

## ANTIPARKINSONIAN EFFECT OF PIOGLITAZONE THROUGH EFFECTIVE ANTIOXIDATIVE PROPERTY IN MPTP MICE MODEL OF PARKINSON'S DISEASE

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### ABSTRACT

Oxidative stress plays a vital role in dopaminergic neurodegeneration of Parkinson's disease (PD). However, an antidiabetic drug, pioglitazone had shown a positive effect against oxidative stress to treat nervous unrest. Objective of this study is to evaluate the protective effect of Pioglitazone (25mg/kg/p.o) against 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice model of PD. The PD induced animals were treated orally with 25 mg/kg bodyweight of *Pioglitazone hydrochloride* for a period of 2 weeks. The Mice in control and experimental groups were subjected to various behavioural tests like rota rod, stride length measurement, olfactory, inverted grip and open-field tests etc. At the end of the experimental period, mice were sacrificed and the mid brain region was isolated. Striatal dopamine level was estimated and Tyrosine hydroxylase positive (TH<sup>+</sup>) cells were counted by flow cytometry and monoamine oxidase levels estimated by fluorimetry. Histopathological studies were performed on mid brain sections. The levels of monoamine oxidase B, total monoamine oxidase levels, lipid peroxidation (LPO), Reactive Oxygen Species (ROS), reduced glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GPx) were analysed. The Dopamine level lowered in MPTP mice was significantly and dose dependently increased with Pioglitazone treatment. TH<sup>+</sup> cell count was significantly lowered in MPTP mice while it was increased with Pioglitazone treatment. H&E stained sections of mid brain of PD mice revealed glial cell infiltration and occasional areas of dystrophic calcification. The histological findings observed in the brain sections of 25mg/kg pioglitazone treatment group were comparable to that of the control group. Oxidative stress (elevated LPO, increased levels of monoamine oxidase B, total monoamine oxidase and decreased activities of antioxidants (GSH, ROS, GR and GPx)) observed in MPTP mice were significantly ameliorated by Pioglitazone treatment. These results specify the anti-parkinsonian effect of Pioglitazone and this neuro-protective effect was executed partly through its anti-oxidant property.

**Keywords:** 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Pioglitazone, monoamine oxidase B, lipid peroxidation (LPO), Reactive Oxygen Species (ROS), reduced glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GPx). *substantianigra pars compacta*.

## INTRODUCTION

Parkinson's disease (PD) is a neurological movement disorder primarily resulting from damage to the nigrostriatal dopaminergic pathway. Pathologically, the hallmarks of PD are the loss of dopaminergic neurons in the *substantianigra pars compacta* and the presence of cytoplasmic protein aggregates, known as Lewy bodies, in remaining dopaminergic cells (Dauer and Przedborski, 2003). Mechanistically, the death of dopaminergic neurons has been linked to mitochondrial dysfunction, oxidative stress, neuroinflammation, and inefficient proteasomal protein degradation (Dauer and Przedborski 2003; Hirsch and Hunot 2009; Martin *et al.*, 2010).

Dopamine metabolism and mitochondrial dysfunction predominantly contributes to oxidative stress in Parkinson's disease. Oxidative stress via the generation of free radicals also play a vital role in pathogenesis of Parkinson's disease and the mitochondria serve as an important source in free radical generation.

Dopamine, as relatively unstable molecule in nature, undergoes auto-oxidation metabolism in the nigrostriatal tract system thereby producing ROS (Slivka *et al.*, 1985). Whereas, link between PD and mitochondria was first established with the detection of paucity in complex I activity in the *substantianigra pars compacta* region of parkinsonian brains (Schapira *et al.*, 1989).

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) in experimental parkinsonian animal models also produce nigrostriatal dopaminergic degeneration by inhibition of Complex I activity resulting in reactive ion species generation (Langston *et al.*, 1983). The oxidative stress and derangements in mitochondrial complex-I lead to the accumulation and aggregation of  $\alpha$ -synnuclein protein and demise of dopaminergic neurons in MPTP mice model of Parkinson's disease (Dawson *et al.*, 2003). At the peak of all, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated as a result of impaired mitochondrial function, serve as an important contributing factor in inducing

oxidative and nitrosative stressed neurodegeneration in PD models (Przedborski *et al.*, 2005; Ischiropoulos *et al.*, 2003).

More recently, PPAR- $\gamma$  agonists have received considerable attention as potential therapeutic agents for a wide range of neurodegenerative diseases (Heneka *et al.*, 2007). Of which, the PPAR- $\gamma$  agonist, pioglitazone may emerge as a potential treatment for Parkinson's disease since pioglitazone treatment offers an opportunity for the disease to slow its progression (Dehmer *et al.*, 2004). In our study, we evaluated the antioxidant property of the PPAR- $\gamma$  agonist, pioglitazone with its post treatment against MPTP induced Parkinson's disease in C57BL6/J mice.

## MATERIALS AND METHODS

### *Animals:*

All experiments and protocols described in present study were approved by the Institutional Animal Ethical Committee (IAEC), Pondicherry University (PU/IAEC/10/32). The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council for Medical Research, Hyderabad, India. Male C57BL6/J mice of body weight 35-40g supplied by Ragavendra suppliers, Bangalore were used in this study. The animals were fed on the standard pellet diet (NPD) for acclimatization. Water was given *ad libitum*. The animals were housed in plastic cages under controlled condition of 12h light / 12h dark cycles and at  $27 \pm 2^\circ\text{C}$ . They were kept on balanced diet and water *ad libitum* in a well-ventilated animal unit.

### *Chemicals:*

The neurotoxin MPTP is purchased from Sigma Aldrich, Bangalore, India. Dopamine ELISA kit was purchased from IBL international, Germany. The antibody for Tyrosine hydroxylase and the secondary antibodies are purchased from Santa Cruz Biotechnology and Merck, India respectively. Other chemicals and standards used are of analytical grade and purchased from Hi Media, India.

**Drug:**

Pioglitazone hydrochloride was obtained as a gift sample from *Bonn Schlerin Biosciences, Puducherry, India*.

**Experimental procedure:**

After 10 days adaptation period, the experimental mice were divided into 3 groups with 4 animals in each group ( $n=4$ ) and the groups were treated for five weeks

*Study was conducted as follows:*

**Group I: Control group** (Only standard diet was given).

**Group II: Diseased group:** Mice were induced with Parkinson's disease by injecting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) at a dose of 25 mg/kg body weight via i.p. for five consecutive days (one dose/day).

**Group III: Treatment group:** Mice were induced with Parkinson's disease and the behavioural tests (Rotarod test, Open field test, Stride length test, Grid hang test and Olfactory test) were continued during these periods and the level of PD progression was assessed by comparing disease and treatment group with control group. Based on the behavioural test results the animals were left for 7 days after disease induction for the onset of PD. After 7 days they were treated orally with 20 mg/kg bodyweight of *Pioglitazone hydrochloride* for a period of 2 weeks. The effects of pioglitazone post-treatment on MPTP-induced behavioural impairments were assessed on the 8<sup>th</sup> day of post MPTP injection and on 11<sup>th</sup> day of post pioglitazone treatment and animals were killed at the end of the experimental period by cervical dislocation.

Their brains were dissected to procure mid brain and striatal region by following the mice brain atlas (Rosen *et al.*, 2000). The brain regions were snap frozen with liquid nitrogen and stored at -80°C and used for determining the dopamine levels, tyrosine hydroxylase levels, levels of monoamine oxidase B (MAO-B), total monoamine oxidase levels, Lipid peroxidation (LPO) and antioxidants (Reactive Oxygen

Species (ROS), reduced glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GPx)). For histopathological analyses brain segments were preserved in 10% formal saline and stored at room temperature.

**Estimation of dopamine levels** (Tanet.*al.*, 2012):

Levels of striatal dopamine were estimated by ELISA technique as per the manual provided with the kit. Following the treatment, cell culture medium was measured directly. For brain tissues, 50 mg of tissue were homogenized in 1 ml HCl (0.01 N) with EDTA (1 mM) and sodium metabisulfite (4 mM). Under this condition, DA is positively charged and has the optimized solubility. The homogenate was centrifuged at 15000 g at 4°C for 15 min and the supernatant was collected for measuring. 20  $\mu$ l of standards and diluted sample were used for measurements. The process included Cis-diol-specific affinity gel extraction, acylation, derivatization enzymatically. Finally, DA was detected by competitive ELISA. Results were obtained from microplate reader set to 450 nm and a reference wavelength between 620 nm and 650 nm. The concentrations of DA in the sample were calculated according to the six standards from 0 to 90 ng. By using the ELISA method, this kit provided very sensitive (lower limits at 0.7 ng/mL) and high throughput measurements of DA.

**Determination of tyrosine hydroxylase positive (TH<sup>+</sup>) cells by flow cytometry** (Wolf *et.al*,1989):

The percentage of TH<sup>+</sup> cells in the mid brain region of mice were analysed using flow cytometry. Briefly a portion of mid brain region was added to collagenase (1U) and 0.25% trypsin solution mixture, incubated at 4°C for a minute, homogenized and centrifuged. The pellet obtained was washed extensively with phosphate buffered saline (PBS) and incubated in PBS containing 0.1% triton X 100 for 10 minutes. After washing with PBS, the pellet was incubated with blocking buffer for 30 minutes and centrifuged. To the pellet, anti-mouse tyrosine hydroxylase antibody was added, left for two hours, washed with PBS. FITC conjugated

secondary antibody was added to the pellet, incubated for 30-45 minutes, washed with PBS, suspended in small quantity of PBS and immediately loaded in flow cytometer.

#### **MAO Assay:**

Monoamine oxidase was estimated in various tissues by the method described by Tiptonet al., 2001 where 100µl of the homogenized supernatant was taken into 3 vials to which two of the inhibitors clorgyline and pargyline were added to measure the activity MAO-B and MAO-A respectively and were left for incubation at -20°C for 1hr. The third vial was used for measuring the total MAO content. After 1hr 870µl of PBS was added to all the three vials following which 30µl of kynuramine was added to all the three vials and was left to incubate in a shaker for 15min. the reaction was terminated by the addition of 600µl of perchloric acid. 1ml of supernatant was taken after centrifugation at 12000 rpm for 5min and the volume was made upto 3ml using 1M NaOH. Readings were taken using spectrofluorometer at an excitation  $\lambda$  315 nm and emission  $\lambda$  380-400nm and the results were expressed as nM of HQ produced/min/100mg tissue.

#### **Antioxidants Estimation:**

##### ***Estimation of Lipid peroxidation (LPO):***

LPO in brain tissue was estimated by the method of Ohkawa et al., 1979. In brief, 0.1ml of homogenate was treated with 2 ml of (1:1:1) ratio of 0.37% thiobarbituric acid -15%, trichloroacetic acid - 0.25 N hydrochloric acid reagent and boiled at 80°C for 30 min, cooled and centrifuged. Then supernatant was measured at 535 nm against reference blank.

##### ***Estimation of reduced glutathione (GSH):***

GSH was determined by the method of Ellman, 1959. In brief, 0.5 ml of brain homogenate was precipitated by adding 2 ml of 5% trichloroacetic acid (TCA). 1ml of supernatant was taken and 0.5 ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 1.5 ml of phosphate

buffer (pH 8.0) were added. The colour developed was read at 412 nm.

##### ***Assay of glutathione peroxidase (GPx), and Glutathione reductase (GR):***

Gpx and GR was assayed by the method of Rotruck et al., 1973 and the activity was expressed as µg of GSH utilized/minute/mg protein. GR activity was measured as followed by Carlberg and Mannervik, 1975.

##### ***Estimation of Reactive Oxygen Species (ROS):***

The formation of ROS was assayed by the method described by Lebel et al., 1990 where the brain regions were homogenized in ice-cold phosphate buffer (0.1 M; pH 7.4) containing 0.1% Triton X -100 (buffer A) and an aliquot of the homogenate was incubated with Dichloro-dihydro-fluorescein diacetate (DCFH-DA) (0.5µM) for 15 min at 37°C. Thereafter, the reaction was terminated by the addition of ice-cold buffer A. The formation of the oxidized fluorescent derivative, 2',7'-dichlorofluorescein was monitored at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Blanks containing brain homogenate, but no Dichloro-dihydro-fluorescein diacetate were processed similarly for the measurement of autofluorescence.

##### **Statistical analysis:**

Statistical analysis of the data was done using SPSS 7.5-Windows Students version software (SPSS Inc., Chicago, IL, USA). The data represents mean  $\pm$  SE. One-way ANOVA followed by Tukey's HSD. Values are considered statistically significant if  $p \leq 0.05$ .

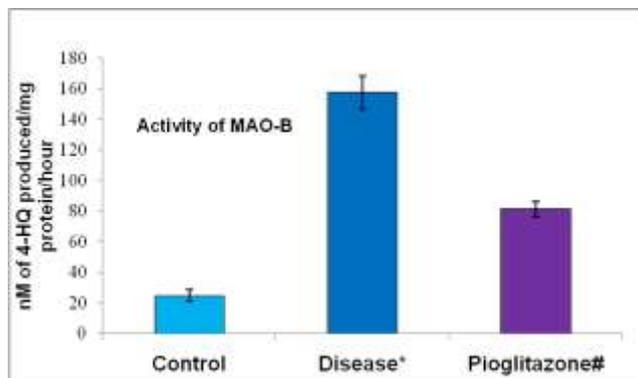
## **RESULTS**

##### ***Levels of striatal dopamine:***

The striatal dopamine levels are presented in Fig. 1. PD mice showed significantly decreased ( $p \leq 0.001$ ) dopamine levels as compared to control mice. As compared with PD mice, pioglitazone treated mice showed mild increase ( $p \leq 0.05$ ) in

the levels of dopamine and this effect was pronounced only with 20 mg/kg of pioglitazone.

**Figure-1. Striatal Dopamine Levels**

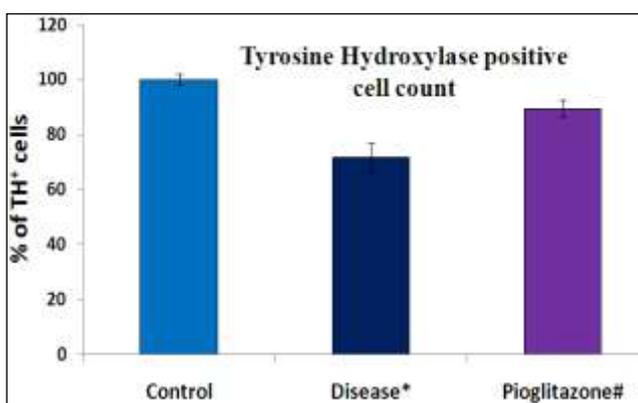


Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's LSD  
 \* indicates value significantly different from the control at P ≤ 0.001;  
 # indicates value significantly different from the PD model at P ≤ 0.05.

**Determination of Tyrosine hydroxylase positive cells:**

TH<sup>+</sup> neurons represent the dopaminergic neurons in brain and the percentage of immunostained TH<sup>+</sup> cells as determined by flow cytometry is presented in Fig.2.

**Figure-2: Percentage of Tyrosine Hydroxylase Positive Cell Count**



Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at P ≤ 0.001;

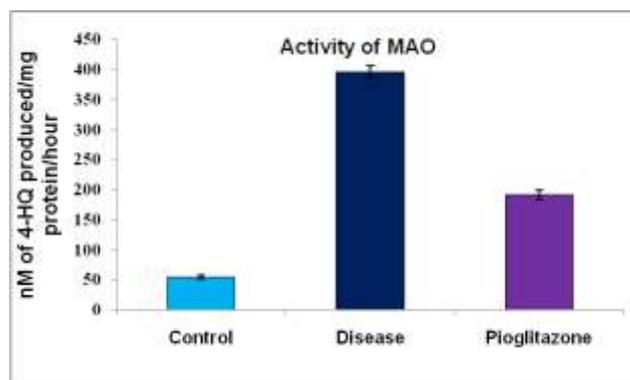
# indicates value significantly different from the PD model at P ≤ 0.05

The percentage of TH<sup>+</sup> cells in control mice was found to be 100% whereas in PD mice it was significantly reduced to 72% (p ≤ 0.001). It was interesting to observe that pioglitazone treatment caused significant increase in TH<sup>+</sup> cell count (p ≤ 0.05) as compared to PD group with 89% of TH<sup>+</sup> cells as compared to control.

**Estimation of Total Monoamine Oxidase Activity:**

The Total Monoamine Oxidase levels in brain are shown in Fig 3. The total MAO activity of diseased group was found to be increased significantly (p ≤ 0.001) than control group. The activity of MAO was found to be significantly increased (p ≤ 0.001) in pioglitazone treated group as compare to PD mice.

**Figure-3 Total Monoamine Oxidase levels**



Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at P ≤ 0.001;  
 # indicates value significantly different from the PD model at P ≤ 0.05.

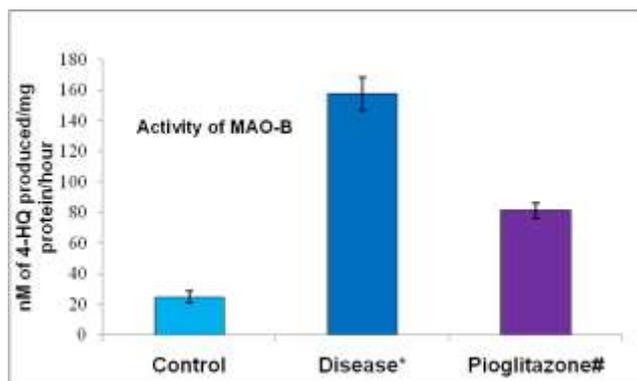
**Estimation of Monoamine Oxidase B activity:**

The Monoamine Oxidase-B levels in brain are shown in Fig 4 .The MAO-B activity was found to be increased significantly (p ≤ 0.001) in PD mice than the control group. The activity of MAO-B was found to be significantly reduced (p ≤ 0.001) in pioglitazone treated group as compared to PD mice.

**Estimation of lipid peroxidation (LPO):**

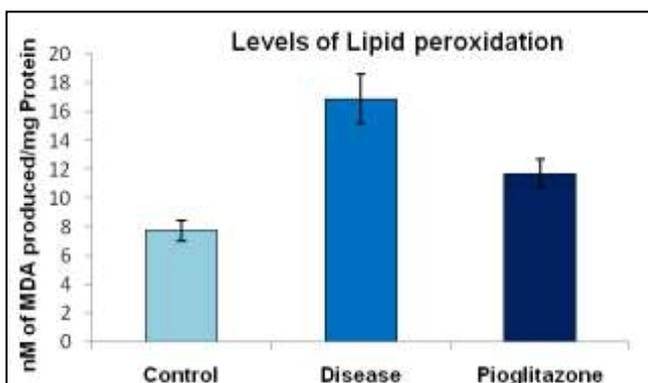
The LPO levels in brain are shown in Fig5. In PD mice, the LPO level was significantly elevated ( $p \leq 0.001$ ) as compared to control mice. Pioglitazone treatment at 20 mg/kg BW caused significant reduction ( $p \leq 0.05$ ) in LPO level as compared to control levels.

**Figure-4. Monoamine Oxidase-B Levels**



Values represent mean  $\pm$  SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at  $P \leq 0.001$ ;  
 # indicates value significantly different from the PD model at  $P \leq 0.05$ .

**Figure-5. Lipid Peroxidation Levels**

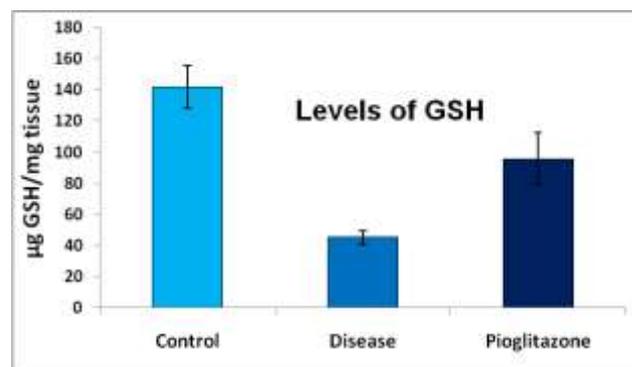


Values represent mean  $\pm$  SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at  $P \leq 0.001$ ;  
 # indicates value significantly different from the PD model at  $P \leq 0.05$ .

**Estimation of reduced glutathione (GSH) levels:**

The GSH levels in brain are shown in Fig6. The mid brain reduced glutathione (GSH) level was found to decrease significantly ( $p \leq 0.001$ ) in PD mice as compared to control. Pioglitazone treatment significantly protected ( $p \leq 0.05$ ) the GSH content of PD induced mice at 20 mg/kg BW doses.

**Figure-6. Reduced Glutathione (GSH) levels**



Values represent mean  $\pm$  SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at  $P \leq 0.001$ ;  
 # indicates value significantly different from the PD model at  $P \leq 0.05$ .

**Estimation of glutathione peroxidase (GPx), and Glutathione reductase (GR) and reactive oxygen species (ROS) levels:**

The glutathione peroxidase (GPx), and Glutathione reductase (GR) and reactive oxygen species (ROS) levels in brain are shown in Figure-7,8,9 respectively. The activities of antioxidant enzymes—GPx, GR, ROS were also found to be significantly reduced ( $p \leq 0.001$ ) in PD mice as compared to control. Encouraging results were observed with pioglitazone treatment at the dose of 20mg/kg BW which caused significant increase in the activities of GPx, GR ( $p \leq 0.05$ ) and decreased ROS levels ( $p \leq 0.001$ ) as compared to PD mice.

## DISCUSSION

Excessive free radical formation or antioxidant enzyme deficiency can result in oxidative stress, a mechanism proposed in the toxicity of MPTP and in the aetiology of Parkinson's disease (PD)(Langston *et al.*, 1984) and the “oxidative stress” hypothesis assumes that the nigrostriatal cell death observed in PD is due to the MPTP-mediated formation of hydroxyl and superoxide radicals (Obata *et al.*, 1992; Singer *et al.*, 1987). Using internal antioxidants, the cells can deal with normal levels of oxygen radical formation. When oxygen free radical formation is greater than normal, or antioxidant levels are lower than normal, oxidative stress occurs which may contribute to cell death (i.e. necrosis) and tissue damage (Ebadi *et al.*, 2001; Stefanis *et al.*, 1997).

There have been several reports during the past decade describing increased lipid peroxidation (Dexter *et al.*, 1986), a decrease in GSH content (Perry *et al.*, 1982), and changes in antioxidant enzyme activity (Kishet *et al.*, 1985; Ambaniet *al.*, 1975), in the brains of both parkinsonian patients and MPTP models emphasizing the importance of oxidative damage and the involvement of free radicals in the pathogenesis of this movement disorder.

MPTP can alter the activity of the antioxidant enzymes like GSH, ROS & GPx. Since it has been shown that MPTP gets converted to MPP<sup>+</sup> by mitochondrial monoamine oxidase (MAO) (Chiba *et al.*, 1984), specifically by MAO B (Heikkila *et al.*, 1984) and is rapidly concentrated in the mitochondria (Ramsay *et al.*, 1984) which eventually leads to cell death, decrease in the activity of SOD, CAT, GSH & GPx and Increased activity of ROS in the diseased group due to the action of MPTP.

The present study also supports the observations on neurodegenerative effects of MPTP inducing oxidative stress. MPTP induced depletion of TH<sup>+</sup> neurons, tyrosine hydroxylase and dopamine levels (Schmidt and Ferger, 2001) have been reported previously. The significant reduction in the levels of dopamine, TH<sup>+</sup> cells and tyrosine

hydroxylase in PD mice observed in this study was positively amended with pioglitazone treatment demonstrating its neuroprotective effect against MPTP induced dopaminergic neurotoxicity.

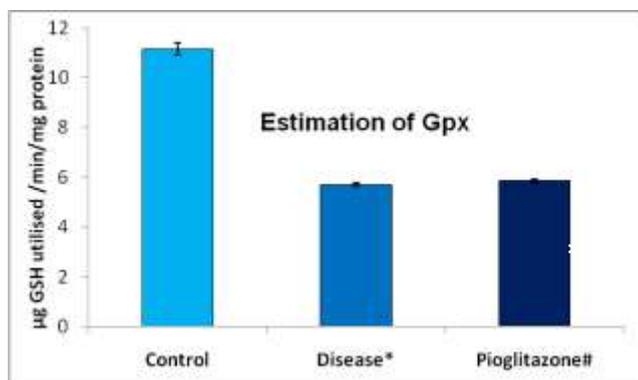
Increased malonaldehyde (Dexter *et al.* 1989) and lipid hydroperoxides (Dexter *et al.* 1994) have been found in the SN in PD. MPP<sup>+</sup> administration have been shown to enhance the striatal LPO levels in mice (Rojas and Rios, 1993). Treatment with MPTP in rodents had been shown to cause significant enhancement in the levels of TBARS in both striatum and midbrain (Hung and Lee 1998; Sankar *et al.* 2007). Elevated level of TBARS was also recorded in the brain of MPTP-treated monkeys (Marzatico *et al.* 1993). These observations correlate with our findings of increased LPO levels in the mid brain of PD induced mice. Interestingly, pioglitazone treatment brought about significant reduction in TBARS levels indicating its protective effect against MPTP induced oxidative damage.

SN neurons have low levels of endogenous antioxidants and are more prone to oxidative stress compared to the other regions of the brain (Calabrese *et al.* 2002). It has been previously reported that MPTP treatment results in reduction of GSH levels in mice (Martin and Teismann 2009). MPTP is also known to alter the activities of GPx, GR and GST (Smeyne and Smeyne 2013). Treatments with antioxidants have been reported to partially protect the neurotoxic effects of MPTP in mice (Albarracin *et al.* 2012; More *et al.* 2013). In this study, the significant reduction in levels of antioxidants including GSH, GPx and GR observed in PD induced mice, were found to increase following pioglitazone treatment.

Also, MPTP administration directly increases the ROS production by inhibiting enzymes of respiratory chain, and by increasing dopamine turnover, increased ROS formation has been shown to contribute to dopaminergic neuron injury after MPTP administration (Teismann and Ferger, 2001; Teismann *et al.*, 2003). In the same way, the present study has shown elevated levels of ROS in MPTP injected PD mice and

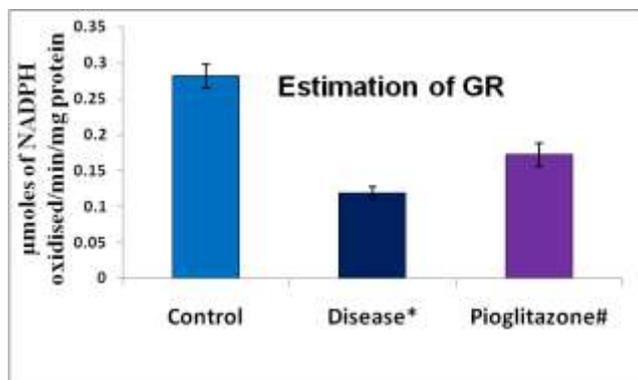
pioglitazone had significantly decreased the ROS levels as compared to PD mice.

**Figure-7 Glutathione Peroxidase (Gpx) Levels**



Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at P ≤ 0.001;  
 # indicates value significantly different from the PD model at P ≤ 0.05.

**Figure-8 Glutathione Reductase (Gr) Levels**

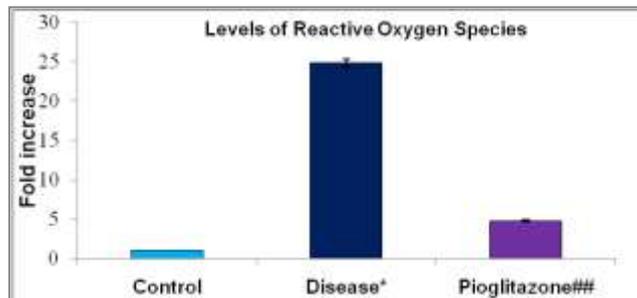


Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at P ≤ 0.001;  
 # indicates value significantly different from the PD model at P ≤ 0.05.

These data suggests that pioglitazone helps in boosting up the antioxidant system protecting the neurons from MPTP induced damage. Thereby, from the results observed, it can be demarcated that the neuro-protective effect of pioglitazone against MPTP insult is partly accomplished

through its antioxidant activity. Further study at molecular level to support this statement is required.

**Figure-9. Reactive Oxygen Species (Ros) Levels**



Values represent mean ± SE (n = 4)  
 One way ANOVA followed by Tukey's HSD  
 \* indicates value significantly different from the control at P ≤ 0.001;  
 # indicates value significantly different from the PD model at P ≤ 0.05.  
 ## indicates value significantly different from the PD model at P ≤ 0.001

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