Carbenoxolone Exerts Neuroprotection in an Animal Model of Parkinson's Disease Induced by Proteasome Inhibitor MG-132

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Abstract

Dysfunctions of ubiquitin proteasome system (UPS), intracellular protein degradation mechanism lead to the aggregation of aberrant proteins, which is a hallmark of Parkinson's disease (PD). Heat shock proteins (HSP) assist in refolding of misfolded proteins and in proper functioning of UPS. Thus, the neuroprotective potentials of carbenoxolone (Cbx), a HSP inducer were evaluated in a PD model generated by a single intranigral injection of a proteasome inhibitor MG-132 into rat brains. Cbx (20 mg/kg body weight) was given as post-treatment for 12 days. MG-132 destroyed dopaminergic neurons causing loss of dopamine and tyrosine hydroxylase (TH) that resulted in impaired motor functions. Elevations in the markers of oxidative stress like LPO (Lipid peroxidation), protein carbonyl, NO (Nitric oxide) and citrulline was also observed in MG-132 treated animals. However, induction of HSP-70 by Cbx helped to combat the toxicity caused by proteasome inhibition. Reduction in oxidative stress was also observed after Cbx co-treatment which helped in preventing neuronal cell death. As a result, improvements in the dopamine levels and associated motor functions were also observed. Thus, all the assessed parameters provide the clear evidence that Cbx is a potential neuroprotector for PD.

Keywords: UPS dysfunction; Carbenoxolone; Oxidative stress; Motor functions.

1. Introduction

Parkinson's disease (PD) is second most common age related neurodegenerative disorder that is highly prevalent in people over the age of 50 [1-2]. Pathologically, it is characterized by the specific loss of dopaminergic neurons within the substantia nigra region. The clinical symptoms include tremors at rest, rigidity, slowness or

absence of voluntary movement, postural instability, and freezing [3]. Another crucial pathological hallmark of PD involves the accumulation of fibrous protein deposits known as Lewy bodies in the neuronal cytoplasm [4]. These deposits interfere with normal neuronal functions, protein-protein interactions and protein homeostasis [5].

Excessive free radical production and impaired anti-oxidant mechanism in the neurons lead to the protein modifications [6]. These alterations of cellular proteins cause them to transform into misfolded forms, which get accumulated in the neurons [7]. Usually the protein aggregation is rectified by the protective system of the cells known as ubiquitin proteasome system (UPS) [8-9]. However, during PD, diminished expression of proteasome subunits and alterations in the proteasome subunits has been reported, which in turn inhibits its activity [10]. Due to the proteasome inhibition, fewer proteins are degraded in the proteasome core, which leads to the buildup of harmful cross-linked proteins and ultimately formation of Lewy bodies [11].

HSPs or molecular chaperones assist in folding and refolding of nascent polypeptide, or partially denatured proteins [12]. When moderately over-expressed in cells, HSPs exhibit beneficial effects in the pathological conditions associated with the protein misfolding and aggregation [12]. HSPs aid in the refolding of damaged and misfolded proteins and thus suppress their aggregation [13-15]. As a result, they exert cytoprotective action during proteotoxic stress [16-18].

Present study was designed with the aim to evaluate the beneficial effects of HSP induction in an animal model of PD. There are several type of HSP inducers reported in the literature, which include non-steroidal anti-inflammatory drugs, HSP-90 inhibitors, prostaglandins, anti-ulcer drugs, and some herbal medicines [19-21]. Carbenoxolone (Cbx) is one such drug that acts as HSP inducer [22-24]. It has been reported to induce the heat shock protein-70 (HSP-70) [25], HSP-40 and HSP-27 [26]. Due to the central role of proteasome dysfunctions in PD pathogenesis, we used a semi-synthetic proteasome inhibitor

MG-132 to induce the toxicity in the nigral dopaminergic neurons in the male SD rats. In comparison to the other proteasome inhibitors such as lactacystin and bortezomib, MG-132 is a cheaper and a potent alternative proteasome inhibitor [27-28]. It effectively escalates the burden of misfolded proteins in the neurons and concomitantly increases the cellular stress [29-32]. Hence, in the current study we evaluated the potential neuroprotective role of Cbx in rat model of PD induced by MG-132.

2. Material and Methods

2.1 Experimental Animals

Healthy male rats of the Sprague Dawley (SD) strain weighing between 200-250 grams and in the age group of 8-9 weeks were purchased from the Central animal house of Panjab University, Chandigarh. Animals were acclimatized in the department animal house for two weeks in polypropylene cages under hygienic conditions and were provided standard animal feed and water ad libitum throughout the treatment period. All procedures were done in accordance with the ethical guidelines for care and use of laboratory animals and were approved by Institutional Animal Ethics Committee (IAEC), Panjab University Chandigarh, India. The standard animal feed was obtained from Aashirwad industries, Punjab, India.

2.2 Chemicals

MG-132 was purchased from Calbiochem and Cbx was purchased from Sigma (St. Louis, MO). Chemicals for HPLC were procured from Merck. Other chemicals were procured from local suppliers (SRL, Hi-Media and CDH).

2.3 Experimental design

Animals are randomly allocated in four different groups (6-8 animals per group). Group 1 animals served as sham. They were unilaterally administered 2 µl of DMSO dissolved in PBS (0.1 M, pH 7.0) into the substantia nigra. Group 2 animals were injected with MG-132 (0.01 mg in 2µl of DMSO dissolved in 0.1M PBS) into the substantia nigra. Group 3 animals were given a combined treatment of MG-132 as described

before and followed by Cbx (20 mg/kg body weight i.p) administration for duration of 12 days. Group 4 animals were given Cbx alone. While the stereotaxic injection was given only once, Cbx treatment was given everyday for a period of 12 days. For the surgeries, rats were anesthetized with the combination of ketamine (80 mg/kg body weight i.p) and xylazine (20 mg/kg body weight i.p). The unilateral injection of either MG-132 dissolved in DMSO or DMSO alone dissolved in 0.1M PBS into the substantia nigra (AP 4.01mm, ML 1.5mm, DV 7.7mm) was then given on the right side of the brain.

Various behavioral assessments were carried out at regular intervals during the study period. First behavioral assessments were carried out 2 days after the stereotaxic surgery of the animals, second behavioral 8 days after surgery and the last behavioral assessment was done 24 hour before the sacrifice of the animals i.e. on 11th day. After the completion of 12 days, animals were sacrificed and midbrain dissected out for various studies.

2.4 General observation of animals

To study the possible outcomes of the drug treatments, general behavior of the animals such as dietary intake, general body activity, movements, lethargy, stiffness or rigidity was monitored. The mortality rate of the rats was also recorded.

2.5 Behavioral analysis

2.5.1 Catalepsy (Bar Test)

The bar test was used for measuring the rigidity in the rats [33]. In the bar test, rats were placed with both front paws on the horizontal bar which was 9 cm above and parallel from the base and time recorded with a stopwatch. Whenever the animals removed one paw off the bar, the time was noted. The maximum cut off for bar test was fixed at 180 seconds.

2.5.2 Measurement of locomotor activity (Actophotometer)

A computerized actophotometer was used to measure the total locomotor activity (ambulations and rearing) of the rats (IMCORP, India). An array of 16 infrared emitter/detector pairs measured animal activity along the single axis of motion and the digital data was displayed on the front panel meters as ambulatory movements. Rats were allowed to acclimatize in the observation chamber for a period of 5 minutes and thereafter activity was monitored continuously for a period of 5 minutes. Locomotion was expressed in terms of total photo beam counts per 5 minutes [34].

2.5.3 Rotarod test

Rota-rod test was used to evaluate the effects of drug treatments on the motor balance and co-ordination of the rats. All rats were given two initial training trials of 300 sec, approximately 10 min apart, to maintain posture on the rota-rod. Bar was 3 cm in diameter and rotation speed was fixed at 20 revolutions per minute. After the initial training trials, a baseline trial of 120 sec was conducted. The time for which each animal remained on the rota-rod was recorded [35].

2.6 Neurotransmitter analysis (HPLC)

Biogenic amines (Dopamine, DOPAC, HVA) were estimated by HPLC by the method of Thakur and Nehru [36]. Analysis was done by using Waters HPLC system equipped with ECD on a C18 reverse phase column. Data was recorded and analyzed with the help of Empower software. Mobile phase consisting of 32 mM NaH_2PO_4, 10 mM sodium citrate, 0.025 mM EDTA and 0.77 mM 1-heptane–sulphonic acid was used and pH of the mobile phase was adjusted to 4.5 with the help of acetic acid. Electrochemical conditions for the experiment were set at +0.800 V, with the sensitivity range of 5-50 nA. Separation was carried out at a flow rate of 0.80 ml/min.

10% homogenates were prepared immediately before analysis in 0.1M perchloric acid and subjected to centrifugation for 20 minutes at 20,000 rpm. The supernatant was further filtered through 0.22 μm nylon mesh and a volume of 20 μl was injected into the column manually. Dopamine turnover was calculated by the formula DOAPC+HVA/Dopamine. All the chemicals used were of HPLC grade and procured from Merck, India. Standards for

dopamine, DOPAC and HVA were procured from Sigma– Aldrich (St. Louis, MO, USA).

2.7 Oxidative stress markers

2.7.1 Lipid peroxidation

MDA is considered as the most informative marker of endogenous intoxication and reflects the degree of oxidative stress [37]. Lipid peroxidation was estimated by the method described by Wills [38]. 0.5 ml of 10% tissue homogenate was added to 0.5 ml Tris HCl buffer. It was incubated at 37° C for 2 hours and 1 ml of cold TCA was added to the reaction mixture. Thereafter, samples were mix thoroughly and centrifuged at 800 g for 10 min. Supernatant was boiled for 15 min with thiobarbituric acid (TBA). The MDA concentration was measured at 532 nm using an extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$. The results were expressed as nM MDA/ mg protein.

2.7.2 Protein carbonyl

It was estimated by method of Burcham [39] using DNPH-(2, 4 dinitrophenylehydrazine). 0.5 ml protein sample was added to reaction mixture containing 0.5 ml of DNPH solution and 2 M HCl. For blank only 2 M HCl was added. Afterwards, the reaction was allowed to proceed at room temperature for 1 hour. Then 500 µl of 20% TCA was added to the mixture and centrifuged for 3 min. After discarding the supernatant, pellet was washed thrice with 1 ml volumes of ethanol:ethyl acetate (1:1). Pellet was re-suspended in 0.6 ml guanidine solution and allowed to dissolve for 15 min at 37° C. Insoluble material was removed by centrifugation. Carbonyl concentration was measured at 370 nm using extinction coefficient 22000 $M^{-1}cm^{-1}$ and the results were expressed as nM of carbonyl/mg protein.

2.7.3 Nitric oxide (NO)

Nitric oxide was estimated by the method of Raddassie [40]. 100 µl of sample and 100 µl of Griess reagent were added in the wells of the ELISA strip. It was incubated for 30 min in dark at room temperature. Thereafter absorbance was read at 540 nm on ELISA reader. Nitric oxide

concentration was determined by plotting a linear standard curve for sodium nitrate with a concentration range of 2.5-20 mM. The result was expressed as µM of NO_2/mg protein accumulated in the sample.

2.7.4 Citrulline

Citrulline was estimated by the method of Boyde and Rahmatullah [41]. 40 µl of sample was added to the 50 µl of 30% $ZnSO_4$ mixed well and then centrifuged at 2000 rpm for 10 min. 40 µl of the supernatant was mixed with 460 µl 0.1 N HCl and 1.5 ml freshly prepared chromogenic solution. It was boiled in water bath for 5 min at 100° C and absorbance was measured at 530 nm. The result was expressed as µM of citrulline/mg protein.

2.8 Antioxidant defense system

2.8.1 Catalase

The catalase activity was estimated by measuring the breakdown of hydrogen peroxide at 240 nm according to the method of Luck [42]. The catalase activity was expressed as mM of H_2O_2 used/min/mg of protein.

2.8.2 Superoxide dismutase

Superoxide dismutase activity was estimated by the method of Kono [43] which is based on the reduction of nitrobluetetrazolium mediated by superoxide anions (generated by photo-oxidation of hydroxylamine) to form formazan at 560 nm. The activity of superoxide dismutase was expressed as International Units per mg protein (IU/mg protein) where 1 IU is defined as the amount of enzyme inhibiting the increase in optical density by 50 %.

2.8.3 Reduced glutathione (GSH)

GSH was estimated as the total non-protein sulfhydryl groups by the method described by Moron [44]. In this method 5,5'-disthiobis-(2-nitrobenzoic acid) (DTNB) is reduced by the -SH groups of GSH to form one mole of 2-nitro-5-mercaptobenzoic acid per mole of –SH. The nitromercaptobenzoic acid anion released has an intense yellow color, which can be used to measure –SH groups at 412 nm. 10%

homogenates in phosphate buffer were precipitated with 100 µl of 25% TCA and the precipitate was removed by centrifugation at 1500 g for 10 minutes. 200 µl of the supernatant was added to 800 µl of phosphate buffer to which 2 ml of DTNB (0.6 mM prepared in 0.2 M phosphate buffer) was added. The absorbance of yellow color complex was read at 412 nm. GSH was used as a standard to calculate the content of GSH which is expressed as µM of GSH/mg of protein.

2.9 Western blots

Immunoblots for tyrosine hydroxylase and HSP70 were performed following the protocols of Thakur and Sanyal [45]. Mid brain region was used to prepare the 20% homogenate in the lysis buffer and centrifuged at 10,000 rpm for 30 min. The resulting supernatants were collected for the protein estimation and analyzing the expressions of HSP-70 (Santa Cruze, 1:1000). 50 µg of protein were resolved on 10% SDS gel (SDS-PAGE) and transferred on to nitrocellulose membrane. The blots were also performed with beta-actin antibody (Sigma Chemicals, St. Louis, MO, 1:5000) to ensure uniform protein loading.

2.10 Histological analysis

Histological analysis of mid-brain sections was done with hematoxylin and eosin staining [46]. Brain tissue was fixed in formaldehyde and embedded in paraffin wax [45]. Coronal sections of 5 µm thickness were cut and picked over albumin coated slides. Sections were then stained with hematoxylin and eosin using standard procedure. Slides were cleared in xylene and finally mounted in DPX.

2.11 Statistical analysis

The results were expressed as mean ± S.D. and were analyzed with the help of SPSSv14 computer software. One way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc analysis was applied to the results. For behavioral test, a multivariate ANOVA with LSD post hoc analysis was applied. A p-value less than or equal to 0.05 was considered to be statistically significant.

3. Results

3.1 Behavioral analysis

3.1.1 Total locomotor activity

A significant decrease ($p<0.05$) in the total locomotor activity of MG-132 treated group was observed in comparison to the control group at 2nd, 8th and 11th day of the experimental study. However, post-treatment of Cbx resulted in a significant improvement ($p<0.05$) in the total locomotor activity in comparison to the animals treated with MG-132 (Fig. 1.A).

3.1.2 Rota-rod test

MG-132 administration was able to induce any significant decline ($p<0.05$) in the rota-rod score as compared to the control at 8th and 11th day of the study (Fig. 1.B). However, at 11th day of study a significant increase ($p<0.05$) was observed in rota-rod score after Cbx post-treatment when compared to MG-132 group. It was noteworthy that all the surgically treated animals (including control) had a significantly lower rotarod score 2 days post-surgery in comparison to the Cbx group where no surgery was done. This observation indicates that surgery itself has some effects in the motor performance of animals. However, as the animals recovered with time, the rotarod score was comparable across various groups.

3.1.3 Catalepsy (Bar Test)

There was a significant increase ($p<0.05$) in the rigidity as indicated by catalepsy score in MG-132 treated group when compared to control group (Fig. 1.C). Prominent effects were observed 8 days after the MG-132 administration and approximately seven-fold increase was seen in cataleptic score at the 11th day. However, Cbx post-treatment significantly inhibited the increase ($p<0.05$) in the cataleptic behavior in comparison to the MG-132 group at the 11th day of the experiment (Fig. 1.C).
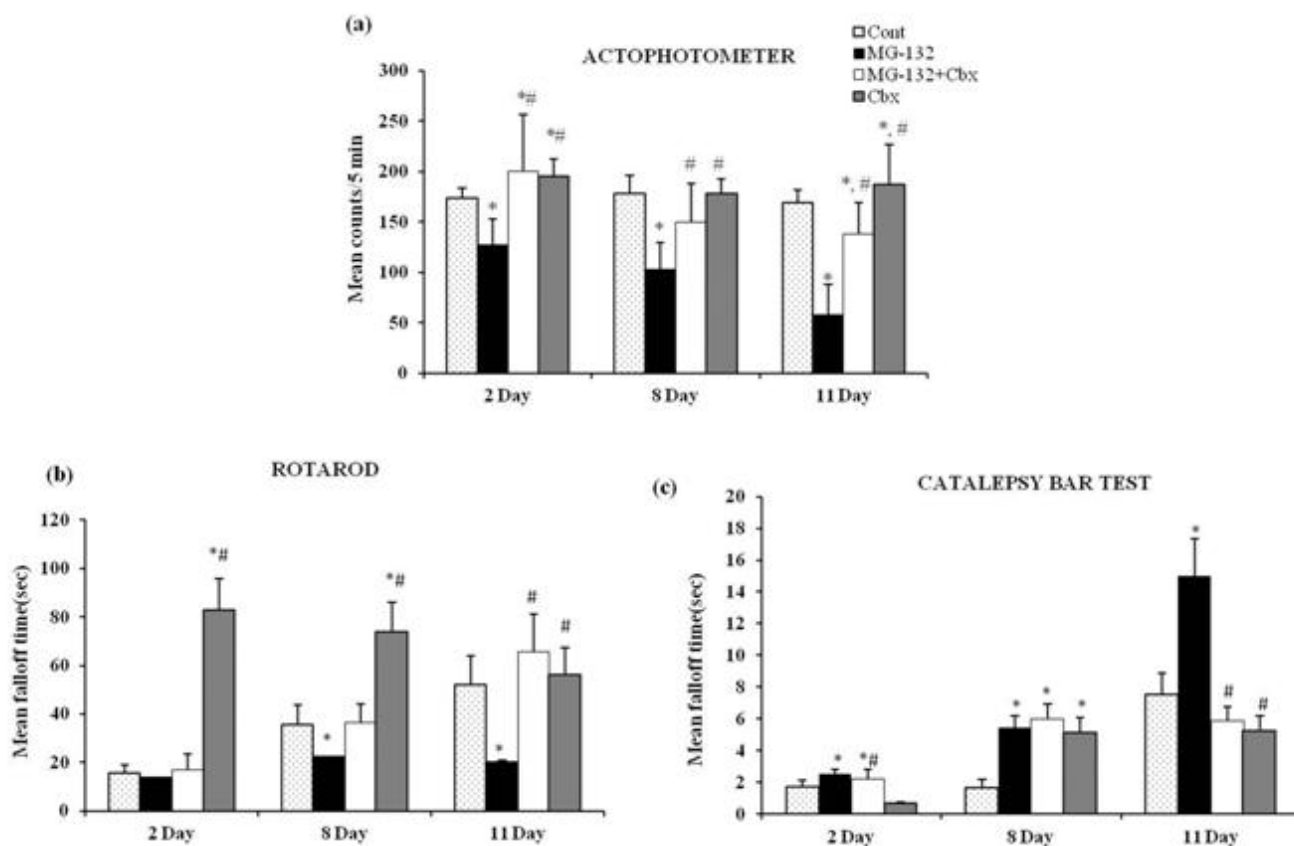
Figure 1 – Effect of various drug treatments on motor functions of animals
Effect of MG-132, MG-132+Cbx and Cbx on (A) Total locomotor activity (using actophotometer) (B) muscle relaxant activity (rota-rod test) and (C) Cataleptic behavioural (bar test) of rats. Values are Mean±S.D. of 6 animals. *$p<0.05$ significant as compared to control of 2nd, 8th and 11th days observation of respective treatment group, #$p<0.05$ significant as compared to MG-132 treated group of 2nd, 8th and 11th days observation of respective treatment group.

3.2 Neurotransmitter Analysis

MG-132 administration resulted in a significant decrease ($p<0.01$) in the dopamine levels as compared to the control counterpart (Fig. 2.A). However, Cbx post-treatment was able to prevent this decline and significantly higher levels of dopamine ($p<0.001$) were observed as compared to MG-132 group. In addition, a significant decrease ($p<0.01$) in the DOPAC levels (Fig. 2.C) along with a significant increase ($p<0.001$) in the HVA levels (Fig. 2.B) was observed in MG-132 treated animals when compared to the control group. On the other hand, Cbx post-treatment showed significantly decreased ($p<0.05$) HVA levels and significantly increased ($p<0.01$) DOPAC levels in comparison to the MG-132 treated group. However, Cbx alone treatment showed significant increase ($p<0.001$) in dopamine levels compared to MG-132 animals. In addition, a significant decrease ($p<0.05$) in the HVA levels and a significant increase ($p<0.01$) in DOPAC levels of Cbx alone treated group was seen when compared to the MG-132 treated group. Overall, MG-132 treated group exhibited significantly higher ($p<0.01$) dopamine turnover when compared to sham group (Fig. 2.D). Similarly, Cbx post-treated ($p<0.01$) and Cbx alone ($p<0.001$) treated group showed lower rate of dopamine turnover when compared to MG-132 group.

3.3 Histopathological analysis and Tyrosine Hydroxylase (TH)

Histopathological examinations were performed in hematoxylin and eosin stained section in which cell shrinkage and degeneration

of neurons in SN of mid brain region of MG-132 treated group was revealed (Fig. 3.A). A significant reduction (p<0.001) in the number of neurons was clearly seen in the MG-132 treated animals as compared to the control (Fig. 3.B). However, Cbx post-treatment significantly prevented (p<0.001) the loss of neurons in the group as compaered to the animals treated with MG-132. To confirm the loss of dopaminergic neurons western blot for TH was also done. It was found to be significantly reduced (p<0.001) in MG-132 treated group as compared to sham (Fig. 3.C). Whereas Cbx post-treatment and Cbx alone treatment led to significant increase (p<0.001) in the expression of TH when compared to the MG-132 treated group.
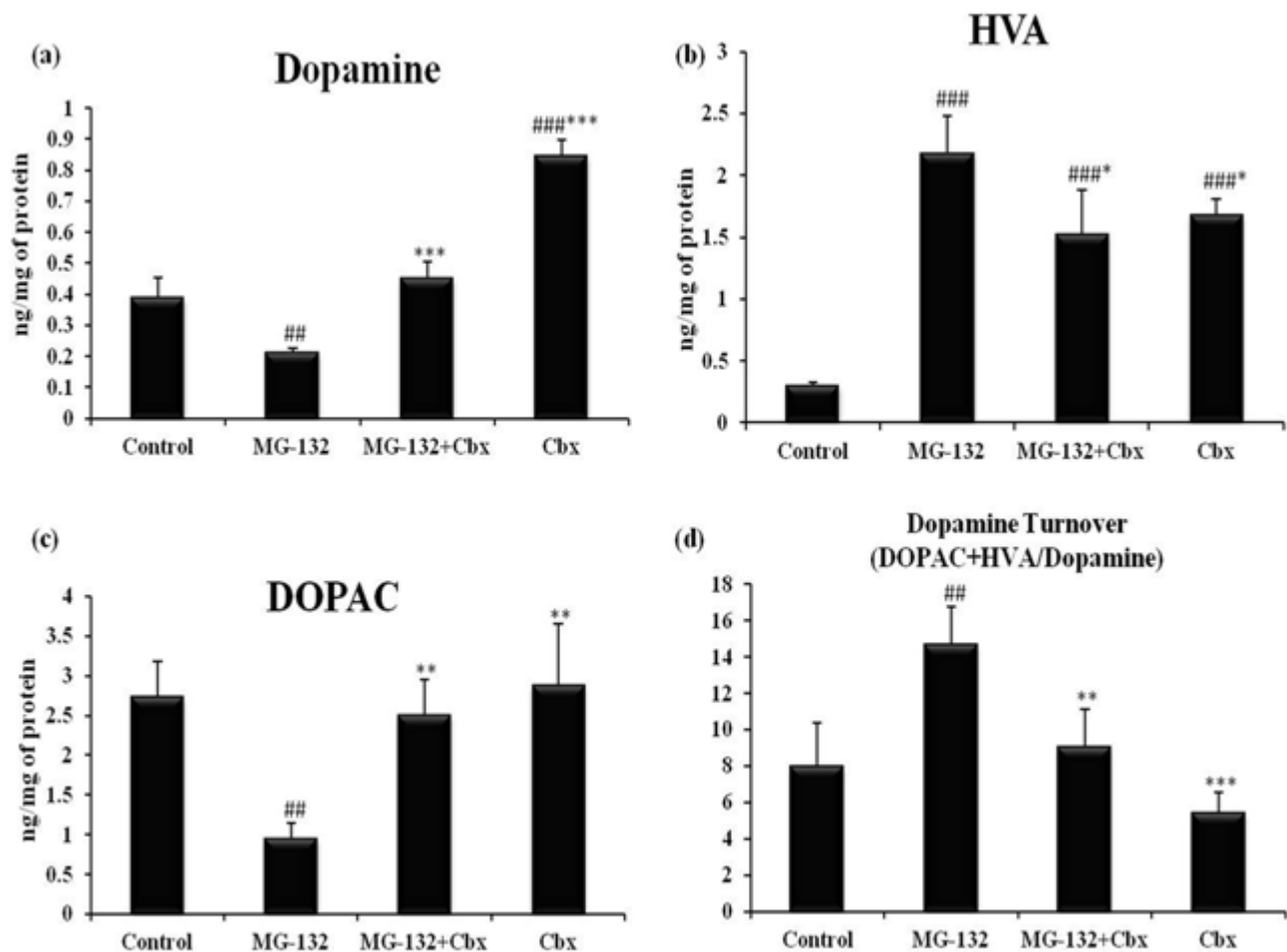
Figure 2 – Alterations in the dopamine metabolism following various drug treatments
Effect of MG-132, Cbx post-treatment and Cbx alone treatment on levels of (A) Dopamine (B) Homovanilic acid (HVA) (C) DOPAC and (D) Dopamine turnover (HVA+DOPAC/Dopamine). Values are Mean±S.D. of 6 animals. [#]p<0.05, [##]p<0.01, [###]p<0.001 significance when compared to sham; *p<0.05, **p<0.01, ***p<0.001 significance when compared with MG-132 treated group.
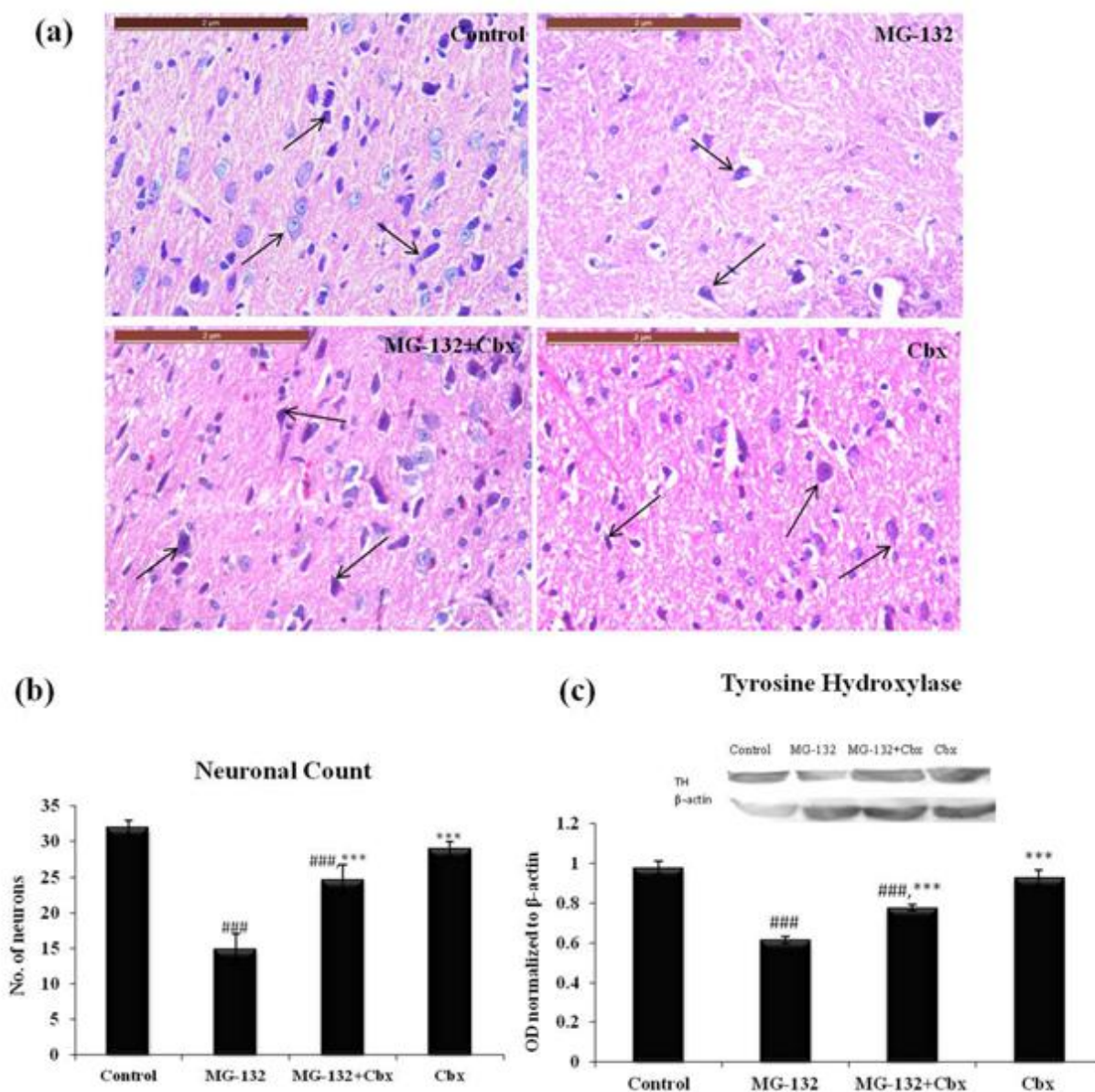
Figure 3 – Changes in the dopaminergic neurons and tyrosine hydroxylase following drug treatments
(A) Histological analysis of cells in the substantia nigra region reveals loss of neurons after MG-132 treatment, which was restored following Cbx. (B) Alteration in the neuronal count in the substantia nigra region after MG-132 and Cbx treatment. (C) Western blot analysis of tyrosine hydroxylase (TH) in the mid brain of rats in the indicated treatment groups, also shows similar results. Values are Mean±S.D. of 3 animals. #p<0.05, ##p<0.01, ###p<0.001 significance when groups compared to sham, *p<0.05, **p<0.01, ***p<0.001 significance when groups compared to MG-132 treated group.

3.4 Oxidative stress markers

3.4.1 LPO and protein carbonyl

Lipid peroxidation and protein carbonyl are the two oxidative stress markers. They indicate the altered levels of the reactive oxygen species in the cellular environment. Levels of MDA (malondialdehyde) in MG-132 treated group was found to be significantly increased (p<0.001) when compared to sham (Fig. 4.A). On the other hand, Cbx supplementation significantly reduced (p<0.001) the MDA levels in comparison to the MG-132 treated animals. Protein carbonyl levels were also significantly high (p<0.001) in the MG-132 group with respect to sham (Fig. 4.B). However, Cbx post-treatment aided in reducing

(p<0.001) the protein carbonyl levels significantly when a comparison was drawn with the MG-132 group.

3.4.2 NO and citrulline

A significant increase (p<0.001) in the levels of the nitric oxide (NO) was observed in MG-132 treated group when compared to the sham (Fig. 4.C). These levels were significantly lowered (p<0.001) in the Cbx post-treated animals as compared to the animals treated with MG-132. Also, a significant increase (p<0.01) in the citrulline levels was observed in the MG-132 treated when compared to the sham (Fig.4.D) whereas in animals subjected to the Cbx post-treatment a remarkable significant decrease (p<0.01) was observed in the levels of citrulline in comparison to MG-132 treated group.
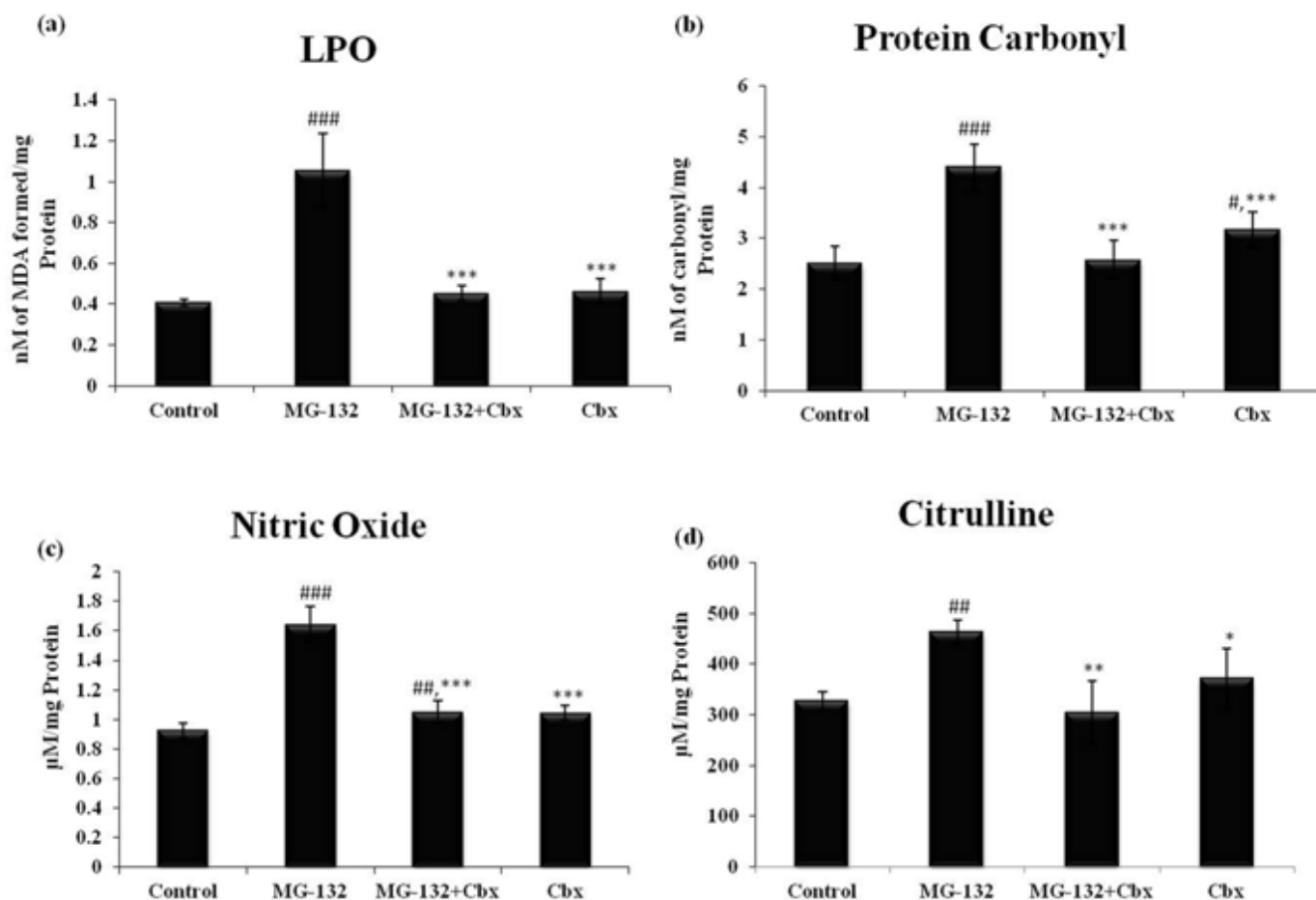
Figure 4 – Alteration in oxidative stress parameters upon drug treatments
Effect of MG-132, Cbx post-treatment and Cbx alone treatment on levels of (A) Lipid peroxidase (LPO) and (B) Protein carbonyl, (C) Nitric oxide (NO) and (D) Citrulline in mid brain of rats. Values are Mean±S.D. of 6 animals. #p<0.05, ##p<0.01, ###p<0.001 significance when compared to sham, *p<0.05, **p<0.01, ***p<0.001 significance when compared to MG-132 treated group.

3.5 Antioxidant defence enzymes

3.5.1 SOD and catalase

The Cu/Zn-SOD activity was significantly increased (p<0.001) in MG-132 treated group as compared to sham (Fig. 5.B). However, Cbx post-treated and Cbx alone treated groups demonstrated significantly lowered (p<0.001) Cu/Zn-SOD activity when compared to the MG-132 treated group. In addition, catalase activity was found to be increased (p<0.001) significantly in MG-132 treated group in contrast to the sham

(Fig. 5.A). Whereas in the case of animals receiving Cbx post-treatment and Cbx alone treatment a significant decrease ($p<0.001$) in the catalase activity in comparison to the MG-132 treated group was observed.
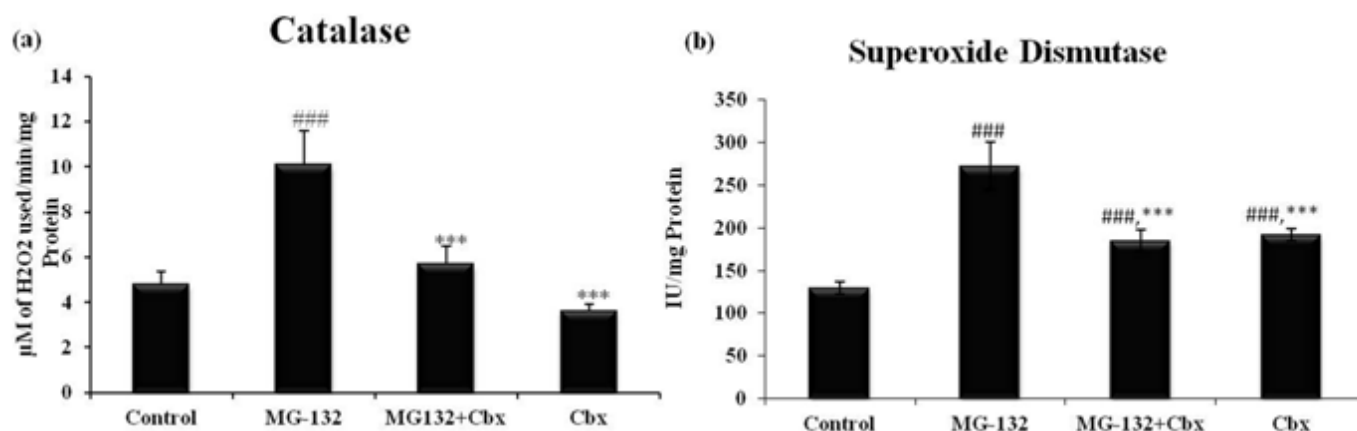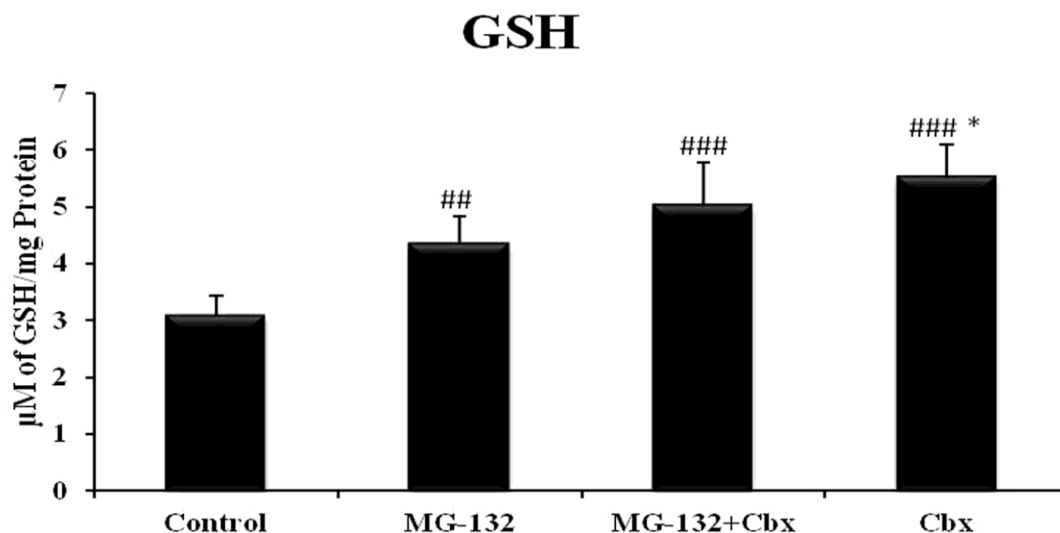

Figure 5 – Effect of drug treatments on the anti-oxidant enzymes
Effect of MG-132, Cbx post-treatment and Cbx alone treatment on levels of (A) Catalase and (B) Superoxide dismutase (SOD) in mid brain of rats. Values are Mean±S.D. of 6 animals. #$p<0.05$, ##$p<0.01$, ###$p<0.001$ significance when compared to sham, *$p<0.05$, **$p<0.01$, ***$p<0.001$ significance when compared to MG-132 treated group.

Figure 6 – Alterations in glutathione homeostasis following various drug treatments
Effect of MG-132, Cbx post-treatment and Cbx alone treatment on levels of GSH in mid brain of rats. Values are Mean±S.D. of 6 animals. #$p<0.05$, ##$p<0.01$, ###$p<0.001$ significance when compared to sham, *$p<0.05$, **$p<0.01$, ***$p<0.001$ significance when compared to MG-132 treated group.

3.5.2 Reduced Glutathione

GSH levels were found to be significantly increased ($p<0.01$) in the MG-132 treated group in comparison to the sham (Fig. 6). Similar results were exhibited by the Cbx post-treated and Cbx alone treated groups when compared to the sham. However, a significant increase ($p<0.05$) in the levels of GSH in case of Cbx alone treated group was observed when compared to the MG-132 treated group (Fig. 6).

3.6 HSP-70

The expression of heat shock protein-70 (HSP-70) was significantly increased ($p<0.001$) in the MG-132 treated group as compared to sham (Fig. 7). Whereas Cbx post-treatment and alone Cbx treatment significantly increased ($p<0.01$, $p<0.001$) the expression of HSP-70 when compared to the MG-132 treated group respectively.
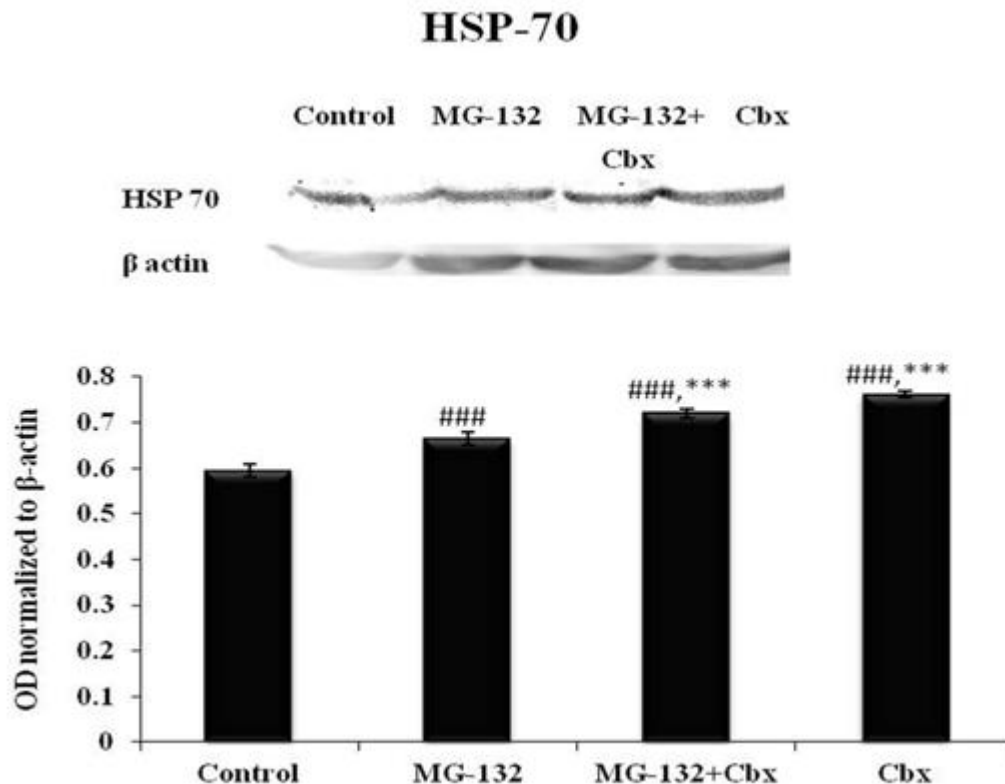
HSP-70

Figure 7 - HSP-70 upregulation following Cbx administration
Western blot analysis of HSP 70 expression in mid brain of rats in indicated treatment groups. Values are Mean±S.D. of 3 animals. [#]$p<0.05$, [##]$p<0.01$, [###]$p<0.001$ significance when compared to sham, *$p<0.05$, **$p<0.01$, ***$p<0.001$ significance when compared to MG-132 treated group.

4. Discussion

Protein misfolding and aggregation is a common pathological characteristic feature of large number of neurodegenerative diseases such as Alzheimer's, Huntington's, and PD. Stimulation of HSP expression has been projected to be beneficial in these disorders due to their ability to assist in the refolding of aberrant proteins [47]. Recently, Cbx has been reported as a HSP inducer in the *in-vitro* studies [48-49, 26]. These HSP inducing effects were also confirmed in our previous in vivo studies [50]. By the virtue of HSP induction, Cbx has shown to exert neuroprotection in protein aggregation associated with PD both in vivo [50] and in vitro [49]. Thereby, we evaluated its potential protective role in the PD model that operates through the inhibition of proteasome system. The PD model was established by intranigral injection of proteasome inhibitor MG-132 that has previously been reported to induce dopaminergic degeneration in the rat brain [51]. Cbx administration at the dose of 20 mg/kg body weight has shown to enhance the expression of various HSPs and subsequently improve the proteasome activity in a rotenone model of PD [50]. Thus, we speculated that it might show neuroprotective effects in the MG-132-based PD model.

A single intranigral injection of MG-132 was able to produce relevant motor dysfunctions that are considered as trademark of PD. Catalepsy, which is a measure of akinesia [52] and immobility [53] was found to increase sharply after MG-132 administration. Further, a substantial decline in the total locomotor activity was also observed in the MG-132 treated group. In addition to catalepsy and total locomotor activity, rotarod test showed a significant decline in the score as compared to sham. Thus, MG-132 induced the akinesia, bradykinesia, rigidity in the animals and motor imbalance in the 12 day duration. The motor dysfunctions arise due to the significant decrease in the dopamine levels, which is the gold hallmark of PD [54]. This depletion of dopamine is an indicator of the loss of dopaminergic neurons.

Cbx post-treatment exhibited a significant improvement in the cataleptic score and total locomotor activity in comparison to MG-132 group. However, improvement was observed in the rotarod score at the later stage of the experiment. In fact, all the groups which underwent surgery (sham, MG-132, and conjunctive group) showed a decline in the rotarod score, which might be due to the effect of the surgery on the animals. Cbx treatment was able to prevent the loss of dopaminergic neurons as demonstrated by a significant increase in the levels of dopamine and TH in Cbx post-treatment group when compared to the MG-132 treated group. Thus, results indicate that Cbx has potency to prevent the degeneration of dopaminergic neurons caused due to the attack of proteasome inhibitor.

Earlier studies from our lab, demonstrated the ability of Cbx to inhibit the oxidative stress caused by rotenone treatment [50]. Proteasome inhibitors are known to evoke the conditions of oxidative stress [55]. Proteasome inhibitors lactacystin and epoxymicin have been shown to cause increase in protein carbonylation, 7-hydroxyguanine, and NO production thus contributing to oxidative stress [56]. This effect is achieved by affecting several processes such as invoking neuroinflammation [55], stimulation of NOS [56] and inhibition of mitochondrial processes or enzymes [57]. Hence, we carried out a detailed study of various markers of oxidative stress as well as antioxidant defenses in the cells to decipher the putative mechanism by which Cbx operates in the MG-132 model.

The inhibition of proteasome results in the reduced clearance of oxidative damaged proteins as evidenced by the increase in the protein carbonyl levels. Concomitantly, an increase in the oxidative damage to lipids was observed in the MG-132 injected animals. Various antioxidant defenses such as GSH, SOD and catalase also get activated in response to the oxidative stress. Increase in the catalase activity observed after MG-132 injection could be due to the excessive production of H_2O_2 [58] while the changes in the SOD activity could be due to the short-term cell response to free radical insult [59]. Along with these changes, the GSH levels were also observed to increase, which might be an adaptive response to increased free radical rush. In addition, activation of iNOS expression as evidenced by the upsurge in the levels of NO and citrulline levels was also observed following MG-132 injection. Prolonged and excessive oxidative stress results in the death of neurons by apoptosis [60]. This was well reflected by a decrease in the cell count in substania nigra region of the MG-132 treated animals.

The Cbx post-treatment was able to reduce the oxidative stress conditions as evident by the decrease in the levels of lipid peroxidation and protein carbonylation products. In addition, increase in the levels of GSH were also observed, which helped in the scavenging of free radicals. Cbx is also known to activate the expression of various cytoprotective HSPs. The decrease in the oxidative stress that was observed following Cbx post-treatment might be due to the HSP-70 induction by Cbx. HSP-70 has been reported to inhibit the iNOS expression and subsequently reduce the NO production [61-62]. Thus, reduced NO levels might be helping to curb the conditions of oxidative stress. Though an adaptive increase in the HSP-70 was also observed in MG-132 treated animals, the levels were insufficient to prevent the cell death as indicated by loss of dopamine. However, the even higher expression of HSP-70 in the Cbx post-treated group was able

to rescue the cells effectively against oxidative stress.

Thus, Cbx offers neuroprotection in the MG-132 induced toxicity. This protection is possibly mediated by its known activation of cytoprotective HSPs. These HSPs help to combat oxidative stress and inhibits apoptosis. The effects of Cbx should be explored further to elucidate the molecular pathways involved in its neuroprotective actions.

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