

# Rotenone: from modelling to implication in Parkinson's disease

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## Abstract

Rotenone (*[(2R-(2 $\alpha$ ,6 $\alpha$ ,12 $\alpha$ ))-1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl)-[1]benzopyran[3,4-b]furo[2,3-h][1]benzopyran-6(6aH)-one)* is a naturally occurring compound derived from the roots and stems of *Derris*, *Tephrosia*, *Lonchocarpus* and *Mundulea* plant species. Since its discovery at the end of the 19<sup>th</sup> century, rotenone has been widely used as a pesticide for controlling insects, ticks and lice, and as a piscicide for management of nuisance fish in lakes and reservoirs. In 2000, Betarbet *et al.* reproduced most of the behavioural, biochemical and pathological features of Parkinson's disease (PD) in rotenone-treated rats. Since that time, rotenone has received much attention as it would be one of the environmental neurotoxins implicated in etiopathogenesis of PD. Moreover, it represents a common experimental model to investigate the underlying mechanisms leading to PD and evaluate the new potential therapies for the disease. In the current general review, we aimed to address recent advances in the hazards of the environmental applications of rotenone and discuss the updates on the rotenone model of PD and whether it is implicated in the etiopathogenesis of the disease.

**Key words:** rotenone, Parkinson's disease, neurodegeneration, pesticides, piscicides.

## Introduction

Rotenone, a naturally occurring substance, is derived from the roots, seeds and stems of some tropical plants including *Derris*, *Tephrosia*, *Lonchocarpus* and *Mundulea* species [26]. Rotenone was discovered hundreds of years ago in South America and Southeast Asia, and nowadays, it is an active ingredient of hundreds of pesticides and piscicides [31]. Most recently, rotenone has attained much attention since 2000 when Betarbet *et al.* reproduced the major features of Parkinson's disease (PD) in rotenone-infused rats [3].

In this review, we aimed to address currently reported hazards of rotenone applications in humans, animals and environment, and recent updates on the rotenone model of PD and to discuss its implication in etiopathogenesis of PD.

## Historical background

The use of rotenone goes back hundreds of years when Peruvian natives used crude extracts of rotenone-containing plants e.g. cubè plant to catch fish for eating. In the early 1900s, botanists looking for useful plants of commercial values in Peruvian jun-

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gles exported large quantities of plant roots and extracts with the major active ingredient being rotenone to the USA for use as insecticides on crops [31]. Rotenone was first isolated between the years 1895 and 1902. While travelling in French Guiana, French botanist Emmanuel Geoffroy isolated an active chemical compound that he called nicouline from the plant *Lonchocarpus nicou*. In 1902, Japanese chemist Nagai Nagayoshi isolated a pure crystalline compound from *Derris elliptica* and he called it rotenone. Nicouline and rotenone were recognized to be chemically the same by 1930 [39]. Since 1932, rotenone has been used extensively in fisheries management as a piscicide in North America. Nowadays, rotenone is recognized as the most environmentally benign pesticide and piscicide worldwide [46].

### Physical and chemical characters

Rotenone is an odourless and colourless to brownish crystalline powder, which belongs to a class of compounds of related molecular structure referred to as isoflavones. Its empirical formula is  $C_{23}H_{22}O_6$  with a molecular weight of 394.41 and a melting point of 165-166°C. It is insoluble in water and on the other hand, it is very soluble in many organic solvents. Rotenone is very sensitive to light and oxygen, decomposes to less toxic products, e.g. rotenolone. The rate of decomposition of rotenone is dependent upon several factors such as temperature, pH, sunlight, depth, dose and presence of organic debris [16].

### Toxicokinetics and metabolism

Rotenone is absorbed in relatively different amounts through the gastrointestinal tract, lungs and skin. Low and incomplete absorption of rotenone occurs in the gastrointestinal tract. Fats and oils enhance the absorption of rotenone from the intestines. Rotenone dust can find its way to the lungs through inhalation; experimental inhalation of rotenone dust in rats and dogs resulted in earlier clinical signs than following oral ingestion. Dermal absorption of rotenone is negligible. Application of a single dose of 5 g/kg rotenone on rabbit's skin resulted in no systemic toxicity or mortalities. Rotenone is metabolized by NADP-linked hepatic microsomal enzymes in the liver. Unabsorbed rotenone is excreted by the faecal route and about 20% of the oral doses are excreted in the urine after 24 h. Several metabolites of rotenone metabolism such as

rotenolone, and hydroxyl- and dihydroxyrotenone are identified in the blood, urine, faeces and liver. These metabolites can serve as a biomarker of rotenone exposure [20,24].

### Current applications of rotenone

#### Fisheries management and marine research

Rotenone is still extensively used as a selective piscicide for fisheries management in the USA, Canada and more than 30 countries worldwide. This includes controlling of undesirable fish, eradication of harmful and exotic fish, quantification of fish population, controlling of fish diseases, and restoration of threatened and endangered fish species [46]. Also, rotenone is considered a unique and efficient tool for sampling fishes for marine research [61]. In 2007, the Environmental Protection Agency (EPA) allowed re-registration of all piscicidal uses of rotenone as none of them posed any adverse effects on the humans and environment [76].

#### Controlling pests

After its discovery in 1848 and for more than 150 years, rotenone had been commonly used as a broad-spectrum insecticide for controlling a wide range of pests in numerous crops and home garden, and lice, ticks and mites in veterinary practices and animal husbandry. Currently, food uses of rotenone have been cancelled in the USA, Canada and the EU. However it continues to be used legally in many other countries [46].

### Implication of rotenone use on humans, animals and environment

#### Rotenone toxicity in humans

The World Health Organization (WHO) classifies rotenone as a moderately hazardous agent (a class II pesticide). Rotenone poisoning in humans is uncommon as: (1) the estimated oral  $LD_{50}$  of rotenone in humans is much higher (300-500 mg/kg b.w.) to expose to it and upon exposure, rotenone is efficiently metabolized in the gut and so little or no rotenone goes to the blood stream [17]. Moreover, the most effective route of exposure, the intravenous one, is difficult to happen and absorption of rotenone through lungs and skin is negligible [20]; (2) Quick decomposition of environmental rotenone

to less toxic products decreases the opportunities of human exposure to toxic rotenone [31]; (3) EPA has cancelled all food uses of rotenone since 2006 and only allowed re-registration of its piscicidal uses with strict regulations [46]. Therefore, fatalities of rotenone in humans were only reported following accidental or intended poisoning. In this context, Holland [29] reported that natives in Papua Guinea were seen to eat the roots of plants known to contain rotenone as a method of deliberate suicide. Three fatalities were reported following ingestion of commercially available rotenone-containing formulations in a 3.5-year-old girl [9], a 47-year-old woman [78] and a 49-year-old Tamil man [12]. The three cases showed a similar course of signs including vomiting, irregular respiration, unconsciousness, hypotension and circulatory failure, and they eventually died after 5 h – 3 days [12]. Implication of rotenone in PD development will be discussed in a separate section.

### Rotenone toxicity in animals

Rotenone toxicity is moderate and widely varies between and within animal species. The oral LD<sub>50</sub> values of rotenone are approx. 1.5 mg/kg b.w., 60-135 mg/kg b.w. and 350 mg/kg b.w. in rabbits, rats and mice, respectively. Systemic uptake of rotenone results in higher toxicity than by the oral route. The estimated intravenous and intraperitoneal LD<sub>50</sub> values in rabbits and mice are 0.35-0.65 mg/kg b.w. and 2.8 mg/kg b.w., respectively [20]. In general, common clinical signs of rotenone poisoning in animals include pharyngitis, gastric pain, vomiting, muscle tremors, chronic convulsions and respiratory stimulation followed by depression. It can also lead to severe signs of hypoglycaemia, liver failure, alterations in arterial blood gases and acid base balance, and hypercapnia and hypoxemia due to seizures and respiratory depression. Death can result from cardio-respiratory failure [20]. Compared to mammalian species, rotenone is highly toxic to fish as it is rapidly absorbed from the gastrointestinal tract and directly uptaken through gills to the blood stream [20].

### Effects of rotenone on the environment

Because rotenone breaks down quickly by exposure to light and temperature, its impact on the environment is considered to be low. It has low mobility in soil and only travels less than one inch through most

soils. It does not leach far into underlying sediment and therefore, it does not affect groundwater supplies [8].

### Rotenone model of Parkinson's disease

In 1985, rotenone was first used by Heikkila *et al.* to model PD by stereotaxic administration of 5 mg of this mitochondrial complex I inhibitor into the rat brain [25]. Drawbacks of Heikkila's study were the use of a higher dose of rotenone, i.e. approximately 500,000-fold higher than the half maximal inhibitory concentration (IC<sub>50</sub>) of 10 nM and the stereotaxic route of administration which is an impracticable route for rotenone exposure. Another trial of using rotenone to model PD was in 1997 when Ferrante *et al.* [13] administered 18 mg/kg/day intravenously to rats. In addition to the higher dose, the results were nonspecific brain lesions and peripheral toxicity [13]. In 2000, Betarbet *et al.* succeeded to reproduce the two pathological hallmarks of PD, i.e. the loss of dopaminergic neurons and the formation of Lewy-like bodies in the surviving dopaminergic neurons, as well as some of the parkinsonian motor deficits by systemic administration of rotenone in rats [3]. Following Betarbet *et al.*'s study, the rotenone model has been widely used by many researchers to investigate both the mechanisms that underlie dopaminergic cell death and to test new potential symptomatic and neuroprotective therapies in PD [34,59]. This is due to: (1) its extreme lipophilicity that enables it to cross cellular membranes independent of any transporter producing systemic inhibition of mitochondrial complex I [19]; (2) its implication in many pathogenic pathways that mediate dopaminergic cell death including oxidative stress,  $\alpha$ -synuclein phosphorylation and aggregation, and Lewy pathology, DJ-1 acidification and translocation, proteasomal dysfunction and nigral iron accumulation [4]; (3) its capability of reproducing some of the non-motor symptoms of PD, most notably disruption of gastrointestinal and olfactory discrimination [34]; (4) its usefulness in assessing some end-points such as  $\alpha$ -synuclein accumulation, ubiquitin-proteasome function and GIT dysfunction in addition to preservation of dopaminergic neurons and related motor functions which are the most commonly used endpoints in neuroprotective studies [7]. Beside the intravenous route of rotenone administration used in Betarbet's study, researchers also adopted oral, subcutaneous and intraperitoneal routes to deliver

rotenone in different animal models of PD. Intra-peritoneal rotenone administration was reported to produce highly reproducible PD-like lesions including L-dopa-responsive locomotor deficits and loss of dopaminergic neurons in substantia nigra associated with  $\alpha$ -synuclein pathology. These features make it a well-suited model for the assessment of pathogenic pathways and experimental therapeutic intervention [7]. The subcutaneous route of rotenone administration has recently attracted some attention as it is more convenient, simple and efficacious in reproducing features of PD in animal models [81]. Use of the oral route is primarily intending to test the effect of rotenone on gastric motility and enteric the nervous system as its absorption from the gastrointestinal tract is low and incomplete to produce systemic mitochondrial inhibition [72]. Rotenone was also used to study PD in some *in vitro* cellular models such as primary mesencephalic cell culture, neuroblastoma cell line (SH-SY5Y) and pheochromocytoma cells (PC12). Apart of motor clinical signs, these rotenone *in vitro* models of PD presented most of the cellular and molecular pathology that occurs in PD patients, most notably dopaminergic cell loss (Fig. 1) [55] and formation of protein aggregates containing  $\alpha$ -synuclein [80]. Unlike MPP<sup>+</sup> which specifically damages dopaminergic neurons, rotenone was shown to injure other neuronal populations in primary mesencephalic cell culture. Similar results were obtained by our laboratory for some other neurotoxins like domoic acid [56] and acrylamide [57].

## Mechanisms of action of rotenone

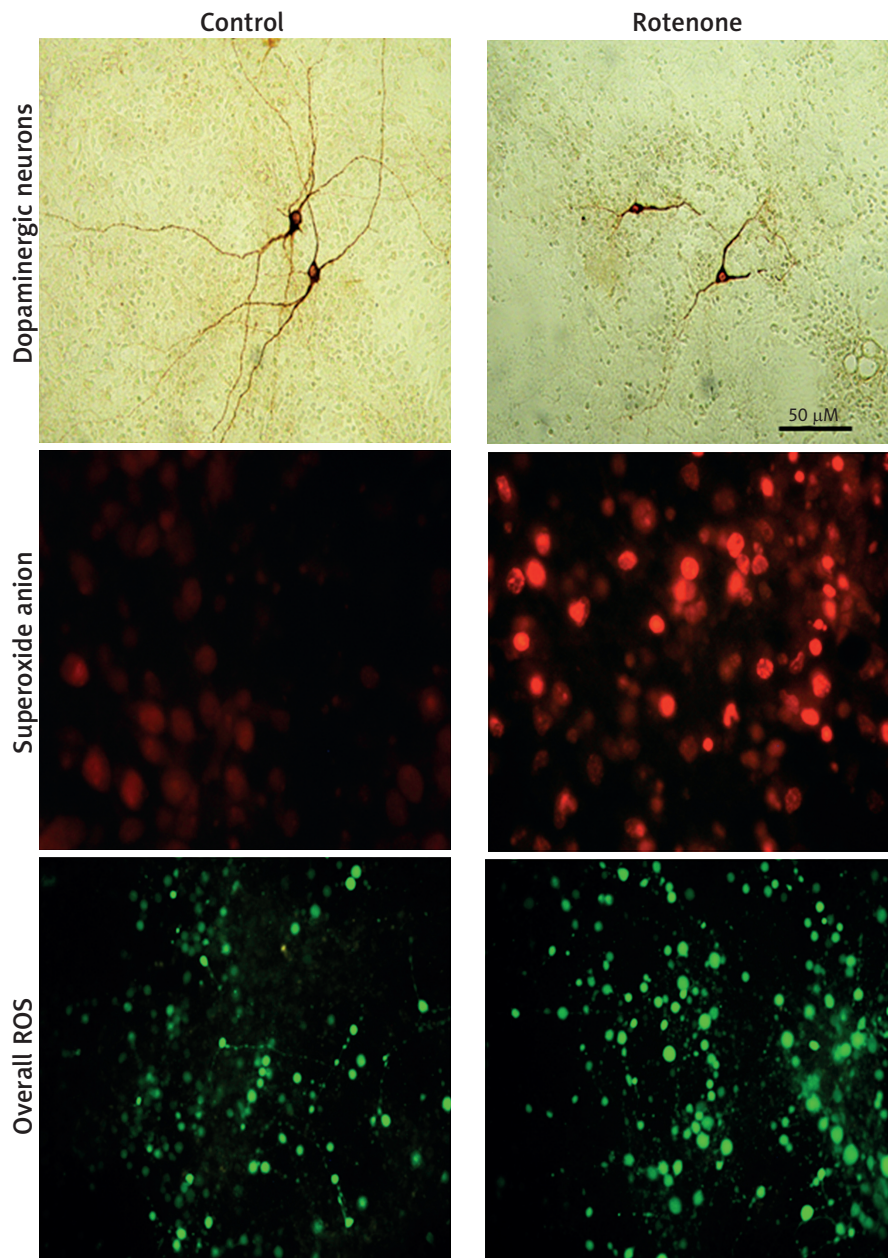
### Complex I inhibition and production of reactive oxygen species

Mitochondria generate ATP via oxidative phosphorylation complexes that are present in their inner membranes. These complexes are known as NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). The process of oxidative phosphorylation is initiated by oxidation of NADH by the complex I enzyme. This results in transferring two electrons, which reduces ubiquinone to ubiquinol. Ubiquinol is re-oxidized by the complex III enzyme and transfers electrons to reduce molecular oxygen to water at complex IV. As a result, the redox energy released during this pro-

cess is used to transfer protons from the mitochondrial matrix to the periplasmic space that generates proton-motive force across the inner mitochondrial membrane at complex I, III, and IV. This proton-motive force is used by complex V to produce ATP from ADP and inorganic phosphate [64]. Therefore, complex I is the major entry point for electrons to the respiratory chain and the rate-limiting step in overall respiration. However it is still the most complex and least understood component of the mitochondrial oxidative phosphorylation system [63,77].

Inhibition of complex I is the strongest action of the pesticide rotenone [26]. This effect of rotenone dates back to the 1960s [73] when Palmer *et al.* [53] found that rotenone inhibited electron transfer from the iron-sulfur centres in complex I to ubiquinone leading to blockade of oxidative phosphorylation with limited synthesis of ATP in submitochondrial particles. Singer and Ramsay [68] described two binding sites for rotenone that must be occupied to completely inhibit NADH oxidation: one is located in the corner between the two arms of the l-shaped protein complex [40] and the other is in the hydrophobic domains in the membrane bond arm of the protein which may be responsible for the formation of superoxide radicals. The sensitivity of complex I to rotenone was reported to be different in-between species and ages. In this context, Ueno *et al.* [75] found that the effect of rotenone is not equal in all species and considerable differences in various taxa are shown. Lenaz *et al.* [41] reported that mitochondria are not identical and their activity alters during aging. For instance, Genova *et al.* [15] observed a decrease in complex I sensitivity to rotenone in liver, heart and muscle mitochondria of 24 more than 4-month-old rats.

Rotenone inhibition of electrons transfer from the Fe-S centres in complex I to ubiquinone results in reducing oxidation of NADH and ATP formation [62]. In addition to decreasing ATP production, electrons that leak at complex I can reduce oxygen that was not reduced at complex IV to reactive oxygen species (ROS) such as superoxide and hydrogen peroxide [67]. Elevation of ROS production by rotenone was observed in some experimental models. For instance, Radad *et al.* [58] found that treatment of primary mesencephalic cell culture with 20 nM of rotenone significantly increased formation of superoxide radical (Fig. 1) and overall ROS (Fig. 1) as measured by dihydroethidium and C-DCEH-DA fluorescent dyes, respectively. Li *et al.* [43] reported that



**Fig. 1.** Treatment of primary mesencephalic cell cultures with rotenone on 10<sup>th</sup> DIV for 48 h results in loss of dopaminergic neurites (TH immunostaining), increases formation of superoxide radicals (DHE fluorescent dye) and rises overall ROS (CDCDHF-DA fluorescent dye) compared to untreated control cultures.

rotenone was able to increase ROS production both in isolated mitochondria from HL-60 cells and the cultured HL-60 cells themselves. Besides production of ROS, rotenone was reported to decrease the activities of antioxidant enzymes. In this context, Ojha *et al.* [52] reported that rotenone administration to rats significantly reduced the activity of superoxide dismutase (SOD) and catalase (CAT), and depleted

glutathione (GSH) concentrations. Elevation of ROS production and reducing activity of antioxidant enzymes lead to oxidative stress, the process that mediates most of the rotenone-induced insults.

### Inflammatory mechanisms

Inflammation was reported as one of important underlying mechanisms that mediate rotenone-induced

damage to neuronal cells. In this context, it was shown that rotenone increased the release of pro-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in the BV2 cells [44] and in rat's brain tissues [33,52,65,74]. Microglial activation was reported to play a central pillar in rotenone-induced neuroinflammation. Zaitone *et al.* [79] found that rotenone upregulates genes encoding CD11bc, a microglial surface antigen, in mice brain. Activated microglia was shown to release cytotoxic inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  [14]. TNF- $\alpha$  activates intracellular death signalling pathways such as nuclear factor  $\kappa$ B (NF- $\kappa$ B), c-Jun N-terminal kinase (JNK) and p38 pathways, and increases cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions which further amplify inflammatory cascades [28]. Activated microglia was also seen to lead to neuronal damage through phagocytic activities without increasing pro-inflammatory cytokines in primary neuronal/glial cultures prepared from rat cerebella [11]. Prevention of such rotenone-induced neuronal loss by inhibition of microglial phagocytic activities indicates that rotenone neurotoxicity is at least partially mediated by microglial phagocytosis [11]. On the other hand, Klintworth *et al.* [38] showed that rotenone did not act directly on microglia in cultures prepared from C57BL/6 mice and rotenone-induced microglial activation may occur as a result of neuronal damage or due to releasing of some factors by other neurons or cells.

### Microtubules depolarizing effects

Rotenone was shown to depolarize microtubules in some *in vitro* studies. For instance, Passmore *et al.* [54] showed that treatment of COS-7 cells with higher rotenone concentrations resulted in a complete depolarization of microtubules. Bisbal *et al.* [5] reported that rotenone decreased microtubule stability in cultured hippocampal neurons. This depolarizing effect of rotenone on microtubules was suggested to be attributed to binding to tubulin, the protein that polymerizes to microtubules [70]. Protection of microtubules against rotenone-induced depolarization by the microtubule-stabilizing drug taxol confirmed the depolarizing effect of rotenone on microtubules [54,60]. As a microtubule-depolarizing agent, rotenone can result in (1) arresting mitosis and inhibition of cell proliferation [70], (2) inhibition of axon

growth by releasing Lfc, a specific GEF for RhoA, from depolarized microtubules [5] and (3) disruption of vesicular transport of dopamine along microtubules leading to accumulation of dopamine in the dopaminergic soma. This can result in increasing oxidative stress due to oxidation of cytosolic dopamine [60]. Moreover, induction of microtubule depolarization in COS-7 cells by rotenone was shown to alter peroxisome morphology and distribution, and on the other hand, treatment of COS-7 cells with rotenone after stabilization of microtubules by paclitaxel resulted in no effects on peroxisome. Peroxisomes are closely linked to the mitochondria and they both maintain a redox-sensitive relationship [54].

### Autophagy inhibition

Autophagy is a highly regulated intracellular catabolic process that mediates the degradation of unnecessary materials and dysfunctional organelles in eukaryotic cells [21]. The process of autophagy is essential in maintaining cellular haemostasis and protection against various physiological and pathological stresses, and on the other hand, can lead to some pathological processes [49]. Rotenone was reported to inhibit the autophagy system both in *in vitro* and *in vivo* experimental models [32,36]. The raised question now is whether rotenone inhibits autophagy process by decreasing autophagy initiation or autophagy-lysosomal pathway (ALP). The answer to this question is still controversial. Suppression of Beclin 1 expression which acts during initiation of autophagosome formation in rotenone-treated SH-SY5Y neuroblastoma cells indicates that rotenone inhibits autophagy process earlier at the initiation step [32,36]. On the other hand, accumulation of autophagic vacuoles as indicated by increasing the expression of microtubule associated protein-light chain 3-II (LC3-II) in rotenone-treated SH-SY5Y neuroblastoma cells and C57BL/6 mice [82] indicates that rotenone either stimulated autophagic vacuoles formation or decreasing ALP. LC3-II is an accepted and selective marker of autophagic vacuoles. Mader *et al.* [49] showed that autophagic accumulation by rotenone in SH-SY5Y neuroblastoma cells resulted not from autophagy induction but rather from a block in the lysosomal degradation of autophagic vacuoles. This is because treatment of SH-SY5Y neuroblastoma cells with bafilomycin A1 prior to rotenone resulted in no increase in levels

of LC3-II. Bafilomycin A1 is a selective inhibitor of vacuolar-type V-ATPase that completely blocks degradation of autophagic vacuoles through inhibition of autophagic vacuole-lysosome fusion [49].

## Rotenone-induced cell death

### Apoptotic cell death

The pesticide rotenone was shown to induce apoptotic cell death in some cellular and animal experimental models. For example, Ahmadi *et al.* [2] reported that exposure of primary dopaminergic neuronal cell culture to rotenone resulted in increasing the number of apoptotic tyrosine hydroxylase positive neurons (TH+) and that was correlated with upregulation of caspase-3 immunoreactivity. Lin *et al.* [45] showed that chronic rotenone intoxication resulted in apoptotic cell death in rat's striatum. Using neuroblastoma SH-SY5Y cells, it was shown that rotenone induced apoptotic cell death through activation of caspases [37,48], and p38 and JNK pathways [50], and upregulation of Bax and downregulation of Bcl-2 [10]. In PC12 cells, Hirata *et al.* [27] showed that rotenone induced apoptosis through activation of JNK and p38 mitogen-activated protein kinase (MAPK). Increasing mitochondrial ROS is implicated in rotenone-induced apoptotic cell death [43]. In this context, the authors showed that rotenone-induced apoptosis was inhibited by antioxidants such as GSH, N-acetylcysteine and vitamin C [43]. Moreover, they observed that HT1080 cells overexpressing magnesium superoxide dismutase were more resistant to rotenone-induced apoptosis than untreated control cells [43].

### Necrotic cell death

Not only apoptosis, rotenone was also reported to induce necrotic cell death. In this context, Kamalden *et al.* [35] found that rotenone induced degeneration of RGC-5 cells by activation of mitogen-activated kinase and not caspase-dependent apoptosis. Hong *et al.* [30] found that rotenone induced necrotic cell death in PC12 cells. Recently, Callizot *et al.* [6] have shown that rotenone treatment resulted in necrotic cell death of primary rat dopaminergic neurons at higher concentrations. According to Skulachev [69], rotenone seems to induce necrotic cell death through ATP depletion. Generally, rotenone was reported to cause apoptosis at low concentrations and necrosis at high concentrations [22].

### Necroptotic cell death

In addition to apoptotic and necrotic cell death, rotenone was reported to lead to necroptosis in primary rat dopaminergic neurons as measured by the upregulation of RIPK3 after 24 h of exposure [6]. Upregulation of RIPK3 is an indicator of necroptotic cell death [47].

## Implication of rotenone in Parkinson disease

For a long time, it has been known that there is an increased risk of PD among people who live in rural areas compared to those who live in cities and this would be attributed to some kind of environmental factors including pesticides [66]. In 2000, successful reproduction of most behavioural, biochemical and pathological features of PD in systemically rotenone-treated rats by Betarbet *et al.* raised significant concerns about rotenone contribution to PD [3]. Following Betarbet *et al.*'s study, researchers showed that rotenone can produce a number of pathological processes such as inflammation [46], apoptotic cell death [2], autophagic impairment [32] and microtubule depolarization [54] in rodents similar to those occurring in parkinsonian human brains. Besides CNS pathology, rotenone was also found to cause progressive functional and pathological changes in the enteric nervous system of rodents mimicking changes found in human PD [18]. All together strengthen the association relationship between the pesticide rotenone and PD, and support Greenamyre's belief that rotenone can produce PD in humans as it did in experimental animals [1]. Supporting animal experiments, some epidemiological studies have linked exposure to pesticides with an increased risk of PD in humans [51]. Of which, Tanner *et al.* [71] in a case-control study nested in the Agricultural Health Study (AHS) found that PD was positively associated with exposure to two groups of pesticides defined experimentally by impairing mitochondrial function (rotenone) and increasing oxidative stress (paraquat). On the other hand, short environmental half-life and limited bioavailability of rotenone, and its detoxification in the gut by enzymatic, bacterial and hydrolytic reactions make its relationship to PD questionable [23,42].

## Conclusions

Rotenone is still used as a non-specific broad-spectrum insecticide and as a piscicide worldwide. Data from experimental and epidemiological studies show

a clear association between rotenone exposure and a higher risk of PD. However the causal relationship between rotenone and PD is still questionable. Rotenone in *in vitro* and *in vivo* models is an invaluable tool for investigating most molecular and cellular pathology that occur in PD patients and therefore can efficiently test new treatment strategies that would be beneficial for patients with PD.

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## Disclosure

The authors report no conflict of interest.

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