Synergistic Effects of Aerobic Exercise after Bone Marrow Stem Cell Transplantation on Recovery of Dopaminergic Neurons and Angiogenesis Markers of Parkinsonian Rats

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Abstract: Parkinson is a progressive neurodegenerative disease in central nervous system. Non-pharmacologic treatment methods such as stem cell transplantation and exercise have been considered as a treatment. The purpose of this study was to evaluate the synergistic effects of aerobic exercise after bone marrow stem cells transplantation on recovery of dopaminergic neurons and promotion of angiogenesis markers in the striatum of parkinsonian rats. 42 rats were divided into six groups: Normal (N), Sham (S), Parkinson’s (P), Stem cells transplanted Parkinson’s (SP), Exercised Parkinson’s (EP) and Stem cells transplanted+Exercised Parkinson’s (SEP). To create a model of Parkinson's, the striatum was destroyed by injection of 6-hydroxy-dopamine into the striatum through stereotaxic apparatus. Stem cells were derived from the bone marrow of femur and tibia of male rats aged 6-8 weeks. After cultivation, approximately 5×10⁵ cells were injected into the striatum of rats through the channel. Aerobic exercise was included 8 weeks of running on treadmill with a speed of 15 meters per minute. At the end of the study, all subjects were decapitated and striatum tissues were separately isolated for measurement of vascular endothelial growth factor (VEGF), dopamine (DA) and tyrosine hydroxylase (TH) levels. VEGF, DA and TH levels in the striatum of parkinsonian rats significantly increased in treatment groups (SP, EP and SEP), especially in SEP group compared to P group after treatment (P<0.05). The BMSCs transplantation in combination with exercise would have synergistic effects leading to functional recovery, dopaminergic neurons recovery and promotion of angiogenesis marker in the striatum of parkinsonian rats.

Keywords: Stem cells, Aerobic exercise, Neurotrophic factors, Parkinson
Introduction

Parkinson’s disease (PD), one of the most common causes of disability among older adults, is a chronic and slowly progressive neurodegenerative disorder that is classically characterized by resting tremors, bradykinesia, rigidity, postural instability and difficulty walking [1,2]. At the physiological level, PD results from a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that causes to reduced dopamine (DA), which is a neurotransmitter that sends messages between nerve cells from midbrain to the striatum in the mammalian brain [3,4,5]. Transmission of these messages creates balance in the body movements. When dopaminergic cells destroyed in the midbrain, other centers of body movements controller are irregular and lack of dopamine release causes Parkinson’s symptoms [6]. As tyrosine hydroxylase (TH) catalyses the formation of L-dopa, the rate-limiting step in the biosynthesis of DA, the disease can be considered as a TH-deficiency syndrome of the striatum [7,8]. However, numerous studies have suggested that PD is a multiple neurotransmitter disorder that involves noradrenergic, cholinergic and serotoninergic systems in addition to the dopaminergic system [9,10].

Dopamine replacement therapy via oral supplementation of the DA precursor levodopa (L-dopa) has been normally used as an effective treatment for PD [3]. Treatment with L-dopa has been shown to alleviate major symptoms of PD [6,9]. However, long-term treatment with levodopa is often accompanied with side effects [10]. There have been additional anti parkinsonian drugs, such as dopamine agonists, but the available therapies do not protect against dopaminergic neurodegeneration. The prevalence of PD is likely to increase in the coming decades as the number of elderly people increases. Therefore, it is of utmost importance to develop new treatment that slow or halt the progression of PD [8].

Recently, two non-drug treatment options (i.e. stem cell transplantation and exercise) have been studied for the treatment of Parkinson's disease especially in animal models [9,11,12]. The replacement of degenerated neurons with effectively functional exogenous potent stem cells is a more promising technique for tissue repair and regeneration in case of PD. Fetal dopaminergic cells originate in the ventral mesencephalic tissue that has been obtained from fetuses and have the potential to improve motor function in animal models of PD. However, technical difficulties in obtaining sufficient graft tissues, ethical consideration and the risk of tumor formation are some of limitations for the application of this therapy [12,13]. Therefore, bone-marrow mesenchymal stem cells (BMSCs) is considered more than embryonic stem cells for treatment of PD. BMSCs are available and have self-replicating ability and differentiation into other cells, including nerve cells. Thus BMSCs have the ability to transform into dopaminergic cells [12].

Moreover, recent studies demonstrated that exercise ameliorated physical and cognitive impairments of patients suffering from CNS disorders, including PD and spinal cord injury [14,15]. Various reports clarified that exercise might exert neuroprotective effects, enhance neurogenesis [14] and increase angiogenesis [15,16]. Several trophic factors might be involved in the rationale for mechanisms of these beneficial effects of exercise [17]. Zigmond et al (2014) reported that exercise improves the substantia nigra and striatum injury through the release of dopamine in the rat brain [11].
One of the markers that is shown to be increased following exercise is vascular endothelial growth factor (VEGF). VEGF is a potent regulator of angiogenesis involved not only in such well-known functions as angiogenesis, accentuation of vessel permeability and glial proliferation, but also more recently acknowledged functions such as neuroprotection and even neurogenesis itself in PD and PD animal models [17]. In PD, new studies have shown that VEGF signaling may result in neuroprotective effects through the enhancement of dopaminergic neuron survival [18]. So an increased VEGF level in the affected areas of the brain in PD might have a potential therapeutic effect [3]. Beyond the neuroprotective role, Yasuhara reported that VEGF may also play a role in neurogenesis itself [19]. In our previous studies on the brain of Parkinsonian mouse, we reported increased angiogenesis on the striatum of exercised PD mice compared to sedentary PD ones [20]. These results were addressed in the way that neuroprotection role of VEGF is mediated indirectly by promoting angiogenesis in exercise [3].

Important and distinguishing feature of the present study compared with others is the focus on the interactive effects of aerobic exercise after BMSCs transplantation. A series of scientific publications have reported that physical activity is linked to the activation, mobilization and differentiation of various types of stem cells [3]. The major problem in stem cell transplantation in parkinsonian rats is tumor formation [13], therefore it seems exercise could have a preventive effect on tumor formation and stimulatory effect on BMSCs and develop them to become dopaminergic cells [21]. In previous studies, the effect of exercise therapy and stem cell transplantation have been examined separately for the treatment of PD, but the effect of exercise on VEGF, DA and TH levels in striatum of parkinsonian rats transplanted with BMSCs is likely to be neglected. Hence, we hypothesized that BMSCs transplantation combined with aerobic exercise could lead to a greater improvement in recovery of dopaminergic neurons and promotion of angiogenesis markers in the striatum of Parkinsonian rats (with 6-hydroxydopamine injection) than BMSCs or exercise treatment alone.

**Materials and Methods**

**Animals**
42 adult male Wistar rats weighing 220-280 g, were maintained in a constant temperature (20-24 °C) and humidity (45-55%) environment under a 12 h light/dark cycle. Food and water were available ad libitum. Rats were assigned randomly into six groups (n=7 in each group): Normal (N), Sham (S), Parkinson’s (P), Stem cells transplanted Parkinson’s (SP), Exercised Parkinson’s (EP), and Stem cells transplanted + Exercised Parkinson’s (SEP). The experimental protocol was approved by the Research and Ethics Committee of Bu-Ali Sina University (Hamedan, Iran).

**Surgical Procedures and 6-HydroxyDopamine Injection**
To create parkinsonian model, the rats striatum in parkinson’s groups destroyed with 6-hydroxydopamine (6-OHDA) injection into the corpus striatum (right side). For this purpose, rats were fixed to a stereotaxic apparatus (Stoelting, Wood Dale, USA) under general anesthesia using intraperitoneal injection of ketamine/xylazine (60 mg/kg ketamine and 3 mg/kg xylazine mixture) [13]. A midline skin incision was made with subsequent drilling of the skull for the guide cannula. Cannula location has been designed according to atlas of Paxinos and Watson, 0.5 mm anterior, 1.0 mm lateral to the bregma and 1.5 mm ventral to the surface of
the brain, with the tooth bar set at 0 mm [22,23]. Then the cannula was fixed to the skull using three stainless steel screws and dental acrylic. 26-gauge stainless steel guide cannula fitted with 33-gauge stainless steel obturators were implanted into the striatum. The day after surgery, rats rested for recovery. 6-OHDA (5 μg/μl dissolved in 0.9% saline containing 0.2 mg/ml ascorbic acid; Sigma, USA) was injected into the right striatum with a 28-gauge Hamilton syringe by cannula. Upon completion of the 6-OHDA injection at a rate of 1 μL/min, the syringe was left in place for additional 5 min before being retracted slowly at a rate of 1 mm/min. Control-operated rats (S group) received the vehicle solution alone. To evaluate the effect of 6-hydroxy-dopamine and confirmation of PD, apomorphine-induced rotation test was applied 21 days after injection.

**Apomorphine-Induced Rotation Test**

After unilateral 6-OHDA injection into the striatum, rats were placed in circular bowls (diameter: 22 cm, height: 26 cm) and allowed 15 min for habituation. Animals were injected subcutaneously by 0.5 mg/kg apomorphine hydrochloride (Sigma, St. Louis, USA) dissolved in saline and then were placed in a round cylinder [6]. The total number of complete 360° rotations within 30 min was counted using the videotape at a later time by a blinded researcher to group assignment. Ipsilateral rotations subtracted from the number of contralateral rotations resulted in a net number of contralateral rotations [6,8,9]. More rotations have been shown to correlate well with lesion severity in terms of dopaminergic cell loss [6]. The test was repeated at the end of experimental protocol.

**Preparation and Transplantation of Rat BMSCs**

In order to obtain the BMSCs, five male Wistar rats aged 6-8 weeks have been scarificed under deep anesthesia with IP injection of 15 ml/kg of 1% ketamine (Bela-Pharm, Vechta, Germany) and 0.2% xylazine (Bayer HealthCare, Leverkusen, Germany). Femur and tibia bones were dissected and removed. Both ends of the bones were removed, bone marrow was separated and suspended in the Alpha-modified MEM (PAA, Pasching, Austria), and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the cell pellets resuspended and cultured in Alpha-modified MEM supplemented with 10% fetal bovine serum (FBS) (PAA) and 1% penicillin/streptomycin (PAA). After three days, non-adherent cells were discarded by exchange of culture medium (passage 0). Ten days later, adhering BMSCs were resuspended by trypsinization, and replaced on culture dishes (passage 1). After 80% confluence, the cells were trypsinized and subcultured for two more passages. Then they were detached and prepared for transplantation (5 × 10⁵ BMSCs in 5 microlitre for each rat). The BMSCs injected in the brain of rats via cannula with velocity of one microlitre per minute [24].

**Exercise Protocol**

Exercise protocol for EP and ESP groups started in the day after stem cells transplantation, according to the protocol described by Landers et al [6]. This exercise condition consisted of forced exercise using a 10-lane rodent treadmill at a frequency of 5 days per week for 8 weeks during the light cycle. Rats were run twice per day at a speed of 15 m/min separated by at least 1 hour between them. Each running session lasted 15 minutes for a total of 30 min per day.

**Narrow beam test**

This test used for measurement of balance behavioral factor after BMSCs transplantation and aerobic exercise. For this aim used a 105 cm long wooden beam, 4 cm wide and 3 cm tall. The beam was suspended 80 cm from the ground by
wooden supports at either end. The wooden supports at the “starting” end of the beam formed a sheer drop while a platform was located at the other end, next to which was placed the home cage of the rat being tested (Fig. 1). Beneath the beam was placed 1m wide foam padding, approximately 12 cm thick to prevent injury to the animals in case of a fall. At the start end of the beam, a line was drawn 20 cm from the end of the beam. During a test the rat was placed entirely within this 20 cm starting zone facing its home cage and a stopwatch started immediately upon release of the animal. The time was recorded when the animal placed a weight bearing step entirely over the start line. This time represented the latency to begin the task. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min. The start line must be crossed within 1 min from release or the test was cancelled and maximum time was recorded for that trial. A fall was also recorded as a maximum time. A testing session consisted of five trials on the beam, recording five latencies to begin the test, and five total times on the beam for each animal [25].

![Fig. 1. Diagram of narrow beam apparatus](image)

**Tissue Processing and Measurement of VEGF, DA and TH**

At the end of protocol (Fig. 2), All subjects were anaesthetized by intraperitoneal injection of 60 mg/kg ketamine and 3 mg/kg xylazine mixture and then were decapitated and the brains were immediately taken out and rinsed in ice-cold isotonic physiologic saline. The striatum tissues were separately isolated and kept frozen in liquid nitrogen before analysis. Striatum tissues were then homogenized and centrifuged at 10,000 × g for 10 min and the supernatant was obtained for measurement of VEGF, DA and TH. At the end of this experiment, striatum VEGF level was measured using rat VEGF Elisa assay kit (Cusabio, Japan). Also, relevant kits (Rat DA Elisa kit and Rat TH Elisa kit respectively) were applied for DA and TH measurement.
Statistical Analyses
All values are expressed as the mean ± standard error of the mean (SEM). For between groups comparisons, one-way analysis of variance (ANOVA) and Scheffe’s post-hoc test were performed with P<0.05 as an indication of statistical significance. The normality of populations and homogeneity of variances were tested before each analysis of variance. Statistical analyses performed with SPSS software (version 20).

Results
Apomorphine-Induced Rotation Analysis
The number of apomorphine-induced rotation 3 weeks after 6-OHDA injection and after 8 weeks of exercise are shown in Fig. 3 and Fig. 4, respectively, for all groups. The number of contralateral rotations significantly increased in parkinson’s groups compared to N and S groups after 6-OHDA injection (P<0.05) (Fig. 3). Thus, PD was confirmed in those groups. This test repeated at the end of the treatment protocol to determine the efficacy of BMSCs transplantation and exercise. As shown in Fig. 4, the number of contralateral rotations significantly decreased in SP, EP and SEP groups compared to P group (P<0.05).
Fig. 3- Analysis of apomorphine-induced rotation test after 6-OHDA injection. *Represents significant increase in the number of rotations in parkinson’s groups compared to N and S groups (P<0.05).

Fig. 4. Analysis of apomorphine-induced rotation test at the end of treatment protocol. *Represents significant decrease in the number of rotations in SP, EP and SEP groups compared to P group (P<0.05).

**Narrow beam test results**

The results of narrow beam test showed that there is significant difference between groups in balane behavioral factor. The total duration of the test decreased in SP, EP and specially SEP groups compared with P group significantly (P<0.05) (Fig. 5). In other words, the rats in three groups performed this test more quickly than P group.
Fig. 5. Analysis of narrow beam test at the end of treatment protocol. *Represents significant decrease in SP, EP and SEP groups compared to P group (P<0.05).

Changes of VEGF, DA And TH
The VEGF levels decreased in N, S, and P group at the end of treatment protocol, but there was no statistically significant difference between them (P>0.05). On the other hand, there was a significant difference between SP, EP and SEP groups compared to P group (P<0.05). It seems that the striatum VEGF levels were significantly increased in treatment groups (SP, EP and SEP) in comparison with P group (Fig. 6) and this increase was higher in SEP group.

Fig. 6- Analysis of VEGF levels in the striatum at the end of treatment protocol.
*Represents significant increase in SP, EP and SEP groups compared to N, S and P groups (P<0.05).
Furthermore, the DA (Fig. 7) and TH (Fig. 8) levels of striatum significantly increased in SP, EP and SEP groups compared to P group at the end of treatment protocol (P<0.05). Results showed that the BMSCs transplantation, 8 weeks of aerobic exercise and their interaction had a positive effect on VEGF, DA and TH levels in the striatum of parkinsonian rats.

![Dopamine](image1)

Fig. 7- Analysis of DA levels in the striatum at the end of treatment protocol.
*Represents significant increase in SP, EP and SEP groups compared to P group (P<0.05).

![Tyrosine hydroxylase](image2)

Fig. 8- Analysis of TH levels in the striatum at the end of treatment protocol.
*Represents significant increase in SP, EP and SEP groups compared to P group (P<0.05).
Discussion
Parkinson’s disease almost affects 2% of all people older than 70 years. Individuals with Parkinson’s disease exhibit tremors at rest, loss of mental function, involuntary function, and psychiatric problems. Two new proposed experimental treatment for PD are the transplantation of BMSCs into the brain [12] and exercise training [14]. Given that the many studies have shown that exercise and BMSCs transplantation separately have positive effects on parkinsonian rats [12,13,14,15], the present study hypothesized that exercise after BMSCs transplantation may have a synergistic effect and restore dopaminergic neurons and increase angiogenesis in parkinsonian rats induced by 6-OHDA.

The data of apomorphine rotational behavior test showed that the number of contralateral rotations significantly reduced in treatment groups, especially in SEP group compared to P group. Also, the results of narrow beam test for measurement of balance behavioral indicator, showed that the BMSCs transplantation, aerobic exercise and especially combination of them, improve the balance behavioral indicator in treatment groups compared to P group. In a behavioral study, Haji Ghasem Kashani et al. [12] reported that the number of apomorphine-induced rotation decreased in parkinsonian rats after transplantation of BMSCs into the striatum, which is consistent with our results. The authors suggested that the BMSCs injected into the lesion site, secrete growth factors such as BDNF, NGF and cytokines influenced by their environment that impact on their and host cells by autocrine or paracrine mechanisms. These cells not only increase survival and cell differentiation, but lead to the re-innervation and neural communication [12]. In addition, the results of this study demonstrated that the VEGF, DA and TH significantly increased in SP, EP and especially SEP groups compared to P group after treatment.

Stem cell and exercise therapy have been broadly proposed for the treatment of neurodegenerative diseases such as PD [11,12,13]. BMSCs have been considered as an alternative treatment, and it has been reported that these cells have the ability to migrate, differentiate into astrocytes and integrate into the brain [26]. Thus, the ability of BMSCs to differentiate into functional neuronal cells such as dopaminergic cells is under contention. The core pathological event of PD is the degeneration of a specific population of dopaminergic substantia nigra neurons and that in most studies the focus has been on delivering DA-producing cells and TH to the striatum [27]. Garcia and co-workers [28] emphasized the positive role of BMSCs in treating neurodegenerative diseases such as PD by restoring lost dopaminergic neurons. Also, Zigmond et al. [11] and Dutra et al. [29] proposed that exercise as well as neurotrophic factors, are likely to be effective neuroprotective strategies in the treatment of PD. To the best of our knowledge, no study has been conducted to investigate the combined effects of exercise and stem cells in PD. In this study, both BMSCs transplantation and 8 weeks of aerobic exercise, separately and in combination, increased the DA, TH and VEGF levels in the striatum of parkinsonian rats.

One of the interesting findings of this study was the additional effect of exercise on BMSCs transplantation. In this regard, Wahl and co-workers suggested that exercise and physical activity can cause mobilization and blossoming of the stem cells [21]. Moreover, recent advances in the field of stem cells have shown that BMSCs are able to become effectively excitable
Therefore, according to the results of the present study, exercise can have a stimulatory effect on BMSCs to become dopaminergic cells and promote angiogenesis markers such as VEGF. In accordance with our results, the research literature indicates that this process does not occur without stimulation [3,20]. For instance, Al-Jarrah and colleagues [20] reported that 4 weeks of treadmill exercise promoted angiogenesis in the striatum of chronic parkinsonian mice, which can partially explain the beneficial role of exercise in patients with PD. Additionally, Yasuhara et al. [19] have found that parkinsonian rats receiving continuous infusion of VEGF into the brain displayed a significant decrease in amphetamine-induced rotational behavior and a significant preservation of TH-positive neurons and fibers compared with control animals.

The underlying mechanism of these changes remain still unclear [19], but there might be some possible reasons. In PD, blood flow to the brain has a functional importance not only to promote the survival of the affected dopaminergic neurons by providing more oxygen and nutrients, but may also help with efficient drug delivery to the affected areas. This is especially important for levodopa since a large proportion of the drug does not reach the brain, due to its rapid conversion to dopamine [3].

In the present study, it has been shown that the BMSCs transplantation in combination with exercise might have synergistic effects leading to functional recovery, dopaminergic neurons recovery and promotion of angiogenesis marker in the striatum of parkinsonian rats.

References


