Chapter 6

To observe the role of nuclear factor erythroid 2-related factor 2 (Nrf2) activity in rebamipide-mediated changes against 6-OHDA toxicity in rats

6.1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) activity is found to be decreased with aging and act as a significant risk factor for the development and progression of PD (Sykiotis and Bohmann, 2010). Nrf2 regulates ARE (antioxidant response element)-containing genes (Alam, 2006). Antioxidant system regulated by Nrf2 includes, but not limited to GSH, SOD and CAT (Joshi and A Johnson, 2012). ARE responses are reported downstream of Nrf2 nuclear translocation in PD. However, the extent of the endogenous activation of Nrf2/ARE responses was not found to be sufficient enough to counterbalance the overload of oxidative stress and protect the neuronal degeneration. This is the reason that several Nrf2 activators which also include antiparkinsonian drugs, such as apomorphine (Hara et al., 2006) and deprenyl (Nakaso et al., 2006) have been found to protect DA neurons against PD neurotoxins both *in vivo* and *in vitro*.

Oxidative stress has been involved in PD and the same is consistent with predominant nuclear translocation of Nrf2 together with mitochondrial dysfunction in the DA neurons (Imaizumi et al., 2012). Nrf2 inhibition promotes α-synuclein aggregation in SK-N-SH neuroblastoma cells (Gan and Johnson, 2014). Nrf2 may be involved in α-synuclein degradation through the UPS because basal mRNA levels of proteasome subunits PSMB7 (proteasome subunit beta type-7), PSMC3 (26S protease regulatory subunit 6A) and PSMC4 (26S proteasome regulatory subunit 6B) are found to be lower in the ventral midbrain of Nrf2 knockout (KO) mice compared to Nrf2 wild-type (WT) mice. Moreover, PSMB7 is only increased in Nrf2 WT mice, not in Nrf2 KO mice, after the overexpression of α-synuclein (Lastres-Becker et al., 2012). Nrf2 also takes part in the regulation of mitochondrial biogenesis by

interacting with molecules, such as PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), Tfam (mitochondrial transcription factor A), and nuclear respiratory factors, all of which are involved in the biogenesis of mitochondria (Tufekci et al., 2011). Mitochondrial dysfunction is followed by apoptosis (Gan and Johnson, 2014).

Rebamipide has been reported to increase nuclear activities of Nrf2 in murine CD4+ T cells and LBRM-33 murine T lymphoma cells against CIA (collagen-induced arthritis) (Moon et al., 2014) and spleens of rebamipide-treated CIA mice (Moon et al., 2013). It also significantly preserved the levels of Nrf2 as potential mechanism of cytoprotection from oxidative stress in reflux esophagitis model *in vivo* (Song et al., 2016). Hence, rebamipide may act through Nrf2-mediated mechanism against 6-OHDA-induced hemiparkinson's rat model as depicted in **Figure 6.1**. The current study was performed to evaluate the underlying mechanism behind the activity of rebamipide against 6-OHDA-induced hemiparkinson's rat model. Specific mechanistic studies were conducted by using pharmacological inhibitor of Nrf2 (Nrf2i; trigonelline) to inhibit its translocation into the nucleus (Arlt et al., 2013). A battery of behavioral tests was performed and nuclear Nrf2 activity was estimated. α-synuclein levels, GCase activity, mitochondrial complex-I activity, DA, TH, DAT levels and Nissl bodies were measured to investigate PD pathology. Antioxidant system regulated by Nrf2 was evaluated by estimating GSH, SOD and CAT.
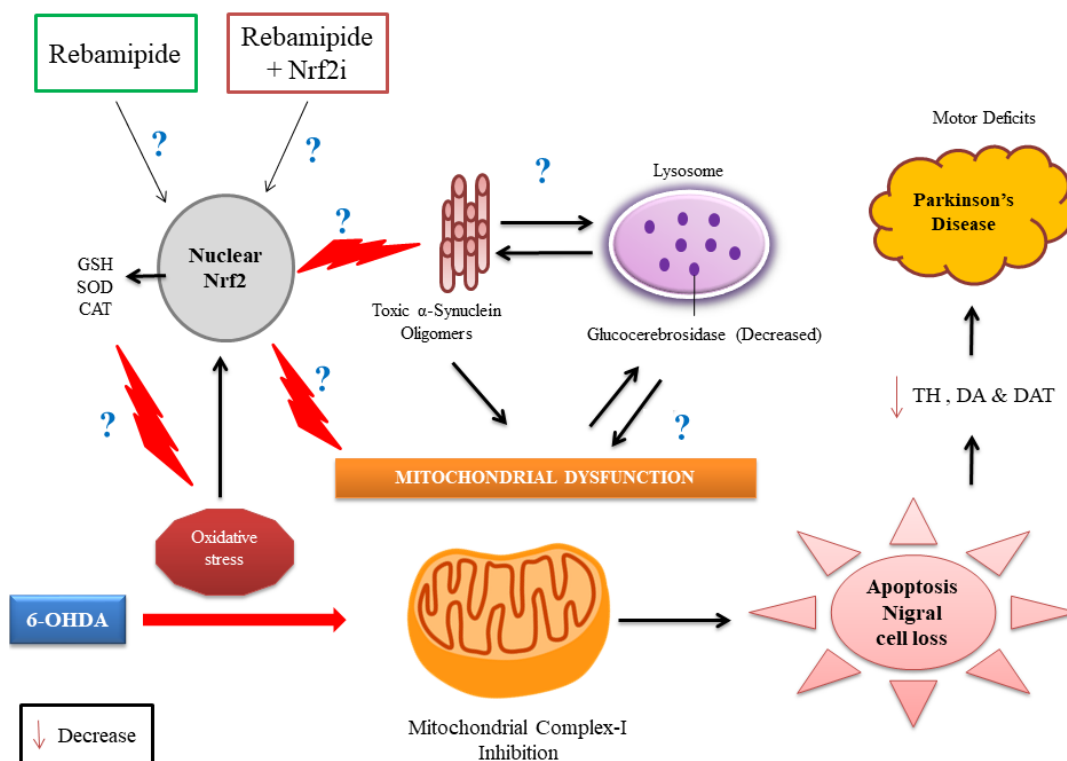
Figure 6.1 The schematic diagram of hypothesis for the role of Nrf2 activity in rebamipide-mediated changes against 6-OHDA toxicity in rats. Co-administration of Nrf2 inhibitor (Nrf2i) may inhibit rebamipide-induced changes in Nrf2, GSH, SOD, CAT, TH, DA, DAT, α-synuclein, GCase activity, mitochondrial complex-I activity, numbers of nigral Nissl bodies and motor behavior against 6-OHDA-induced hemiparkinson's rat model.

6.2. Materials and Methods

6.2.1. 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.

6.2.2. Materials

Nrf2 inhibitor (trigonelline) was received as a gift sample from Indus Biotech Private Limited, Pune, India. Potassium ferricyanide, citric acid, and sodium carbonate were acquired from Hi-media (Mumbai). Rat dopamine (DA) ELISA kit (catalogue no.: CSB-E08660r) and Rat dopamine transporter (DAT) ELISA Kit (Catalog No: MBS701143) was obtained from Cusabio, USA. and MyBioSource Inc., San Diego, CA respectively. Nuclear factor, erythroid derived 2 like protein 2 (NFE2L2; Nrf2) ELISA kit (SEL947Ra) for rats were purchased from Cloud-Clone Corp., USA. Please refer Chapter 3 (page 18) and Chapter 4 (page 29) for the source of remaining materials.

6.2.3. Stereotaxic Surgery

Please refer Chapter 4 (page 30).

6.2.4. Experimental Design

Experimental animals were randomly divided into seven groups, each containing twenty animals. The groups were control, sham, 6-OHDA, 6-OHDA+Selegiline (positive control), 6-OHDA+R-80 (rebamipide at 80 mg/kg), 6-OHDA+Nrf2i (Nrf2 inhibitor, trigonelline), 6-OHDA+R-80+Nrf2i. The experimental design is depicted in **Figure 6.2**. On D-1, animals were administered unilaterally with 6-OHDA intrastriatal injection, except control and sham groups as described in Chapter 4 (page 31). Sham group received only 4 µL of normal saline with 0.2 mg/mL ascorbic acid (Kumar et al., 2012). Drug administration was initiated to their respective groups after the onset of motor deficits from D-4. In the previous study it was found that 80 mg/kg oral dose of rebamipide compared to 40 mg/kg significantly attenuated 6-OHDA-induced motor deficits, α-synuclein pathology, mitochondrial dysfunction and nigral GCase deficiency. However, 20 mg/kg dose of rebamipide was not effective at all and only highest dose (R-80) decreased apoptotic proteins and upregulated striatal DA concentration in 6-OHDA-induced hemiparkinson's rat model, as discussed in Chapter 5 (Mishra and Krishnamurthy, 2019). On the basis of above, the dose of rebamipide was selected as 80 mg/kg twice a day (*p.o.*) and given to animals at every 12 hour. Rebamipide was given as a suspension in 0.5% CMC whereas control group were administered with 0.5 % CMC only, same as Chapter 5 (page 70) (Mishra and Krishnamurthy, 2019). Selegiline was administered as 10 mg/kg *p.o.* daily as the same dose attenuated 6-OHDA-induced striatal DA deficiency and motor deficits in rats in Chapter 4 and 5 (Mishra et al., 2018). Selegiline has also been reported to protect DA neurons against PD neurotoxins through Nrf2 pathway (Nakaso et al., 2006), and therefore was used as positive control in present study. Trigonelline, an

alkaloid is reported to prevent Nrf2 translocation into the nucleus in human ovarian carcinoma cells (Sirota et al., 2015) and is a known pharmacological inhibitor of Nrf2 (Arlt et al., 2013). Trigonelline was given as 0.02 mg/kg i.p. in 0.9% NaCl twice a day (Abdo et al., 2014). Once initiated on D-4, the treatment schedule was continued up to D-27 after 6-OHDA intrastriatal injection. Behavioral tests were conducted at every week on D-0, 7, 14, 21 and 28. Training sessions for behavioral parameters were performed as discussed in Chapter 4 (page 32-33). Apomorphine-induced rotational behavior was also performed on D-4 in order to confirm the motor deficits before drug administration. ANY-MAZE behavioral tracker version 4.72 (USA) was used to record the open field behavior. The observers blind to the study protocol recorded remaining of the behavioral tests with a video camera. Three animals from each group were assigned for Nissl's staining (n = 3) and rest of the animals were killed by decapitation on D-28. Ipsilateral striatal and nigral tissues were micro dissected from the hemispheres (Paxinos and Watson, 1998) and stored at -80^0C for further estimations. DA and DAT levels were estimated in striatal tissues (n = 6). Nigral tissues were used to measure TH levels, GCase activity, soluble α-synuclein concentration, GSH, SOD, CAT, mitochondrial complex-I activity (n = 6) and nuclear Nrf2 (n = 5).
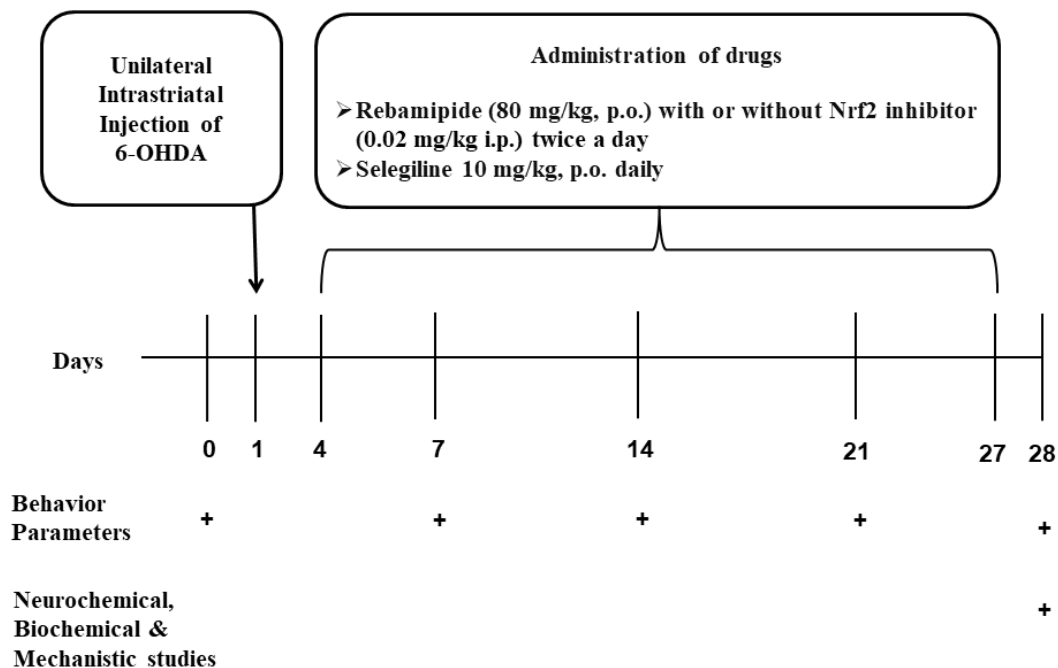
Figure 6.2 The experimental design of study for the role of Nrf2 activity in rebamipide-mediated changes against 6-OHDA toxicity in rats. "+" indicates the days at which the tests were performed.

6.2.5. Behavioral Parameters

6.2.5.1. Apomorphine-induced head rotation

Please refer Chapter 4 (page 34).

6.2.5.2. Open field test

Please refer Chapter 4 (page 34).

6.2.5.3. Rotarod Test

Please refer Chapter 4 (page 34).

6.2.5.4. Grip Strength test

Please refer Chapter 4 (page 35).

6.2.5.5. Bar Catalepsy Test

Please refer Chapter 4 (page 35).

6.2.6. Estimation of TH, DA and DAT levels

The replenishment of DA cell was observed by estimating TH (Voutilainen et al., 2017) and DAT (Gainetdinov et al., 1998). Commercially available ELISA kits were used for the measurement of TH (E-EL-R1437) in nigral tissues as well as DA (CSB-E08660r) and DAT (MBS701143) in striatal tissues. ELISA plate reader was used to read the absorbance at 450 nm and normalized to protein content (Lowry et al., 1951) the results are expressed as ng/mg protein.

6.2.7. Measurement of nuclear factor erythroid 2-related factor 2 (Nrf2)

The level of Nrf2 in the nuclear fraction of ipsilateral nigral tissues was measured using commercial ELISA kit (SEL947Ra) according to the manufacturer's instructions (Cloud-Clone Corp., USA). The absorbance of samples was read at 450 nm by microplate reader (BioTek, USA) which was normalized to protein content. Final values were expressed as Nrf2 (pg/mg protein).

6.2.8. Measurement of SOD and CAT activity

SOD activity was assessed in nigral tissues of rats by measuring the reduction of NBT to blue colored formazan in the presence of phenazine-methosulphate and NADH at 560 nm using n-butanol as blank (Kakkar et al., 1984). Single unit of enzyme was denoted as 50% inhibition of NBT reduction per min per mg protein. CAT activity in nigral tissues was estimated by measuring H_2O_2 degradation at 240 nm. Mitochondrial sample (100 µL) was added to 900 µL of 10 mM H_2O_2 – 10 mM

phosphate buffer (pH 7.0) and CAT activity was measured at 25^0C (Aebi, 1984). Single unit was expressed as 1 nmol of H_2O_2 consumed per min. SOD and CAT enzyme activities were denoted as units/min/mg protein.

6.2.9. Assessment of GSH levels

Nigral brain tissues of rat were homogenized in ice-cold normal saline (5% of tissue) and centrifuged at 4^0C. The obtained supernatant was aliquotted and GSH levels were measured spectrophotometrically as previously described (Ellman, 1959; Sedlak and Lindsay, 1968). Trichloroacetic acid (5%) was used to centrifuge the supernatant and 0.1 mL of the homogenate was added to 2 mL of phosphate buffer (pH 8.4). 0.5 mL of DTNB [5,5'- dithiobis (2-nitrobenzoic acid)] and 0.4 mL of distilled water was added. The mixture was further vortexed and absorbance was measured at 412 nm within 5 min. The protein concentration was determined as previously described method (Lowry et al., 1951). The specific activity of GSH was expressed as µmol/mg protein.

6.2.10. Estimation of GCase activity and α-synuclein concentration

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

6.2.11. Measurement of mitochondrial complex-I activity

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

6.2.12. Nissl's staining

Please refer Chapter 4 (page 38) for the quantification of Nissl-stained neurons in SNc of rats.

6.2.13. Statistical Analysis

All the datasets were expressed as mean ± SD. The statistical significance for the effects on experimental parameters were analyzed by one-way ANOVA followed by post hoc Student Newman-Keuls test, except for the behavioral parameters which were analyzed by two-way ANOVA followed by post hoc Bonferroni test. $p < 0.05$ was considered statistically significant throughout the experimental data analysis. Further, Pearson's correlation analysis was performed to correlate nuclear Nrf2 levels with different behavioral parameters, SOD activity, CAT activity, GSH levels, GCase activity, mitochondrial complex-I activity, α-synuclein concentration, number of nigral neurons, DA, DAT and TH levels and on D-28. In correlation analysis also, the criterion for statistical significance was $p < 0.05$.

6.3. Results

6.3.1. Behavior Parameters

6.3.1.1. The combination of rebamipide with Nrf2i partly blocked attenuation of 6-OHDA-induced motor deficits by rebamipide in apomorphine-induced rotation, cataleptic behavior and grip strength score

Repeated measures of two-way ANOVA revealed significant differences in rotational and cataleptic behavior as well as grip strength scores in rats among groups ([$F_{(6, 665)} = 314.5$; $p < 0.05$], [$F_{(6, 665)} = 264.9$; $p < 0.05$], [$F_{(6, 665)} = 267.4$; $p < 0.05$]

respectively), time ([F (4, 665) = 213.4; p < 0.05], [F (4, 665) = 384.2; p < 0.05], [F (4, 665) = 282.0; p < 0.05] respectively) and an interaction ([F (24, 665) = 43.47; p < 0.05], [F (24, 665) = 62.85; p < 0.05], [F (24, 665) = 33.23; p < 0.05] respectively) between group and time **(Table 6.1).** 6-OHDA increased apomorphine-induced rotational and cataleptic behavior up to 38% from D-7 and 60% from D-14 respectively and decreased grip strength scores up to 72% from D-7 compared to sham groups. 6-OHDA-induced abnormalities in motor deficits were progressive. Control and sham groups were not found to be significantly different. Rebamipide progressively attenuated 6-OHDA-induced loss of motor behavior with the onset of action at D-21 in cataleptic behavior (38%) and D-14 in rotational behavior and grip strength scores (22% and 14% respectively). Nrf2i did not aggravate 6-OHDA-induced motor deficits. Nrf2i co-administration with rebamipide significantly decreased 6-OHDA-induced motor deficits from D-21 in bar catalepsy and grip strength test and D-14 in apomorphine-induced rotation. However, this reduction was also found to be significantly different than rebamipide-administered 6-OHDA group, indicating the decreased potency of rebamipide in presence of Nrf2i.

6.3.1.2. The combination of rebamipide with Nrf2i abolished the attenuation of 6-OHDA-induced motor deficits by rebamipide in rotarod retention time

Due to the role of DA in movement-encoding (Parker et al., 2016), its deficiency in PD is responsible for motor-incoordination as shown by rotarod retention time in rats (Fernandez et al., 1998; Rozas et al., 1997). Statistical analysis by repeated measures of two-way ANOVA indicated that there were significant differences in rotarod retention time among groups ([F (6, 665) = 324.4; p < 0.05], time ([F (4, 665) =

312.7; $p < 0.05$], and an interaction between group and time ([$F_{(24, 665)} = 34.88$; $p < 0.05$] as shown in **Table 6.1**. 6-OHDA decreased rotarod retention time in rats up to 48% from D-7 compared to sham group. Control and sham groups were not found to be significantly different in post-hoc analysis. Rebamipide increased the rotarod retention time up to 31% from D-21 in rats against 6-OHDA group. Nrf2i did not aggravate 6-OHDA-induced motor deficits, but it completely blocked the progressive increase in rotarod retention time caused by rebamipide in 6-OHDA-infused rats.

6.3.1.3. The combination of rebamipide with Nrf2i partly blocked the attenuation of 6-OHDA-induced motor deficits by rebamipide in number of central squares crossed, and abolished the reduction of 6-OHDA-induced changes by rebamipide in ambulation, grooming and rearing of open field test

Spontaneous locomotor activity is reported to be adversely affected in rats due to 6-OHDA administration (Van Den Buuse et al., 1986) and can be measured by open field test (Denenberg, 1969). 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 ([$F_{(6, 665)} = 126.8$; $p < 0.05$], [$F_{(6, 665)} = 541.7$; $p < 0.05$], [$F_{(6, 665)} = 213.2$; $p < 0.05$], [$F_{(6, 665)} = 331.3$; $p < 0.05$] respectively), time ([$F_{(4, 665)} = 206.6$; $p < 0.05$], [$F_{(4, 665)} = 485.3$; $p < 0.05$], [$F_{(4, 665)} = 159.4$; $p < 0.05$], [$F_{(4, 665)} = 263.8$; $p < 0.05$] respectively) and an interaction ([$F_{(24, 665)} = 26.33$; $p < 0.05$], [$F_{(24, 665)} = 71.79$; $p < 0.05$], [$F_{(24, 665)} = 23.51$; $p < 0.05$], [$F_{(24, 665)} = 37.28$; $p < 0.05$] respectively) between group and time in open field test **(Table 6.2).** No significant differences were observed between control and sham groups. 6-OHDA significantly decreased the number of

central squares crossed from D-14 (58%) and rest of the open field parameters were decreased from D-7 because 55%, 57% and 82% reduction was observed in number of grooming, rearing and ambulation respectively compared to sham group. Rebamipide significantly attenuated 6-OHDA-induced reduction in open field behavior with the onset of action at D-14 for rearing (21% increase) and D-21 for the remaining open field test parameters (39%, 49% and 76% increase in number of central squares crossed, grooming and ambulation respectively). Rebamipide progressively attenuated 6-OHDA-induced loss of motor deficits. Nrf2i-administered 6-OHDA group was not found to be significantly different than 6-OHDA group. However, when given with Nrf2i, rebamipide significantly increased (28%) the number of central squares crossed from D-21 compared to 6-OHDA group, the extent of which is significantly lower compared to rebamipide. Moreover, Nrf2i completely blocked the rebamipide-induced recovery of remaining of the open field parameters against 6-OHDA-infused rats, suggesting the action of rebamipide through Nrf2.

Table 6.1 Effects of rebamipide and Nrf2i on 6-OHDA-induced alterations 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	Retention Time in Rotarod Test (sec)
DAY 0				
Control	5.68 ± 1.04	1.85 ± 0.51	4.45 ± 0.96	180.30 ± 14.04
Sham	5.82 ± 0.95	1.92 ± 0.49	4.36 ± 0.90	181.30 ± 13.83
6-OHDA	5.56 ± 1.05	1.67 ± 0.43	4.49 ± 0.72	181.50 ± 19.26
6-OHDA+Selegiline	5.87 ± 1.05	1.88 ± 0.44	4.43 ± 0.83	180.70 ± 16.25
6-OHDA+R-80	5.84 ± 0.94	2.01 ± 0.44	4.34 ± 0.78	175.20 ± 15.81
6-OHDA+Nrf2i	6.18 ± 0.82	1.74 ± 0.63	4.38 ± 0.98	176.40 ± 18.81
6-OHDA+R-80+Nrf2i	5.93 ± 0.75	1.71 ± 0.49	4.51 ± 0.83	180.50 ± 16.25
DAY 7				
Control	6.17 ± 1.11	1.74 ± 0.46	4.36 ± 0.73	180.70 ± 10.31
Sham	5.57 ± 0.50	1.94 ± 0.39	4.19 ± 0.78	168.20 ± 8.27
6-OHDA	9.01 ± 1.61^a	1.99 ± 0.22	1.17 ± 0.22^a	88.13 ± 25.99^a
6-OHDA+Selegiline	9.02 ± 1.52^a	1.87 ± 0.25	1.34 ± 0.26^a	89.86 ± 28.74^a
6-OHDA+R-80	8.58 ± 1.40^a	2.10 ± 0.34	1.31 ± 0.17^a	81.28 ± 22.57^a
6-OHDA+Nrf2i	9.23 ± 1.61^a	2.04 ± 0.43	1.15 ± 0.21^a	86.16 ± 23.47^a
6-OHDA+R-80+Nrf2i	9.22 ± 1.34^a	1.92 ± 0.49	1.29 ± 0.20^a	87.63 ± 23.26^a
DAY 14				
Control	5.76 ± 1.18	1.64 ± 0.43	4.20 ± 0.75	181.80 ± 11.31
Sham	5.92 ± 0.95	1.69 ± 0.44	4.11 ± 0.98	169.70 ± 10.01
6-OHDA	11.91 ± 1.01^a	4.25 ± 0.98^a	1.09 ± 0.18^a	79.92 ± 19.37^a
6-OHDA+Selegiline	$10.27 \pm 1.81^{a,b}$	$3.07 \pm 0.79^{a,b}$	$2.90 \pm 0.39^{a,b}$	$121.60 \pm 13.97^{a,b}$
6-OHDA+R-80	$9.23 \pm 1.22^{a,b}$	3.99 ± 0.81^a	1.27 ± 0.20^a	90.12 ± 12.16^a
6-OHDA+Nrf2i	$12.55 \pm 2.04^{a,c}$	3.90 ± 0.80^a	1.28 ± 0.31^a	$69.18 \pm 17.56^{a,c}$
6-OHDA+R-80+Nrf2i	$10.72 \pm 1.13^{a,b,c,d}$	3.87 ± 0.78^a	1.32 ± 0.15^a	$72.41 \pm 17.73^{a,c}$
DAY 21				
Control	5.81 ± 0.82	1.89 ± 0.48	4.28 ± 0.92	180.20 ± 14.03
Sham	5.97 ± 0.76	1.95 ± 0.39	4.15 ± 1.06	171.30 ± 13.65
6-OHDA	13.98 ± 1.08^a	6.20 ± 1.01^a	1.32 ± 0.16^a	93.95 ± 24.58^a
6-OHDA+Selegiline	6.44 ± 1.08^b	2.06 ± 0.37^b	4.23 ± 0.79^b	168.70 ± 12.59^b
6-OHDA+R-80	$7.87 \pm 1.26^{a,b}$	$3.86 \pm 0.81^{a,b}$	$3.00 \pm 0.57^{a,b}$	$135.60 \pm 22.11^{a,b}$
6-OHDA+Nrf2i	$14.59 \pm 1.90^{a,c}$	$5.88 \pm 0.52^{a,c}$	$1.35 \pm 0.30^{a,c}$	$84.88 \pm 23.29^{a,c}$
6-OHDA+R-80+Nrf2i	$12.76 \pm 1.21^{a,b,c,d}$	$5.39 \pm 0.69^{a,b,c,d}$	$1.91 \pm 0.22^{a,b,c,d}$	$96.10 \pm 25.07^{a,c}$
DAY 28				
Control	6.16 ± 0.92	1.99 ± 0.37	4.22 ± 1.03	180.00 ± 15.33
Sham	6.23 ± 1.14	1.93 ± 0.38	4.22 ± 0.78	171.30 ± 14.12
6-OHDA	14.61 ± 2.26^a	5.82 ± 0.57^a	1.20 ± 0.17^a	87.05 ± 23.16^a
6-OHDA+Selegiline	6.51 ± 0.97^b	2.24 ± 0.35^b	4.25 ± 0.52^b	167.20 ± 13.86^b
6-OHDA+R-80	6.48 ± 1.05^b	2.30 ± 0.37^b	4.11 ± 0.86^b	165.00 ± 12.40^b
6-OHDA+Nrf2i	$15.08 \pm 2.52^{a,c}$	$5.76 \pm 1.06^{a,c}$	$1.42 \pm 0.20^{a,c}$	$78.81 \pm 22.00^{a,c}$
6-OHDA+R-80+Nrf2i	$12.89 \pm 2.01^{a,b,c,d}$	$5.25 \pm 0.36^{a,b,c,d}$	$1.80 \pm 0.10^{a,b,c}$	$89.64 \pm 23.75^{a,c}$

All values are mean ± SD; n = 20; [a]$p < 0.05$ compared to sham, [b]$p < 0.05$ compared to 6-OHDA, [c]$p < 0.05$ compared to 6-OHDA+R-80, [d]$p < 0.05$ compared to 6-OHDA+Nrf2i [Repeated measures of two-way ANOVA followed by Bonferroni test].

Table 6.2 Effects of rebamipide and Nrf2i 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	4.52 ± 0.80	45.14 ± 8.65	15.04 ± 1.48	6.60 ± 1.27
Sham	4.60 ± 0.86	46.41 ± 7.54	15.14 ± 2.44	6.70 ± 1.23
6-OHDA	4.58 ± 0.96	45.99 ± 8.10	15.19 ± 1.92	6.55 ± 1.21
6-OHDA+Selegiline	4.63 ± 0.64	45.09 ± 7.20	15.03 ± 2.39	6.51 ± 1.20
6-OHDA+R-80	4.66 ± 0.90	45.02 ± 9.31	14.86 ± 2.48	6.50 ± 1.19
6-OHDA+Nrf2i	4.64 ± 0.61	46.38 ± 8.26	14.77 ± 2.29	6.48 ± 1.26
6-OHDA+R-80+Nrf2i	4.61 ± 0.80	45.51 ± 7.38	14.90 ± 2.10	6.51 ± 1.20
DAY 7				
Control	4.53 ± 0.94	45.66 ± 8.03	14.93 ± 2.31	6.44 ± 1.43
Sham	4.43 ± 0.74	46.35 ± 7.38	14.54 ± 2.38	6.50 ± 0.94
6-OHDA	4.40 ± 0.68	8.27± 1.80[a]	6.22 ± 0.94[a]	2.94± 0.48[a]
6-OHDA+Selegiline	4.42 ± 0.84	9.24 ± 2.00[a]	6.22 ± 1.05[a]	3.21 ± 0.59[a]
6-OHDA+R-80	4.55 ± 0.60	9.08 ± 1.83[a]	6.73± 0.85[a]	3.17 ± 0.52[a]
6-OHDA+Nrf2i	4.44 ± 0.71	7.90 ± 1.06[a]	6.30 ± 1.20[a]	3.04 ± 0.58[a]
6-OHDA+R-80+Nrf2i	4.47 ± 0.72	7.99 ± 1.11[a]	6.14 ± 0.99[a]	3.09 ± 0.41[a]
DAY 14				
Control	4.42 ± 0.45	45.63 ± 6.36	15.07 ± 2.80	6.36 ± 1.40
Sham	4.57 ± 0.72	45.85 ± 8.36	15.06 ± 2.78	6.43 ± 1.08
6-OHDA	1.90 ± 0.27[a]	6.50 ± 1.70[a]	5.92 ± 0.36[a]	2.72 ± 0.31[a]
6-OHDA+Selegiline	3.87 ± 0.40[a,b]	11.46 ± 2.10[a,b]	10.06 ± 1.61[a,b]	5.36 ± 0.89[a,b]
6-OHDA+R-80	2.09 ± 0.40[a]	7.33 ± 1.59[a]	7.46 ± 0.84[a,b]	2.99 ± 0.10[a]
6-OHDA+Nrf2i	2.12 ± 0.31[a]	6.58 ± 1.10[a]	5.47 ± 0.81[a,c]	2.17 ± 0.11[a,c]
6-OHDA+R-80+Nrf2i	2.11 ± 0.44[a]	6.22 ± 1.26[a]	5.42± 0.75[a,c]	2.72 ± 0.48[a]
DAY 21				
Control	4.54 ± 0.19	45.85 ± 7.22	14.91 ± 2.00	6.43 ± 1.37
Sham	4.61 ± 0.38	45.46 ± 9.63	14.77 ± 2.72	6.50 ± 1.35
6-OHDA	2.12 ± 0.39[a]	8.15 ± 2.16[a]	5.95 ± 1.04[a]	2.73 ± 0.50[a]
6-OHDA+Selegiline	3.88± 0.10[a,b]	44.11 ± 6.41[b]	14.42 ± 2.34[b]	6.25 ± 1.00[b]
6-OHDA+R-80	3.48 ± 0.44[a,b]	33.71 ± 5.39[a,b]	10.90 ± 1.73[a,b]	5.38 ± 0.95[a,b]
6-OHDA+Nrf2i	2.32 ± 0.28[a,c]	8.76 ± 0.83[a,c]	5.05 ± 0.18[a,c]	2.20 ± 0.10[a,c]
6-OHDA+R-80+Nrf2i	2.94± 0.38[a,b,c,d]	8.81 ± 2.32[a,c]	6.71 ± 0.56[a,c,d]	3.07 ± 0.11[a,c,d]
DAY 28				
Control	4.36 ± 0.75	43.96 ± 7.12	14.58 ± 2.68	6.37± 1.31
Sham	4.45 ± 0.82	44.61 ± 8.93	14.63 ± 1.99	6.51 ± 1.28
6-OHDA	1.80 ± 0.32[a]	6.43 ± 1.10[a]	5.59± 0.97[a]	2.68 ± 0.41[a]
6-OHDA+Selegiline	4.44 ± 0.13[b]	43.62 ± 8.69[b]	14.41± 2.36[b]	6.25 ± 1.58[b]
6-OHDA+R-80	4.02 ± 0.42[b]	43.27 ± 7.67[b]	14.27 ± 2.31[b]	6.29 ± 0.83[b]
6-OHDA+Nrf2i	1.81 ± 0.15[a,c]	6.02 ± 1.20[a,c]	4.70 ± 0.20[a,c]	2.45 ± 0.22[a,c]
6-OHDA+R-80+Nrf2i	2.37 ± 0.32[a,b,c,d]	6.30 ± 1.30[a,c]	6.23 ± 0.35[a,c,d]	2.60 ± 0.28[a,c]

All values are mean ± SD; n = 20; [a]$p < 0.05$ compared sham, [b]$p < 0.05$ compared to 6-OHDA, [c]$p < 0.05$ compared to 6-OHDA+R-80, [d]$p < 0.05$ compared to 6-OHDA+Nrf2i [Repeated measures of two-way ANOVA followed by Bonferroni test].

6.3.2. The combination of rebamipide with Nrf2i partly blocked the elevation of nigral TH levels by rebamipide against 6-OHDA-infused rats

DA biosynthesis takes place in the presence of TH, which gets reduced in PD patients (Haavik and Toska, 1998). One way ANOVA showed significant differences among groups in TH levels [$F_{(6, 35)}$ = 21.26; $p < 0.05$] in nigral tissues as shown in **Figure 6.3**. Control and sham groups were not found to be significantly different. 6-OHDA significantly reduced TH levels up to 66% compared to sham group, which was significantly increased (60%) by rebamipide compared to 6-OHDA-infused rats. No changes were observed in case of Nrf2i co-administration in 6-OHDA rats compared to 6-OHDA-infused rats. When Nrf2i and rebamipide were administered together in 6-OHDA-infused rats, TH levels were found to be 44% and 39% higher than that of 6-OHDA and 6-OHDA+Nrf2i groups respectively. However, this increment in TH levels was significantly lower than that of rebamipide alone in 6-OHDA-infused rats, suggesting the role of Nrf2i in reducing the efficacy of rebamipide.

6.3.3. The combination of rebamipide with Nrf2i abolished the elevation of striatal DA and DAT levels by rebamipide against 6-OHDA-infused rats

DAT level is the determinant of extracellular DA concentration and indicates viability of dopaminergic cells (Gainetdinov et al., 1998; Nutt et al., 2004). DAT and DA were significantly decreased up to 58% by 6-OHDA in ipsilateral striatal tissues in rats compared to sham group. One way ANOVA showed significant differences among groups in DA [$F_{(6, 35)}$ = 52.16; $p < 0.05$] and DAT [$F_{(6, 35)}$ = 24.11; $p < 0.05$] levels in striatal tissues as shown in **Figure 6.4**. Nr2i administration in 6-OHDA rats did not show any changes compared to 6-OHDA-infused rats.

Rebamipide significantly increased striatal DA and DAT levels up to 45% and 52% respectively against 6-OHDA group. However, when administered with Nrf2i, rebamipide failed to do so. Control and sham groups were not found to be significantly different.
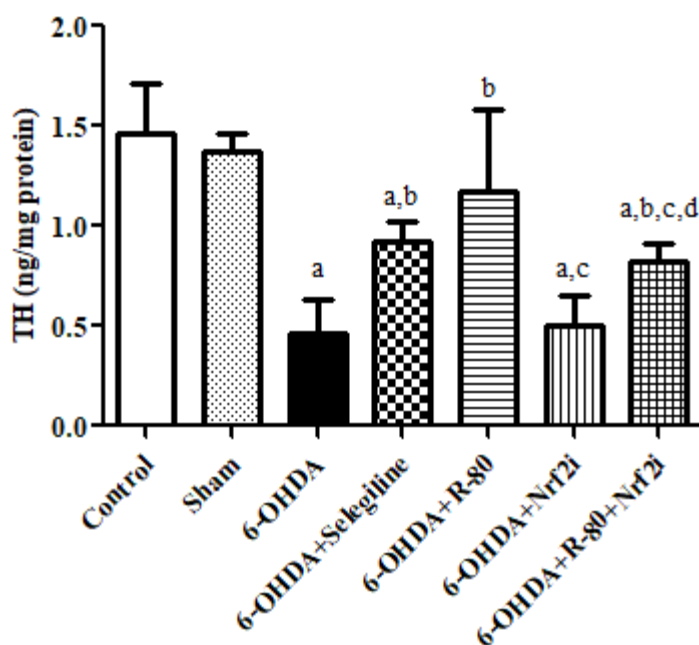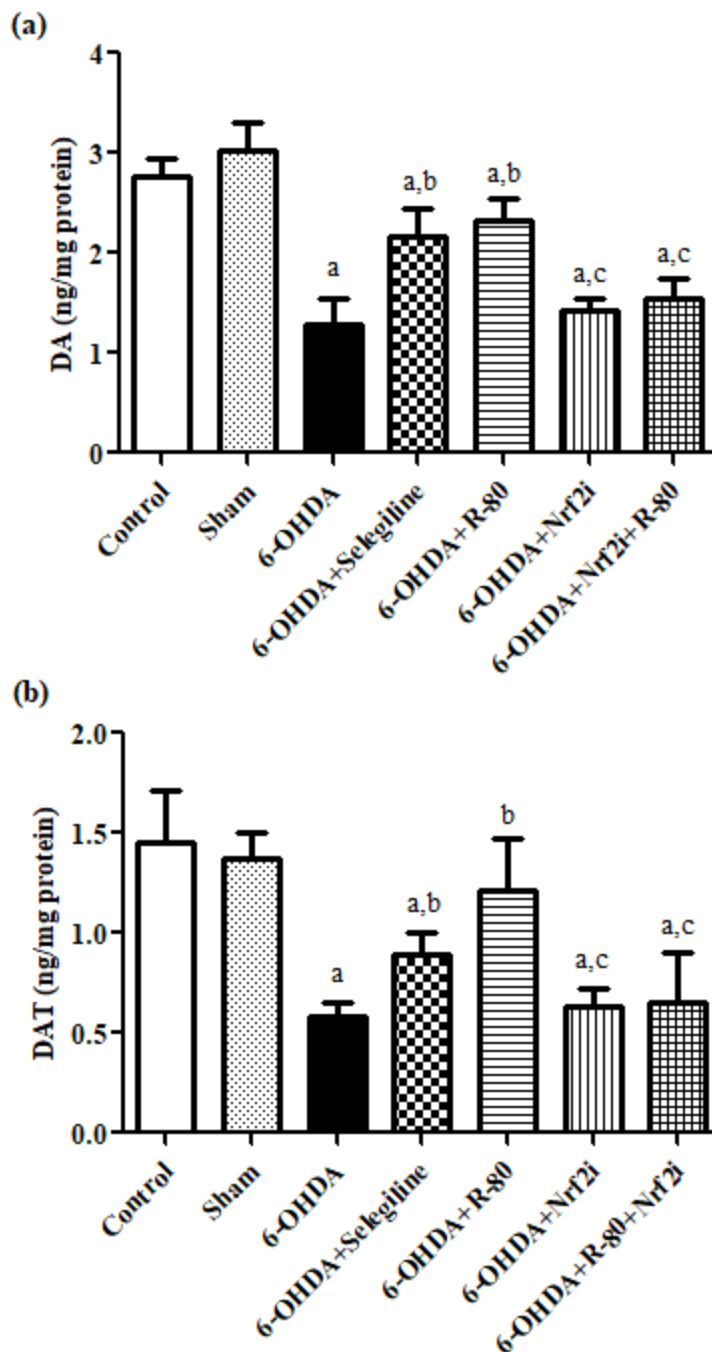

Figure 6.3 Effect of rebamipide and Nrf2i on 6-OHDA-mediated loss of TH levels in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; [a]p < 0.05 compared to sham, [b]p < 0.05 compared to 6-OHDA, [c]p < 0.05 compared to 6-OHDA+R-80 and [d]p < 0.05 compared to 6-OHDA+Nrf2i [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

Figure 6.4 Effect of rebamipide and Nrf2i on 6-OHDA-mediated loss of DA (a) and DAT (b) levels in ipsilateral striatal tissues of rats. All values are mean $\pm$ SD; n = 6; [a]p < 0.05 compared to sham, [b]p < 0.05 compared to 6-OHDA and [c]p < 0.05 compared to 6-OHDA+R-80 [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

6.3.4. The combination of rebamipide with Nrf2i abolished the elevation of nuclear Nrf2 by rebamipide against 6-OHDA-infused rats

Significant differences in nuclear Nrf2 were observed in ipsilateral nigral tissues of rats among groups [$F_{(6, 28)}$ = 11.68; $p < 0.05$] by using one-way ANOVA. No significant differences were observed between control and sham groups **(Figure 6.5)**. 6-OHDA significantly decreased nigral Nrf2 up to 42% in nuclear fractions compared to sham groups, and it was significantly increased by rebamipide up to 28% compared to 6-OHDA-infused rats. However, when administered with Nrf2i, rebamipide did not increase nuclear Nrf2 against 6-OHDA group, indicating the role of nuclear Nrf2 activity in rebamipide action.
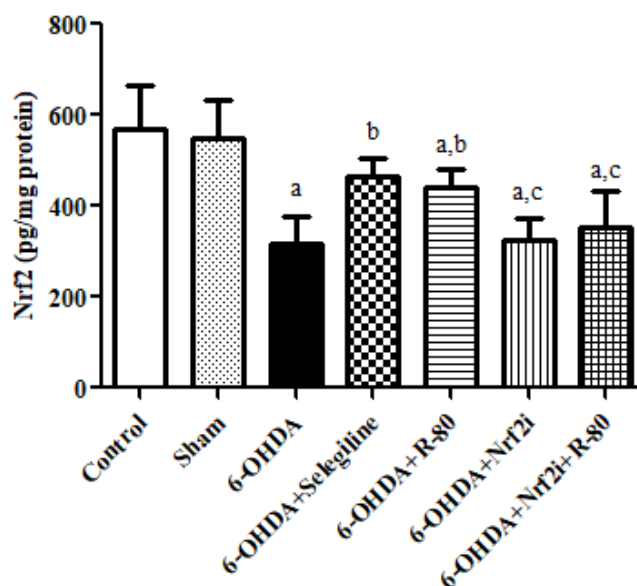

Figure 6.5 Effect of rebamipide and Nrf2i on 6-OHDA-mediated loss of Nrf2 in the nuclear fraction in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 5; [a]$p < 0.05$ compared to sham and [b]$p < 0.05$ compared to 6-OHDA and [c]$p < 0.05$ compared to 6-OHDA+R-80 [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

6.3.5. The combination of rebamipide with Nrf2i partly blocked the elevation of nigral SOD and CAT activity as well as GSH level by rebamipide against 6-OHDA-infused rats

Enzymatic activity of CAT is reported to be reduced in nigral region of parkinsonian brains (Ambani et al., 1975). GSH, a major tissue antioxidant is diminished in nigral tissues of PD subjects (Sofic et al., 1992). One-way ANOVA revealed significant differences in SOD activity [$F_{(6, 35)}$ = 33.05; $p < 0.05$], CAT activity [$F_{(6, 35)}$ = 15.82; $p < 0.05$] and GSH levels [$F_{(6, 35)}$ = 27.40; $p < 0.05$] among groups as shown in **Figure 6.6**. No significant differences were found between control and sham groups. 6-OHDA auto-oxidation takes part in generation of toxic ROS (Blum et al., 2001), therefore decreased SOD (up to 55%) and CAT (up to 60%) activity against sham group in rat nigral tissues. Rebamipide significantly increased SOD and CAT activity up to 50% and 56% respectively compared to 6-OHDA group. When administered with Nrf2i, rebamipide significantly increased SOD (32%) and CAT activity (40%) compared to 6-OHDA group. However, the extent was significantly lower than that of rebamipide in 6-OHDA group. Similarly, rebamipide increased GSH concentrations up to 50% against 6-OHDA group. However, in presence of Nrf2i, the increment was not observed up to similar extent as that of rebamipide alone. GSH level was significantly increased only up to 34% compared to 6-OHDA group.
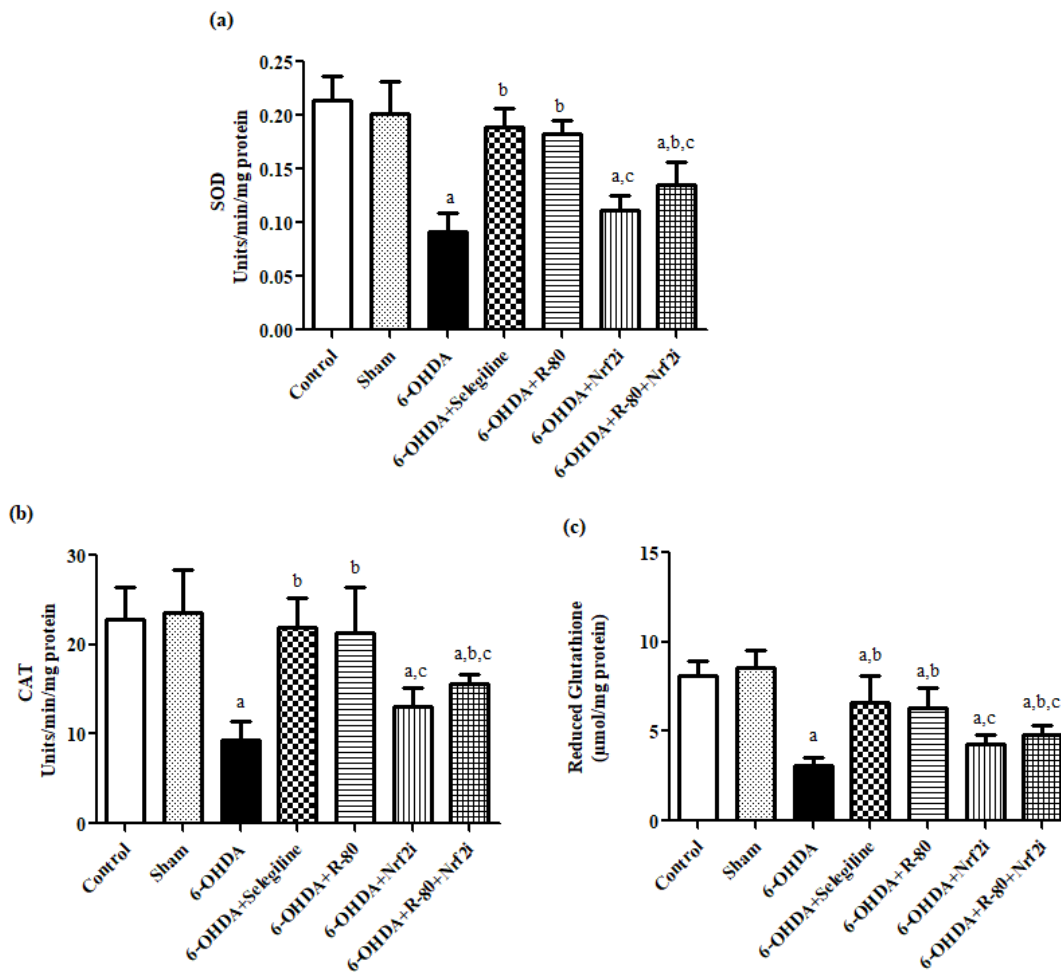
Figure 6.6 Effect of rebamipide and Nrf2i on 6-OHDA-mediated loss of SOD activity (a), CAT activity (b) and amount of GSH (c) in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; [a]$p < 0.05$ compared to sham, [b]$p < 0.05$ compared to 6-OHDA and [c]$p < 0.05$ compared to 6-OHDA+R-80 [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

6.3.6. The combination of rebamipide with Nrf2i abolished the elevation of mitochondrial complex-I activity, GCase activity and soluble α-synuclein concentration in nigral tissues by rebamipide against 6-OHDA-infused rats

Mitochondrial dysfunction is considered as one of the important predisposing factor in the pathogenesis of PD (Mizuno et al., 1998) which was decreased up to 76% by 6-OHDA compared to sham group. One-way ANOVA showed significant differences in mitochondrial complex-I activity [$F_{(6, 35)} = 33.06$, $p < 0.05$] among groups. Rebamipide significantly increased the same compared to 6-OHDA group. However, when administered with Nrf2i, rebamipide did not show any increment in mitochondrial complex-I activity. Control and sham groups were not found to be significantly different as shown in **Figure 6.7 (a).**

One-way ANOVA showed significant differences in GCase enzymatic activity [$F_{(6, 35)} = 43.11$; $p < 0.05$] and soluble α-synuclein concentration [$F_{(6, 35)} = 20.04$; $p < 0.05$] among groups. There was no significant difference observed between control and sham groups as shown in **Figure 6.7 (b) and (c).** Rebamipide significantly increased GCase activity and soluble α-synuclein concentrations up to 70% and 63% respectively compared to 6-OHDA group. However, this increment was not observed in case of combined administration of rebamipide and Nrf2i against 6-OHDA group, suggesting Nrf2-mediated mechanism of rebamipide.
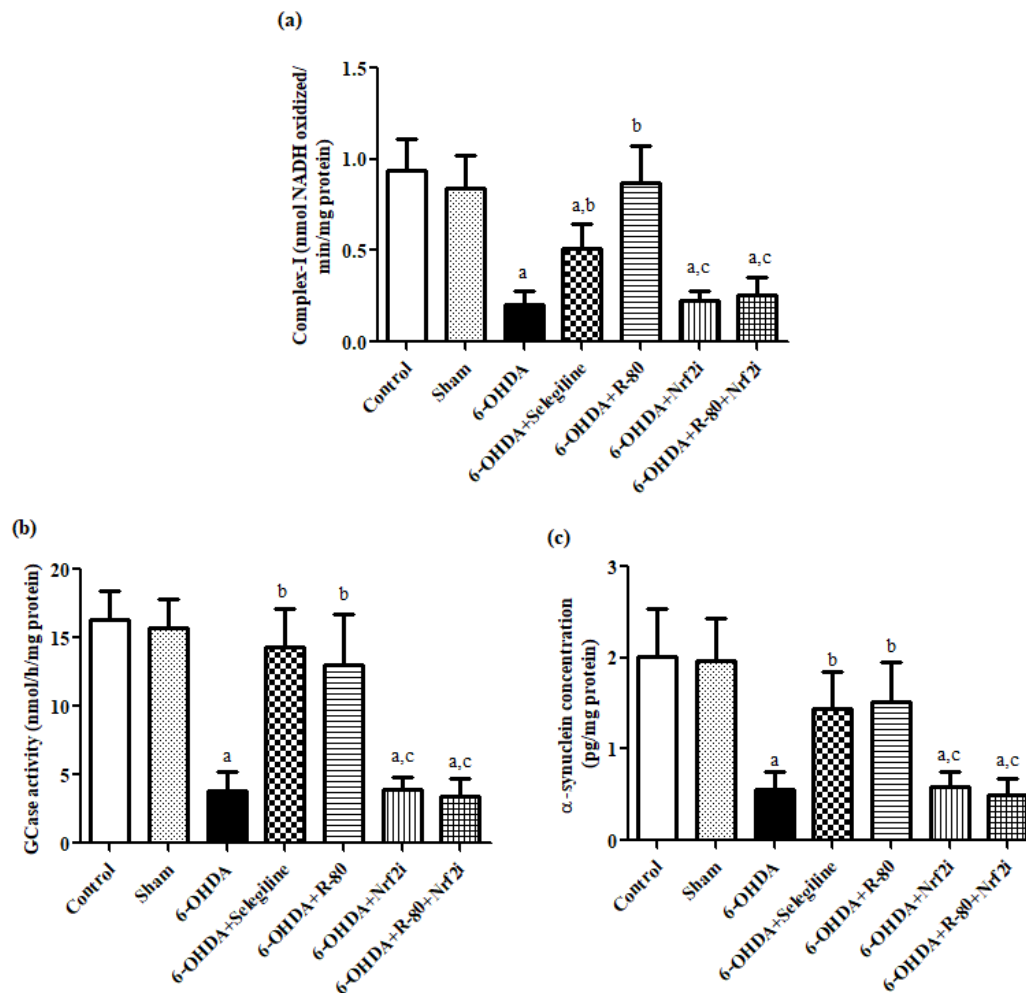
Figure 6.7 Effect of rebamipide and Nrf2i on 6-OHDA-induced alterations in mitochondrial complex-I activity (a), GCase enzymatic activity (b) and α-synuclein protein concentration (c) in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; [a]$p < 0.05$ compared to sham, [b]$p < 0.05$ compared to 6-OHDA and [c]$p < 0.05$ compared to 6-OHDA+R-80 [One-way ANOVA followed by Student Newman–Keuls Post-hoc test].

6.3.7. The combination of rebamipide with Nrf2i abolished the elevation in number of nigral cells by rebamipide against 6-OHDA-infused rats

Significant differences in the percentages of Nissl bodies were observed in nigral tissues among groups [F (6, 14) = 26.33; p < 0.05] by using one-way ANOVA. In the present study, 6-OHDA caused upto70% and 66% loss of Nissl bodies compared to control and sham groups respectively **(Figure 6.8)**. Rebamipide significantly increased the number of Nissl-positive cells up to 68% compared to 6-OHDA-infused rats. However, when it was administered with Nrf2i, no rebamipide-induced increase in number of nigral cells was observed. Control and sham groups were not found to be significantly different.
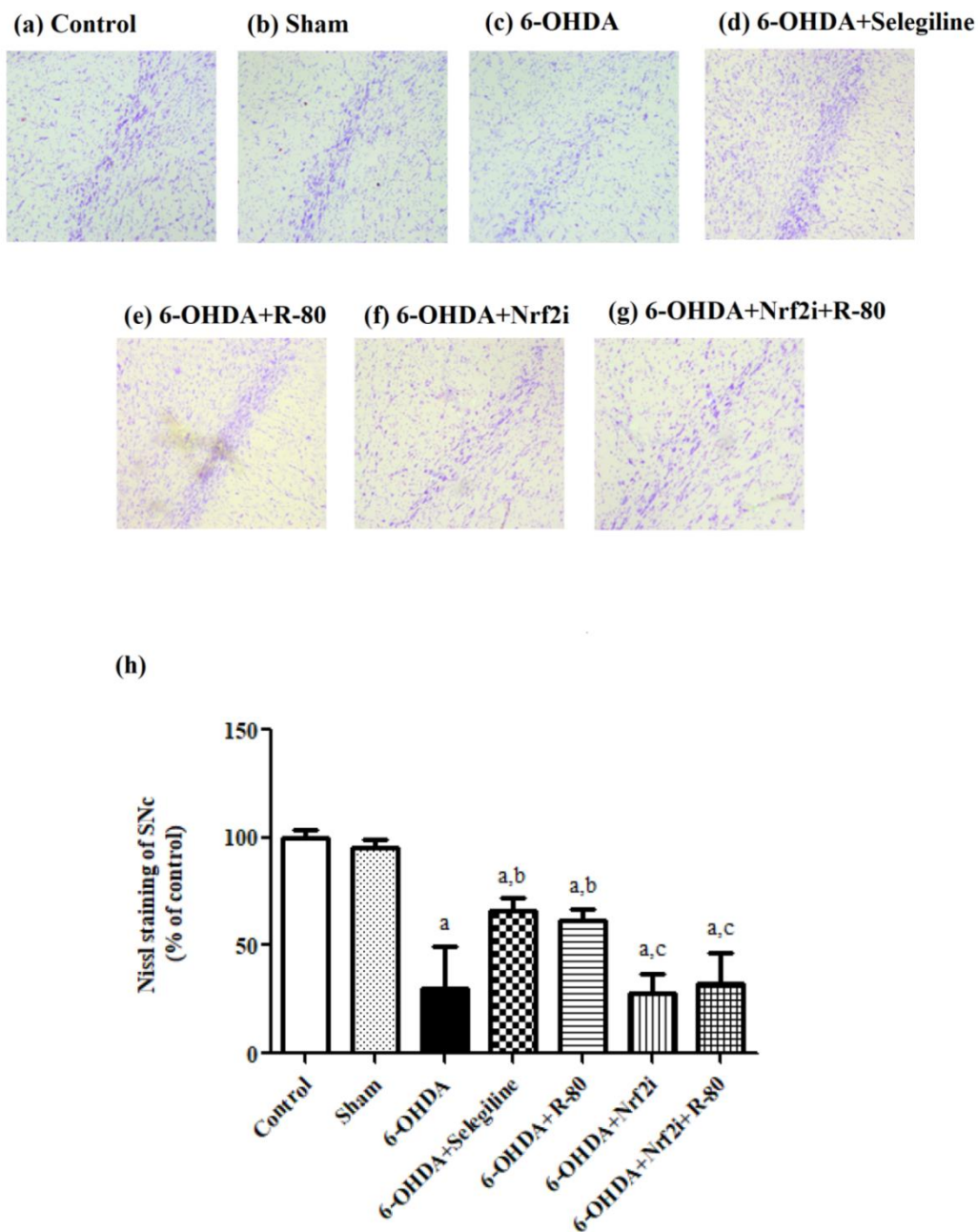
Figure 6.8 Nissl's staining of SNc in rats. Control (a); Sham (b); 6-OHDA (c); 6OHDA+Selegiline (d); 6-OHDA+R-80 (e); 6-OHDA+Nrf2i (f); 6-OHDA+Nrf2i+R-80 (g); Data of counting cells (h). All values are mean ± SD; n = 3; [a]$p < 0.05$ compared to sham, [b]$p < 0.05$ compared to 6-OHDA and [c]$p < 0.05$ compared to 6-OHDA+R-80 [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

6.3.8. Correlation analysis between the Nuclear Nrf2 levels and different PD parameters

The correlation analysis between nuclear Nrf2 and different behavioral, neurochemical and mechanistic parameters observed on D-28 of experimental protocol are depicted in **Figure 6.9, 6.10 and 6.11.** Statistically significant correlation was observed between nuclear Nrf2 and behavioral observations such as rotarod retention time (positive; r^2 = 0.4985, Pearson's r = 0.7061), apomorphine-induced contralateral rotations (negative; r^2 = 0.4880, Pearson's r = -0.6986), catalepsy behavior (negative; r^2 = 0.6669, Pearson's r = -0.8167) and grip strength scores (positive; r^2 = 0.4754, Pearson's r = 0.6895) as well as open field behavior parameters like ambulation (positive; r^2 = 0.5358, pearson's r = 0.7320), rearing (positive; r^2 = 0.4671, Pearson's r = 0.6835), central square crossed (positive; r^2 = 0.5282, Pearson's r = 0.7268) and grooming (positive; r^2 = 0.08006, Pearson's r = 0.2830).

Statistically significant correlation was observed between nuclear Nrf2 and mitochondrial complex-I activity (positive; r^2 =0.6324, Pearson's r = 0.7952), SOD (positive; r^2 = 0.5326, Pearson's r = 0.7298), CAT (positive; r^2 = 0.5714, Pearson's r = 0.7559), GSH (positive; r^2 = 0.5839, Pearson's r = 0.7641), GCase activity (positive; r^2 = 0.5602, Pearson's r = 0.7485), soluble α-synuclein concentration (positive; r^2 = 0.3898, Pearson's r = 0.6243), TH (positive; r^2 = 0.6807, Pearson's r = 0.8250), DAT (positive; r^2 = 0.4735, Pearson's r = 0.6881), number of nigral neurons in Nissl's staining (positive; r^2 = 0.6824; Pearson's r = 0.8261) and DA levels (positive; r^2 = 0.5628, Pearson's r = 0.7502).
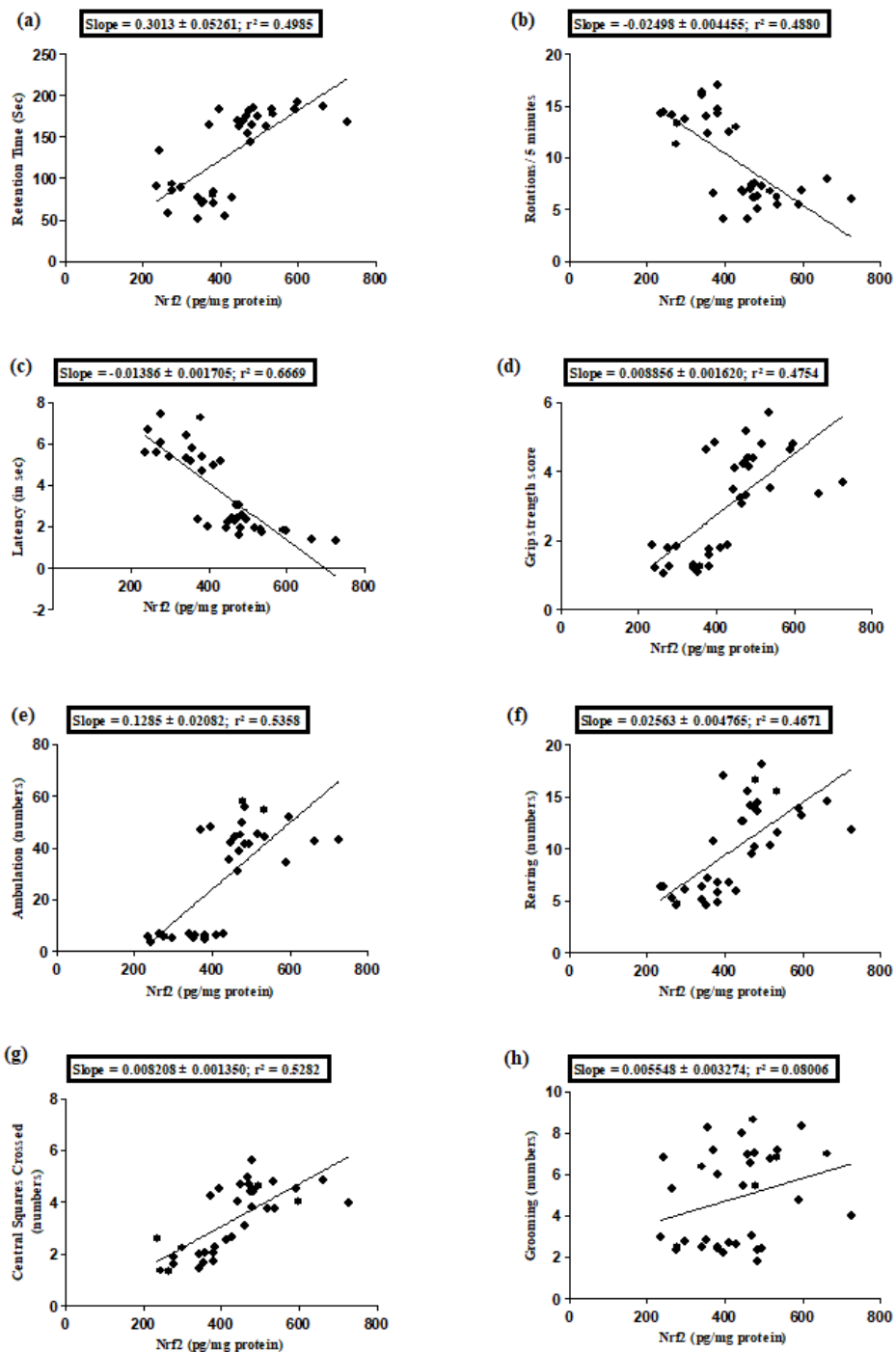
(a) Slope = 0.3013 ± 0.05261; r² = 0.4985

(b) Slope = -0.02498 ± 0.004455; r² = 0.4880

(c) Slope = -0.01386 ± 0.001705; r² = 0.6669

(d) Slope = 0.008856 ± 0.001620; r² = 0.4754

(e) Slope = 0.1285 ± 0.02082; r² = 0.5358

(f) Slope = 0.02563 ± 0.004765; r² = 0.4671

(g) Slope = 0.008208 ± 0.001350; r² = 0.5282

(h) Slope = 0.005548 ± 0.003274; r² = 0.08006

Figure 6.9 The correlation between nuclear Nrf2 and behavior parameters [rotarod retention time (a); apomorphine-induced rotations (b); catalepsy behavior (c); grip strength scores (d); ambulation (e), rearing (f), central squares crossed (g) and grooming (h) during open field test] [Pearson's correlational analysis at p < 0.05].
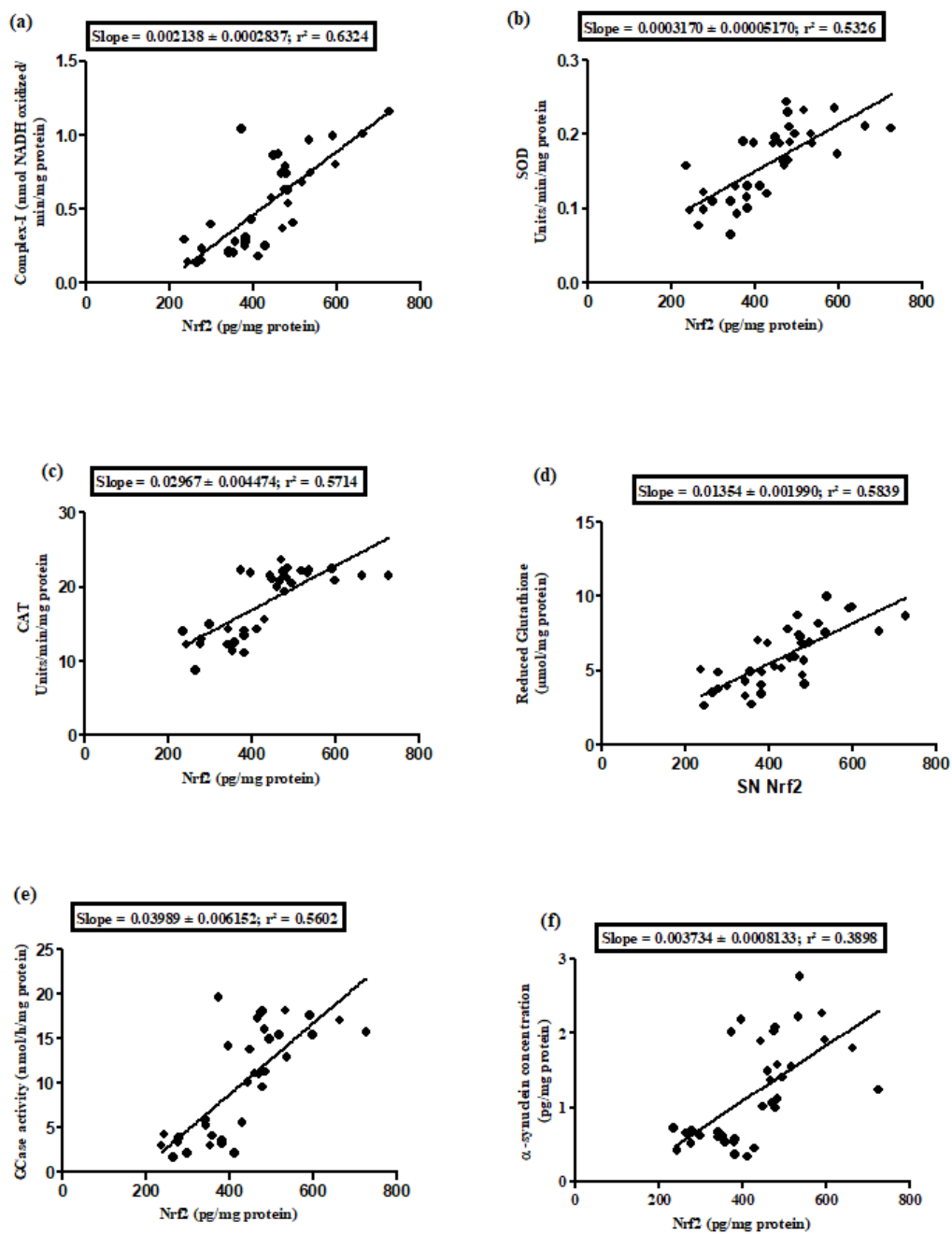
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Figure 6.10 The correlation between nuclear Nrf2 and PD parameters such as mitochondrial complex-I activity (a), SOD activity (b), CAT activity (c), GSH levels (d), GCase activity (e) and α-synuclein concentration (f) [Pearson's correlational analysis at $p < 0.05$].

(a) Slope = 0.002506 ± 0.0002988; r² = 0.6807

(b) Slope = 0.001500 ± 0.0002753; r² = 0.4735

(c) Slope = 1.142 ± 0.1787; r² = 0.6824

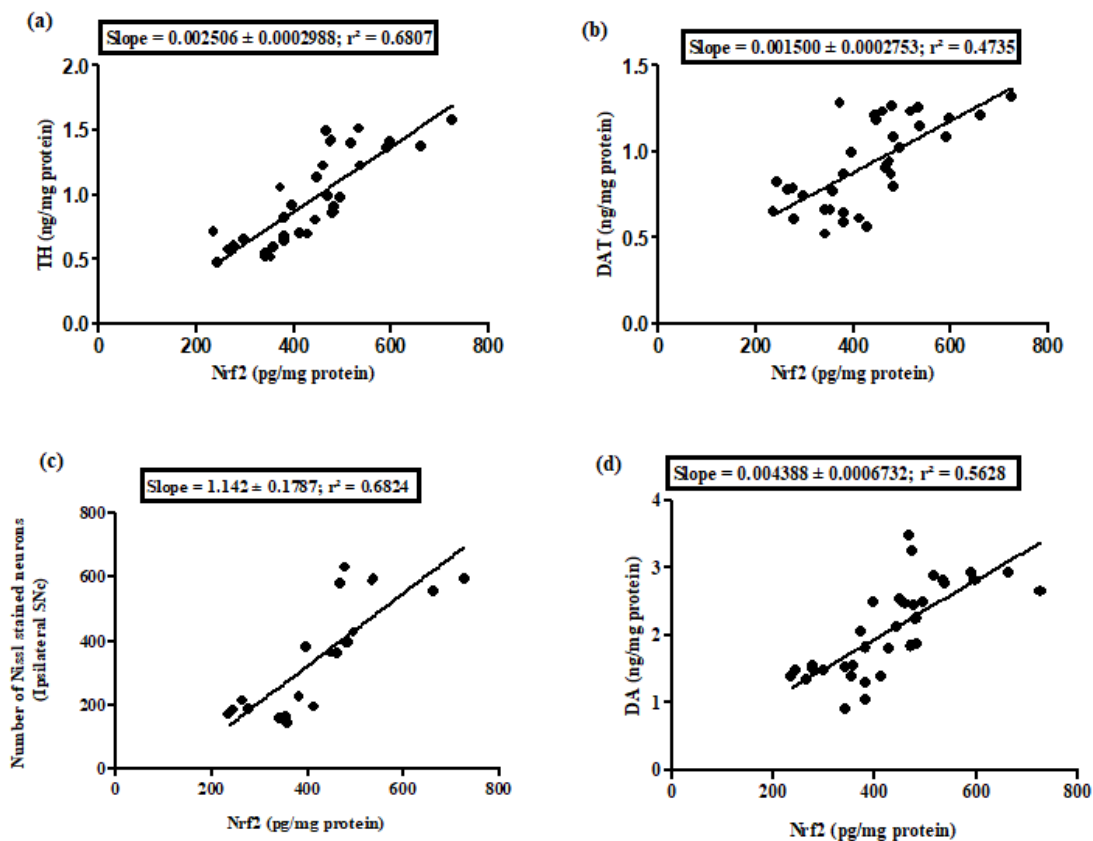**(d)** Slope = 0.004388 ± 0.0006732; r² = 0.5628

Figure 6.11 The correlation between nuclear Nrf2 and PD parameters such as TH levels (a), DAT levels (b), number of Nissl-stained neurons in rat nigral tissues (c) and DA levels (d) [Pearson's correlational analysis at $p < 0.05$].

6.4. Discussion

The current study showed that the activity of rebamipide against 6-OHDA-induced hemiparkinson's model in rats was dependent on Nrf2 activation. Rebamipide at a dose of 80 mg/kg twice a day ameliorated motor deficits, DA deficiency, α-synuclein pathology, mitochondrial dysfunction and GCase deficiency with concomitant increase in endogenous nuclear Nrf2. Antioxidant defense system regulated by Nrf2 consisting of GSH, SOD and CAT along with markers of dopaminergic cell, such as

TH and DAT were also increased. Rebamipide inhibited the nigral cell loss in 6-OHDA-infused rats. Co-administration of Nrf2 inhibitor (Nrf2i; trigonelline) attenuated the effects of rebamipide on 6-OHDA-induced dopaminergic toxicity.

PD patients show motor deficits like tremor, loss of grip strength and gait (Vervoort et al., 2016). Behavioral tests were performed to measure the motor activity in rats such as, catalepsy, grip strength, rotarod, apomorphine-induced head rotation and open-field tests. In the present study, 6-OHDA-infused animals showed motor deficits from D-7 assessed by increase in apomorphine-induced head rotation (Ungerstedt, 1971). There was decrease in locomotor activity (Van Den Buuse et al., 1986), grip strength score (Kumar et al., 2017) and retention time on rotarod (Rozas et al., 1997). 6-OHDA increased cataleptic behavior (Kumar et al., 2017) and decreased the number of central squares crossed in open field test from D-14. 6-OHDA-induced motor deficits were found to be progressive till D-28. Thereafter, rebamipide-administration alleviated the motor deficits progressively in 6-OHDA-infused rats indicating its anti-PD action, as discussed in Chapter 5 also (Mishra and Krishnamurthy, 2019).

Rebamipide is reported to act through activation of nuclear Nrf2 in collagen-induced arthritis model both *in vivo* and *in vitro* (Moon et al., 2013; Moon et al., 2014) and reflux esophagitis model *in vivo* (Song et al., 2016). Reduction in Nrf2 activity with aging contributes to the PD progression (Gan and Johnson, 2014). Therefore, Nrf2 activity may be involved in the anti-PD mechanism of rebamipide. To confirm this possibility, pharmacological evalaution was done by the simultaneous administration of Nrf2 inhibitor (Nrf2i; trigonelline) (Arlt et al., 2013) with rebamipide. When Nrf2i was given in 6-OHDA-infused rats, it did not aggravate

the effects of 6-OHDA in any of the parameters in present study. This is probably due to ceiling effect of 6-OHDA-induced dopaminergic toxicity (Sauer and Oertel, 1994). The same was reported earlier with another PD toxin MPTP, where Nrf2i given post-treatment did not further increase MPTP-induced motor deficits in the rodent model of PD (Gaur et al., 2013). Grooming signifies displacement behavior (Smolinsky et al., 2009) and rearing is interpretated as directed exploration in the adult phase of life (Coronel-Oliveros and Pacheco-Calderón, 2018; Lever et al., 2006). Ambulation shows the exploratory behavior of rats (Lamprea et al., 2003). Catalepsy indicates fine motor control including akinesia (Walther and Strik, 2012; Whishaw et al., 1990). Open field parameters (Qian et al., 2010), bar cataleptic behavior (Walther and Strik, 2012; Whishaw et al., 1990) and grip strength test (Pradhan et al., 2010) denote fine motor skills. Gross motor coordination is characterized by rotarod test (Qian et al., 2010; Rozas et al., 1997). Co-administration of Nrf2i significantly inhibited rebamipide-induced attenuation of behavioral deficits including rotational behavior and some of the fine motor skills, such as catalepsy, grip strength and numbers of central squares crossed in open field test against 6-OHDA-infused rats. Moreover, rebamipide-induced attenuation of remaining behavioral deficits against 6-OHDA-infused rats was completely blocked by Nrf2i. These motor deficits include gross motor skills characterized by rotarod retention time and fine motor skills, including open field related parameters, such as number of ambulation, grooming and rearing. This indicates the role of Nrf2 behind the pharmacological action of rebamipide against 6-OHDA toxicity.

PD is also considered as TH-deficiency syndrome. Tyrosine is a primary amino acid, involved in the biosynthesis of DA and gets converted to L-DOPA due to

the action of TH enzyme (Haavik and Toska, 1998). DA depletion in the nigrostriatal region is involved in the motor dysfunction of PD. Therefore, TH-deficiency is used to signify the presence of dopaminergic cells in various studies (Voutilainen et al., 2017). In the current study, there is marked reduction of TH and DA in the ipsilateral nigral tissues after the intrastriatal injection of 6-OHDA in rats (Mirfakhrai et al., 2017). Rebamipide significantly increased TH and DA levels suggesting the increased availability of dopaminergic cells in the nigral region. Extracellular DA content is maintained due to DA release from SNc to striatum and indicated by severe decrease of DAT in PD cases (Gainetdinov et al., 1998; Nutt et al., 2004). 6-OHDA decreased DAT levels in the ipsilateral striatal tissues of rats as reported earlier (Chotibut et al., 2012). Rebamipide increased DAT levels in 6-OHDA-infused group. This suggests increased viability of dopaminergic cells (Gainetdinov et al., 1998). However, Nrf2i co-administration with rebamipide inhibited the rebamipide-induced increase in TH, DA and DAT levels in 6-OHDA-infused rats. This indicates the involvement of Nrf2 activity in maintaining homeostasis of dopaminergic system by rebamipide.

Nrf2 is present in the cytosol bound to its inhibitor kelch-like ECH-associated protein (Keap1), an adaptor for the cullin-3-based E3 ligase (Gan and Johnson, 2014). Under normal unstressed condition, the ubiquitylation of Nrf2 is promoted in a constitutive manner and leads to Nrf2 degradation (half-life ~20 min) in the cytoplasm by ubiquitin-proteasome system (E3-ubiquitin ligase-like domain of Keap1) followed by 26S proteasomal degradation. Therefore, Keap1 regulates Nrf2 negatively by promoting Nrf2 sequestration and degradation. In the case of PD, during oxidative stress, elevated amount of ROS are produced by mitochondria.

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Keap1 inhibition by stress signals disrupts the interaction between Keap1 and Nrf2 in the cytoplasm, thus stimulating Nrf2 translocation into the nucleus (Bryan et al., 2013). Nrf2 deficiency exasperates experimental parkinsonism and Nrf2 activation is reported to protect DA neurons from 6-OHDA and MPP+ (1-methyl-4-phenylpyridinium) toxicity, both *in vitro* and *in vivo* (Tufekci et al., 2011). In present study, 6-OHDA decreased the concentration of nuclear Nrf2 in nigral tissues as reported earlier (Ryu et al., 2013). In normal cases, Nrf2 is present in cytoplasm and gets translocated to nucleus during PD pathology; therefore Nrf2 becomes nuclear in nigral neurons in the brains of PD patients (Ramsey et al., 2007). Nuclear Nrf2 was increased by rebamipide whereas Nrf2i significantly abolished rebamipide-induced increase in Nrf2 against 6-OHDA-infused rats. These results indicate that Nrf2 is responsible for the pharmacological action of rebamipide.

Nrf2 translocation to the nucleus activates the antioxidant defense system (Gan and Johnson, 2014; Tufekci et al., 2011). Decreased Nrf2 transcriptional activity caused age-related loss of GSH synthesis (Gan and Johnson, 2014). GSH acts as high capacity detoxification agent and maintains the cellular redox balance as well as protects against oxidative damage (Sofic et al., 1992). Reduced glutathione was found to be decreased in the nigral region of parkinsonian patients. The enzymatic activity of free radical scavengers regulated by Nrf2 such as, CAT and SOD are also decreased in PD patients (Ambani et al., 1975; Gan and Johnson, 2014; Zhu et al., 2005). In the present study, 6-OHDA decreased SOD, CAT and GSH in rat nigral tissues (Kumar et al., 2012; Kumar et al., 2017). Rebamipide administration showed antioxidant effects in 6-OHDA model as observed from increased activity of antioxidant enzymes SOD and CAT in nigral tissues. This may be due to the

conversion of toxic ROS into H_2O_2 by SOD, and later into water and molecular oxygen by CAT (Aebi, 1984; Kakkar et al., 1984). Rebamipide also increased GSH in 6-OHDA-infused rats. However, in the presence of Nrf2i, rebamipide-induced increment in SOD, CAT and GSH was not observed up to similar extent as that of rebamipide alone against 6-OHDA-infused rats, showing the involvement of Nrf2 in the antioxidant activity of rebamipide to alleviate 6-OHDA toxicity.

Overexpressing transcription factor Nrf2 is reported to increase lifespan and decreased α-synuclein aggregation in αSynA53T transgenic mice due to Nrf2-mediated stimulation of the lysosomal degradation of misfolded α-synuclein (Gan et al., 2012; Manecka et al., 2017). Therefore, Nrf2 overexpression delayed α-synuclein-mediated dopaminergic neuronal loss and motor dysfunction in *Drosophila* model of PD with α-synuclein (Barone et al., 2011). In the present study, 6-OHDA decreased the concentration of soluble α-synuclein, as discussed in Chapter 4 and 5 (Mishra and Krishnamurthy, 2019). Soluble α-synuclein with monomeric structures converts into insoluble oligomeric aggregates during toxic conditions and aging (Budi et al., 2012). 6-OHDA has been reported to cause aggregation of α-synuclein oligomers in rat model of PD in earlier studies (Gu et al., 2016). In present study, rebamipide increased the soluble α-synuclein in the ipsilateral nigral tissues of 6-OHDA-infused rats, indicating reduced aggregation of insoluble α-synuclein oligomers (Budi et al., 2012; Mishra et al., 2018; Mishra and Krishnamurthy, 2019). This was mediated through Nrf2 because Nrf2i administration abolished rebamipide-induced increase in soluble α-synuclein concentration, indicating accumulation of α-synuclein oligomeric aggregates against 6-OHDA-infused rats.

α-synuclein oligomeric aggregates are reported to inhibit GCase enzymatic function by inhibiting GCase trafficking from ER to Golgi apparatus and finally to lysosome (Mazzulli et al., 2011). In the present study, GCase enzymatic activity was decreased by 6-OHDA (Mishra et al., 2018), and increased by rebamipide (Mishra and Krishnamurthy, 2019). Involvement of Nrf2 was confirmed by the inhibitory effects of Nrf2i on rebamipide-induced increase in GCase activity against 6-OHDA-infused rats. GCase inhibition is also involved in triggering the accumulation of α-synuclein oligomers (Cleeter et al., 2013) and both of these increase mitochondrial dysfunction (Cleeter et al., 2013; Di Maio et al., 2016). Altered glutathione metabolism due to deregulation of Nrf2 is also associated with impaired mitochondrial bioenergetics (Gan and Johnson, 2014; Raza and John, 2012). Mitochondrial complex I deficiency is widely found in SNc region of PD patients (Cuadrado et al., 2009). In the present study, 6-OHDA being mitochondrial toxin decreased mitochondrial complex I activity (Glinka and Youdim, 1995) which was significantly increased by rebamipide indicating proper regulation of oxidative phosphorylation because either complex-I or complex-II are responsible for the entry of electrons in mitochondrial inner membrane (Kayser et al., 2004; Mishra and Krishnamurthy, 2019). Administration of Nrf2i abolished the pharmacological activity of rebamipide on mitochondrial complex I enzyme activity, suggesting the role of Nrf2 in the mitochondrial mechanism of rebamipide. Mitochondrial impairment also leads to reduction in GCase enzymatic activity in SNc and cerebellum of sporadic PD patients (Gegg et al., 2012). The regulation of mitochondrial biogenesis has also been reported through Nrf2 (Holmström et al., 2016), supporting the present study.

Mitochondrial dysfunction is followed by apoptosis (Elmore, 2007). 6-OHDA decreased the number of Nissl-stained cell bodies upto 70% in SNc. Rebamipide administration in 6-OHDA-infused animals increased Nissl-stained cell bodies (68%) compared to 6-OHDA group. Nigrostriatal dopaminergic pathway comprises of SNc and striatal regions which contain dopaminergic cell bodies and axons respectively (Dauer and Przedborski, 2003). Nissl's staining in SNc indicates the density of dopaminergic neurons (Domesick et al., 1983; Zaitone et al., 2012); therefore, rebamipide-induced increase in Nissl-stained cell bodies against 6-OHDA toxicity denotes the high numbers of DA neurons in nigral tissues. This effect was inhibited by the use of Nrf2i (trigonelline), which is previously reported to inhibit the cytoprotective effect and cell viability induced by dimethyl fumarate against MPTP toxicity in SH-SY5Y cells (Campolo et al., 2017). Hence, Nrf2 plays an important role in increasing the number of nigral cells by rebamipide in present study. However, the stereological assessment of TH neurons along with counterstaining with Nissl can measure specific dopamine neurons. The current study proves that nuclear Nrf2 activity is involved in in attenuating 6-OHDA-induced neurotoxicity by rebamipide in animal model of PD.

6.5. Conclusions

Rebamipide-induced attenuation of 6-OHDA toxicity was inhibited by co-administration of Nrf2i in rats. This can be observed by attenuation of rebamipide-induced increase in SOD and CAT activity, GSH, and TH levels by Nrf2i against 6-OHDA-infused rats. Additionally, co-administration of Nrf2i completely blocked the action of rebamipide against 6-OHDA-induced motor and some non-motor deficits.

This includes rotarod retention time, open field related parameters, DAT levels, nuclear Nrf2, soluble α-synuclein concentration, GCase activity, mitochondrial complex-I activity, DA levels and numbers of nigral Nissl bodies. It indicates the involvement of Nrf2 activity in rebamipide-induced anti-PD effect against 6-OHDA toxicity. Additionally, significant statistical correlation was observed between nuclear Nrf2 and 6-OHDA-induced motor deficits. A statistical positive correlation was also observed between 6-OHDA-induced pathological parameters and nuclear Nrf2. Hence, the present study demonstrates that rebamipide acts as Nrf2 activator and exhibited anti-PD like effects to strengthen dopaminergic system, as shown in **Figure 6.12.**
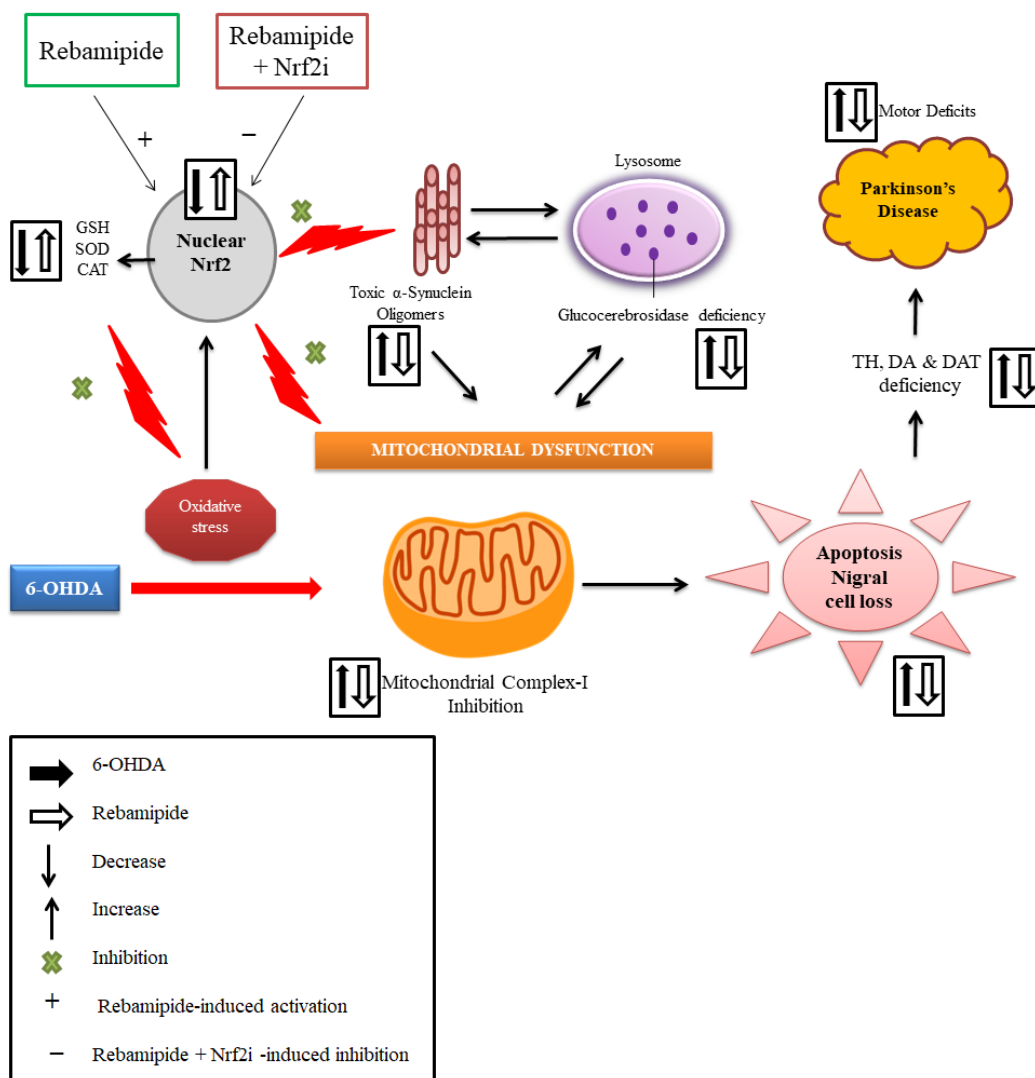
Figure 6.12 The outcome of specific objective for the role of Nrf2 activity in rebamipide-mediated changes against 6-OHDA toxicity in rats. Co-administration of Nrf2i inhibits rebamipide-induced attenuation of 6-OHDA toxicity. Nrf2 regulates ARE, which includes GSH, SOD and CAT to counterbalance the overload of oxidative stress. Nrf2 inhibition promotes α-synuclein aggregation. Nrf2 also takes part in the regulation of mitochondrial biogenesis. Rebamipide increases the levels of Nrf2, GSH, SOD, CAT, TH, DA, DAT, soluble α-synuclein, numbers of nigral Nissl bodies, and activities of GCase enzyme along with mitochondrial complex-I. This leads to improved motor behavior by rebamipide. However, Nrf2i co-administration inhibits rebamipide induced changes, indicating the involvement of Nrf2 activity in rebamipide-induced anti-PD effect against 6-OHDA toxicity.