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A promising therapeutic potential of cerebrolysin in 6-OHDA rat model of Parkinson's disease

Neveen A. Noor a,⁎, Haitham S. Mohammed b, Iman M. Mourad a, Yasser A. Khadrawy c, Heba S. Aboul Ezz a

a Zoology Department, Faculty of Science, Cairo University, Egypt
b Biophysics Department, Faculty of Science, Cairo University, Egypt
c Medical Physiology Department, Medical Division, National Research Center, Egypt

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A B S T R A C T

Aims: Parkinson's disease (PD) is the second most prevalent neurodegenerative disease affecting the population. The present study investigates the potential therapeutic effect of cerebrolysin (CBL), as a neurotrophic factor mimic, on the behavioral and biochemical alterations induced in 6-hydroxydopamine (6-OHDA) – lesioned rats as a model of PD.

Main methods: The animals were divided into 3 experimental groups; control group, Parkinsonian model group through bilateral microinjection of 6-OHDA into substantia nigra (SN) and CBL-treated group which received a daily intraperitoneal administration of CBL (2.5 ml/kg) initiated 24 h after induction of Parkinsonism for 21 days.

Key findings: Treatment of Parkinsonian animals with CBL succeeded in restoring the midbrain and striatum dopamine levels. In addition, it normalized the increased MDA and NO levels recorded in the Parkinsonian animals and replenished the decreased level of midbrain GSH. In addition to the recorded recovery of the biochemical parameters, there was a parallel improvement in the animal's behavioral aspects.

Significance: The findings of the present study provide evidence for the promising therapeutic effect of CBL in the present 6-OHDA rat model of PD through counteracting oxidative stress, replenishing dopamine content and enhancing behavioral outcomes.

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1. Introduction

Neurodegenerative diseases are characterized by progressive damage of neurons [1]. The etiology of neurodegenerative diseases is still not fully understood. However, oxidative stress has been implicated as a common mechanism underlying various neurodegenerative disorders [2].

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease affecting the population [3]. PD is caused by a progressive loss of dopaminergic neurons in the substantia nigra (SN) and depletion of striatal dopamine levels [4]. This is accompanied by the hallmark motor symptoms including rigidity, resting tremor, bradykinesia and postural instability [5]. Moreover, accumulating evidence suggests that mitochondrial dysfunction and oxidative stress play a role in the pathogenesis of PD [4]. It has been reported that reduced activity in complex I of the respiratory chain occurs in the substantia nigra pars compacta (SNc) of PD patients leading to excessive generation of reactive oxygen species (ROS) and apoptosis [6].

Oxidative stress is a state of imbalance between the production of ROS and antioxidant scavenging systems, leading to accumulation of ROS [7]. This can induce tissue injury and may activate apoptosis processes [8]. The brain is more sensitive to oxidative damage due to the high levels of polyunsaturated fatty acids, high utilization of oxygen and the abundance of redox-active transition metal ions [9]. Moreover, there are relatively low levels of reduced glutathione (GSH), which plays an antioxidant role in the elimination of ROS [10].

Till now, treatment with Levodopa (L-Dopa) is the most effective therapy for PD as it compensates the depletion of dopamine [11]. Nevertheless, L-Dopa does not stop PD progression and long term treatment produces side effects such as dyskinesia [12] and promotes neuronal degeneration through oxidative stress [13]. Therefore, it is necessary to search for a more effective therapy that impedes PD progression and possesses regenerative properties and few side effects.

Cerebrolysin (CBL) is a purified porcine brain-derived peptide preparation, consisting of approximately 15% low molecular weight peptides and 85% amino acids, based on total nitrogen [14]. CBL has the ability to cross the blood–brain barrier [15] and exert neurotrophic factor-like activity [16]. It has been reported that CBL is able to promote neurogenesis [17] and synaptogenesis [18]. In addition, CBL has been reported to possess neuroprotective properties which are correlated...
with its effectiveness in the treatment of neurodegenerative diseases such as PD, multiple sclerosis, Alzheimer’s disease, dementia, and acute or chronic stroke [19]. The neurotrophic effect of CBL interferes with excitotoxicity, free radical formation, and inflammatory responses [20–22].

In view of the neurotrophic and neuroprotective properties of CBL, it was prompted to investigate the potential therapeutic effect of CBL on the behavioral and biochemical alterations induced in 6-hydroxydopamine – lesioned rats as model of Parkinsonism.

2. Materials and methods

2.1. Experimental animals

The experimental animals used in the present investigation were adult male Wistar albino rats weighing 250–350 g. They were maintained under fixed appropriate conditions of housing and handling and were given food and water ad libitum. All experiments were carried out in accordance with the research protocols established by the Animal Care Committee of the National Research Center, Egypt which followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

6-hydroxydopamine hydrochloride (6-OHDA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in saline containing 0.2% ascorbic acid (SAS Chemicals Co. Mumbai-India). Imipramine hydrochloride was obtained from Alexandria Co. for: ACIMA International. CBL was purchased from EVER Neuropharma GmbH A-4866 Unterach, Austria. All other reagents were analytical grade reagents purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Experimental design

At the beginning of the experiment 30 animals were divided into three groups (n = 10). Group (1) represented the control animals which received a single bilateral intranigral microinjection of saline and then received a daily intraperitoneal injection of saline 24 h after surgery for 21 days. Group (2) represented the Parkinsonism model in which the animals received a single bilateral intranigral microinjection of 15 μg of 6-OHDA [23] and then received a daily intraperitoneal injection of saline 24 h after surgery for 21 days. The animals of group (3) received a single bilateral intranigral microinjection of 6-OHDA (15 μg) and then received a daily intraperitoneal injection of CBL (2.5 ml/kg, each ml contains 215.2 mg CBL) [16] 24 h after 6-OHDA injection for 21 days.

2.4. Stereotaxic surgery and induction of Parkinsonism

Under sodium pentobarbital (40 mg/kg, i.p.) anesthesia, rats were positioned in the stereotaxic device (David Kopf instruments, Tujunga, California, USA). Animals were pre-treated orally with imipramine (25 mg/kg) 30 min prior to 6-OHDA injection in order to protect noradrenergic neurons. Bilateral microinjections of 15 μg of 6-OHDA (dissolved in 3 μl of 0.2% ascorbic acid saline solution) into the substantia nigra (SN) were applied at the following coordinates, according to the stereotaxic atlas of Paxinos S., amp. S.amp; Watson, relative to the bregma [AP: −5.3 mm, ML: ±2.3, V: (−8.3 mm from the skull surface)] using a10 μl Hamilton syringe. The infusion rate was 1 μl/min, and the injection syringe was kept in place for a further 5 min after injection for complete absorption of the toxin.

2.5. Behavioral assessments

The behavioral tests were carried out on the 20th day post induction of Parkinsonism.

2.5.1. Open field (OF) test

To access the general activity in different rat groups, the open field (OF) test was used. The enclosure was a square 66 × 66 cm² with surrounding walls of 30 cm height. The base of the OF was marked with black parallel lines in both X and Y-axes, forming a grid (36 squares, each square 11 × 11 cm). The central zone was marked with a red color representing 25% of the total arena. All attempts were made to minimize the background noise and stress. All animals to be tested were handled in the same manner.

Each rat was placed in the central zone and observed for 10 min. The OF parameters measured were: the number of squares crossed, the time spent in the central zone, the rearing frequency (the number of vertical movement when the rat stood vertically on its hind paws on the floor and forepaws on the wall), the number of grooming (the number of times the animal spent licking or scratching itself while stationary), the number of stretch attend posture, the freezing duration and the number of defecation [24].

2.5.2. Forelimb hanging test

The wire hanging test was carried out according to Fan et al. [25] with modification to measure the strength and endurance. Each rat was suspended grasping by its forelimb a wire 50 cm above a foam cushion and the latency to drop was recorded. This procedure was repeated 3 times for each rat. Then the mean was calculated for each rat. A greater latency was taken as an indicator of better strength or motor endurance.

2.5.3. Traction test

The traction test was performed as described by Dai et al. [26]. The rats were suspended by their front paws to a wire placed horizontally. The score was determined as follows: 3: the rat grasps the wire with two hind paws, 2: the rat grasps the wire with one hind paw, 1: the rat cannot grasp the wire with neither hind paw.

2.6. Handling of tissue samples

The animals were sacrificed by sudden decapitation at the end of the experiment. The control animals were sacrificed simultaneously with the Parkinsonian and cerebrolysin-treated groups. The brain of each animal was quickly removed and rapidly transferred to an ice-cold Petri dish and dissected to obtain the midbrain and striatum. Each brain sample was divided into two equal halves, weighed and kept at −58 °C until analyzed. The right half of each brain sample was homogenized in acidified butanol and used for the determination of dopamine. The left half was homogenized in 5% w/v 20 mM phosphate buffer, pH 7.6, centrifuged and the supernatant was used for the analysis of the levels of malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO).

2.6.1. Determination of dopamine content

The quantitative determination of dopamine levels was performed according to the method of Ciarlone [27] using a spectrophotometer (Jasco FP-777, with a source of xenon arc lamp 150 W, JASCO Ltd., Tokyo, Japan). The excitation and emission wavelengths are 320 and 375 nm, respectively.

2.6.2. Determination of lipid peroxidation

Malondialdehyde (MDA), as a measure of lipid peroxidation, was determined by measuring the thiobarbituric acid-reactive substances according to the method of Ruiz-Larrea et al. [28], in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce...
a pink colored complex whose absorbance is read at 532 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

2.6.3. Determination of reduced glutathione (GSH) level

Reduced glutathione (GSH) was measured according to Ellman’s method [29]. Ellman’s reagent is reduced by —SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid. The nitromercaptobenzoic acid anion has an intense yellow color whose absorbance is measured spectrophotometrically at 412 nm.

2.6.4. Determination of nitric oxide (NO) level

Nitric oxide (NO) level was assayed as nitrite using Griess reagent according to the method of Moshage et al. [30]. After the addition of Griess reagent, nitrite is converted to a deep purple azo compound whose absorbance is measured spectrophotometrically at 450 nm.

2.7. Statistical analysis

The data were expressed as means ± S.E.M. Analysis of data was carried out by analysis of variance (ANOVA) followed by Bonferroni multiple range test when the F-test was significant (p < 0.05). All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a compatible computer.

3. Results

The intranigral microinjection of 6-OHDA induced significant decreases in midbrain and striatal dopamine levels in Parkinsonian animals, recording 30.81% and 18.33% below the control level, respectively (Figs. 1 and 2). Treatment of Parkinsonian animals with CBL succeeded in restoring the midbrain dopamine level to nearly control value and significantly increased striatal dopamine content as compared with control level.

Regarding oxidative stress parameters (Figs. 1 and 2), the intranigral injection of 6-OHDA resulted in significant increases in midbrain and striatal MDA levels in the Parkinsonian group in comparison to control group. However, treatment of Parkinsonian animals with CBL restored MDA levels to nearly control value in the midbrain. Meanwhile, a significant decrease in GSH level occurred in the midbrain of Parkinsonian rats. This was accompanied by significant increases in NO levels in both midbrain and striatum in the Parkinsonian group with reference to the control group. However, these changes in GSH and NO levels became nonsignificant in the CBL-treated group in comparison to the control group. In the striatum, CBL induced a significant increase in GSH level with reference to both control and Parkinsonian groups.

Concerning the behavioral experiments, data of the hanging and score tests showed nonsignificant changes between the investigated groups (Table 1). However, data of the open field test (Table 1) showed significant increases in the central square and freezing durations in the Parkinsonian animals in comparison to control animals. These significant alterations have not been observed in the Parkinsonian animals treated with CBL. However, the number of squares crossed by Parkinsonian and CBL-treated rats was significantly decreased relative to control rats. Regarding grooming behavior, treatment of Parkinsonian animals with CBL resulted in a significant increase in the number of grooming compared with both control and Parkinsonian animals.

![Fig. 1. Effect of cerebrolysin on dopamine, malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) levels in the midbrain of rat model of PD. Control Rat model of Parkinson’s disease Rat model of Parkinson’s disease treated with cerebrolysin a: significant in comparison to control group. b: significant in comparison to PD group.](image-url)
4. Discussion

Experimental animal models of neurodegenerative diseases are becoming increasingly more relevant to the investigation of disease mechanisms and pathogenesis and the development of new therapeutic strategies [31]. The hydroxylated analogue of dopamine 6-OHDA, a specific dopaminergic neurotoxin, has been widely used to induce Parkinsonism in animals [32]. The microinjection of 6-OHDA directly into the brain causes the degeneration of nigrostriatal dopaminergic neurons [33]. It has been reported that the unilateral administration of 6-OHDA leads to an asymmetric motor behavior that can be quantified and equivalent to the degree of lesion; nevertheless, bilateral models resemble more closely the human case [34].

In the present study, the animal model of Parkinsonism was induced by the bilateral microinjection of 6-OHDA into the SN of adult male Wistar rat. This mimics the pathological features of the late stage of human PD [35]. Consistent with the dopaminergic theory of PD, the present data recorded significant decreases in midbrain and striatal dopamine levels after 6-OHDA injection.

6-Hydroxydopamine induces degeneration of dopaminergic neurons through a combination of mitochondrial respiratory impairment and oxidative stress [36]. 6-OHDA enters dopaminergic neurons

### Table 1

<table>
<thead>
<tr>
<th>Behavioral parameters</th>
<th>Control</th>
<th>Parkinsonism</th>
<th>CBL</th>
<th>F-test</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanging duration in sec.</td>
<td>8.20 ± 1.18</td>
<td>7.79 ± 0.65</td>
<td>8.05 ± 0.47</td>
<td>n.s.</td>
<td>0.072</td>
</tr>
<tr>
<td>Score</td>
<td>1.17 ± 0.31</td>
<td>1.09 ± 0.21</td>
<td>1.47 ± 0.13</td>
<td>n.s.</td>
<td>1.255</td>
</tr>
<tr>
<td>Central square duration in min.</td>
<td>0.04 ± 0.01</td>
<td>0.24 ± 0.05*</td>
<td>0.03 ± 0.01b</td>
<td>*</td>
<td>19.537</td>
</tr>
<tr>
<td>Number of crossed squares</td>
<td>57.75 ± 8.00</td>
<td>10.13 ± 2.31*</td>
<td>23.00 ± 3.63*</td>
<td>*</td>
<td>35.592</td>
</tr>
<tr>
<td>Rearing frequency</td>
<td>5.83 ± 1.68</td>
<td>4.33 ± 0.78</td>
<td>5.00 ± 0.71</td>
<td>n.s.</td>
<td>0.436</td>
</tr>
<tr>
<td>Number of stretches</td>
<td>4.29 ± 1.01</td>
<td>2.88 ± 0.39</td>
<td>5.57 ± 0.82</td>
<td>n.s.</td>
<td>3.205</td>
</tr>
<tr>
<td>Number of grooming</td>
<td>3.00 ± 0.58</td>
<td>1.50 ± 0.65</td>
<td>7.00 ± 1.20*</td>
<td>*</td>
<td>11.719</td>
</tr>
<tr>
<td>Freezing duration in min.</td>
<td>5.12 ± 0.61</td>
<td>7.04 ± 0.31*</td>
<td>6.41 ± 0.26</td>
<td>*</td>
<td>6.135</td>
</tr>
<tr>
<td>Number of defecation</td>
<td>2.17 ± 0.54</td>
<td>1.67 ± 0.24</td>
<td>2.80 ± 0.20</td>
<td>n.s.</td>
<td>2.598</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. Number of animals in each group (n = 10).

n.s.: nonsignificant.

* p < 0.05 significant.

Significant in comparison to control group.

Significant in comparison to Parkinsonian group.
through the high affinity DA transporter and accumulates in the cytosol, where it inhibits mitochondrial respiratory chain complexes I and IV [37]. Moreover, 6-OHDA undergoes robust auto-oxidation generating cytotoxic H₂O₂, ROS and catecholamine quinones which attack intracellular nucleophilic groups [38,39]. This increase in ROS levels results in a depletion of intracellular antioxidant enzymes causing abnormalities in cell structure and metabolism and eventually leading to neuronal degeneration [40]. The dopaminergic neurons in the SN are particularly susceptible to oxidative stress [41]. This increased vulnerability of dopaminergic neurons to oxidative damage is mainly due to their reduced antioxidant capacity, high concentration of iron and DA which is susceptible to oxidative modification [42].

In the present investigation, intranigral microinjection of 6-OHDA caused significant increases in midbrain and striatal MDA levels accompanied by a significant decrease in midbrain GSH level and significant increases in both midbrain and striatal NO contents. These results are consistent with the aforementioned studies that revealed that the neurotoxic effects of 6-OHDA occurs through the induction of severe oxidative stress in the dopaminergic neurons. Therefore, the significant decreases in the midbrain and striatal dopamine content observed in the present study in the Parkinsonian group is due to the loss of dopaminergic neurons in the SN with a consequent degeneration of their axonal terminals in the striatum. Confirming the present dopaminergic degeneration, Inden et al. [33] found a marked decrease in the nigral and striatal tyrosine hydroxylase (TH) immunoreactivity and loss of nigral TH-positive neurons in rats receiving intranigral injections of 6-OHDA.

The symptoms of PD become evident when about 60–80% of the dopamine (DA) content is lost, which corresponds to about 50–60% loss of DA neurons in the substantia nigra pars compacta (SNpc) [43]. The present behavioral data revealed significant increases in the central square and freezing durations accompanied by a significant decrease in the number of crossed squares in the Parkinsonian animals. These results are consistent with the cardinal motor symptoms of PD [5] and agree with the previous studies which indicated that animals treated bilaterally with 6-OHDA in SNpc traveled significantly less distance than sham rats [44,23].

The present study investigates the potential therapeutic effect of CBL as a neurotrophic factor mimic [16] containing brain-derived neurotrophic factor (BDNF) in its composition [45]. The present data revealed that treatment of Parkinsonian animals with CBL succeeded in restoring the midbrain and striatal dopamine levels. In addition, it normalized the increased MDA and NO levels recorded in the Parkinsonian animals and replenished the decreased level of midbrain GSH.

The antioxidant and neurotrophic activities of CBL have been reported in other models. Abdel-Salam et al. [46] demonstrated that CBL exerted inhibitory effects on the elevation of brain MDA and nitric oxide levels and attenuated the decrease in reduced glutathione level induced by peripherally injected lipopolysaccharide in rats. In addition, Patočková et al. [47] showed that a single injection of CBL significantly attenuated brain lipid peroxidation in a mouse-model of subacute insulin treatment. In the same way, Ahmed et al. [48] found significantly increased serum BDNF level, brain TH gene expression and brain DA level in ovarioectomized rat model of Parkinsonism treated with CBL.

In addition to the present recovery of the biochemical parameters, there is a parallel improvement in the behavioral aspects. Treatment of the Parkinsonian animals with CBL decreased the central square and freezing durations and increased grooming behavior. These results are in accordance with several studies that indicated that CBL improved the motor function in rats and transgenic mice [49–51] and increased the locomotor activity in aged mice [52,53].

It has been reported that nigral microinjection of 6-OHDA causes degeneration of dopaminergic neurons within 12 h preceding a significant loss of striatal terminals, which takes place 2–3 days later [54,55]. In the present investigation, treatment with CBL started 24 h after lesion with 6-OHDA i.e. after the initiation of neuronal death in SN. This emphasizes the therapeutic potential of CBL in the present Parkinsonian model. This suggestion is supported by the study of Zhang et al. [16] who demonstrated that CBL treatment initiated 24 and 48 h after experimental stroke enhanced neurogenesis in the ischemic brain and improved functional outcome. In conclusion, the present biochemical and behavioral data emphasize the promising therapeutic effect of CBL in the present 6-OHDA rat model of Parkinsonism through counteracting oxidative stress, replenishing dopamine content and enhancing behavioral outcomes. However, further investigations are needed to establish CBL treatment as one of the main interventions used in PD.

Conflict of interest

The authors declare that they have no conflict of interest.

References
