

The Rotenone Model of Parkinson's Disease in Studying the Mechanisms of Nigrostriatal Cell Death

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Received: 09-27-2015

Accepted: 11-19-2015

Published: 12-12-2015

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Abstract

Parkinson's disease is a progressive neurodegenerative disorder of the basal ganglia for which no specific cause could be found in the majority of cases. For a long time a possible role for an environmental toxin (s) in a genetic background has been thought. Pesticides and insecticides were implicated in the increased prevalence of PD in rural areas. Rotenone is a plant derived pesticide and a complex I (NADH-quinone oxidoreductase) inhibitor. In rodents, the pesticide administered *via* systemic routes as well as locally into specific brain areas eg., the substantia nigra or striatum, resulted in nigrostriatal cell loss, α -synuclein like cytoplasmic inclusions, and motor impairments. Rotenone induces decreased dopamine and tyrosine hydroxylase immunoreactive neurons in the striatum and substantia nigra. Changes in serotonergic, noradrenergic, and cholinergic neurotransmitters also occur. Parkinsonian like α -synuclein pathology also develops inside the spinal cord, dorsal motor nucleus of the vagus as well as in the enteric neurons in rotenone-treated rodents. Oxidative and nitrosative stress, mitochondrial dysfunction, dysfunction of the ubiquitin-proteasome pathway, protein aggregation, microtubule depolymerization, excitotoxicity, microglia activation, and neuroinflammation have been identified as important mechanisms underlying the rotenone-induced death of dopaminergic neurons. Research into the rotenone model of PD offers many possibilities for studying the pathways of cell death in PD and developing new drug therapies.

Keywords: Parkinson's Disease; Pesticides; Rotenone; Rodents; Nigrostriatal Cell Death

Introduction

Parkinson's disease (PD), also known as idiopathic PD or paralysis agitans, is one of the most common neurodegenerative disorders. It ranks the second after Alzheimer's disease. The disorder is that of old age affecting about 1% of the population over the age of 60 [1,2]. Clinically, PD is characterized by bradykinesia or akinesia, muscular rigidity, rest tremor of the hands and less commonly the feet and by postural instability [3,4].

In PD, the dopamine producing cells of the substantia nigra pars compacta (SNpc) and striatum of the midbrain die [5,6]. The loss of these neurons produces the motor features of the disease [7]. The process is a slowly progressive one and motor symptoms start to appear after a substantial percentage of cells degenerate (~50-60% of the SNpc

dopaminergic neurons and 70-80% of striatal nerve terminals) [8]. The pathological hallmarks of the disease are those of nigrostriatal dopaminergic cell loss and the presence of intraneuronal eosinophilic proteinaceous inclusions, known as "Lewy bodies", in the cytoplasm of surviving dopaminergic neurons [9,10].

The cause of PD remains largely unknown. Genetic or familial forms account for ~5% of cases, occur in young age, and these are inherited as an autosomal recessive or autosomal dominant traits. The remaining 95% of the cases are sporadic for which no specific etiology can be found [11, 12]. Parkinsonian symptoms can also occur as a side effect to dopamine blocking drugs eg, neuroleptics, especially the classic drugs eg, haloperidol, cinnarizine, follow repeated head trauma, viral infection, or poisoning due to manganese or carbon monoxide. This is termed secondary parkinsonism [11, 13].

For long time, the involvement of an environmental toxin in causing idiopathic PD has been suspected. Supporting evidence, however, came, when in several illicit drug users features of L-dopa responsive chronic parkinsonism developed after intravenous use of a meperidine analog [14-16]. 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) which developed as a byproduct during the meperidine congener synthesis process was identified as the agent selectively damaging neurons in the substantia nigra [15]. In brains of victims, there were moderate to severe depletion of the pigmented nerve cells in the substantia nigra but no Lewy bodies [17]. This accidental discovery of a selective nigrostriatal neurotoxin has paved the way towards developing animal models of PD and which in turn has resulted in a remarkable progress towards understanding the pathogenetic mechanisms underlying nigrostriatal cell death and studying possible therapeutic interventions.

Another important contribution towards identifying possible environmental toxins causing PD came this time from epidemiological studies. In these studies, there was a consistent and significant increase in the risk for developing PD in subjects exposed to environmental factors such as rural environment and farming, drinking well water and the use of pesticides [18-20]. In particular, one pesticide which is rotenone, a complex I (NADH-quinone oxidoreductase) inhibitor, has been shown to reproduce some of the pathological features of PD in rodents [21-23]. Rotenone is a naturally occurring pesticide, derived from the roots of tropical plants of the Leguminosae family [24]. It is widely used as a garden insecticide and in organic farming. It is also used for eradication of non-native fish in lakes and streams in USA [25].

Rotenone Reproduces the Features of PD

Studies in Rodents

Subcutaneous, Intraperitoneal and Intravenous Rotenone

Ferrante et al. [26] administered rotenone intravenously (i.v.) in rats. The authors reported selective damage in the striatum and the globus pallidus, but not in the substantia nigra. Betarbet et al. [21] used minipumps to infuse 2–3 mg/kg of rotenone i.v. Rotenone caused selective substantia nigra degeneration, α -synuclein inclusions in the affected neurons as well as hypokinesia and rigidity after 28–36 days of injection. Höglinger et al. [27] infused 2.5 mg/kg/day i.v. for 28 days and found loss of striatal dopaminergic fibers, and nigral dopaminergic neurons. Alam and Schmidt [22] reported loss of dopaminergic neurons after 60 days of intraperitoneal (i.p.) treatment with 1.5 and 2.5 mg/kg/day rotenone. Other neurotransmitters were not or were much less affected. Catalepsy, a hunchback posture and reduced locomotion were observed and these were L-dopa responsive. Using the subcutaneous (s.c.) route, Sherer et al. [23] administered rotenone at 2-3 mg/kg daily to rats via os-

motric minipumps. The authors reported selective nigrostriatal dopaminergic toxicity manifested as reduced tyrosine hydroxylase immunoreactivity (TH-ir) with sparing of globus pallidus and subthalamic nucleus neurons after 4 weeks of rotenone injection. In cytoplasm of nigral neurons, aggregates positive for α -synuclein were detected. Sherer et al. [23] noted that not all rats showed nigrostriatal dopaminergic lesions and these were present in only 48.6% of surviving rats as determined by TH-ir. Lapointe et al. [28] observed significant loss of dopaminergic fibers in the striatum but not in the SN of rotenone-treated rats (following 8 days of subcutaneous rotenone delivery at 2.5 mg/kg/day). There was increased α -synuclein and ubiquitin protein expression at the 8th day. These changes were encountered in less than 20% of their rats. Fleming et al. [29] found markedly decreased TH-ir in the striatum in a few of the surviving rats following 3.5 mg/kg/day rotenone for 21 days. In this study, no TH-ir loss was observed following 2.0 mg/kg/day for 21 days. Other researchers, however, reported decreased TH-ir in striatum and SN and Lewy body-like aggregates in SN following s.c. administration of 2 mg/kg/day to rats [30].

In their study, Marella et al. [31] injected rotenone microspheres subcutaneously (100 mg of rotenone/kg) into rats. Thirty days later, there was substantial decrease in the number of SN neurons, decreased striatal dopamine content (by 45%) as well as α -synuclein and ubiquitin aggregates (Lewy body like inclusions). Cannon et al. [32] treated rats with 3 mg/kg/day intraperitoneally daily and observed significant loss of TH-ir in SN neurons and loss of striatal dopamine content. Intracellular aggregates of α -synuclein and poly-ubiquitin positive inclusions were seen in SN of rotenone-treated rats as well. Rats showed bradykinesia, postural instability and rigidity.

In mice, Thiffault et al. [33] reported a slight decrease in striatal dopamine and its metabolites (homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) following s.c. rotenone 1.5 mg/kg three times/week for 3 consecutive weeks. Rotenone at high doses of 5-15 mg/kg was also tested, but with considerable mortality. In this study, no significant change was observed in striatal dopamine content after 24 h or 14 days of high dose rotenone [33]. In another study, mice that received 2.5, 4 or 5 mg/kg of rotenone s.c. daily for 30-45 days exhibited decreased spontaneous motor activity. No significant change in the density of nigral dopaminergic neurons was observed, but old mice given 4 mg/kg of rotenone, however, exhibited a tendency for decreased nigral neurons density [34]. Other studies have shown that rotenone given to mice at 1.5 mg/kg/day, three times a day for two consecutive weeks (a total of 6 injections) was capable of inducing nigrostriatal degeneration. Shrunken, distorted neurons, pericellular haloes and inflammation in the striatum as well as loss of pigmented neurons in the SN were seen. TH-ir decreased in the striatum [35].

Studies also examined the effects of single dose administration. In one study, a single systemic dose of 15 mg/kg s.c. in rats, increased striatal and nigral lactate levels, and caused hypokinesia. Rotenone did not increase dopamine release but enhanced glutamate-induced dopamine release [36]. Similar observations were reported by Antkiewicz-Michaluk et al. [37] who observed no change in striatal dopamine metabolism after a single dose of 10 mg/kg s.c. Li et al. [38], however, reported loss of SN dopaminergic neurons after 1 day and decreased TH-ir after 4 days of s.c. rotenone injection (2 mg/kg/day). Changes progressed gradually over 28 days.

Rotenone induced changes in striatal dopamine appeared to depend on environmental temperature. Single injection of 1.3 or 2.6 mg/kg rotenone at 28°C caused significant decrease in dopamine and DOPAC. This contrasted with the effect seen when mice were maintained at 21°C. Here, rotenone increased dopamine and DOPAC concentrations [39].

Intragastric Rotenone

Rotenone was also given orally to rodents. Inden et al. [40] treated mice with rotenone (30 mg/kg, p.o.) daily for 28 days. The authors reported loss of dopaminergic neurons in the SNc, motor deficits and increased α -synuclein immunoreactivity in some of the surviving neurons. In another study, mice that received oral administration of rotenone (30 mg/kg/day) showed decreased neuromuscular strength and increased brain oxidative stress after 4 weeks of daily rotenone treatment. Jejunal α -synuclein expression and decreased TH-immunoreactive neurons in the SN were reported in mice given intragastric rotenone for 28 days [42]. Pan-Montojo et al. [43] provided data that intragastrically administered low doses of rotenone were able to induce the accumulation of α -synuclein in SN, spinal cord, dorsal motor nucleus of the vagus as well as in the enteric neurons of mice. This last observation is particularly important in view of the involvement of the gastrointestinal tract in the disease process [44,45]. In PD, abnormal salivation, the gastrointestinal symptoms of dysphagia, nausea, constipation, and defecatory dysfunction do occur more frequently compared to controls [44]. In cases staged for sporadic PD-related brain pathology, α -synuclein immunoreactive aggregates were found in neurons of the gastric myenteric and submucosal plexuses [45].

Inhaled Rotenone

The inhalation route has also been tested, but without success. Rotenone-treated mice or rats were asymptomatic. In contrast the MPTP-treated mice showed motor deficits, depletion of striatal dopamine and loss of TH-ir in striatum and SN [46].

Rotenone Administered into the Rat Brain

Other researchers provided data that intrastriatal rotenone is associated with nigrostriatal degeneration whilst being less toxic compared to the systemic route of administration. Antkiewicz-Michaluk et al. [37] administered rotenone (2 μ g) into the median forebrain bundle of rats and observed a decrease in dopamine content in the striatum and SN by 70% and 35%, respectively. There was no affection of the serotonergic system. These changes were observed 21 days after rotenone injection. Saravanan et al. [47] administered rotenone (2-12 μ g/rat) into SNc of rats and observed dose-dependent and significant decrease in dopamine but not serotonin in the ipsilateral striatum by the 5th day of injection. Changes persisted for up to 90 days. Rotenone infused into right medial forebrain bundle resulted in nigrostriatal damage (decreased TH-ir). Changes were most pronounced on day 14 after infusion [48]. Rotenone (2-12 μ g/rat) infused into the right ventral tegmental area and SNc caused more dopamine depletion (TH-ir) in ipsilateral striatum at the 4th week compared to systemic administration of 2 mg/kg/day for 4 weeks (75.8% vs. 43.7%) [49]. Increased α -synuclein expression was also seen in the lesioned brain (~125-156.2 μ g/mice). Rats developed bradykinesia and hypokinesia. Mulcahy et al. [50] infused rotenone solutions (0.4-10.8 μ g/rat) into the striatum and observed a dose-dependent degeneration of the nigrostriatal neurons (TH-ir), but no change in the expression of α -synuclein. Klein et al. [51] reported a dose-dependent impairment in skilled and non-skilled motor function following single rotenone infusion into medial forebrain bundle in order of 4 μ g > 8 μ g or 12 μ g. It was suggested that the changes in motor function do not reflect dopamine cell loss per se or that there is a ceiling effect beyond it the dopaminergic system is unable to compensate for the dopamine depletion. The effect of a single injection of rotenone into the striatum of rats was also investigated. Rats were euthanized after 30 days. Rotenone (1-5 mM/rat; 1.95-9.75 μ g/rat) increased brain oxidative stress in a dose-dependent manner. There were decreased dopamine content and TH-ir in ipsilateral striatum and substantia nigra in the rotenone-treated compared to the vehicle-treated rats. Rotenone increased the number of degenerated cells in SN and depletion of pigment granules from cells [52].

Extra-Nigral Pathology in Rotenone-Treated Rats

The α -synuclein pathology (Lewy pathology) associated with sporadic PD does not develop only in the central nervous system but also occurs in the peripheral, and enteric nervous systems [53]. Similar pathomechanisms appear to drive both cerebral and extracranial Lewy body pathology [54]. Parkinsonian α -synuclein pathology in the enteric neurons has also been reported in rats given rotenone (2.0 mg/kg, i.p.; 5 days/week) for 6-weeks. Myenteric plexus α -synuclein aggregate pathology and cytoplasmic inclusions that resembled enteric Lewy-bodies in idiopathic PD were detected. This occurred

along with moderate and a permanent loss of small intestine myenteric neurons and a slowing of gastrointestinal motility 6-months post-rotenone [55]. Other researchers reported a delay in gastric emptying in 45% of rotenone-treated rats (3 mg/kg/day for 22-28 days by osmotic minipump). Electrophysiological studies indicated a functional defect in enteric inhibitory neurons. However, no change in the number of enteric neurons or their morphology was observed [56]. Delayed gastric emptying is also a finding in early and advanced PD [57]. Biehlmaier et al. [58] reported not only depletion of dopamine in striatum and SN but also decreased number of retinal dopaminergic amacrine cells. In this study, rats were treated with 2.5 mg/kg i.p. daily for 60 days (Biehlmaier et al. 2006). Moreover, Samantaray et al. [59] reported degeneration of cervical and lumbar spinal cord motoneurons following rotenone injection in rats.

Other Neuronal Systems Affected by Rotenone

In PD, the main pathology is that of the selective loss of SNpc neurons which results in the motor features of the disease [57]. Other neuronal populations eg., serotonergic, noradrenergic, and cholinergic are also affected in the course of the disease [60,61]. Changes in these neurotransmitter systems are likely to contribute for many of the non-motor features seen in PD such as cognitive impairment, depression, sleep disturbances, autonomic and visual dysfunctions [62,63]. Degeneration of basal forebrain cholinergic cells occurs early in the disease and is associated with cognitive decline and deficits of odor identification [64]. Moreover, cholinergic denervation might contribute to non-dopaminergic impairments in gait and balance [64,65].

Studies reported changes in serotonin and norepinephrine content in striatum of rotenone treated-rodents. In rats, there was decreased serotonin in striatum after infusion of 12 µg rotenone into right ventral tegmental area and SNc [49]. Changes in hippocampal levels of serotonin and norepinephrine metabolites were also reported after bilateral infusion of rotenone into the SN in rats [66]. Morais et al. [67] reported increments in the serotonin and norepinephrine turnovers in the striatum and hippocampus following rotenone (2.5 mg/kg/day, i.p.) for 10 consecutive days. Moreover, rats treated with (2.5 mg/kg/day, i.v.) for 28 days exhibited loss of serotonergic fibers, and cholinergic interneurons in the striatum. There was also loss of cholinergic neurons in the pedunculopontine tegmental nucleus and of noradrenergic neurons in the locus ceruleus [27].

In PD, there is loss of nigrostriatal dopaminergic neurons which results in an imbalance between dopamine and acetylcholine [68]. In striatum, dopamine control acetylcholine (ACh) release through muscarinic autoreceptors located directly on cholinergic interneurons and thus loss of dopamine potentiates acetylcholine release leading to interruption of the cortico-basal ganglia-thalamocortical loop circuits [69]. It

is through these circuits, that the basal ganglia controls and smoothes the intensity of motor activity initiated by the cortex [70]. Before the advent of L-dopa and dopaminergic agonists, anticholinergic drugs e.g., benzotropine and diphenhydramine were the agents used in the treatment of PD, particularly the associated resting tremor [71]. Studies indicated that rotenone inhibits acetylcholinesterase (AChE) activity in brain of treated rats [72,73] and thus increase the level of ACh.

Other Brain Regions Affected by Rotenone

The rotenone-induced damage is not confined to the striatum and substantia nigra. Other brain areas were also affected whether rotenone was administered systemically or directly into specific brain regions [51, 52, 74]. Klein et al. 2011 [51] reported that the damage caused by rotenone infused into medial forebrain bundle extended beyond the boundaries of the targeted area. We in addition found that the development of oxidative stress due to systemic or intracerebral rotenone involved several brain areas [35, 52, 72,73,74]. In rats that received a single injection of rotenone into the striatum and their brains examined 30 days later, increased levels of oxidative stress and neuronal degenerative changes were observed not only in SN and striatum but also in the cerebral cortex, and hippocampus [52,74]. Moreover, in rats treated with subcutaneous rotenone 1.5 mg/kg three times per week for 2 weeks, degenerated neurons were observed in the SN, striatum as well as in cerebral cortex and hippocampus. In cerebral cortex, gliosis was also seen [72,73]. Similar observations were found in mice treated with rotenone subcutaneously, 1.5 mg/kg three times per week for 2 weeks [35]. Widespread brain damage thus develops as a consequence of rotenone.

Systemic Effects of Rotenone

Rotenone causes systemic toxicity [34, 49]. Peripherally, the liver of rotenone-treated rats showed fatty changes [34]. In another study, following systemic administration of the toxicant, pathological changes were reported in lung, liver, kidney, and spleen of rats, thereby, indicating a systemic toxic effect for rotenone [49]. Lapointe et al. [28] observed hypokinesia after 3-5 days and dystonia after 8 days of s.c. rotenone. Significant loss of dopaminergic fibers occurred on the 8th day of rotenone injection. This led the authors to suggest that general health problems rather than a specific motor deficit have accounted for the hypokinesia observed between 3 and 5 days.

Mortality Due to Rotenone

Rotenone injection in rodents is associated with substantial mortality. Sherer et al. [23] reported severe, systemic toxicity in 36% of their rats. Fleming et al. [29] reported a survival rate of 47%, 44% and 29% following intravenous infusion of 2.0, 2.5 and 3 mg/kg/day of rotenone, respectively. The corresponding survival rates in rats treated subcutaneous-

ly with the above doses of the toxicant were 67%, 36% and 9%, respectively. Biehlmaier et al. reported 33% mortality rate which began after 30 days of rotenone treatment [58]. Other researchers reported a mortality of ~ 10% [32].

Studies in *Drosophila Melanogaster*

The fruit fly, *Drosophila melanogaster* has become an increasingly used model of neurological disorders including toxic and genetic PD [75, 76]. In their study, Coulom and Birman [77], placed flies in contact with a feeding medium supplemented with sublethal concentrations of rotenone. L-dopa responsive, severe locomotor deficits and loss of dopaminergic (but not 5-HT) neurons were encountered after one week. Melatonin, an antioxidant, improved locomotion and protected dopaminergic neurons from rotenone. Shu et al. [78] reported loss of dopaminergic neurons in the brain and severe locomotor deficits. Rotenone also caused marked learning and memory impairment in flies. Sudati et al. [79] reported impaired climbing capability, mobility time and high mortality in rotenone-fed flies. In analyzing the effect of toxic (rotenone and paraquat) and genetic models of PD in *Drosophila*, Navarro et al. [80] found no dopaminergic neuronal loss. The flies, however, exhibited marked decrease in a green-fluorescent protein reporter gene fluorescence in dopaminergic neurons which correlated with the phenotypes observed. Parkinson's disease also results from mutations in a number of genes. For example, mutations in the gene for leucine-rich repeat kinase 2 (LRRK2) cause autosomal dominant PD, while mutations in the DJ-1 cause autosomal-recessive parkinsonism [12]. Studies in fruit flies showed that flies lacking LRRK2 or DJ-1 function were more sensitive to rotenone (and also paraquat) which links genetic susceptibility and environmental toxins in the pathogenesis of PD [81]. Liao et al. [82] suggested that the short-term startle-induced locomotion and long-term spontaneous locomotion represent sensitive assays of rotenone induced toxicity in fruit flies.

In Vitro Studies

Rotenone induced apoptotic cell death of PC12 cells [83], human dopaminergic SH-SY5Y cells [84,85] and reduced the number of TH-ir in primary ventral mesencephalic cultures from rats [86]. In midbrain organotypic slices from rats, rotenone caused the destruction of neuron processes, morphologic changes, some neuronal loss, decreased TH protein levels [87] and increased the release of lactate dehydrogenase, superoxide, and nitric oxide [88]. Rotenone caused dopaminergic cell loss in mouse embryonic mesencephalic cultures. The release of lactate dehydrogenase into the culture medium, and the number of necrotic cells and of apoptotic nuclei increased by rotenone [89]. Rotenone also caused significant toxicity in differentiated PC12 cells, which was accompanied by decreases in ATP levels, changes in catechol levels, and increased dopamine oxidation [90]. In their study, Borland et al. [91] exposed

differentiated SH-SY5Y neural cells to 50 nM rotenone for 21 days. The authors reported death of ~60% of the cells, decreased mitochondrial movements, and Lewy neuritis in cell processes, but no cytoplasmic inclusions resembling Lewy bodies. The model is said to reproduce Lewy neuritic changes of early PD pathology.

Mechanisms of Rotenone-Induced Cell Damage

Mitochondrial Complex I Inhibition

Mitochondrial complex I inhibition has been implicated in the degeneration of midbrain dopaminergic neurons in sporadic PD [92,93]. Rotenone inhibits complex I activity in isolated brain mitochondria [94]. The single-subunit nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase (Ndi1) of the baker's yeast *Saccharomyces cerevisiae* acts as an alternative enzyme that replaces complex I [95]. In their study, Marel-la et al. [31] investigated whether increased expression of Ndi1 would rescue dopaminergic cells in the rotenone model. The expression of the Ndi1 protein in SN (using recombinant adeno-associated virus carrying the NDI1 gene) resulted in prevention of oxidative DNA damage and dopaminergic cell death. Similar conclusions were drawn from the transgenic strains of *Drosophila* that express yeast NDI1. Moreover, the decline in respiratory capacity and increase in mitochondrial reactive oxygen species production associated with aging was reduced by NDI1 expression [96].

Oxidative Stress

In humans with sporadic PD, evidence for increased levels of oxidative stress has been detected in their brains post-mortem [97,98]. Parkinson's disease patients with inadequately controlled motor symptoms who received intravenous glutathione showed symptomatic benefit [99]. Studies suggested that oxidative stress is involved in the rotenone neurotoxicity [22, 35,41, 44, 52, 72,73,74, 100, 101]. In mouse embryonic mesencephalic cultures, Radad et al. [89] provided data that rotenone causes dopaminergic cell loss by decreasing the mitochondrial membrane potential, and increasing the generation of reactive oxygen species. In rats treated with systemic or intrastriatal rotenone, the concentrations of nitric oxide, reactive oxygen species, and lipid peroxidation products rose in brain [22, 35, 41, 44, 52] while reduced glutathione and catalase activity decreased in several brain regions [47, 52, 72,73,74]. In *Drosophila*, rotenone caused marked decrease in the total thiol content in brain homogenates [79]. *In vitro*, rotenone increased the release of reactive oxygen species [85]. Rotenone also caused prominent inducible nitric oxide synthase (iNOS) immunostaining in the striatum and substantia nigra after systemic administration in rats and mice [72,73,102]. Increased generation of nitric oxide *via* iNOS is associated with development of neuronal damage [103]. In rats treated with rotenone, the neuronal NOS inhibitor 7-nitroindazole decreased the in-

creased NOS activity and 3-nitrotyrosine along with decreased nigrostriatal damage [102]. Observations from several *in vivo* and *in vitro* experiments also indicated that the rotenone-induced cytotoxicity could be counteracted with antioxidants. The glutathione precursor N-acetyl-cysteine, attenuated the rotenone-induced accumulation of reactive oxygen species and cell death [83]. In organotypic substantia nigra cell cultures, the rotenone-induced oxidative stress (increased protein carbonyl levels) and the reductions in TH protein and TH-ir were markedly decreased by α -tocopherol (vitamin E) [87].

Paraoxonases are a group of detoxifying enzymes which comprise three isoforms: PON1, PON2 and PON3. These enzymes hydrolyze the toxic metabolites (oxon) of several organophosphorus insecticides and nerve agents and hence their name [104]. Recent studies suggest a possible association between PON1 and neurodegenerative diseases including PD. The risk for developing PD appears to be increased with exposure to insecticides, especially, organochlorines and organophosphorus compounds [105]. Moreover, decreased PON1 activity due to genetic polymorphism might increase the risk of PD in subjects exposed to organophosphates [106]. The activity of the enzyme decreases also in neurological disorders e.g., multiple sclerosis, Alzheimer's disease or other dementias [107-109] where oxidative stress is considered to contribute to the neurodegenerative process [110], thereby suggesting a link between PON1 activity and oxidative stress. Our findings indicated marked and significant decreased in PON1 activity in the brain of mice given systemic rotenone and in rats following the intrastriatal injection of the pesticide [35,52]. Protection against nigrostriatal cell death by agents such as cerebrolysin or methylene blue was associated with recovery of the enzyme activity [72,73]. It is not clear whether the inhibition of PON1 activity by rotenone is the result of a direct toxic effect for the pesticide or caused by increased oxidative stress. There is evidence that PON1 activity decreases with elevated levels of oxidative stress [111].

Neuro-Inflammation

Neuro-inflammation is an important contributor to neurodegeneration in PD [112]. Striatal degeneration in rodents can be induced by injection of lipopolysaccharide (LPS). Gao et al. [113] reported synergistic neurotoxicity for mild toxic concentrations of rotenone and LPS in primary mesencephalic cultures. In a study by Ling et al. [114] rats exposed to LPS prenatally developed higher degree of TH-ir cell loss in response to post-natal rotenone challenge (1.25 mg/kg per day for 14 days) than did their counterparts treated with saline at prenatal time. The mechanism is likely to be increased reactive microglia and the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α). This indicated that the effect of neurotoxins in adult life is enhanced by neuroinflammation at an early age. Sherer et al. [23] reported extensive microglial activation (OX-42-ir) in striatum and substantia nigra of rotenone-treated

rats. Microglial activation was less pronounced in cortex. Chang et al. [115] found that rotenone activates microglia to release oxygen intermediates via myeloperoxidase. Interestingly, however, myeloperoxidase deficiency potentiated rather than inhibited the rotenone effect. Rotenone increased TNF- α , interleukin-6 (IL-6), iNOS mRNA levels in microglia. Rotenone treated rats also exhibited prominent TNF- α immunostaining in the striatum [72]. In rats treated with rotenone (2 mg/kg s.c.), the loss of dopaminergic neurons was associated with microglia activation (OX₄₂ positive cells) and increased expression of interleukin-1 β in SN [38]. The above data implicate TNF- α and interleukin-1 β in the substantia nigra damage due to rotenone. Significant and marked increase in the concentration of monocyte chemoattractant protein-1 (MCP-1) was also observed in the striatum of rats that received systemic rotenone injection [72]. This chemokine is involved in the recruitment and activation of phagocytes, lymphocytes and microglia which exacerbates inflammation and neurodegeneration [116,117]. Spinal cord motoneuron degeneration following rotenone injection in rats was also associated with increased immunoreactivity of glial fibrillary acidic protein and OX-42, suggesting an inflammatory component being involved in the process of neurodegeneration [118].

Intracellular Dopamine

Studies have suggested a role for intracellular dopamine in mediating rotenone toxicity. In culture, the rotenone induced apoptotic cell death of PC12 cells is due to caspase-3 activation. It increased reactive oxygen species, and caused lactic acid accumulation. The effects of rotenone decreased when intracellular dopamine was depleted by prior treatment with reserpine [83]. In their study, Dukes et al. [116] found that methamphetamine potentiated the rotenone-induced PC12 cell toxicity. This effect was markedly reduced when PC12 cells were depleted of dopamine prior to methamphetamine and rotenone co-treatment. In human dopaminergic SH-SY5Y cells, inducing a 40% decrease of dopamine content resulted in suppression of rotenone-induced apoptosis [84]. Rotenone induced dopamine distribution from vesicles to the cytosol [120]. Moreover, in fruit flies lacking vesicular monoamine transporter (VMAT) dopaminergic neurons loss was observed. This increased by rotenone (and paraquat). The loss of dopaminergic neurons by rotenone could be blocked with over expression of VMAT protein [121].

Protein Aggregation

The accumulation of abnormal protein aggregates is the hallmark of several neurodegenerative diseases [122]. In PD, there is accumulation of Lewy bodies. These are composed mainly of aggregates of the synaptic protein α -synuclein as well as other proteins such as parkin, ubiquitin, synphilin, and neurofilaments [123]. The protein degradation pathways eg, molecular chaperones, the ubiquitin-proteasome system and

the autophagy-lysosomal pathway function to prevent the accumulation of misfolded and damaged proteins inside the cell and hence preventing their cytotoxicity [124, 125]. Several *in vivo* and *in vitro* studies reported the accumulation of α -synuclein and ubiquitin aggregates following rotenone exposure [21,23,30,31,32,101]. In their study, Chaves et al. [126] provided data that low concentrations of rotenone could induce protein aggregation. In cultures of hippocampus, substantia nigra and locus coeruleus from newborn rats, rotenone (0.5 nM) caused α -synuclein (in cell bodies and extensions of the locus coeruleus and hippocampus). Moreover, beta-amyloid aggregates, and increased tau hyperphosphorylation (in cells from the hippocampus) were observed. High concentrations of rotenone (1 nM) promoted cell death before protein aggregation. Chou et al. [127] suggested that decreased proteasome activity by rotenone is mediated through increased nitric oxide production, and peroxynitrite resulting in oxidative stress, mitochondrial dysfunction, and degradation of the proteasome protein components. The effect of rotenone was attenuated with antioxidant treatment as well as with nitric oxide synthase inhibitors. Xiong et al. [85] provided data that in human neuroblastoma cell line SH-SY5Y, autophagy enhancers prevented while autophagy inhibitors accentuated the rotenone-induced toxicity. The study implicate impaired autophagy in the rotenone-induced neurotoxicity.

Other Mechanisms

Rotenone could also mediate its neurotoxic effects through apoptotic pathways. Rats treated with systemic rotenone exhibited decreased the levels of the antiapoptotic protein Bcl-2 in the striatum [72,73]. Rotenone also caused prominent immunostaining of cleaved caspase-3 in the striatum of treated rats and mice [35, 72, 73]. *In vitro*, nanomolar concentrations of rotenone induced caspase-3-mediated apoptosis in dopaminergic neurons [84, 128,129,130]. Other researchers provided data suggestive of disassembly of the Golgi apparatus in the early stages of the apoptotic process due to rotenone [131]. In midbrain neuronal cultures, rotenone selectively killed serotonergic neurons. This effect decreased by the microtubule-stabilizing drug taxol and mimicked by microtubule-depolymerizing agents such as colchicine and nocodazole [131]. In primary mesencephalic cultures, rotenone caused microtubule depolymerization [133]. Studies also suggested an excitotoxic mechanism in the rotenone-induced neurotoxicity. In which rotenone potentiates N-methyl-D-aspartate (NMDA) currents by inhibiting voltage-dependent Mg^{2+} block of NMDA-gated channels [134,135].

Conclusions

Rotenone is a plant derived pesticide that has been shown to model human PD when injected into rodents. Collectively, studies indicate that rotenone given via intravenous, subcuta-

neous or intracerebral routes targets the nigrostriatal neurons and causes the appearance of α -synuclein positive inclusions in nigral neurons. The latter is a pathological hallmark of PD. Rotenone causes dopaminergic cell death in the striatum and substantia nigra, decreasing dopamine content and tyrosine hydroxylase immunoreactive neurons. The involvement of other neurotransmitter systems as well as extra-nigral pathology occur in rotenone-treated animals. Despite several limitations, the rotenone model of PD simulates in many aspects human PD and provides a simple model for evaluating possible therapeutic interventions.

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