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Cytotoxicity of mitochondrial Complex I inhibitor rotenone: a complex interplay of cell death pathways

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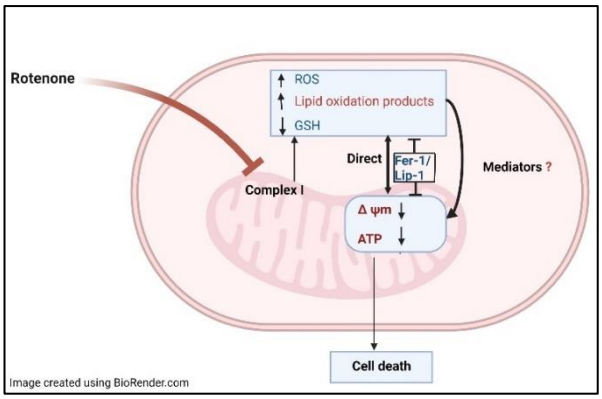
Abstract



Ferroptosis has been identified as a type of regulated cell death triggered by a diverse set of agents with implications in various diseases like cancer and neurodegenerative diseases. Ferroptosis is iron-dependent and accompanied by an accumulation of reactive oxygen species (ROS) and lipid oxidation products, a depletion of reduced glutathione, mitochondrial morphological alterations and the rupture of cell membrane; the process is inhibited by specific antioxidants like ferrostatin-1 and liproxstatin-1 and by other general antioxidants like the iron-chelator deferoxamine, vitamin E and N-acetylcysteine. However, the mechanism of cell death in ferroptosis subsequent to the accumulation of ROS and lipid oxidation products is not clearly established.

We show here that the classical mitochondrial Complex I inhibitor rotenone (0.5 μM) causes death of SH-SY5Y cells (a human neuroblastoma cell line) over a period of 48 h accompanied by mitochondrial membrane depolarization and intracellular ATP depletion. This is associated with an intracellular accumulation of ROS and the lipid oxidation product malondialdehyde or MDA and a decrease in reduced glutathione content. All these processes are inhibited very conspicuously by specific inhibitors of ferroptosis such as ferrostatin-1 and liproxstatin-1. However, the decrease in Complex I activity upon rotenone-treatment of SH-SY5Y cells is not significantly recovered by ferrostatin-1 and liproxstatin-1. When the rotenone-treated cells are analyzed morphologically by Hoechst 33258 and propidium iodide (PI) staining, a mixed picture is noticed with densely fluorescent and condensed nuclei indicating apoptotic death of cells (Hoechst 33258) and also significant numbers of necrotic cells with bright red nuclei (PI staining).

1. Introduction

An important sub-type of regulated cell death (RCD) called ferroptosis has been under intensive investigation over the last few years, and the process has been implicated in different diseases, including cancer and neurodegenerative diseases [Li et al., 2020; Jiang et al., 2021]. Ferroptosis is an iron-dependent pathway of RCD which is accompanied by an excess production of reactive oxygen species (ROS) and lipid oxidation products, depletion of glutathione, mitochondrial morphological alterations, and the rupture of the cell membrane; the process is inhibited by iron-chelator like deferoxamine, vitamin E and some specific antioxidants known as ferrostatin-1 and liproxstatin-1 [Xie et al., 2016; Stockwell et al., 2017]. Ferroptosis can be triggered by many different pharmacological agents, which cause a depletion or decreased synthesis of the major intracellular antioxidant like reduced glutathione (GSH) or inhibit the antioxidant enzyme glutathione peroxidase 4 (Gpx4), or cause an increase in the intracellular iron content [Jiang et al., 2021; Li et al., 2020]. Several pathways regulating or initiating ferroptosis in cells have been identified primarily by genetic studies, but definite mechanisms or mediators executing cell death following the accumulation of ROS and lipid oxidation products are yet to be established in ferroptosis. Mitochondria being the most important source of ROS generation *in vivo*, it is also important to investigate how mitochondrial bioenergetic alterations are involved in ferroptotic death of cells. In fact, mitochondrial respiratory chain inhibitors and TCA cycle intermediates have been implicated in complex and contradictory ways with ferroptosis [Gao et al., 2019; Yao et al., 2021; Gan 2021]

Linking the process of ferroptosis with neuronal death in neurodegenerative diseases like Alzheimer's disease (AD) or Parkinson's disease (PD) appears to be an attractive proposition because both increased iron accumulation and oxidative damage in specific brain regions are key pathological features of these diseases [Ganguly et al.,

2017]. Unlike AD, several toxins-based animal models are quite popular in analyzing the PD pathogenesis or screening for potential anti-parkinsonian drugs [Duty and Jenner, 2011; Betarbet et al., 2002]. Despite the limitations of cell-based models to recreate the pathologic scenario of a complex disease like PD, the cytotoxic mechanisms of several PD-related toxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, rotenone have been extensively studied in different cell lines and primary cultures of neurons [Ke et al., 2021]. Rotenone, the classical inhibitor of mitochondrial complex I, causes cell death in different experimental models, but the mechanisms and the nature of cell death are quite varied in published papers [Moon et al., 2005; Radad et al., 2006; Callizot et al., 2019]. Because of the known toxicity of rotenone on mitochondria and its ability to generate ROS, we attempted to study if rotenone induces ferroptotic death in SH-SY5Y cells; this catecholaminergic human neuroblastoma cell line is widely used to study PD neurodegeneration [Xicoy et al., 2017].

2. Materials and Methods

2.1. Cell culture and treatment protocol

SH-SY5Y cells (purchased from NCCS, Pune, India) were maintained in a medium composed of 2 volumes of Dulbecco's modified Eagle's medium (DMEM) and 1 volume of Ham's F12 medium containing 10 % fetal bovine serum; penicillin 50 units/mL, streptomycin 50 µg/mL, and amphotericin B 2.5 µg/mL were also present in the medium. For experimental purposes, the cells were grown and manipulated in plastic tissue culture flasks or multi-well tissue culture plates, or poly-L-lysine coated coverslips under a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. The cells were treated without (control) or with rotenone (0.5 µM) for 48 h in the presence or absence of ferrostatin-1 (Fer-1, 1 µM), liproxstatin-1 (Lip-1, 1 µM), or other additions followed by the analysis of various biochemical and microscopic parameters.

2.2. Measurement of cell viability

The cell viability was assessed by Trypan blue assay as well as by LDH release assay [Ganguly et al., 2020]. In Trypan blue assay, the dead (blue-stained) and live cells were counted by an automated cell counter (Thermo Fisher Scientific, USA). In addition, the LDH released in the medium in each well was assayed spectrophotometrically by monitoring the oxidation of NADH at 340 nm using pyruvate as the substrate; the activity of the enzyme released in each well was normalized with the protein content and then expressed as a percentage of LDH released in reference wells with 100 % cell death (cell treated with 10 µM antimycin) [Ganguly et al., 2020].

2.3. Measurements of oxidative stress parameters

ROS production and the cellular levels of malondialdehyde (MDA) and reduced glutathione were measured as oxidative stress parameters in control and various groups of treated SH-SY5Y cells. For ROS measurement, the fluorogenic ROS probe, 2', 7'-dichlorodihydrofluorescein diacetate or H_2DCFDA, was utilized; intracellular esterases hydrolyzed H_2DCFDA to release $DCFH_2$, which reacted with ROS including H_2O_2 to produce a fluorescent product (λ_{ex} 485 nm, λ_{em} 535 nm) [Jana et al., 2011]. The assay was conducted with the cells in 96-well plates; after removal of the medium, each well was

gently rinsed twice with phosphate-buffered saline, pH 7.4 (PBS), removing dead cells and debris. The adherent cells were incubated with a serum-free medium containing 10 µM H_2DCFDA at 37 °C for 30 min in the dark, followed by removal of the medium and two washings with the PBS. The cells in each well were finally covered with 200 µL of PBS, and the fluorescence intensity was measured in a multi-mode plate reader (Molecular Devices, USA).

The cellular content of GSH was determined by 5,5´-dithio-bis-nitrobenzene (DTNB) assay. After brief washings in PBS, the cells were re-suspended in 100 mM potassium phosphate buffer / 5 mM EDTA, pH 7.5 (KPE buffer), extracted by brief vortexing with an equal volume of 0.1 % Triton X-100 in 5 % trichloroacetic acid in KPE buffer and centrifuged at 4 °C at 10000 rpm for 5 min. The supernatant was used to measure GSH by allowing it to react with DTNB in KPE buffer at 37 °C for 10 min, and the color developed was read at 412 nm. The method was adapted from the published procedures [Rahman et al., 2006; Look et al., 1997].

The measurement of MDA was based on 2-thiobarbituric acid (TBA) assay as obtained from published procedures [Draper et al., 1993].

2.4. Measurement of mitochondrial parameters

The mitochondrial membrane potential and ATP content of control and treated SH-SY5Y cells were measured by tetramethylrhodamine ethyl ester (TMRE) and luciferin-luciferase-based assays, respectively earlier by our group [Ganguly et al., 2020]. The fluorescence or chemiluminescence signals were measured in a multi-mode plate reader (Molecular Devices, USA).

For the assay of complex I-III, mitochondria were isolated from the cells as described earlier [Jana et al., 2011]. Complex I-III assay was performed spectrophotometrically using NADH as the electron donor and ferricytochrome *c* as the electron acceptor. The reaction was monitored by following the rate of reduction of ferricytochrome *c* at 540 nm [Bagh et al., 2011].

2.5. Nuclear staining by Hoechst and propidium iodide (PI)

Hoechst 33258 dye (Invitrogen, USA) was used for the identification of apoptotic nuclei [Jana et al., 2011]. SH-SY5Y cells were grown on poly-L-lysine coated coverslips and treated without (control) or with rotenone (0.5 µM) for 48 h. The cells were washed twice with PBS, fixed with 4 % paraformaldehyde for 10 min, and re-washed twice with PBS. The fixed cells were then incubated with 1 µg/mL Hoechst 33258 dye for 20 min at 37 °C. After staining, the coverslips were rinsed with PBS, mounted on glass slides, and observed under a fluorescence microscope (Carl Zeiss, Germany). For PI staining, control and treated cells grown on 24-well plates were trypsinized into suspensions, centrifuged and the pelleted cells fixed with 70 % ethanol for 10 min. The cells were then washed twice with PBS and re-suspended in PBS with PI (1 µM) for 20 min at 37 °C. The stained cells were collected by centrifugation, washed with PBS twice to remove the excess dye, and observed under a fluorescence microscope (Carl Zeiss, Germany).

2.6. Protein quantification

Protein quantification was performed by bicinchoninic acid (BCA) assay. The samples were solubilized in 1 % sodium dodecyl sulfate (SDS) before estimation of protein.

2.7. Statistical analysis

Statistical significance among three or more groups was performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value of ≤ 0.05 was considered statistically significant. Values are represented as the medians with range and scatterplots reflected the individual data points.

3. Results

Results presented in Figure 1 show that rotenone (0.5 µM) caused marked loss of cell viability after 48 h of incubation; comparable and more than 5.5 fold increase of cell death with respect to control cells could be seen both in Trypan blue and LDH release assays. The cell death was very significantly prevented when the cells were co-incubated with Fer-1 (1 µM) or Lip-1 (1 µM) along with rotenone (Figure 1).

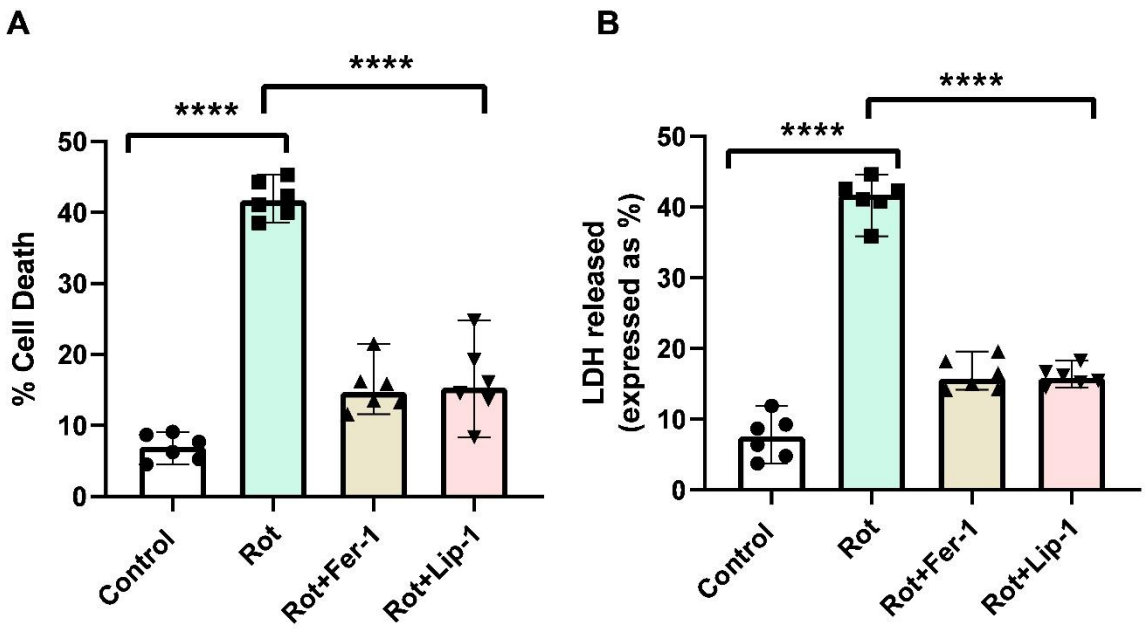


Figure 1. Ferrostatin-1 and Liproxstatin-1 prevent rotenone-induced cell death
SH-SY5Y cells were treated without (Control) or with Rotenone (Rot, 0.5 µM) in the presence or absence of ferrostatin-1 (Fer-1, 1 µM) or liproxstatin-1 (Lip-1, 1 µM) for 48 h. The cell viability was analyzed by (A) Trypan blue assay and (B) LDH release assay as described in Materials and Methods. Values are medians with range of six observations (independent) with scatterplots of individual data points. Statistically significant, ****p<0.0001.

When SH-SY5Y cells were incubated with rotenone (0.5 μM) for 48 h, nearly 4.5 folds increase in the level of MDA, an end product of lipid peroxidation, could be seen compared to that in control cells which was abolished nearly completely by co-treatment with Fer-1 and Lip-1. The generation of ROS on rotenone–treatment of SH-SY5Y cells increased very significantly over the control value (by about 4.5 folds) which could be prevented markedly by Fer-1 or Lip-1 (Figure 2). The major cellular antioxidant GSH was depleted significantly on rotenone-treatment of SH-SY5Y cells for 48 h which could be rescued remarkably by the co-treatment with Fer-1 and Lip-1 (Figure 2).

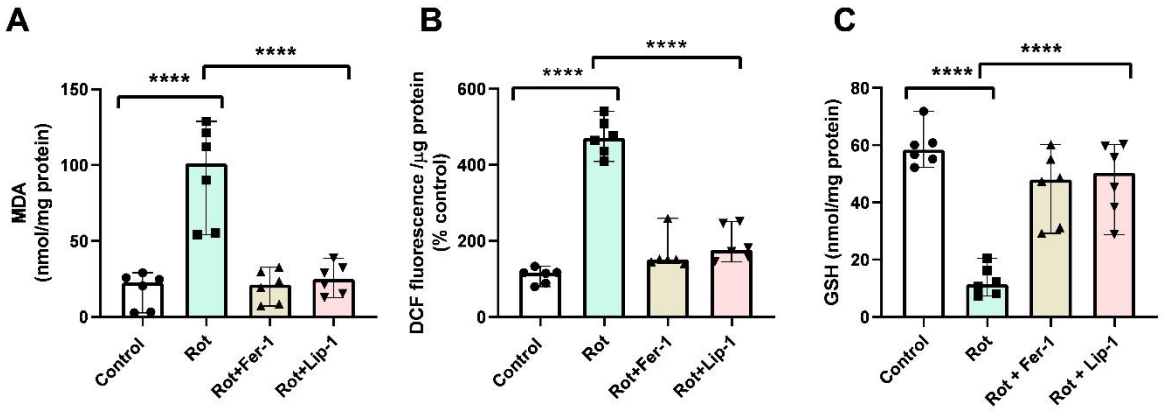


Figure 2. Rotenone-mediated oxidative stress in SH-SY5Y cells
SH-SY5Y cells were treated without (Control) or with Rotenone (Rot, 0.5 μM) in the presence or absence of ferrostatin-1 (Fer-1, 1 μM) or liproxstatin-1 (Lip-1, 1 μM). After 48 h of incubation, the cells were analyzed for MDA (A), ROS (B), and GSH (C) content as described in Materials and Methods. Values are represented as medians with range of six independent experiments. ****p<0.0001 denotes the level of significance.

Three important parameters related to mitochondrial functions were affected by the rotenone-treatment of SH-SY5Y cells for 48 h as presented in Figure 3. Mitochondrial transmembrane potential and cellular ATP content were decreased by nearly 60 % when SH-SY5Y cells were incubated for 48 h with 0.5 μM rotenone and both these phenomena were markedly, but not completely, prevented by the co-treatment of the cells with Fer-1 and Lip-1 (Figure 3). Complex I-III (NADH-cytochrome c reductase) activity was inhibited by about 50 % after the rotenone-treatment of SH-SY5Y cells for 48 h, but Fer-1 or Lip-1 did not show any statistically significant protection against rotenone (Figure 3).

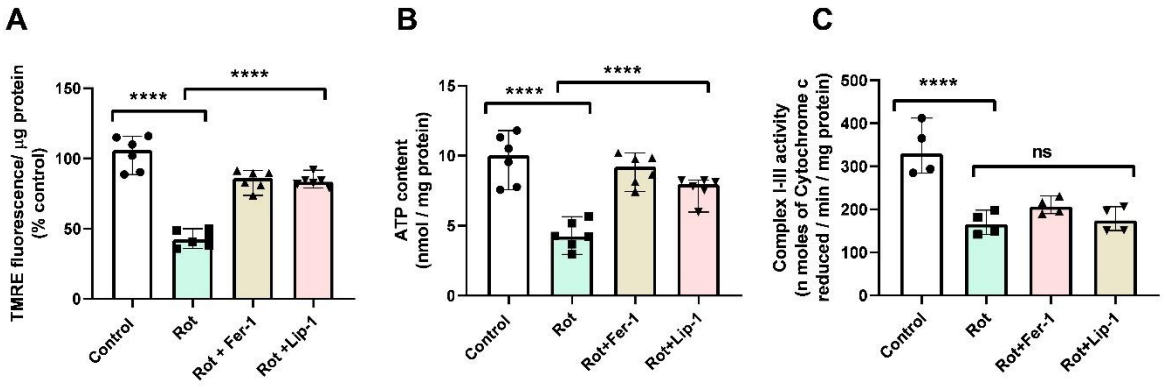


Figure 3. Effects of Ferrostatin-1 and Liproxstatin-1 on rotenone-mediated mitochondrial dysfunction

SH-SY5Y cells were either untreated (Control) or treated with rotenone (Rot, 0.5 µM) with or without ferrostatin-1 (Fer-1, 1 µM) or liproxstatin-1 (Lip-1, 1 µM) for 48 h. The cells were analyzed for (A) mitochondrial membrane potential using TMRE assay, (B) intracellular ATP content, or (C) mitochondrial complex I-III activity. The experiments were performed in six biological replicates for (A) and (B) and four biological replicates for (C). Values are represented as medians with range indicating individual data points in scatterplots. Statistically significant, ****p<0.0001. ns, non-significant.

When SH-SY5Y cells were treated with rotenone (0.5 µM) for 48 h and the cells examined under fluorescence microscope after staining with Hoechst 33258, many brightly fluorescent and condensed nuclei could be noticed indicating apoptotic cells; likewise, an examination of PI-stained cells after similar rotenone-treatment revealed many bright red fluorescent nuclei indicative of cell membrane rupture and necrosis (Figure4).

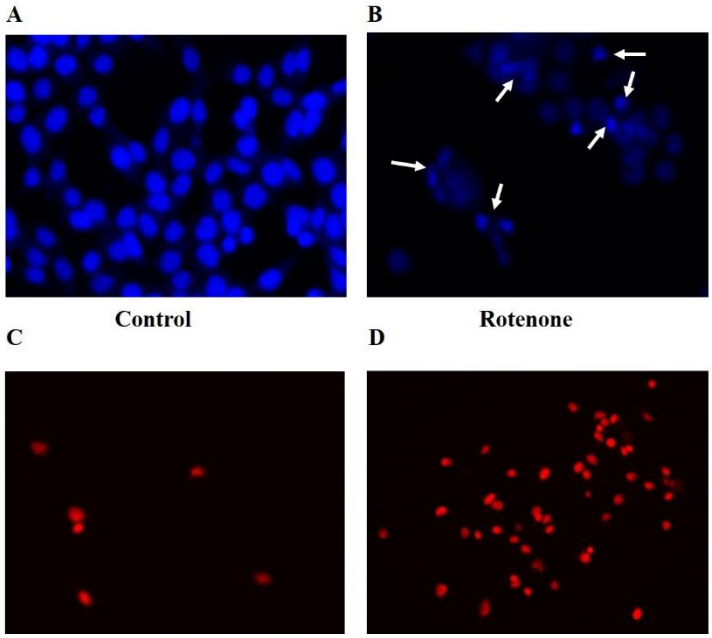


Figure 4. Nuclear staining Hoechst 33258 and PI

SH-SY5Y cells were treated without (Control) or with Rotenone (Rot, 0.5 µM) for 48 h. The cells were stained by Hoechst 33258 or PI as described in the Materials and Methods. A representative image from a set of 4 independent experiments. The white arrows indicate the apoptotic nuclei.

4. Discussion

The inhibition of mitochondrial Complex I (measured as NADH-ubiquinone reductase) as well as NADH-cytochrome c reductase was initially observed in the substantia nigra of postmortem PD brains, and subsequently, other mitochondrial anomalies were also reported in this disease from similar postmortem studies [Schapira et al., 1990; Toulorge et al., 2016]. At the experimental level, Complex I inhibitors like MPTP and rotenone are popular toxins to develop animal models of PD [Duty and Jenner, 2011; Richardson et al., 2007]. The mechanisms of rotenone cytotoxicity are not, however, definitely established, and a simple bioenergetic failure of the cell from mitochondrial Complex I inhibition might not be the dominant factor as was elegantly shown in dopaminergic neurons obtained from Ndufs4 (a gene necessary for Complex I assembly)-deficient mice [Choi et al., 2008]. The increased ROS production in rotenone-treated cells was reported earlier, which could trigger cell death in multiple ways [Li et al., 2003; Mohammed et al., 2020]. Our current study demonstrated that rotenone could induce cell death and an increased accumulation of ROS and lipid oxidation products, and depletion of GSH, which were all prevented markedly by typical inhibitors of ferroptosis like Fer-1 and Lip-1; thus, these observations are indicative of rotenone-induced ferroptosis under our experimental conditions. Although mitochondrial morphological changes have been characteristically described in ferroptosis, the precise involvement of mitochondria in ferroptosis has remained controversial in many respects [Gao et al., 2019; Gan 2021; Wang et al., 2020]. For example, mitochondrial membrane hyperpolarization was reported by classical inducers of ferroptosis such as erastin [Gao et al., 2019]. On the other hand, t-butyl hydroperoxide was shown to trigger ferroptosis in PC12 cells with mitochondrial membrane depolarization and loss of ATP, and our results are consistent with this study [Wu et al., 2018]. In melanoma cells also, the mitochondrial Complex I inhibitor BAY 87-2243 was reported to cause ferroptosis and necroptosis with mitochondrial membrane depolarization [Basit et al., 2017]. Our results further show that Fer-1 and Lip-1 rescued the rotenone-induced loss of mitochondrial membrane potential and ATP production, but not Complex I inhibition implying that the latter phenomenon *per se* was not responsible for mitochondrial bioenergetic impairment. Instead, it appears that the accumulation of ROS and lipid oxidation products in rotenone-treated cells was responsible for mitochondrial dysfunction and cell death. It is not known if ROS directly damage the mitochondria triggering cell death, or other mediators are involved in this process. Interestingly, the Hoechst 33258 and PI staining of cells indicate a mixed pattern of necrosis and apoptosis after rotenone treatment; this is again in conformity with earlier studies wherein evidence in favor of apoptosis and necrosis after rotenone treatment of cells was documented [Moon et al., 2005; Hong et al., 2014; Li et al., 2003]. In brief, our study implicates rotenone as a ferroptotic inducer, and further implies that the typical biochemical features of ferroptosis may be just early events, and the final pathway of cell death may follow apoptosis or different forms of necrosis. This, however, needs more elaborate studies.

Abbreviations

AD	Alzheimer's disease	MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
GSH	reduced glutathione	PD	Parkinson's disease
MDA	malondialdehyde	ROS	reactive oxygen species

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