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Research report

Dipeptide mimetic of BDNF ameliorates motor dysfunction and striatal apoptosis in 6-OHDA-induced Parkinson's rat model: Considering Akt and MAPKs signaling

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ABSTRACT

Parkinson's disease (PD) is a progressive and debilitating neurodegenerative disorder associated with motor and non-motor complaints. Dysregulation of neurotrophic factors and related signaling cascades have been reported to be common events in PD which is accompanied by dopaminergic (DA) neuron demise. However, the restoration of neurotrophic factors has several limitations. Bis-(N-monosuccinyl-L-methionyl-L-serine) heptamethylenediamide (BHME) is a dipeptide mimetic of brain-derived neurotrophic factor (BDNF) with reported antioxidant and neuroprotective effects in several experimental models. The current study has investigated the effect of BHME on 6-hydroxydopamine (6-OHDA)-caused motor anomalies in Wistar rats. In this regard, rats were treated daily with BHME (0.1 or 1 mg/kg) 1 h after 6-OHDA-caused damage until the twelfth day. Afterwards, motor behavior and DA neuron survival were evaluated via behavioral tests and immunohistochemistry (IHC) staining, respectively. Moreover, the activity of Akt, mitogen-activated protein kinases (MAPKs) family, and Bax/Bcl-2 ratio were evaluated by Western blotting. Our results indicated that BHME prevents motor dysfunction and DA cell death following 6-OHDA injection, and this improvement was in parallel with an enhancement in Akt activity, decrement of P38 phosphorylation, along with a reduction in Bax/Bcl-2 ratio. In conclusion, our findings indicated that BHME, as a mimetic of BDNF, can be considered for further research and is a promising therapeutic agent for PD therapy.

Abbreviations: PD, Parkinson's disease; AD, Alzheimer's disease; DA, dopaminergic; i.p, intraperitoneal; 6-OHDA, 6-hydroxydopamine; MFB, medial forebrain bundle; BHME, bis-(N-monosuccinyl-L-methionyl-L-serine) heptamethylenediamide; SN, substantia nigra; SNpc, substantia nigra pars compacta; NTs, neurotrophic factors or neurotrophins; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; LBs, Lewy bodies; LNs, Lewy neuritis; Trk, tropomyosin receptor kinase; CNS, central nervous system; PNS, peripheral nervous system; BBB, blood brain barrier; ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; Akt, a serine/threonine protein kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c- Jun N-terminal kinase; ALS, Amyotrophic lateral sclerosis; MCAO, middle cerebral artery occlusion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MKP-1, mitogen-activated protein kinase phosphatase-1; ECL, enhanced chemiluminescence; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; BSA, Bovine Serum Albumin; TH, tyrosine hydroxylase; RIPA, Radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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1. Introduction

Parkinson's disease (PD) is an incapacitating and the second most prevalent nervous system disorder after Alzheimer's disease (AD) that predominantly affects the nigrostriatal pathway. This neurodegenerative disorder typically occurs in the elderly over the age of 60, of whom approximately 1 % are affected [1]. The symptoms develop progressively over many years and ultimately become more severe. Disturbance in the nigrostriatal pathway is due to the selective and irreversible death of dopaminergic (DA) neurons of substantia nigra pars compacta (SNpc) which results in dopamine dysregulation in the striatum. Moreover, the abnormal accumulation of the protein α-synuclein in remaining neurons, called Lewy bodies (LBs) and Lewy neuritis (LNs), is the well-known neuropathological hallmark of PD, all of which bring about several motor and non-motor manifestations [2–4]. The most common early motor symptoms are resting tremor, bradykinesia, muscle stiffness, and postural abnormalities. As the disease advances, symptoms can also include non-motor manifestations (cognitive dysfunction, sleep disorders, dementia, hyposmia, etc.) that worsen over time [2,5,6]. Despite many efforts, the cause of PD remains unclear, and it is well documented that both genetic and environmental factors have a central role in its etiology [3]. Several lines of evidence suggest that defective protein clearance pathways, mitochondrial dysfunction, gene mutations, neuro-inflammation, oxidative stress, and neurotrophic factors (NTs) dysregulation are implicated in the development of PD [7–11]. For the first time in 1999, Mogi et al. indicated that brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) concentrations were significantly decreased in the substantia nigra (SN) of parkinsonian patients [12]. Later on, a few other researchers also expressed that alterations in NTs levels may participate in the pathogenesis of neurodegenerative disorders [7,9,13], and they might be potential candidates for therapy.

The neurotrophic factors or neurotrophins, as a family of secreted proteins including NGF, BDNF, neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5), support cell survival, growth, maturation, and differentiation of both developing and mature nervous system via interactions with two types of receptors: the high-affinity tropomyosin receptor kinase (Trk) and the low-affinity $p75^{NTR}$ receptor. Their interactions can trigger at least two main signaling cascades containing phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways [14–16]. Dysregulation of these mentioned pathways has been reported to be a common event in various neurodegenerative disorders, particularly PD [17–19].

Regarding NTs neuroprotective effects in the central and peripheral nervous systems (CNS and PNS) on one hand and dysregulation of NTs levels in neurodegenerative disorders on the other hand [9,20], several research were conducted to apply these growth factors, particularly NGF and BDNF, as therapeutic agents in nervous system disorders [21–24]. However, due to the weak pharmacokinetic properties of NTs, problems in the delivery of these large polypeptides into the CNS, weak capability to cross the blood-brain barrier (BBB), short half-life, and unendurable side effects (such as hyperalgesia and severe weight loss), researchers have begun to develop small molecule mimetics which can expectantly accomplish the clinical potential of NTs attitude towards the treatment of some neurodegenerative disorders [25–28].

Among the abundant efforts was the development of mimetic molecules, which were designed based on the structure of NTs. These mimetics can interact with Trk receptors and trigger related downstream signaling cascades without causing associated side effects [29,30]. One of these mimetic molecules is the dimeric dipeptide bis-(N-monosuccinyl-L-methionyl-L-serine) heptamethylenediamide (BHME) which was synthesized based on the BDNF loop 1 β-turn sequence. So far, in vitro studies have shown that this compound, which was referred to as GSB-214, has neuroprotective activity under oxidative stress conditions, like BDNF. It can interact with the TrkB receptor and trigger the PI3K/Akt signaling pathway. Additionally, this mimetic has antidiabetic properties [31] and can reduce cerebral infarct volume in an animal model of stroke [32,33]. However, less evidence about the possible effectiveness of this dimeric mimetic against PD is available. Considering the effects of NTs mimetics as mentioned earlier, and the lack of investigations about the possible effectiveness of BHME against PD, in the present research, we aim to investigate if BHME is effective in reverting the PD-like behavioral and molecular deficits in an experimental animal model of 6-hydroxydopamine (6-OHDA) neurotoxicity.

2. Material and methods

2.1. Drugs, reagents, and antibodies

6-OHDA was purchased from Sigma-Aldrich, Germany (Cat # 28094–15–7). Apomorphine hydrochloride hemihydrate (Cat # sc-253341) was purchased from Santa Cruz Biotechnology Company, USA. Desipramine-HCl was from Pars daroo company, Iran. Primary antibodies, including β-actin (Cat # 4970), Bax (Cat # 2772), and the phosphorylated form of antibodies, including extracellular signal-regulated kinase (ERK) (Cat # 4377), P38 (Cat # 9211), c- Jun N-terminal kinase (JNK) (Cat # 4671), Akt (Ser473) (Cat # 4060) and anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cat # 7074) were obtained from Cell Signaling Technology (Danvers, MA, USA), and Bcl-2 (Cat # ab59348) was purchased from Abcam (Waltham, MA,USA). Tyrosine hydroxylase (TH) antibody (Cat # orb333893) was from Generon Clinisciences Group, Ireland. Amersham™ ECL™ Select (RPN2235) Detection Reagent kit was purchased from GE healthcare. PVDF (Immobilon-P polyvinylidene difluoride) Membrane (IPVH00010) and Bovine Serum Albumin (BSA, 1120180100) were from Millipore. Salts were bought from Merck. Halt protease/phosphatase inhibitor cocktail (78440) was obtained from Thermofisher Scientific.

2.2. Animals

Experiments were conducted on male Wistar rats weighing 230–270 g. Rats were taken from the animal house of the Neuroscience Research Center, Shahid Beheshti University of Medical Sciences. The rats were randomly housed five per cage in a temperature-controlled (23 ± 2 °C) room that was kept on a 12 h light/dark cycle. Water and food were available ad libitum. Treatments and tests were performed at the same time to prevent circadian variations. All experiments and methods were carried out based on the standards of the Ethics committee of the Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1398.819).

2.3. Experimental groups and surgical procedures

In the present study, 40 male rats were divided into five groups: (a) control (sham/vehicle, n = 8), (b) 6-OHDA (20 μg/rat, stereotaxic surgery, n = 8), (c) 6-OHDA + BHME (0.1 mg/kg, intraperitoneal injection or i.p., n = 8), (d) 6-OHDA + BHME (1 mg/kg, i.p., n = 7, one of the rats died before the behavioral tests started) and (e) BHME (0.1 mg/kg, i.p., n = 8). These doses were chosen based on previous in vivo studies on dipeptide mimetics [32,33]. Two consecutive days before the surgery, all the animals underwent training for behavioral tests; rotarod treadmill and beam walking test. On the day of surgery, desipramine (25 mg/kg) was administered intraperitoneally 1 h prior to the surgery to prevent the noradrenergic neurons damage. Afterwards, the animals were anesthetized with the injection of ketamine/ xylazine (100/10 mg/kg, i.p.) and placed in a stereotaxic apparatus. Control and BHME groups received a unilateral injection of the vehicle, and other experimental groups were injected with 6-OHDA (total concentration of 20 μg 6-OHDA dissolved in 2 μl normal saline 0.9 % containing 0.02 % ascorbic acid). The microinjection of 6-OHDA was done into the right medial forebrain bundle (MFB) using a 10 μl Hamilton syringe at the following coordinates from the atlas of Paxinos and Watson: AP = −4.3,

ML = 1.6, DV = −8.2 [34]. The rate of the injection was 1 μl/min, and the needle was left for an additional 5 min before being removed slowly.

2.4. BHME administration

BHME treatments were started 1 h after the surgery. In the treatment groups, rats received i.p. injections of BHME for 12 days at two various doses: 0.1 and 1 mg/kg. Control and 6-OHDA groups received an equal volume of BHME solvent (distilled water). On the 13th day, behavioral tests were performed.

2.5. Behavioral testing

All behavioral experiments were carried out 13 days after the stereotaxic surgery, with 1 h intervals. In all behavioral procedures, silence was maintained in the room.

2.5.1. Rotarod treadmill

Motor performance, including motor coordination, motor control, and balance, was appraised using the rotarod treadmill apparatus as described elsewhere [35]. The rotarod apparatus consists of a hanging rod that can rotate at constant or at accelerating velocity. Each rat individually received two consecutive training days before the surgery for free adaptation. On the first day, rats were situated on the rotating cylinder with a steady velocity of 10 rpm. On the second day, the cylinder velocity increased from 5 to 20 rpm. On the test day, the apparatus was set to hasten slowly from 5 to 40 rpm for all test sessions. Both training and testing days consisted of 5 trials, and the entire time of each trial was 5 min. Finally, the average elapsed time of all sessions was calculated and documented for each rat as the final score.

2.5.2. Beam walking test

The beam walking test assessed the rodent gait, motor coordination, and balance. The beam apparatus consisted of a 4 cm wide, 100 cm long, and 3 cm thick wooden beam. A home cage was placed on one side of the beam, and the starting point was on the other side. The whole assembly was 80 cm above the ground. Each rat was situated on the starting point away from the home cage, and allowed to walk toward it. All the animals were individually trained for two consecutive training days before the surgery. Both training and testing sessions had five trials with 2 min cut-off on testing days. The trials ended when the rat placed its hindlimbs into the cage. Eventually, the elapsed time on the beam was recorded, and the average elapsed time of all trials was documented as the final score [34].

2.5.3. Open field test

Open field test appraised general locomotor activity in a novel environment, as described previously [36]. Briefly, each rat was separately situated in the corner of a closed box (60 cm × 60 cm) for 1 min habituation period. Then, two parameters including total distance traveled and velocity, were video-recorded automatically for 5 min and analyzed by a computerized system (Noldus, EthoVision XT 11) (Noldus Information Technology, Wageningen, Netherlands). At the end of each trial, the box was washed with 70 % ethanol solution and dried to remove odors left by the previous animal.

2.5.4. Apomorphine-induced rotations test

One day after the last injection of BHME, apomorphine-induced rotations test was performed. Accordingly, each rat was individually situated in the cylinder for habituation. Then, animals were tested for turning behavior by subcutaneous injection of apomorphine hydrochloride (0.25 mg/kg) into their necks. Video recording was begun 5 min after the injection, and contralateral rotations were recorded and counted for 10 min [36].

2.6. Immunohistochemistry

One day after completing the behavioral tests, the density of TH-positive neurons in the SNpc of the rats was evaluated. In this regard, three rats from each group were randomly selected for further immunohistochemistry (IHC) assay. The animals were deeply anesthetized with ketamine/xylazine (100/10 mg/kg) and transcardially perfused with normal saline, followed by a 4 % paraformaldehyde solution. Next, brains were extracted, fixed with 4 % paraformaldehyde, and then embedded in paraffin. Tissue processing (fixation, dehydration, clearing, and embedding) was performed by an automatic tissue processor (DS2080/H, Did sabz Co.). Afterwards, 5 μm thick coronal sections were prepared from the SN region. Briefly, the sections were incubated with an anti-TH primary antibody at 4 °C overnight in blocking Tris-buffered saline (TBS) containing 2 % normal serum and 5 % BSA. TH-immunoreactivity was identified by HRP-linked secondary antibody and visualized using liquid DAB, followed by counterstaining with Mayer's hematoxylin. Finally, all stained samples were analyzed using a light microscope. To evaluate the number of TH-positive neurons, Image J software from NIH (Bethesda, MD, USA) was utilized for counting.

2.7. Western blot analysis

The Western blotting method was carried out as previously described [37]. Briefly, the animals underwent mild anesthesia using CO_2 inhalation and then have been decapitated. Their right striatum tissues were removed, homogenized, and lysed in RIPA (Radioimmunoprecipitation assay) lysis buffer containing protease and phosphatase inhibitor cocktail. To determine the total protein concentration, the Bradford test was applied, and 40 μg protein of each sample was loaded on 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. After blocking with 5 % BSA at room temperature for 1 h, the membranes were probed overnight with these primary antibodies: Akt, ERK, P38, JNK, Bcl-2, Bax, and β-actin (as internal standard) at 4 °C. Next, blots were incubated with HRP-conjugated secondary antibody for 90 min. Subsequently, the immunoreactivity was assessed using enhanced chemiluminescence (ECL select; GE Healthcare) reagents, and the density of protein bands was quantified using Image J software from NIH (Bethesda, MD, USA).

2.8. Statistical analysis

All data are presented as mean ± S.D. GraphPad Prism version 6.0 was used for data analysis. The Kolmogorov-Smirnov normality test was also applied to evaluate the normality of behavioral results. All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. In all cases, $p < 0.05$ was taken as the criterion of statistically significant difference.

3. Results

3.1. BHME attenuated motor dysfunction in 6-OHDA-treated rats

In the current study, motor coordination and balance were measured using the rotarod treadmill and beam walking test. Statistical analysis showed that there was a significant difference between the groups in both tests [$F(4, 34) = 103.5$, $p < 0.0001$] and [$F(4, 34) = 30.36$, $p < 0.0001$], respectively.

A) According to post-hoc Tukey's test, the latency time to fall off from the cylinder meaningfully diminished (76 % reduction) in the 6-OHDA-treated groups compared to the control group. However, BHME injection at a dose of 0.1 mg/kg significantly enhanced (108 % elevation) the latency time compared to the model animals (Fig. 1 A).

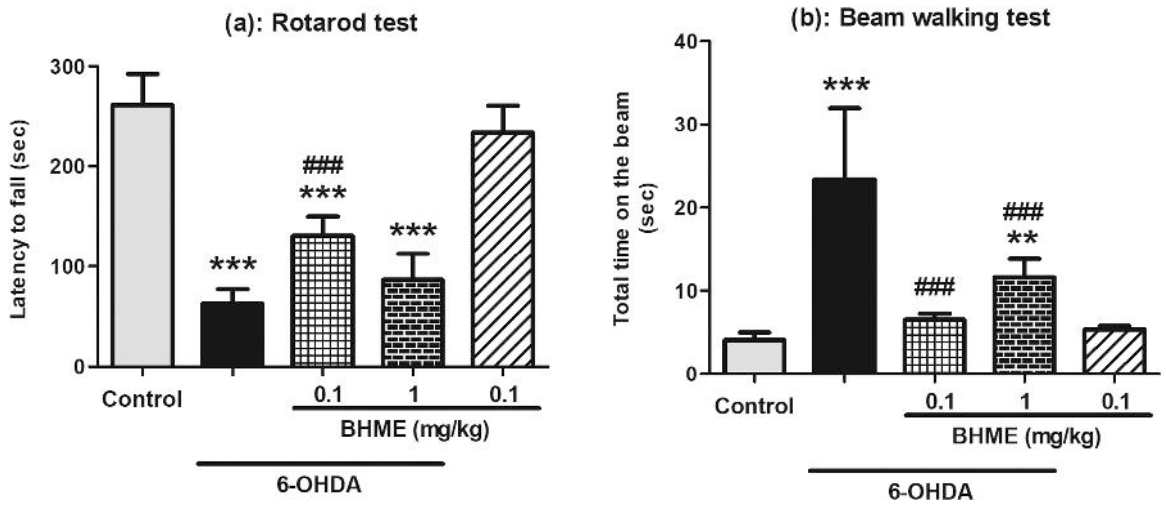

Fig. 1. shows the results of motor coordination and balance tests. (A): shows the effect of 6-OHDA and/or BHME treatment on latency time to fall off from the rotating rod. As is clear, 6-OHDA meaningfully induces motor impairments. Intraperitoneal injection of BHME at a dose of 0.1 mg/kg significantly attenuates motor dysfunction following 6-OHDA injection. However, it still has a significant difference from the control group. (B): shows the effect of 6-OHDA and/or BHME treatment on the beam walking test. After the 6-OHDA injection, the total time on the beam meaningfully increases, whereas BHME treatment significantly decreases the total time compared to the 6-OHDA group. Values are expressed as means ± S.D. (** $p < 0.01$, *** $p < 0.001$ compared to the control group, ### $p < 0.001$ compared to the 6-OHDA group, n = 7–8/group).

B) To appraise the bradykinesia as an indicator of motor dysfunction in PD animal models, the beam walking test was performed. According to post-hoc Tukey's test, a considerable enhancement (468 %) in total time on the beam was observed in the 6-OHDA animals compared to the control group. In contrast, BHME-injected rats behaved similarly to the control group (73 % at a dose of 0.1 mg/kg and 51 % at a dose of 1 mg/kg) (Fig. 1B).

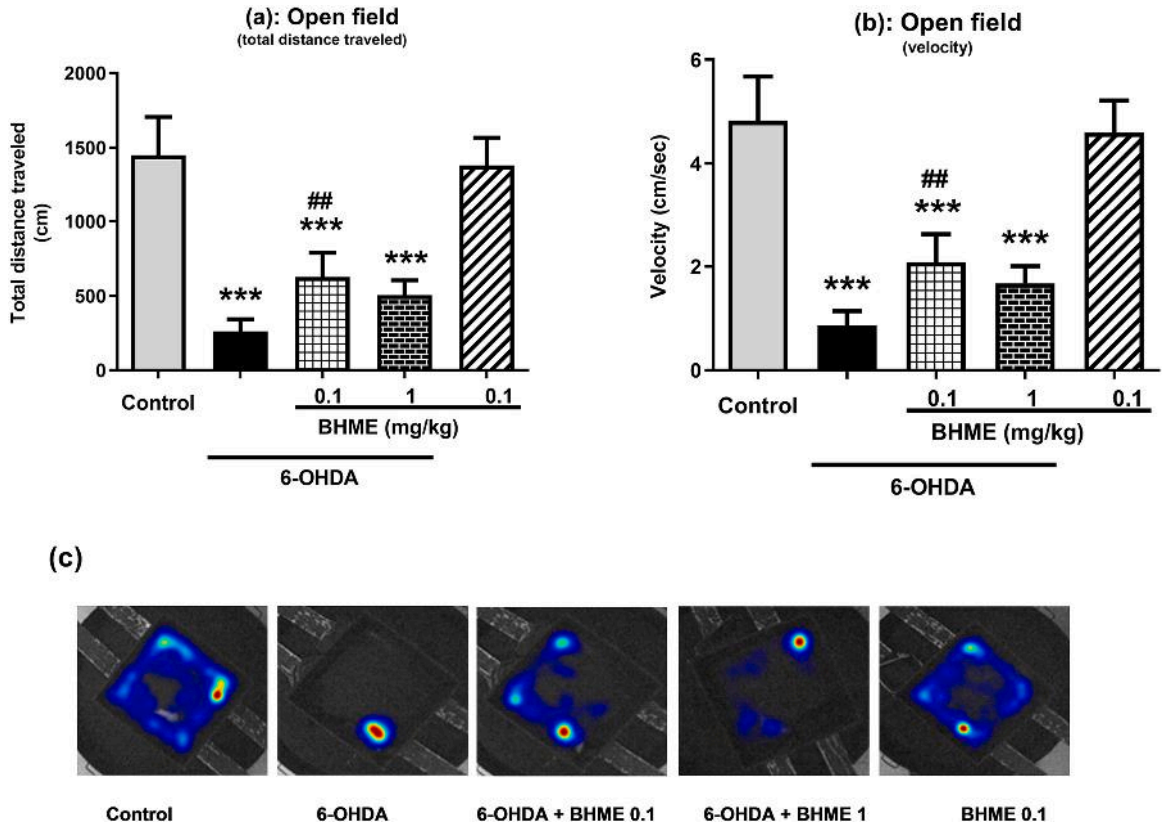

Fig. 2. shows the effect of 6-OHDA and/or BHME treatment on (A) total distance traveled and (B) velocity in the open field test. Following 6-OHDA injection, the total distance traveled and velocity significantly decrease compared to the control animals. BHME treatment at a dose of 0.1 mg/kg meaningfully improves the mentioned locomotor activities. (c) Heatmap images of the total distance traveled are in accordance with the graphs. Values are expressed as means ± S.D. (*** $p < 0.001$ compared to the control group, ## $p < 0.01$ compared to the 6-OHDA group, n = 7–8/group).

3.2. BHME improved general locomotor activity in 6-OHDA-injected rats

The open field test was accomplished to measure the general locomotor activity. Two parameters, including total distance traveled and velocity, were determined in this test. Data analysis revealed a significant difference between the groups [F (4, 34) = 78.35, $p < 0.0001$] and [F (4, 34) = 78.66, $p < 0.0001$], respectively. Both mentioned parameters significantly reduced in the 6-OHDA-treated animals compared to the control animals (82 % and 83 % reduction, respectively). According to post-hoc Tukey's test, the administration of BHME at a dose of 0.1 mg/kg significantly improved the tested parameters. (139 % and 140 % enhancement, respectively) (Fig. 2).

3.3. BHME treatment decreased apomorphine-caused rotations in 6-OHDA-damaged rats

In the case of the apomorphine rotation test as a main test which is used to prove the induction of the rat PD model, one-way ANOVA analysis showed a significant difference between the groups [F (4, 34) = 102.6, $p < 0.0001$]. In this test, 6-OHDA-treated rats indicated a significant enhancement in the number of contralateral rotations compared to the control animals, whereas BHME treatment considerably reduced the rotations (41 % reduction at a dose of 0.1 mg/kg and 32 % reduction at a dose of 1 mg/kg). However, BHME treatment groups still had a significant difference from the control animals (Fig. 3).

3.4. BHME treatment protected TH-positive cells in the SNpc against 6-OHDA toxicity

TH-positive immunohistochemistry staining was used as a well-known histological staining for the determination of DA neurons. Since BHME at a dose of 0.1 mg/kg showed better results in all the behavioral studies, we used this dose in the immunohistological study. According to data analysis, 6-OHDA caused 84 % DA neuron death compared to the control animals [F (2, 6) = 37.72, $p = 0.0004$]. In contrast, intraperitoneal injection of BHME 237 % protected them against 6-OHDA toxicity (Fig. 4).

3.5. Effects of BHME treatment on Akt activity in the striatum

Western blot analysis displaying the effects of treatments in our study on the amount of phosphorylated Akt (Ser473) in the striatum of rats is depicted in Fig. 5. A single band for p.Akt at 60 kDa was detected in striatum extraction. One-way ANOVA analysis indicated a significant difference between the groups [F (3, 12) = 9.270, $p = 0.0019$]. According to post-hoc Tukey's test, 6-OHDA significantly suppressed Akt activation, but BHME treatment at a dose of 0.1 mg/kg reversed the 6-OHDA-induced decrement of Akt activation.

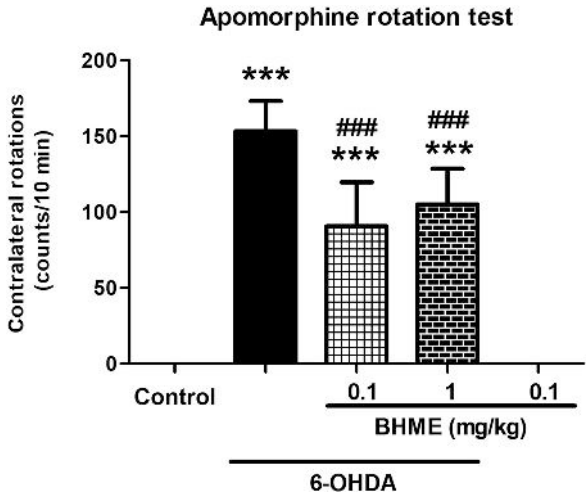

Fig. 3. Effect of 6-OHDA and/or BHME treatment on apomorphine rotation behavior. As is evident, contralateral rotations were considerably enhances in 6-OHDA-treated groups compared to the control group. BHME treatment meaningfully reduces the rotations induced by 6-OHDA. Values are expressed as means ± S.D. (*** $p < 0.001$ compared to the control group, ### $p < 0.001$ compared to the 6-OHDA group, n = 7–8/group).

3.6. Effects of BHME treatment on P38 phosphorylation levels in the striatum

A single band for p.P38 at 43 kDa was detected in striatum extraction. As it is evident in Fig. 6, the statistical analysis reported a significant difference between the groups [F (3, 12) = 14.80, $p = 0.0002$]. Consequently, post-hoc by Tukey's test indicated that 6-OHDA significantly increased p.P38/β-actin ratio compared to the control rats. Treatment with BHME meaningfully reduced the 6-OHDA-induced elevation of P38 phosphorylation levels.

3.7. Effects of BHME treatment on JNK phosphorylation levels in the striatum

Western blot results of treatments on the striatal JNK phosphorylation levels are shown in Fig. 7. Anti p.JNK antibody detected two bands: JNK1 at 46 kDa and JNK2 at 54 kDa. According to data analysis, JNK phosphorylation had no significant difference between the groups [F (3, 12) = 1.863, $p = 0.1897$].

3.8. Effects of BHME treatment on ERK phosphorylation levels in the striatum

Western blot results of treatments on the striatal ERK phosphorylation levels are presented in Fig. 8. Anti p.ERK antibody visualized two bands at 42 kDa and 44 kDa. According to data analysis, ERK phosphorylation had no significant difference between the groups [F (3, 12) = 1.987, $p = 0.1698$].

3.9. Effects of BHME treatment on Bax/Bcl2 ratio in the striatum

Western blot results on Bax/Bcl-2, Bax/β-actin, and Bcl-2/β-actin ratios are presented in Fig. 9. Antibodies against Bax and Bcl-2 visualized a band at 20 kDa and a band at 26 kD, respectively. One-way ANOVA analysis displayed a significant difference between the groups [F (3, 12) = 8.418, $p = 0.0028$]. Subsequently, post-hoc by Tukey's test revealed that while 6-OHDA injection significantly enhanced the ratios of Bax/Bcl-2 and Bax/β-actin, BHME treatment at a dose of 0.1 mg/kg reversed this 6-OHDA-induced elevation of Bax/Bcl-2 and Bax/β-actin ratios.

4. Discussion

The results of the current research work revealed that i.p. injection of BHME preserved the demise of nigrostriatal DA neurons against 6-OHDA toxicity. It also attenuated motor impairments and led to a significant enhancement in the density of DA neurons in the SNpc of PD-like model rats. These results are in accordance with and confirmatory to our molecular results demonstrating that BHME neuroprotective role was correlated with the regulation and/or modulation of some intracellular signaling pathways, including apoptosis, PI3K/Akt, and MAPKs.

According to the acquired results, 6-OHDA-treated rats showed motor deficiencies due to the degeneration of impaired DA neurons. These findings are in accordance with the previous investigations [38–40]. In contrast, daily treatment of BHME at a dose of 0.1 mg/kg meaningfully attenuated motor impairments and protected DA neuron survival, which were clearly seen in the behavioral and immunohistological studies. Since dipeptide mimetics of BDNF can cross BBB and due

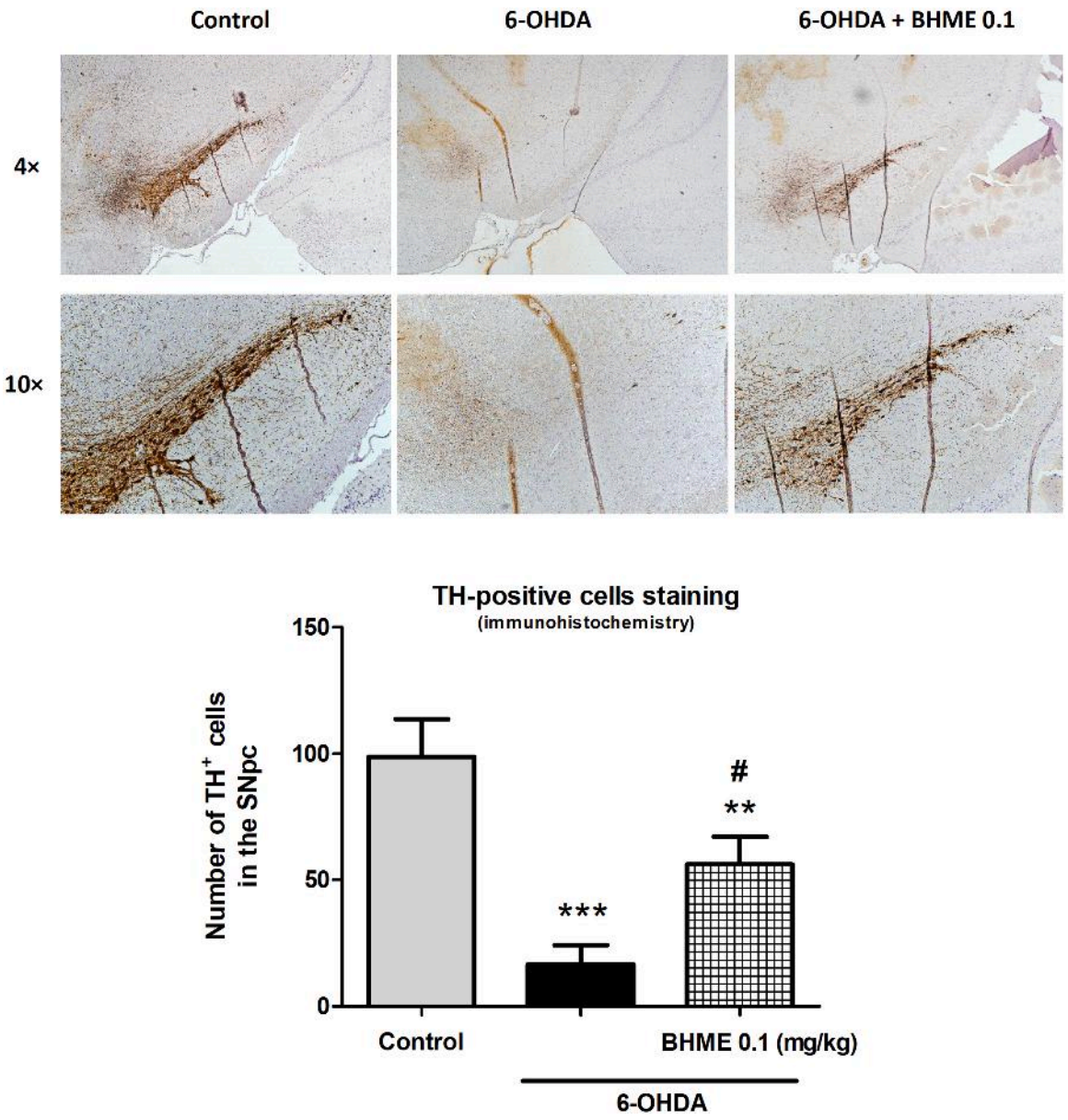

Fig. 4. Effect of 6-OHDA and/or BHME treatment on TH-positive cells number in the SNpc. The 6-OHDA injection causes extensive DA neuron death compared to the control animals. BHME treatment at a dose of 0.1 mg/kg protects DA neurons against 6-OHDA toxicity. Values are expressed as means ± S.D. (** $p < 0.01$, *** $p < 0.001$ compared to the control group, # $p < 0.05$ compared to the 6-OHDA group, n = 3/group).

to the nigrostriatal pathway involvement in the accurate movements, it seems that BHME protects DA neurons from 6-OHDA-induced nigrostriatal function deterioration. Formerly, it has been shown that BHME at a dose of 0.1 mg/kg exhibits neuroprotective activity against middle cerebral artery occlusion (MCAO) in rats [32,33]. In another in vivo research, this mimetic at the same dose, prevented memory impairments in the streptozotocin-induced AD model [41]. Moreover, in vitro studies indicated that BHME preserves HT-22 hippocampal neuronal cell line under the oxidative stress conditions [42]. While NTs play a vital role in modulating the function, survival, proliferation, and differentiation of neuronal cells [43], it is important to note that excessive amounts of NTs can potentially have destructive effects (such as neuronal cell demise, learning and memory deficits) due to different receptor-dependent mechanisms [44,45]. Additionally, based on a survey conducted by Thoenen and Sendtner, various side effects of NTs, which prevent the use of higher doses, are the main reasons why clinical trials of NTs have failed [46]. Hyperalgesia, weight loss, sleep disturbance, diarrhea, and etc. are among the side effects of NTs [46]. However, there have been no studies on the possible harmful effects of NTs mimetics, like the one in our study. Based on the evidence mentioned earlier, we suggested that at higher doses of BHME, the side effects mentioned above may outweigh the benefits. This could be the reason why we observed weaker protective effects at the higher dose in our study.

It has been well established that the striatum is a critical structure involved in the voluntary movement control. So, in the next step, the right striatum of each rat was extracted for evaluating molecular alterations study associated with these effects. In line with earlier investigations [47,48], our present research work confirmed that p-Akt (ser473) amounts in 6-OHDA-lesioned rats were significantly lower than in the control animals. However, BHME was effective in the increment of p-Akt (ser473) levels in the striatum of 6-OHDA-damaged rats. Accumulating evidence intensely suggests that a defective PI3K/Akt signaling pathway is associated with PD [49,50]. Akt, also known as PKB (protein kinase B), is a serine/threonine kinase that mediates various biological functions such as cell proliferation, growth, and survival/apoptosis. Besides, PI3K/Akt pathway plays a crucial role in neuroprotection, and

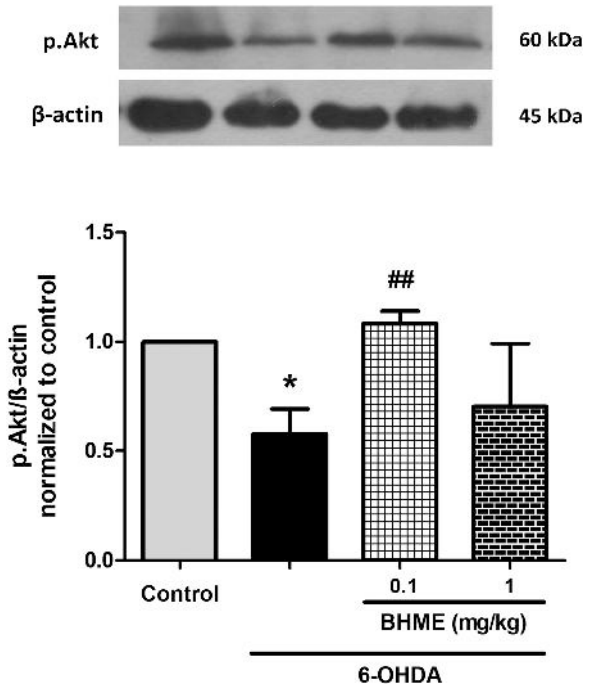

Fig. 5. Effect of 6-OHDA and/or BHME (0.1 and 1 mg/kg) treatment on the striatal phosphorylated Akt (Ser473) protein. In the bar graph, the normalized values of the control group were set at 1. As is obvious, 6-OHDA significantly suppresses the Akt activation compared to the control group, while BHME treatment at a dose of 0.1 mg/kg meaningfully reverses the decrement of p-Akt levels. Data are represented as mean ± S.D. (* $p < 0.05$ compared to the control group, ## $p < 0.01$ compared to the 6-OHDA group, n = 4/group).

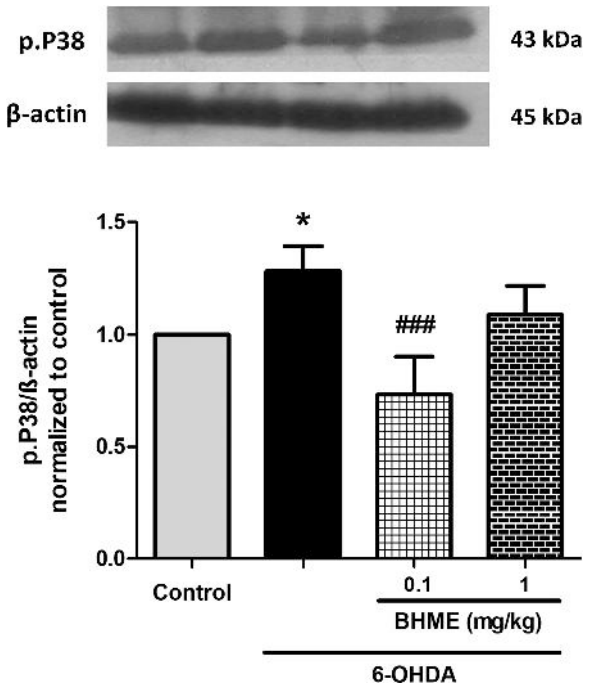

Fig. 6. Effect of 6-OHDA and/or BHME (0.1 and 1 mg/kg) treatment on the striatal phosphorylated P38 protein. In the bar graph, the normalized values of the control group were set at 1. According to the analysis, 6-OHDA significantly enhances p.P38 levels compared to the control group. In contrast, BHME treatment meaningfully decreases the enhancement of p.P38 levels. Data are represented as mean ± S.D. (* $p < 0.05$ compared to the control group, ### $p < 0.001$ compared to the 6-OHDA group, n = 4/group).

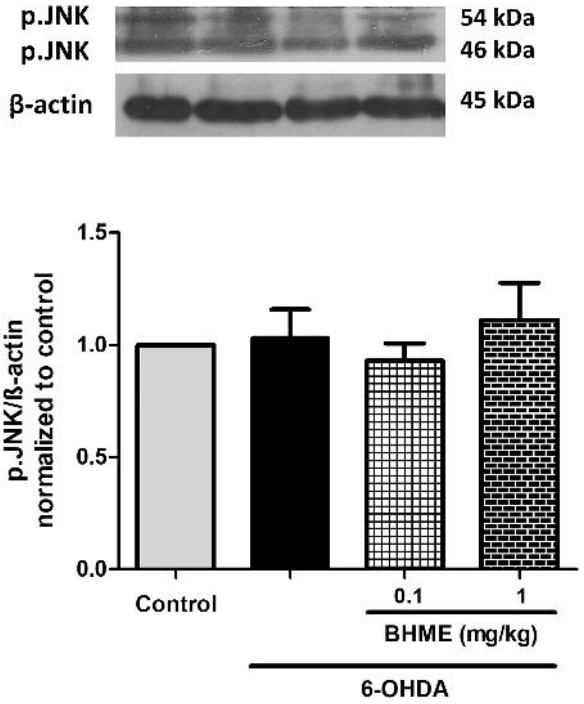

Fig. 7. Effect of 6-OHDA and/or BHME treatment (0.1 and 1 mg/kg) on the striatal phosphorylated JNK protein. In the bar graph, the normalized values of the control group were set at 1. No significant difference was seen between the groups. Data are represented as mean ± S.D. (n = 4/group).

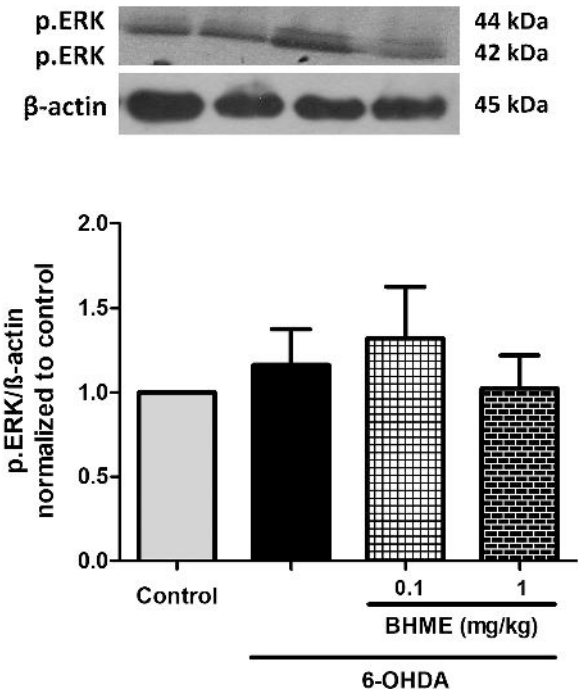

Fig. 8. Effect of 6-OHDA and/or BHME (0.1 and 1 mg/kg) treatment on the striatal phosphorylated ERK protein. In the bar graph, the normalized values of the control group were set at 1. No significant difference was seen between the groups. Data are represented as mean ± S.D. (n = 4/group).

it has been reported that impaired Akt signaling is related to common nervous system diseases like AD and PD [51]. It has been well-documented that 6-OHDA, a widely recognized neurotoxin that induces PD models and causes DA neuron degeneration, exerts its

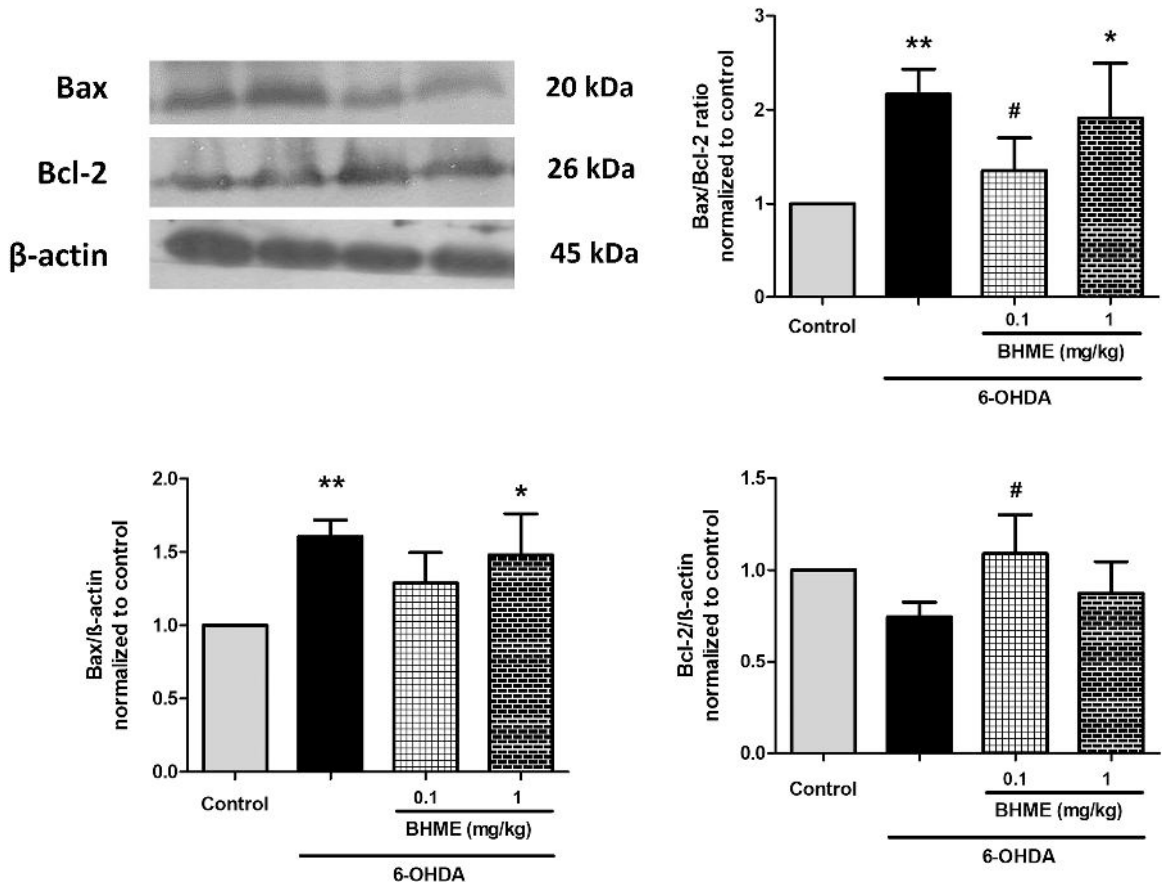

Fig. 9. Effect of 6-OHDA and/or BHME (0.1 and 1 mg/kg) treatment on the striatal Bax/Bcl-2, Bax/β-actin, and Bcl-2/β-actin ratios. In the bar graph, the normalized values of the control group were set at 1. As is clear, 6-OHDA significantly increases Bax/Bcl-2 and Bax/β-actin ratios compared to the control group, while BHME treatment at a dose of 0.1 mg/kg reverses the enhancement of these ratios. Data are represented as mean ± S.D. (* $p < 0.05$, ** $p < 0.01$ compared to the control group, # $p < 0.05$ compared to the 6-OHDA group, n = 4/group).

detrimental effects primarily through the generation of reactive oxygen species (ROS), cytotoxic species, and oxidative stress, ultimately leading to neuronal apoptosis [52–54]. Akt signaling pathway can decrease 6-OHDA-induced ROS production [55]. There are several evidence demonstrating the association between 6-OHDA and disrupted Akt signaling [47,49,56], whereby 6-OHDA significantly attenuates p-Akt levels [57]. This is in agreement with our findings. Additionally, previous documents have shown that Akt activation prevents apoptosis via the inhibition of pro-apoptotic factors or modulation of transcription factors involved in apoptosis [58]. Phosphorylated Akt supports cell survival by increasing the phosphorylation levels of downstream molecules including GSK3β, Bad, and caspase-9 [49]. Accordingly, previous investigations demonstrated that BHME increases the TrkB phosphorylation levels followed by the activation of the PI3K/Akt pathway [32,42]. For example, Gudasheva et al. have shown that this compound is capable of elevating the TrkB receptor phosphorylation similar to BDNF and can selectively trigger PI3K/Akt pathway. They also have established that this mimetic does not activate MAPK/ERK pathway [42]. Taken together, based on previous studies and our experimental findings, we can deduce that BHME applies protective impacts via activating the PI3K/Akt signaling pathway.

Multiple bodies of evidence implicate the disruption of kinase activities and related phosphorylation pathways in the pathogenesis of neurodegenerative disorders, particularly PD. This has been shown in numerous studies [59,60]. Accordingly, we evaluated the activity of MAPKs signaling molecules (P38, JNK, and ERK) to figure out if they contribute to the neuroprotective impacts of BHME against 6-OHDA--generated motor impairments. Our results indicated that BHME treatment significantly reversed the 6-OHDA-mediated elevation of P38 phosphorylation levels. In support of our findings, former investigations have revealed that persistent activation of JNK and P38 can lead to neuronal apoptosis in the brain of AD, PD, and Amyotrophic lateral sclerosis (ALS) patients [59,61,62]. The P38 MAPKs are particularly activated through a series of inflammatory cytokines and cytotoxic stress insults and can promote critical cellular processes, including survival, proliferation, stress-mediated apoptosis, and neuro-inflammation [63,64]. Multiple lines of evidence obtained from in vivo and in vitro studies proposed that neurotoxins (including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or MPTP, 6-OHDA, etc.)-induced P38 activation stimulates cell apoptosis in DA neurons [63,65,66]. Moreover, several studies have also indicated that inhibitors of P38 enhance DA neurons survival against different toxic insults in PD models [63,65,67,68]. Besides, it has been reported that MKP-1 (mitogen-activated protein kinase phosphatase-1) overexpression, which negatively regulates MAPKs activity, in DA neurons protects them against 6-OHDA-induced neurotoxicity [69]. It must be noted that oxidative stress, as the main mechanism underlying 6-OHDA toxicity, can trigger P38 signaling pathway that promotes apoptosis in DA neurons [67]. On the other hand, numerous other reports have demonstrated that treating SH-SYS5 neuroblastoma cells with N-acetyl-L-cysteine impressively inhibits 6-OHDA-produced ROS and restrains the activation of JNK and P38 by 6-OHDA, and hence decreases cell apoptosis [70,71]. Because neuro-inflammation and oxidative stress are important features of PD, and P38 signaling is involved in both processes, we can conclude that the inhibitory effects of BHME on P38 signaling are, at least in part, due to its anti-oxidant and

anti-inflammatory properties.

To further understand the implicated downstream molecular mechanism, the active form of ERK and JNK proteins were also assessed. Their phosphorylation levels had no significant difference between the control and treated groups in this research. However, mounting evidence has stated that the activated form of JNK provokes neuronal apoptosis in AD and PD patients [59,60]. It has been well-documented that JNKs adjust a series of processes in the brain (including neuro-inflammation, development, plasticity, etc.) and promote apoptosis via nuclear and non-nuclear downstream pathways [72,73]. Studies by Pan et al. reported that 6-OHDA-activated JNK3 results in DA neuron death by enhancement of caspase-3 activation and mitochondrial cytochrome c release [74,75]. Other in vitro and in vivo studies of PD have revealed that JNK signaling suppression with inhibitors (such as CEP-1347) protects cell survival [74,76,77]. However, the clinical use of CEP-1347 has failed [78]. This contradiction shows the necessity for more preclinical investigations regardless of previous findings.

ERK signaling has a pivotal role in the modulation of cell survival/death, differentiation, and proliferation. Dysregulation of the ERK signaling cascade results in the pathogenesis of different neurodegenerative disorders, particularly AD and PD [49,79]. As mentioned above, according to a survey done by Gudasheva et al. in 2012, this mimetic of loop 1 BDNF does not increase ERK phosphorylation levels following binding to the TrkB receptor [42]. The activated form of ERK in the lesioned animals had no significant difference with the control group in our experiments. For many years it has been accepted that ERK1/2 promotes cell survival, but a mounting number of investigations have recently suggested a dual role for ERK1/2 in cell fate. In this regard, temporary ERK activity is protective, whereas its constant activation results in cell demise [49]. For instance, in contrast to the ERK protective effect against oxidative stress, some in vitro studies have shown that 6-OHDA or glutamate-caused chronic activation of ERK contributes to neurodegeneration [80]. However, in our study, due to inconclusive results, we did not obtain strong evidence to confirm the previous findings of others. Nonetheless, we can not completely disregard the potential involvement of JNK and ERK based on our results. The discrepancy observed may be attributed to various factors such as the duration of treatment, type of the study (in vitro or in vivo), the specific brain region studied, different toxic agents used, and other variables. Furthermore, the kinetics and duration of ERK activation, as well as its subcellular localization, may determine whether downstream targets elicit beneficial or detrimental effects on neuronal cells.

In the context of apoptosis, abundant studies have shown that members of the Bcl-2 family adjust apoptosis or programmed cell death by either stimulating pro-apoptotic or inhibiting anti-apoptotic proteins. Among these family members, Bcl-2 is categorized as an anti-apoptotic protein, whereas Bax is a pro-apoptotic protein, and their comparative expression ascertains cell destiny [81]. Our results indicated that Bax/Bcl-2 ratio in 6-OHDA-treated animals was considerably higher than in the control animals. However, BHME treatment at a dose of 0.1 mg/kg showed outstanding protection and significantly diminished Bax/Bcl-2 ratio. In agreement with our findings, there are studies concluding harmful effects for 6-OHDA and disrupting signaling pathways which finally leads to cell apoptosis [82,83]. Various in vitro and in vivo studies have indicated that 6-OHDA induces cell death in a way leading to the Bax activation [84,85]. Besides, it has been shown that this neurotoxin causes mitochondrial damage through p38 MAPK-mediated Bax activation. During apoptosis in DA neurons, Bax activation triggers intracellular events that eventually results in the mitochondrial cytochrome c release [83]. In 2008, Ikeda et al. reported that astaxanthin, an antioxidant agent, inhibits 6-OHDA-generated apoptosis via blockade of P38 activation in SH-SY5Y neuroblastoma cells [86]. On the other hand, there are studies indicating that Bcl-2 expression in neurons protects them against 6-OHDA neurotoxicity [87,88]. Although the precise mechanism underlying BHME-modulating these proteins is unknown, it can be concluded that this mimetic anti-apoptotic action, similar to BDNF, might be responsible. It is well-established that BDNF has anti-oxidant and anti-apoptosis effects and modulates different elements of these molecular pathways [89,90].

5. Conclusion

In general, the outcome of our study shows the protective effects of BHME administration against 6-OHDA neurotoxicity. In the 6-OHDA group, motor deficits are accompanied by a reduction of TH-positive cell number in the SNpc and the elevation of p.P38 levels along with the increment of the Bax/Bcl-2 ratio on one hand and the decrement of p.Akt on the other hand. BHME treatment, for the first time, has been shown to reverse these effects. Therefore, to our knowledge, BHME can be considered as a potential healing agent for PD therapy. Nonetheless, further investigations are required to discover the precise molecular mechanisms of this mimetic protection in the various model of PD, such as transgenic and in vitro models.

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Ethical statement

All of the experimental procedures were approved by the Ethical Committee for Research at Shahid Beheshti University of Medical Sciences (ID: IR.SBMU.MSP.REC.1398.819).

CRediT authorship contribution statement

Bita Firouzan: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Validation, Writing - review & editing. **Farideh Iravanpour:** Methodology, Validation, Writing – review & editing. **Fatemeh Abbaszadeh:** Methodology, Resources, Validation, Writing – review & editing. **Valery Akparov:** Validation, Writing – review & editing. **Jalal Zaringhalam:** Supervision, Validation, Writing – review & editing. **Rasoul Ghasemi:** Conceptualization, Supervision, Methodology, Formal analysis, Investigation, Resources, Validation, Project administration, Writing – review & editing. **Nader Maghsoudi:** Conceptualization, Supervision, Methodology, Investigation, Resources, Validation, Project administration, Writing – review & editing.

Data Availability

Data will be made available on request.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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