The intracellular cysteine storage limits the synthesis of GSH, while WP is rich in cysteine and it can work as an effective precursor for glutathione synthesis.[24,25]

Following these reports, our data also revealed a decreased level of GSH in the striatum of the rotenone-induced PD model, which was associated with locomotor dysfunction. Interestingly, WP supplement but SP increased GSH concentration in the striatum of the rotenone-treated animals and also improved locomotor dysfunctions. Thus, cysteine-containing WP supplements might have an important involvement in our findings. A previous study has shown oral administration of cysteine rich WP ameliorates neural oxidative stress and glutathione deficits in a mouse model of schizophrenia.[26]

Conclusion

The presented data revealed the neuroprotective effects of WP by increasing the GSH levels and reducing the apoptotic caspases 8 and 9 and cytochrome C in the striatum. Through this neuroprotection and/or other possible mechanisms, WP enhanced the dopamine concentration in the striatum and reversed the rotenone-induced motor dysfunctions. Based on the results, daily WP supplement might have a potential preventive value in the control of PD, however, the effective dosage and the other guidelines for administration for patients need future investigations.

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Conflicts of interest

There are no conflicts of interest.

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