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ATTENUATION OF 1-METHYL-4-PHENYL-1, 2,3,6-TETRAHYDROPYRIDINE INDUCED NIGROSTRIATAL TOXICITY IN MICE BY N–ACETYL CYSTEINE

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Abstract – The present study was designed to investigate the effects of N-acetyl cysteine (NAC), an antioxidant on 1-methyl 4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) induced neurotoxicity in the nigrostriatal dopaminergic system of mice. MPTP treatment caused 80% decrease of the dopamine levels in the striatum of C57BL/ 6J mice. A marked increase in the extent of lipid peroxidation, superoxide dismutase (SOD) and g-glutamyl transpeptidase (g-GTP) was seen, while a significant decrease in the levels of glutathione (GSH), total thiols and glutathione peroxidase (GPx) activity was observed in the substantia nigra pars compacta (SNpc) of MPTP treated animals. As compared to control animals, Co-administration of NAC with MPTP restored the depleted dopamine, GSH, total tissue thiol levels and GPx activity in SNpc of treated mice brain. Moreover, NAC treatment also provided protection against lipid peroxidation and superoxide dismutase activity. The results of present study suggested that NAC attenuates MPTP neurotoxicity in mice brain and this protection by the NAC might be contributing to the regeneration of GSH, a major antioxidant.

Key words: 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP), N-acetyl cysteine (NAC), Parkinson's disease, oxidative stress, glutathione

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder, progressive with mean age at onset of 55 and with an incidence that increases markedly with age [10]. PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the brain [6]. The symptoms (tremor, rigidity and slowness of movement) appear when 70 % of the dopaminergic neurons are lost, demonstrating that the degenerative process is active long before the patients become aware of the disease. The major problem concerning a better therapeutic approach to the treatment and prevention of the disease is the enigma of its underlying cause. This has remained obscure in spite of many approaches and efforts made so far [18,30].

Although the pathogenesis of PD remains obscure, oxidative stress to dopaminergic neurons of SNpc is believed to be one of the leading causes of neurodegeneration in PD [1, 5]. Although the human body in general has evolved several defense mechanisms to counteract oxidative stress, the brain appears to be more susceptible to this damage than other organs. Reactive oxygen species (ROS) are generated during both enzymatic and non-enzymatic (autoxidation) metabolism of dopamine. Changes in the antioxidants defenses also support the evidence of oxidative stress in the substantia nigra of PD patients [17]. The most significant alteration is the decrease in the level of reduced glutathione (GSH). The cause of this reduction of GSH in SNpc in Parkinson's disease patients is unknown but may be related to alterations in its synthesis, utilization or degradation [34]. The activity of g-glutamyl transpeptidase (g-GTP) has also been shown to be increased in PD, a change that is specific to substantia nigra and

Abbreviations: **GPx**, glutathione peroxidase; **GSH**, reduced glutathione; **g-GTP**, g-glutamyl transpeptidase; **LPO**, lipid peroxidation; **MDA**, malondialdehyde; **MPTP**, 1-methyl 4-phenyl 2,3,6-tetrahydropyridine; **NAC**, N-acetyl cysteine; **OS**, oxidative stress; **PD**, Parkinson's disease; **ROS**, Reactive oxygen species; **SNpc**, substantia nigra pars compacta; **SOD**, superoxide dismutase;

does not occur in other neurodegenerative illnesses [34].

Much of the knowledge about dopaminergic neurodegeneration has come from studies with toxins like 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), Methamphetamine (MA) and 6-hydroxy dopamine (6-OHDA) that have been used in rodents, primates and other species to study Parkinsonism [7]. The neurotoxin MPTP has been used in different animal studies especially in mice to recapitulate the hallmark of PD cellular pathology [16]. MPTP has been shown to cause the degeneration of nigrostriatal dopaminergic neurons in several species, including baboons, cats and mice [3,16,32,]. In view of the oxidative stress associated with PD, animal models infer that free radical scavengers and GSH analogues could be a valid therapeutic approach (15). Therefore the present study was designed with an aim to assess the potential of Nacetyl cysteine as an antioxidant for Parkinson's disease by evaluating its protective efficacy in MPTP induced mice model of Parkinson's disease.

MATERIAL & METHODS

Chemicals

All the chemicals used in this study were of analytical reagent grade. The chemicals MPTP, oxidised and reduced glutathione, nicotinamide adenine dinucleotide phosphate (reduced), nitrobluetetrazolium, 5-5'-'dithiobis-2-nitrobenzoic acid, bovine serum albumin, Tris-HCl, dopamine, thiobarbituric acid and trichloro acetic acid were purchased from Sigma Chemical Co (St Louis, MO, USA). Alumina and ethanol were purchased from E.merck, Munich, Germany.

Animals

C57BL/6J mice, 25-35 g were procured from National Institute of Nutrition, Hyderabad (India). The animals were housed in polypropylene cages and were kept in well-ventilated rooms. Animals were provided standard mice pellet diet and water ad libitum.

Experimental Design

Four groups of 9 animals in each were set up.

Group I: Control group: In this group the animals received an equal volume of normal saline as administered to the animals of the MPTP treated group.

Group II: MPTP treated group: in this group the animals received four injections of 20mg/kg b.wt/day of MPTP-HCl intraperitoneally at 2-hr intervals for ten days [2].

Group III: MPTP and NAC treated group: Animals received MPTP as above and NAC intraperitoneally (3 injections of 150 mg / kg b. wt at 12 hr intervals) after the first dose of MPTP.

Group IV: NAC treated group: Animals received only NAC as in group .. At the end of treatment, animals were fasted overnight, next morning they were anesthesized and sacrificed by decapitation. The mice brains were removed immediately after decapitation and rinsed in ice-cold physiological saline (0.9% NaCl). The corpus striatum, and substantia nigra were isolated as per the guidelines of Glowinski and Iversen [14].

Preparation of tissue homogenate

Tissue homogenate (1:10) was prepared using phosphate buffer (66 mM, pH 7.0). The crude homogenate was used for the estimation of lipid peroxidation. The homogenate was centrifuged at 1000 x g for 20 minutes at 4ºC. The pellet was discarded. The supernatant was centrifuged at 20,000 x g for 20 minutes at 4ºC to obtain the post mitochondrial supernatant (PMS). It was used to assay superoxide dismutase, glutathione peroxidase, glutathione reductase, g-glutamyl transpeptidase.

Striatal dopamine

It was assayed by the spectrofluorimetric method of Cox and Perlach [9]. The brain samples were homogenized and the homogenate was centrifuged at 800 x g for 5 min. Supernatant was transferred to a glass centrifuge tube containing DDW and heptane and centrifuged at 800 x g for 5 min. The aqueous phase containing alumina and sodium acetate were centrifuged at 800 x g for 5 min. After adding acetic acid (0.1 N) to the alumina, again it was centrifuged at 800 x g for 10 minutes. Then aqueous phase was transferred to a small test tube for fluorescence assay. The fluorescence was measured in spectrofluorometer at activation and emission wavelengths of 320 & 370 nm for dopamine.

Lipid Peroxidation

Lipid peroxidation was measured by thiobarbituric acid method as described by Wills [38]. The reaction mixture contained (0.1M) Tris-HCl buffer (pH 7.4), (10% w/v) TCA, and requisite amount of sample. The reaction mixture was centrifuged at 500 rpm for 10 minutes. Supernatant was collected and TBA (0.67%) was added. Colour was developed by placing the tubes for 10 minutes in a boiling water bath. Absorbance was read at 532 nm against appropriate blank. The amount of malondialdehyde formed was calculated on the basis of molar – extinction coefficient of MDA (1.56 x 105 M-1 cm-1) and the results were expressed as nmol MDA / mg protein.

Reduced Glutathione

Glutathione was estimated by the method of Sedlack and Lindsay [33]. The reaction mixture contained DTNB (0.1M in absolute methanol), (0.4M), Tris buffer (pH 8.9), EDTA (0.02M), TCA (50% w/v). Change in absorbance was measured at 412 nm. The results were expressed as mg of glutathione/mg protein.

Total Tissue Thiols

Total tissue thiols were estimated by the method of Sedlak and Lindsay [33]. To the homogenate EDTA (0.2 M), Tris buffer (0.2 M), DTNB (0.01 M) and absolute methanol were added and reaction mixture was centrifuged at 3000 rpm for 15 minutes. Supernatant was read at 412 nm.

Glutathione Peroxidase

Glutathione peroxidase activity was measured by the method of Necheles et al [27]. The reaction mixture contained phosphate buffer (0.4M), sodium azide (10mM), GSH (8mM), 2.5mM H_2O_2 and requisite amount of sample. This was incubated at 37ºC for 3 minutes. After addition of TCA (10%), reaction mixture was centrifuged at 3000 rpm

for 15 min. Supernatant was collected and disodium hydrogen phosphate (0.3M) and DTNB (4mg% in sodium citrate) were added to it.Absorbance at 412 nm was recorded. The results were expressed as mg of GSH consumed/min/mg protein.

Glutathione Reductase

Glutathione Reductase was assayed by the method of Worthington et al [41]. The reaction mixture contained 0.5g/ml serum albumin, (200mM) KCl, (1mM) EDTA, (1mM) oxidized glutathione in (50mM) phosphate buffer. Change in absorbance was observed at 340 nm. Enzyme activity was calculated on the basis of molar extinction coefficient for NADPH at 340 nm (6.22 x 10-6 M-1 cm-1). Specific activity of the enzyme was expressed as mmole of NADPH utilized/min/mg/protein.

g-glutamyl transpeptidase

It was assayed by the method of Naftalin et al [26]. The reaction mixture consisted of buffered substrate, 22mg gamma glutamyl-p-nitroanilide in (0.1M) buffer glycylglycine reagent (pH 8.2), 10% acetic acid, 0.1% sodium nitrite and 1% ammonium sulphamate and NEED (1naphthylethylene diamine dihydrochloride) 50mg%. Change in absorbance was monitored at 405 nm. From these absorbance values, DA/min was calculated and the activity in the specimen was expressed as U/L.

Superoxide dismutase

Superoxide dismutase activity was measured by the method of Asada et al [4]. The reaction mixture contained carbonate buffer (100mM), NBT (96µM), Triton-X-100 (0.6%). The reaction was then initiated by the addition of hydroxylamine-hydrochloride (20mM, pH 6.0). Change in absorbance at 560 nm was observed after addition of sample. The enzyme activity was expressed as U/mg protein, where 1 unit of enzyme is defined as the amount of enzyme required to produce 50% inhibition.

Stastical analysis

The results were compared using students' t-test and ANOVA.

RESULTS

To make animal models of Parkinsonism, earlier primates and rats were used [9]. Studies have shown that C57BL/6J mice are the most appropriate strains for MPTP toxicity and formation of model of Parkinsonism [38]. Dopaminergic neurons have been shown to degenerate in patients with Parkinson's disease, resulting in striatal dopamine depletion. There is loss of more than 80% dopaminergic neurons before the symptoms of Parkinson's disease appear [6,43] The present study shows that the striatal dopamine levels were greatly reduced by 80% in MPTP treated group. Further, when the animals were co exposed with MPTP+NAC, there was a slight decrease in dopamine levels but it was not statistically significant as compared to control (Fig 1).

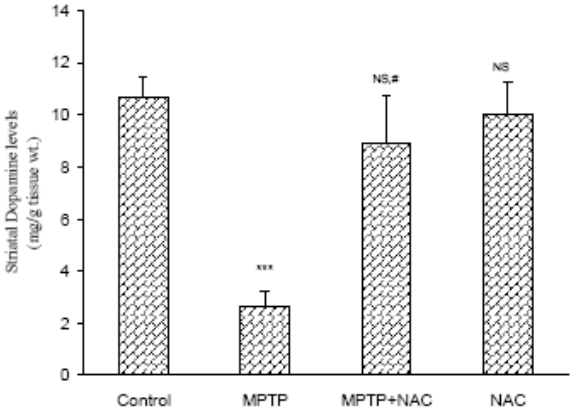

Figure 1. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on striatal dopamine levels in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value *p<0.05, all groups are significantly different from each other.
***p<0.001, significantly different from the control group.
#p< 0.05 significantly different from the MPTP treated group.
NS -Not significant from control group.

In the N-acetyl cysteine treated group, there was no change in the dopamine levels. Therefore the results suggested that N-acetyl cysteine was effective in protecting SNpc from the toxicity of MPTP, in terms of dopamine depletion.

Since lipid peroxidation has been identified as the major deteriorative reaction due to oxidative stress, therefore it was imperative to study lipid peroxidation in vivo. There was an almost two fold increase in LPO in the MPTP treated mice as compared to the control. This increase in lipid peroxidation is in consistent with the previous studies [11]. On the other hand in mice, co-exposed to MPTP+NAC, there was no significant decrease (p<0.05) in the extent of lipid peroxidation as compared to the MPTP treated group as shown in (Fig 2).

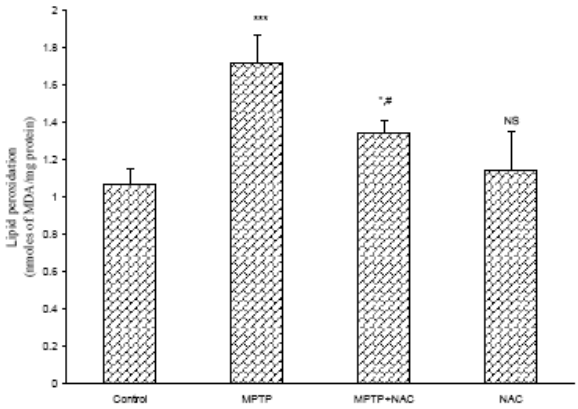

Figure 2. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on lipid peroxidation in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value *p<0.05, all groups are significantly different from each other.
***p<0.001, *p< 0.05 significantly different from the control group.
#p< 0.05 significantly different from the MPTP treated group.
NS -Not significant from control group.

Moreover there was also no significant difference in the extent of lipid peroxidation between the NAC treated group and control. These results indicate that MPTP neurotoxicity causes oxidative stress in the Parkinsonian mice model. We can also infer from the data that NAC by its ability to scavenge free radicals, has decreased the oxidative stress and hence the lipid peroxidation to some extent. Glutathione offers an important line of defense against free radical damage. The data presented in (Fig 3) demonstrates that there was marked decrease (38.77%) in the glutathione levels in the MPTP treated mice. Alternations in the distribution of glutathione in the substantia nigra in Parkinson's disease have been studied earlier [29]. On MPTP+ NAC co-exposure, there was an increase in GSH levels as compared to MPTP treated group and the levels reached the control value in this group. NAC treatment alone did not have any effect on the glutathione levels.

A similar trend as that observed in glutathione levels was observed in the total tissue thiol levels. Total tissue thiol levels were 50% lower in the MPTP treated group as compared to the control. On co-exposure to MPTP + NAC the levels reached the control value(Fig 4).

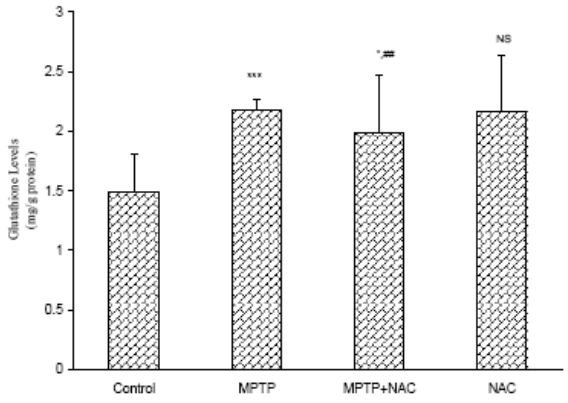

Figure 3. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on glutathione levels in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value *p<0.05, all groups are significantly different from each other.
***p<0.001, *p< 0.05 significantly different from the control group.
##p<0.01 significantly different from MPTP treated group.
NS -Not significantly different from control group.

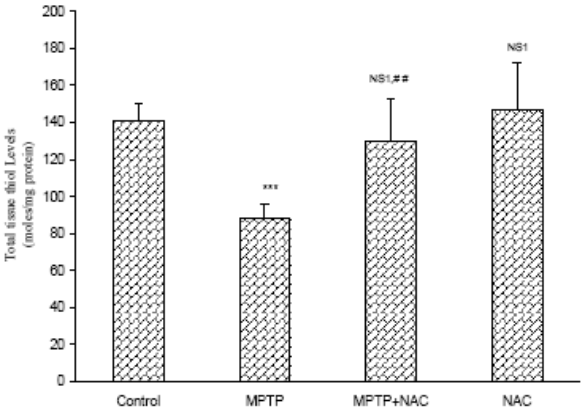

Figure 4. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on total tissue thiol levels in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value *p<0.05, all groups are significantly different from each other.
***p<0.001, significantly different from the control group.
#p<0.01 significantly different from MPTP treated group.
NS1 -Not significantly different from control group.

However NAC exposure alone had no effect on total tissue thiol levels. On MPTP treatment a slight decrease (14.21%) in the activity of GPx was observed as compared to the controls, which is in accordance with earlier studies, which have found 10-15% reductions in the activity of this enzyme [20]. This decrease in the activity of GPx was reduced to 3% on exposure to MPTP+ NAC. Treatment with NAC alone had no effect on GPx activity. The results indicate that treatment with MPTP had detrimental affect on the activity of GPx (Fig5).

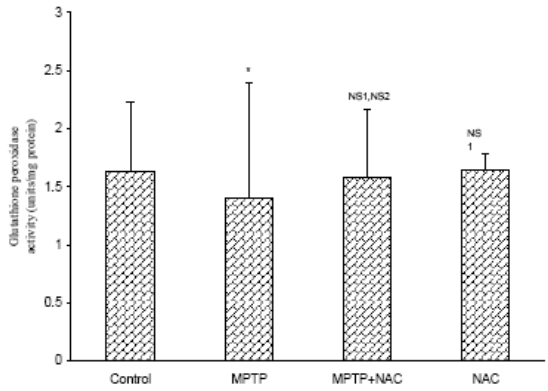

Figure 5. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on glutathione peroxidase activity in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value NS -groups are not significantly different from each other.
*p< 0.05 significantly different from the control group.
NS1-Not significantly different from the control group.
NS2 -Not significantly different from the MPTP treated group.

The activity of GR was unaltered in all the four groups (Fig 6)

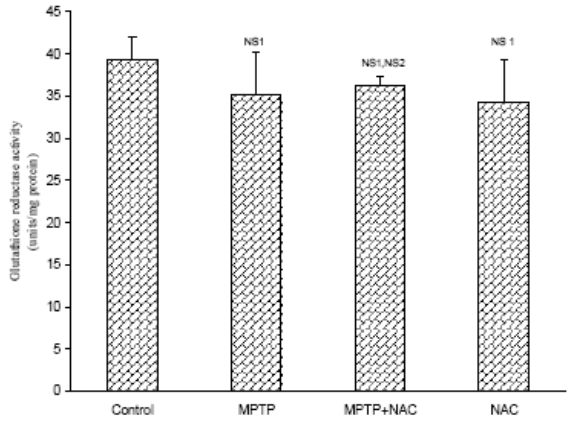

Figure 6. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on glutathione reductase activity in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value NS -groups are not significantly different from each other.
NS1-Not significantly different from the control group.
NS2 -Not significantly different from the MPTP treated group.
Treatment with neither MPTP nor NAC or their co-exposure had any effect on glutathione reductase activity. The absence of any change in GR activity could just be a compensatory mechanism of the body against oxidative stress.

There was 46% increase in superoxide dismutase activity in the MPTP treated group as compared to the control (Fig 7).

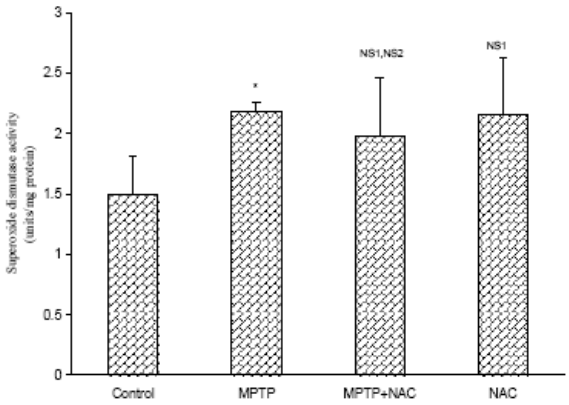

Figure 7. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on superoxide dismutase activity in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value NS -groups are not significantly different from each other.
*p< 0.05 significantly different from the control group.
NS1-Not significantly different from the control group.
NS2 -Not significantly different from the MPTP treated group.

Moreover it has been shown that both the mitochondrial Mn and the cytoplasmic Cu+2/Zn+2 isoforms of SOD expression to be increased in the SNpc in PD [31]. When MPTP +NAC were given together, the increase in SOD activity observed in MPTP treated mice alone decreased from 46 % to 32%. There was no difference in the SOD activity between the NAC treated group and control in SNpc. g-GTP, the enzyme that has a role in cellular translocation and degradation of GSH was evaluated in the post-mitochondrial supernatant. Its

activity was found to be significantly increased in the MPTP treated group ($p<0.001$) as compared to the control group. In the MPTP+NAC treated group the activity continued to remain high as compared to the control group. There was no difference in g-GTP activity in the NAC treated group as compared to the control (Fig 8).

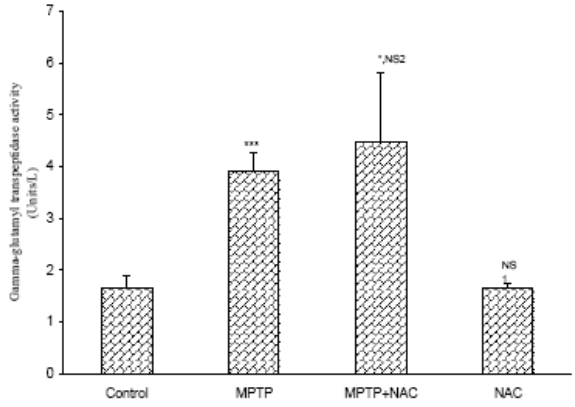

Figure 8. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-acetyl cysteine (NAC) and their co-exposure on gamma-glutamyl transpeptidase activity in substantia nigra of mice
The values are mean + S.D. of 9 animals.
F-value *p<0.05, all groups are significantly different from each other.
***p<0.001, *p< 0.05 significantly different from the control group.
NS1 -Not significantly different from control group.
NS2-Not significantly different from MPTP treated group.

DISCUSSION

Dopaminergic neurons have been shown to degenerate in patients with Parkinson's disease, resulting in striatal dopamine depletion. The present study showed a marked decrease in striatal dopamine levels in mice; almost 80% after MPTP exposure and treatment with N-acetyl cysteine attenuated the depletion of dopamine. MPTP has been used in different animal studies, especially in mice to recapitulate the hallmark of PD cellular pathology, namely the degeneration of the nigrostriatal dopaminirgic pathway [10,16]. MPTP exerts its toxicity, by conversion to MPP+ by Monoamine oxidase B (MAO-B). MPP+ is taken up by the dopaminergic nerve terminals via the dopamine uptake system and is accumulated in the nigrostriatal cells. MPP+ carrier then transfers MPP+ to the mitochondrial matrix. Increased MPP+ in the matrix inhibits NADH dehydrogenase in the electron transport chain (ETC). Malfunction of the ETC leads to electron leakage and hence increased production of superoxide anions [30]. Increased SOD activity seen in our study may be a compensatory mechanism of the body to dismutate these excess superoxide anions. Dismutation of the superoxide anions leads to H2O2 formation. In the SNpc H2O2 interacts with Fe+2 in the Fenton reaction to produce cytotoxic hydroxyl radicals, which lead to lipid peroxidation. In the brain, lipid peroxidation gains importance as brain is particularly rich in polyunsaturated fatty acids (PUFA) and on the other hand concentration of antioxidant enzymes, catalase and superoxide dismutase, which help in the detoxification of peroxyradicals, is not so high in the brain. Higher levels of malondialdehyde (MDA), a marker of oxidative stress (OS) have been reported in the substantia nigra pars compacta (SNpC) of PD patients [11]. Dexter et al have also shown elevated levels of lipid hydro peroxide, an earlier component of the lipid peroxidation cascade in the Parkinsonian substantia nigra, which indicates damage of cell membrane structure due to elevated reactive oxygen species (ROS) [12,25]. Thus these increased lipid peroxidation products suggest that ROS have an important role to play in the pathogenesis of neurodegenerative diseases. Our study also observed increased MDA levels in the SNpc of MPTP treated mice indicating that OS is being generated in the MPTP induced mice Parkinsonian model. Treatment with N-acetyl cysteine (NAC) decreased the level of lipid peroxidation to some extent but not as significant as observed in the control group, which suggested that NAC helps in decreasing the oxidative stress generated by MPTP to some extent.

Cause and effect links between GSH metabolism and diseases such as cancer, neurodegeneration and aging have been shown [36]. GSH normally acts through a combination of various reduction and conjugation reactions to protect cells against both exogenous toxicants and the reaction of endogenous compounds [22]. The decrease in GSH seen in SNpc is specific, as GSH levels are not reduced in other areas of the PD brain. One possible explanation for decreased level of GSH is that there may be defective synthesis, utilization and degradation of GSH. Early reductions in the total glutathione levels have also been detected in animal models of the disease [19]. Changes in the activity of glutathione peroxidase and reductase may also be responsible for this decrease. Therefore we evaluated these antioxidant enzymes in our animal model. In the MPTP + NAC treated group, GSH levels almost returned to control values, indicating that Nacetyl cysteine, which is a precursor of glutathione might have helped in

the regeneration of GSH and restored GSH levels in the mice SNpc.

Denier et al suggested that the GSH deficit in SN of Parkinson's disease patients could be a consequence of its oxidation to GSSG by glutathione peroxidase to inactivate cytotoxic species and H2O2 [13]. Mice deficient in cellular GPx has been shown increased vulnerability to MPTP [21]. In our study, we found a decrease in Glutathione Peroxidase (GPx) activity in the substantia nigra of mice, after MPTP treatment. This decrease may be due to the detrimental effect of ROS, which are generated during OS. The co-exposure of MPTP+NAC also could not ameliorate the

MPTP induced decrease in GPx activity. There was no change in the activity of GPx on treatment with NAC alone.

As the exact cause for GSH depletion was not known in Parkinson's disease patients, Sian et al decided to evaluate the activity of g -GTP in the SNpc of PD patients [34]. In PD, the activity of g -GTP is doubled in the SNpc and, like the change in GSH itself, this does not occur in any other brain region and is not found in any other neurodegenerative disease. Although the relevance of the increase in g -GTP is not clear, it may represent an attempt by the cell to conserve the peptide precursors of GSH and thus restore normal GSH levels. Alternatively, gamma-GTP can clear GSSG, which in high concentrations can be toxic to surrounding tissues [37]. This might also account for the absence of an absolute increase in GSSG in PD under conditions of oxidative stress. In our study we found a higher g-GTP activity in the SNpc of mice treated with

MPTP. On co exposure to MPTP+NAC, the activity was still higher as compared to control, indicating that treatment with NAC could not bring g-GTP activity to normal. There was no change in g-GTP activity on treatment with NAC alone. As g-GTP has been shown to cause increased clearance of GSSG, the increase in g-GTP could be a compensatory mechanism of the body to protect from the toxic actions of ROS and GSSG. This increase in g-GTP, in turn would lead to increased GSH degradation and hence may be one of the major contributors to GSH depletion, as observed in our study. So it can be concluded from present study that NAC when administered along with MPTP was able to restore the changes in the oxidant/ antioxidant status almost to normal level and thus helped in decreasing the oxidative stress generated in mice model

of PD. Further, it can be reasonably argued that NAC may serve as a therapeutic approach in ameliorating the Oxidative stress in Parkinson's patients also.

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