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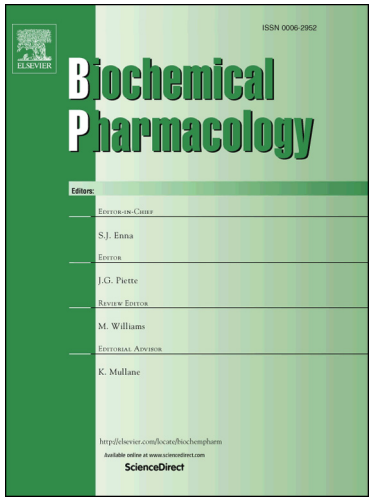

Ambroxol modulates 6-Hydroxydopamine-induced temporal reduction in Glucocerebrosidase (GCase) enzymatic activity and Parkinson's disease symptoms

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Ambroxol modulates 6-Hydroxydopamine-induced temporal reduction in Glucocerebrosidase (GCase) enzymatic activity and Parkinson's disease symptoms

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Abstract

Reduced glucocerebrosidase (GCase) enzymatic activity is found in sporadic cases of Parkinson's disease making GCase a serious risk factor for PD. GCase gene mutations constitute a major risk factor in early-onset PD cases but only account for 5-10%. Having enough evidence for construct and face validity, 6-OHDA-induced hemiparkinson's model may be useful to assess the GCase-targeting drugs in order to have new therapeutic leads in PD. Ambroxol (AMB) is reported to increase GCase activity in different brain-regions. Therefore, we investigated anti-PD like effects of AMB as well as GCase activity in striatal and nigral tissues of rats in hemiparkinson's model. .AMB was given as 400 mg/kg per oral twice daily and SEL used as positive control was given in the dose of 10 mg/kg per oral daily from D-4 to D-27 after 6-OHDA administration. 6-OHDA reduced GCase activity in striatal and in a progressive manner in nigral tissues. AMB and SEL attenuated 6-OHDA-induced motor impairments, dopamine (DA) depletion and GCase deficiency. AMB and SEL also ameliorated 6-OHDA-induced mitochondrial dysfunction in terms of MTT reduction, α-synuclein pathology, loss of nigral cells, and intrinsic pathway of apoptosis by modulating cytochrome-C, caspase-9, and caspase-3 expressions. The results suggest that AMB attenuated 6-OHDA-induced GCase deficiency and PD symptoms. Therefore, the regenerative effects of AMB in dopamine toxicity may be due to its effects on GCase activity and mitochondrial function. Results indicate that SEL also has regenerative effect in the 6-OHDA model. Thus, GCase enzymatic activity is likely to be involved in the development of PD symptoms, and 6-OHDA-induced hemiparkinson's model may be used to evaluate compounds targeting GCase activity for management of PD symptoms.

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Keywords: Glucocerebrosidase; 6-hydroxydopamine; Parkinson's disease; Ambroxol; α-synuclein; Mitochondrial dysfunction

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1. Introduction

Parkinson's disease (PD), one of the widely reported neurodegenerative movement disorders is characterized by the loss of neurons in the nigrostriatal dopaminergic (DA) pathway. The pathway consists of the substantianigra pars compacta (SNc), where DA neuronal cell bodies are located and their nerve terminals and axons project to the striatum [1, 2]. DA participates in motivation, learning and is directly involved in encoding movement. Therefore, PD symptoms mainly comprise of resting tremors, rigidity, postural instability and bradykinesia [1, 3]. Genetics, environmental toxins, and immune factors are some of the known reasons for the pathophysiology of PD. Some neurotoxins like MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine (6-OHDA) are also used to induce experimental PD in animals [4]. Unilateral intrastriatal infusion of 6-OHDA in rats is an established and well-validated experimental model of PD [5]. Being structurally similar to dopamine, 6-OHDA binds to DA transporters (DAT) and transported into DA neurons, followed by its auto oxidation causing generation of highly toxic hydrogen peroxide, paraquinone, and reactive oxygen species (ROS). Besides, 6-OHDA directly inhibits the mitochondrial respiratory chain, which leads to mitochondrial dysfunction and oxidative stress to the cell and results intodopaminergic cell death [6]. Currently, PD affects millions of people around the world [7] and is incurable to date as all the available drugs provide only symptomatic relief [8]. Hence, there is a need for novel PD targets along with suitable experimental models to investigate new therapeutic compounds for PD.

Glucocerebrosidase (GBA1) gene mutation numerically constitutes the major risk factor for PD [9] which is more prevalent in early than late-onset PD cases [10]. Animals with

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GBA1 gene mutation also showed symptoms of neurodegeneration [11]. GCase is synthesized in the endoplasmic reticulum (ER)-bound polyribosomes and trafficked to lysosome by LIMP-2 (lysosomal integral membrane protein type-2) receptor [12, 13]. GCase, a lysosomal enzyme is responsible for the metabolism of glucocerebroside (GC) [14]. GCase deficiency results into accumulation of GC in the lysosome in different cell types like macrophages and neurons [14, 15]. Reduced mitochondrial function was observed in GCase-deficient neurons and cellular models with high mitochondrial volume and low turnovers [16]. This relationship is bidirectional as loss of mitochondrial function by PINK1 [PTEN (phosphatase and tensin homolog)-induced putative kinase 1] knockdown also results into GCase deficiency [17]. Most of the PD cases are sporadic, whereas genetic form of the disease is only found in 5-10% of total PD patients [18]. Therefore GBA1 mutation may only present in 5-10 % of PD patients, but decreased GCase enzymatic activity is found in sporadic patients also, making GCase a serious risk factor for PD [17]. 58% and 33% decrease in GCase enzyme activity was found in SNc of PD patients with mutant GBA and sporadic PD (non-GBA mutation) respectively [17]. GCase protein was reported to be lower in SNc [17]and cerebrospinal fluid (CSF) of sporadic PD brains with reduced enzymatic activity[19]. Reduced GCase activity was also found in cerebellum and putamen in sporadic PD patients [17]. GCase activity is not only decreased in PD patients but also in healthy subjects gradually with age (30-50 %) which becomes comparable to PD patients by about 70 years of life and make individuals prone to PD [20]. There is a continuous decrease in lysosomal functions with normal aging causing accumulation of misfolded proteins and dysfunctional mitochondria with α-synuclein pathology which turns out pathological by the seventh decade of life [20]. Therefore, reduced GCase activity stimulates PD symptoms in non-GBA carriers [20]. α-synuclein is a significant component of lewy bodies and its oligomeric

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aggregation are found in brains of PD patients [4]. GCase deficiency also causes accumulation of α-synuclein toxic oligomers in lysosomes which is reported to inhibit mitochondrial protein import in PD [11, 21]. Different underlying mechanisms of PD like oxidative stress, mitochondrial dysfunction and α-synuclein aggregation which are also reported to be caused by 6-OHDA, take part in development and progression of PD cases with GCase deficiency [4, 6, 22, 23]. Therefore, due to high degree of construct validity for 6-OHDA model [24], 6-OHDA might have an effect on GCase activity in rats. However, treatment of cells with rotenone, a mitochondrial complex I inhibitor did not affect GCase protein levels whereas PINK1 knockdown cells showed decreased GCase activity [17]. Moreover, tracking GCase activity during aging and earlier PD stages can also be beneficial to understand PD pathophysiology in depth and for the development of new therapeutics [20]. There is no established non-genetic animal model to validate GCase enzymatic activity in PD. Therefore, in the present study, we focused on the temporal effects of 6-OHDA on GCase activity in striatum and SNc region of the rat. Due to high degree of the face and predictive validity [24], 6-OHDA model may be further utilized to target GCase for the development of novel neuroprotective drugs in PD.

Ambroxol (AMB), an FDA-approved drug for the treatment of respiratory diseases [25] is currently under investigation in PD patients [26].AMB acts as a chaperone to convert GCase to its full-length form [27] and facilitates trafficking of GCase through the ER [28]. AMB is currently in a clinical trial to stimulate GCase activity in Phase 2 study for PD (ClinicalTrials.gov Identifier NCT02914366 [29] and NCT02941822). AMB is reported to increase GCase activity in brainstem, midbrain and cortex of α-synuclein transgenic mice [30], improved lysosomal biochemistry and rescued defective GCase in GBA1 mutation-linked PD cells [31, 32]. AMB not only increased GCase activity in wild-type micebut also reduced α-

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synuclein levels and restored GCase activity in mice overexpressing human α-synuclein [30]. PD phenotype was alleviated in flies carrying misfolded mutant GCase by growing them in the presence of AMB [33]. GCase-Lysosome-SNCA pathway has been targeted to treat PD [34] and AMB may also upregulate GCase activity in patients without GBA1 mutation [9]. However, there are no reports showing the effect of AMB in well-characterized models of PD in vivo perhaps due to the absence of non-genetic models of PD for evaluating GCase activity. Therefore, in the present study, we used AMB as a specific GCase activator to investigate its anti-PD like effects along with the temporal effects of 6-OHDA on GCase activity in striatum and SNc region of rats in 6-OHDA-induced hemiparkinson's model. We performed different behavioral parameters like apomorphine-induced rotation, open field, rotarod, grip strength and bar catalepsy tests to evaluate the motor deficits in PD. Neurochemical measure of PD was done by estimating striatal monoamines and their metabolites. Mitochondrial functions were assessed by MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] reduction. Glucocerebrosidase activity and α-synuclein concentration was estimated. Loss of nigral cells was examined by nissl's staining. Cytochrome-C, caspase-9, andcaspase-3 proteins were expressed to evaluate mitochondrial–linked apoptosis.

2. **Materials and Methods**

2.1. **Animals**

Charles-Foster strain of adult albino rats male (260 $\pm$ 20 g) was procured from Central Animal House; Institute of Medical Sciences (IMS-BHU) and acclimatized at a temperature of 25 $\pm$ 1^0C and 45-55% relative humidity with light/dark cycle of 12:12 h by keeping them in polypropylene

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cages. Commercial food pellets (DoodhdharaPashuAhar, India) and water was made available ad libitum and no experiments were performed for one week in order to let the animals adapt to the laboratory conditions. All the experimental procedures were carried out in compliance with the principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the care and Use of Laboratory Animals 2011) guidelines and approved by the Institutional animal ethical committee, Banaras Hindu University (Dean/2016/CAEC/33). The experiments were performed between 9:00 h and 16:00 h.

2.2. **Materials**

GCase activator (Ambroxol hydrochloride) was received as a gift sample from MerrilPharma Pvt. Ltd. (Roorkee, India). 6-OHDA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), apomorphine-hydrochloride, MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide], 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferone, cresyl violet acetate, protease inhibitor cocktail,selegiline hydrochloride and buprenorphine hydrochloride were acquired from Sigma-Aldrich (St. Louis, MO, USA). Paraformaldehyde, glycine, HEPES buffer acid-free [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], EGTA [ethylene glycol-bis (2-aminoethylether)-N,N,N’,N’-tetraacetic acid], sodium carbonate, mannitol, sucrose, bovine serum albumin (BSA), xylene, sodium dodecyl sulphate, ascorbic acid and ethyl alcohol were procured from Hi-media (Mumbai). Rat SNCα (Synuclein Alpha) ELISA Kit (Catalog No: E-EL-R1217) was purchased from Elabscience Biotechnology Co., Ltd, US. Antibodies of caspase-3, caspase-9, cytochrome-C, and β-actin were obtained from Santa Cruz Biotechnology Inc. Santa Cruz, California, USA.

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The other reagents of high-performance liquid chromatography (HPLC) and analytical grades were acquired from local suppliers.

2.3. **Surgery and Microinjection**

Rats were anesthetized with pentobarbitone sodium (35 mg/kg, i.p.) and their furs were shaved from the scalp using a trimmer. Rat was mounted on the stereotaxic frame in a way that the head can no longer be moved. The anterior-posterior incision on the scalp was made. Bregma and lambda points on the skull were placed in a same horizontal plane. Guide cannula was positioned over the bregma and the coordinates were set for the left striatum at 1.0 mm anterior, 3.0 mm lateral, and 5.0 mm ventral (A/P +1.0, L/M +3.0, D/V -5.0 relative to the bregma and dura) with the tooth bar at 0 mm [34]. A hole of 1.5 mm depth was drilled and 6-OHDA was injected through a 5 µl Hamilton syringe via polyethylene tube into the left striatum in order to induce unilateral striatal DA degeneration. The injection rate was set at 1µl/min and the needle was left there for an additional five minutes for total diffusion of 6-OHDA [35]. Homeothermic blankets were used to maintain body temperature of animals at 37^0C throughout the surgery and buprenorphine (0.05 mg/kg, s.c.) was injected for postoperativeanalgesia. All the microinjections were performed with Quintessential Stereotaxic Injector (Stoelting, USA). During anesthesia period, the animals were housed separately with proper ventilation and later four animals were kept together per cage. For an initial week after surgery, water and food were provided within the cage to avoid any surgery-induced stress.

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2.4. **Experimental Design**

Animals were randomly divided into five groups for each five time points namely D-0, D-7, D-14, D-21 and D-28. The groups were control, sham, 6-OHDA, 6-OHDA+Ambroxol (AMB; GCase activator), and 6-OHDA+Selegiline (SEL; positive control). On the basis of the previous studies, 20µg of 6-OHDA (4 µl of 5 µg/µl dissolved in normal saline containing 0.2 mg/ml ascorbic acid) was injected into the left striatum (A/P +1.0, L/M +3.0, D/V -5.0 relative to the bregma and dura) in all the animals except sham group which received only 4 µl of normal saline with 0.2mg/ml ascorbic acid [34, 36]. AMB is reported to be neuroprotective in Oxaliplatin-induced peripheral neuropathic pain in rats at the dose of 1000 mg/kg p.o. for 21 days [37]. Since half-life of AMB is approx. 10 h [38], we chose 800 mg/kg/day dose of AMB and administered it orally (per os, p.o.) as 400 mg/kg twice daily from D-4 to D-27, since this dose was also found to be effective against 6-OHDA-induced motor deficits in our pilot study. AMB was prepared in 0.9% saline and administered in a volume of 5 ml/kg whereas control group was given with similar volume of 0.9% saline [39, 40]. SEL has shown multimodal effects in various experimental models of PD [41-45] and therefore was used as positive control at the dose of 10 mg/kg p.o. daily. SEL has shown post-toxin effect in MPTP and rotenone models to regenerate dopamine neurons and also attenuated mitochondrial dysfunction [41, 44]. These parameters were of particular interest to the present hypothesis. The drugs AMB and SEL were administered by oral gavage.

6-OHDA was infused on day 1 (D-1) and drugs were given to their respective groups from D-4 after the onset of motor deficits and continued up to D-27. Behavior parameters were performed on D-0, 7, 14, 21 and 28. Apomorphine-induced rotational behavior was conducted on D-4 also. Open field parameters were recorded using ANY-MAZE behavioral

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tracker version 4.72 (USA). Apomorphine-induced rotation, cataleptic behavior, grip strength score and rotarod observations were recorded with a video camera by observers blind to the study protocol. The experiment was designed to study the temporal changes in GCase activity. The animals were killed at designated days for temporal studies. Therefore, on D-0, each group had thirty nine animals, out of which six from each group were killed by cervical dislocation after behavioral analysis (n = 39). Striatal and SNc tissues were microdissected on ice from ipsilateral hemispheres [34] and stored at -80^{0}C to perform GCase activity and MTT reduction assay (n = 6). Thus, thirty three animals remained in each group for D-7 behavioral estimation (n = 33), followed by killing six animals from each group on D-7. Twenty seven animals/ group were evaluated for behavioral parameters on D-14. Similarly on D-21 (n = 21) and D-28 (n =15) motor deficits were evaluated and six animals in each group were killed. Therefore, fifteen animals/ group were left out on D-28, three animals from each group were randomly assigned to nissl's staining (n = 3). Twelve animals/ group were left which were killed at 24 hours after the last drug dosing. Striatum and SNc tissues were collected on ice from ipsilateral hemispheres in all the animals and tissues were immediately stored at -80^{0}C for further studies.GCase activity and MTT reduction assay (n = 6) was performed on striatal and SNc tissues. Striatal monoamines were estimated by HPLC (n = 6). SNc tissues were used for α-synuclein estimation (n = 3) and western blots for apoptotic proteins expression (n = 3). The detailed experimental design is depicted in **Fig. 1.**

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2.5. **Behavior Parameters**

2.5.1. Apomorphine-induced rotational behavior

Apomorphine-induced rotational behavior is considered as an authentic physiological measure of DA depletion and asymmetric DA receptor stimulation [46]. Apomorphine-hydrochloride was dissolved in normal saline and administered to rats as 1mg/kg by single intraperitoneal injection (i.p.). Net rotations towards the contralateral side were scored for 5 minutes continuously. Rats were also monitored for any basal level of contralateral rotations before surgery.

2.5.2. Open field test (OFT)

OFT, an important parameter for locomotor activity consists of a square wooden open field (60×60 cm) with the white surface divided into 36 squares (10×10 cm) and enclosed by continuous 25 cm high walls. 20 squares adjacent to the wall are known as 'arena periphery' while remaining 16 squares represent 'arena center'. The animal was placed in the middle of the arena and was allowed to move freely for 5 minutes under moderate illumination. The behavior was recorded and ambulation (total number of squares crossed by animal on all four paws), rearing (number of times the animal stood on its hind-paws), the number of central squares crossed and grooming (number of times the rat licked/scratched its fur, washed its face while stationary) were observed. After each test, the arena was cleaned with alcohol and rinsed with water carefully [47].

2.5.3. Rotarod test

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Rotarod test gives an insight into motor coordination ability [48, 49]. Rats were trained on the rotarod (IKON Instrument New Delhi, India) for two consecutive days twice daily with the rotation speed set at 8 revolutions per minute (rpm) on D-1 and 10 rpm on D-2 in order to get stable performances. On D-3, rotational speed was increased up to 15 rpm in the test session. Time for each rat to maintain its balance on the rotating rod was recorded. As soon as rat falls from the rod, counting is stopped and data was represented as retention time on the rotarod over three test trials.

2.5.4. Grip Strength Test

Neuromuscular strength is recorded by hanging the animal with its fore-paws in the middle position of a 90 cm long metal wire (1 mm diameter). The horizontally fixed metal wire was supported by two vertical supports at 50 cm height. Control animals were able to grasp the wire and climbed up within 5 seconds. Grip strength was scored as follows: 0- fall off; 1- hangs onto string by two fore-paws; 2- as for 1 but also attempts to climb on string; 3- hangs onto string by two fore-paws plus one or both hind-paws; 4- hangs onto string by all four paws plus tail wrapped around the string and 5- escape from the apparatus and fall down on flat surface (cut-off time = 60 seconds) [50].

2.5.5. Bar Catalepsy Test

Catalepsy, also known as adopting and maintaining abnormal posture was performed by using bar test [51, 52]. The rats were gently placed by their fore-paws on a horizontal bar placed at 10 cm height from the flat surface. The duration of time in which animal maintained its position on

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the bar was recorded and the measurement was stopped as soon as rat removed any of its fore-paws from the bar. The mean of three consecutive trials was taken with cut-off time = 60 sec.

2.6. **Estimation of striatal monoamines and their metabolites**

The levels of neurotransmitter DA and its metabolites DOPAC (3,4-Dihydroxyphenylacetic acid) and HVA (homovanillic acid) were detected in the striatal tissues of rats in all the groups by using HPLC with an electrochemical detector (ECD) as described in standard protocol [53]. Protein concentration was estimated as described previously [54].

2.7. **Measurement of glucocerebrosidase (GCase) activity**

GCase activity was measured as described earlier with some modifications [55]. Rat brain tissues of the striatum and SNc (~5 mg) were homogenized in 300 µl of water. Samples were diluted in 2 mg/ml BSA, citric acid sodium phosphate buffer (pH 5). 10 µl of sample was added to 75 µl of 10 mM 4-methylumbelliferyl-β-D-glucopyranoside substrate and incubated for 60 minutes at 37^0C. 200 µl of stop solution (0.3 M glycine/0.2 M sodium carbonate, pH 10.7) was added to terminate the reaction. Plates were read at Ex 360/Em 460 by using spectrofluorophotometer. The standard curve of 4-methylumbelliferone (4-MU) was plotted to assess the enzymatic activity and normalized to protein content in each sample as determined using standard method [54]. GCase activity was expressed as nanomoles of 4-MU released/hour/mg of protein (nmoles/hr/mg protein).

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2.8. **Estimation of mitochondrial function**

Firstly, mitochondria were isolated from rat striatal and SNc tissues as previously described protocol with slight modifications [56]. Isolation buffer (215 mMmannitol, 75 mM sucrose, 0.1% w/v BSA, 20 mM HEPES buffer and 1 mM EGTA in 100 ml distilled water, pH 7.2) was used to homogenize the tissues followed by centrifugation at 1300×g for 5 min at 4^0C. The supernatant was then topped off with isolation buffer with EGTA and centrifugation was carried out at 14,000×g for 10 min at 4^0C to get tighter mitochondrial pellets. Washing step was performed by making suspension of pellets in isolation buffer without EGTA and centrifuged at 14,000×g for 10 min at 4^0C to remove EGTA. Mitochondrial proteins were estimated by standard protocol [54] using microplate reader (Biotek, USA).

Mitochondrial function was assessed in terms of MTT reduction [57]. 50 µg mitochondrial suspension was incubated with 0.1 mg/ml MTT for 30 minutes at 37^0C and centrifuged. The obtained formazan pellets were dissolved in 1 ml of absolute ethanol followed by centrifugation. The supernatant was collected and absorbance was taken at 595 nm. Results were expressed as µg formazan formed/min/mg protein using blue-formazan as standard.

2.9. **Rat Alpha-Synuclein (α-synuclein) measurement**

Rat α-synuclein concentration was measured in the rat SNc tissues using enzyme-linked immunosorbent assay (ELISA) plate reader from commercially available ELISA kit (E-EL-R1217). Protein concentration was measured as standard protocol [54] and results were expressed as α-synuclein concentration in pg/mg protein.

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2.10. **Nissl's staining**

On D-28, 24 hours after the last drug dosing, three animals from each group were deeply anesthetized using pentobarbitone sodium (35 mg/kg, i.p.). Animals were perfused transcardially with 200 ml of precooled 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4), followed by 200 ml precooled fixative solution containing 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4). The brains were removed from the skull and postfixed overnight in the same paraformaldehyde fixative at 4^0C and then transferred to 10% sucrose solution. Next day, the brains were immersed in 20 % sucrose solution for 24 hours followed by 30 % sucrose for 2-3 days at 4^0C. The blocks of the brain were prepared and sections were cut at a thickness of 40 µm on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany). Free-floating sections were washed with 0.01 mol/L PBS three times for 10 minutes each at room temperature. The brain sections were mounted on glass slides. Sections were nissl-stained with 0.125% cresyl violet, dehydrated twice through graded alcohols (70%, 95 %, and 100%), and cleared in xylene 3 times for 5 min each. In the end, the slides were coverslipped with resinous mountant and observed with a light microscope Magnus MLXi-TR Plus (SN 16B1394) from Olympus Opto Systems India Pvt. Ltd. Noida, India with Magcam DC5 sensor [58, 59]. The number of nissl-stained nigral neurons was quantified with the cell counter tool of NIH ImageJ software [60]. The data was expressed as percentage of control rats.

2.11. **Western blot for cytochrome-C, caspase-9, and caspase-3 protein expressions**

The nigral tissues were collected and lysed in protease inhibitor cocktail - containing buffer for western blot as previously described [61]. BSA was used as a standard protein and protein concentrations were determined [54]. 10 % concentration of SDS-PAGE gels was taken for

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electrophoresis of each sample aliquot of three proteins. It was then transferred to polyvinylidene fluoride membranes to probe with respective antibodies. Polyclonal primary antibodies of rabbit-anti-cytochrome-C, anti-caspase-9, andanti-caspase-3 were diluted (1:1000, 1:500 and 1:500 respectively) for overnight incubation of membrane. The antibodies were detected against the protein of interest. Stripping buffer (25 mM glycine pH 2.0, 2% SDS) was used to strip the membrane at room temperature for 30 minutes. Polyclonal primary antibody of Rabbit-anti-β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) was diluted as 1:500 and the membrane was reprobed overnight to confirm the equal loading of protein. The membrane was then reprobed with corresponding secondary antibodies. Immunoreactive band of proteins was detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Densitometric scan of the films was performed to quantify the results and to measure the immunoreactive area using biovis gel documentation software.

2.12. **Statistical Analysis**

The experimental results were expressed as mean ± standard deviation (SD). Non-repeated measures of two-way ANOVA were performed for the data analysis of behavior parameters, mitochondrial studies and GCase activity followed by Bonferronipost-hoc test. For the other datasets, one-way ANOVA was performed followed by Student-Newman-Keulspost-hoc test. $p < 0.05$ was considered significant in the overall data analysis.

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3. **Results**

3.1. **Behavior Parameters**

3.1.1. *AMB decreased catalepsy and apomorphine-induced changes in rotational behavior in 6-OHDA rats*

Unilateral DA reduction can be accurately measured by using apomorphine-induced rotation test in animals [46].Non-repeated measures of two-way ANOVA indicated significant differences in rotational behavior among groups [$F(4, 650) = 469.8$; $p < 0.05$], time [$F(4, 650) = 177.5$; $p < 0.05$] and an interaction between group and time [$F(16, 650) = 75.58$; $p < 0.05$].Significant differences were also found among groups [$F(4, 650) = 314.4$; $p < 0.05$], time [$F(4, 650) = 136.0$; $p < 0.05$] and an interaction between group and time [$F(16, 450) = 66.68$; $p < 0.05$] for latency (catalepsy behavior) in bar test **[Table 1].** Unilateral 6-OHDA infusion in striatum caused neuronal death in ipsilateral dopaminergic nigrostriatal pathway due to which animals showed head rotation in response to systemic injection of apomorphine from D-4 (data not shown). In 6-OHDA group, we observed 31% more rotations on D-14 than D-7, 42% and 41% more rotations on D-21 and 28 compared to D-7 and 17% and 16% more rotations on D-21 and 28 compared to D-14. However, rotational behavior started to return towards normal level in AMB-administered 6-OHDA group from D-14 with 21% decrease from 6-OHDA group which became similar to control group on D-28. AMB statistically decreased head rotations progressively from D-14 to D-28.

Catalepsy is used to model akinesia in PD [62] and contribute to fine motor control [63]. 6-OHDA took more time to reduce control on fine motor movements and increased cataleptic behavior (65%) from D-14. However, once initiated, 6-OHDA-induced increase was

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continuous and was 71% and 69% higher on D-21 and D-28 respectively than control animals. AMB exhibited noteworthy reduction in cataleptic behavior against 6-OHDA administration from D-14 (37% reduction compared to 6-OHDA group). The effect was maximal on D-28. AMB-induced attenuation in cataleptic behavior in 6-OHDA rats was 23% and 28% on D-28 compared to D-14 and D-21 respectively. SEL also attenuated 6-OHDA induced cataleptic and rotational behavior in rats.

3.1.2. *AMB improved spontaneous locomotor activity in open field test (OFT) in 6-OHDA rats*

General locomotor activity of animals was measured by open field test [64]. Since, DA neurons play an important role in encoding movement [3], 6-OHDA administered animals showed severe reduction in number of ambulation, grooming and rearing from D-7. However, number of central squares crossed were reduced by 6-OHDA from D-14. This reduction in central squares crossed was 18% more severe on D-28 than D-21. Non-repeated measures of two-way ANOVA revealed significant differences in number of central square crossed, ambulation, grooming and rearing among groups ([$F(4, 650) = 172.6$; $p < 0.05$], [$F(4, 650) = 502.4$; $p < 0.05$], [$F(4, 650) = 502.4$; $p < 0.05$], [$F(4, 650) = 472.2$; $p < 0.05$] respectively), time ([$F(4, 650) = 163.2$; $p < 0.05$], [$F(4, 650) = 868.6$; $p < 0.05$], [$F(4, 650) = 868.6$; $p < 0.05$], [$F(4, 650) = 259.9$; $p < 0.05$] respectively) and interaction between group and time ([$F(16, 650) = 38.13$; $p < 0.05$], [$F(16, 650) = 113.8$; $p < 0.05$], [$F(16, 450) = 113.8$; $p < 0.05$], [$F(16, 450) = 76.34$; $p < 0.05$] respectively) in OFT **[Table 2**]. AMB decreased 6-OHDA-induced reduction in number of central squares crossed, ambulation, grooming and rearing as 23%, 46%, 37% and 30% respectively from D-14. This indicates the efficacy of AMB to enhance exploration in the open field, denoted as rearing [65, 66] and displacement behavior as interpreted by grooming

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[67].Temporal significant effects of AMB were observed against 6-OHDA induced motor deficits from D-14 to D-28. SEL was also found to decrease 6-OHDA induced motor deficits in open field parameters.

3.1.3. *AMB increased rotarod retention time and grip strength scores in 6-OHDA rats*

Motor coordination ability and regulation of neuromuscular strength can be specifically measured by rotarod retention time [48, 49] and grip strength scores [50, 68] respectively. Due to rapid degeneration of neurons in DA nigrostriatal pathway, toxic effects of 6-OHDA were observed from D-7 in the form of 51% and 73% reduction in rotarod retention time and grip strength scores. However, it was not declined progressively. Statistical analysis by non-repeated measures of two-way ANOVA revealed that there were significant differences in rotarod retention time and grip strength scores among groups ([$F(4, 650) = 859.3$; $p < 0.05$], [$F(4, 650) = 1245$; $p < 0.05$] respectively), time ([$F(4, 650) = 616.1$; $p < 0.05$], [$F(4, 650) = 742.4$; $p < 0.05$] respectively) and an interaction between group and time ([$F(16, 450) = 140.5$; $p < 0.05$], [$F(16, 650) = 742.4$; $p < 0.05$] respectively) as observed in **Table 1**. AMB reduced both the motor deficits and attenuated 6-OHDA-induced reduction in rotarod retention time (26%) and grip strength scores (56%) from D-14. AMB-induced attenuation was progressive and motor deficits were recovered on D-28 against 6-OHDA infused rats. SEL also elicited similar effects in the rotorod test and grip strength scores.

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3.2. **AMB increased DA and its metabolites DOPAC and HVA in striatal tissues of 6-OHDA rats**

Intrastriatal injection of 6-OHDA which exhibited apomorphine-induced rotational behavior on D-7 probably due to reduction in DA neurons, decreased striatal DA up to 68%, DOPAC (54%), and HVA (49%) and upgraded DOPAC/DA and HVA/DA ratios up to 34% and 39% correspondingly compared to control groups as shown in **Fig. 2**. One way ANOVA denoted significant differences among groups in the levels of DA [$F(4, 25) = 84.63$; $p < 0.05$], DOPAC [$F(4, 25) = 26.13$; $p < 0.05$], HVA [$F(4, 25) = 20.03$;$p < 0.05$], DOPAC/DA [$F(4, 25) = 3.811$; $p < 0.05$] and HVA/DA [$F(4, 25) = 7.801$; $p < 0.05$] in striatal tissues of rats. AMB increased the levels of DA (50%) and its metabolites DOPAC (32%) and HVA (33%) and downregulated DOPAC/DA (28%) and HVA/DA (26%) ratios in 6-OHDA infused rats. SEL also improved dopamine concentration after 6-OHDA infusion.

3.3. **6-OHDA decreased and treatment with AMB increased GCase activity and mitochondrial function in terms of MTT reduction in rat striatal and nigral tissues**

Non-repeated measures of two-way ANOVA showed that there were significant differences in GCase enzymatic activity in striatal and nigral tissues among groups ([$F(4, 125) = 42.29$; $p < 0.05$], [$F(4, 125) = 101.2$; $p < 0.05$] respectively), time ([$F(4, 125) = 21.52$; $p < 0.05$], [$F(4, 125) = 28.06$; $p < 0.05$] respectively) and interaction between group and time ([$F(16, 125) = 4.984$; $p < 0.05$], [$F(16, 125) = 12.58$; $p < 0.05$] respectively) as depicted in **Fig. 3 and Fig. 4.** 6-OHDA elicited inhibitory effects on GCase enzymatic activity in both the striatal (48%) and nigral tissues (27%) from D-7. It is noteworthy that 6-OHDA caused a progressive reduction in GCase enzymatic activity in nigral tissues without causing a significant reduction in the striatum

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after D-7. Day-dependent aggravation of 6-OHDA inhibitory effects on enzymatic activity was noted in nigral tissues on D-14 (44%) compared to D-7. GCase activity was also reduced on D-21 (68% and 42%) and D-28 (70% and 47%) compared to D-7 and D-14. AMB, a GCase activator significantly attenuated 6-OHDA-induced decrease in GCase enzymatic activity from D-14 in both the striatal and nigral tissues up to 32% and 37% respectively. The effects of AMB were maximum on D-21 in striatum and D-28 in nigral tissues. Moreover, progressive elevation in AMB-induced enzymatic activity in 6-OHDA group was observed on D-21 and 28 (31% and 33% respectively) compared to D-7 and (19% and 21% respectively) compared to D-14 in striatal tissues. In nigral tissues also, significant increase (24%) was noted on D-28 compared to D-14.

MTT reduction was used to assess mitochondrial function [57]. 6-OHDA impaired mitochondrial function and decreased MTT reduction in striatal (36%) and nigral (31%) tissues from D-7. On D-28, 56% and 65% decrease was observed in striatal and nigral tissues compared to control rats. Non-repeated measures of two-way ANOVA showed that there were significant differences in striatal and nigral tissues among groups ([$F(4, 125) = 74.03$; $p < 0.05$], [$F(4, 125) = 74.13$; $p < 0.05$] respectively), time ([$F(4, 125) = 21.93$; $p < 0.05$], [$F(4, 125) = 24.02$; $p < 0.05$] respectively) and interaction between group and time ([$F(16, 125) = 8.219$; $p < 0.05$], [$F(16, 125) = 10.41$; $p < 0.05$] respectively) in MTT reduction. The effect of 6-OHDA was found to be more severe on D-14 (27%), D-21 (29%) and D-28 (31%) in striatal tissues and on D-21 (41%) and D-28 (43%) in nigral tissues compared to D-7. AMB ameliorated 6-OHDA-induced changes in MTT reduction in 6-OHDA administered rats from D-14 in both the striatal (29%) and nigral (27%) tissues. AMB-induced effects were increased progressively

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from D-14 to D-28 and found to be similar to control group on D-28. GCase activity and MTT function were found to increase with SEL treatment against 6-OHDA model.

3.4. **AMB increased soluble α-synuclein concentration in nigral tissues of 6-OHDA rats**

Under pathophysiological conditions, α-synuclein forms oligomeric aggregates which are insoluble in nature and are important component of toxic lewy bodies [4]. However, under normal physiology, α-synuclein is water-soluble [69] which is estimated in present study. Analysis by one-way ANOVA showed significant differences among groups in α-synuclein concentration in SNc [$F(4, 10) = 6.343$; $p < 0.05$]. 6-OHDA decreased the soluble α-synuclein concentration up to 75% on D-28 compared to control group which was significantly increased (65%) by AMB as observed in **Fig. 5**. Soluble α-synuclein concentration was also increased by SEL.

3.5. **AMB increased nigral cells in 6-OHDA rats**

Unilateral intrastriatal 6-OHDA injection causes retrograde degeneration of DA neurons causing death of neuronal cell bodies present in SNc [70]. This is the reason why a remarkable loss (68%) of nissl's body was observed in the 6-OHDA lesioned SNc compared to control group.One-way ANOVA revealed that there were significant differences among groups in the percentage (%) of nissl's body in nigral tissue [$F(4, 10) = 24.63$; $p < 0.05$]. AMB substantially reduced the loss of nigral cells up to 34% **(Fig. 6).** However, AMB-induced increase in nigral neurons was also significantly different than control and sham groups. Similarly, nigral cells were also recovered by SEL.

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3.6. **AMB decreased apoptotic proteins in nigral tissues of 6-OHDA rats**

Apoptotic proteins like cytochrome-C, caspase-9 and caspase-3 were expressed and significant differences were found among groups in cytochrome-C [$F(4, 10) = 39.8$; $p < 0.05$], caspase-9 [$F(4, 10) = 53.3$; $p < 0.05$] and caspase-3 [$F(4, 10) = 35.1$; $p < 0.05$] as shown by one-way ANOVA. 6-OHDA upregulated apoptotic proteins compared to control and sham groups which were significantly decreased by AMB and SEL [**Fig. 7].**

4. **Discussion**

The salient findings of the current study are: 1. Modulation of GCase activity in striatal and nigral tissues by 6-OHDA. 2. Progressive time-dependent decrease of GCase activity in the substantia nigra. 3. Anti-PD like effects of GCase activator Ambroxol (AMB). The tracking of GCase activity, a major risk factor for PD can be beneficial in the development of new therapeutic potential [20]. However, there are no reports showing the effects of intrastriatal infusion of 6-OHDA, an established experimental PD model on GCase enzymatic activity. AMB, a GCase activator [9] is currently being investigated in PD patients [26] but there is no preclinical study on the pharmacology of AMB in well-validated non-genetic PD models *in vivo.* Therefore, the primary objective of the study is to establish a well-characterized PD model in order to target GCase-acting drugs followed by investigating the usage of GCase activator in PD management.

PD, a progressive movement disorder causes several motor deficits such as tremors, gait and rigidity in patients [4]. In the present study, 6-OHDA infused rats showed impaired motor movement in different behavioral parameters. Apomorphine-induced contralateral rotations [46] and cataleptic behavior [71] were increased whereas open field

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parameters like numbers of central squares crossed, ambulation, grooming and rearing [64], rotarod retention time [48]and grip strength scores [71] were decreased by 6-OHDA. Apomorphine-induced rotation is considered as a reliable physiological measure of DA depletion and asymmetric dopamine receptor stimulation [46]. Grip strength test gives an insight into the regulation of neuromuscular strength [50, 68] and rotarod retention time characterizes motor coordination ability [48, 49]. 6-OHDA-induced alterations in behavioral performance were observed from D-7 except for central squares crossed and bar catalepsy test which was noticed from D-14. Open field test indicates spontaneous locomotor activity of animals [64]. It includes parameters such as grooming, rearing, ambulation and central squares crossed. Rearing signifies expression of directed exploration in the adult phase of life [65, 66] and displacement behavior is interpreted as grooming [67]. The number of central squares crossed indicates further exploration of the open field by animal [72]. Bar catalepsy test assesses acceptance and retention of abnormal posture [73]and is used to model akinesia in PD[62]. Akinesia occurs due to the loss of fine motor control [63].This indicates that 6-OHDA took more time to impair some fine motor movements. There were time-dependent alterations in apomorphine-induced rotations, central squares crossed and cataleptic behavior which shows that 6-OHDA induces progressive loss of exploration and fine motor movement in animals. SEL was found to decrease 6-OHDA-induced motor impairment from D-14. It has been reported thatSEL decreased apomorphine-induced rotational behavior caused by 6-OHDA in rats when given after 6-OHDA [43]. Ambroxol (AMB) alleviated the motor deficits in the behavioral parameters in 6-OHDA infused rats with the onset of action on D-14 and maximal effect on D-28. There was progressiveattenuation of motor deficits induced by 6-OHDA, suggesting that AMB has significant effects against 6-OHDA-induced impairment in motor behavior of the animals.

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PD-related motor dysfunction results from the depletion of the neurotransmitter DA in the striatum. 6-OHDA intrastriatal injection causes the destruction of DA nerve terminals and 6-OHDA is then transported towards cell body causing retrograde degeneration [5, 70]. In the present study, 6-OHDA decreased the levels of DA and its metabolites as well as upregulated the DA turnover in rat striatal tissues [71] which was reversed by AMB and SEL. The regenerative effects of SEL were earlier observed against rotenone [41] and MPTP-induced [44, 45] striatal DA depletion in rats. However, the regenerative effects of SEL on striatal DA content in rats post-6-OHDA toxicity are reported for the first time in present study. Pharmacological inhibition of GCase also led to decreased striatal DA release in mice [74]. Therefore in the present study, marked improvement in motor activities with AMB may be due to the improvement in striatal dopamine concentration.

Mitochondrial dysfunction plays a pivotal role in PD pathogenesis. 6-OHDA impairs mitochondrial complex enzyme function, mitochondrial integrity and respiration due to the production of ROS in 6-OHDA-induced hemiparkinson's rats [71]. In our study, mitochondrial function was decreased from D-7 in terms of MTT reduction in 6-OHDA infused rats in both the striatum and SNc tissues. AMB and SEL increased MTT reduction in both the tissues with onset of action at D-14, indicating improvement in mitochondrial function in 6-OHDA infused rats. SEL has been reported to attenuate mitochondrial dysfunction induced by rotenone and MPTP in rats [41, 44]. Mitochondrial dysfunction is also crucial for PD pathogenesis [4] and loss of mitochondrial function by PINK1 knockdown results in GCase deficiency [17]. In our study, we found that 6-OHDA decreased GCase activity in rat striatal and nigral tissues from D-7. From D-7 the GCase activity was reduced in a progressive manner only in nigral tissues of 6-OHDA infused rats. The underlying reasons may consist of the mode of

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synthesis and distribution of GCase and other being the mode of dopamine toxicity by 6-OHDA. GCase is formed in ER-bound polyribosomes to get transported to lysosome [12, 13]. Most of the protein synthesis machinery and genetic material are localized to the cell body; therefore axonal transport is necessary to provide the structural and functional materials along the length of axon. Axonal transport is ongoing process in neurons and is bidirectional [75]. GCase-related organelles such as ER- bound ribosomes are undetectable in axons [75], whereas lysosomes in approximately equal proportion undergo either anterograde or retrograde transport [76]. In PD, there is damage to nigrostriatal DA neurons whose cell bodies are located in SNc and axons with nerve terminals are projected to the striatum [1, 2]. Unilateral intrastriatal injection of 6-OHDA causes retrograde degeneration of nigrostriatal DA neurons in rats [70]. Hence, striatal terminal damage is produced within one day of injection whereas nigral cell loss is lowest at one week. Within 2-3 weeks, nigral cell loss reach maximum [77]. This may be the reason; 6-OHDA caused more severe reduction in GCase activity in striatal tissue (48%) on D-7 compared to SNc (27%) and later, no progressive decrease in GCase activity was observed in striatum. However, GCase activity was reduced in a progressive manner upto D-21 in nigral tissues of 6-OHDA infused rats. Further as discussed earlier, 6-OHDA may decrease stored GCase in the striatum, however inhibition of synthesis of GCase in the cell body may take time. GCase enzymatic activity is also found to be consistently low in SNc of sporadic PD patients (non-GBA mutation, 33%) across sixth to eighth decade of life [17, 20]. GCase gene therapy increased GCase protein level in the substantianigra more significantly than striatum in mice [78].

AMB, a pH-dependent GCase inhibitor facilitate trafficking of GCase through the ER to lysosome [28, 79]. AMB pharmacological treatment in cell culture models is reported to restore GCase activity [80]. In our study, AMB and SEL increased GCase enzymatic activity in

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6-OHDA infused rats from D-14 both in the striatum and SNc tissues As mitochondrial impairment by PINK1 deficiency resulted in GCase reduction in SH-SY5Y cells [17], there may be a possible involvement of mitochondrial function in the regulation of GCase enzymatic activity in present study also. The relationship between GCase and mitochondrial dysfunction is bidirectional as pharmacological inhibition of GCase also lead to changes in mitochondrial function in cellular models [81]. The clearance capacity of lysosome gets deranged which leads to the formation of fragmented and dysfunctional mitochondria having impaired respiratory chain [81,82]. In our study also, GCase activation by AMB may be responsible for mitigating mitochondrial dysfunction in 6-OHDA-induced dopaminergic toxicity. SEL increased GCase activity which may also be related to its effect on mitochondrial function [44].

Pharmacological inhibition of GCase is reported to cause accumulation of α-synuclein [81]. α-synuclein, a component of lewy bodies forms insoluble oligomeric aggregates which arecharacteristic markers of PD and appeared in PD patients having decreased GCase activities [4, 83]. GCase deficiency causes accumulation of its substrate glucocerebroside (GC) in SNc which prolong the lag phase of α-synuclein fibril growth [11]. In the present study, we measured the soluble α-synuclein concentration. Under normal physiology, α-synuclein is water-soluble with monomeric structure and present in the cytosol of the neuron. However, under toxic conditions, α-synuclein turns water-insoluble and forms aggregates. The water solubility of α-synuclein is reported to decrease with age, indicating the formation of aggregate proteins [69]. In our study, 6-OHDA decreased the concentration of water-soluble α-synuclein in nigral tissues, suggesting the formation of α-synuclein aggregates as stated previously [23]. AMB and SEL increased the concentration of water-soluble α-synuclein in 6-OHDA infused rats, indicating decreased aggregation of α-synuclein. SEL decreased α-synuclein in SNc of mice infused with

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MPTP [43]. However, we report decrease in α-synuclein oligomers by SEL after 6-OHDA induced dopaminergic toxicity. AMB has been reported to reduce α-synuclein levels and restored GCase activity in mice overexpressing human α-synuclein [30]. There is a bidirectional relationship between α-synuclein and GCase because α-synuclein is reported to impair GCase trafficking from ER to golgi apparatus and thus to the lysosome[11]. α-synuclein interacts with GCase and makes α-synuclein-GCase complex, thereby inhibiting GCase enzyme function [84]. Therefore, in our study also there is a scope to believe that α-synuclein aggregation may have a role in aggravating GCase deficiency in 6-OHDA-induced DA toxicity.

Moreover, α-synuclein toxic oligomers are reported to inhibit mitochondrial protein import in PD, which results into mitochondrial dysfunction [11, 21]. Mitochondrial impairment could further lead to apoptosis via intrinsic pathway due to the release of mitochondrial cytochrome-C which activates other apoptotic proteins like caspase-9 and caspase-3 [6]. In the present study, these apoptotic proteins were upregulated in rat nigral tissues by 6-OHDA [85] and decreased by AMB and SEL. We also examined the loss of nigral cells by nissl's staining and found that 6-OHDA decreased nigral cells [86]. AMB and SEL treatment substantially increased nigral cell count. Therefore, decreased GCase activity may be responsible for cell loss in 6-OHDA group and ability of AMB to increase the 6-OHDA-induced decrease in GCase activity may be partly responsible for attenuating cell loss. SEL is previously reported to increase anti-apoptotic proteins and decrease apoptotic cells in MPTP model in mice [42, 45]. However, the present study provides the evidence of SEL-induced decrease in apoptosis after 6-OHDA toxicity.

In conclusion, the current study showed inhibitory effects of 6-OHDA on GCase enzymatic activity in striatal and nigral tissues of rats. Reduction in GCase activity was found to

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be progressive in nigral tissues. GCase deficiency is probably due to the mitochondrial dysfunction and α-synuclein aggregation because these two factors can regulate GCase enzymatic activity. AMB, a GCase activator up regulated striatal DA content and attenuated motor impairment, mitochondrial dysfunction, α-synuclein pathology, loss of nigral cells and mitochondrial-linked apoptosis. Therefore, enhancing GCase activity may have the capacity to regenerate the dopaminergic system. SEL improved behavioral deficits, mitochondrial function and increased soluble α-synuclein. It also showed regenerative effects as observed from increase in striatal DA content and number of nigral cells in the 6-OHDA model. The current study shows anti-PD like effects of AMB as well as indicates the use of 6-OHDA-induced hemiparkinson's model to evaluate GCase-targeting drugs for management of PD.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Author contribution.

SK and AM conceived and designed the study. AM was responsible for acquisition of data. SK and AM analyzed and interpreted the data. LPC and SKT were involved with the histology

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experiments. SK and AM drafted the work for intellectual content and context. SK did the final approval and takes overall responsibility of the published work.

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Figure legends

Fig. 1. The experimental design of the study. "+" indicates days on which parameters were performed, number of animals/ group on each time points are included in a separate row (after the "+" rows).

Fig. 2. Effect of AMB on 6-OHDA-induced alterations on the levels of DA (a), DOPAC (b), HVA (c), DOPAC/DA (d), and HVA/DA (e) in striatal tissues of rats at D-28. All values are mean ± SD; n = 6; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham and $^{c}p< 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-KeulsPost-hoc test].

Fig. 3. Effect of 6-OHDA and treatment with AMB on GCase enzyme activity in striatal (a) and nigral (b) tissues of rats. All values are mean ± SD; n = 6; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham and $^{c}p< 0.05$ compared to 6-OHDA. $^{x}p< 0.05$ compared to D-0, $^{y}p< 0.05$ compared to D-7 and $^{z}p< 0.05$ compared to D-14 [Non-repeated measures of two-way ANOVA followed by Bonferroni test].

Fig. 4. Effect of 6-OHDA and treatment with AMB on mitochondrial function in terms of MTT reduction in striatal (a) and nigral (b) tissues of rats. All values are mean ± SD; n = 6; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham and $^{c}p< 0.05$ compared to 6-OHDA. $^{x}p< 0.05$ compared to D-0, $^{y}p< 0.05$ compared to D-7 and $^{z}p< 0.05$ compared to D-14 [Non-repeated measures of two-way ANOVA followed by Bonferroni test].

Fig. 5. Effect of AMB on 6-OHDA-induced changes on α-Synuclein concentration in rat nigral tissues at D-28. All values are mean ± SD; n = 3; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham and $^{c}p< 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-KeulsPost-hoc test].

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Fig. 6. Nissl's staining of SNc in rats on D-28. Control (a); Sham (b); 6-OHDA (c); 6-OHDA+Ambroxol (d); 6-OHDA+Selegiline (e); Data of counting cells (f). All values are mean ± SD; n = 3; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham and $^{c}p< 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-KeulsPost-hoc test].

Fig. 7. Effect of AMB on 6-OHDA-induced changes on the protein levels of cytochrome-C, caspase-9 and caspase-3 in rat nigral tissues. Proteins are represented in blots (a) and histograms express the ratio of the relative intensity of protein levels of cytochrome-C (b), caspase-9 (c) and caspase-3 (d) to Beta-actin. All values are mean ± SD; n = 3; $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham; $^{c}p< 0.05$ compared to 6-OHDA and $^{d}p< 0.05$ compared to 6-OHDA+Ambroxol [One-way ANOVA followed by Student Newman-KeulsPost-hoc test].

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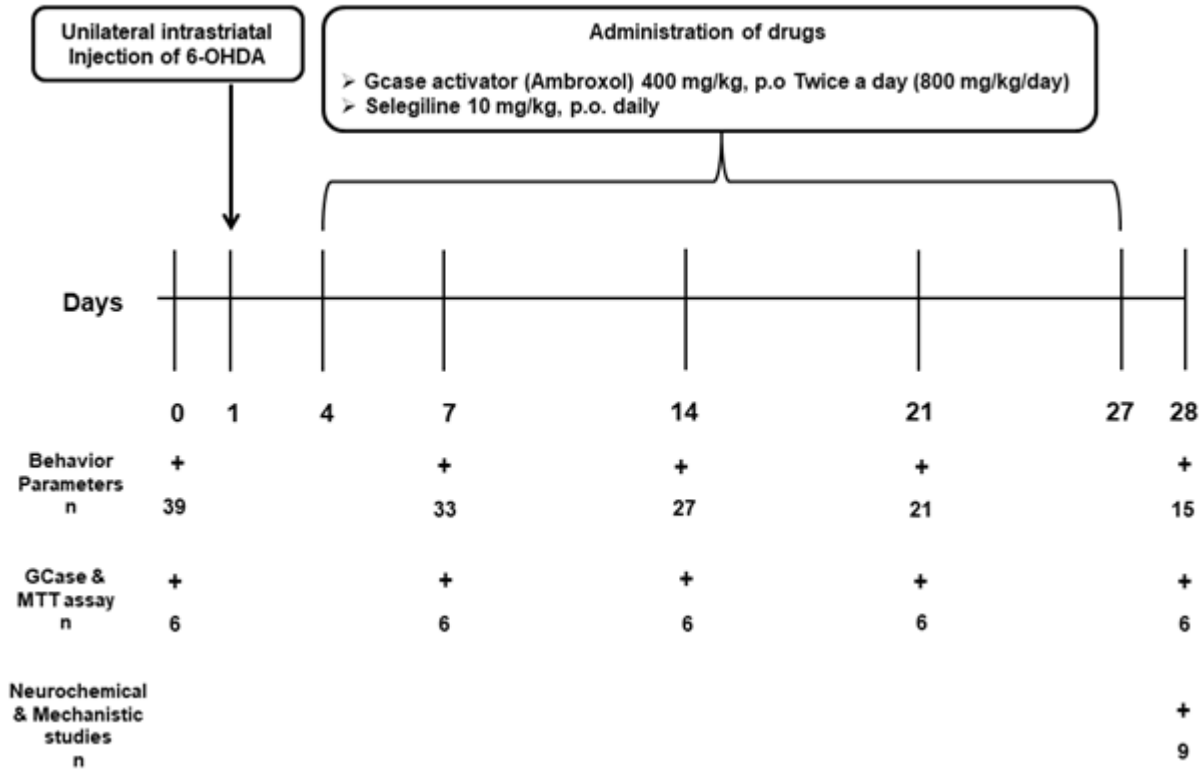

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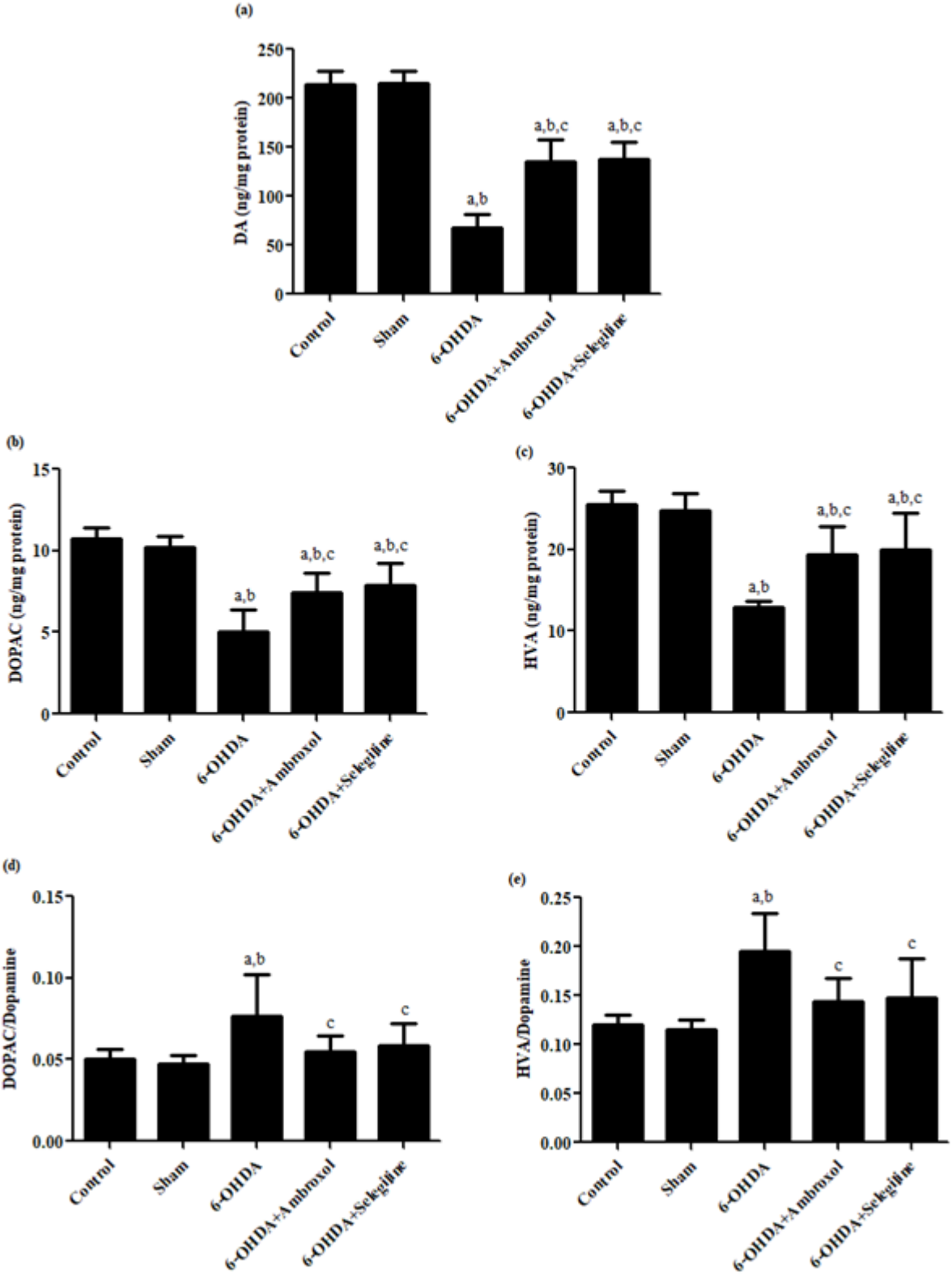
(a)
DA (ng/mg protein)
250
200
150
100
50
0
a,b
a,b,c
a,b,c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
(b)
DOPAC (ng/mg protein)
15
10
5
0
a,b
a,b,c
a,b,c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
(c)
HVA (ng/mg protein)
30
20
10
0
a,b
a,b,c
a,b,c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
(d)
DOPAC/Dopamine
0.15
0.10
0.05
0.00
a,b
c
c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
(e)
HVA/Dopamine
0.25
0.20
0.15
0.10
0.05
0.00
a,b
c
c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline

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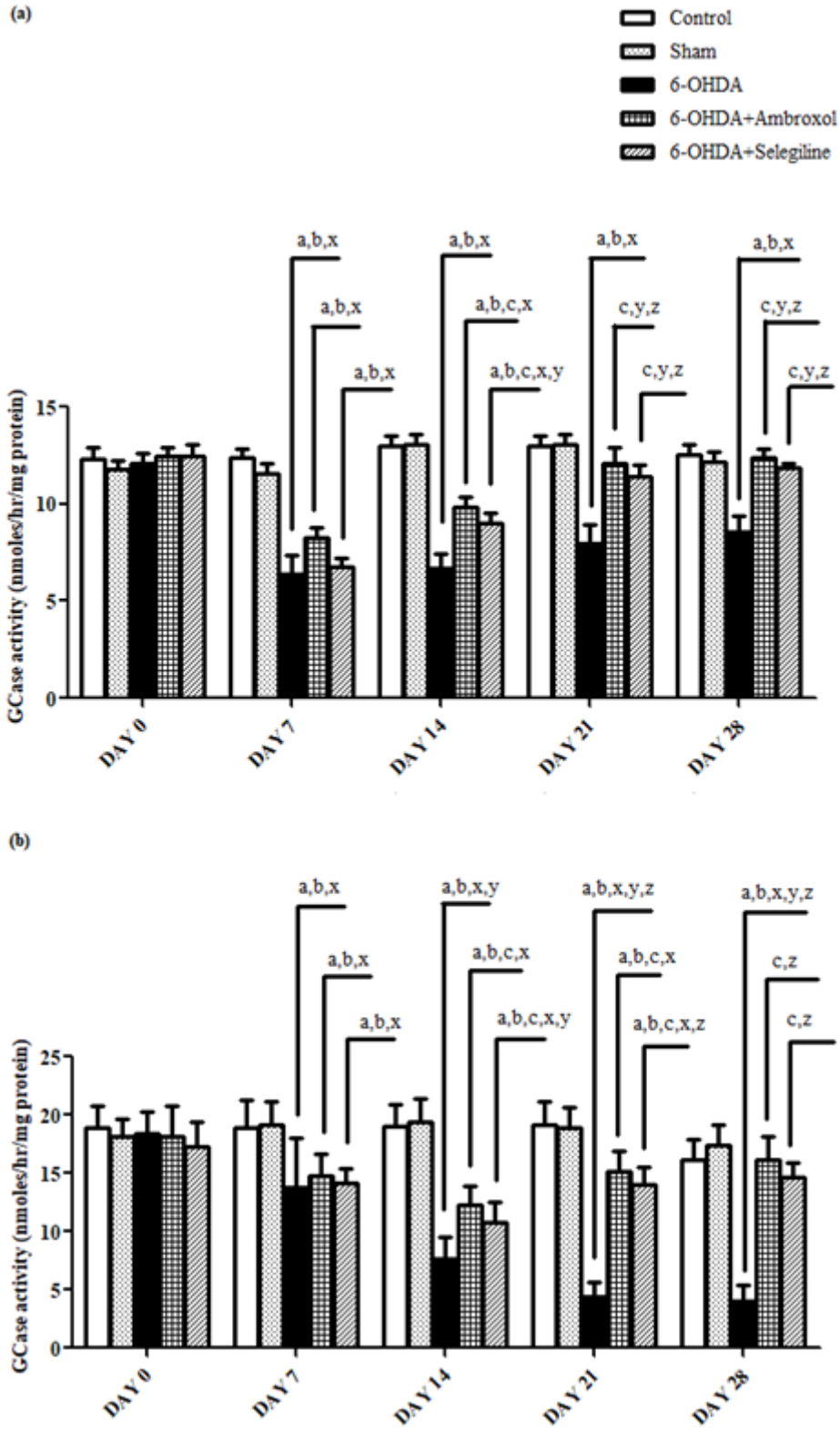
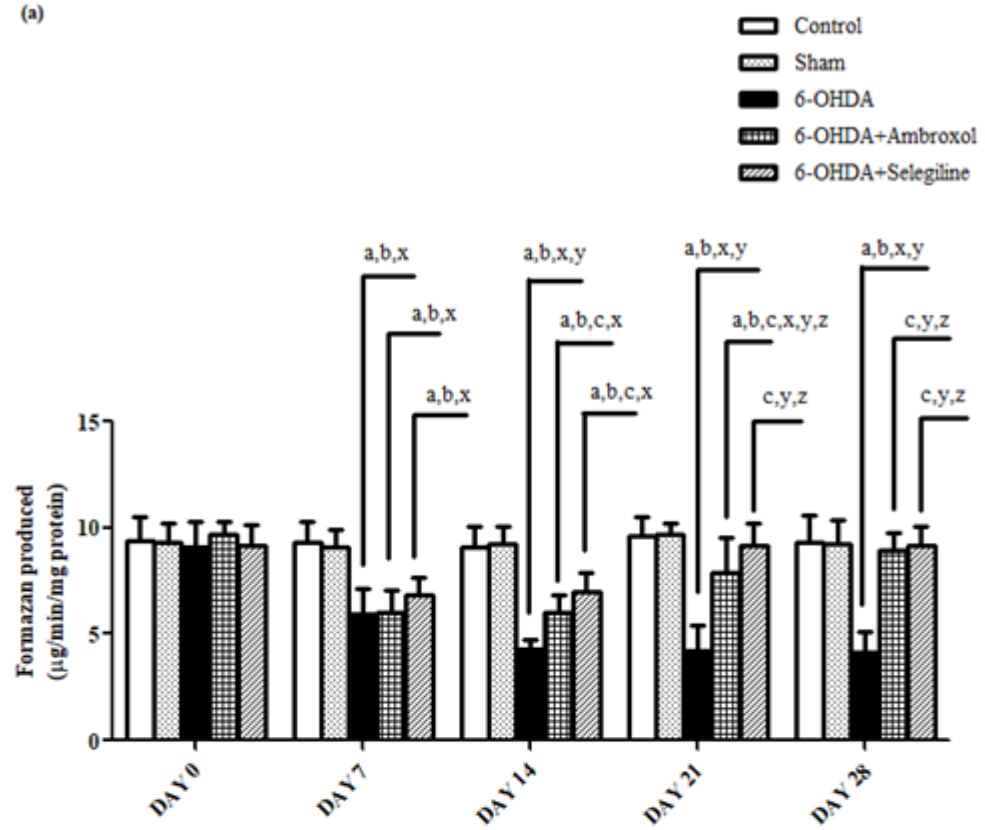
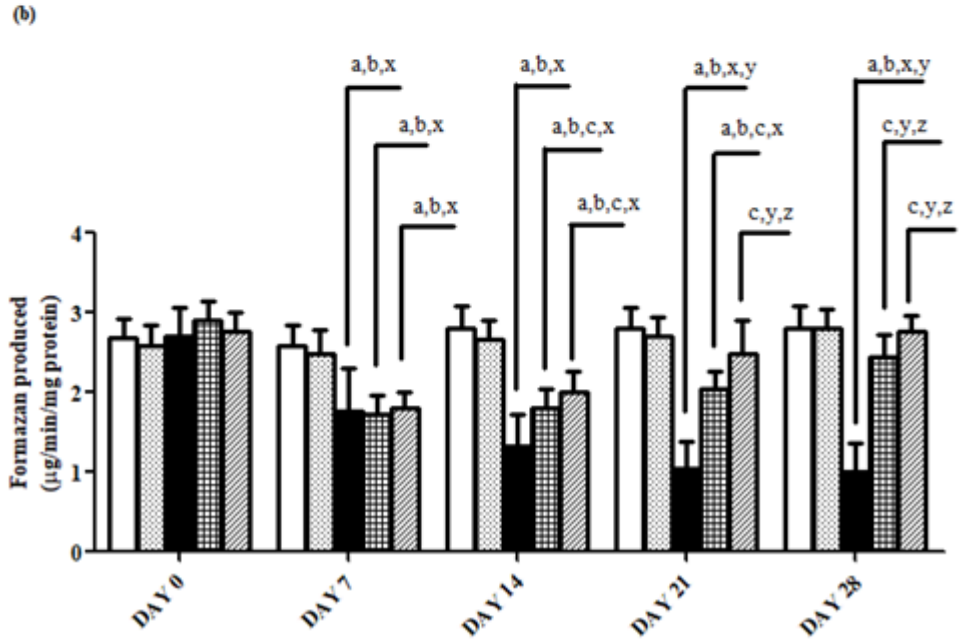



(a)
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
Formazan produced (µg/min/mg protein)
15
10
5
0
a,b,x
a,b,x
a,b,x
a,b,x,y
a,b,c,x
a,b,c,x
a,b,x,y
a,b,c,x,y,z
c,y,z
a,b,x,y
c,y,z
c,y,z
DAY 0
DAY 7
DAY 14
DAY 21
DAY 28


(b)
Formazan produced (µg/min/mg protein)
4
3
2
1
0
a,b,x
a,b,x
a,b,x
a,b,x
a,b,c,x
a,b,c,x
a,b,x,y
a,b,c,x
c,y,z
a,b,x,y
c,y,z
c,y,z
DAY 0
DAY 7
DAY 14
DAY 21
DAY 28

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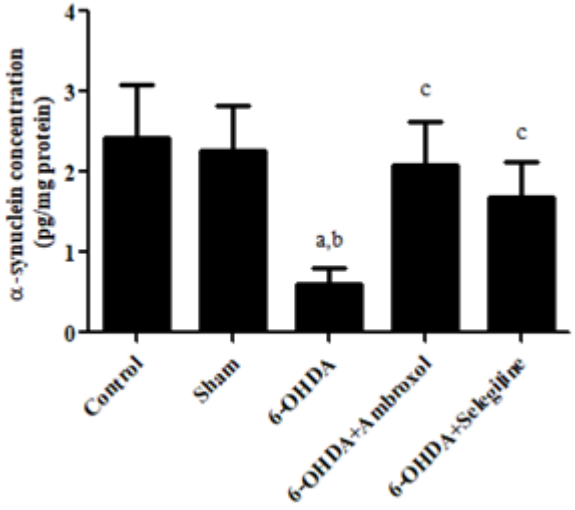

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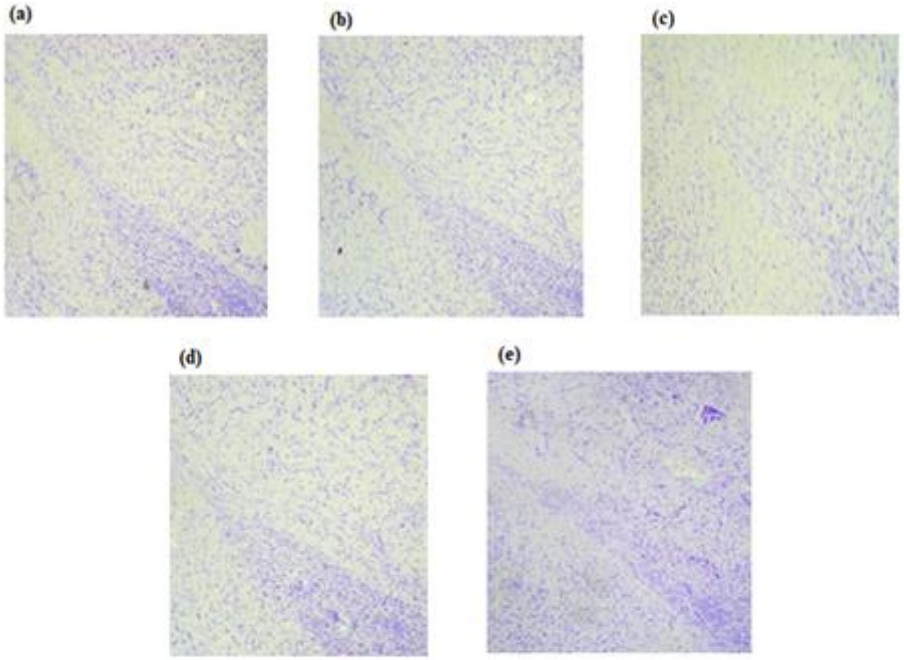
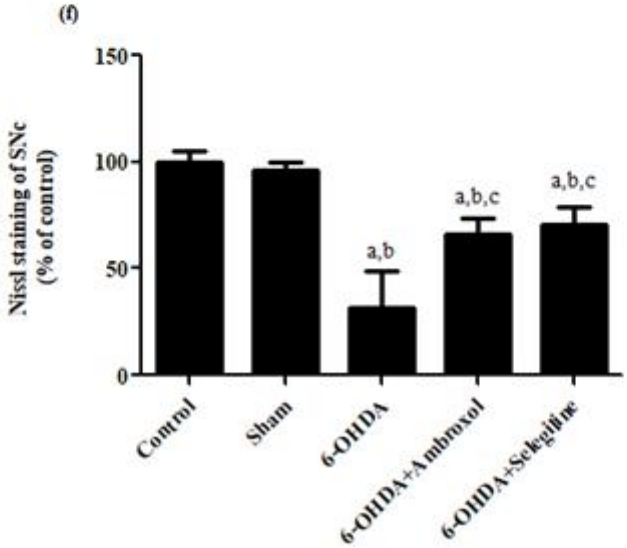

(a)
(b)
(c)
(d)
(e)


(f)
Nissl staining of SNc
(% of control)
150
100
50
0
a,b
a,b,c
a,b,c
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Sekegiline

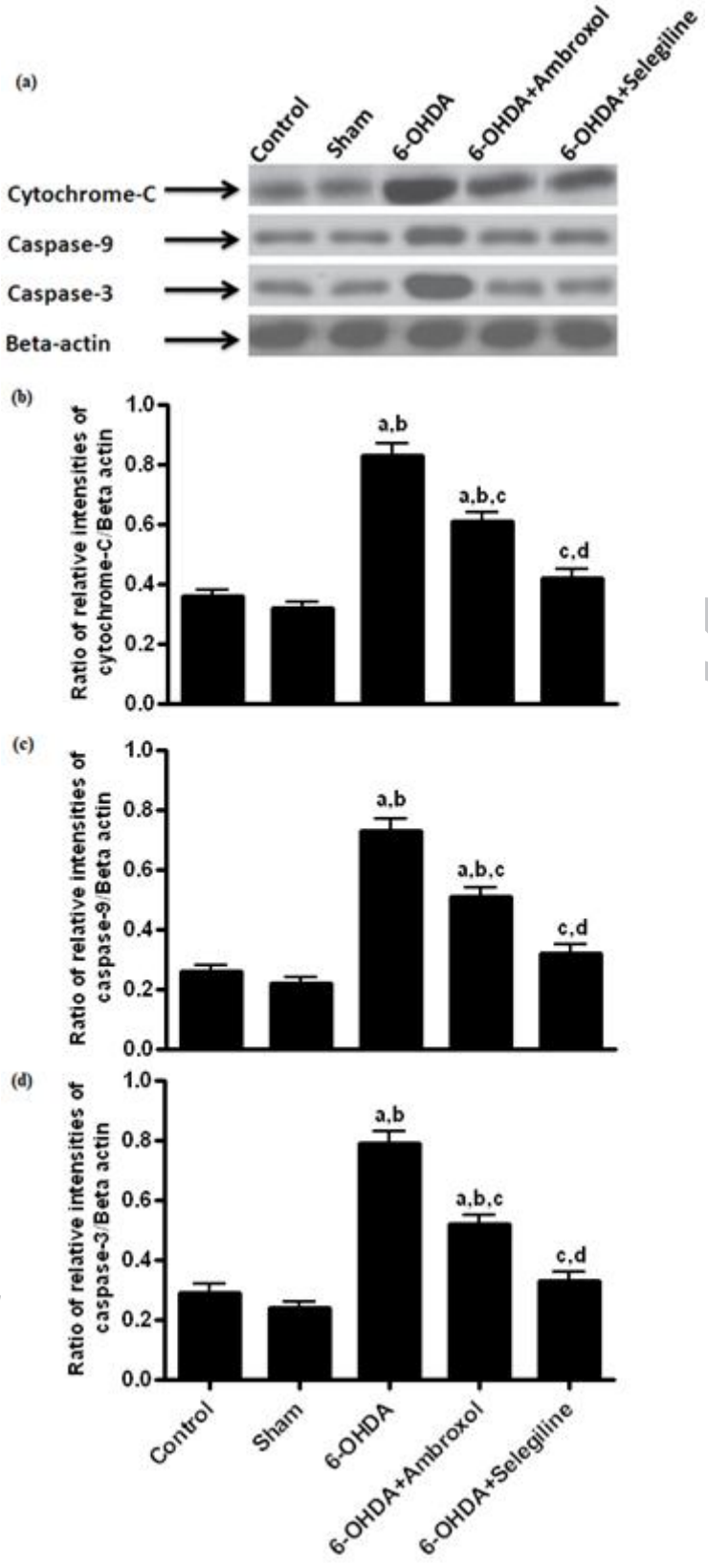

(a)
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline
Cytochrome-C
Caspase-9
Caspase-3
Beta-actin
(b)
Ratio of relative intensities of cytochrome-C/Beta actin
1.0
0.8
0.6
0.4
0.2
0.0
a,b
a,b,c
c,d
(c)
Ratio of relative intensities of caspase-9/Beta actin
1.0
0.8
0.6
0.4
0.2
0.0
a,b
a,b,c
c,d
(d)
Ratio of relative intensities of caspase-3/Beta actin
1.0
0.8
0.6
0.4
0.2
0.0
a,b
a,b,c
c,d
Control
Sham
6-OHDA
6-OHDA+Ambroxol
6-OHDA+Selegiline

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Table 1 Effects of AMB on 6-OHDA-induced changes in motor functions as assessed by apomorphine-induced rotations, latency in cataleptic behavior, grip strength score and rotarod retention timein rats

Groups	Apomorphine-induced rotations (counts/5 minutes)	Cataleptic Behavior (seconds)	Grip Strength Score (numbers)	Retention Time In Rotarod Test (seconds)
DAY 0				
Control	5.683 ± 0.436	1.865 ± 0.314	4.393 ± 0.299	180.30 ± 12.99
Sham	5.824 ± 0.519	1.902 ± 0.221	4.270 ± 0.294	181.30 ± 14.24
6-OHDA	5.800 ± 0.616	1.610 ± 0.163	4.325 ± 0.372	181.50 ± 10.41
6-OHDA+Ambroxol	5.613 ± 0.314	1.678 ± 0.195	4.386 ± 0.363	180.70 ± 15.86
6-OHDA+Selegiline	5.537 ± 0.706	1.542 ± 0.175	4.275 ± 0.284	180.70 ± 13.37
DAY 7				
Control	6.168 ± 0.637	1.753 ± 0.284	4.512 ± 0.337	180.70 ± 12.71
Sham	5.397 ± 0.864	1.983 ± 0.201	4.324 ± 0.463	176.60 ± 13.93
6-OHDA	9.732 ± 1.570^{a,b,x}	2.007 ± 0.557	1.217 ± 0.294^{a,b,x}	88.17 ± 7.98^{a,b,x}
6-OHDA+Ambroxol	10.500 ± 1.090^{a,b,x}	1.692 ± 0.101	1.411 ± 0.189^{a,b,x}	91.83 ± 11.70^{a,b,x}
6-OHDA+Selegiline	10.00 ± 1.126^{a,b,x}	1.570 ± 0.213	1.421 ± 0.399^{a,b,x}	89.67 ± 9.81^{a,b,x}
DAY 14				
Control	5.762 ± 0.579	1.427± 0.145	4.327 ± 0.239	181.80 ± 10.91
Sham	6.305 ± 0.649	1.418 ± 0.269	4.180 ± 0.283	174.60 ± 14.83
6-OHDA	14.100 ± 4.194^{a,b,x,y}	4.114 ± 1.276^{a,b,x,y}	0.998 ± 0.353^{a,b,x}	80.74 ± 4.88^{a,b,x}
6-OHDA+Ambroxol	11.090 ± 1.482^{a,b,c,x}	2.595 ± 0.309^{a,b,c,x,y}	2.255 ± 0.305^{a,b,c,x,y}	109.40 ± 15.75^{a,b,c,x,y}
6-OHDA+Selegiline	11.49 ± 1.242^{a,b,c,x,y}	2.792 ± 0.382^{a,b,c,x,y}	2.478 ± 0.384^{a,b,c,x,y}	121.80 ± 8.38^{a,b,c,d,x,y}
DAY 21				
Control	5.477 ± 0.625	1.752 ± 0.331	4.382 ± 0.373	180.20 ± 13.60
Sham	5.265 ± 0.579	1.848 ± 0.219	4.314 ± 0.438	178.40 ± 10.26
6-OHDA	16.890 ± 4.007^{a,b,x,y,z}	6.080 ± 1.774^{a,b,x,y,z}	1.243 ± 0.207^{a,b,x}	89.36 ± 4.69^{a,b,x}
6-OHDA+Ambroxol	7.992 ± 0.577^{a,b,c,x,y,z}	2.790 ± 0.221^{a,b,c,x,y}	3.670 ± 0.392^{a,b,c,x,y,z}	157.20 ± 7.84^{a,b,c,x,y,z}
6-OHDA+Selegiline	7.037 ± 0.657^{a,b,c,x,y,z}	2.438 ± 0.082 ^{a,b,c,x,y}	3.931 ± 0.320^{a,b,c,x,y,z}	167.20 ± 5.12^{a,b,c,d,x,y,z}
DAY 28				
Control	5.274 ± 0.569	1.622 ± 0.209	4.290 ± 0.275	181.50 ± 14.11
Sham	5.339 ± 0.744	1.892 ± 0.271	4.450 ± 0.155	179.30 ± 9.78
6-OHDA	16.700 ± 3. 482^{a,b,x,y,z}	5.252 ± 1.616^{a,b,x,y,z,v}	1.028 ± 0.278^{a,b,x}	87.50 ± 6.29^{a,b,x}
6-OHDA+Ambroxol	6.636 ± 0.700^{c,y,z,v}	2.010 ± 0.166^{c,z,v}	4.165 ± 0.326^{c,y,z,v}	171.90 ± 6.152^{c,y,z,v}
6-OHDA+Selegiline	6.581 ± 0.453^{c,y,z}	2.380 ± 0.146^{c,z}	4.152 ± 0.405^{c,y,z}	172.20 ± 4.13^{c,y,z}

All values are mean ± SD; (n = 39 for D-0, n = 33 for D-7, n = 27 for D-14, n = 21 for D-21 and n = 15 for D-28); $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham, $^{c}p< 0.05$ compared to 6-OHDA and $^{d}p< 0.05$ compared to 6-OHDA+Ambroxol. $^{x}p< 0.05$ compared to D-0, $^{y}p< 0.05$ compared to D-7, $^{z}p< 0.05$ compared to D-14 and $^{v}p< 0.05$ compared to D-21 [Non-repeated measures of two-way ANOVA followed by Bonferroni test].

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Table 2 Effects of AMB on 6-OHDA-induced alterationsin motor functions as assessed by number of central squares crossed, ambulation, rearing and grooming behavior in open field test in rats

Groups	Central Squares crossed (numbers)	Ambulation (numbers)	Rearing (numbers)	Grooming (numbers)
DAY 0				
Control	5.117 ± 0.516	45.850 ± 3.172	14.580 ± 1.242	6.888 ± 0.722
Sham	4.839 ± 0.463	45.360 ± 5.340	14.840 ± 0.969	6.837 ± 0.817
6-OHDA	5.090 ± 0.518	44.490± 3.610	15.250 ± 1.136	6.661 ± 0.725
6-OHDA+Ambroxol	4.880 ± 0.353	44.140 ± 4.115	15.070 ± 0.303	6.538 ± 0.473
6-OHDA+Selegiline	4.968 ± 0.435	44.090 ± 5.362	14.520 ± 1.521	6.850 ± 0.878
DAY 7				
Control	4.675 ± 0.516	44.890 ± 5.298	14.480 ± 1.336	6.488 ± 0.582
Sham	4.416 ± 0.391	43.180 ± 4.997	15.090 ± 1.597	6.698 ± 0.867
6-OHDA	4.376 ± 0.477	14.200 ± 3.005^{a,b,x}	7.280 ± 2.146^{a,b,x}	3.121 ± 0.919^{a,b,x}
6-OHDA+Ambroxol	4.595 ± 0.614	16.290 ± 2.054^{a,b,x}	7.958 ± 1.069^{a,b,x}	3.545± 0.451^{a,b,x}
6-OHDA+Selegiline	4.414 ± 0.541	16.400 ± 2.379^{a,b,x}	8.013 ± 0.810^{a,b,x}	3.499 ± 0.496^{a,b,x}
DAY 14				
Control	4.725 ± 0.471	46.200 ± 5.912	15.060 ± 1.532	6.803 ± 0.690
Sham	4.773 ± 0.393	45.430 ± 2.574	15.610 ± 1.884	6.867 ± 0.546
6-OHDA	2.472 ± 0.742^{a,b,x,y}	11.500± 1.791^{a,b,x}	6.474 ± 1.317^{a,b,x}	2.820 ± 0.736^{a,b,x}
6-OHDA+Ambroxol	3.213 ± 0.464^{a,b,c,x,y}	21.340± 3.986^{a,b,c,x,y}	9.246 ± 1.053^{a,b,c,x,y}	4.487 ± 0.577^{a,b,c,x,y}
6-OHDA+Selegiline	3.667 ± 0.487^{a,b,c,d,x,y}	25.290 ± 6.716^{a,b,c,d,x,y}	10.35 ± 1.252^{a,b,c,d,x,y}	4.867 ± 0.430^{a,b,c,x,y}
DAY 21				
Control	4.785 ± 0.561	44.06 ± 3.612	14.950 ± 1.458	6.707 ± 0.616
Sham	4.572 ± 0.433	42.910 ± 3.728	14.850 ± 1.151	6.497 ± 0.387
6-OHDA	2.278 ± 0.743^{a,b,x,y}	11.740 ± 3.330^{a,b,x}	7.365 ± 2.130^{a,b,x}	2.845 ± 0.890^{a,b,x}
6-OHDA+Ambroxol	3.710 ± 0.389^{a,b,c,x,y,z}	30.200 ± 2.681^{a,b,c,x,y,z}	12.820 ± 1.508^{a,b,c,x,y,z}	5.655 ± 0.683^{a,b,c,x,y,z}
6-OHDA+Selegiline	4.047 ± 0.486^{a,b,c,x,y,z}	35.620 ± 3.031^{a,b,c,d,x,y,z}	13.350 ± 1.128^{a,b,c,x,y,z}	5.863 ± 0.739^{a,b,c,x,y,z}
DAY 28				
Control	4.480 ± 0.523	44.400 ± 3.435	15.050 ± 1.369	6.554 ± 0.536
Sham	4.411 ± 0.354	43.870 ± 3.290	15.150 ± 1.274	6.323 ± 0.581
6-OHDA	1.872 ± 0.633^{a,b,x,y,z}	11.050 ± 1.895^{a,b,x}	6.257 ± 1.075^{a,b,x}	2.830 ± 0.441^{a,b,x}
6-OHDA+Ambroxol	4.478 ± 0.415^{c,z,v}	41.390 ± 4.535^{c,y,z,v}	14.450 ± 0.9253^{c,y,z,v}	6.089 ± 0.381^{c,y,z}
6-OHDA+Selegiline	4.242 ± 0.456^{c,z}	40.940 ± 4.252^{c,y,z,v}	14.060 ± 1.475^{c,y,z}	6.698 ± 0.737^{c,d,y,z}

All values are mean ± SD; (n = 39 for D-0, n = 33 for D-7, n = 27 for D-14, n = 21 for D-21 and n = 15 for D-28); $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to sham, $^{c}p< 0.05$ compared to 6-OHDA and $^{d}p< 0.05$ compared to

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6-OHDA+Ambroxol. $^{x}p< 0.05$ compared to D-0, $^{y}p< 0.05$ compared to D-7, $^{z}p< 0.05$ compared to D-14 and $^{v}p< 0.05$ compared to D-21 [Non-repeated measures of two-way ANOVA followed by Bonferroni test].

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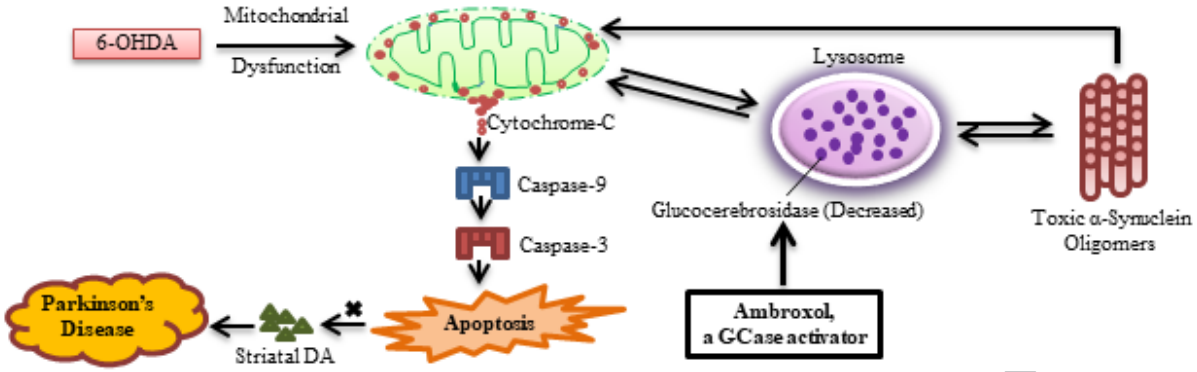

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