Chapter 4

(a) Validation of GCase as a target in 6-OHDA-induced model of PD in rats

(b) Assessing the effects of sub-acute administration of ambroxol in 6-OHDA-induced model of PD in rats

4.1. Introduction

GCase, a lysosomal enzyme is responsible for the metabolism of glucocerebroside (GC) (Grabowski, 2008) and degrades it to glucose and ceramide (Beavan and Schapira, 2013). GCase deficiency results into accumulation of GC in the lysosomes in different cell types like macrophages and neurons (Grabowski, 2008; Santos and Tiscornia, 2017). 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 (Gegg et al., 2012). There was no accumulation of lipid substrates found in the former case (Gegg et al., 2015), so other mechanisms must be responsible for neurodegeneration whereas in latter case accumulation of GC was found in SNc in PD brain (Rocha et al., 2015a). GCase protein was reported to be lower in SNc and CSF of sporadic PD brains with reduced enzymatic activity (Chiara et al., 2007; Gegg et al., 2012). Reduced GCase activity was also found in cerebellum region of sporadic PD patients (Gegg et al., 2012). Additionally, GCase expression is reported to rescue amyloid-β (Aβ) 1-42 oligomer-induced neuronal cell death in primary hippocampal neurons (Choi et al., 2015), suggesting the protective effect of GCase in neurodegeneration.

Reduced mitochondrial function is reported in GCase-deficient animal and cellular models with high mitochondrial volume and low turnovers (Osellame et al., 2013). 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 (Gegg et al., 2012). GCase deficiency also causes accumulation of α-synuclein toxic oligomers in lysosomes (Cleeter et al., 2013;

Mazzulli et al., 2011) and reduced striatal DA release (Ginns et al., 2014). α-synuclein is a significant component of lewy bodies and its oligomeric aggregation are found in brains of PD patients (Moore et al., 2005). When GCase bind to α-synuclein at lysosomal pH (5.5), α-synuclein degradation occurs. Such homeostatic processes that keep the feedback loops in check may fail with time, supporting age as the major risk factor in neurodegenerative diseases (Yap et al., 2011). α-synuclein is natively unfolded and intrinsically unstructured protein, having conformational plasticity. Depending on the environment, α-synuclein may form monomeric or oligomeric species, which may convert into amyloidogenic filaments and serve as major structural component of lewy bodies, suggesting the significant role of α-synuclein in pathogenesis of PD (Moore et al., 2005). During normal physiology, α-synuclein regulates vesicle size and membrane curvature (Yap et al., 2011). However, its oligomeric aggregates are toxic which inhibit the mitochondrial protein import and decrease mitochondrial respiration, complex enzyme system and membrane potential (Di Maio et al., 2016). Mice with presynaptic accumulation of α-synuclein are also found with less striatal DA release (Garcia-Reitböck et al., 2010). α-synuclein is also reported to make complex with GCase enzyme and inhibit its function (Mazzulli et al., 2011; Yap et al., 2011).

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 (Blum et al., 2001; Gu et al., 2016; Migdalska-Richards and Schapira, 2016; Moore et al., 2005). Therefore, due to high degree of construct validity for 6-OHDA model (Duty and Jenner, 2011), 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 (Gegg et al., 2012). Previous studies indicated the possibility of earlier PD onset in case of GBA mutation which is approximately six years earlier than non-GBA carriers of PD. 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 (Rocha et al., 2015a). There is no established non-genetic animal model to validate GCase enzymatic activity in PD. Therefore, considering the majority of non-genetic PD cases (90-95%), the prime focus of the present study is to observe the temporal effects of 6-OHDA on GCase activity in striatum and SNc region of the rat in hemiparkinson's model. Due to high degree of the face and predictive validity (Duty and Jenner, 2011), 6-OHDA model may be further utilized to target GCase for the development of novel neuroprotective drugs in PD.

Ambroxol is reported to increase GCase activity in brainstem, midbrain and cortex of α-synuclein transgenic mice (Migdalska-Richards et al., 2016), improved lysosomal biochemistry and rescued defective GCase in GBA1 mutation-linked PD cells (Ambrosi et al., 2015; McNeill et al., 2014). Ambroxol not only increased GCase activity in wild-type mice but also reduced α-synuclein levels and restored GCase activity in mice overexpressing human α-synuclein (Migdalska-Richards et al., 2016). PD phenotype was alleviated in flies carrying misfolded mutant GCase by growing them in the presence of ambroxol (Maor et al., 2016). Ambroxol may also upregulate GCase activity in patients without GBA1 mutation (Schapira, 2015). However, there are no reports showing the effect of ambroxol in well-characterized

models of PD *in vivo* perhaps due to the absence of non-genetic PD models for evaluating GCase activity. Therefore, in the present study, ambroxol was used to stimulate GCase activity and its anti-PD like effects were investigated 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. Ambroxol administration was initiated before the full development of motor deficits in rats. Ambroxol may increase GCase activity, followed by decrement in α-synuclein oligomers, mitochondrial dysfunction and apoptosis against 6-OHDA toxicity as shown in **Figure 4.1.** Different behavioral parameters were performed, such as apomorphine-induced rotation, open field, rotarod, grip strength and bar catalepsy tests to evaluate the motor deficits in PD. Neurochemical measure of PD was performed by estimating striatal monoamines and their metabolites. Mitochondrial function was assessed by MTT reduction. GCase 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.

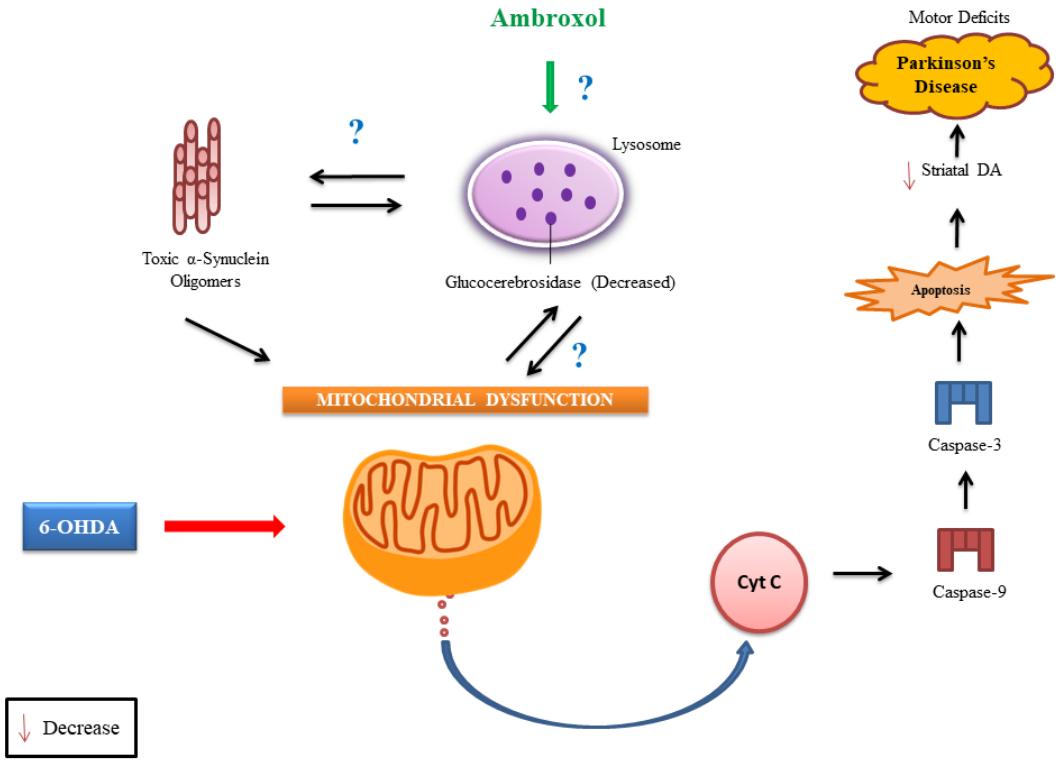

Figure 4.1 The schematic diagram of hypothesis for the validation of GCase as a target in 6-OHDA-induced model of PD in rats and the effects of sub-acute administration of ambroxol against 6-OHDA toxicity. Effect of 6-OHDA on GCase enzymatic activity may be observed due to the involvement of 6-OHDA-induced mitochondrial dysfunction and α-synuclein aggregation. Ambroxol due to its GCase-stimulating activity may act against 6-OHDA-induced toxicity by attenuating mitochondrial dysfunction, α-synuclein pathology, loss of nigral cells, mitochondrial-linked apoptosis and motor impairment along with the upregulation of striatal DA content.

4.2. Materials and Methods

4.2.1. Animals

Charles-Foster strain of adult albino rats male (260 ± 20 g) was procured from Central Animal House; Institute of Medical Sciences, Banaras Hindu University

(IMS-BHU) and acclimatized at a temperature of 25 ± 1^0C and 45-55% relative humidity with light/dark cycle of 12:12h by keeping them in polypropylene cages. Commercial food pellets (Doodhdhara Pashu Ahar, India) and water was made available *ad libitum.* 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 Institutes of Health guide for the care and use of Laboratory animals (NIH Publication No. 8023, revised 1978)] guidelines and approved by the Institutional animal ethical committee, BHU (Dean/2016/CAEC/33). The experiments were performed between 9:00h and 16:00h.

4.2.2. Materials

Ambroxol hydrochloride was received as a gift sample from Merril Pharma Pvt. Ltd. (Roorkee, India). 6-OHDA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), apomorphine-hydrochloride, MTT, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferone (4-MU), 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, EGTA, 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 (enzyme-linked immunosorbent assay) 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. The other reagents of HPLC and analytical grades were acquired from local suppliers.

4.2.3. Surgery and Microinjection

Rats were anesthetized with pentobarbitone sodium (35 mg/kg) intraperitoneally (i.p.) and their furs were shaved from the scalp using a trimmer. Rat was mounted on the stereotaxic frame in a way that its 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) (Paxinos and Watson, 1998). 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 additional 5 min for total diffusion of 6-OHDA (Ambrosi et al., 2017; Kumar et al., 2017). Homoeothermic blankets were used to maintain body temperature of animals at 37^0C throughout the surgery. Buprenorphine (0.05 mg/kg, subcutaneous.) was injected for postoperative analgesia. 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.

4.2.4. Experimental Design

Animals were randomly divided into five groups namely control, sham, 6-OHDA, 6-OHDA+Ambroxol (to stimulate GCase activity), and 6-OHDA+Selegiline (positive control). 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 4 µL of normal saline containing 0.2 mg/mL ascorbic acid (Kumar et al., 2012; Paxinos and Watson, 1998). Ambroxol 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 (Bhardwaj et al., 2016). Since half-life of ambroxol is approx. 10h (Malerba and Ragnoli, 2008), the dose of ambroxol was chosen as 800 mg/kg/day and was administered orally (*per os, p.o.)* as 400 mg/kg twice daily from D-4 to D-27. The same dose was also found to be effective against 6-OHDA-induced motor deficits in the pilot study. Ambroxol 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 (Hama et al., 2010; Sanders et al., 2013). Selegiline has shown multimodal effects in various experimental models of PD (Bisht et al., 2017; Liu et al., 2017; Saravanan et al., 2006; Zhao, Q. et al., 2013; Zhao, X. et al., 2013) and therefore was used as positive control at the dose of 10 mg/kg *p.o.* daily. Selegiline has shown post-toxin effect in MPTP and rotenone models to regenerate dopaminergic neurons and attenuated mitochondrial dysfunction (Bisht et al., 2017; Saravanan et al., 2006), which are of particular interest to the present hypothesis. The drugs ambroxol and selegiline were administered by oral gavage.

6-OHDA was infused on 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. For grip strength and bar catalepsy test, training session was performed on animals two days before D-0 in order to make them habituated for the test (Kheradmand et al., 2016; Meyer et al., 1979). However, for rotarod test training was performed for two consecutive days before D-0 (Fernandez et al., 1998; Rozas et al., 1997). Apomorphine-induced rotational behavior was conducted on D-4 also. Open field parameters were recorded using ANY-MAZE behavioral 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 decided 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 micro dissected on ice from ipsilateral hemispheres (Paxinos and Watson, 1998) 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 24h 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 cytochrome-C, caspase-9 and caspase-3 proteins expression (n = 3). The detailed experimental design is depicted in **Figure 4.2.**

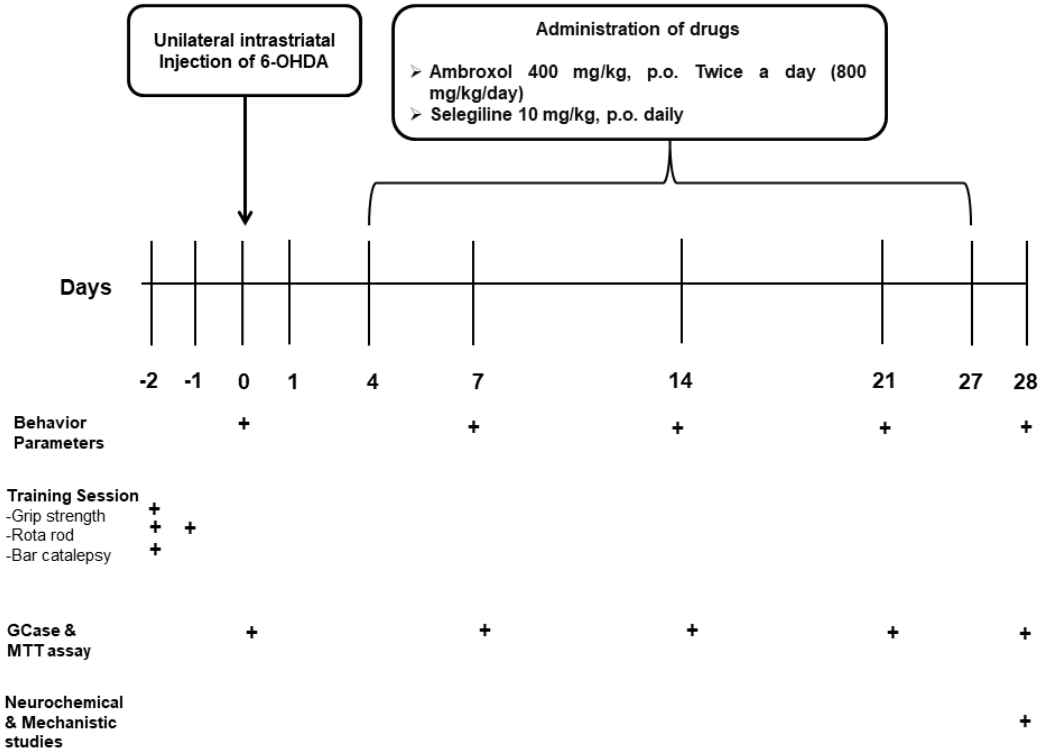

Figure 4.2 The experimental design of the study for the validation of GCase as a target in 6-OHDA-induced model of PD in rats and the effects of sub-acute administration of ambroxol against 6-OHDA toxicity. "+" indicates days on which parameters were performed.

4.2.5. Behavior Parameters

4.2.5.1. Apomorphine-induced rotational behavior

Apomorphine-hydrochloride was dissolved in normal saline and administered to rats as 1mg/kg by single i.p. injection. Animals were observed for basal contralateral rotations before the surgery on D-1. Net rotations towards the contralateral side were scored for 5 min continuously (Ungerstedt, 1971).

4.2.5.2. Open field test

Apparatus for open field test 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'. This task is based on the novelty of the environment, therefore animals do not require any pre-training (Seibenhener and Wooten, 2015). The animal was placed in the middle of the arena and was allowed to move freely for 5 min 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 (Bronstein, 1972).

4.2.5.3. Rotarod test

Animals were trained so that they can retain for 180 seconds (sec) on the rod (IKON Instrument New Delhi, India). Training was performed twice daily and only those

animals were selected for experiments that could retain at least for 60 sec initially. The rotation speed of the rod was set at 8 and 10 revolutions per min (rpm) for the training sessions of first and second day respectively. Thereafter, rotarod tests were performed with higher rotational speed (15 rpm) on D-0, 7, 14, 21 and 28. As soon as the animal fell from the rod, recording was stopped and the time during which animal retained its position on the rod was noted. Data is expressed as retention time on the rotarod over three test trials (Rozas et al., 1997).

4.2.5.4. 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 sec. Only the animals fulfilling this criterion during training session were included in the study. The average of three successive trials was taken. 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 sec) (Meyer et al., 1979).

4.2.5.5. Bar Catalepsy Test

Catalepsy, also known as adopting and maintaining abnormal posture was performed by using bar test (Geed et al., 2014; Sanberg et al., 1988). 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 the bar was recorded

and the measurement was stopped as soon as rat removed any of its fore-paws from the bar. Animals with elapsed time in normal range during training sessions were included in the study (Kheradmand et al., 2016). The mean of three consecutive trials was taken with cut-off time = 60 sec.

4.2.6. Estimation of striatal monoamines and their metabolites

The levels of neurotransmitter DA and its metabolites DOPAC and HVA 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 (Kim et al., 1987). Protein concentration was estimated as described previously (Lowry et al., 1951).

4.2.7. Measurement of GCase enzymatic activity

GCase activity was measured as described earlier with some modifications (Rocha et al., 2015c). 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 min 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-MU was plotted to assess the enzymatic activity and normalized to protein content in each sample as determined using standard method (Lowry et al., 1951). GCase activity was expressed as nanomoles of 4-MU released/hour/mg of protein (nmol/h/mg protein).

4.2.8. Estimation of mitochondrial function

Firstly, mitochondria were isolated from rat striatal and SNc tissues as previously described protocol with slight modifications (Berman and Hastings, 1999). Isolation buffer (215 mM mannitol, 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 (Lowry et al., 1951) using microplate reader (Biotek, USA).

Mitochondrial function was assessed in terms of MTT reduction (Liu et al., 1997). 50 µg mitochondrial suspension was incubated with 0.1 mg/mL MTT for 30 min 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.

4.2.9. Rat α-synuclein measurement

Rat α-synuclein concentration was measured in the rat SNc tissues using ELISA plate reader from commercially available ELISA kit (E-EL-R1217). Protein concentration was measured as standard protocol (Lowry et al., 1951) and results were expressed as α-synuclein concentration in pg/mg protein.

4.2.10. Nissl's staining

On D-28, 24h 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 24h 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 three times with PBS (0.01 mol/L), for 10 min 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 cover slipped 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 (Li et al., 2008; Sedaghat et al., 2014). The number of Nissl-stained nigral neurons was quantified with the cell counter tool of NIH ImageJ software (Hambright et al., 2017). The data was expressed as percentage of control rats.

4.2.11. 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 (Burnette, 1981). BSA was used as a standard protein and protein concentrations were determined (Lowry et al., 1951). 10% concentration of SDS-PAGE gels (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) was taken for 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, and anti-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 min. 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.

4.2.12. Statistical Analysis

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

4.3. Results

4.3.1. Behavior Parameters

4.3.1.1. Ambroxol decreased catalepsy and apomorphine-induced changes in rotational behavior in 6-OHDA-infused rats

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 4.1].** Unilateral 6-OHDA injection 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, 31% more rotations were observed 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 ambroxol-administered 6-OHDA group from D-14 with 21% decrease from 6-OHDA group which became similar to control group on D-28. Ambroxol statistically decreased head rotations progressively from D-14 to D-28.

6-OHDA increased cataleptic behavior (65%) from D-14. 6-OHDA-induced increase was observed to be 71% and 69% higher on D-21 and D-28 respectively than control animals. Ambroxol 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. Ambroxol-induced attenuation in cataleptic behavior in 6-OHDA rats was 23% and 28% on D-28 compared to D-14 and D-21 respectively. Selegiline also attenuated 6-OHDA induced cataleptic and rotational behavior in rats.

4.3.1.2. Ambroxol increased rotarod retention time and grip strength scores in 6-OHDA-infused rats

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 4.1**. Ambroxol reduced both the

motor deficits and attenuated 6-OHDA-induced reduction in rotarod retention time (26%) and grip strength scores (56%) from D-14. Ambroxol-induced attenuation was progressive and motor deficits were recovered on D-28 against 6-OHDA-infused rats. Selegiline also elicited similar effects in the rotorod test and grip strength scores.

4.3.1.3. Ambroxol improved spontaneous locomotor activity in open field test in 6-OHDA-infused rats

General locomotor activity of animals was measured by open field test (Van Den Buuse et al., 1986). Since, DA neurons play an important role in encoding movement (Parker et al., 2016), 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 open field test **[Table 4.2**]. Ambroxol 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. Temporal significant effects of ambroxol were observed against 6-OHDA induced motor deficits from D-

14 to D-28. Selegiline was also found to decrease 6-OHDA induced motor deficits in open field parameters.

4.3.2. Ambroxol increased DA and its metabolites DOPAC and HVA in striatal tissues of 6-OHDA-infused 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 **Figure 4.3**. 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. Ambroxol 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. Selegiline also improved DA concentration after 6-OHDA injection.

Table 4.1 Effects of ambroxol on 6-OHDA-induced changes in motor functions as assessed by apomorphine-induced rotations, cataleptic behavior, grip strength score and rotarod retention time in rats

Groups	Apomorphine-induced rotations (counts/5 min)	Cataleptic Behavior (sec)	Grip Strength Score (numbers)	Retention Time In Rotarod Test (sec)
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].

Table 4.2 Effects of ambroxol on 6-OHDA-induced alterations in motor functions as assessed by number of central squares crossed, ambulation, rearing and grooming 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 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].

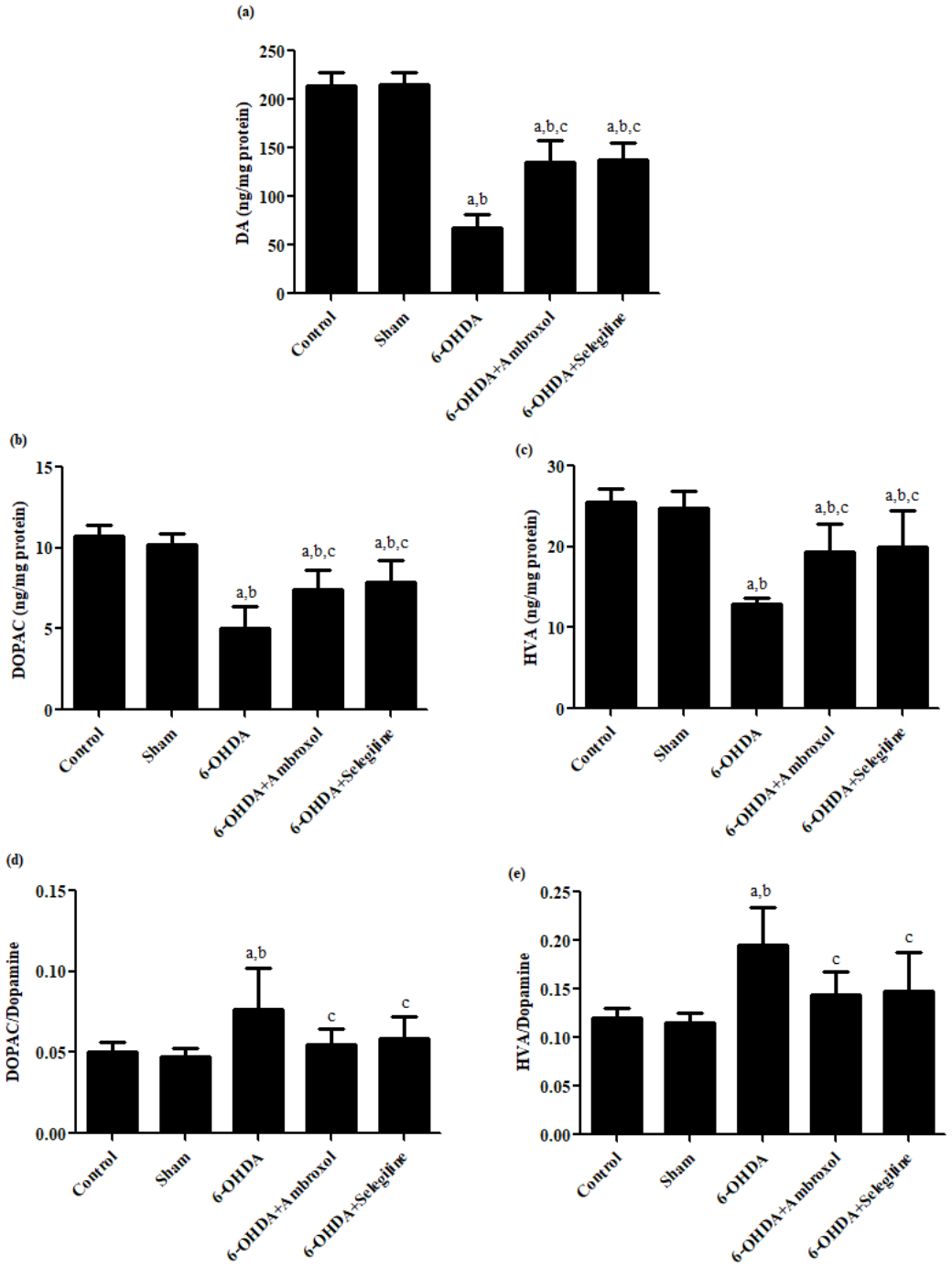

Figure 4.3 Effect of ambroxol on 6-OHDA-induced alterations on the levels of DA (a), DOPAC (b), HVA (c), DOPAC/DA (d), and HVA/DA (e) in striatal tissues of rats at D-28. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to sham and $^{c}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

4.3.3. 6-OHDA decreased and treatment with ambroxol 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 **Figure 4.4.** 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 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. Ambroxol 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 ambroxol were found to be maximum on D-21 in striatum and D-28 in nigral tissues. Moreover, progressive elevation in ambroxol-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 (Liu et al., 1997). 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 **[Figure 4.5].** 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. Ambroxol 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. Ambroxol-induced effects were increased progressively 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 selegiline treatment against 6-OHDA model.

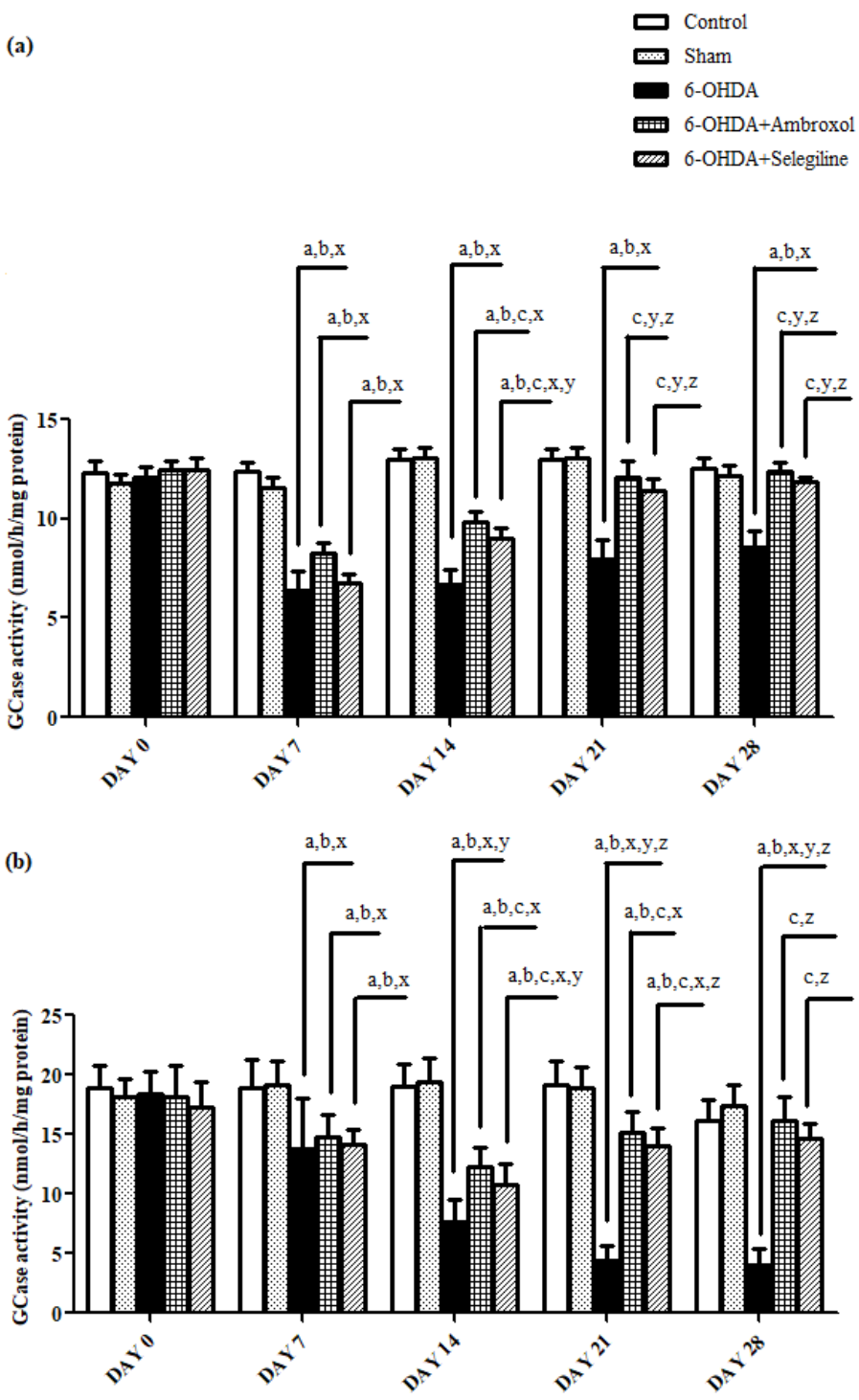

Figure 4.4 Effect of 6-OHDA and treatment with ambroxol on GCase enzyme activity in striatal (a) and nigral (b) tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to sham and $^{c}p < 0.05$ compared to 6-OHDA. $^{x}p < 0.05$ compared to D-0, $^{y}p < 0.05$ compared to D-7 and $^{z}p < 0.05$ compared to D-14 [Non-repeated measures of two-way ANOVA followed by Bonferroni test].

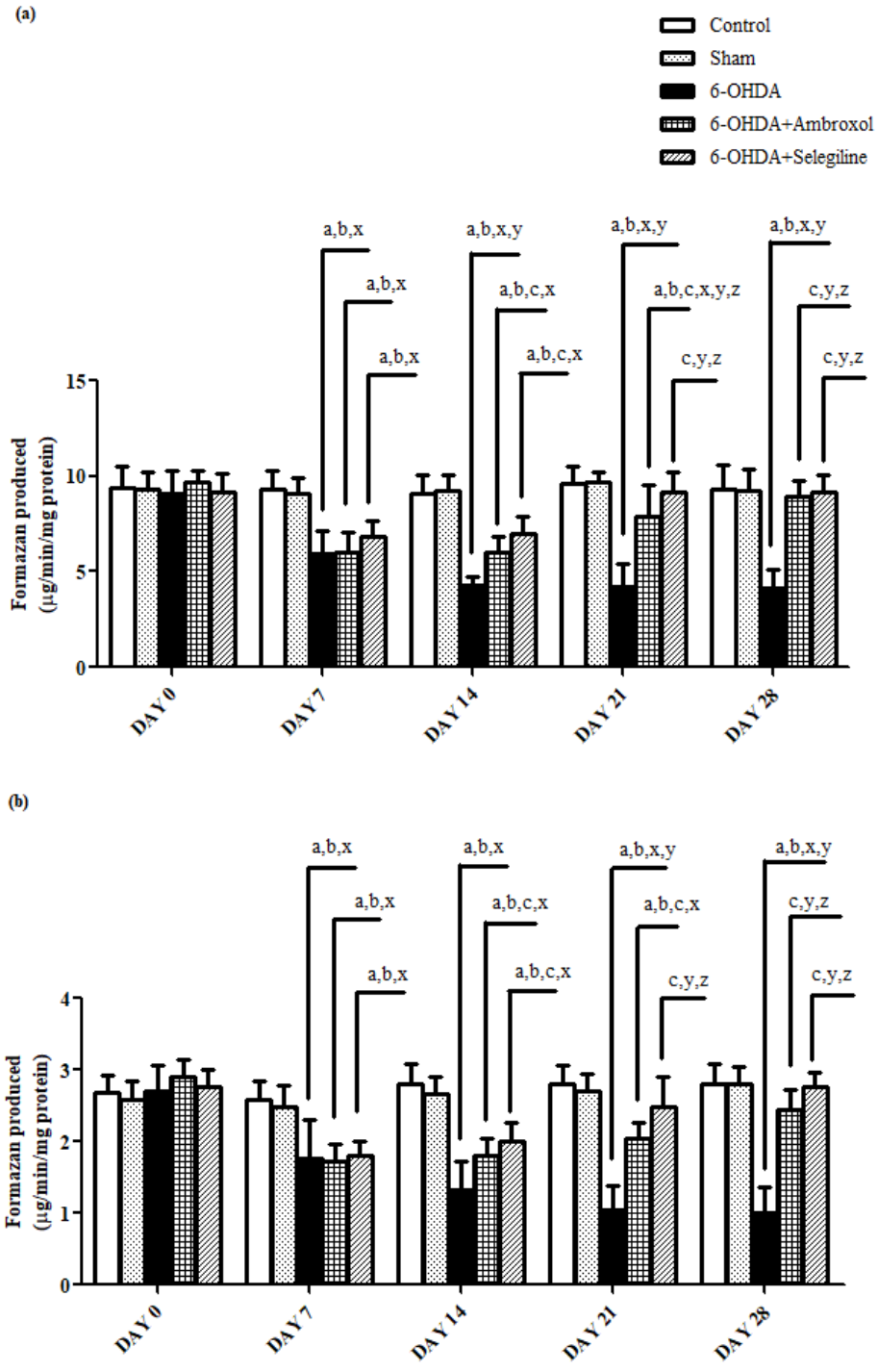

Figure 4.5 Effect of 6-OHDA and treatment with ambroxol on mitochondrial function in terms of MTT reduction in striatal (a) and nigral (b) tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to sham and $^{c}p < 0.05$ compared to 6-OHDA. $^{x}p < 0.05$ 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].

4.3.4. Ambroxol increased soluble α-synuclein concentration in nigral tissues of 6-OHDA-infused rats

Under pathophysiological conditions, α-synuclein forms oligomeric aggregates which are insoluble in nature and are important component of toxic lewy bodies (Moore et al., 2005). However, under normal physiology, α-synuclein is water-soluble (Budi et al., 2012) 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 ambroxol as observed in **Figure 4.6**. Soluble α-synuclein concentration was also increased by selegiline.

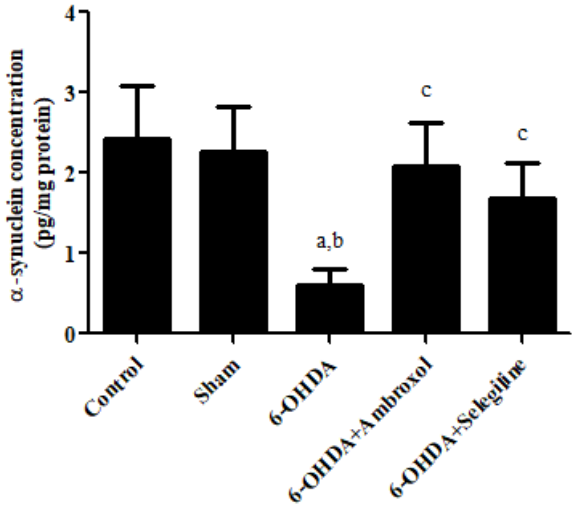

Figure 4.6 Effect of ambroxol on 6-OHDA-induced changes on α-synuclein concentration in rat nigral tissues at D-28. All values are mean ± SD; n = 3; $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to sham and $^{c}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

4.3.5. Ambroxol increased nigral cells in 6-OHDA-infused rats

A remarkable loss (68%) of Nissl bodies 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 bodies in nigral tissue [$F(4, 10) = 24.63$; $p < 0.05$]. Ambroxol substantially reduced the loss of nigral cells up to 34% **(Figure 4.7).** However, ambroxol-induced increase in nigral neurons was also significantly different than control and sham groups. Similarly, nigral cells were also recovered by selegiline.

4.3.6. Ambroxol decreased the expressions of cytochrome-C, caspase-9 and caspase-3 proteins in nigral tissues of 6-OHDA-infused rats

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 these proteins compared to control and sham groups which were significantly decreased by ambroxol and selegiline [**Figure 4.8].**

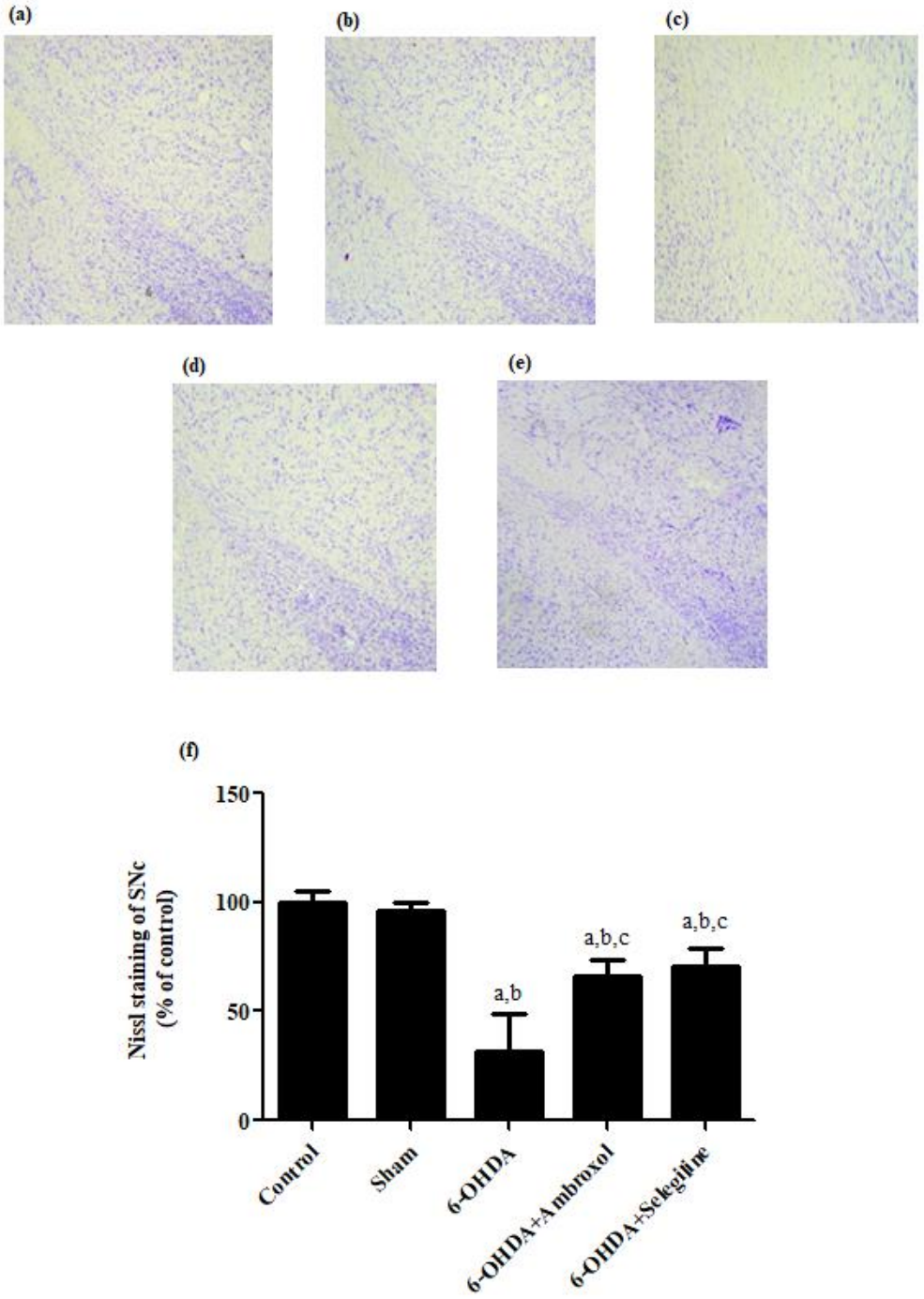

Figure 4.7 Nissl's staining of SNc in rats on D-28. Control (a); Sham (b); 6-OHDA (c); 6-OHDA+Ambroxol (d); 6-OHDA+Selegiline (e); Data of counting cells (f). All values are mean ± SD; n = 3; $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to sham and $^{c}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

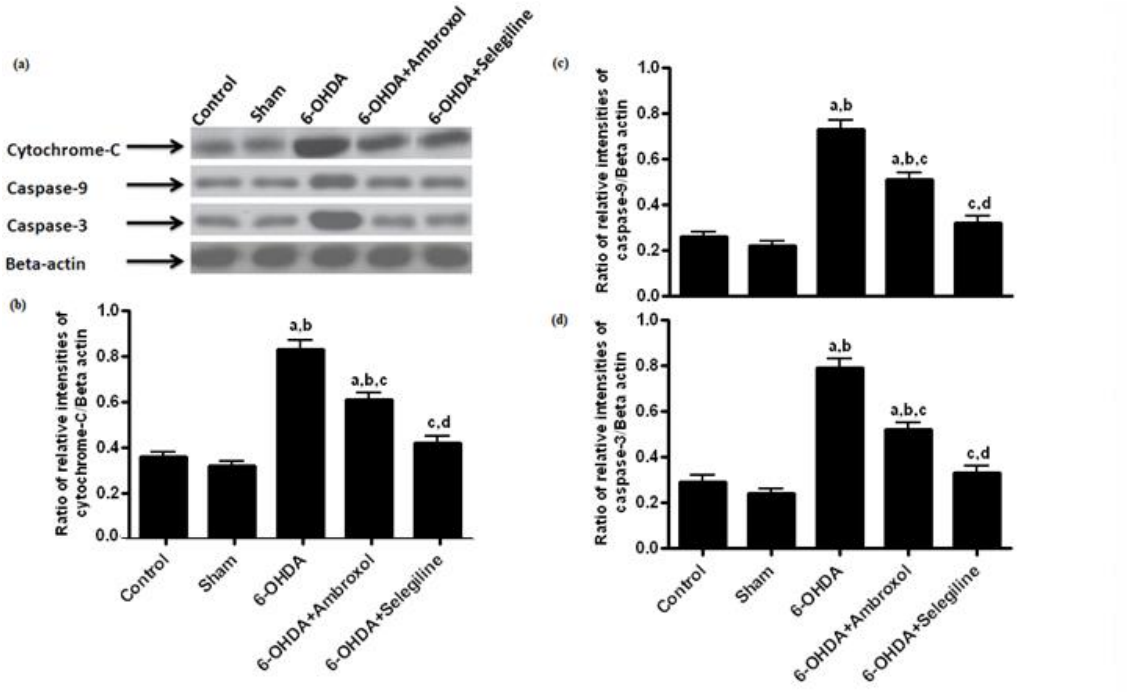

Figure 4.8 Effect of ambroxol on 6-OHDA-induced changes on the protein levels of cytochrome-C, caspase-9 and caspase-3 in rat nigral tissues. Proteins are represented in blots (a) and histograms express the ratio of the relative intensity of protein levels of cytochrome-C (b), caspase-9 (c) and caspase-3 (d) to β-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-Keuls Post-hoc test].

4.4. 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 ambroxol, which stimulate GCase activity. The tracking of GCase activity, a major risk factor for PD can be beneficial in the development of new therapeutic potential (Rocha et al., 2015a). However, there are no reports showing the effects of intrastriatal injection of

6-OHDA, an established experimental PD model on GCase enzymatic activity. Ambroxol due to its GCase stimulating activity (Schapira, 2015) is currently being investigated in PD patients (Girolamo et al., 2017) but there is no preclinical study on the pharmacology of ambroxol 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 (Moore et al., 2005). In the present study, 6-OHDA-infused rats showed impaired motor movement in different behavioral parameters. Apomorphine-induced contralateral rotations (Ungerstedt, 1971) and cataleptic behavior (Kumar et al., 2017) were increased whereas open field parameters like numbers of central squares crossed, ambulation, grooming and rearing (Van Den Buuse et al., 1986), rotarod retention time (Rozas et al., 1997) and grip strength scores (Kumar et al., 2017) were decreased by 6-OHDA. Apomorphine-induced rotational behavior is considered as an authentic physiological measure of DA depletion and asymmetric DA receptor stimulation (Ungerstedt, 1971). Grip strength test gives an insight into the regulation of neuromuscular strength (Meyer et al., 1979; Takeshita et al., 2017) and rotarod retention time characterizes motor coordination ability (Fernandez et al., 1998; Rozas et al., 1997). 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 (Van Den Buuse et al., 1986). It includes parameters such as grooming, rearing, ambulation and central squares

crossed. Rearing signifies expression of directed exploration in the adult phase of life (Coronel-Oliveros and Pacheco-Calderón, 2018; Lever et al., 2006) and displacement behavior is interpreted as grooming (Smolinsky et al., 2009). The number of central squares crossed indicates further exploration of the open field by animal (Lamprea et al., 2003). Bar catalepsy test assesses acceptance and retention of abnormal posture (Batool and Haleem, 2008) and is used to model akinesia in PD (Walther and Strik, 2012). Akinesia occurs due to the loss of fine motor control (Whishaw et al., 1990). 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 induced progressive loss of exploration and fine motor movement in animals. Selegiline was found to decrease 6-OHDA-induced motor impairment from D-14. It has been reported that selegiline decreased apomorphine-induced rotational behavior caused by 6-OHDA in rats when given after 6-OHDA (Zhao, X. et al., 2013). Ambroxol 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 progressive attenuation of motor deficits induced by 6-OHDA, suggesting that ambroxol has significant effects against 6-OHDA-induced impairment in motor behavior of the animals.

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 (Berger et al., 1991; Kirik et al., 1998). 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 (Kumar et al., 2017) which was reversed by ambroxol and selegiline. The regenerative effects of selegiline were earlier observed against rotenone (Saravanan et al., 2006) and MPTP-induced (Bisht et al., 2017; Liu et al., 2017) striatal DA depletion in rats. However, the regenerative effects of selegiline 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 (Ginns et al., 2014). Therefore in the present study, marked improvement in motor activities with ambroxol may be due to the improvement in striatal DA 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 (Kumar et al., 2017). In the present 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. Ambroxol and selegiline increased MTT reduction in both the tissues with onset of action at D-14, indicating improvement in mitochondrial function in 6-OHDA-infused rats. Selegiline has been reported to attenuate mitochondrial dysfunction induced by rotenone and MPTP in rats (Bisht et al., 2017; Saravanan et al., 2006). Mitochondrial dysfunction is also crucial for PD pathogenesis (Moore et al., 2005) and loss of mitochondrial function by PINK1 knockdown results in GCase deficiency (Gegg et al., 2012). In the present study, 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 synthesis and distribution of GCase

and other being the mode of dopaminergic toxicity by 6-OHDA. GCase is formed in ER-bound polyribosomes to get transported to lysosome (Erickson et al., 1985; Reczek et al., 2007). 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 (Morfini et al., 2012). GCase-related organelles such as ER- bound ribosomes are undetectable in axons (Morfini et al., 2012), whereas lysosomes in approximately equal proportion undergo either anterograde or retrograde transport (Maday et al., 2014). 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 (Bernheimer et al., 1973; Dauer and Przedborski, 2003). Unilateral intrastriatal injection of 6-OHDA causes retrograde degeneration of nigrostriatal DA neurons in rats (Berger et al., 1991). 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 to maximum (Blandini et al., 2007). 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 up to 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 (Gegg et al., 2012; Rocha et al., 2015a). GCase gene therapy increased GCase protein

level in the substantia nigra more significantly than striatum in mice (Rocha et al., 2015b).

Ambroxol facilitate trafficking of GCase through the ER to lysosome (Bendikov-Bar et al., 2011; Maegawa et al., 2009) and is reported to restore GCase activity in cell culture models (Sanchez-Martinez et al., 2016). In the present study, ambroxol and selegiline increased GCase enzymatic activity in 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 (Gegg et al., 2012), 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 (Cleeter et al., 2013). The clearance capacity of lysosome gets deranged which leads to the formation of fragmented and dysfunctional mitochondria having impaired respiratory chain (Cleeter et al., 2013; Dehay et al., 2013). In the present study also, GCase activation by ambroxol may be responsible for mitigating mitochondrial dysfunction in 6-OHDA-induced dopaminergic toxicity. Selegiline increased GCase activity which may also be related to its effect on mitochondrial function (Bisht et al., 2017).

Pharmacological inhibition of GCase is reported to cause accumulation of α-synuclein (Cleeter et al., 2013). α-synuclein, a component of lewy bodies forms insoluble oligomeric aggregates which are characteristic markers of PD and appeared in PD patients having decreased GCase activities (Creese et al., 2017; Moore et al., 2005). GCase deficiency causes accumulation of its substrate GC in SNc which prolong the lag phase of α-synuclein fibril growth (Mazzulli et al., 2011). In the

present study, the soluble α-synuclein concentration was measured. 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 (Budi et al., 2012). In the present study, 6-OHDA decreased the concentration of water-soluble α-synuclein in nigral tissues, suggesting the formation of α-synuclein aggregates as stated previously (Gu et al., 2016). Ambroxol and selegiline increased the concentration of water-soluble α-synuclein in 6-OHDA-infused rats, indicating decreased aggregation of α-synuclein. Selegiline decreased α-synuclein in SNc of mice infused with MPTP (Zhao, X. et al., 2013). However, in the present study, a decrease in α-synuclein oligomers by selegiline after 6-OHDA induced dopaminergic toxicity was reported. Ambroxol has been reported to reduce α-synuclein levels and restored GCase activity in mice overexpressing human α-synuclein (Migdalska-Richards et al., 2016). GCase gene therapy is also reported to decrease α-synuclein oligomers in the midbrain DA neurons of mice (Rocha et al., 2015b). 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 (Mazzulli et al., 2011). α-synuclein interacts with GCase and makes α-synuclein-GCase complex, thereby inhibiting GCase enzyme function (Yap et al., 2011). Therefore, in the present 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 (Di Maio et al., 2016; Mazzulli et al., 2011). Mitochondrial impairment could further lead to apoptosis via intrinsic pathway due to the release of mitochondrial cytochrome-C which activates other proteins like caspase-9 and caspase-3 (Blum et al., 2001). In the present study, these proteins were upregulated in rat nigral tissues by 6-OHDA (Prajapati et al., 2017) and decreased by ambroxol and selegiline. The loss of nigral cells was also examined by Nissl's staining and it was found that 6-OHDA decreased nigral cells (Cheng et al., 2009). However, the stereological assessment of TH neurons evaluated by counterstaining with Nissl would be better measure of dopaminergic neurons. Ambroxol and selegiline treatment substantially increased nigral cells. Therefore, decreased GCase activity may be responsible for cell loss in 6-OHDA group and ability of ambroxol to increase the 6-OHDA-induced decrease in GCase activity may be partly responsible for attenuating cell loss. Selegiline is previously reported to increase anti-apoptotic proteins and decrease apoptotic cells in MPTP model in mice (Liu et al., 2017; Zhao, Q. et al., 2013). However, the present study provides the evidence of selegiline-induced decrease in apoptosis after 6-OHDA toxicity.

4.5. Conclusions

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 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. Ambroxol in sub-acute doses due to its GCase-stimulating activity upregulated 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. Selegiline also showed regenerative effects as observed from the behavioral parameters, striatal DA content, mitochondrial function, GCase activity, α-synuclein concentration, number of nigral cells and apoptotic proteins in the 6-OHDA model. The current study shows anti-PD like effects of ambroxol as well as indicates the use of 6-OHDA-induced hemiparkinson's model to evaluate GCase-targeting drugs for PD management, as shown in **Figure 4.9.**

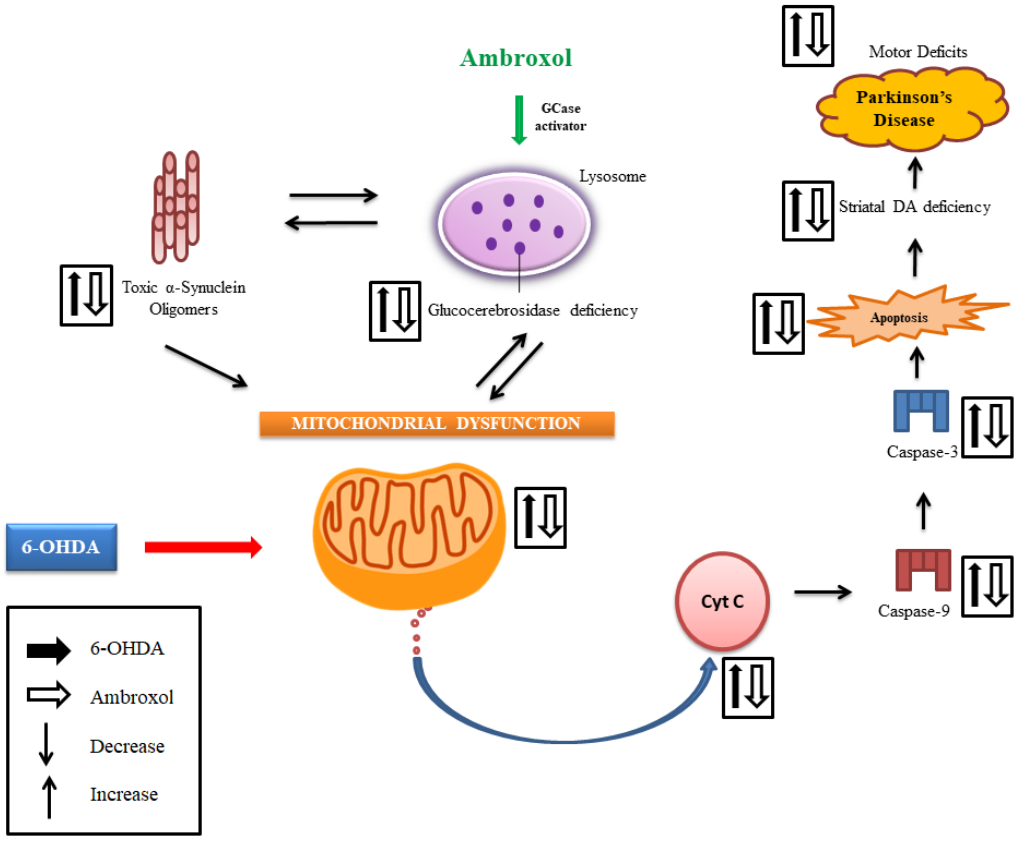

Figure 4.9 The outcome of specific objective for the validation of GCase as a target in 6-OHDA-induced model of PD in rats and the effects of sub-acute administration of ambroxol against 6-OHDA toxicity. Mitochondrial dysfunction and α-synuclein aggregation may be responsible for 6-OHDA-induced inhibition of GCase enzymatic activity. Ambroxol stimulates GCase activity, which is followed by attenuation of mitochondrial dysfunction, α-synuclein pathology, loss of nigral cells, mitochondrial-linked apoptosis and motor impairment along with the upregulation of striatal DA content against 6-OHDA mediated toxicity.