



Pharmacological and Biochemical Interventions of Cigarette Smoke, Alcohol, and Sexual Mating Frequency on Idiopathic Rat Model of Parkinson's Disease

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ABSTRACT

Parkinson's Disease (PD) is a neurodegenerative disorder in the nigrostriatal pathway of animals and humans and is responsible for most of the movement disorders, including rigidity. The present study aimed to determine the effect of chronic cigarette smoke, alcohol intake, and frequent sexual mating on 1-Methyl-4-phenyl-1,2,3,6-tertahydro pyridine (MPTP)-induced rat model of PD. After treatment, the effect of these factors was determined by biochemical and molecular evaluation. Dopamine (DA) concentration, antioxidant enzymes, and mitochondrial activity decreased after treatment with cigarette smoke, alcohol, and frequent sexual mating when compared to the values in the control group. Excessive exposure of these factors may lead to neurodegeneration, dopaminergic toxicities, and, ultimately, clinical parkinsonism. Earlier literature from different publisher suggested that nicotine and cigarette smoke can protect the dopaminergic neurons in the substantia nigra against MPTP toxicity. In this study, we assessed the effect of the above three factors on an MPTP-treated rat model and concluded that they have a neurodegenerative effect and were found to be toxic to dopaminergic neurons in the substantia nigra. Further investigation is required to understand the exact etiology of clinical parkinsonism.

Key words: Dopamine, mitochondria, MPTP, nigrostriatal pathway

INTRODUCTION

Parkinson's Disease (PD) has a lifetime risk of 2%,

making it the second most common neurodegenerative disease after Alzheimer's disease. Current demographic trends predict a doubling in the number of cases by 2050. Parkinson's disease is mainly observed due to degeneration of dopaminergic pathway in the brain.^[1]

However, other neurotransmitter systems (eg, cholinergic, adrenergic, and serotonergic) also degenerate and cell loss is seen in other brain stem nuclei and the cortex. This nondopaminergic degeneration is a major cause of the nonmotor symptoms of PD (eg, cognitive decline,

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autonomic dysfunction). Although sometimes preceding the motor symptoms, these are predominant in the later stages of the disease.^[2]

Several studies have sought to define the environmental contribution to the etiology of PD. A rural residency and exposure to pesticides appear to increase the risk of the development of PD, particularly of 'young-onset PD'.^[3-5] Although an environmental contribution to the cause of PD seems likely, no specific agent has been clearly identified as causative.

There is evidence for a specific defect of 35% in the activity of mitochondrial complex I in substantia nigra.^[6,7] In addition to the mitochondrial defect in PD, there is substantial evidence of free-radical-mediated damage to proteins and lipid in the substantia nigra,^[8] proteasomal dysfunction,^[9] and inflammatory change.^[10]

Exposure of experimental animals to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides a valuable model of neurotoxicity with behavioral, pathological, and neurochemical features remarkably similar to those of PD.^[11]

Injection of MPTP causes rapid onset of Parkinsonism. MPTP by itself is not toxic, and as a lipophilic compound can cross the blood-brain barrier. Once inside the brain, MPTP is metabolized into toxic cation 1-Methyl-4-Phenylpyridinium (MPP⁺) by enzyme L-Monoamine Oxidase (MAO)-B of glial cells. MPP⁺ kills primarily Dopamine (DA)-producing neurons in a part of the brain called the pars compacta of the substantia nigra. MPP⁺ interferes with complex I of the electron transport chain, a component of mitochondrial metabolism, which leads to cell death and causes the buildup of free radicals and toxic molecules that contribute further to cell destruction. The group of enzymes that form complex I seem to be impaired in PD.^[6,12]

Because MPTP itself is not directly harmful, the toxic effects of MPP⁺ can be mitigated by the administration of Monoamine Oxidase Inhibitors (MAOIs), such as selegiline. MAOIs prevent the metabolism of MPTP to MPP⁺ by inhibiting the action of MAO-B, minimizing toxicity and preventing neural death. MPP⁺ has quite selective abilities to cause neuronal death in dopaminergic cells; it is presumed to be through a high-affinity uptake process in nerve terminals normally used to reuptake DA after it has been released into the synaptic cleft. The DA transporter moves MPP⁺ inside the cell.^[12]

MATERIALS AND METHODS

Animals

Healthy, adult Wistar rats of both sexes (180-220 gm) were obtained from the Central animal house facility from J S S College of Pharmacy, Ootacamund, Tamil Nadu. The animals were kept in a well ventilated room and were exposed to 12 hr day and 12 hr night cycle with a temperature of 25±3°C; humidity 35-60%. The animals were housed in large, spacious, hygienic polypropylene cages during the experimental period. The animals were fed with water and rat feed *ad libitum*. All experiments were performed after obtaining prior approval from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC).

Chemicals and reagents

The following chemicals and drug were used: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) (Merck India Ltd, Mumbai), Dopamine (Sd-Fine Chemicals, Mumbai), Glutathione reductase, Ubiquinone, NADH, Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (Sigma Aldrich, USA), Hexane sulfonic acid, Rotenone, Thiobarbituric acid, Sodium dodecyl sulfate, Phenazine methosulfate, Nitro blue tetrazolium (Loba chemicals, Mumbai).

Grouping of animals

Animals were divided into five groups with 5 male and 5 female rats in each group.

Group I	: Control-I (Negative control)
Group II	: Sham operated control (Positive Control)
Group III	: Cigarette smoke-treated group
Group IV	: Alcohol-treated group
Group V	: Frequent sexual mating group

Induction of Parkinsonism by MPTP

On the zero days to each animal intraperitoneal (ip) injections of 1-Methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP) (20 mg/kg) in normal saline were given. MPTP is a neurotoxin, which after absorption is converted to MPP⁺ radical, which specifically degenerates DA-producing neurons in the *substantia nigra*-a part of the midbrain.^[13] Due to degeneration of dopaminergic neurons, the amount of DA production will reduce and lead to Parkinsonism. Subsequently, 48 hours after induction of Parkinsonism, the animals were treated with cigarette smoke, alcohol and frequent sexual mating continuously for 60 days.

Cigarette smoke treatment

The exposure system consists of two chambers separated by a perforated wall. In the first chamber (combustion), the cigarette was burned passively and the animals were placed in the second chamber (inhalation). During exposure, the compressed air fed the combustion and directed the smoke flow into the inhalation chamber and then to an exit.^[14,15] The animals were subjected to inhalation for 15 min, twice a day with a 12-hour interval. This procedure was done daily up to 60 days.

Alcohol treatment

Daily about 3.0 g/kg body weight of ethanol (alcohol) was ingested orally up to 60 days. Ethanol was administered once a day employing a 20% solution prepared by mixing 26.9 ml of 95% ethanol plus 73.1 ml distilled water (20 g ethanol in 100 ml water). The effect of ethanol on the contents of DA, in the Central Nervous System (CNS) regions of the rat was examined after 60 days.^[16,17]

Frequent sexual mating

Daily mating of the animal with opposite sex up to 60 days by using a harem method (1 male: 3 female) and vice versa. A male was placed in a clear Perspex box (45 cm × 25 cm × 40 cm) for 5 min before a receptive female was introduced; male sexual behaviour was then monitored and recorded for 30 min. If the male failed to display a mount within the first 15 min, the female was removed and replaced with another receptive female and the 30 min session resumed.^[18,19]

BIOCHEMICAL EVALUATION

Estimation of dopamine

The previously reported HPLC method was followed for DA content analysis.^[20] Dissected striata were immediately frozen on dry ice and stored at -80°C. Striatal tissues were sonicated in 0.1 M of perchloric acid (about 100 µl/mg tissue). The supernatant fluids were taken for measurements of levels of dopamine by HPLC. Briefly, 20 µl of supernatant fluid was isocratically eluted through a 4.6-mm C18 column containing paracetamol (100 mg/ml) as the internal standard with a mobile phase containing 50 mM ammonium phosphate pH 4.6, 25 mM hexane sulfonic acid pH 4.04, and 5% acetonitrile and detected by a UV spectrophotometer detector. The flow rate was 1 ml/min. Concentration of DA was expressed as nanograms per milligram of protein. The protein concentrations of tissue homogenates were measured by Lowry's method.

Estimation of total protein by Lowry's method

Protein levels were estimated with the Lowry's method, from similar rat brain slices, which were used for DA assay and anti-oxidant enzymes estimations.^[21]

Lipid peroxidation assay

Lipid peroxidation in the rat brain homogenate was carried out essentially as described earlier.^[22] Rat forebrain (stored at -80°C for less than 8 days) was homogenized in 20 mM Tris-HCl, pH 7.4 (10 ml) at 4°C using a Polytron homogenizer. The homogenate was centrifuged at 1000×*g* for 10 min at 4°C, and the supernatant collected. Further, acetic acid 1.5 ml (20%; pH 3.5), 1.5 ml of thiobarbituric acid (0.8%), and 0.2 ml of sodium dodecyl sulfate (8.1%) were added to 0.1 ml of the supernatant and heated at 100°C for 60 min. The mixture was cooled and 5 ml of *n*-butanol-pyridine (15:1) mixture, 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance measured at 532 nm using Elisa plate reader. Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen–thiobarbituric acid reactive substance.

Estimation of catalase

Catalase (CAT) measurement was done by the ability of CAT to oxidize hydrogen peroxide (H₂O₂). Potassium phosphate buffer (2.25 ml) (65 mM, pH 7.8) and 100 µl of the brain homogenate were incubated at 25°C for 30 min. An aliquot of 650 µl H₂O₂ (7.5 mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT µmol/min mg of protein.^[22]

Estimation of superoxide dismutase assay

Superoxide Dismutase (SOD) activity was analyzed by the method described earlier.^[23] The assay mixture contained 0.1 ml supernatant, 1.2 ml sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 ml phenazine methosulfate (186 µm), 0.3 ml nitro blue tetrazolium (300 µM), and 0.2 ml NADH (750 µM). The reaction was started by addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 ml of *n*-butanol. Colour intensity of the chromogen in butanol was measured spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein.

Analysis of glutathione/glutathione reductase

GSH was measured enzymatically by the method described above.^[24] The striata were homogenized in ice-cold perchloric acid (0.2 M) containing 0.01% EDTA. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min. The enzymatic reaction was started by adding 200 µl of clear supernatant in a spectrophotometric cuvette containing 500 µl 0.3 mM NADPH, 100 µl 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), and 10 µl 25 units/ml Glutathione reductase (all the above three reagents were freshly prepared in phosphate buffer at pH 7.5). The absorbance was measured over a period of 3 min at 412 nm at 30°C. The GSH level was determined by comparing the change of absorbance (ΔA) of test solution with the ΔA of standard GSH.

MOLECULAR PHARMACOLOGY STUDY

Isolation of mitochondria

Tissue was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris HCl, 1 mM EDTA; pH 7.4) and suspensions were centrifuged at $800\times g$, at 4°C, for 10 min. The supernatant fluids were centrifuged at $13000\times g$, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at $13,000\times g$, 4°C, for 10 min to obtain the crude mitochondrial fraction.^[25]

Complex I activity assay

NADH: Ubiquinone oxidoreducase (Complex I) activity was measured in the substantia nigra as described in the literature.^[25] Brain mitochondria, isolated as above, were lysed by freeze-thawing in a hypotonic buffer (25 mM KH₂PO₄, 5 mM MgCl₂; pH 7.4). The reaction was initiated by the addition of 50 µg mitochondria to the assay buffer [hypotonic buffer containing 65 µM ubiquinone, 130 µM NADH, 2 µg/ml antimycin A and 2.5 mg/mL defatted bovine serum albumin (BSA)]. The oxidation of NADH by Complex I was monitored spectrophotometrically at 340 nm for 2 min at 30°C. The activity was monitored for a further 2 min following the addition of rotenone (2 µg/ml). The difference between the rate of oxidation before and after the addition of rotenone was used to calculate Complex I activity.

Statistical analysis

Data are expressed as mean \pm SEM of values. The collected data were subjected to appropriate statistical test such as

one-way analysis of variance (ANOVA), followed by an appropriate Bonferroni multiple comparisons test. P values of less than 0.01 were considered significant.

RESULTS

Estimation of dopamines from brain homogenate

The brain DA level was estimated by using HPLC method. When compared with the control group, Group 2 to 5 showed a significant reduction in the DA levels. When compared with the sham-operated group, Group 3 to 5 showed a significant increase in DA concentration. For the cigarette smoke-treated group, the level of DA was higher than that of alcohol and the frequent sexual mating group [Table 1].

Estimation of protein

The protein estimation was performed by Lowry's method. When compared with control animals, the concentration of protein was significantly reduced for other treatment groups. But when compared with sham-operated control, it significantly increased the protein concentration for Groups 3, 4, and 5 [Table 2].

In vivo antioxidant parameters

The level of lipid peroxidation was estimated. When compared with control animals, the level of lipid peroxidation was more for groups 2 to 4. It was less for group 5 with the significance of ($P<0.01$). When compared with sham-operated control, the cigarette smoke-treated group showed a higher level of lipid peroxidation, and the alcohol-treated group had the maximum level. For frequent sexual-mating group, the level of lipid peroxidation was less [Table 3].

When compared with control animals, a significant reduction was seen in the level of superoxide dismutase for treatment groups 2 to 5. When compared with sham-operated control, the superoxide dismutase level significantly reduced for groups 3 and 4, but at the same time, it increased for group 5 [Table 3].

The control group showed a significant reduction in the glutathione concentration when compared with groups 2 to 5. When compared with sham-operated control, the level of glutathione reductase was significantly increased for groups 2 to 3 [Table 3].

For catalase activity, the control group showed less

Table 1: Effect of cigarette smoke, alcohol, and frequent sexual mating on concentrations of dopamine in the brain homogenate of treatment groups

Treatment Group	Dopamine concentration (ng/mg of protein)
Control group	1754.287 ± 5.432
Sham-operated control	248.78 ± 2.322***
Cigarette smoke-treated group	386.927 ± 2.124*** ##
Alcohol-treated group	156.164 ± 1.470*** ##
Frequent sexual-mating group	178.387 ± 2.249*** ##

***P<0.001 vs. Control group; ##P<0.001 vs. Sham-operated control; One-way ANOVA followed by Bonferroni multiple comparisons test

Table 2: Effect of cigarette smoke, alcohol, and frequent sexual mating on total protein concentrations in the brain homogenate of treatment groups by using Lowry's method

Treatment Group	Concentration of protein (mg/100 mg of tissue)
Control group	30.24 ± 0.2049
Sham-operated control	6.905 ± 0.2041***
Cigarette smoke-treated group	8.695 ± 0.0523*** ##
Alcohol-treated group	9.94 ± 0.08131*** ##
Frequent sexual-mating group	20.57 ± 0.0878*** ##

***P<0.001 vs. Control group; ##P<0.001 vs. Sham-operated control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Table 3: Effect of cigarette smoke, alcohol, and frequent sexual mating, on in vivo antioxidant parameters of the brain homogenate of treatment groups

Treatment Group	Lipid peroxidation (nmol/mg protein)	Superoxide dismutase (U/ml)	Glutathione reductase (nmol/mg of protein)	Catalase (μmol/min mg protein)
Control group	13.180 ± 0.1452	103.71 ± 1.281	1.020 ± 0.005	0.969 ± 0.071
Sham-operated control	46.957 ± 1.117***	65.521 ± 0.4867***	0.593 ± 0.019***	0.482 ± 0.021***
Cigarette smoke-treated group	44.130 ± 0.6254***, ns	43.984 ± 0.726*** ##	0.767 ± 0.005*** ##	0.581 ± 0.03***, ns
Alcohol-treated group	71.613 ± 1.953*** ##	48.894 ± 0.620*** ##	0.731 ± 0.009*** ##	0.559 ± 0.014***, ns
Frequent sexual-mating group	18.804 ± 0.2957** ##	74.475 ± 0.491*** ##	0.806 ± 0.002*** ##	0.721 ± 0.03*** ##

***P<0.001, **P<0.01 vs. Control group; ##P<0.001, **P<0.01, nsP>0.05 vs. Sham-operated control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Table 4: Effect of cigarette smoke, alcohol, and frequent sexual mating on mitochondrial Complex I activity in the brain homogenate of treatment groups

Treatment Group	Complex I activity (nm/min/mg protein)
Control group	99.292 ± 1.903
Sham-operated control	46.012 ± 4.073***
Cigarette smoke-treated group	42.947 ± 1.537*** ns
Alcohol-treated group	45.571 ± 1.626*** ns
Frequent sexual-mating group	74.025 ± 1.271*** ##

***P<0.001vs. Control group; ##P<0.001, nsP>0.05 vs. Sham-operated control; One-way ANOVA followed by Bonferroni multiple comparisons test.

concentration when compared with treatment groups 2 to 5. But when compared with sham-operated control, the catalase activity was significantly increased for frequent sexual-mating groups. Groups 3 and 4 did not show significant variation in the catalase activity [Table 3].

Molecular estimation

The complex I activity was measured from the mitochondrial fraction of brain homogenate. When compared with the control group, it showed a significant reduction in the mitochondrial complex I activity for groups 2 to 5. When compared with sham-operated control, the complex I activity was significantly increased for frequent sexual-mating group and without any significant alteration for groups 3 and 4 [Table 4].

DISCUSSION

Various studies were conducted to access and evaluate the

influence of cigarette smoke, alcohol intake and active sex and their relationship with Parkinsonism, or dopaminergic activities in the brain.

The DA concentration in the midbrain region showed a less level for sham operated control group, obviously it was higher in cigarette smoke treated group and low for frequent sex and alcohol-treated groups. It was previously reported that alcohol administration and sexual stimulation leads to release of DA and increase in the metabolism of dopamine to Dihydroxy Phenyl Acetic Acid (DOPAC) and Homovanillic Mandelic Acid (HVA), respectively.^[26] The increase in DOPAC and HVA in the repeated sexual satiety period could reflect the enhanced dopaminergic transmission.

Furthermore, it was evident from earlier studies that the neurochemical changes found during the intervening state of sexual inactivity (ie, increased levels of DA metabolites) are reminiscent of the effects of DA receptor blockers; this suggests a possible neurochemical mechanism for sexual refractoriness. It was also seen that as the DA level increased, sexual mating frequency in animals increased subsequently.^[27] In the case of alcohol treatment, it was proved that the conversion level of DOPAC and HVA was elevated according to the concentration of alcohol in the blood.^[28] And it was earlier reported that ethanol had no effect on endogenous release of DOPAC, HVA, or DA. However, ethanol did enhance the potassium stimulation, calcium-dependent release of glutamate and aspartate from the striatal region when compared with the normal brain.

These excitatory mediators followed by DA release could have led to neurotoxicity and further neuronal damage of dopaminergic neurons. But in our studies, we could analyze the level of ultimate concentration of DA after 60 days by HPLC method. This states that either cigarette smoke, alcohol, or frequent sexual mating could reduce the DA concentration, and this may be due to an exhaust mechanisms followed by chronic discharge and catabolism of DA up to 60 days, followed by MPTP treatment.

The estimation of total protein concentration was carried out to assess two factors—one was to know the extent of protein degradation or catabolism that has taken place in the brain and the other is for estimation of DA present in the brain homogenates. The level of protein concentration in cigarette smoke and alcohol-treated groups was drastically reduced compared to that in the frequent sexual-mating group. These results suggest that higher cigarette smoke or alcohol intake could induce the relative catabolism of protein or hastens its denaturation.

Oxidative stress and oxidative damage to critical biomolecule is an important process mediating cell death in PD. However, it has not yet been proven that this is the primary event to initiate nigral cell degeneration.^[29] The studies showed an increase in lipid peroxidation for cigarette smoke and alcohol-treated groups, but not for frequent sexual-mating group and the activities of Glutathione reductase, superoxide dismutase, and catalase were reduced by cigarette smoke, alcohol, and frequent sexual-mating groups. Reduction in glutathione might impair H_2O_2 clearance, promote formation of OH radical, and produce oxidative stress. All the antioxidant defence mechanisms were related and a disturbance on one might damage the balance in all. The depletion in Glutathione reductase content and enhancement of lipid peroxidation leads to degeneration of nigrostriatal neurons and, consequently, leads to a reduction in the content of catecholamine.

Earlier studies have proven that MPTP inactivates oxygen-sensitive mitochondrial aconitase in the substantia nigra; further, MPTP mobilises a novel early pool of chelatable mitochondrial ions that balance mitochondrial aconitase inactivation and presides over neurotoxicity. Finally, MPTP-induced mitochondrial aconitase inactivation, chelatable ion, and neurotoxicity lead to degeneration of dopaminergic neurons. Mitochondrial dysfunction and bio-energetic decline are directly related with a reduction in the complex I activities.^[30] Our studies demonstrate that the complex I activity was significantly reduced for the cigarette smoke and alcohol-treated groups as

compared to that of the frequent sexual-mating group. It has been assumed that the dopaminergic cells in the substantia nigra are lost through a cell necrosis and even possibly precipitated by oxidative damage or complex I deficiency, which might have happened because of cigarette smoke and alcohol and less with frequent sexual mating.

In conclusion, we have demonstrated that cigarette smoke, alcohol intake, and frequent sexual mating all proved to have a neurodegenerative effect and were found to be toxic to dopaminergic neurons in the substantia nigra. This suggests that these three factors may lead to clinical parkinsonism through all or any of the diseased parameters. Thus, there is still a need for detailed and specific pharmacological or biochemical or molecular level of discourse to understand the exact etiology or causative factors of clinical parkinsonism to resolve the mystery of neurodegenerative diseases.

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