Chapter 7

To assess the sub-chronic dose of ambroxol for neurorestorative effects against 6-OHDA-induced model of PD in rats

7.1. Introduction

Disease modification may be achieved by either restraining the primary events leading to neurodegeneration, namely "neuroprotection", or upgrading the regenerative and compensatory phenomena in the related brain region, the process is defined as "neurorestoration" (Francardo et al., 2017). Currently available cure for PD is the symptomatic treatment which is achieved by increasing the concentration of DA using L-DOPA as stated earlier. L-DOPA was discovered about five decades ago and drug is prescribed to PD patients till today as DA precursor (Bourque et al., 2018; Olanow et al., 2009). However, as disease progresses, fluctuations are observed in striatal DA due to loss of buffering capacity which causes dyskineisa (Vijayakumar and Jankovic, 2016). All the marketed drugs for PD can only delay severe motor symptoms in patients and improve their overall quality of life (Pires et al., 2017). However, neurorestoration which can cure PD is still under investigation (Francardo et al., 2017).

Some compounds are being investigated for their therapeutic potential in PD due to GCase-stimulating activity (McMahon et al., 2016). Ambroxol hydrochloride is one such drug, which acts as GCase chaperone as stated earlier (Bendikov-Bar et al., 2011). It is currently undergoing phase-II clinical trials to investigate its disease-modifying property [ClinicalTrials.gov Identifier NCT02941822] and in order to repurpose this drug for PD (Lang and Espay, 2018). The other clinical trial for ambroxol is under process to establish it as a novel disease-modifying agent for the treatment of dementia and cognitive impairment in PD [ClinicalTrials.gov Identifier NCT02914366] (Silveira et al., 2019). GCase is not only decreased in brain regions of PD patients (Gegg et al., 2012), but also found to be deficient in healthy older

subjects, which makes them prone to PD. GCase enzymatic activity is reported to become comparable to PD patients by the 70^{th} years of life (Rocha et al., 2015a). GCase deficiency is involved in mitochondrial impairment (Osellame et al., 2013) and formation of oligomeric α-synuclein in rodents (Mazzulli et al., 2011). Both of these are characteristic markers of PD (Spillantini et al., 1998) and widely involved in the disease pathogenesis (Greenamyre et al., 2001). Therefore, restoration of GCase activity may have a significant role in reducing the disease progression in PD.

Chapter 4 includes the modulation of 6-OHDA-induced PD symptoms by ambroxol when its administration was initiated to rats from D-4 after 6-OHDA injection before the full development of motor deficits. Intrastriatal 6-OHDA injection takes 2-3 weeks to show maximum nigral cell loss (Sauer and Oertel, 1994). However, a critical question remains as to whether ambroxol can exert neurorestorative effects on the dopaminergic system after the disease progresses to full development of motor symptoms. This would indicate the disease-modifying property of ambroxol. Therefore, the neurorestorative properties of ambroxol were evaluated in 6-OHDA-induced hemiparkinson's rat model **(Figure 7.1)**. Treatment with ambroxol was started after the development of full motor symptoms i.e., 4 weeks after 6-OHDA intrastriatal injection. Mitochondrial complex-I activity was measured as a marker for mitochondrial function. Motor symptoms of the model were evaluated by a battery of behavioral tests. PD pathology and markers of neurorestorative treatment were evaluated by estimating TH levels, α-synuclein concentration, GCase enzymatic activity, DAT levels and Nissl's staining of substantia nigra pars compacta (SNc).

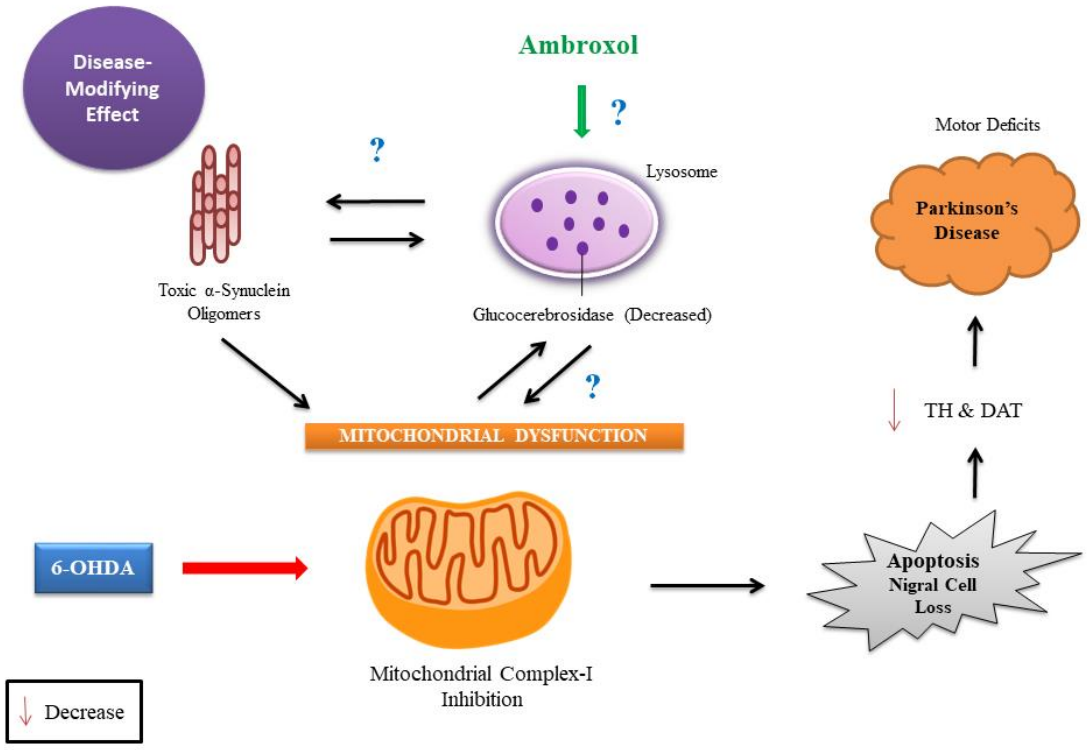

Figure 7.1 The schematic diagram of hypothesis for the assessment of the sub-chronic dose of ambroxol for neurorestorative effects against 6-OHDA-induced model of PD in rats. Ambroxol due to its GCase-stimulating activity may inhibit mitochondrial dysfunction and α-synuclein pathology, which may be followed by increase in Nissl bodies, TH, DAT and recovery of motor deficits. Ambroxol is administered after the full development of 6-OHDA induced motor deficits to observe its neurorestorative potential.

7.2. Material and Methods

7.2.1. 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.

7.2.2. Materials

Please refer Chapter 4 (page 29) and 6 (page 102) for the source of used materials.

7.2.3. Intrastriatal administration of 6-OHDA

Please refer Chapter 4 (page 30).

7.2.4. Experimental Procedure

Animals were randomly selected to make four groups, fifteen animals in each; control, sham, 6-OHDA and 6-OHDA+Ambroxol. 6-OHDA and 6-OHDA+Ambroxol groups received unilateral intrastriatal injections of 6-OHDA on D-1 as shown in Chapter 4 (page 31). Sham group was administered with stereotaxic intrastriatal injections of 0.2 mg/mL ascorbic acid-normal saline solution (vehicle of 6-OHDA; 4 µL) on D-1 (Kumar et al., 2012). Behavioral parameters were performed for animals of each group on D-0 and continued at every week up to D-70, except for the narrow beam walk test which was performed only on D-70. Ambroxol was given at the dose of 400

mg/kg *p.o.* twice daily (at every 12 h) from D-28 after the development of motor deficits. The dose was selected based on half-life (approx. 10 h) and an earlier report (Malerba and Ragnoli, 2008; Mishra et al., 2018). Ambroxol was prepared as discussed previously in Chapter 4 (page 31). The drugs were given by oral gavage up to D-70 of experimental schedule **(Figure 7.2)**. Control group was administered with the normal saline, as discussed earlier in Chapter 4 (page 31) (Mishra et al., 2018). Behavioral parameters were performed in the sequence of grip strength, rotarod, bar catalepsy, open field, apomorphine-induced head rotation and narrow beam walk test, with a 10 min interval between experiments. Behavioral assessments were conducted by investigator blind to the study protocol. Open field parameters were recorded by ANY-MAZE behavioral tracker version 4.72 (USA). Remaining of the behavioral observations was recorded with video camera by observers in a blinded manner. 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). Training sessions were conducted two times for narrow beam walk test on D-69 with cut-off time as 120s (Allbutt and Henderson, 2007; Geed et al., 2014). On D-71, three animals from each group were randomly chosen for Nissl's staining (n = 3) and the others were killed by decapitation. Brains were separated from the skull. Striatal and nigral tissues from the ipsilateral region were micro dissected on ice (Paxinos and Watson, 1998) by investigator unaware of the animals' treatment group. Tissues were stored at -80^0C till further studies. GCase enzymatic activity, α-synuclein

concentration, TH levels and mitochondrial complex-I activity were estimated in nigral tissues (n = 6). DAT levels were assessed in striatum (n = 6).

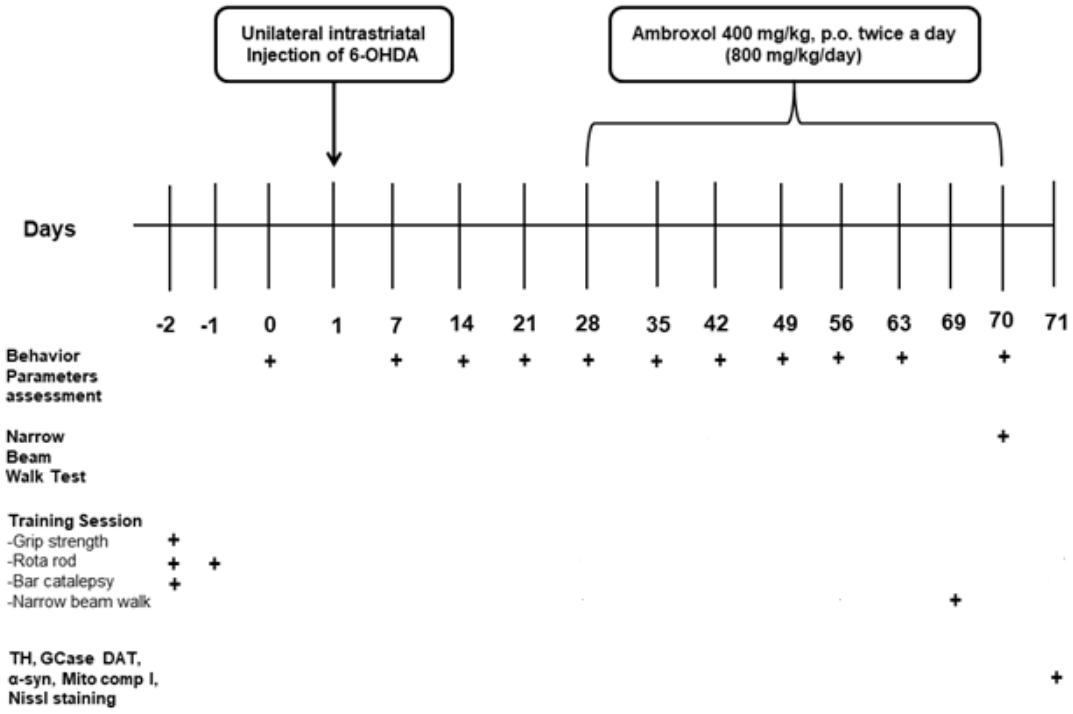

Figure 7.2 The experimental design of study for the assessment of the sub-chronic dose of ambroxol for neurorestorative effects against 6-OHDA-induced model of PD in rats. "+" indicates days on which parameters were performed, "-" indicates days on which parameters were not performed.

7.2.5. Behavioral Parameters

7.2.5.1. Open field test

Please refer Chapter 4 (page 34).

7.2.5.2. Grip strength test

Please refer Chapter 4 (page 35).

7.2.5.3. Rotarod test

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 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, 28, 35, 42, 49, 56, 63 and 70 (Rozas et al., 1997). Please refer Chapter 4 (page 34).

7.2.5.4. Apomorphine-induced rotational behavior

Single i.p. injection of apomorphine-hydrochloride at 1 mg/kg dose was administered once at every 7^{th} day starting from D-0 to D-70 in order to observe the rotational behavior in rats of all the groups (Bianchi et al., 1986). Animals were observed for basal contralateral rotations before the surgery on D-1 (Ungerstedt, 1971). Please refer Chapter 4 (page 34).

7.2.5.5. Bar Catalepsy Test

Please refer Chapter 4 (page 35).

7.2.5.6. Narrow Beam Walk Test

The apparatus consists of 120 cm long wooden beam (3 cm diameter), placed horizontally 60 cm above the flat surface. Animals were placed at one end of the beam to go towards the other edge of the beam which ends at a darkened goal box (25×20×18 cm). Akinesia was assessed by recording the time taken by animal to

initiate the movement when placed on beam, whereas total time taken by animal to cross the beam to reach at open area characterized postural instability, bradykinesia and balance (Allbutt and Henderson, 2007; Geed et al., 2014). The average of three successive trials is expressed. Once animal reached the darkened goal box, it was allowed to rest for 15 sec.

7.2.6. Measurement of TH and DAT levels

Please refer Chapter 6 (page 106) for the measurement of TH in nigral tissues and DAT in striatal tissues.

7.2.7. Estimation of GCase activity and soluble α-synuclein concentration

Please refer Chapter 4 (page 36-37) for the estimation of GCase enzymatic activity and soluble α-synuclein concentration in rat nigral tissues.

7.2.8. Measurement of mitochondrial complex-I activity

Please refer Chapter 4 (page 37) for the isolation of mitochondria from rat nigral tissues and Chapter 5 (page 72) for measurement of mitochondrial complex-I activity.

7.2.9. Nissl's staining

Please refer Chapter 4 (page 38).

7.2.10. Statistical Analysis

The results were expressed as mean ± SD. Behavior parameters were analyzed by repeated measures of two-way ANOVA, followed by Bonferroni post-hoc test, except for narrow beam walk test. This along with other parameters was analyzed by one-

way ANOVA followed by Student-Newman-Keuls post-hoc test. $p < 0.05$ was considered significant throughout the analysis.

7.3. Results

7.3.1. Behavioral Parameters

7.3.1.1. Ambroxol attenuated 6-OHDA-induced increase in rotational behavior caused by apomorphine and cataleptic behavior in rats

Repeated measures of two-way ANOVA revealed significant differences in rotational and cataleptic behavior in rats among groups ([$F (3, 616) = 1800$; $p < 0.05$], [$F (3, 616) = 1154$; $p < 0.05$] respectively), time ([$F (10, 616) = 95.57$; $p < 0.05$], [$F (10, 616) = 95.12$; $p < 0.05$] respectively) and an interaction ([$F (30, 616) = 55.82$; $p < 0.05$], [$F (30, 616) = 50.17$; $p < 0.05$] respectively) between group and time, as shown in **Table 7.1.** No significant differences were observed between control and sham groups. Both the cataleptic behavior and head rotations were increased progressively up to D-21 by 6-OHDA. The onset of action for head-rotations and catalepsy was D-7 and D-14 respectively. 6-OHDA-induced motor symptoms were maintained till D-70. Ambroxol initiated the decrease in motor deficits significantly from D-42 (head rotations) and D-49 (catalepsy). Ambroxol-induced attenuation of motor symptoms was found to be progressive.

7.3.1.2. Ambroxol inhibited 6-OHDA-induced changes in rotarod retention time and grip strength score

Statistical analysis by repeated measures of two-way ANOVA indicated that there were significant differences in rotarod retention time and grip strength scores among

groups ([F (3, 616) = 1166; $p < 0.05$], [F (3, 616) = 1333; $p < 0.05$] respectively), time ([F (10, 616) = 60.59; $p < 0.05$], [F (10, 616) = 55.14; $p < 0.05$] respectively) and an interaction between group and time ([F (30, 616) = 32.87; $p < 0.05$], [F (30, 616) = 28.42; $p < 0.05$] respectively) as shown in **Table 7.1.** Control and sham groups were not found to be significantly different in both the parameters. The decrease caused by 6-OHDA in both the rotarod retention time and grip strength score was found to be maximum on D-7. Ambroxol significantly increased both the rotarod retention time and grip strength score in 6-OHDA-infused rats in a progressive manner from D-42.

7.3.1.3. Ambroxol attenuated 6-OHDA-induced decrease in number of central squares crossed, ambulation, grooming and rearing in open field test

Repeated measures of two-way ANOVA showed that there were significant differences in number of central squares crossed, ambulation, grooming and rearing behavior among groups ([F (3, 616) = 675.5; $p < 0.05$], [F (3, 616) = 2065; $p < 0.05$], [F (3, 616) = 1161; $p < 0.05$], [F (3, 616) = 1441; $p < 0.05$] respectively), time ([F (10, 616) = 50.58; $p < 0.05$], [F (10, 616) = 84.03; $p < 0.05$], [F (10, 616) = 58.58; $p < 0.05$], [F (10, 616) = 58.36; $p < 0.05$] respectively) and an interaction ([F (30, 616) = 21.87; $p < 0.05$], [F (30, 616) = 47.27; $p < 0.05$], [F (30, 616) = 27.02; $p < 0.05$], [F (30, 616) = 32.18; $p < 0.05$] respectively) between group and time in open field test **(Table 7.2)**. Maximum decrease by 6-OHDA in the parameters of open field test was observed on D-7, except for the number of central squares crossed which was significantly decreased from D-14. Ambroxol progressively increased the number of central squares crossed and rearing from D-49, and the remaining parameters of open

field test from D-42 significantly against 6-OHDA-infused rats. No significant differences were observed between the parameters of control and sham groups.

7.3.1.4. Ambroxol inhibited 6-OHDA-induced changes in beam performance in narrow beam walk test

One way ANOVA showed significant differences among groups in the latency to initiate the task [$F(3, 56) = 74.54$; $p < 0.05$] and total time taken to cross the beam [$F(3, 56) = 40.96$; $p < 0.05$] as shown in **Figure 7.3**. Both the parameters including latency to start the movement by animal when placed on beam and total time taken by animals to cross the beam were found to be significantly increased in 6-OHDA-infused rats compared to sham group. Ambroxol significantly decreased the latency to initiate the task as well as total time taken to cross the beam compared to 6-OHDA-infused rats. Control and sham groups were not found to be significantly different for both the parameters.

7.3.2. Ambroxol has Restorative Effects on 6-OHDA-induced reduction in nigral TH levels

One way ANOVA showed significant differences among groups in the levels of TH [$F(3, 20) = 73.27$; $p < 0.05$] in nigral tissues as shown in **Figure 7.4.** No significant differences were observed in TH levels between control and sham groups. 6-OHDA significantly decreased TH levels (72%) in ipsilateral nigral tissues of rats compared to sham group. Ambroxol significantly increased TH levels compared to 6-OHDA group.

Table 7.1 Ambroxol-induced recovery of motor functions as assessed by apomorphine-induced rotations, cataleptic behavior, grip strength score and rotarod retention time against 6-OHDA-induced motor deficits in rats

Groups	Apomorphine-induced rotations (Counts/5 min)	Cataleptic Behavior (Sec)	Grip Strength Score	Retention Time In Rotarod Test (Sec)
DAY 0				
Control	4.78 ± 0.36	1.74 ± 0.38	4.38 ± 0.92	180.08 ± 12.97
Sham	4.42 ± 0.78	1.79 ± 0.36	4.30 ± 0.51	180.68 ± 12.43
6-OHDA	4.74 ± 0.55	1.57 ± 0.41	4.26 ± 0.43	180.28 ± 17.55
6-OHDA+Ambroxol	5.05 ± 0.92	1.62 ± 0.39	4.18 ± 0.83	180.33 ± 14.67
DAY 7				
Control	5.06 ± 0.50	1.58 ± 0.13	4.23 ± 0.76	181.68 ± 9.27
Sham	4.41 ± 0.34	1.82 ± 0.34	4.10 ± 0.53	166.58 ± 7.47
6-OHDA	8.19 ± 0.94[a]	1.90 ± 0.19	1.07 ± 0.19[a]	87.09 ± 27.23[a]
6-OHDA+Ambroxol	8.53 ± 0.80[a]	1.84 ± 0.23	1.05 ± 0.20[a]	85.69 ± 22.92[a]
DAY 14				
Control	4.86 ± 0.65	1.66 ± 0.52	4.12 ± 0.35	179.68 ± 9.84
Sham	4.75 ± 0.40	1.54 ± 0.35	4.09 ± 0.68	167.18 ± 8.69
6-OHDA	11.09 ± 1.77[a]	3.92 ± 0.46[a]	1.04 ± 0.22[a]	79.21 ± 17.03[a]
6-OHDA+Ambroxol	11.51 ± 1.04[a]	3.83 ± 0.28[a]	1.03 ± 0.09[a]	78.48 ± 11.28[a]
DAY 21				
Control	4.80 ± 0.49	1.58 ± 0.48	4.11 ± 0.76	179.08 ± 13.75
Sham	4.68 ± 0.71	1.88 ± 0.44	4.12 ± 0.55	167.38 ± 12.19
6-OHDA	13.95 ± 2.07[a]	5.97 ± 0.94[a]	1.19 ± 0.24[a]	92.83 ± 21.72[a]
6-OHDA+Ambroxol	14.11 ± 1.04[a]	5.66 ± 0.70[a]	1.20 ± 0.25[a]	79.58 ± 10.69[a]
DAY 28				
Control	5.14 ± 0.49	1.54 ± 0.38	4.19 ± 0.57	180.28 ± 13.48
Sham	4.75 ± 0.60	1.84 ± 0.39	4.37 ± 0.63	171.78 ± 13.13
6-OHDA	14.01 ± 1.17[a]	5.89 ± 0.61[a]	1.12 ± 0.19[a]	86.47 ± 21.48[a]
6-OHDA+Ambroxol	14.69 ± 1.26[a]	6.04 ± 1.09[a]	1.15 ± 0.24[a]	83.24 ± 10.48[a]
DAY 35				
Control	4.81 ± 0.47	1.67 ± 0.54	4.41 ± 0.52	183.26 ± 12.58
Sham	4.49 ± 0.65	1.83 ± 0.32	4.37 ± 0.48	178.53 ± 16.58
6-OHDA	14.33 ± 1.26[a]	6.01 ± 1.18[a]	1.07 ± 0.21[a]	92.07 ± 13.93[a]
6-OHDA+Ambroxol	13.52 ± 1.90[a]	5.72 ± 0.87[a]	1.40 ± 0.42[a]	103.14 ± 23.37[a]
DAY 42				
Control	5.04 ± 0.51	1.72 ± 0.34	4.17 ± 0.75	181.01 ± 9.24
Sham	4.70 ± 0.35	1.57 ± 0.44	4.38 ± 0.61	171.97 ± 15.39
6-OHDA	14.42 ± 2.27[a]	6.27 ± 0.96[a]	0.98 ± 0.21[a]	87.91 ± 16.41[a]
6-OHDA+Ambroxol	12.95 ± 1.86[a,b]	5.84 ± 0.84[a]	1.59 ± 0.56[a,b]	119.67 ± 17.38[a,b]
DAY 49				
Control	5.21 ± 0.60	1.81 ± 0.67	4.16 ± 0.67	183.26 ± 15.00
Sham	4.76 ± 0.45	1.78 ± 0.39	4.41 ± 0.64	180.31 ± 12.65
6-OHDA	14.51 ± 2.05[a]	6.28 ± 1.43[a]	0.98 ± 0.34[a]	86.54 ± 19.27[a]
6-OHDA+Ambroxol	11.57 ± 1.85[a,b]	5.39 ± 0.83[a,b]	1.61 ± 0.66[a,b]	138.60 ± 21.43[a,b]
DAY 56				
Control	4.87 ± 0.74	1.77 ± 0.71	4.42 ± 0.39	182.16 ± 14.16
Sham	4.43 ± 0.49	1.79 ± 0.44	4.36 ± 0.23	179.23 ± 11.46
6-OHDA	14.46 ± 1.94[a]	6.29 ± 1.27[a]	0.96 ± 0.42[a]	82.36 ± 13.41[a]
6-OHDA+Ambroxol	8.01 ± 0.86[a,b]	3.88 ± 0.54[a,b]	2.48 ± 0.61[a,b]	160.68± 14.86[a,b]
DAY 63				
Control	4.81 ± 0.45	1.80 ± 0.30	4.09 ± 0.50	179.71 ± 13.64
Sham	4.90 ± 0.72	1.62 ± 0.26	4.16 ± 0.42	177.33 ± 14.68
6-OHDA	14.47 ± 2.02[a]	6.29 ± 0.91[a]	0.94 ± 0.37[a]	80.05 ± 17.46[a]
6-OHDA+Ambroxol	5.51 ± 1.15[b]	2.09 ± 0.37[b]	3.79 ± 0.57[b]	171.45 ± 20.37[b]
DAY 70				
Control	5.00 ± 0.81	1.67 ± 0.36	4.40 ± 0.72	179.45 ± 9.47
Sham	4.70 ± 0.58	1.78 ± 0.54	4.26 ± 0.65	176.89 ± 11.44
6-OHDA	14.39 ± 2.74[a]	6.28 ± 0.95[a]	0.96 ± 0.53[a]	79.93 ± 8.46[a]
6-OHDA+Ambroxol	5.18 ± 0.87[b]	1.62 ± 0.58[b]	4.02 ± 0.71[b]	173.02 ± 23.38[b]

Ambroxol-administration was initiated from D-28 and continued up to D-70. All values are mean ± SD; n = 15; [a]$p < 0.05$ compared to sham and [b]$p < 0.05$ compared to 6-OHDA [Repeated measures of two-way ANOVA followed by Bonferroni test].

Table 7.2 Ambroxol-induced recovery of motor functions as assessed by the number of central squares crossed, ambulation, rearing and grooming in open field test against 6-OHDA-induced motor deficits in rats

Groups	Central Squares crossed (numbers)	Ambulation (numbers)	Rearing (numbers)	Grooming (numbers)
DAY 0				
Control	4.35 ± 0.65	44.29 ± 7.21	14.03 ± 1.95	6.51 ± 1.10
Sham	4.41 ± 0.57	44.04 ± 5.37	13.37 ± 1.74	6.39 ± 0.92
6-OHDA	4.46 ± 0.50	46.02 ± 6.78	14.05 ± 1.34	6.48 ± 0.84
6-OHDA+Ambroxol	4.42 ± 0.42	45.80 ± 5.45	14.14 ± 1.80	6.32 ± 1.08
DAY 7				
Control	4.46 ± 0.59	44.83 ± 5.50	13.90 ± 1.75	6.51 ± 0.93
Sham	4.37 ± 0.77	43.87 ± 6.38	13.52 ± 1.93	6.04 ± 1.20
6-OHDA	4.30 ± 0.80	6.09 ± 1.22[a]	4.94 ± 0.84[a]	3.01± 0.53[a]
6-OHDA+Ambroxol	4.55 ± 0.73	7.17 ± 1.10[a]	5.02 ± 1.02[a]	3.23 ± 0.49[a]
DAY 14				
Control	4.42 ± 0.66	45.09 ± 7.24	14.04 ± 1.78	6.21 ± 0.80
Sham	4.48 ± 0.53	43.72 ± 6.06	13.34 ± 1.52	6.07 ± 1.08
6-OHDA	1.90 ± 0.21[a]	5.86 ± 1.14[a]	4.92 ± 0.73[a]	2.62 ± 0.32[a]
6-OHDA+Ambroxol	1.88 ± 0.14[a]	7.88 ± 1.44[a]	4.86 ± 0.75[a]	2.81 ± 0.28[a]
DAY 21				
Control	4.33 ± 0.60	45.51 ± 8.87	13.63 ± 1.65	6.31 ± 1.05
Sham	4.37 ± 0.49	44.35 ± 6.41	13.38 ± 1.82	6.08 ± 0.79
6-OHDA	1.88 ± 0.17[a]	6.40 ± 1.60[a]	4.75 ± 0.57[a]	2.66± 0.15[a]
6-OHDA+Ambroxol	1.80 ± 0.19[a]	6.82 ± 1.50[a]	5.19 ± 0.72[a]	2.74 ± 0.20[a]
DAY 28				
Control	4.41 ± 0.80	43.96 ± 6.60	13.74 ± 1.63	6.28 ± 1.18
Sham	4.34 ± 0.61	43.51 ± 6.19	14.00 ± 1.86	6.07 ± 1.22
6-OHDA	1.75 ± 0.25[a]	6.01 ± 2.76[a]	4.45 ± 0.74[a]	2.52 ± 0.32[a]
6-OHDA+Ambroxol	1.82 ± 0.21[a]	7.98 ± 0.92[a]	5.02 ± 0.87[a]	2.71 ± 0.24[a]
DAY 35				
Control	4.41 ± 0.59	44.25 ± 6.34	14.26 ± 1.78	6.17 ± 0.87
Sham	4.47 ± 0.72	45.13 ± 5.01	13.52 ± 1.35	6.30 ± 0.94
6-OHDA	1.70 ± 0.43[a]	5.08 ± 2.46[a]	4.46 ± 0.85[a]	2.84± 1.38[a]
6-OHDA+Ambroxol	1.91 ± 0.69[a]	6.99 ± 1.89[a]	5.08 ± 1.47[a]	3.44 ± 1.04[a]
DAY 42				
Control	4.33 ± 0.52	44.64 ± 6.57	13.85 ± 1.67	6.36 ± 1.03
Sham	4.48 ± 0.73	44.94 ± 6.81	13.54 ± 1.85	6.22 ± 0.80
6-OHDA	1.72 ± 0.37[a]	4.80 ± 2.54[a]	4.41 ± 0.68[a]	2.64 ± 1.37[a]
6-OHDA+Ambroxol	1.88 ± 0.19[a]	10.86 ± 2.47[a,b]	5.14 ± 0.85[a]	3.66 ± 0.46[a,b]
DAY 49				
Control	4.31 ± 0.68	45.57 ± 6.84	14.26 ± 1.77	6.19 ± 0.90
Sham	4.29 ± 0.53	43.65 ± 4.22	13.55 ± 1.95	6.07 ± 1.24
6-OHDA	1.69 ± 0.50[a]	4.85 ± 2.77[a]	4.26 ± 0.62[a]	2.67 ± 0.80[a]
6-OHDA+Ambroxol	2.37 ± 0.51[a,b]	21.89 ± 3.71[a,b]	7.84 ± 0.85[a,b]	4.09 ± 0.65[a,b]
DAY 56				
Control	4.43 ± 0.71	44.05 ± 5.33	14.07 ± 1.89	6.46 ± 1.29
Sham	4.47 ± 0.55	45.09 ± 7.04	13.72 ± 1.63	6.18 ± 1.03
6-OHDA	1.70 ± 0.31[a]	4.66 ± 2.48[a]	4.30 ± 0.63[a]	2.55 ± 1.27[a]
6-OHDA+Ambroxol	2.46 ± 0.20[a,b]	28.74 ± 2.50[a,b]	9.48 ± 1.25[a,b]	4.93 ± 0.53[a,b]
DAY 63				
Control	4.48 ± 0.82	44.55 ± 5.42	13.63 ± 2.08	6.26 ± 1.12
Sham	4.30 ± 0.49	44.93 ± 7.34	13.96 ± 1.75	6.09 ± 1.05
6-OHDA	1.68 ± 0.41[a]	4.64 ± 2.71[a]	4.31 ± 0.85[a]	2.64 ± 1.46[a]
6-OHDA+Ambroxol	3.02 ± 0.52[a,b]	33.84 ± 4.11[a,b]	10.83 ± 1.51[a,b]	5.83 ± 0.61[b]
DAY 70				
Control	4.42 ± 0.73	43.95 ± 6.53	13.56 ± 1.58	6.48 ± 0.97
Sham	4.44 ± 0.89	44.30 ± 6.77	13.89 ± 1.67	6.20 ± 1.08
6-OHDA	1.67 ± 0.30[a]	4.66 ± 2.82[a]	4.28 ± 1.16[a]	2.76 ± 1.35[a]
6-OHDA+Ambroxol	4.18 ± 0.48[b]	42.40 ± 5.25[b]	13.46 ± 1.25[b]	6.27 ± 0.52[b]

Ambroxol-administration was initiated from D-28 and continued up to D-70. All values are mean ± SD; n = 15; [a]$p < 0.05$ compared to sham and [b]$p < 0.05$ compared to 6-OHDA [Repeated measures of two-way ANOVA followed by Bonferroni test].

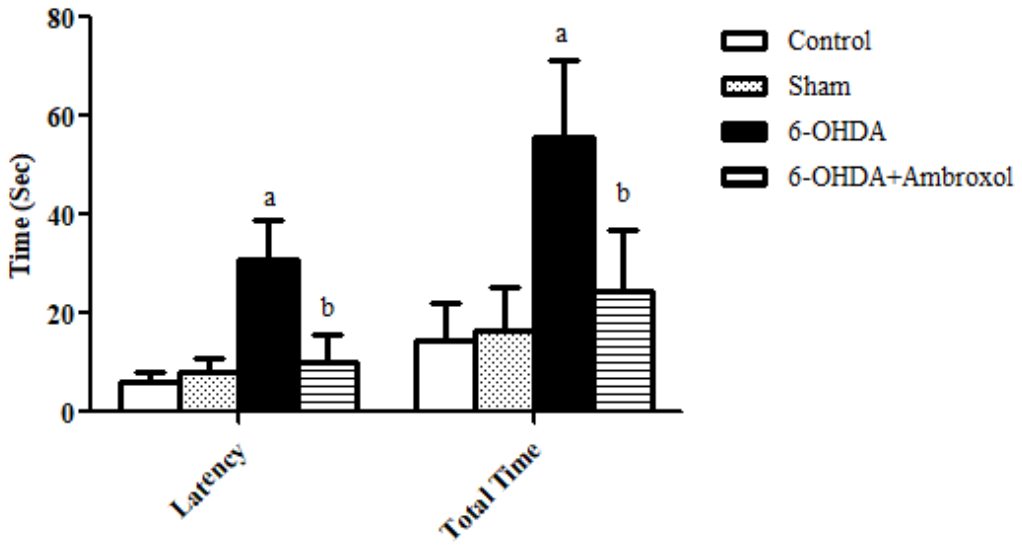

Figure 7.3 Effect of ambroxol on 6-OHDA-induced alterations in beam performance as assessed by latency to begin the task and total time taken to cross the beam in narrow beam walk test in rats. All values are mean ± SD; n = 15; [a]p < 0.05 compared to sham and [b]p < 0.05 compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

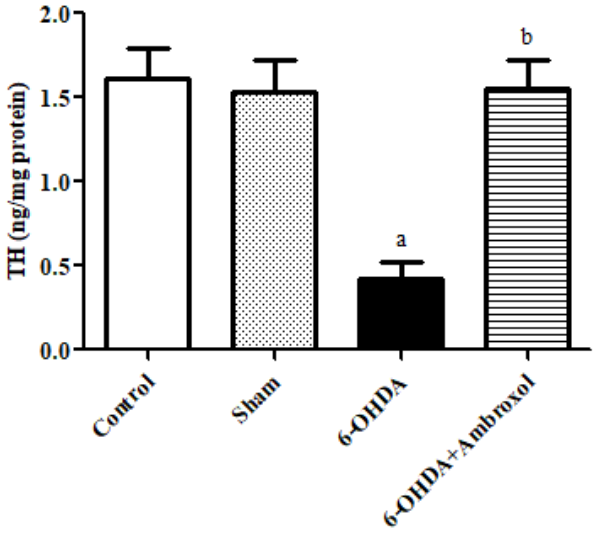

Figure 7.4 Effect of ambroxol on 6-OHDA-induced changes in the TH levels in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; [a]p < 0.05 compared to sham and [b]p < 0.05 compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

7.3.3. Ambroxol attenuated 6-OHDA-induced changes in mitochondrial complex-I, GCase enzymatic activity and soluble α-synuclein concentration in rat nigral tissues

One-way ANOVA showed significant differences in mitochondrial complex-I activity [$F(3, 20) = 61.17$; $p < 0.05$], GCase enzymatic activity [$F(3, 20) = 25.75$; $p < 0.05$] and soluble α-synuclein concentration [$F(3, 20) = 16.91$; $p < 0.05$] among groups as depicted in **Figure 7.5** and **Figure 7.6**. There was no significant difference observed in soluble α-synuclein concentration, mitochondrial complex-I and GCase enzymatic activity between control and sham groups. 6-OHDA significantly decreased mitochondrial complex-I (65%), GCase enzymatic activity (64%) and soluble α-synuclein concentration (70%) in ipsilateral nigral tissues of rats compared to sham group. Ambroxol significantly increased mitochondrial complex-I and GCase enzymatic activities up to 56% and soluble α-synuclein concentration up to 55% compared to 6-OHDA-infused rats.

7.3.4. Ambroxol has Restorative Effects on 6-OHDA-induced decrease in DAT levels in rat striatal tissues

One way ANOVA showed significant differences among groups in the levels of DAT [$F(3, 20) = 25.30$; $p < 0.05$] in striatal tissues as shown in **Figure 7.7.** 6-OHDA significantly decreased DAT levels up to 36% in ipsilateral striatal tissues of rats compared to sham group. Striatal DAT levels were increased by ambroxol compared to 6-OHDA-infused rats. No significant difference in DAT levels was observed between control and sham groups.

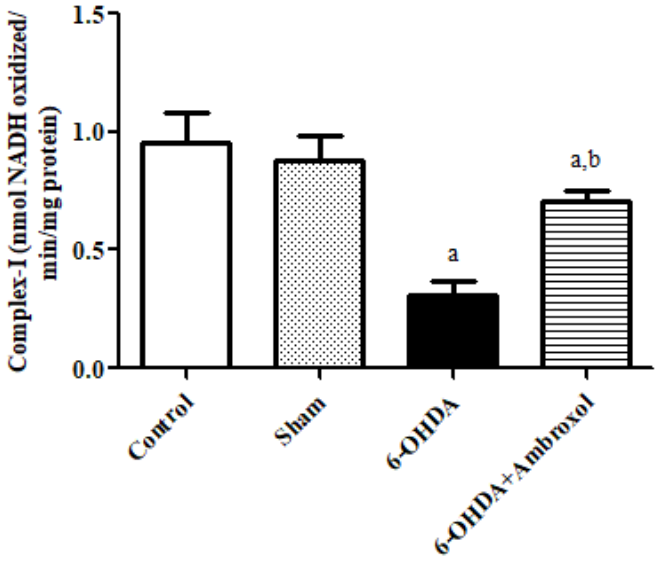

Figure 7.5 Effect of ambroxol on 6-OHDA-induced alterations in mitochondrial complex-I in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

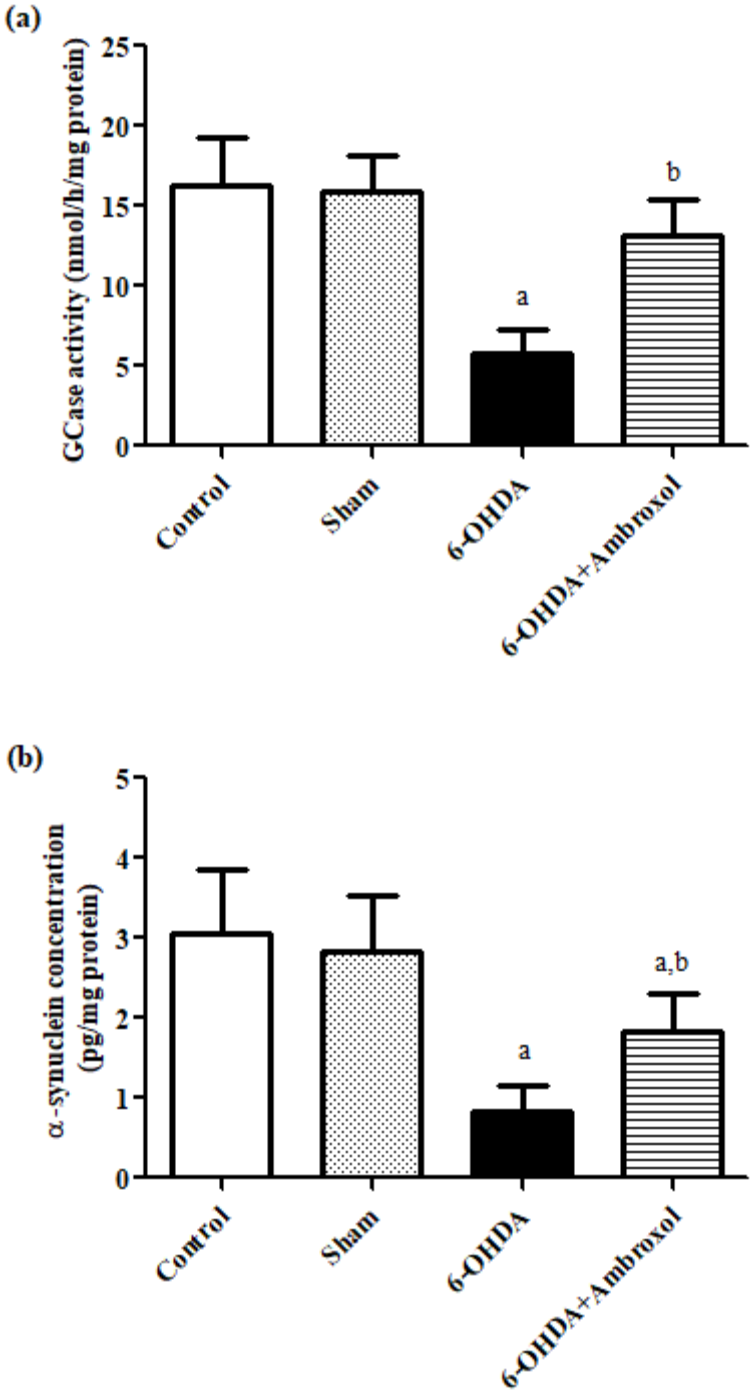

Figure 7.6 Effect of ambroxol on 6-OHDA-induced alterations in GCase enzymatic activity (a) and α-synuclein protein concentration (b) in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

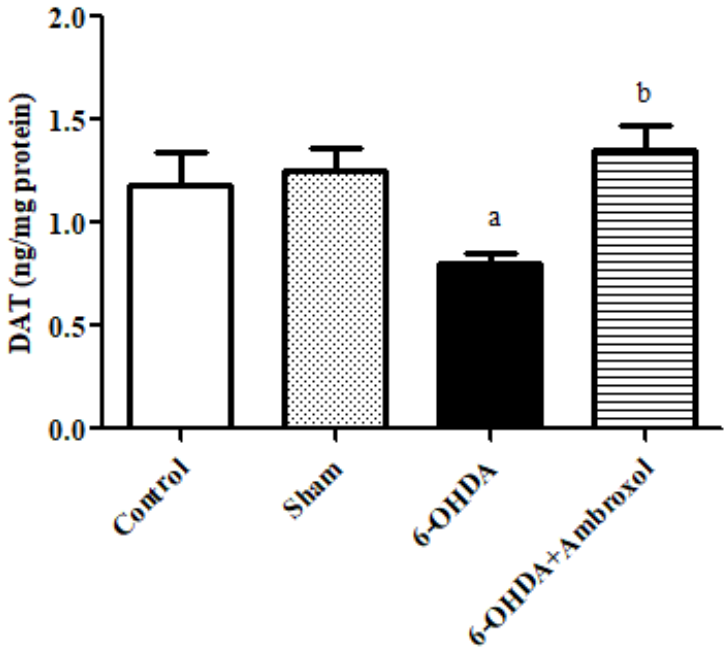

Figure 7.7 Effect of Ambroxol on 6-OHDA-induced changes in the DAT levels in ipsilateral striatal tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

7.3.5. Ambroxol has Restorative Effects on 6-OHDA-induced nigral cell loss

Significant differences in the percentages of Nissl bodies were observed in nigral tissues among groups [$F(3, 8) = 30.73$; $p < 0.05$] by using one-way ANOVA. The numbers of Nissl-positive cells were significantly decreased in nigral tissues of 6-OHDA-infused rats compared to control and sham groups **(Figure 7.8)**. Ambroxol significantly increased the number of Nissl-positive cells compared to 6-OHDA-infused rats. No significant difference was found in number of Nissl-positive cells between control and sham groups.

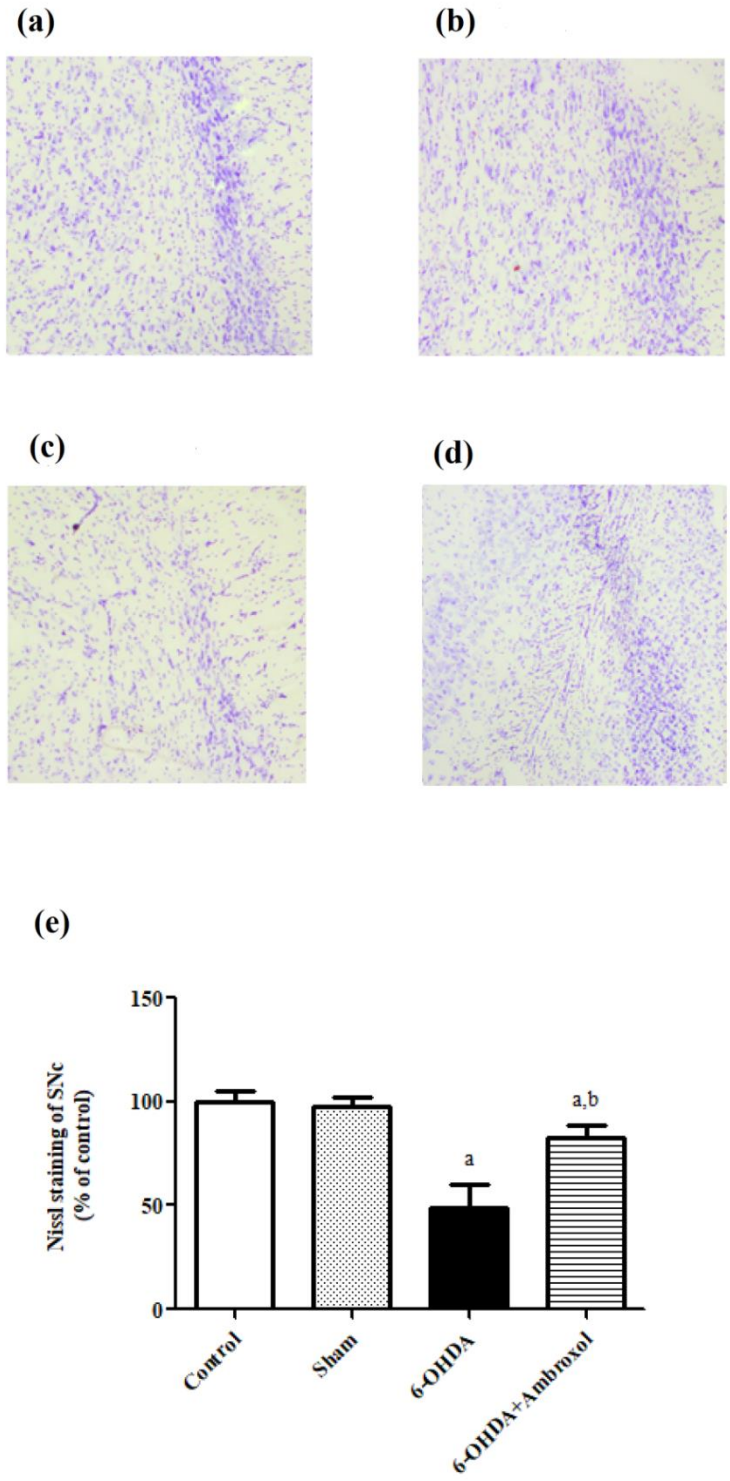

Figure 7.8 Nissl's staining of SNc in rats. Control (a); Sham (b); 6-OHDA (c); 6-OHDA+Ambroxol (d); Data of counting cells (e). All values are mean ± SD; n = 3; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

7.4. Discussion

Ambroxol showed neurorestorative potential on the dopaminergic system of nigrostriatal pathway, after 4 weeks of 6-OHDA intrastriatal injection. This is the period when nigral cell loss was found to be maximum (Sauer and Oertel, 1994) and motor deficits were fully developed (Coulombe et al., 2016; Lindholm et al., 2007; Voutilainen et al., 2017). Ambroxol restored the dopaminergic deficits, as observed by restoration of TH and DAT levels and normalization of Nissl bodies. Additionally, GCase enzymatic activity, mitochondrial complex-I and α-synuclein – like factors which take part in pathogenesis of PD, were also counterbalanced and behavioral function was recovered by ambroxol.

Motor deficits dominate the clinical picture of PD (Stern et al., 1983). 6-OHDA caused behavioral deficits in rats up to D-70 of experimental protocol as reported previously (Coulombe et al., 2016; Lindholm et al., 2007; Voutilainen et al., 2017). Ambroxol recovered some of the motor symptoms earlier at D-42 whereas the other motor deficits were recovered later at D-49. There was recovery of retention time in the rotarod test from D-42, which characterizes gross motor coordination (Qian et al., 2010; Rozas et al., 1997). The earlier recovery is probably due to the fact that gross motor skills recover earlier than fine motor skills. In humans too fine motor skills takes more time to develop than gross motor skills (Hamel and Pelphrey, 2009). However, some of the fine motor skills such as grip strength (Pradhan et al., 2010), grooming and ambulation parameters of open field behavior (Qian et al., 2010) were also recovered by ambroxol earlier (D-42), compared to other fine motor skills including cataleptic behavior, number of central squares crossed and rearing which were recovered by ambroxol from D-49. The underlying reason behind the late onset

of action of ambroxol in these fine motor skills may be due to the involvement of exploratory behavior. The entry in central squares indicates wider exploration of open field by the animal (Lamprea et al., 2003). Rearing is also considered to be exploratory activity (Coronel-Oliveros and Pacheco-Calderón, 2018). Bar catalepsy test indicates akinesia which involves fine motor control (Walther and Strik, 2012; Whishaw et al., 1990). Therefore, ambroxol took more time to recover fine motor movements involving wider exploration ability and akinesia. Ambroxol recovered grip strength and apomorphine-induced rotational behavior earlier (D-42). This may be due to the fact that grip strength test involves primary motor cortex (Ismail et al., 2014), located in the frontal lobe (Dum and Strick, 2002) which contains most of the DA-sensitive neurons in cerebral cortex (Emson and Koob, 1978). Apomorphine, a DA agonist leads to direct stimulation of DA receptors causing rotations contralateral to DA-depleted hemisphere, thereby characterizing DA depletion (Ungerstedt, 1971). Therefore, ambroxol potentially recoverd DA neurons sufficiently enough to affect grip strength and rotational behavior earlier.

Neuroprotective and neurotrophic effects of apomorphine (10 mg/kg/day) were observed when it was administered for 11 days daily in the striatal 6-OHDA-lesion rat model of PD (Yuan et al., 2004). However, in the current study, apomorphine (half-life ~10 min) (Bianchi et al., 1986) was administered to rats at a very low dose (1 mg/kg) once every week which may not produce any pharmacological activity. Moreover, sham and 6-OHDA groups did not show any pharmacological effects indicating apomorphine may not be factor to influence treatment outcomes. Ambroxol-induced recovery of motor movements was increased temporally and motor symptoms were found to recover fully till the last day of

experimental protocol. Narrow beam walk test was used to assess some of the most relevant PD symptoms such as, bradykinesia, akinesia and postural instability (Allbutt and Henderson, 2007). Ambroxol decreased 6-OHDA-induced aggravation of these symptoms, indicating the effectiveness of ambroxol in restoring motor functions.

Degeneration of nigrostriatal DA neurons is closely associated with the motor symptoms of PD (Bernheimer et al., 1973). Biosynthesis of DA consists of the conversion of tyrosine to L-DOPA and this rate-limiting step is catalyzed by the enzyme TH. DA depletion in PD is the direct downstream event of TH reduction (Haavik and Toska, 1998), therefore, TH is considered as dopaminergic cell marker in neurorestorative studies (Voutilainen et al., 2017). In the present study, 6-OHDA decreased TH levels in ipsilateral nigral tissues on D-70 as reported earlier (Lindholm et al., 2007; Voutilainen et al., 2017). Apart from being directly involved in DA synthesis and thereby motor behavior (Joshua et al., 2009), TH also takes part in pathogenesis of PD at several other levels, including the generation of ROS and thereby it is the target for radical-mediated oxidative injury (Haavik and Toska, 1998). Therefore, drugs modulating TH levels may be promising candidates among treatment strategies for PD. Ambroxol-induced increase in the TH levels in SNc against 6-OHDA toxicity suggests the restoration of TH-expressing dopaminergic cells. This indicates that ambroxol has potential to affect PD pathogenesis at several levels, and thereby target the disease progression. However to further clarify the effect of drug, TH positive neurons in substantia nigra could have been estimated by additional techniques. Therefore, the further experiments are required to precisely validate the increase in TH with ambroxol. TH is previously reported to be increased

by ambroxol in the posterior brain regions of transgenic flies expressing mutant GCase (Maor et al., 2016).

Release of neuronal DA from SNc to striatum forms extracellular DA concentration, which is reported to be decreased by 6-OHDA (Robinson and Whishaw, 1988). DAT is the most important determinant of extracellular DA concentration (Nutt et al., 2004) and is expressed abundantly in nigrostriatal DA neurons (Shimada et al., 1992). DAT reduction is found to be 50-70% in severe PD cases (Nutt et al., 2004). In present study, striatal DAT levels were decreased following ten weeks of unilateral intrastriatal injection of 6-OHDA as reported previously (Coulombe et al., 2016; Voutilainen et al., 2017). DAT is specific for DA neurons and has been used as an index of DA cell viability in various studies (Gainetdinov et al., 1998). In case of surviving dopaminergic neurons, an overall reduction was found in the intensity of DAT mRNA expression in the brains of PD patients (Counihan and Penney, 1998). This suggests the importance of DAT levels in improving the condition of surviving dopaminergic neurons. Ambroxol restored DAT levels in the ipsilateral striatum indicating the improved health of existing dopaminergic neurons. DAT restoration can augment dopaminergic transmission and decrease DA turnover in the nigrostriatal neurons leading to better functional output (Gainetdinov et al., 1998; Perez-Pardo et al., 2017). Increase in functional capability of existing dopaminergic neurons with ambroxol treatment may underlie the currently observed improvement in behavioral deficits.

GCase enzymatic deficiency, α-synuclein oligomeric aggregation, and mitochondrial impairment are some of the important factors involved in PD pathogenesis (Di Maio et al., 2016; Rocha et al., 2015a), which are inter-connected to

each-other and observed in PD patients (Rocha et al., 2015a; Schapira et al., 1990; Yap et al., 2011). GCase activating action of ambroxol is reported to be the underlying mechanism in attenuating 6-OHDA toxicity in Chapter 4 (Mishra et al., 2018). Therefore, in the current study also restoration of GCase activity by ambroxol may have a significant role in reducing the disease progression against 6-OHDA-induced toxicity. Ambroxol due to its role as GCase chaperone would be one of the first disease-modifying agents for the treatment of PD dementia on successful completion of its ongoing clinical trial (Silveira et al., 2019). In the present study also, GCase activity was restored by the administration of ambroxol against 6-OHDA toxicity. GCase deficiency leads to mitochondrial dysfunction both *in vivo* and *in vitro* (Cleeter et al., 2013; Osellame et al., 2013) as clearance capacity of lysosome get disturbed leading to accumulation of fragmented and dysfunctional mitochondria with impaired respiratory chain (Cleeter et al., 2013; Dehay et al., 2013). In the present study, ambroxol attenuated 6-OHDA-induced impairment in mitochondrial complex-I activity and there may be possible involvement of GCase activation by ambroxol in the regulation of mitochondrial complex-enzyme activity. Healthy mitochondria are necessary to regulate energy in the form of ATP production for neuronal development (Son and Han, 2018). Therefore, ambroxol-induced restoration of GCase activity and thereby mitochondrial complex enzyme activity indicates the improved health of existing neurons in nigral tissues, which leads to improve motor behavior in rats.

Ambroxol is previously reported to decrease aggregates of α-synuclein toxic oligomers and increase GCase activity in the treatment protocol of 6-OHDA-induced hemiparkinson's model, as discussed in Chapter 4 (Mishra et al., 2018) and in mice

overexpressing human α-synuclein (Migdalska-Richards et al., 2016). α-synuclein aggregates are neurotoxic and characteristic markers of PD, which reflects pathological inclusion (Li et al., 2019; Spillantini et al., 1998). Decreased water-soluble concentration of α-synuclein in the nigral region due to 6-OHDA observed in the present study indicates the accumulation of toxic α-synuclein oligomers as discussed in Chapter 4 and 5 (Budi et al., 2012; Gu et al., 2016; Mishra et al., 2018). α -synuclein oligomeric aggregates and GCase negatively affects each other as discussed in previous chapters (Mazzulli et al., 2011; Yap et al., 2011). Therefore, in the current study, inhibition of oligomeric aggregates of α-synuclein by ambroxol as shown by increase in soluble α-synuclein concentration may be due to its GCase-stimulating activity. An additional technique, such as co-staining with anti-synuclein antibody to observe the effect on pathological inclusions will be important. This would further confirm the increase of synuclein level in the model, as well as its subsequent reduction following a drug treatment. However, this is the limitation of the present study. Presynaptic terminals are enriched with α-synuclein, which regulates presynaptic function (Chandra et al., 2004; Fortin et al., 2005) and maintains the integrity of presynaptic terminals (Bonini and Giasson, 2005). Therefore, ambroxol induced restoration of soluble α-synuclein reflects the reduction of pathologic inclusion in the surviving dopaminergic neurons of nigral region due to restoration of terminal integrity.

Abnormal accumulation of α-synuclein is reported to cause reduction in mitochondrial protein import as negative downstream effect (Di Maio et al., 2016). Thus, both GCase deficiency and α-synuclein pathology caused by 6-OHDA may be responsible for mitochondrial impairment. Mitochondrial dysfunction is the

significant upstream event of apoptosis (Elmore, 2007). Dopaminergic neuronal cell bodies of SNc are destroyed due to retrograde degeneration caused by intrastriatal injection of 6-OHDA in rats (Berger et al., 1991). Dopaminergic nigrostriatal pathway consists of dopaminergic cell bodies-containing SNc and axons-containing striatum (Dauer and Przedborski, 2003). Nissl substance, a large granular body found in the neuron, denotes rough ER with rosettes of free ribosome. Its primary function includes protein synthesis (Byrne et al., 2014). Nissl's staining in SNc indicates the density of dopaminergic neurons (Domesick et al., 1983; Zaitone et al., 2012); therefore, ambroxol-induced normalization of Nissl bodies against 6-OHDA toxicity in the present study indicates the rescue of existing dopaminergic neurons in SNc tissues. This may be due to improved protein synthesis in existing dopaminergic neurons as well as restored terminal integrity as indicated by increased concentration of soluble α synuclein. TH neurons may further be stereologically assessed by counterstaining with Nissl. In the present study, both the GCase-stimulating and α-synuclein pathology-diminishing effects of ambroxol may be responsible for restoration of mitochondrial function followed by dopaminergic cells and motor behavior, therefore may act as significant mechanisms for disease-modifying potential of ambroxol.

7.5. Conclusions

The present study demonstrated for the first time the neurorestorative potential of ambroxol in 6-OHDA-induced hemiparkinson's model in rats. TH and DAT are markers for dopaminergic cells and extracellular DA concentration respectively. Restoration of these markers and normalization of Nissl bodies by ambroxol in 6-

OHDA-infused rats indicates the improved health of existing dopaminergic neurons. Ambroxol was administered after the full development of motor deficits (from D-28 of 6-OHDA intrastriatal administration) and continued up to D-70. Then also, it was able to restore dopaminergic deficits. Hence, there are possibilities of neuronal replacement by ambroxol. Moreover, restoration of DAT levels in striatum may serve as a mechanism to increase dopaminergic transmission in the neurons of nigrostriatal pathway for better functional output as observed from recovery of motor deficits. Mitochondrial dysfunction was also attenuated by ambroxol. Additionally, ambroxol induced restoration of soluble α-synuclein indicates the restoration of terminal integrity in nigral dopaminergic neurons. Overall, the findings show that ambroxol augments the dopaminergic system by modifying the disease-progression due to its GCase-stimulating and α-synuclein pathology-reducing effects. Hence, preclinical evidence indicates that ambroxol has neurorestorative properties and may be successfully used as disease-modifying agent in the treatment of PD, as depicted in **Figure 7.9**.

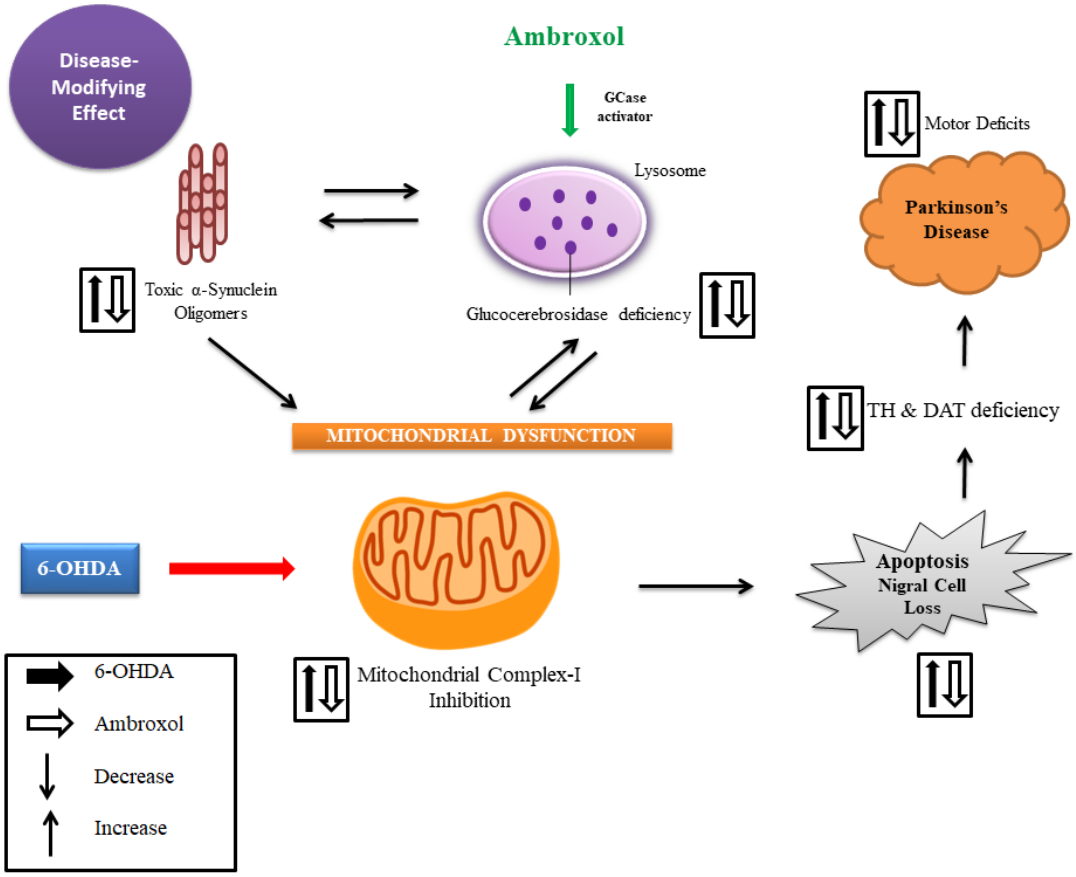

Figure 7.9 The outcome of specific objective for the assessment of the sub-chronic dose of ambroxol for neurorestorative effects against 6-OHDA-induced model of PD in rats. Ambroxol due to its GCase-stimulating activity inhibits mitochondrial dysfunction and α-synuclein pathology, which is followed by increase in Nissl bodies, TH, DAT and recovery of motor deficits. Ambroxol restores dopaminergic deficits even after its administration is initiated after the full development of 6-OHDA induced motor deficits, indicating neurorestorative potential of ambroxol.