Life Sciences 229 (2019) 219–224

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Comparing the effects of progressive and mild intensity treadmill running protocols on neuroprotection of parkinsonian rats

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ARTICLE INFO

Keywords:
Parkinson's disease
Mesencephalic astrocyte-derived neurotrophic factor
Cerebral dopamine neurotrophic factor
Neurotrophic growth factor
Progressive exercise
Mild-intensity exercise

ABSTRACT

Aims: Parkinson's disease (PD) is characterized by progressive loss of dopamine cells. It is suggested that exercise could be employed as a non-pharmacological approach for reducing the risk of PD incidence. The purpose of this study was to compare the effects of 4-week Mild-intensity (MIEx) and progressive exercise (PEx) protocols on rotational behavior, GFAP, DA, TH, MANF, CDNF and NGF levels in striatum of parkinsonian rats induced by 6-hydroxydopamine.

Methods: 42 Wistar male rats were divided into 6 groups including, healthy and PD controls, MIEx, PEx, healthy MIEx, and healthy PEx. MIEx protocol was performed as follows: 5 days a week, 2 sessions a day of 15 min at a speed of 15 m/min. PEx protocol encompassed a training regimen of 5 days a week initiating by 20 min in the first day reaching 50 min on the fifth day and 60 min in the next 3 weeks. PD was induced after training protocol by injection of 6-OHDA into the striatum of rats. For confirming PD, apomorphine rotational test was employed.

Key findings: The MIEx protocol did not have any positive impacts on the variables except for CDNF ($P < 0.0001$). Levels of DA ($P < 0.0001$) and TH ($P = 0.0004$) increased significantly after performing PEx protocol. Moreover, PEx protocol considerably reduced rotational behavior of rats ($P = 0.0244$).

Significance: The findings of this research confirm positive effects of PEx in protecting against PD. This progressive training protocol has explicitly shown a neuroprotective effect against PD-inducing nervous toxin through increasing neurotrophins.

1. Introduction

Parkinson's disease (PD) is featured by degeneration of neurons in the substantia nigra [1]. This neurodegenerative disorder, the second most common motor impairment, results in certain dysregulation of the basal ganglia, such as rest tremor, muscle stiffness, akinesia, rigidity, and postural imbalance [2,3]. Several studies have proven exercise to be a potential non-pharmacological therapy for lowering the risk of neurodegenerative disorders incidence, although the molecular and cellular basis is rather unclear [4–6].

It is hypothesized that exercise exerts its neuroprotective effects in PD through increasing several neurotrophic factors. It has been reported that exercise training increases glial cell-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF)-1, fibroblast growth factor (FGF)-2, and nerve growth factor (NGF) levels [7,8]. For example, injecting GDNF can decrease the effects of neurotoxins on dopamine (DA) neurons [9]. Similarly, cerebral dopamine neurotrophic factor (CDNF) injection protected DA neurons against the toxic effects of 6-hydroxydopamine (OHDA), a neurotoxin used to induce PD in rats [10].

In contrast, there are reports about the ineffectiveness of exercise training on preventing degeneration or death of striatal DA and tyrosine hydroxylase (TH) [11,12]. To an extent, exercise can act as a moderate stressful factor, and enable DA neurons to resist higher stressful conditions in future [13]. This hypothesis is supported by a study in which exercise led to increases in plasma corticosterone and HsP70 anti-apoptosis chaperon in the striatum [14]. Both of them are classic stress responses which are observed in preconditioning. So far, the exact elements and optimal dosage of exercise intervention for PD patients have not been determined.

Recent studies have demonstrated the possible involvement of astrocyte dysfunction and PD [15]. A key cytoskeletal protein of astrocytes is glial fibrillary acidic protein (GFAP). GFAP maintains the integrity of astrocytes, myelination and blood-brain barrier integrity.

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https://doi.org/10.1016/j.lfs.2019.05.036
Received 26 February 2019; Received in revised form 12 May 2019; Accepted 13 May 2019
Available online 14 May 2019
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GFAP also plays an important role in cell-cell communication [16]. In case of an injury in the nervous system, astrocytes start to increase GFAP expression [15]. The rapid expression of these structural proteins leads to reactive gliosis, the condition that involves the proliferation or hypertrophy of astrocytes and it results in the glial scar at the location of the impaired tissue. The glial scar prevents neuronal regrowth as well. According to Sofroniew, M.V., extraneous gliosis has negative effects on structural and functional recovery in the brain [17]. Therefore, GFAP has become an important biomarker for neurological conditions including PD [16]. This elevated expression of GFAP can be normalized with physical exercise [18]. Bernardi and colleagues have reported that mild treadmill exercise reduces GFAP concentrations [19) Conversely, Saur and colleagues (2014) have reported the opposite, showing that a four-week treadmill running was capable of increasing the GFAP expression [20]. The lack of consensus in the literature requires continued research in this area [19].

Until now, the majority of studies concerning the prevention or treatment of neurodegenerative diseases via exercise have been performed through the classic method, mild intensity exercise (MIEx), and there is no study investigating the progressive exercise (PEx) as a potential approach. Therefore, this study is designed to compare the preventative effects of MIEx and PEx on PD by measuring striatal levels of GFAP, DA, TH, mesencephalic astrocyte-derived neurotrophic factor (MANF), cerebral dopamine neurotrophic factor (CDNF) and Nerve growth factor (NGF) in rat model of PD. We hypothesized that PEx protocol, through increasing the levels of these neurotrophins, is more capable of exerting neuroprotective effects against 6-OHDA toxicity than MIEx protocol.

2. Materials and methods

2.1. Animals

Forty-two 12-week Wistar male rats were provided from Pasteur Institute of Amol, Iran. All the procedures involving animal experiments were approved by the ethical committee of University of Mazandaran, Iran, and were performed in accordance with the ethical principles of the committee (protocol 2256741). Animals were housed in a temperature-controlled room under a 12:12-hour dark/light cycle with food and water access ad libitum. The overall design of this research is presented in Fig. 1.

2.2. Training protocol

The animals were randomly divided into 6 groups, 7 per group, including: healthy control (HC), control + Parkinson (PC), Mild-intensity exercise + Parkinson (MIEx), progressive exercise + Parkinson (PEx), healthy mild-intensity exercise (HMIEx), healthy progressive exercise (HPEx). Training groups were subjected to treadmill running for 4 weeks. After one week of adaptation, the MIEx protocol was performed as follows: 5 days a week, 2 sessions a day of 15 min at a speed of 15 m/min with at least 1 h rest in between [12]. The PEx protocol encompassed a training regimen of 5 days a week initiating by 20 min in the first day and it reached 50 min on the fifth day and 60 min in the next 3 weeks. These protocols were conducted prior to the injection of 6-OHDA [21]. Furthermore, none of the animals died before or during stereotaxic surgery.

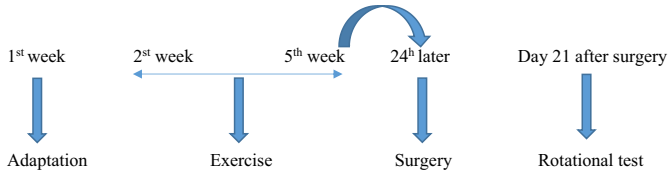

Fig. 1. The overall design of the study.

2.3. Stereotaxic surgery to develop the PD rat model

For producing PD model in rats, 6-OHDA was injected into the brain striatum through stereotaxic surgery twenty-four hours after the final exercise session. Animals were first anesthetized with an intraperitoneal injection of 60% ketamine and xylazine. Then, with a 27-gauge syringe, a 9 mm cannula was inserted in the striatum (2.5 mm lateral to the right side, 1 mm downwards from Bregma and 4.5 mm in depth). The cannula was connected to a 2-μL Hamilton syringe by a polyethylene pipe. Ten μg 6-OHDA/2 μL in 0.1% ascorbic acid-saline was injected into the right striatum at the rate of 0.5 μL/min [22].

2.4. Apomorphine rotational test

At 21 days, rats were evaluated for progression of PD. To evaluate the effectiveness of 6-OHDA injection and confirm the occurrence of PD in rats, rotational behavior on the apomorphine rotational test following apomorphine induction (0.5 mg/kg) was used. The apomorphine rotational test was carried out in a 22 cm diameter and 26 cm height cylinder. The total number of complete rotations (360°) in opposite direction of the injured striatum following the intraperitoneal injection of apomorphine was counted during a period of 30 min recorded by a video camera. The number of injured side rotations was subtracted from the total number of rotations providing actual number of rotations towards opposite side. More rotations were indicative of lesion intensity of dopaminergic neurons [12].

2.5. Biochemical assays

After performing behavioral tests, rats were anesthetized by the combination of ketamine and xylazine and euthanized by decapitation. The striatum tissue was quickly isolated from the brain and snap frozen in liquid nitrogen until homogenization. Tissue was homogenized in phosphate-buffered-saline (PBS; pH 4.7) then centrifuged at 10,000*g* for 20 min. The supernatant was used to measure concentrations of GFAP, DA, TH, MANF, NGF, and CDNF using commercially available ELISA assays following the manufacturer's instructions (CUSABIO Corporation kits, China).

2.6. Statistical analysis

Results were presented as means ± standard deviation (SD). Comparison between experimental and control groups was performed by one-way analysis of variance (ANOVA) which was followed by Tukey's multiple-range post hoc test in order to investigate intergroup differences. To determine the normal distribution of data in different variables Kolmogorov–Smirnoff's test was used. $P < 0.05$ was considered as statistically significant. All statistical analysis and plotting graphics were performed using the GraphPad Prism software (version 6.07).

3. Results

3.1. Progressive treadmill running decreases PD severity

Evaluation of apomorphine-induced changes in rotational behavior was performed 21 days after injecting 6-OHDA into the striatum. The number of pure rotations was 106.2 ± 15.7 turns/min in the 6-OHDA-injection group (PC). ANOVA revealed significant differences between the groups with respect to rotational behavior in the striatum ($F(2, 12) = 4.805$, $P = 0.0293$). Mild-intensity exercise (MIEx) showed no significant difference compared to Parkinson control (PC) group. On the other hand, performing the progressive exercise (PEx) resulted in a significant decrease of apomorphine-induced rotations ($P = 0.024$; Table 1).

Table 1
Effect of 4-week of moderate intensity (MIEx) and progressive (PEx) treadmill running protocols on number of rotations in the apomorphine rotational test followed by apomorphine injection in 6-OHDA challenged mice.[a]

Groups	HC	PC	MIEx	PEx	HMIEx	HPEx
Mean ± SD	5.5 ± 6.7	106.2 ± 15.7[b]	93.2 ± 18.2	72 ± 18.74[c]	5.8 ± 4.95	6.7 ± 6.3

[a] The values were analyzed by one-way ANOVA and Tukey comparison test.
[b] $P < 0.001$ when comparing Parkinson control group (PC) with healthy control (HC) group.
[c] $P = 0.024$ when compared PEx with PC.

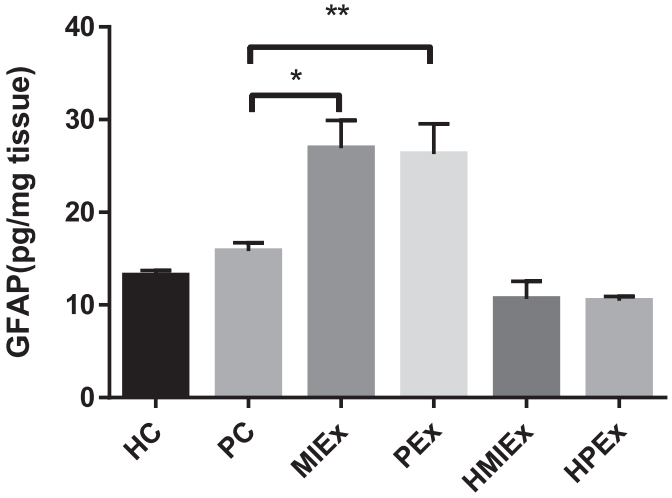

Fig. 2. Both moderate (MIEx) and progressive (PEx) treadmill running increases GFAP concentrations in striatum homogenates ($F(5, 35) = 94.57$, $P < 0.0001$). *Significant difference between MIEx and PC. **Significant difference between PEx and PC ($P < 0.0001$).

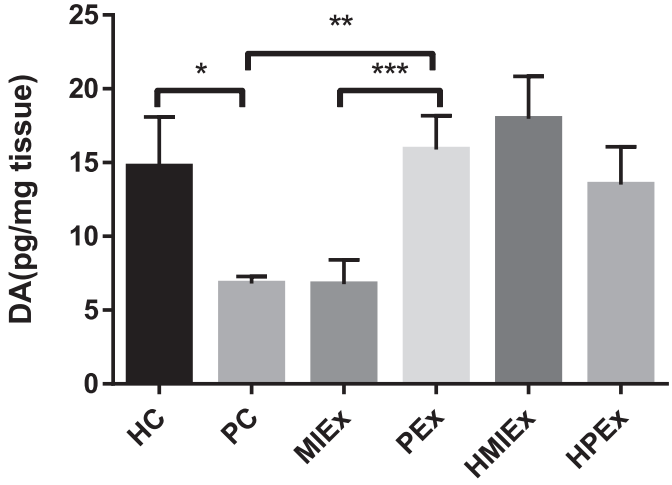

Fig. 3. PEx prevents the loss of DA in 6-OHDA challenged rats greater than MIEx ($F(5, 29) = 22.03$, $P < 0.0001$). *Significant difference between HC and PC. **Significant difference between PEx and PC. ***Significant difference between PEx and MIEx ($P < 0.0001$).

3.2. Progressive treadmill running increases neuroprotective proteins to healthy levels

No significant difference was observed in the striatum levels of GFAP in PC group compared to HC group. Significantly higher concentration of GFAP was observed in both the MIEx (70.3%) and PEx (66.2%) groups compared to PC, respectively ($P < 0.0001$). No significant difference between MIEx and PEx groups ($P < 0.05$) (Fig. 2).

DA levels in the striatum showed a significant reduction (−53.8%) in the PC group compared to the HC group ($P < 0.0001$). Only PEx increased DA concentration ($P < 0.0001$) compared to PC (133.7%). A significant difference between the PEx and MIEx groups ($P < 0.0001$) existed suggesting a greater benefit of progressive running training over mild intensity training (Fig. 3). Moreover, increases in DA mimics concentrations in HMIEx and HPEx groups, suggesting that PEx can help normalize DA cells in the context of Parkinson's disease.

Concentrations of TH and MANF were similarly higher in the PEx group compared to that of the PC (129.9%; 53.1% respectively) and MIEx groups ($P < 0.01$; Figs. 4 and 5). The striatum CDNF levels decreased (−65.3%) significantly in PC group ($P < 0.0001$) compared to HC. This reduction was significantly reversed with both MIEx and PEx significantly increasing (83.04%; 213.12% respectively) concentration of CDNF ($P < 0.0001$; Fig. 6). Finally, concentrations of NGF increased in the PEx group (128.1%) in comparison with the PC group ($P = 0.0031$; Fig. 7).

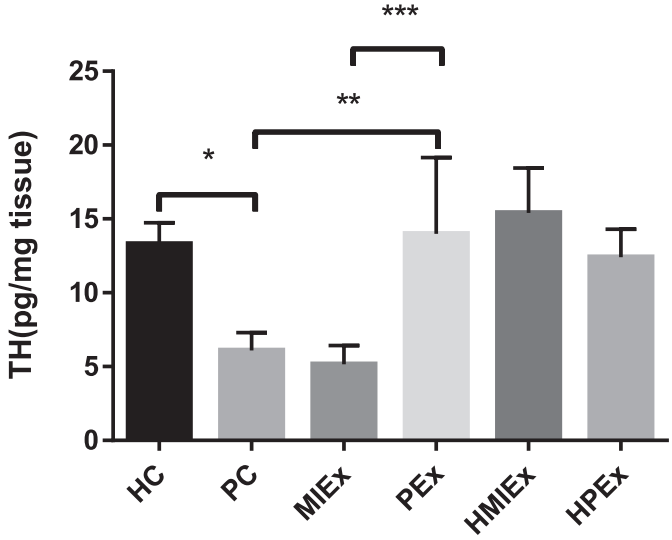

Fig. 4. Progressive exercise (PEx) protects against TH loss ($F(5, 29) = 13.78$, $P < 0.0001$). *Significant difference between HC and PC ($P = 0.0012$). **Significant difference between PEx and PC ($P = 0.0004$). ***Significant difference between PEx and MIEx ($P < 0.0002$).

4. Discussion

Previous studies have reported contradictory results on various modalities effects of exercise in protecting neurons against 6-OHDA induced neurotoxicity [23–25]. The purpose of this present study was to compare 4 weeks of progressive treadmill running and mild intensity treadmill running exercise on striatum concentrations of GFAP, DA, and TH and several neurotrophins in rats exposed to 6-OHDA. Briefly, the MIEx protocol was performed in 2–15 min bouts of exercise per day at a steady rate of 15 m/min. The PEx protocol encompassed a training regimen of 5 days a week initiating by 20 min in the first day, reaching 50 min on the fifth day and 60 min in the next 3 weeks at the same rate of work. Toxicity associated with 6-OHDA administration was evident with decreased levels of neurotrophins, DA, and TH in the striatum

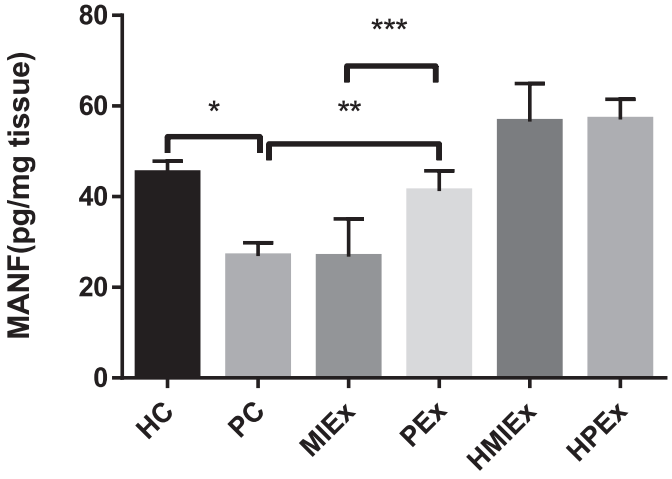

Fig. 5. MANF concentrations are maintained with progressive exercise (PEx) ($F(5, 31) = 33.62$, $P < 0.0001$). PC ($P = 0.0022$). ***Significant difference between PEx and MIEx ($P < 0.0013$).

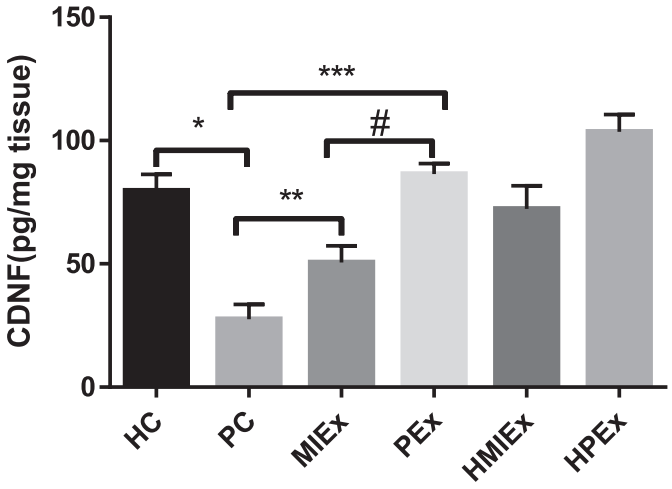

Fig. 6. Both progressive (PEx and moderate (MIEx)) treadmill running protect against CDNF loss associated with Parkinson's disease ($F(5, 29) = 92.99$, $P < 0.0001$). *Significant difference between HC and PC ($P < 0.0001$). **Significant difference between MIEx and PC ($P < 0.0001$). ***Significant difference between PEx and PC ($P < 0.0001$). #Significant difference between PEx and MIEx ($P < 0.0001$).

manifesting with increased rotational behavior of the treated rats. The results of our study suggest that PEx has greater protective effects compared to MIEx defending against 6-OHDA toxicity. Specifically, majority of the proteins measured were significantly increased in 6-OHDA-challenged rats, with many concentrations representing levels similar to non-challenged, healthy controls. Furthermore, this neuroprotective effect of PEx was confirmed by the behavioral test in which the subjects of this group had significantly fewer cylindrical rotations. The results of our study conclude that sustained activity has a greater protective effect than intermittent exercise. It is likely that this protective effect is exerted through elevated MANF, CDNF, and NGF neurotrophins.

Briefly, mesencephalic astrocyte-derived neurotrophic factor (MANF) is a secretory protein that has neuroprotective properties on dopaminergic neurons and protects the brain from insults [26]. Cerebral dopamine neurotrophic factor (CDNF) is a secretory protein which prevents the degeneration of dopaminergic neurons in the rat model of Parkinson's disease induced by 6-OHDA [27]. Our study is the first of its kind to measure the levels of CDNF and MANF in the striatum of

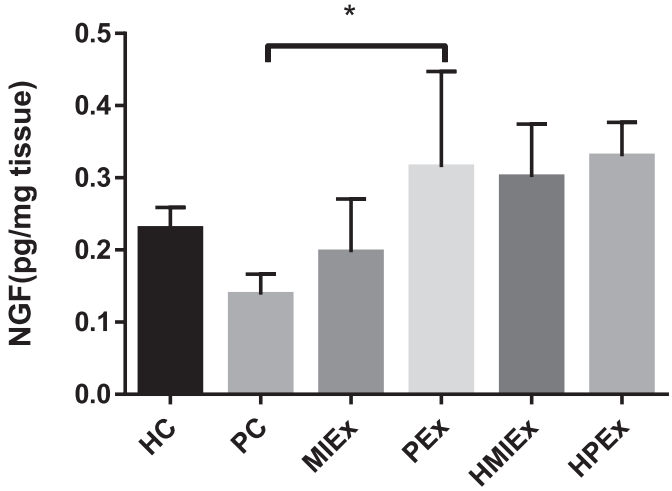

Fig. 7. Progressive exercise (PEx) significantly increases NGF in the striatum of parkinsonian rats ($F(5, 29) = 6.359$, $P = 0.0004$). *Significant difference between PEx and PC ($P = 0.0031$).

parkinsonian rats following the pre-treatment with two different types of exercise training. We found that performing progressive treadmill exercise, sustained at 60 mins for 3 weeks resulted in increased concentrations of these both secretory proteins. Notwithstanding, mild-intensity exercises (MIEx group) did increase CDNF level, though the neuroprotective effect was not translated to behavioral performance in the apomorphine rotational test. It is interesting to note that when comparing levels of these neurotrophic proteins with respect to exercise, the PEx group had significantly greater concentrations of the measured proteins compared to the MIEx group. Further, concentrations of the measured proteins were at levels similar to healthy controls. This supports our rationale that the PEx training is a more effective and promising approach for protecting against Parkinson's disease.

The results of our study suggest that PEx is able to increase striatal NGF concentrations and protecting against 6-OHDA toxicity. NGF was the first member of neurotrophins family to be discovered and it has several roles including trophic, protection, differentiation and maturity of neurons. We observed a non-significant reduction in NGF concentration in 6-OHDA-challenged rats supporting a previous report that showed similar reductions of NGF in the CNS of parkinsonian rats [28]. The reversal of this loss with PEx has tremendous implications of CNS health and plasticity [29,30]. Other exercise interventions have found similar effects in increasing NGF concentrations. For example, an 8-week swimming training protocol led to increases in NGF levels in the hippocampus of adult rats. This increase was more obvious in swimming training group in comparison with treadmill running training group from our study. This is likely given that swimming training imposes less physical stress than run training, increasing the NGF levels in the hippocampus [31]. Similarly, an 8-week progressive treadmill running protocol in which the running speed had been increased gradually resulted in an increase in NGF levels of rats' hippocampus following social isolation [32]. In summary, the results of our study support previous reports suggesting that exercise increases NGF concentration in the CNS.

Although the effects of exercise on neurotrophic factors in the hippocampus have been explicitly confirmed, there are only limited numbers of studies that investigate the impact of exercise on neurotrophins in the nigrostriatal dopaminergic neurons. In this study, the levels of endogenous neurotrophins in the striatum of PEx and MIEx groups were observed at concentrations comparable to the healthy control group. Specifically, concentrations of DA, TH, MANF, and NGF in the MIEx group revealed little difference compared to concentrations in the PC group. This suggests that the short-term mild intensity

endurance training is ineffective at exerting the neuroprotective effects of exercise. The noticeable change of CDNF in the training protocol of our study supports an earlier publication [33] demonstrating that the neurotrophic response to short-term mild-intensity exercise is selective and could be specific to different regions in the brain. There appears to be no such restrictions concerning progressive exercise. Short-term mild intensity treadmill running protocol led to improvements in brain indices and increases in GFAP of the rats' hippocampus, and this increase was observed while there were increased levels of corticosterone [34]. Corticosterone can have mixed effects in the CNS. In one case, corticosterone can impede neurotrophin physiology and hamper neurogenesis. However, relative levels of glucocorticoids are necessary for maintaining neurogenesis [35].

Chen et al. have shown that voluntary and forced exercise activates disparate signaling pathways which could provide mechanistic explanations for the differential between the MIEx and the PEx groups in our study [36]. For example, 3 to 6 weeks of forced treadmill running increased GFAP levels and astrocytic density in both cortex and striatum of rats [37]. In another study, 7 days of voluntary wheel running increased GFAP expressing cells in the subgranular zone of the CNS [38]. A likely explanation for this observation centers on the improved neurovascularization derived through exercise and the associated improvements in the blood-brain barrier which has protective roles, particularly during the post-injury in the brain [37].

In this study, we report increases in GFAP levels in both PEx and MIEx parkinsonian rat groups. However, this increase was not observed in healthy training groups. One probable reason for this observation is the inflammatory response associated with 6-OHDA injection and the creation of sclerosis tissue triggering increases in GFAP as a defensive mechanism. Inexplicably, levels of this protein in PC group did not change. Currently, there are not any conclusive answers to this phenomenon. Perhaps, a paradoxical effect of exercise promotes an environment of chronic inflammations in the brain which overstimulates the inflammation needed for neuroplasticity [39]. On the other hand, chronic inflammation at basal levels is necessary for basic neurogenesis and synaptogenesis [40]. Therefore, it can be hypothesized that the inflammatory impact of 6-OHDA may promote neurogenesis/synaptogenesis followed by a defensive reaction of astrocytes which results in an increase in GFAP levels [41]. For example, three-week treadmill running (5 days a week, 30 min/day, at the rate of 30 m/min) prior to the cerebral ischemia increased TNF-α which was associated with decreased disruption of the blood-brain barrier [42]. There are increasing observations showing the important role of TNF-α in tissue repair and as a fundamental mechanism for neuroprotection [43–45]. Though not measured in this study, several other studies have reported increased TNF-α in association with exercise in neurologically challenged models [46]. Based on this information, it can be presumed that performing PEx increases inflammatory factors, like TNF-α, leading to increases in GFAP levels. Future research is needed to test this hypothesis.

5. Conclusion

The results of our study suggest that treadmill exercise protocol prevents the loss of dopaminergic neurons associated with Parkinson's disease by maintaining neurotrophins including MANF, CDNF, and NGF. We further observed that performing PEx resulted in more prominent neuroprotective effects in comparison to MIEx. As a result, our findings suggest a training protocol with the features similar to PEx as a protective approach for dopaminergic neurons. However, more studies are required for investigating the effects of exercise on GFAP.

Acknowledgements

The authors are grateful to the University of Mazandaran for supporting this research.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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