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Genotoxic effects of rotenone on cultured lymphocytes

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ABSTRACT. Rotenone is a heterocyclic compound widely used as an insecticide, acaricide and piscicide. Its toxicity is mainly caused by the inhibition of mitochondrial respiratory processes and ATP production, resulting in the generation of reactive oxygen species. Reactive oxygen species can interact with DNA, RNA and proteins, leading to cell damage, followed by death. We used the Comet assay, and we analyzed

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chromosome aberrations, in order to evaluate the genotoxic and clastogenic effects of rotenone on the different phases of the cell cycle. Cultured human lymphocytes were treated with 1.0, 1.5 and 2.0 µg/mL rotenone during the G1, G1/S, S (pulses of 1 and 6 h), and G2 phases of the cell cycle. Rotenone induced DNA damage and was clastogenic, but the clastogenicity was detected only with treatments conducted during the G1/S and S phases of the cell cycle. Rotenone also induced endoreduplication and polyploidy in treatments made during G1, while it significantly reduced the mitotic index in all phases of the cell cycle.

Key words: Rotenone, Chromosome aberration, Comet assay

INTRODUCTION

Rotenone (rotenone) is a heterocyclic five-ringed compound, with the molecular formula $C_{23}H_{22}O_6$ (Rahde, 1990; IPCS, 1992; NIOSH, 1994). This compound is extracted from plants of the Leguminosae (*Derris* spp and *Lonchocarpus* spp), and it is widely used as an insecticide, acaricide and piscicide (Rahde, 1990).

The main mechanism for the toxicity of rotenone involves inhibition of complex I of the mitochondrial respiratory chain. More specifically, rotenone blocks NADH oxidation by the NADH-ubiquinone oxide reductase enzymatic complex, which results in the inhibition of mitochondrial respiration and a reduction in the synthesis of ATP (Blandini et al., 1998; Fang and Casida, 1999; Sestili et al., 1999; Chauvin et al., 2001). Rotenone treatment also results in the production of reactive oxygen species, which can interact with proteins, DNA and RNA, altering their functions or inducing lipidic peroxidation, eventually leading to cell death (Mizuno et al., 1998; Suzuki et al., 1999; Barrientos and Moraes, 1999; Betarbet et al., 2000; Gao et al., 2002; Li et al., 2003).

Deficiency in complex I is associated with a great variety of symptomatic phenotypes, varying from fatal infantile lactic acidosis to Leigh disease and a number of neurodegenerative diseases, such as Leber's hereditary optic neuropathy, focal dystonia and Parkinson's disease (Barrientos and Moraes, 1999). It has been suggested that exposure to rotenone is a risk factor for the development of the neurodegenerative processes that characterize Parkinson's disease (Betarbet et al., 2002).

Evidence for biological activity of plant-derived compounds is steadily growing (Konstantopoulou et al., 1992). Since most plants are abundant in nature, any mutagenic, teratogenic and carcinogenic properties that they may have can have a significant impact on human health.

We examined the genotoxic activity of rotenone in cultures of human peripheral blood lymphocytes treated in various phases of the cell cycle. Previous studies with cultured human cells have made treatments only during the S phase of the cell cycle (Ahmed et al., 1977; Guadano et al., 1998). In addition, other studies that have evaluated the genotoxicity of rotenone in rodent and plant cells have measured micronuclei and sister-chromatid exchanges as endpoints (Amer and boul-ela, 1985; Anderson et al., 1990; Matsumoto and Ohta, 1992).

MATERIAL AND METHODS

Test agent

Stock solutions were made by dissolving fine crystals of rotenone (Sigma, St. Louis, MO, USA) in the solvent dimethylsulfoxide (DMSO; Merck-Schuchardt) immediately prior to the experiment. According to Preston et al. (1987), DMSO concentrations should not exceed 1% in the culture medium. The maximum concentration of DMSO in our experiments was 0.5%; there were no differences in the frequency of chromosome aberrations or DNA damage between solvent controls and untreated cultures (data not shown).

Lymphocyte culture

Peripheral blood was collected from four normal, healthy donors, two women and two men, aged 21 to 26 years, with no history of smoking/drinking or chronic drug use. Ten millimeters of venous blood was collected from each donor into heparinized vials (5,000 IU/mL; Liquémine; Roche). Short-term lymphocyte cultures were initiated according to a standard protocol (Preston et al., 1987). The culture medium consisted of 5 mL Hams-F10 (76.8%) (Sigma), heat-inactivated fetal calf serum (19.2%) (Cultilab), phytohemagglutinin-M (2%) (Gibco-Invitrogen, Carlsberg, CA, USA) and antibiotics (0.01 mg/mL penicillin (Sigma) and 0.005 mg/mL streptomycin (USB, Cleveland, OH, USA). The culture tubes were incubated at 37ºC in a humidified atmosphere composed of 5% CO_2/95% air.

Treatments and biological tests

Preliminary experiments indicated high levels of cytotoxicity for rotenone treatments above 2 µg/mL, as evidenced by inhibition of cell division (data not shown). At G1, lymphocytes in complete culture medium were treated with a combination of 0.2 mL phytohemagglutinin-M and 1, 1.5 or 2 µg/mL rotenone. The cells were fixed following 52 h of incubation at 37°C. At G1/S, the cultures were treated with rotenone 24 h after phytohemagglutinin stimulation and were fixed 52 h after the initiation of the culture. To determine the specific effects of rotenone in the S phase, pulse treatments with rotenone for 1 h and 6 h were made 24 h after stimulation. Following each pulse treatment, the cells were washed once in serum-free medium, re-incubated in complete medium, and fixed after 52 h of incubation. In the G2 treatments, 69-h cultures were treated with rotenone for 3 h, and then the cells were fixed immediately (72 h total incubation) (Table 1).

Cytogenetic studies

In order to obtain a sufficient number of analyzable metaphases, colchicine (Sigma) was added at a final concentration of 0.0016%, 2 h prior to the termination of cultures. The cells were harvested by centrifugation and treated with 0.075 M KCl at 37ºC for 20 min. The cells were then centrifuged and fixed in 1:3 (v/v) acetic acid-methanol. Finally, slides were prepared, air-dried and stained with 3% Giemsa solution, pH 6.8, for 8 min (Moorhead et al., 1960).

The slides were analyzed with an optical microscope and structural and numerical chro-

Table 1. Treatment protocols of rotenone applied to short-term cultures of human lymphocytes.

Treatment	PHA	Rotenone	Without FBS	Wash	COL	HAR
G1	0 h	0 h	-	-	50 h	52 h
G1/S	0 h	24 h	-	-	50 h	52 h
S1 (1-h pulse)	0 h	24 h	24 h	24 h	50 h	52 h
S1 (6-h pulse)	0 h	24 h	24 h	24 h	50 h	52 h
G2	0 h	69 h	-	-	70 h	72 h

PHA: phytohemagglutinin; FBS: fetal bovine serum; COL: colchicine; HAR: harvest.

mosome alterations (CAs) were examined in metaphases from the rotenone-treated cultures and from the respective controls. The frequency of CAs (in 100 metaphases per culture) and the mitotic index (MI) (number of metaphases per 2,000 lymphoblasts per culture) were determined.

Comet assay

Peripheral venous blood was collected in heparinized vials as above from two normal, healthy donors, one 21-year-old male and one 26-year-old female, with no history of smoking/ drinking or chronic use of medication. Whole blood and peripheral blood lymphocytes were separately incubated for 5 min with different concentrations of rotenone and then mixed with low-melting point agarose.

The alkaline version of the Comet assay (single-cell gel electrophoresis) was performed as described by Singh et al. (1988). Slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each duplicate slide) with a fluorescence microscope (Zeiss) equipped with a 515-560-nm excitation filter, a 590-nm barrier filter, and a 40X objective. Undamaged cells appeared as intact nuclei without tails, whereas damaged cells had the appearance of a comet. Comets were classified visually as belonging to one of five classes according to tail intensity and given a score of 0, 1, 2, 3, or 4 (from undamaged = 0, to maximally damaged = 4). Thus, the total damage score for 100 comets ranged from 0 (all undamaged) to 400 (all maximally damaged) (Speit and Hartmann, 1999).

Statistical analysis

The Student *t*-test was used to compare the frequencies of CAs observed in cells exposed to the various concentrations of rotenone with the respective controls. The F test (ANOVA) was used to detect significant differences in the MI between cells exposed to rotenone and the respective controls. The level for statistical significance was established at 5% (Ayres et al., 2000).

RESULTS

Chromosome aberrations and mitotic index

Treatments with 1.0 and 1.5 μg/mL rotenone during G1 significantly induced endoreduplication and polyploidy. At G1/S, the frequency of CAs was significantly increased

with all the tested concentrations of rotenone (Table 2). Rotenone treatment during S phase resulted in highly significant increases in the frequency of CAs ($P < 0.01$); however, there were no significant differences between S-phase treatments of 1 and 6 h (Table 3). The treatment at G2 did not induce a significant increase in the frequency of CAs (Table 4). Chromatid gaps and chromatid breaks were the most frequent CAs.

Table 2. Chromosome aberrations (CA) and mitotic index (MI) in cultured human lymphocytes treated with rotenone during the G1 and G1/S phases.

Rotenone treatment	MI (%)	CA			CA/100 cells	Polyp	End
		Gaps	Breaks	Total			
G1							
Control	4.5	1	0	1	0.25	1	0
1.0 µg	4.4	2	0	2	0.5	2	12
1.5 µg	4.3*	2	0	2	0.5	0	9
2.0 µg	4.3*	0	0	0	0	2	17
G1/S							
1.0 µg	2.8**	14	0	14*	3.5	0	0
1.5 µg	2.7**	13	5	18*	4.5	0	0
2.0 µg	2.5**	11	12	23*	5.7	0	0

MI: number of metaphases per 2,000 lymphoblasts per culture; Polyp: polyploid cells; End: endoreduplication.
*$P < 0.05$, **$P < 0.01$.

Table 3. Chromosome aberrations (CA) and mitotic index (MI) in human lymphocytes treated with rotenone during the S phase of the cell cycle.

Rotenone treatment	MI (%)	CA			CA/100 cells	Polyp	End
		Gaps	Breaks	Total			
1 h							
Control	4.5	1	0	1	0.25	1	0
1.0 µg	1.6*	18	4	22*	5.5	0	0
1.5 µg	1.3*	15	12	27*	6.7	0	0
2.0 µg	1.2*	20	12	32*	8.0	0	0
6 h							
1.0 µg	1.2*	17	9	26*	6.5	0	0
1.5 µg	1.1*	13	17	30*	7.5	0	0
2.0 µg	1.0*	27	17	44*	11	0	0

Polyp: polyploid cells; End: endoreduplication.
*$P < 0.01$.

The cytotoxic effects of rotenone were observed as decreases in the MI of lymphocyte in cultures treated during the G1, G1/S, S, and G2 phases of the cell cycle. Only the 1.0 µg/mL rotenone treatment during the G1 phase failed to produce a significant reduction in MI (Tables 2-4).

Table 4. Chromosome aberrations (CA) and mitotic index (MI) in human lymphocytes treated with rotenone during the G2 phase of the cell cycle.

Rotenone treatment	MI (%)	CA			CA/100 cells	Polyp	End
		Gaps	Breaks	Total			
Control	4.5	1	0	1	0.25	1	0
1.0 µg	4.1*	1	1	2	0.5	0	0
1.5 µg	4.0*	4	0	4	1.0	1	0
2.0 µg	3.9*	3	0	3	0.75	2	0

Polyp: polyploid cells; End: endoreduplication.
*$P < 0.05$.

Comet assay

All rotenone treatments, both of whole blood and of isolated lymphocytes, resulted in significant increases in DNA damage (Tables 5 and 6). However, the frequency of cells in comet classes 3 and 4 was higher in the isolated lymphocytes than in whole blood.

Table 5. Comet assay damage score and class distribution of comets in rotenone-treated human whole blood.

Treatment	Comet class					DNA damage score
	0	1	2	3	4	
Control	94	6	0	0	0	6
1.0 µg/mL	82	12	4	2	0	26
1.5 µg/mL	80	10	6	4	0	34
2.0 µg/mL	6	28	8	0	0	44

Table 6. Comet assay damage score and class distribution of comets in rotenone-treated human lymphocytes.

Treatment	Comet class					DNA damage score
	0	1	2	3	4	
Control	76	18	6	0	0	30
1.0 µg/mL	38	16	20	20	6	140
1.5 µg/mL	24	30	20	20	6	154
2.0 µg/mL	8	18	18	14	42	264

DISCUSSION

There are thousands of types of pesticides currently in use, and humans are exposed to them at work, or as a result of contamination of food and water, as well as by spraying and fumigation. The clinical symptoms and the possible mutagenic effects produced by acute poi-

soning and chronic exposure to pesticides are of considerable interest (Le Couteur et al., 1999; Ritz and Yu, 2000). Rotenone was chosen because it is a natural pesticide widely used in agriculture and it may contribute to some of the symptoms of patients with Parkinson's disease (Di Monte, 2001).

The MI results indicated that as little as 1 µg/mL rotenone during the G1/S, S and G2 phases of the cell cycle resulted is cytotoxic. These data are consistent with the findings of Guadano et al. (1998), who also reported a significant, concentration-related reduction of MI in rotenone-treated cells. This toxicity probably occurs due to the inhibition of the cell cycle by this pesticide.

Only 1.5 and 2 µg/mL rotenone were cytotoxic when treatments were conducted during the G1 phase. It is possible that 1 µg/mL rotenone was not toxic to the cells because the half-life of rotenone may be less than 24 h; the same concentration, when applied during the G1/S phase, was cytotoxic. This argument leads us to believe that degradation of rotenone concentrations above 1 µg/mL takes more than 24 h, and that a dose sufficient to induce cytotoxicity remains when the cells reach the S phase of the cell cycle. It is also possible that functional degradation of concentrations less than or equal to 1 µg/mL in less than 24 h may be limited to the rotenone exposure of lymphocytes *in vitro*; in other cell and environmental systems, the rate of degradation is probably different. The half-life of rotenone has been estimated to be one to three days in aquatic and terrestrial environments. This relatively rapid degradation may limit the phenomenon of biomagnification, thus making rotenone a relatively non-toxic pesticide (Lai et al., 1989; Rahde, 1990; IPCS, 1992; EXTOXNET, 1996).

All the test concentrations of rotenone were also cytotoxic at G2 phase, but the cytotoxicity was less than when treatments were made during the other phases of the cell cycle. This lower sensitivity may result from the fact that this pesticide acts during the interval between G1 and S. Treatments during the G2 phase may reduce MI by chemical carry-over in the culture medium. It is also possible that the reduction of MI in G2-treated cells can be explained by alternative mechanisms of rotenone toxicity. Armstrong et al. (2001) observed that the induction of apoptosis in a human type B cell line, as measured by the release of mitochondrial cytochrome C and by the activation of caspase, was associated with a delay of the cells in G2/M phase of the cell cycle and occurred only at concentrations 10 to 50 times higher than those required to block the electron flow through mitochondrial complex I. These results indicate that rotenone-induced apoptosis is more likely a consequence of the rupture of microtubules than of inhibition of electron transport. On the other hand, Li et al. (2003) demonstrated that apoptosis induction results from the production of reactive oxygen species generated by the interaction of rotenone with the mitochondria.

The significant increase in endoreduplications and polyploid cells that was found only with treatment of 1 and 1.5 µg/mL rotenone during the G1 phase indicates that rotenone exposure has an effect on the mitotic apparatus. These observations are consistent with previous descriptions of the antipolymerizing effect of rotenone on tubulin dimers, a protein that is responsible for the formation of the mitotic spindle (Cunningham et al., 1995). Matsumoto and Ohta (1992) also reported that rotenone induced endoreduplication in Chinese hamster cells. The observation that rotenone induces endoreduplication is an indication that rotenone can have an antitumor effect, as suggested by Cunningham et al. (1995), Fang and Casida (1998) and Rowlands and Casida (1998). Treatment of G1 lymphocytes with 2 µg/mL rotenone did not induce significant alterations in ploidy, possibly because this concentration caused a decrease in the number of analyzable cells due to toxicity.

We scored chromosome and/or chromatid breaks and gaps as CAs. There has been a great deal of discussion regarding the inclusion of gaps as true chromatid lesions. Some authors argue that the counting of gaps can be subjective, and that they may be the result of technical artifacts, of variability within the same culture, and of variability in the culture conditions (Schinzel and Schmid, 1976; Brogger, 1982). Yet, Paz-y-Mino et al. (2002) obtained results indicating that gaps are indicative of DNA damage, which supports their inclusion in the analysis of CAs.

A high frequency of CAs was found when rotenone was applied to the G1/S and S phases of the cell cycle. However, more breaks and gaps were seen in the S phase than in the G1/S phase. This may be due to the fact that the S phase treatments were carried out using short “pulses”, i.e., rotenone was in contact with the cells exclusively during this phase; immediately after treatment (1 or 6 h), the pesticide was removed, and the cell cycle continued. This finding reinforces our hypothesis that rotenone has a primary action on DNA.

The absence of significant CA induction with treatments conducted during the G1 and G2 phases of the cell cycle confirms the hypotheses that we described earlier, i.e., when the drug is introduced in conjunction with phytohemagglutinin stimulation in G1 phase cells, the lymphocytes undergo dedifferentiation for an average period of 24 h (Janossy and Greaves, 1972; Preston et al., 1987). During this time, the drug can be metabolized by the cell and/or degraded, but rotenone may not directly interact with DNA. In G2, the level of cytotoxicity was lower compared to the other phases; this parallels the absence of clastogenicity in cells treated during this phase.

We also evaluated the DNA-damaging effects of rotenone detected by the Comet assay in whole blood and in peripheral blood lymphocytes. This assay has been widely used to detect single- and double-strand DNA breaks. The high sensitivity of this test is due to the fact that it detects DNA breaks, alkali-labile lesions and genomic lesions that are subject to repair (Gontijo and Tice, 2003). Although all the rotenone treatments increased the levels of DNA damage, we found that assays conducted with isolated peripheral blood lymphocytes resulted in higher levels of DNA damage (type-4 comets) than did the trials with whole blood (type 1, 2 and 3 comets). This finding may be due to the rotenone interacting with all the myeloid cells in blood, thereby reducing its overall activity.

In conclusion, although rotenone produced DNA damage and was cytotoxic during all phases of the cell cycle, its clastogenicity was limited to exposures made during the G1/S and S phases. Treatment of G1 cells resulted in polyploidy and endoreduplication, consistent with rotenone interacting with the mitotic spindle apparatus. These data indicate that rotenone is genotoxic and should be used with caution.

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